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Abstract

Ammonia oxidizing archaea (AOA) are among the most abundant organisms on this planet and serve a critical function in the nitrogen cycle by oxidizing ammonia to nitrite. Due to the importance of the nitrogen cycle as the most anthropogenically disturbed geobiochemical cycle, AOA have gained much attention in the field of microbial ecology and nitrification over the last two decades. AOA are chemolithoautotrophs that gain energy from the oxidation of ammonia to nitrite and fix carbon dioxide by a modified version of the 3-hydroxypropionate/4-hydroxybutyrate pathway. Even though much scientific effort has gone into elucidating the ecological niches and function of these organisms, biochemical insights into the archaeal ammonia oxidation pathway are still limited. This is mostly due to a lack of AOA biomass, because these organisms are notoriously difficult to cultivate. Thermophilic AOA of the family Nitrosocaldaceae are of particular interest because they form a sister clade to all moderate AOA. They are considered to possess many ancestral features as different phylogenies based on 16S rRNA, ribosomal proteins and amoA genes all point towards a thermophilic origin of the class of Nitrososphaeria, to which all AOA belong. This thesis consists of three major sections: (i) the enrichment, phylogeny and physiology of an extremely thermophilic AOA, (ii) the isolation and physiological characterization of the first axenic culture of a member of the family *Nitrosocaldaceae*, *Nitrosocaldus cavascurensis*. (iii) The development of a lab-scale biomass production process for *Nitrososphaera viennensis*, a model soil AOA, and the characterization of growth-relevant parameters in continuous cultures. The results of this thesis contribute to our understanding of the physiology of AOA and provide the first pure culture of an extremely thermophilic AOA and a biomass production process that enables biochemical research of these important organisms.

Zusammenfassung

Ammoniak oxidierende Archaea (AOA) gehören zu den häufigsten Organismen auf diesem Planeten und üben eine zentrale Rolle im Stickstoffzyklus durch die Oxidation von Ammoniak zu Nitrit aus. Aufgrund der Wichtigkeit des Stickstoffzyklus, den durch den Menschen am meisten gestörten geochemischen Stoffzyklus, haben AOA viel Aufmerksamkeit in der mikrobiellen Ökologie und Nitrifizierung in den letzten zwei Jahrzehnten bekommen. AOA sind chemolithoautotrophe Organismen die Energie aus der Oxidation von Ammoniak zu Nitrit gewinnen und Kohlenstoffdioxid durch eine modifizierte Variante des 3-Hydroxypropionat/4-Hydroxybutyrat Stoffwechselweges fixieren. Obwohl bereits viele Anstrengungen unternommen wurden die ökologischen Nischen und Funktion dieser Organismen zu verstehen, sind die Erkenntnisse über die archaeelle Ammoniak Oxidation im Bereich der Biochemie noch äußerst beschränkt, welches dem Mangel an AOA Biomasse geschuldet ist, da die Organismen äußerst schwer zu kultivieren sind. Thermophile AOA aus der Familie der Nitrosocaldaceae sind von besonderem Interesse, da diese eine Schwesterlinie zu allen moderaten AOA bilden. Sie gelten als besonders ursprünglich, da unterschiedliche Phylogenien basierend auf 16S rRNA, ribosomalen Proteinen und amoA Genen alle auf einen thermophilen Ursprung der Klasse Nitrososphaeria, zu der alle AOA zählen, hindeuten. Diese Arbeit gliedert sich in drei Abschnitte: (i) Anreicherung, Phylogenie und Physiologie eines extrem thermophilen Archaeons, (ii) Isolierung und physiologische Charakterisierung der ersten Reinkultur eines Vertreters der Familie der Nitrosocaldaceae, *Nitrosocaldus cavascurensis*. (iii) Die Entwicklung eines Biomasse-Produktionsprozesses für das Bodenarchaeon *Nitrososphaera viennensis* und die Charakterisierung wachstumsrelevanter Parameter in kontinuierlicher Kultur. Die Ergebnisse dieser Dissertation tragen nicht nur zum besseren physiologischen Verständnis von AOA bei, sondern stellen auch die erste Reinkultur eines extrem thermophilen AOA zur Verfügung, sowie einen Biomasse-Produktionsprozess, welcher in weiterer Folge biochemische Studien dieser wichtigen Organismen ermöglicht.

Chapter I

Introduction

Background

"Organisms are resilient patterns in a turbulent flow - patterns in an energy flow."

(Woese 2004)

Elemental nitrogen (N_2) is the major component of the earths' atmosphere making up about 78 %, but due to its almost inert nature, the availability of reactive nitrogen (N_r, ammonia (NH_3) and NO_x) is usually limited in natural ecosystems. Much of the observed diversity in ecosystems actually derives from the limitation of N_r (Dise, Ashmore et al. 2011), as microbial nitrogen fixation is a very energy intensive process, requiring 4 e^-, 4 H^+ and 8 ATP per NH_3 molecule. With the invention of the Haber-Bosch process (the main industrial nitrogen fixation process), humans started to vastly increase the input of N_r to the biosphere, thereby exponentially increasing food production and, as a consequence, the population. In the current state of industrial agriculture, the Haber-Bosch process is accounted to feed more than 50 % of the human population (Streatfeild 2002, Erisman, Sutton et al. 2008), but due to its energy intensive nature it also heavily relies on fossil fuels, an energy source that becomes increasingly problematic. In addition, free NH_3 is oxidized by nitrifying microorganisms to nitrate (NO_3^-), which leaches from the agricultural soil into the ground water and river systems thereby causing eutrophication. Another undesirable effect of the excessive use of N-fertilizers is the production of nitrous oxide (N_2O) by nitrifiers (and denitrifiers), which is the single most important ozone-depleting substance in the 21st century with an additional global warming potential of approximately 300 times that of CO_2 (Griffis, Chen et al. 2017). Therefore the understanding of the N-cycle and its participants is crucial for improving agricultural practices and environmental management.

Nitrification

As a part of the biogeochemical N-cycle, nitrification is the oxidation of NH_3 to NO_3^- via the intermediate nitrite (NO_2^-). Ammonia oxidation is the first and usually rate limiting step performed by ammonia oxidizing bacteria (AOB) and archaea (AOA), producing NO_2^- which is further oxidized to NO_3^- by nitrite oxidizing bacteria (NOB) (Winogradsky 1892, Daims, Lücker et al. 2016). Another group of bacteria that can perform the complete oxidation of NH_3 to NO_3^- named Comammox was discovered rather recently (Daims, Lebedeva et al. 2015, van Kessel, Speth et al. 2015). AOB have been known for over a century and consist of the bacterial genera *Nitrosomonas*, *Nitrosococcus* and *Nitrospira* that belong to the classes of Betaproteobacteria and Gammaproteobacteria (Winogradsky 1892, Norton 2011). The discovery of AOA came quite as a surprise and was the result of advances in metagenomic sequencing technology. First sequences of the marker gene ammonia monooxygenase (amo) where found in a survey study of the Sargosso sea (Venter, Remington et al. 2004) and complete amo genes were shown to belong to archaeal genomes in a metagenomic study of soil samples (Schleper, Jurgens et al. 2005, Treusch, Leininger et al. 2005). Final proof of archaeal ammonia oxidation came with the isolation of the first AOA *Nitrosopumilus maritimus* in 2005 by Könneke *et al* (Könneke, Bernhard et al. 2005).

Ammonia Oxidation

Even though nitrification has been known for over a century, biochemical insights are still rather limited. This is due to the inability to express many nitrification enzymes heterologously, because either the recombinantly produced enzymes loose function or are even toxic to the expression strain (Klotz and Norton 1998, Gilch, Meyer et al. 2010).To make things even harder, enzymes involved in nitrification are often membrane proteins, which are inherently difficult to purify in a functional form and biomass production of nitrifiers is also a rather tedious task and therefore very limiting. Thus most assays used to investigate the biochemical nature of nitrification are based on whole cells or cell lysates, but at the cost of gaining deeper insights into the mechanistic nature of those processes. Until recently the bacterial pathway of ammonia oxidation was believed to be resolved, NH_3 gets hydroxylated by the ammonia monooxygenase (AMO) enzyme complex (encoded by amoA, amoB and amoC genes) to hydroxylamine (NH_2OH), which was thought to be further oxidized to the end product NO_2^- by the multiheme enzyme hydroxylamine oxidoreductase (HAO). However, a recently study provided evidence that NO_2^- is not the product of HAO but rather nitric oxide (NO) which would then be oxidized by a so far unknown enzyme to NO_2^- (Caranto and Lancaster 2017).

In archaea the ammonia oxidation is far less understood. It also starts with the hydroxylation of NH_3 to NH_2OH by an AMO complex, encoded by amoA, amoB, amoC, amoX, amoY and amoZ genes(Hodgskiss, Melcher et al. 2022), very distantly related to the bacterial AMO and pMMO (particulate methane monooxygenase) (Alves, Minh et al. 2018), which is then somehow further oxidized to NO_2^-. NO appears to serve a critical function in AOA, because archaea show a much higher sensitivity to NO-scavengers like 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) than AOB (Martens-Habbena, Qin et al. 2015, Kozlowski, Stieglmeier et al. 2016). There are two models that try to explain the ammonia oxidation in archea, one in analogy to the bacterial NH_3 oxidation where an unknown enzyme catalyses the oxidation of NH_2OH to NO which would then be further oxidized to NO_2^- by another unknown enzyme (Lancaster, Caranto et al. 2018). The higher sensitivity of AOA to PTIO is thereby explained by a slower turnover of NO compared to AOB, which would lead to an accumulation of NO in a steady state and thus make AOA more susceptible to NO-scavengers like PTIO. The other model suggests that NH_2OH is further oxidized in a 5-electron reaction together with NO by a putative “Cu-HAO” to form two molecules of NO_2^-. The required NO for this reaction would be supplied by the reduction of one NO_2^- molecule by a proposed copper dependent nitrite reductase (NirK) which has been shown to be highly expressed in moderate AOA (Hollibaugh, Gifford et al. 2011, Shi, Tyson et al. 2011, Lund, Smith et al. 2012, Kerou, Offre et al. 2016).

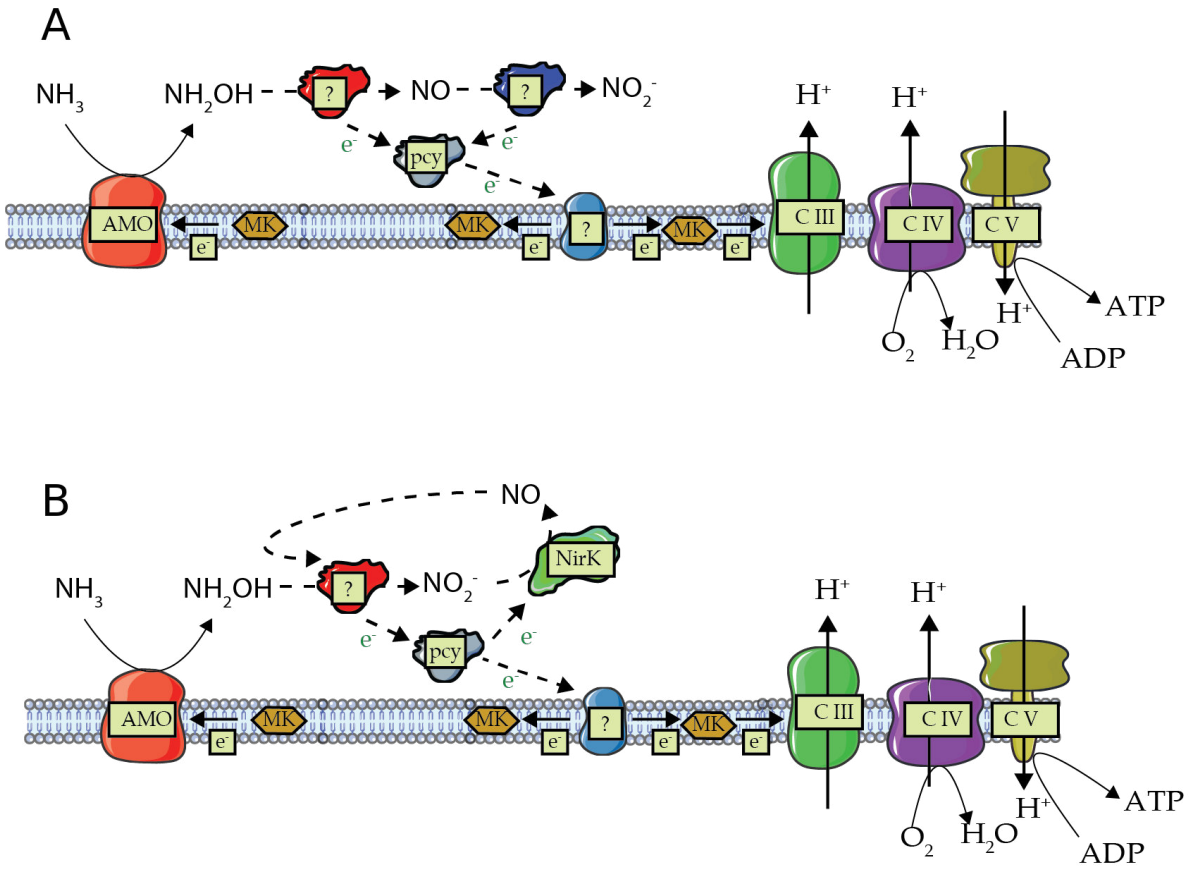

Figure 1: Schematic representation of the two hypothetical models for archaeal ammonia oxidation. A) Based on suggestions in Lancaster et al. (2018)(Lancaster, Caranto et al. 2018) ammonia is oxidized to hydroxylamine followed by two successive oxidations to nitric oxide and nitrite catalyzed by two unidentified proteins. **B)** Based on the proposed model of Kozlowski et al. (2016) (Kozlowski, Stieglmeier et al. 2016) ammonia is oxidized to hydroxylamine which is then further oxidized together with nitric oxide by an unknown protein to produce two molecules of nitrite. Nitrite is reduced by a proposed copper-dependent nitrite reductase (NirK) to nitric oxide, which acts as a cosubstrate for the previous reaction. In both models electrons are passed to plastocyanins and are transferred by an unkown mechanism to the quinone pool. Abbreviations: AMO, ammonia monooxygenase; e^- , electrons; MK, menaquinone; pcy, plastocyanin; C III, complex III (cytochrome c reductase); C IV, complex IV (cytochrome c oxidase); C V, complex V (ATP synthase); NirK, nitrite reductase. Note: Although CIII and CIV are named by their interaction with cytochrome c, AOA do not possess cytochrome c. Cytochrome c is hypothesized to be replaced by plastocyanins or copper containing proteins (Walker, de la Torre et al. 2010, Spang, Poehlein et al. 2012, Kerou, Offre et al. 2016). The figure was taken with permission from the author from Hodgskiss (2022)(Hodgskiss 2022).

Ammonia oxidizing Archaea

All AOA belong to the former phylum Thaumarchaeota that was recently reclassified and are now in the order Nitrososphaerales in the class Nitrososphaeria of the phylum Thermoproteota (Rinke, Chuvochina et al. 2021). Among all archaea, AOA are by far the most widespread with habitats including oceanic water columns (Santoro, Dupont et al. 2015, Qin, Heal et al. 2017, Bayer, Vojvoda et al. 2019), deep sea sediments (Francis, Roberts et al. 2005, Park, Park et al. 2008,

Nunoura, Chikaraishi et al. 2018, Vuillemin, Wankel et al. 2019, Zhao, Hannisdal et al. 2019, Kerou, Ponce-Toledo et al. 2021), oxygen minimum zones (Bristow, Dalsgaard et al. 2016), freshwater systems (French, Kozlowski et al. 2012, Sauder, Engel et al. 2018, French, Kozlowski et al. 2021), waste water treatment plants (WWTP) (Mußmann, Brito et al. 2011, Sauder, Albertsen et al. 2017), soils (Tourna, Stieglmeier et al. 2011, Jung, Park et al. 2014, Zhalnina, Dias et al. 2014, Lehtovirta-Morley, Ross et al. 2016, Alves, Kerou et al. 2019), hot springs (De La Torre, Walker et al. 2008, Reigstad, Richter et al. 2008, Dodsworth, Hungate et al. 2011, Abby, Melcher et al. 2018, Daebeler, Herbold et al. 2018, Luo, Narsing Rao et al. 2020), and even human skin (Probst, Auerbach et al. 2013, Moissl-Eichinger, Probst et al. 2017). This discovery caused a profound change in the perception of archaea, that were previously conceived as mere specialists for specific environment, like thermophiles, acidophiles, halophiles or methanogens. AOA have managed to populate a vast variety of habitats by using a metabolism that -in the bacterial realm- is restricted to only a few genera. The discovery of AOA was made possible by the technological advances in metagenomic sequencing and they would have probably been overlooked by sole cultivation approaches, as they have been for over a century, because AOA are slow growing, do not reach high cell densities and are very sensitive to certain substances and conditions that are not typically thought to be detrimental for microorganisms like e.g. certain rubber stoppers (Stieglmeier, Klingl et al. 2014), traces of detergents (Abby, Melcher et al. 2018) and high gassing rates (Melcher *et al.*, in preparation).

AOA are usually conceived as chemolithoautotrophs with very high substrate affinities (K_S) for NH_3 and rather low specific growth rates (μ). AOB usually have lower substrate affinities for NH_3 and grow faster and are therefore considered ecological R-strategists while AOA are considered k-strategists. This was nicely demonstrated in a chemostat study (French, Kozlowski et al. 2021). Based on environmental marker gene surveys with 16S rRNA or amoA gene amplicons AOA outnumber AOB by orders of magnitude in many habitats (Karner, DeLong et al. 2001, Leininger, Urich et al. 2006, Adair and Schwartz 2008, Nicol, Leininger et al. 2008, Hollibaugh, Gifford et al. 2011), but their contribution to the nitrification process is still not completely resolved. The unusual high abundance and wide distribution of AOA in oligotrophic environments has sparked questions about alternative energy metabolisms of AOA that would enable ammonia oxidation-independent growth.

Alternative Energy Sources of AOA

Beside the chemolithoautotrophy of AOA, there are several genetic and physiological properties that point towards a more flexible metabolic nature. Genomes of AOA for instance encode transporters for a wide variety of organic compounds (Walker, de la Torre et al. 2010, Spang, Poehlein et al. 2012), marine archaea have been shown to incorporate amino acids (Ouverney and Fuhrman 2000) and radiocarbon analyses of marine thaumarchaeal membranes suggest that the communities are composed of a combination of autotrophs and heterotrophs and/ or mixotrophs (Ingalls, Shah et al. 2006). Other indications for heterotrophic metabolisms come from a study of WWTP where the measured NH_3 oxidation could not account for the observed AOA cell number and incorporation of $^{13}CO_2$ was missing (Mußmann, Brito et al. 2011), or erratic increase in AOA cell number decoupled from NH_3 oxidation in enrichment cultures (Alves, Kerou et al. 2019).

Growth enhancing effect of Organics on AOA

While cultivated AOA can usually grow strictly autotrophically, pyruvate and other α-keto acids have been shown to increase ammonia oxidation activity and cell growth. This was originally interpreted as evidence for mixotrophy (Tourna, Stieglmeier et al. 2011, Stieglmeier, Klingl et al. 2014), although less than 10 % of the biomass measured by nanoscale secondary ion mass spectrometry (NanoSIMS) was incorporating carbon from ^{13}C-labeled pyruvate. A rather recent study (Kim, Park et al. 2016) was able to show convincingly that α-keto acids function as H_2O_2 scavenger by nonenzymatic decarboxylation and thus detoxify endogenously produced H_2O_2. Interestingly, different AOA vary in their level of H_2O_2 generation and therefore are more or less dependant on α-keto acids or other H_2O_2 scavengers such as catalase or dimethylthiourea. The same study also investigated the distribution of peroxidase genes in AOA, and while all available genomes contained superoxide dismutase (SOD), they did not contain genes encoding for catalase in general. The exceptions to the rule are *Nitrososphaera gargensis* which was isolated from a hot spring and has one putative but truncated Mn-catalase gene, and *Ca.* Nitrosocosmicus exaquare, the first AOA representative from a municipal WWTP, which has several genes encoding for enzymes associated with detoxification of reactive oxygen species (ROS), including catalase, peroxidase, superoxide dismutase, alkyl hydroperoxide reductase/ peroxiredoxins and thioredoxins (Sauder, Albertsen et al. 2017). Despite the rather elaborate ROS detoxification machinery of *Ca.* N. exaquare the same phenomenon of decreasing growth and activity with increasing level of enrichment, like for *Nitrososphaera viennensis* (Tourna, Stieglmeier et al. 2011), was observed. In case of *Ca.* N. exaquare malate and succinate, followed by pyruvate, provided the most stimulation, although it was not clear if that effect was the result of ROS-scavenging alone or stimulation of contaminating bacteria that might be beneficial for the growth of AOA as well.

Nitrososphaera viennensis

N. viennensis is the type strain of the class Nitrosospheria, a mesophilic AOA isolated from garden soil at the University of Vienna (Tourna, Stieglmeier et al. 2011). Like all other cultivated AOA it grows chemolithoautotrophically by the oxidation of NH_3 to NO_2^- and assimilates CO_2 by a modified version of the hydroxypropionate/hydroxybutyrate cycle (HP/HP cycle) (Könneke, Schubert et al. 2014). It has an optimal growth temperature of 42 °C and NO_2^- production is usually used as a proxy for cell growth as both are tightly coupled and biomass concentrations are far too low to be measurable as optical density. To maintain ammonia oxidation, *N.viennensis* is dependent on the addition of pyruvate or other α-keto acids (Tourna, Stieglmeier et al. 2011) which function as ROS-scavenger (Kim, Park et al. 2016). Raising the incubation temperature from 37 °C to 42 °C also increased the pyruvate demand from 0.1 mmol L^{-1} to 1 mmol L^{-1} (Stieglmeier, Klingl et al. 2014), probably as a result of a higher specific growth rate (μ) and therefore increased ROS production. Due to high μ and good NO_2^- tolerance, *N. viennensis* was chosen for this thesis to develop a lab-scale biomass production process that would enable biochemical studies of the organism and help elucidate the archaeal ammonia oxidation.

Thermophilic AOA

In geothermal environments NH_3 oxidation appears to be solely performed by thermophilic AOA of the family “Ca. Nitrosocaldaceae”, which were detected in environmental studies using molecular marker genes (Reigstad, Richter et al. 2008, Zhang, Ye et al. 2008, Cole, Peacock et al. 2013). *In situ* nitrification activities were measured up to 84 °C by using isotopic techniques in Iceland

(Reigstad, Richter et al. 2008), Yellowstone National Park (Dodsworth, Hungate et al. 2011), a Japanese geothermal water stream (Nishizawa, Sakai et al. 2016), and in enrichment cultures from Tengchong Geothermal Field in China (Li, Yang et al. 2015). Recent metagenomic surveys have led to a number of metagenome-assembled genomes (MAGs), the candidate genus *Ca.* Nitrosothermus with the *Candidatus* species *Ca*. N. koenneckii and two candidate species in the *Nitrosocaldus* genus, namely, *Ca.* Nitrosocaldus schleperae and N. tengchongensis (Luo, Narsing Rao et al. 2020). Cultivation efforts have yielded three enrichment cultures, namely *Ca.* Nitrosocaldus yellowstonensis HL72 (De La Torre, Walker et al. 2008), *Ca.* Nitrosocaldus islandicus 3F (Daebeler, Herbold et al. 2018) and *Nitrosocaldus cavascurensis* SCU2 (Abby, Melcher et al. 2018) of which the enrichment process up to an axenic culture and further physiological characterization is part of this thesis (Abby, Melcher et al. 2018) (Melcher *et al*., in preparation).

Similar to *Ca*. N. islandicus, *N. cavascurensis* also possesses a complete set of genes that should enable it to perform anaerobic fermentation of aromatic amino acids (Abby, Melcher et al. 2018, Daebeler, Herbold et al. 2018) as well as hydrogenotrophic sulfur reduction, due to full sets of genes encoding for the four subunits (and maturation factors) of a soluble 3b [NiFe]-hydrogenase. This oxygen tolerant and bidirectional group of hydrogenases is typically found among thermophilic archaea and can couple the oxidation of H_2 to the reduction of NAD(P), while some have been proposed to have sulfhydrogenase activity (Kanai, Matsuoka et al. 2011, Peters, Schut et al. 2015, Greening, Biswas et al. 2016). Recently *Conexivisphaera calidus* the first member of the former phylum Thaumarchaeota that does not perform ammonia oxidation was isolated and characterized as a thermoacidophilic, sulfur- and iron-reducing organoheterotroph (Kato, Itoh et al. 2019).

Based on phylogenetic studies of 16S rRNA, ribosomal proteins and amoA a thermophilic origin of AOA was suggested (De La Torre, Walker et al. 2008, Abby, Melcher et al. 2018, Alves, Minh et al. 2018, Abby, Kerou et al. 2020). The thermophilic clade Nitrosocaldaceae forms a sister clade to all moderate AOA and can therefore serve as an outgroup to test hypotheses. One particular intriguing example is the absence of a proposed nirK gene in all members of the family *Ca.* Nitrosocaldaceae, except the recently proposed candidate genus *Ca.* Nitrosothermus (Luo, Narsing Rao et al. 2020). In all moderate AOA, except for the MAG of the sponge symbiont *Ca.* Cenarchaeum symbiosum (Bartossek, Nicol et al. 2010), this gene, encoding nitrite reductase is present and also highly expressed based on environmental and pure culture studies (Hollibaugh, Gifford et al. 2011, Shi, Tyson et al. 2011, Lund, Smith et al. 2012, Kerou, Offre et al. 2016) and a pivotal role of the enzyme for the archaeal ammonia oxidation is hypothesized (see above and Kozlowski *et al.*, 2016) (Kozlowski, Stieglmeier et al. 2016).

The gap between genomics and cultivation-based insights

The aim of this thesis was to to improve and develop enrichment and cultivation techniques for novel extremophilic and terrestrial ammonia oxidizing archaea, to allow characterization and comparison of their ecophysiology and to produce biomass for biochemical investigations. The need for reducing the gap between genomics and cultivation based insights is particularly prevalent with archaea, as many of them are not yet cultivated or are very tedious to grow. Thus, hypotheses about the metabolisms of archaea are often formed solely based on genomic information but experimental verification remains absent. Even in well curated genomes the genomic “dark matter” (un- or falsely annotated genes) can only be reduced to about 20 %, but typically is around 30 to 80 % (Makarova, Wolf et al. 2019) and thus genomic predictions are prone to error. AOA provide a

good case to tackle this problem, as some representatives are already cultivated, but many questions, even about their central metabolisms are still not resolved. In addition, AOA are also ecologically highly relevant, because they are among the most abundant microorganisms on this planet and have a critical role in the N-cycle, the most anthropogenically disturbed biogeochemical cycle of the planet (Steffen, Richardson et al. 2015).

Major Goals and Contributions

A major goal of this thesis was the enrichment, purification and physiological characterization of the extremely termophilic AOA *Nitrosocaldus cavascurensis*. With the isolation of *N. cavascurensis* hypotheses regarding the importance of NO and nitrite reductase (NirK) in the archaeal ammonia oxidation could be tested with incubation experiments using NO-scavengers with and without NO-donor, as well as nitrite reductase assays with crude cell extracts. Proposed alternative energy metabolisms like the fermentation of aromatic amino acids were tested in batch cultures with different media setups.

To tackle the problem of limited AOA biomass which hampers biochemical research of these microorganisms, the second goal of this thesis was to establish a lab-scale biomass production process for *N. viennensis*. Even though still limited in its scale it enabled biochemical research of the archaeal AMO complex deciphering its structure (Hodgskiss, Melcher et al. 2022). Unexpectedly, *N. viennensis* also revealed a unique growth behavior in continuous culture that might be connected to earlier observations of AOA in WWTP (Mußmann, Brito et al. 2011).

Below are more detailed descriptions of goals and own contributions on the three manuscripts of this cumulative thesis.

Chapter II

***Candidatus* Nitrosocaldus cavascurensis, an Ammonia Oxidizing, Extremely Thermophilic Archaeon with a Highly Mobile Genome.**

Sophie S. Abby[#], Michael Melcher[#], Melina Kerou, Mart Krupovic, Michaela Stieglmeier, Claudia Rossel, Kevin Pfeifer and Christa Schleper*

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Major Goals

The goal of this study was to enrich an extremely thermophilic AOA of the *Nitrosocaldus* clade to study its physiology and phylogenetic placement based on ribosomal proteins. Metabolic and genomic features and adaptations were reconstructed from its genome. The enrichment process included sampling and establishing ammonia oxidizing cultures, verification of AOA abundance by PCR of the 16S rRNA and thermophilic archaeal amoA gene, for which specific primers had to be developed. The composition of the microbial community was detected by 16S rRNA amplicon sequencing of V2/V4 region with general prokaryotic primers to get an idea about the bacterial

contaminants. Fluorescence *in situ* hybridization (FISH) was used to determine the morphology of *Nitrosocaldus cavascurensis*, which was then further used to select cultures with high AOA abundance for propagation based on cell counts with phase contrast microscopy. As part of the enrichment process and physiological characterization the growth temperature was optimized. The specific growth rate was further increased by avoiding the cultures to enter stationary phase, thereby decreasing the batch cultivation time from about 10-14 days to 4 days. The microbial diversity was also reduced to an almost binary system because slower growing organisms could not keep up and vitamins were omitted from the medium to minimize heterotrophic substrates. Antibiotic treatments as well as filtration either enhanced bacterial growth or sterilized the cultures, which was interpreted as a possible dependency of *N. cavascurensis* on the main remaining contaminant from the Thermus genus which contained a NirK gene based on metagenome binning. Cell number and relative abundance of a batch culture were determined by qPCR of the bacterial and archaeal 16S rRNA gene, Urease activity was confirmed by growth in urea containing medium and sensitivity to the NO-scavenger PTIO was demonstrated by inhibition of NO_2^- production.

Contributions

I was responsible for the enrichment process after the initial culture setup and preliminary growth temperature determination. This included the regular culture maintenance, further optimization of growth temperature, volume of inoculum, passaging rhythm and omitting vitamins from the medium which resulted in an increased growth rate, reduction of microbial diversity and a relative abundance of up to 92 % based on microscopic cell counts. I also performed the FISH labeling to determine the morphology and relative abundance which was further used to select cultures with high AOA abundance for propagation. Data processing and analysis of amplicon sequencing data of 16S rRNA gene from primers for the V2/V4 region to investigate the microbial diversity and relative abundance were also done by me as well as qPCR of 16S rRNA gene for absolute and relative abundance. I applied different antibiotics and filtration but without succeeding in further enriching or purifying the AOA. The PTIO-experiment and urea growth experiment were also done by me. I further wrote the parts of the manuscript regarding cultivation and physiology.

Chapter III

***Nitrosocaldus cavascurensis* gen. nov., sp. nov., the first pure culture of a thermophilic ammonia-oxidizing archaeon**

Michael Melcher, Kevin Pfeifer, Christa Schleper and Melina Kerou*

*corresponding author

Status: Manuscript Draft

Major Goals

This study describes the process from enrichment to an axenic culture for *N. cavascurensis*, its physiology and investigation of potential alternative energy metabolisms as well as nitrite reductase activity. As part of the physiological characterization the pH optimum was determined in

bioreactors, which resulted in a strong increase in relative abundance from 60 % to over 90 %. As no other buffer system allowed AOA to grow, a CO_3^{2-} buffer system was established and optimized to ensure stable growth conditions in batch cultures. Together with treatments of antibiotics with reduced concentrations a pure culture was established. Effect of NH_4^+, NO_2^- and O_2 concentrations on growth as well as optimal pH were determined. α-keto acids were tested for their growth enhancing effect due to ROS-scavenging, but surprisingly only minor benefits for the organism were observed. On the other hand a positive effect of pyruvate on the reduction of the lag phase after cryo preservation of cultures grown with pyruvate or addition of pyruvate to the culture before freezing were observed. Growth curves with NO_2^-, NH_4^+ and cell number based on qPCR of 16S rRNA gene were done which showed an increase in the specific growth rate and cell number compared to enrichments. Aromatic amino acid fermentation and hydrogenase activity were tested with various media compositions but no growth or growth enhancing effect was observed. The effect of NO-scavengers on the growth was reinvestigated by the application of PTIO and 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) in different concentrations with and without the NO-donor Spermine NONOate which should neutralize the negative effect on growth, however no difference was observed. NO_2^- reductase activity was tested with crude cell extracts of *N. cavascurensis*, *N. viennensis* and Thermus sp. as control, but was only observed for the *N. viennensis* and Thermus sp. extracts. Therefore the role of NO and NirK for the archaeal ammonia oxidation remains still ambiguous.

Contributions

My responsibilities in this study were the establishment of an axenic culture and physiological characterization. The enrichment process proceeded with the determination of optimal pH in bioreactors, testing of various buffer systems, optimizing CO_3^{2-} buffer and application of antibiotics in low concentrations which resulted in the first pure culture of an extremely thermophilic AOA. I verified the purity of the culture by PCR of bacterial 16S rRNA gene and addition of pyruvate to cultures without antibiotics. I did all the physiological characterization of the pure culture including optimal pH, NH_4^+, NO_2^- and O_2 concentrations, tested the effect of α-keto acids on the growth rate and established cryo stocks. A growth curve with NH_4^+, NO_2^- and cell number determined by qPCR was also done by me, as well as growth experiments for aromatic amino acid fermentation and hydrogenase activity. PTIO and cPTIO experiments with and without the NO-donor Spermine NONOate were also done by me, as well as the enrichment and cultivation of Thermus sp. under NO_2^- reducing conditions to produce biomass for the NO_2^- reductase assays of which I performed preliminar exeriments. I made major contributions to the manuscript for all parts regarding the cultivation and physiology of the strain.

Chapter IV

Analysis of biomass productivity and physiology of *Nitrososphaera viennensis* grown in continuous culture

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Major Goals

The aim of this study was to develop a biomass production process by investigating the growth behavior of *N. viennensis* in continuous culture. The produced biomass would enable biochemical studies of the organism to further elucidate the energy metabolism of AOA. Growth conditions were optimized to ensure that only NH_3 acts as a limiting substrate while general process parameters were characterized for the bioprocess development: such as K_S, biomass to substrate yield ($Y_{(X/NH3)}$) and the critical dilution rate (D_{crit}) at which the organism will be washed out. To further increase the biomass productivity per reactor volume a continuous culture at a higher substrate concentration was also established.

Contributions

I wrote almost the entire manuscript and was responsible for all experimental design and nearly all laboratory work. This included setting up the bioreactors and maintaining the continuous cultures over months, with daily sampling, determining NH_4^+ and NO_2^- concentrations, measuring and adjusting the pump rates, preparing and exchanging feed medium and phase contrast microscopy to check for contamination. DNA of selected samples was extracted, measured by Qbit and qPCR was performed to determine the cell number. Analysis and interpretation of cultivation relevant parameters was also done by me as well as parts of the biofilm analysis.

Futher publications on the topic, but not included in this thesis:

Proteomics and comparative genomics of *Nitrososphaera viennensis* reveal the core genome and adaptations of archaeal ammonia oxidizers

Melina Kerou, Pierre Offre, Luis Valledor, Sophie S. Abby, **Michael Melcher,** Matthias Nagler, Wolfram Weckwerth and Christa Schleper (2016), PNAS 113 (49) E7937-E7946

Nitrogen Isotope Fractionation During Archaeal Ammonia Oxidation: Coupled Estimates From Measurements of Residual Ammonium and Accumulated Nitrite.

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Unexpected Complexity of the Ammonia Monooxygenase in Archaea

Logan H. Hodgskiss, **Michael Melcher**, Melina Kerou, Weiqiang Chen, Rafael I. Ponce-Toledo, Savvas N. Savvides, Stefanie Wienkoop, Markus Hartl, Christa Schleper, bioRxiv 2022.04.06.487334;

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References of chapter I and chapter V are combined at the end of this thesis.

Chapter II

Candidatus Nitrosocaldus cavascurensis, an Ammonia Oxidizing, Extremely Thermophilic Archaeon with a Highly Mobile Genome

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Candidatus Nitrosocaldus cavascurensis, an Ammonia Oxidizing, Extremely Thermophilic Archaeon with a Highly Mobile Genome

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Ammonia oxidizing archaea (AOA) of the phylum Thaumarchaeota are widespread in moderate environments but their occurrence and activity has also been demonstrated in hot springs. Here we present the first enrichment of a thermophilic representative with a sequenced genome, which facilitates the search for adaptive strategies and for traits that shape the evolution of Thaumarchaeota. *Candidatus* Nitrosocaldus cavascurensis has been enriched from a hot spring in Ischia, Italy. It grows optimally at 68°C under chemolithoautotrophic conditions on ammonia or urea converting ammonia stoichiometrically into nitrite with a generation time of approximately 23 h. Phylogenetic analyses based on ribosomal proteins place the organism as a sister group to all known mesophilic AOA. The 1.58 Mb genome of *Ca.* N. cavascurensis harbors an *amo*AXCB gene cluster encoding ammonia monooxygenase and genes for a 3-hydroxypropionate/4-hydroxybutyrate pathway for autotrophic carbon fixation, but also genes that indicate potential alternative energy metabolisms. Although a *bona fide* gene for nitrite reductase is missing, the organism is sensitive to NO-scavenging, underlining the potential importance of this compound for AOA metabolism. *Ca.* N. cavascurensis is distinct from all other AOA in its gene repertoire for replication, cell division and repair. Its genome has an impressive array of mobile genetic elements and other recently acquired gene sets, including conjugative systems, a provirus, transposons and cell appendages. Some of these elements indicate recent exchange with the environment, whereas others seem to have been domesticated and might convey crucial metabolic traits.

Keywords: ammonia oxidation, archaea, Thaumarchaeota, nitrification, mobile genetic elements, thermophilic adaptations, *amo*A gene, DNA repair enzymes

INTRODUCTION

Ammonia oxidizing archaea (AOA) now collectively classified as *Nitrososphaeria* within the phylum Thaumarchaeota (Brochier-Armanet et al., 2008; Spang et al., 2010; Stieglmeier et al., 2014; Kerou et al., 2016a) represent the sole archaeal group that is globally distributed in oxic environments efficiently competing with aerobic bacteria. Because of their large numbers in the

ocean plankton, in marine sediments, in lakes and in soils, AOA are considered one of the most abundant groups of prokaryotes on this planet (Schleper et al., 2005; Prosser and Nicol, 2008; Erguder et al., 2009; Schleper and Nicol, 2010; Pester et al., 2011; Hatzenpichler, 2012; Stahl and de la Torre, 2012; Offre et al., 2013). All currently cultivated AOA strains gain energy exclusively through the oxidation of ammonia to nitrite, i.e., they perform the first step in nitrification. They grow chemolithoautotrophically from inorganic carbon supply. Some strains show growth only in the presence of small organic acids (Tourna et al., 2011; Qin et al., 2014) which seem to catalyze degradation of reactive oxygen species (ROS) from the medium (Kim et al., 2016). Different from their bacterial ammonia oxidizing counterparts, AOA are often adapted to rather low levels of ammonia for growth, which seems to favor their activities in oligotrophic environments, such as the marine pelagic ocean and marine sediments, but also in acidic environments, where the concentration of ammonia decreases in favor of ammonium (Nicol et al., 2008). However, AOA occur also in large numbers in terrestrial environments, including fertilized soils and some waste water treatment plants, and several studies indicate alternative energy metabolisms (Mussmann et al., 2011; Alves et al., 2013). Although ammonia oxidation in archaea has not been biochemically resolved, the presence of genes for an ammonia monooxygenase in all AOA with remote similarity to methane and ammonia monooxygenases of bacteria implies involvement of this complex in the process (Konneke et al., 2005; Treusch et al., 2005; Nicol and Schleper, 2006). Hydroxylamine has been suggested to be the first product of ammonia oxidation (Vajrala et al., 2013), but further conversion to nitrite is performed in an unknown process, as no homolog of the bacterial hydroxylamine dehydrogenase has been found in the genomes of AOA. However, nitric oxide (NO) has been suggested to be involved in the process, because NO production and re-consumption have been observed (Martens-Habbena et al., 2015; Kozlowski et al., 2016b) and the NO scavenger PTIO was shown to inhibit AOA at very low concentrations (Yan et al., 2012; Shen et al., 2013; Martens-Habbena et al., 2015).

Ammonia oxidation by AOA has also been documented to occur in hot springs. With the help of molecular marker genes, diverse ammonia oxidizing thaumarchaea were found in hot terrestrial and marine environments (Reigstad et al., 2008; Zhang et al., 2008; Jiang et al., 2010; Cole et al., 2013). Furthermore, *in situ* nitrification activities up to 84°C were measured using isotopic techniques in AOA-containing habitats in Iceland (Reigstad et al., 2008), in Yellowstone National park (Dodsworth et al., 2011), in a Japanese geothermal water stream (Nishizawa et al., 2016), and in enrichment cultures from Tengchong Geothermal Field in China (Li et al., 2015). In addition, an enrichment of an ammonia oxidizing thaumarchaeon, *Ca.* Nitrosocaldus yellowstonensis, from a hot spring in Yellowstone National park grew at temperatures of up to 74°C confirming that archaeal ammonia oxidation, different from bacterial ammonia oxidation, indeed occurs at high temperatures (de la Torre et al., 2008).

A remarkable diversity of archaea with different metabolisms has been described from terrestrial and marine hot springs and hyperthermophilic organisms tend to be found at the base of almost all lineages of archaea. Therefore, it has often been proposed that the ancestor of all archaea and the ancestors of mesophilic lineages of archaea were hyperthermophilic or at least thermophiles (Barns et al., 1996; Lopez-Garcia et al., 2004; Gribaldo and Brochier-Armanet, 2006; Brochier-Armanet et al., 2012; Eme et al., 2013). This has been supported by sequence analyses which suggested a parallel adaptation from hot to moderate temperatures in several lineages of archaea (Groussin and Gouy, 2011; Williams et al., 2017). Indeed, the thermophilic strain *Ca.* Nitrosocaldus yellowstonensis emerged in 16S rRNA phylogeny as a sister group of all known mesophilic AOA (de la Torre et al., 2008) indicating that AOA also evolved in hot environments.

However, until now, no genome of an obligate thermophilic AOA was available to analyze in depth the phylogenetic placement of thermophilic AOA and to investigate adaptive features of these ammonia oxidizers that have a pivotal position in understanding the evolution of Thaumarchaeota.

In this work we present the physiology and first genome of an extremely thermophilic AOA of the *Nitrosocaldus* lineage that we cultivated from a hot spring in Southern Italy. We analyze its phylogeny based on ribosomal proteins and reconstruct metabolic and genomic features and adaptations.

MATERIALS AND METHODS

Sampling and Enrichment

About 500 mL of mud were sampled from a terrestrial hot spring on the Italian island Ischia at "Terme di Cavascura" and stored at 4°C until it arrived at the laboratory in Vienna (after about one week, October 2013). A temperature of 77°C and pH 7–8 were measured *in situ* using a portable thermometer (HANNA HI935005) and pH stripes. Initial enrichments (20 mL final volume) were set up in 120 mL serum flasks (two times autoclaved with MilliQ water) containing 14 mL of autoclaved freshwater medium (FWM) (de la Torre et al., 2008; Tourna et al., 2011) amended with non-chelated trace element solution (MTE) (Konneke et al., 2005), vitamin solution, FeNaEDTA (20 μL each), 2 mM bicarbonate, 1 mM ammonium chloride and 2 mL of 0.2 μm filtrated hot spring water. Serum flasks were inoculated with 4 mL (20%) of sampled mud, sealed with gray rubber stoppers (6x boiled and autoclaved) and incubated aerobically at 78°C while rotating at 100 rpm.

Enrichment Strategies

The temperature was changed after one week to 70°C and medium amendment with filtrated hot spring water was discontinued after four transfers as there was no effect observed. Growth was monitored by microscopy, nitrite production and ammonia consumption. Cultures were transferred at a nitrite concentration of ∼700 μM and in case ammonia was depleted before the desired nitrite concentration was reached, cultures were fed with 1 mM ammonium chloride. For a preliminary

growth temperature test, cultures were incubated at 55, 60, 65, 70, 72, 74, 76, and 80°C, with cultures at 70°C showing the highest nitrite production rate. The antibiotics Sulfamethazine, Rifampicin and Novobiocin (100 μg mL^{-1}) were used alone and in combination with pyruvate, glyoxylate and oxalacetate (0.1 mM), but with little effect on enrichment. The use of the mentioned organic acids or N,N′-Dimethylthiourea (DMTU, all 0.1 mM) as ROS scavengers increased the abundance of heterotrophic bacteria and even reduced nitrite production. Filtration of enrichments with 1.2 μm filters had no or even detrimental effect on AOA abundance when done repeatedly and 0.45 μm filtration sterilized the cultures.

As nitrite production rate increased over time, inoculum size was decreased from 20 to 10% and finally to 5%. Omitting vitamin solution from the medium led to an increase in nitrite production rate and AOA abundance. Most crucial for increasing thaumarchaeal abundance was keeping an exact timing on passaging cultures in late exponential phase (after 4 days) and setting up multiple cultures from which the best (based on microscopic observations) was used for further propagation. Based on cell counts maximal enrichments of up to 92% were achieved (see Results for more details).

Cultivation

Cultures are routinely grown at 68°C using 5% inoculum in 20 mL FWM amended with MTE and FeNaEDTA solutions, 1 mM NH_4Cl, 2 mM $NaHCO_3$ and are transferred every 4 days once they reach about 700 μM NO_2^-. 1 mM NH_4Cl was substituted by 0.5 mM urea to confirm urease activity, while the rest of the medium components and conditions remained unchanged.

Inhibition Test with 2-phenyl-4, 4, 5, 5,-tetramethylimidazoline-1-oxyl 3-oxide (PTIO)

Cultures were grown in 20 mL FWM with 5% inoculum under standard conditions and different amounts of an aqueous PTIO stock solution were added when early to mid-exponential growth phase was reached (ca. 63 h after inoculation; 20, 100, and 400 μM final PTIO concentration). Ammonia oxidation ceased immediately at all applied PTIO concentrations, but cultures with 20 μM PTIO were able to resume nitrite production 72 h after the addition of PTIO.

DNA Extraction

DNA was extracted from cell pellets by bead beating in pre-warmed (65°C) extraction buffer [40.9 g L^{-1} NaCl, 12.6 g L^{-1} Na_2SO_3, 0.1 M Tris/HCl, 50 mM EDTA, 1% sodium dodecyl sulfate (SDS)] and phenol/chloroform/isoamylalcohol [25:24:1 (v/v/v), Fisher BioReagents], in a FastPrep-24 (MP) for 30 s. After centrifugation (10 min, 4°C, 16000 *g*) the aqueous phase was extracted with chloroform/isoamylalcohol [24:1 (v/v)], prior to DNA precipitation with 1 μL of glycogen (20 mg mL^{-1}) and 1 mL of PEG-solution (93.5 g L^{-1} NaCl, 300 g L^{-1} polyethylene glycol 6000) overnight at 4°C. Nucleic acid pellets were washed, dried, resuspended in nuclease-free water and stored at −20°C until further use (adapted from Zhou et al., 1996; Griffiths et al., 2000).

Amplification of *amoA* Gene

Specific primers were designed for the amplification of the *amo*A gene from *Candidatus* N. cavascurensis: ThAOA-277F: CCA TAY GAC TTC ATG ATA GTC and ThAOA-632R: GCA GCC CAT CKR TAN GTC CA (Alves and Schleper, unpublished). They were based on an alignment of *amo*A gene sequences from available genomes, all PCR-amplified sequences related to Nitrosocaldus identified in GenBank through BLAST searches, and several reference sequences from the orders *Nitrososphaerales, Nitrosopumilales,* and *Nitrosotaleales*. ThAOA-277F targets a gene region conserved only among *Ca.* Nitrosocaldus spp., and primer ThAOA-632R was a modification of CrenamoA616r(48x) (Nicol et al., 2008) to match the *amo*A of *Nitrosocaldus* spp.; ThAOA-632R contains additional degenerated bases that account for the most common substitutions among all *amo*A genes, since only two sequences containing this region were available for *Ca.* Nitrosocaldus spp.

PCR conditions were 95°C for 10 min as initialization, followed by 35 cycles of 30 s denaturing at 95°C, 45 s primer annealing at 55°C, and elongation at 72°C for 45 s, finishing with 10 min final elongation at 72°C.

Quantitative PCR

Archaeal and bacterial 16S rRNA genes were quantified in triplicate 20 μL reactions containing 10 μL GoTaq® qPCR Master Mix 2x (Promega), 0.2 mg mL^{-1} BSA and 1 μM of the appropriate primers : Arch931F [5′-AGG AAT TGG CGG GGG AGC A-3′ (Jackson et al., 2001)] and Arch1100R [5′-BGG GTC TCG CTC GTT RCC-3′ (Ovreas et al., 1997)] for the archaeal 16S rRNA gene, P1 (5′-CCT ACG GGA GGC AGC AG-3′) and P2 (5′-ATT ACC GCG GCT GCT GG-3′) (Muyzer et al., 1993) for the bacterial 16S rRNA gene. Reactions were performed in a realplex^2 (Mastercycler ep gradient S, Eppendorf AG) with the following cycling conditions: 95°C for 2 min, followed by 40 cycles of 30 s denaturing at 95°C, 30 s joint annealing-extension at 60°C, and extension with fluorescence measurement at 60°C for 30 s. Specificity of qPCR products was confirmed by melting curve analysis. Standards were prepared by cloning 16S rRNA genes from *Ca.* N. cavascurensis and *Escherichia coli* into pGEM®-T Easy Vectors (Promega). These were amplified using M13 primers and concentration was determined with Qubit™ dsDNA BR Assay Kit (Thermo Fisher Scientific) before preparing serial dilutions. The efficiencies of archaeal and bacterial 16S rRNA standards were 95 and 99%, respectively.

Fluorescence *in Situ* Hybridization

2 mL samples were centrifuged at 4°C, 16000 *g* for 40 min, washed in PBS-buffer and fixed with 4% paraformaldehyde for 3 h using standard protocols (Amann et al., 1990). Cells were washed two times with 1 mL PBS and finally resuspended in 200 μL of 1:1 PBS:ethanol mix before storing them at −20°C. After dehydration in ethanol cells were hybridized overnight in hybridization buffer with 20% formamide using probes Eub338 (Fluos) 5′-GCT GCC TCC CGT AGG AGT-3′ (Amann et al.,

1990) and Arch915 (dCy3) 5′-GTG CTC CCC CGC CAA TTC CT-3′ (Stahl and Amann, 1991).

Scanning Electron Microscopy

For scanning electron microscopy, late exponential cells were harvested from 40 mL of culture by centrifugation (16000 *g*, 4°C, 30 min). The cells were resuspended and washed three times (0.02 mM sodium cacodylate) prior to prefixation (0.5% glutaraldehyde in 0.02 mM sodium cacodylate) overnight, after which the concentration of glutaraldehyde was increased to 2.5% for 2 h. Fixed cells were spotted onto 0.01%-poly-L-lysine coated glass slides (5 mm) and allowed to sediment for 15 min. Samples were dehydrated using an ethanol (30–100%, 15 min each) and acetone (2 × 100%) graduated series and dried using HMDS (2 × 50% HMDS in acetone, 2 × 100%). The slides were subsequently placed on conductive stubs, gold coated for 30 s (JEOL JFC-2300HR) and analyzed (JEOL JSM-IT200).

Genome Assembly

DNA was prepared from 480 mL of culture using standard procedures and sequenced using a PacBio Sequel sequencer at the VBCF (Vienna BioCenter Core Facilities GmbH) with a SMRT Cell 1Mv2 and Sequel Sequencing Kit 2.1. Insert size was 10 kb. Around 500000 reads were obtained with an average size of ∼5 kb (N50: 6866 nt, maximal read length: 84504 nt).

The obtained PacBio reads were assembled using the CANU program (version 1.4, parameters "genomeSize = 20 m, corMhapSensitivity = normal, corOutCoverage = 1000, errorRate = 0.013") (Koren et al., 2017), and then "polished" with the arrow program from the SMRT analysis software (Pacific Biosciences, United States). The *Ca.* N. cavascurensis genome consisted of two contiguous, overlapping contigs of 1580543 kb and 15533 kb, respectively. The resulting assembly was compared to a previous version of the genome bin we had obtained by using IDBA-UD and Newbler for assembly, and differential coverage binning on ∼150 nt reads from five runs of IonTorrent sequencing (use of GC% and reads coverage from IonTorrent PGM reads) (Albertsen et al., 2013; Nurk et al., 2013). The new assembly confirmed the previous version as all nine contigs from the previous aligned with MUMMER to the largest of the two contigs, except for a ca. 21 kb region we had not manually selected using differential coverage binning because of a variable read coverage between runs. Interestingly, this region belongs to an integrated conjugative element (ICE1, see results) that might perhaps be excised occasionally. A repeated region between both extremities of the longest contig, and the 2nd small contig obtained was found, and analyzed using the nucmer program from the MUMMER package (see Supplementary Figure 1) (Kurtz et al., 2004). This region was merged using nucmer results, and the longest contig obtained was "circularized" using the information of the nucmer program, as sequence information from the 2nd contig was nearly identical (>99.5%) to that of the extremities of the long contig. It coincided with a repeat-rich adhesin (Supplementary Figure 1). The origin of replication was predicted using the Ori-Finder 2 webserver (Luo et al., 2014). By analogy with *Nitrosopumilus maritimus*, it was placed after the last annotated genomic object, before the ORB repeats and the *cdc* gene. The annotated genome sequence has been deposited to the European Nucleotide Archive (ENA) with the study accession number: PRJEB24312.

Genome Dataset

Thaumarchaeota and Aigarchaeota complete or nearly complete genomes were downloaded from the NCBI, Genbank database, or IMG database when not available in Genbank. We included the genomes of 27 Thaumarchaeota: Thaumarchaeota archaeon BS4 (IMG_2519899514); Thaumarchaeota group 1.1c (bin Fn1) (IMG_2558309099); Thaumarchaeota NESA-775 (Offre and Schleper, unpublished); Thaumarchaeota archaeon DS1 (IMG_2263082000); *Cenarchaeum symbiosum* A (DP000238); Thaumarchaeota archaeon CSP1 (LDXL00000000); *Nitrosopumilus adriaticus* NF5 (CP011070); *Nitrosocosmicus arcticus* Kfb (Alves and Schleper unpublished); *Nitrosopelagicus brevis* CN25 (CP007026); *Nitrosotenuis chungbukensis* MY2 (AVSQ00000000); *Nitrosotalea devanaterra* Nd1 (LN890280); *Nitrososphaera evergladensis* SR1 (CP007174); *Nitrosocosmicus exaquare* G61 (CP017922); *Nitrososphaera gargensis* Ga9.2 (CP002408); *Nitrosoarchaeum koreensis* MY1 (GCF_000220175); *Nitrosopumilus salaria* BD31 (GCF_000242875); *Nitrosopumilus koreensis* AR1 (CP003842); *Nitrosoarchaeum limnia* BG20 (GCF_000241145); *Nitrosoarchaeum limnia* SFB1 (CM001158); *Nitrosopumilus maritimus* SCM1 (CP000866); *Nitrosocosmicus oleophilus* MY3 (CP012850); *Nitrosopumilus piranensis* D3C (CP010868); *Nitrosopumilus sediminis* AR2 (CP003843); *Nitrosotenuis uzonensis* N4 (GCF_000723185); *Nitrososphaera viennensis* EN76 (CP007536); *Nitrosotenuis cloacae* SAT1 (CP011097); *Nitrosocaldus cavascurensis* SCU2 (this paper).

Two Aigarchaeota genomes were included: *Calditenuis aerorheumensis* (IMG_2545555825) and *Caldiarchaeum subterraneum* (BA000048). Additionally, we selected 11 Crenarchaeota genomes from Genbank to serve as an outgroup in our analysis: *Sulfolobus solfataricus* P2 (AE006641); *Pyrobaculum aerophilum* IM2 (AE009441); *Hyperthermus butylicus* DSM 5456 (CP000493); *Thermofilum pendens* Hrk 5 (CP000505); *Acidilobus saccharovorans* 345-15 (CP001742); *Desulfurococcus fermentans* DSM 16532 (CP003321); *Caldisphaera lagunensis* DSM 15908 (CP003378); *Fervidicoccus fontis* Kam940 (CP003423); *Metallosphaera sedula* DSM 5348 (CP000682); *Aeropyrum pernix* K1 (BA000002) and *Thermoproteus tenax* Kra 1 (FN869859).

Genome Annotation

The first annotation of the *Ca.* N. cavascurensis genome was obtained by the automatic annotation pipeline MicroScope (Vallenet et al., 2009, 2017; Medigue et al., 2017). Annotations from *Nitrososphaera viennensis* EN76 were carried over when aligned sequences were 35% identical (or 30% in syntenic regions) and covered 70% of the sequence. All subsequent manual annotations were held on the platform. Putative transporters were classified by screening against the Transporter Classification Database (Saier et al., 2014). To assist the annotation process, several sets of proteins of interest from Kerou et al. (2016b) (EPS, metabolism), Offre et al. (2014) (transporters), Makarova et al. (2016) (archaeal pili and flagella) and ribosomal proteins were screened specifically within our set of genomes using HMMER

and HMM protein profiles obtained from databases (TIGRFAM release 15, PFAM release 28, SUPERFAMILY version 1.75), or built from arCOG families (Gough et al., 2001; Haft et al., 2003; Finn et al., 2014; Makarova et al., 2015). In the former case, the trusted cutoff "—cut_tc" was used if defined in the profiles. In the latter case, for each family of interest, sequences were extracted from the arCOG2014 database, aligned using MAFFT ("linsi" algorithm) (Katoh and Standley, 2014), and automatically trimmed at both extremities of conserved sequence blocks using a home-made script relying on blocks built by BMGE v1.12 (BLOSUM 40) (Criscuolo and Gribaldo, 2010). HMMER was then used to screen genomes for different sets of specialized families (Eddy, 2011).

In order to visualize and compare the genetic organization of sets of genes (AMO, Urease – Ure, type IV pili), we built HMM profiles (as above except alignments were manually curated for AMO and Ure), based on the corresponding arCOG families and integrated the respective sets of profiles in the MacSyFinder framework that enables the detection of sets of co-localized genes in the genome (parameters inter_gene_max_space = 10, min_nb_mandatory_genes = 1 for AMO and Ure, inter_gene_max_space = 10, min_nb_mandatory_genes = 4 for type IV pili) (Abby et al., 2014). MacSyFinder was then run with the models and profiles on the genome dataset, and the resulting JSON files were used for visualization in the MacSyView web-browser application (Abby et al., 2014). The SVG files generated by MacSyView from the predicted operons were downloaded to create figures.

Reference Phylogeny

Ribosomal proteins were identified in genomes using a set of 73 HMM profiles built for 73 arCOG families of ribosomal proteins (see Genome Annotation). When multiple hits were obtained in a genome for a ribosomal protein, the best was selected. The sequences were extracted and each family was aligned using the MAFFT program ("linsi" algorithm) (Katoh and Standley, 2014). Alignments were curated with BMGE (version 1.12, default parameters and BLOSUM 45) (Criscuolo and Gribaldo, 2010) and then checked manually. In several cases, sequences identified as part of a given ribosomal protein family did not align well and were excluded from the analysis. In the end, 59 families with more than 35 genomes represented were selected to build a phylogeny. The corresponding family alignments were concatenated, and a tree was built using the IQ-Tree program version 1.5.5 (Nguyen et al., 2015) (model test, partitioned analysis with the best model selected per gene, 1000 UF-Boot and 1000 aLRT replicates). A tree drawing was obtained with the Scriptree program (Chevenet et al., 2010), and then modified with Inkscape.

Protein Families Reconstruction and Phyletic Patterns Analysis

Homologous protein families were built for the set of genomes selected. A blast similarity search was performed with all sequences against themselves, and the results used as input for the Silix and Hifix programs to cluster the sets of similar sequences into families (Miele et al., 2011, 2012). For sequences to be clustered in a same Silix family, they had to share at least 30% of identity and the blast alignment cover at least 70% of the two sequences lengths. The distribution of protein families of interest was analyzed across the genome dataset.

Phylogenetic Analyses

Sequences from Silix families of interest were extracted in fasta files, and a similarity search was performed against a large database of sequences which consisted of 5750 bacterial and archaeal genomes (NCBI Refseq, last accessed in November 2016), and the genomes from our genome dataset. Sequences with a hit having an *e*-value below 10^{-10} were extracted, and the dataset was then de-replicated to remove identical sequences. An alignment was then obtained for the family using MAFFT ("linsi" algorithm), filtered with BMGE (BLOSUM 30) and a phylogenetic tree was reconstructed by maximum-likelihood using the IQ-Tree program (version 1.5.5, best evolutionary model selected, 1000 UF-Boot and 1000 aLRT replicates). A tree drawing was obtained with the Scriptree program (Chevenet et al., 2010), and modified with Inkscape.

Identification and Annotation of Integrated MGE

Integrated mobile genetic elements (MGE) were identified as described previously (Kazlauskas et al., 2017). Briefly, the integrated MGE were identified by searching the *Ca.* N. cavascurensis genome for the presence of the hallmark genes specific to mobile genetic elements, such as integrases, casposases, VirB4 ATPases, large subunit of the terminase, major capsid proteins of achaeal viruses, etc. In the case of a positive hit, the corresponding genomic neighborhoods were analyzed further. IS elements were searched for using ISfinder (Siguier et al., 2006). The precise borders of integration were defined based on the presence of direct repeats corresponding to attachment sites. The repeats were searched for using Unipro UGENE (Okonechnikov et al., 2012). Genes of integrated MGE were annotated based on PSI-BLAST searches (Altschul et al., 1997) against the non-redundant protein database at NCBI and HHpred searches against CDD, Pfam, SCOPe and PDB databases (Soding, 2005).

RESULTS AND DISCUSSION

Enrichment of *Ca.* Nitrosocaldus cavascurensis

An ammonia oxidizing enrichment culture was obtained from thermal water outflow of the *Terme di Cavascura* on the Italian island Ischia. After repeated transfers into artificial freshwater medium supplemented with 1 mM ammonium and 2 mM bicarbonate, highly enriched cultures were obtained that exhibited almost stoichiometric conversion of ammonia into nitrite within 4–5 days (**Figure 1A**). A single phylotype of AOA but no AOB or Nitrospira/commamox was identified in the enrichment via amplification and sequencing of *amo*A and 16S rRNA gene fragments (data not shown) and the presence of a single AOA phylotype was confirmed by metagenomic

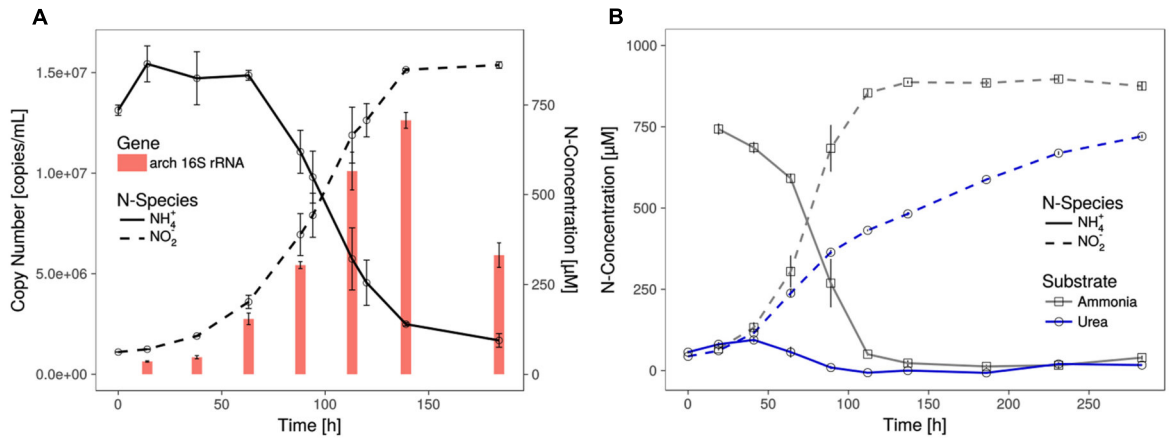

FIGURE 1 | Growth of *Ca.* N. cavascurensis. **(A)** Enrichment cultures with near stoichiometric conversion of ammonia to nitrite were paralleled by cell growth, as estimated by archaeal 16S rRNA gene copies in qPCR. Gene copies declined once ammonia oxidation ceased. Data points for ammonia and nitrite are averages of biological duplicates. Technical triplicates of each biological duplicate were assayed to determine the gene copy number. Error bars represent the standard error of the mean. **(B)** Ammonia and successive nitrite production of enrichment cultures with 0.5 mM urea as nitrogen source. On urea as substrate ammonia accumulated during the early growth phase and the cultures showed decreasing nitrite production rate indicating growth limitation. Ammonia cultures served as control and all data points show the mean of biological triplicates with error bars representing the standard error of the mean.

sequencing (see below). Its closest relative in the 16S rRNA database was fosmid 45H12 isolated from a gold mine metagenome with 99% identity and the next closest cultivated relative was *Ca.* Nitrosocaldus yellowstonensis with 96% identity (Nunoura et al., 2005; de la Torre et al., 2008). The AOA has been propagated for 4 years within a stable enrichment culture and its relative enrichment usually ranged between 75 and 90% based on qPCR or cell counts respectively. Different strategies were tested in order to obtain a better enrichment (heat treatment, antibiotics, dilution to extinction, filtration) which resulted in a maximal enrichment of 92% (based on cell counts) but not in a pure culture. In high throughput sequencing analyses using general prokaryotic primers to amplify the V2/V4 region of the 16S rRNA gene we identified phylotypes of the *Deinococcus/Thermus* group comprising up to 97% of the bacterial contaminating sequences (with 96% identity to sequences from the *Thermus* genus) and to minor extent sequences related to the Chloroflexi and Armatimonadetes (not shown). Although Aigarchaeota had been detected in earlier enrichments, the AOA was the only archaeon discovered in the high-level enrichments used in this study. *Ca.* N. cavascurensis does not encode a catalase, and in the light of current knowledge on the auxiliary role of ROS scavengers in the growth of AOA (Kim et al., 2016), it is possible that a ROS detoxification mechanism is provided by the remaining bacteria in the enrichment culture, although supplementation with α-keto acids did not have a positive effect as it had on other AOA enrichments (see Materials and Methods).

The shortest generation time of the thermophilic AOA was approximately 23 h (±1.1), as measured by nitrite production rates and in qPCR, which is comparable to the generation time of *Ca.* N. yellowstonensis (de la Torre et al., 2008). It grew in a temperature range from about 55 to 74°C with an apparent (but relatively wide) temperature optimum around 68°C (**Figure 2**).

In fluorescence *in situ* hybridizations (FISH) with archaea- and bacteria-specific probes, all coccoid-shaped cells of less than 1 μm in diameter were assigned to the AOA, while all shorter and longer rod-shaped morphotypes were clearly assigned to bacteria (**Figures 3A,B**). Scanning electron microscopy revealed the typical spherical and irregular shape of cocci, as seen, e.g., for the ammonia oxidizing archaeon *Nitrososphaera viennensis* (Tourna et al., 2011) or hyperthermophilic and halophilic coccoid archaea strains with a diameter of around 0.7 μm (**Figures 3C,D**). Based on its relationship with *Ca.* Nitrosocaldus yellowstonensis (de la Torre et al., 2008), and the location it was sampled from (*Terme di cavascura*, Italy) we named this organism provisionally *Candidatus* Nitrosocaldus cavascurensis.

Ca. Nitrosocaldus cavascurensis Represents a Deeply Branching Lineage of AOA

We gathered all complete or nearly complete 27 genome sequences available for Thaumarchaeota. These included 23 genomes of closely related cultivated or uncultivated AOA all harboring *amo* genes, and four genomes obtained from metagenomes that do not have genes for ammonia oxidation. Among the latter were two assembled genomes from moderate environments (Fn1 and NESA-775) and two from hot environments (BS4 and DS1) (Beam et al., 2014; Lin et al., 2015). In addition, two Aigarchaeota genomes, and a selection of representative genomes of Crenarchaeota (11) were included to serve as outgroups (Petitjean et al., 2014; Raymann et al., 2015; Adam et al., 2017; Williams et al., 2017). This resulted in a 40 genomes dataset (see Materials and Methods). A maximum likelihood analysis based on 59 concatenated ribosomal proteins (in one copy in at least 35 of the 40 genomes) resulted in a highly supported tree: UF-boot and aLRT supports were maximal or

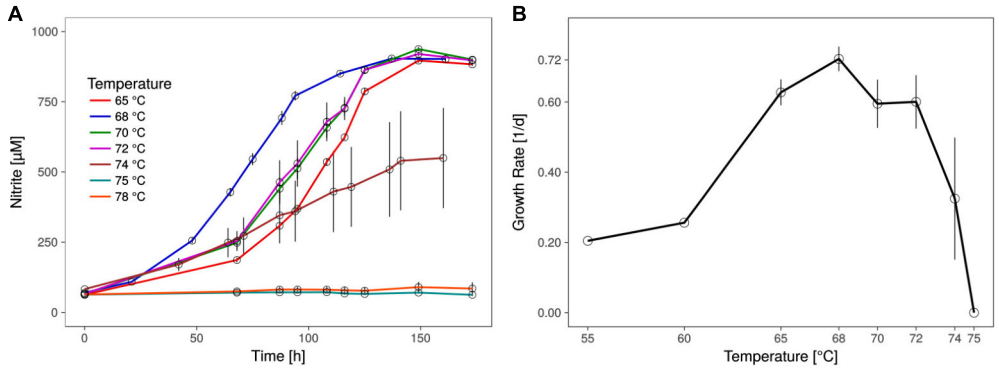

FIGURE 2 | Optimal growth temperature of *Ca.* N. cavascurensis. **(A)** Nitrite production occurs within the tested temperature range (65 – 78°C) up to 74°C, with an optimum at 68°C. Culture quadruplets were incubated for each tested temperature and the error bars represent the standard error of the mean which increases with temperature, indicating an increase of stochasticity within the microbial community. **(B)** Temperature dependance of growth rates showing a maximum of 0.72 ± 0.03 d^{-1} (23.0 ± 1.1 h generation time) at 68°C and the highest growth temperature at 74°C. Growth rates for 55 and 60°C derive from a preliminary temperature test with single cultures, while values for temperatures above 60°C show the mean of culture quadruplets and error bars represent the standard deviation of the mean. Calculations were done by linear regression of semi-logarithmically plotted nitrite values during the exponential growth phase (min. five different time points, neglecting starting value).

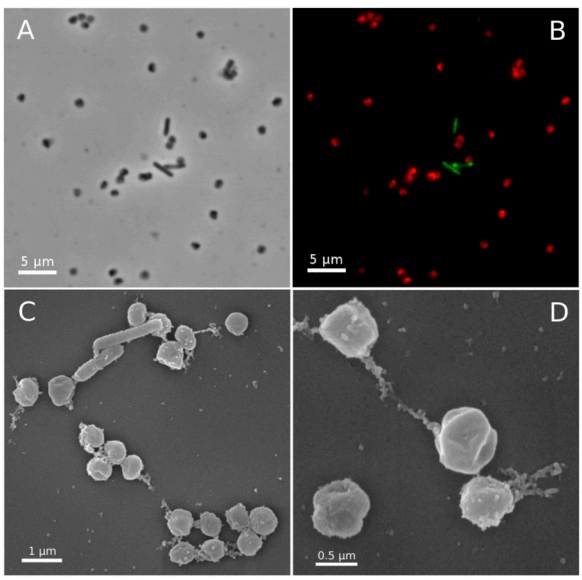

FIGURE 3 | Micrographs of *Ca.* N. cavascurensis. Light **(A)** and epifluorescence micrographs **(B)** of a late exponential enrichment culture analyzed with FISH. *Ca.* N. cavascurensis cells (red) and bacterial cells (green) were labeled with ARCH 915 and EUB 338 probes respectively, to give a representative picture of the enrichment state. Scanning electron micrographs **(C,D)** show the spherical nature of *Ca.* N. cavascurensis cells, having a diameter of 0.6 – 0.8 µm, small rods are visible that belong to the remaining bacterial consortium.

above 99, except for two nodes (>90) in the Thaumarchaeota subtree and one node (>70) in the Crenarchaeota subtree (**Figure 4**). The tree confirms the monophyly of Thaumarchaeota and Nitrososphaeria (AOA), and the deeply branching position of *Ca.* N. cavascurensis as a sister-group of all other (mesophilic) AOA, as initially indicated with a single gene (16S rRNA) phylogeny of *Ca.* Nitrosocaldus yellowstonensis (de la Torre et al., 2008).

Genome and Energy Metabolism of *Ca.* Nitrosocaldus cavascurensis

The genome of *Ca.* N. cavascurensis contains 1.58 Mbp, with 1748 predicted coding sequences, one 16S/23S rRNA operon and 29 tRNA genes. It has a G + C content of 41.6%.

Similar to the genomes of most marine strains (*Nitrosopumilales*) and to *Ca.* Nitrosocaldus yellowstonensis, but different from those of the terrestrial organisms (*Nitrososphaerales*) it encodes all putative subunits of the ammonia monooxygenase in a single gene cluster of the order *amoA*, *amoX*, *amoC*, *amoB* (**Figure 4**) indicating that this might represent an ancestral gene order. Different from several other AOA it has a single copy of *amoC*. The genome contains a cluster of genes for the degradation of urea, including the urease subunits and two urea transporters (**Figures 4**, **5**) with a similar structure to the urease locus of *Nitrososphaerales*. Accordingly, growth on urea, albeit slower than on ammonia, could be demonstrated (**Figure 1B**). Urease loci were so far found in all *Nitrososphaerales* genomes, and in some *Nitrosopumilales* (Hallam et al., 2006; Park et al., 2012; Spang et al., 2012; Bayer et al., 2016). No urease cluster could be found in non-AOA Thaumarchaeota, nor in Aigarchaeota. In Crenarchaeota, only one *Sulfolobales* genome harbored a urease (*Metallosphaera sedula* DSM 5348). A specific protein family of a putative chaperonin (Hsp60 homolog, arCOG01257) was found to be conserved within the urease loci of *Ca.* N. cavascurensis and the *Nitrososphaerales* ("soil-group" of AOA) (**Figure 4**) as also observed in some bacteria (e.g., in *Haemophilus influenzae*). Unlike the conserved "thermosome" Hsp60 (also part of arCOG01257), this particular Hsp60 homolog was not found in any *Nitrosopumilales* genome. Given the conservation of the urease loci between *Ca.* Nitrosocaldus and *Nitrosophaerales*, it

FIGURE 4 | Phylogenetic position of *Ca.* N. cavascurensis, and distribution of the ammonia monooxygenase (AMO) and urease gene clusters in Thaumarchaeota genomes. This maximum-likelihood tree built with IQ-tree is based on 59 ribosomal proteins found in at least 35 genomes out of the 40 genomes dataset. AMO and urease genes clusters found are displayed along the tree. Colors of gene boxes indicate the gene families involved in AMO or urease (*amoABCX* and *UreABCDEFG* respectively). Gray boxes correspond to other genes. Gene clusters are displayed in the orientation which maximizes the vertical alignment of the conserved genes. Genes are represented on an uninterrupted cluster when less than five genes separated them, otherwise, genes separated by more than five gene positions are displayed on another cluster. For AMO clusters, the number of different *amoC* homologs found in the genome outside of the main AMO cluster is indicated in the *amoC* box. Putative urea transporters are indicated by gray boxes outlined in blue for SSS-type urea transporter, and pink for UT urea transporter. The chaperonin Hsp60 homolog found in the urease cluster and discussed in the main text is indicated with a red box underlining its conserved position over multiple loci. Note that *Nitrososphaera evergladensis* and *Nitrososphaera viennensis* both have an extra set of three urease genes (*UreABC*) not displayed here.

is possible that these genes have a common origin, and were acquired *en bloc* from bacteria by the ancestor of AOA. It is likely that the Hsp60 homolog is involved in stabilization of the urease, as demonstrated in the deltaproteobacterium *Helicobacter pylori* (Evans et al., 1992).

Intriguingly, a gene encoding a nitrite reductase that is present in all genomes of AOA (except the sponge symbiont, *Ca.* Cenarchaeum symbiosum, Bartossek et al., 2010) and which is highly transcribed during ammonia oxidation in *Nitrososphaera viennensis* (Kerou et al., 2016b) and in meta-transcriptomic datasets (Shi et al., 2011) is missing from the genome. Nitrite reductases have been postulated to be involved in ammonia oxidation by providing nitric oxide (NO) for the oxidation step to nitrite (Kozlowski et al., 2016a). We therefore tested, if the organism was affected by the NO-scavenger PTIO which inhibited at low concentrations all AOA tested so far (Martens-Habbena et al., 2015; Kozlowski et al., 2016a). Ammonia oxidation of *Ca.* N. cavascurensis was fully inhibited at concentrations as low as 20 μM, similar to those affecting *Nitrososphaera viennensis* and *Nitrosopumilus maritimus* (**Figure 6**). This indicates that NO is also an important intermediate in the ammonia oxidation of this thermophile and it might be produced by an unknown nitrite reductase or perhaps by an unidentified hydroxylamine dehydrogenase (HAO), as recently shown for the HAO of the ammonia oxidizing bacterium *Nitrosomonas europaea* (Caranto and Lancaster, 2017). Alternatively, NO might be supplied through the activity of other organisms in the enrichment culture. Indeed we found a *nirK* gene in the genome of the *Deinococcus* species of our enrichment culture. The sensitivity to PTIO reinforces the previously raised hypothesis that NO represents an important intermediate or cofactor in ammonia oxidation in archaea (Schleper and Nicol, 2010; Simon and Klotz, 2013; Kozlowski et al., 2016b). However, the full understanding of ammonia oxidation in AOA and the role of NO awaits further biochemical investigations.

Pathways of central carbon metabolism and -fixation of *Ca.* N. cavascurensis were found to be generally similar to those of other AOA, underlining the strikingly high similarity and conservation of metabolism within the Nitrososphaeria (**Figure 5**) (Kerou et al., 2016b).

Key enzymes of the 3-hydroxypropionate/4-hydroxybutyrate carbon fixation pathway were identified in the genome of *Ca.* N. cavascurensis (Supplementary Table 1, Könneke et al., 2014). Intriguingly, and similar to *Sulfolobales*, we find two copies of the gene encoding for 4-hydoxybutyryl-CoA dehydratase located in tandem. The first copy (NCAV_0127) is homologous to the other AOA sequences which are more closely related to fermenting Clostridia rather than their crenarchaeal counterparts ("Cren Type-1," Supplementary Figure 2), while the second copy (NCAV_0126) exhibits high similarity to genes from bacterial candidate division NC10 and

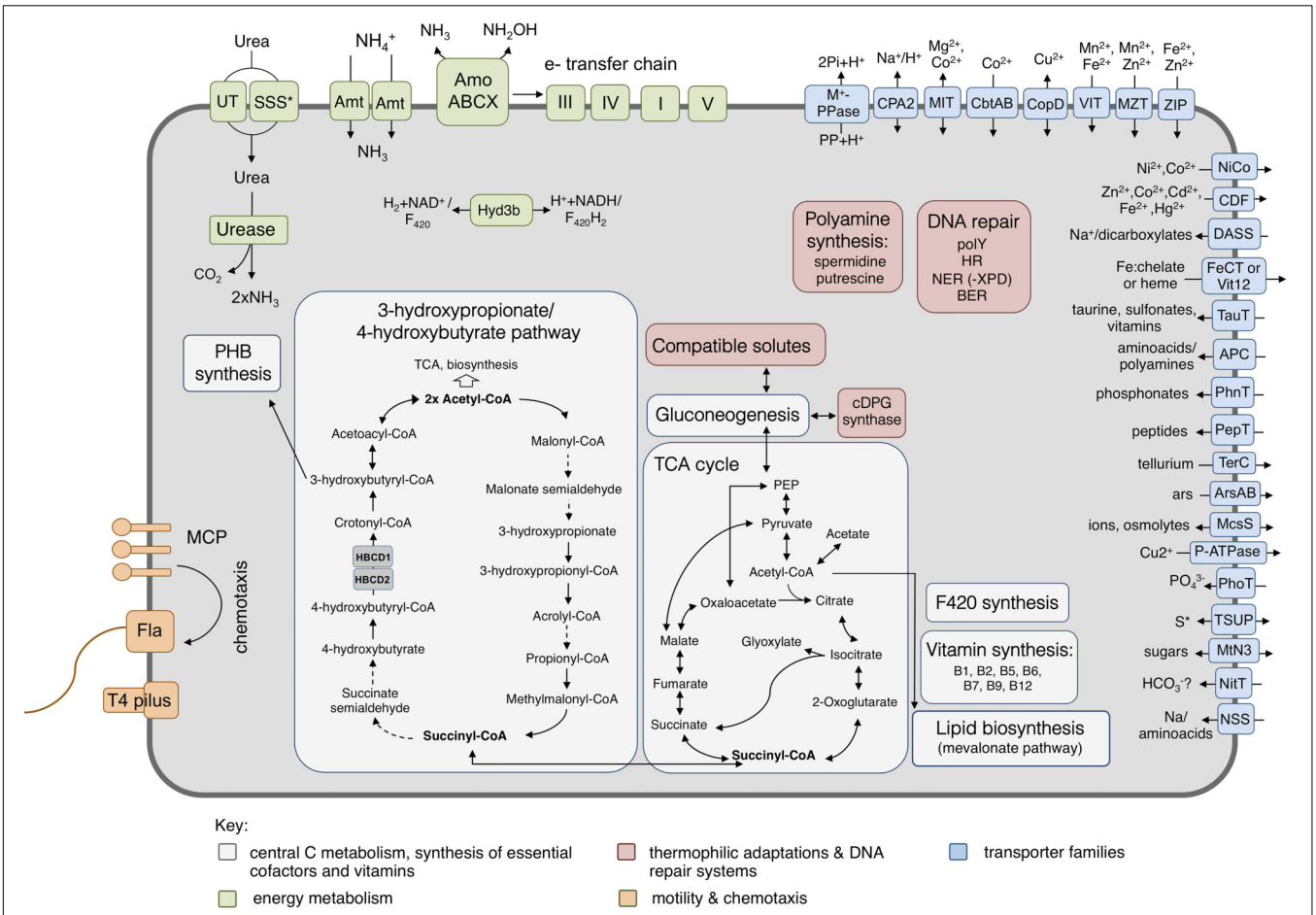

FIGURE 5 | Metabolic reconstruction of *Ca*. N. cavascurensis. Schematic reconstruction of the predicted metabolic modules and other genome features of *Ca.* N. cavascurensis, as discussed in the text. Dashed lines indicate reactions for which the enzymes have not been identified. Candidate enzymes, gene accession numbers and transporter classes are listed in Supplementary Table 1. HBCD, 4-hydoxybutyryl-CoA dehydratase; cDPG, cyclic 2, 3-diphosphoglycerate; MCP, methyl-accepting chemotaxis proteins; Fla, archaellum; Hyd3b, type 3b hydrogenase complex; polY, Y-family translesion polymerase; HR, homologous recombination; NER: nucleotide excision repair; BER, base excision repair; S*, organosulfur compounds, sulfite, sulfate.

Handelsmanbacteria (49% identity). In a phylogenetic tree of the protein family, it clusters together with the second group of crenarchaeal genes ("Cren Type-2," Supplementary Figure 2, see Materials and Methods) which lack essential catalytic residues and whose function remains unknown (Ramos-Vera et al., 2011). Könneke et al. (2014) suggested an independent emergence of the cycle in Thaumarchaeota and autotrophic Crenarchaeota based on the unrelatedness of their respective enzymes (Könneke et al., 2014). But the existence of the second gene in this deep-branching lineage rather indicates that both genes could have been present in a common ancestor of Crenarchaeota and Thaumarchaeota and that their CO_2 fixation pathways could have a common origin. No homolog of the experimentally characterized malonic semialdehyde reductase from *Nitrosopumilus maritimus* (Otte et al., 2015), or any other iron-containing alcohol dehydrogenase protein family member, was found in the *Ca.* N. cavascurensis genome. Since this enzyme (Nmar_1110) is so far the only characterized malonic semialdehyde reductase active in mesophilic conditions (Otte et al., 2015), we searched for homologs of the corresponding (but unrelated) thermophilic enzymes from Crenarchaea (*Metallosphaera sedula*) (Kockelkorn and Fuchs, 2009) and Chloroflexi (Hügler et al., 2002). The best hits of Msed_1993, a member of the 3-hydroxyacyl-CoA dehydrogenase family, were the 3-hydroxybutyryl-CoA dehydrogenase homologs of the thaumarchaeal genomes, as observed already by Könneke et al. (2014). The reduction of malonic semialdehyde in the 3-hydroxypropionate bi-cycle of *Chloroflexus aurantiacus* is carried out by a bifunctional protein (Caur_2614) with an N-terminal short-chain alcohol dehydrogenase domain and a C-terminal aldehyde dehydrogenase domain (Hügler et al., 2002). No homolog of this bifunctional enzyme was identified in the genome of *Ca.* N. cavascurensis, but a number of short-chain alcohol dehydrogenases with low identities (~30%) to Caur_2614 exist (Supplementary Table 1) that could serve as candidates in the absence of a canonical malonic semialdehyde reductase. Whether temperature adaptation leads to non-orthologous gene replacement

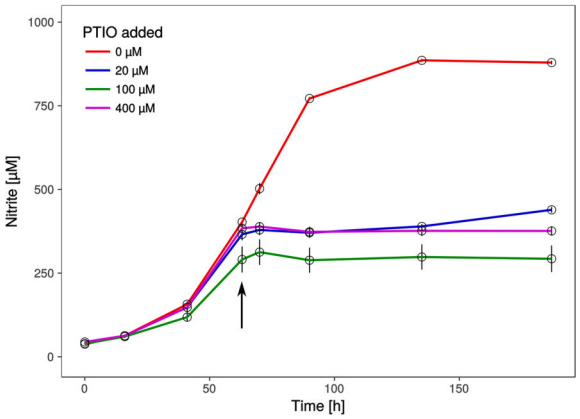

FIGURE 6 | Effect of the NO scavenger 2-phenyl-4, 4, 5, 5,-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) on nitrite production. Different concentrations of PTIO were added after 63 h in early to mid-exponential growth phase indicated by the arrow. Cultures with 20 µM PTIO resumed nitrite production 72 h after the addition of PTIO, while higher concentrations resulted in a complete arrest. Nitrite curves of control and 20 µM PTIO cultures show mean values from duplicates, whereas 100 and 400 µM curves are from triplicates and error bars represent the standard error of the mean.

we observe in mesophilic AOA remains to be seen upon experimental characterisation of the thermophilic thaumarchaeal enzyme.

A full oxidative TCA cycle is present in *Ca.* N. cavascurensis, including a malic enzyme and a pyruvate/phosphate dikinase connecting the cycle to gluconeogenesis. Additionally, and like most mesophilic AOA, *Ca.* N. cavascurensis encodes a class III polyhydroxyalkanoate synthase (phaEC) allowing for the production of polyhydroxyalkanoates, carbon polyester compounds that form during unbalanced growth and serve as carbon and energy reserves (Poli et al., 2011; Spang et al., 2012) (**Figure 5** and Supplementary Table 1).

The presence of a full set of genes encoding for the four subunits (and maturation factors) of a soluble type 3b [NiFe]-hydrogenase, uniquely in *Ca.* N. cavascurensis, indicates the ability to catalyze hydrogen oxidation potentially as part of the energy metabolism. This group of hydrogenases is typically found among thermophilic archaea, is oxygen-tolerant and bidirectional, and can couple oxidation of H_2 to reduction of NAD(P) in order to provide reducing equivalents for biosynthesis, while some have been proposed to have sulfhydrogenase activity (Kanai et al., 2011; Peters et al., 2015; Greening et al., 2016) (and references therein). Although classified by sequence analysis (Sondergaard et al., 2016) and subunit composition as a type 3b hydrogenase, the alpha and delta subunits belong to the arCOG01549 and arCOG02472 families respectively, which contain coenzyme F_{420}-reducing hydrogenase subunits, so far exclusively found in methanogenic archaea. Given the fact that Thaumarchaeota can synthesize this cofactor and encode a number of F_{420}-dependent oxidoreductases with a yet unknown function, it is interesting to speculate whether oxidized F_{420} could also be a potential substrate for the hydrogenase. Expression of the hydrogenase is likely regulated through a cAMP-activated transcriptional regulator encoded within the hydrogenase gene cluster (Supplementary Table 1).

Putative S-layer subunits were identified in the genome of *Ca.* N. cavascurensis (NCAV_0187 and NCAV_0188, Supplementary Table 1). Although the overall identity among thaumarchaeal S-layer subunits is very low (<30% between different genera), investigations of the genomic neighborhood reveals conserved synteny of this region.

Adaptations to Thermophilic Life

The molecular adaptations that enable survival and the maintenance of cell integrity at high temperatures have been the subject of intense studies since the discovery of thermophilic organisms. The issue of extensive DNA damage occurring at high temperatures has led to the study of systems of DNA stabilization and repair in thermophilic and hyperthermophilic archaea. Among them, the reverse gyrase, a type IA DNA topoisomerase shown to stabilize and protect DNA from heat-induced damage, is often (but not always (Brochier-Armanet and Forterre, 2007)) found in thermophiles, and is even considered a hallmark of hyperthermophilic organisms growing optimally above 80°C (Bouthier de la Tour et al., 1990; Forterre, 2002). However, the gene might not always be essential for survival at high temperature in the laboratory (Atomi et al., 2004; Brochier-Armanet and Forterre, 2007). Interestingly, we could not identify a gene encoding for reverse gyrase in the genome of *Ca.* N. cavascurensis. This might either reflect that its growth optimum is at the lower end of extreme thermophiles or that there is a separate evolutionary line of adaptation to thermophily possible without reverse gyrase.

The following DNA repair mechanisms were identified in the genome of *Ca.* N. cavascurensis, in agreement with the general distribution of these systems in thermophiles (for reviews see Rouillon and White, 2011; Grasso and Tell, 2014; Ishino and Narumi, 2015):

(a) Homologous recombination repair (HR): Homologs of RadA and RadB recombinase, Mre11, Rad50, the HerA-NurA helicase/nuclease pair, and Holliday junction resolvase Hjc are encoded in the genome. These genes have been shown to be essential in other archaeal (hyper)thermophiles, leading to hypotheses regarding their putative role in replication and more generally the tight integration of repair, recombination and replication processes in (hyper)thermophilic archaea (Grogan, 2015).

(b) Base excision repair (BER): the machinery responsible for the repair of deaminated bases was identified in the genome, including uracil DNA glycosylases (UDG) and putative apurinic/apyrimidinic lyases. Deletion of UDGs was shown to impair growth of (hyper)thermophilic archaea (Grogan, 2015).

(c) Nucleotide excision repair (NER): Homologs of the putative DNA repair helicase: nuclease pair XPB-Bax1 and nuclease XPF were found in the genome, but repair nuclease XPD could not be identified. XPD is present in all so far analyzed mesophilic AOA, but it is also absent in other thermophiles (Kelman and White, 2005). It should be noted that NER functionality in archaea is still unclear, and deletions of the

respective genes were shown to have no observable phenotype (Grogan, 2015).

(d) Translesion polymerase: A Y-family polymerase with low-fidelity able to perform translesion DNA synthesis is encoded in *Ca.* N. cavascurensis.

Key enzymes of all the above-mentioned systems are also found in mesophilic AOA. Given their extensive study in the crenarchaeal (hyper)thermophiles, it would be interesting to characterize their respective functions and regulation in both mesophilic and thermophilic Thaumarchaeota.

(e) Bacterial-type UvrABC excision repair: In contrast to mesophilic AOA and in agreement with the known distribution of the system among mesophilic archaea and bacteria but its absence in (hyper)thermophiles, *Ca.* N. cavascurensis does not encode homologs of this repair machinery.

Ca. N. cavascurensis could potentially produce the polyamines putrescine and spermidine, which have been shown to bind and stabilize compacted DNA from thermal denaturation, acting synergistically with histone molecules, also present in AOA (Higashibata et al., 2000; Oshima, 2007). Although a putative spermidine synthase is also found in mesophilic AOA, the gene encoding for the previous step, *S*-adenosylmethionine decarboxylase (NCAV_0959), is only found in *Ca.* N. cavascurensis, located in tandem with the former. The biosynthesis of putrescine (a substrate for spermidine synthase) is unclear, since we could not identify a pyruvoyl-dependent arginine decarboxylase (ADC, catalyzing the first of the two-step biosynthesis of putrescine). However, it was shown that the crenarchaeal arginine decarboxylase evolved from an *S*-adenosylmethionine decarboxylase enzyme, raising the possibility of a promiscuous enzyme (Giles and Graham, 2008).

The production of thermoprotectant compounds with a role in stabilizing proteins from heat denaturation seems to be a preferred strategy of heat adaptation in *Ca.* N. cavascurensis. Firstly, the presence of mannosyl-3-phosphoglycerate synthase (NCAV_1295) indicates the ability to synthesize this compatible solute, shown to be involved in heat stress response and protection of proteins from heat denaturation in *Thermococcales* (Neves et al., 2005). Homologous genes are also present in mesophilic members of the order *Nitrososphaerales* and other (hyper)thermophiles (Spang et al., 2012; Kerou et al., 2016b). Secondly, only *Ca*. N. cavascurensis, but no other AOA encodes a cyclic 2, 3-diphosphoglycerate (cDPG) synthetase (NCAV_0908), an ATP-dependent enzyme which can synthesize cDPG from 2,3-biphosphoglycerate, an intermediate in gluconeogenesis. High intracellular concentrations of cDPG accumulate in hyperthermophilic methanogens, where it is required for the activity and thermostability of important metabolic enzymes (Shima et al., 1998).

Notable Features of the DNA Replication and Cell Division Systems in *Ca.* N. cavascurensis

Strikingly, only one family B replicative polymerase PolB was identified in the genome of *Ca.* N. cavascurensis (NCAV_1300), making it the only archaeon known so far to encode a single subunit of the replicative family B polymerase, as in Crenarchaeota multiple paralogs with distinct functions coexist (Makarova et al., 2014). Both subunits of the D-family polymerases PolD present in all other AOA and shown to be responsible for DNA replication in *Thermococcus kodakarensis* (also encoding both polD and polB families) (Cubonova et al., 2013) were absent from the genome, raising intriguing questions about the role of the polB family homolog in mesophilic Thaumarchaeota. Sequence analysis indicated that the *Ca.* N. cavascurensis homolog belongs to the polB1 group present exclusively in the TACK superphylum and shown recently by Yan and colleagues to be responsible for both leading and lagging strand synthesis in the crenarchaeon *Sulfolobus solfataricus* (Yan et al., 2017). However, the activity of *Sulfolobus solfataricus* PolB1 is dependent on the presence and binding of two additional proteins, PBP1 and PBP2, mitigating the strand-displacement activity during lagging strand synthesis and enhancing DNA synthesis and thermal stability of the holoenzyme, respectively. No homologs of these two additional subunits were identified in *Ca.* N. cavascurensis, raising the question of enzymatic thermal stability and efficiency of DNA synthesis on both strands.

Homologs of genes for the Cdv cell division system proteins CdvB (3 paralogs) and CdvC, but not CdvA, were identified in *Ca.* N. cavascurensis. This is surprising given that all three proteins were detected by specific immuno-labeling in the mesophilic AOA *Nitrosopumilus maritimus*, where CdvA, CdvC and two CdvB paralogs were shown to localize mid-cell in cells with segregated nucleoids, indicating that this system mediates cell division in Thaumarchaeota (Pelve et al., 2011). The *Ca.* N. cavascurensis CdvB paralogs (as all other thaumarchaeal CdvBs) all share the core ESCRT-III with the crenarchaeal CdvB sequences, contain a putative (but rather unconvincing) MIM2 motif necessary for interacting with CdvC in Crenarchaeota, while they all lack the C-terminal winged-helix domain responsible for interacting with CdvA (Samson et al., 2011; Ng et al., 2013). Interestingly, one of the *Ca.* N. cavascurensis paralogs (NCAV_0805) possesses a 40 amino-acids serine-rich C-terminal extension right after the putative MIM2 motif absent from other thaumarchaea. It is worth noting that CdvA is also absent from the published Aigarchaeota genomes *Ca.* Caldiarchaeum subterraneum (Nunoura et al., 2011) and *Ca.* Calditenuis aerorheumensis (Beam et al., 2016), while both phyla (Thaumarchaeota and Aigarchaeota) encode an atypical FtsZ homolog. *Thermococcales*, albeit they presumably divide with the FtsZ system, also encode CdvB and CdvC homologs, while no CdvA homolog is detectable (Makarova et al., 2010). Given the emerging differences in the molecular and regulatory aspects of the Cdv system between Crenarchaeota and Thaumarchaeota (Pelve et al., 2011; Ng et al., 2013), the intriguing additional roles of the system (Samson et al., 2017) and the fact that only CdvB and CdvC are homologous to the eukaryotic ESCRT-III system and therefore seem to have fixed roles in evolutionary terms, this observation raises interesting questions regarding the versatility of different players of the cell division apparatus.

Ca. N. cavascurensis Has a Dynamic Genome

The *Ca.* N. cavascurensis genome contains large clusters of genes that showed deviations from the average G+C content of the genome (i.e., 41.6%), indicating that these regions might have been acquired by lateral gene transfer (**Figure 7**).

Two of the larger regions were integrative and conjugative elements (ICE-1 and ICE-2 in **Figures 7**, **8**) of 65.5 and 43.6 kb, respectively. Both are integrated into tRNA genes and flanked by characteristic 21 and 24 bp-long direct repeats corresponding to the attachment sites, a typical sequence feature generated upon site-specific recombination between the cellular chromosome and a mobile genetic element (Grindley et al., 2006). ICE-1 and ICE-2 encode major proteins required for conjugation (colored in red in **Figure 8A**), including VirD4-like and VirB4-like ATPases, VirB6-like transfer protein and VirB2-like pilus protein (in ICE-2). The two elements also share homologs of CopG-like proteins containing the ribbon-helix-helix DNA-binding motifs and beta-propeller-fold sialidases (the latter appears to be truncated in ICE-1). The sialidases of ICE-1 and ICE-2 are most closely related to each other (37% identity over 273 aa alignment). It is not excluded that ICE-1 and ICE-2 have inherited the conjugation machinery as well as the genes for the sialidase and the CopG-like protein from a common ancestor, but have subsequently diversified by accreting functionally diverse gene complements. Indeed, most of the genes carried by ICE-1 and ICE-2 are unrelated. ICE-1, besides encoding the conjugation proteins, carries many genes for proteins involved in DNA metabolism, including an archaeo-eukaryotic primase-superfamily 3 helicase fusion protein, Cdc6-like ATPase, various nucleases (HEPN, PD-(D/E)XK, PIN), DNA methyltransferases, diverse DNA-binding proteins and an integrase of the tyrosine recombinase superfamily (yellow in **Figure 8A**). The conservation of the attachment sites and the integrase gene as well as of the conjugative genes indicates that this element is likely to be a still-active Integrative and Conjugative Element (ICE) able to integrate into the chromosome and excise from it as a conjugative plasmid.

ICE-2 is shorter and encodes a distinct set of DNA metabolism proteins, including topoisomerase IA, superfamily 1 helicase, ParB-like partitioning protein, GIY-YIG and FLAP nucleases as well as several DNA-binding proteins. More importantly, this element also encodes a range of proteins involved in various metabolic activities as well as a Co/Zn/Cd efflux system that might provide relevant functions to the host (green in **Figure 8A**). In bacteria, ICE elements often contain cargo genes that are not

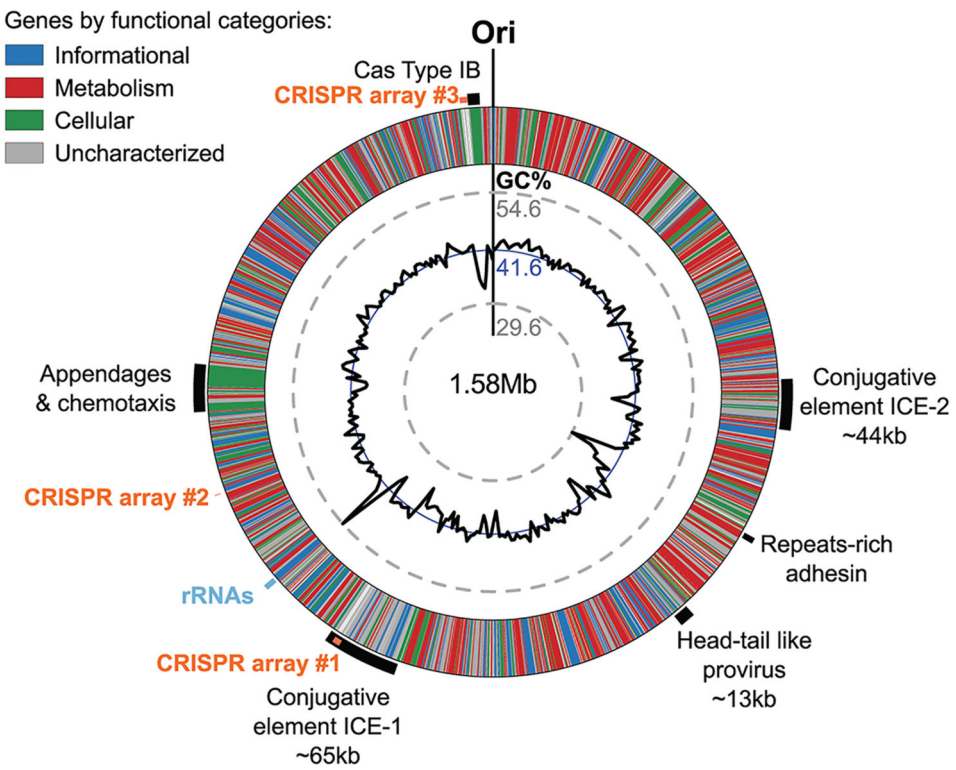

FIGURE 7 | Genomic regions of atypical GC% content contain mobile genetic elements in *Ca.* N. cavascurensis. The genome is displayed in the form of a ring where genes are colored by functional categories (arCOG and COG), with the mean GC% content (blue circle) and local GC% as computed using 5 kb sliding windows (black graph) in inner rings. The minimal and maximal values of local GC% are displayed as dashed gray concentric rings. The predicted origin of replication is indicated with "Ori." Several large regions show a deviation to the average GC% that correspond to mobile genetic elements, i.e., ICEs (integrated conjugated elements), a specific pilus (see **Figure 8**), and a defense system (CRISPR-Cas Type IB and three CRISPR arrays (see text).

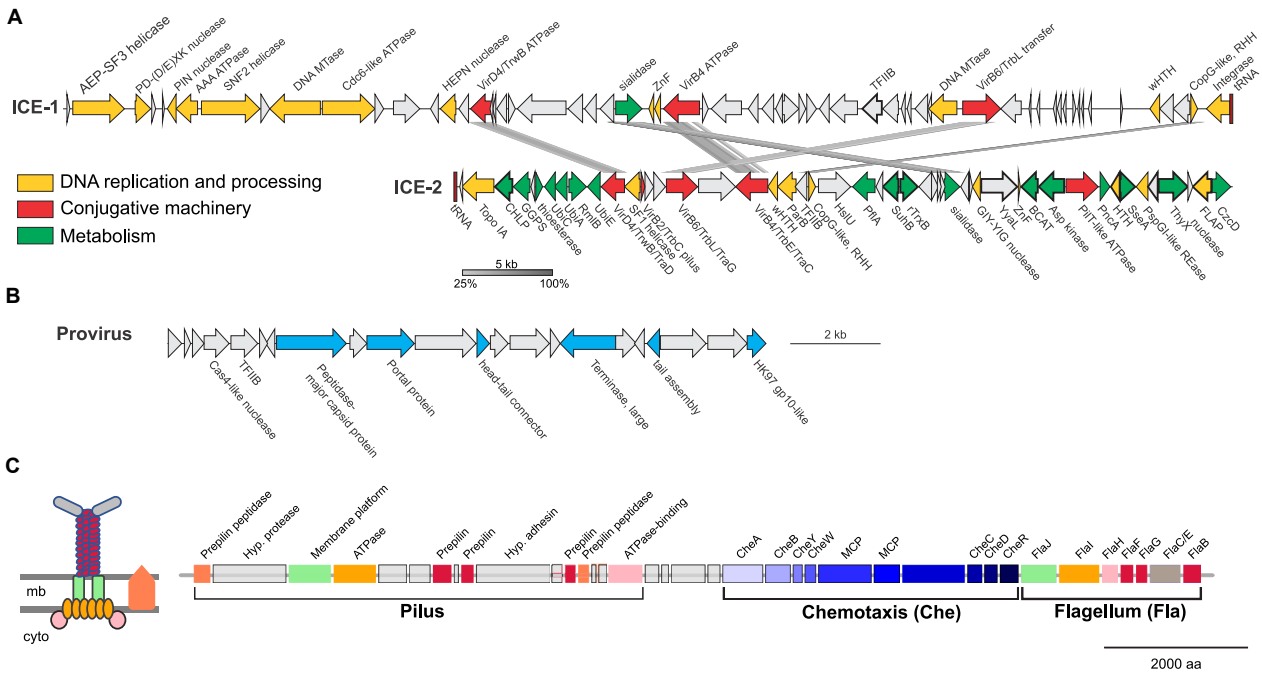

FIGURE 8 | Mobile genetic elements and cell appendages encoded in the genome of *Ca.* N. cavascurensis. **(A)** Two putative Integrative Conjugative Elements (ICE) with sequence similarities shown by connections between the two. Genes are colored by function (see legend). CHLP, geranylgeranyl diphosphate reductase; GGPS, geranylgeranyl pyrophosphate synthetase; UbiC, chorismate lyase; UbiA, 4-hydroxybenzoate octaprenyltransferase; RmlB, dTDP-glucose 4,6-dehydratase; UbiE, Ubiquinone/menaquinone biosynthesis C-methylase; HslU, ATP-dependent HslU protease; PflA, Pyruvate-formate lyase-activating enzyme; SuhB, fructose-1,6-bisphosphatase; TrxB, Thioredoxin reductase; YyaL, thoiredoxin and six-hairpin glycosidase-like domains; BCAT, branched-chain amino acid aminotransferase; PncA, Pyrazinamidase/nicotinamidase; SseA, 3-mercaptopyruvate sulfurtransferase; ThyX, Thymidylate synthase; CzcD: Co/Zn/Cd efflux system. **(B)** A head-tail like provirus from the *Caudovirales* order with genes in blue indicating putative viral functions. **(C)** *Ca.* N. cavascurensis specific type IV pilus locus next to a set of chemotaxis and flagellum (archaellum) genes. Homologs shared between the flagellum and the T4P are displayed with the same colors. A scheme of the putative type IV pilus is shown on the left with the same color code (inspired by drawings in Makarova et al., 2016). mb, membrane; cyto, cytoplasm; MCP, methyl-accepting chemotaxis proteins.

related to the ICE life cycle and that confer novel phenotypes to host cells (Johnson and Grossman, 2015). It is possible that under certain conditions, genes carried by ICE-1 and ICE-2, and in particular the metabolic genes of ICE-2, improve the fitness of *Ca.* N. cavascurensis. Notably, only ICE-1 encodes an integrase, whereas ICE-2 does not, suggesting that ICE-2 is an immobilized conjugative element that can be vertically inherited in AOA. Given that the attachment sites of the two elements do not share significant sequence similarity, the possibility that ICE-2 is mobilized *in trans* by the integrase of ICE-1 appears unlikely.

The third mobile genetic element is derived from a virus, related to members of the viral order *Caudovirales* (**Figure 8B**) (Prangishvili et al., 2017). It encodes several signature proteins of this virus group, most notably the large subunit of the terminase, the portal protein and the HK97-like major capsid protein (and several other viral homologs). All these proteins with homologs in viruses are involved in virion assembly and morphogenesis. However, no proteins involved in genome replication seem to be present. The element does not contain an integrase gene, nor is it flanked by attachment sites, which indicates that it is immobilized. Interestingly, a similar observation has been made with the potential provirus-derived element in *Nitrososphaera viennensis* (Krupovic et al., 2011). Given that the morphogenesis genes of the virus appear to be intact, one could speculate that these elements represent domesticated viruses akin to gene transfer agents, as observed in certain methanogenic euryarchaea (Eiserling et al., 1999; Krupovic et al., 2010), or killer particles (Bobay et al., 2014), rather than deteriorating proviruses. Notably, ICE-1, ICE-2 and the provirus-derived element all encode divergent homologs of TFIIB, a transcription factor that could alter the promoter specificity to the RNA polymerase.

A fourth set of unique, potentially transferred genes encodes a putative pilus (**Figure 8C**). All genes required for the assembly of a type IV pilus (T4P) are present, including an ATPase and an ATPase binding protein, a membrane platform protein, a prepilin peptidase, and several prepilins (Makarova et al., 2016). Based on the arCOG families of the broadly conserved ATPase, ATPase-binding protein, membrane-platform protein, and prepilin peptidase (arCOG001817, -4148, -1808, and -2298), this pilus seems unique in family composition, but more similar to the archetypes of pili defined as clades 4 (A, H, I, J) by Makarova and colleagues, which are mostly found in *Sulfolobales* and *Desulfurococcales* (Figure 4 from Makarova et al., 2016).

Yet, the genes associated to this putative pilus seem to be more numerous, as the locus consists of approximately 16 genes (versus ∼5 genes for the *aap* and *ups* in *Sulfolobus*). Such a combination of families is not found either in the T4P or flagellar loci found in analyzed Thaumarchaeota genomes. Prepilins are part of the core machinery of T4P, but display a high level of sequence diversity. The three prepilins found in *Ca*. N. cavascurensis T4P locus correspond to families that are not found in any other genome from our dataset (arCOG003872, -5987, -7276). We found a putative adhesin right in between the genes encoding the prepilins, and therefore propose that this *Ca*. N. cavascurensis-specific type IV pilus is involved in adhesion (**Figure 8C**). This pilus thus appears to be unique in protein families composition when compared to experimentally validated T4P homologs in archaea (flagellum, *ups*, *aap*, bindosome (Szabo et al., 2007; Zolghadr et al., 2007; Frols et al., 2008; Tripepi et al., 2010; Henche et al., 2012), bioinformatically predicted ones (Makarova et al., 2016), and the pili found in other Thaumarchaeota, which correspond to different types. Interestingly, this pilus gene cluster lies directly next to a conserved chemotaxis/archaellum cluster as the one found in *Nitrososphaera gargensis* or *Nitrosoarchaeum limnia* (four predicted genes separate the T4P genes and *cheA,* **Figure 8C**) (Spang et al., 2012). This suggests that this pilus might be controlled by exterior stimuli through chemotaxis. The interplay between the archaellum and pilus expression would be interesting to study in order to comprehend their respective roles.

The genome of *Ca.* N. cavascurensis also carries traces of inactivated integrase genes as well as transposons related to bacterial and archaeal IS elements, suggesting that several other types of mobile genetic elements have been colonizing the genome. Collectively, these observations illuminate the flexibility of the *Ca.* N. cavascurensis genome, prone to lateral gene transfer and invasion by alien elements. Accordingly, we found a CRISPR-Cas adaptive immunity system among the sets of genes specific to *Ca*. N. cavascurensis that we could assign to the subtype I-B (Abby et al., 2014). We detected using the CRISPRFinder website (Grissa et al., 2007) at least three CRISPR arrays containing between 4 and 101 spacers presumably targeting mobile genetic elements associated with *Ca.* N. cavascurensis, reinforcing the idea of a very dynamic genome. Interestingly, the second biggest CRISPR array (96 spacers) lies within the integrated conjugative element ICE-1, which we hypothesize to be still active. This suggests that ICE-1 may serve as a vehicle for the horizontal transfer of the CRISPR spacers between *Ca*. N. cavascurensis and other organisms present in the same environment through conjugation, thus spreading the acquired immunity conferred by these spacers against common enemies.

CONCLUSION

We present an obligately thermophilic ammonia oxidizing archaeon from a hot spring on the Italian island of Ischia that is related to, but also clearly distinct from *Ca.* Nitrosocaldus yellowstonensis. It contains most of the genes that have been found to be conserved among AOA and are implicated in energy and central carbon metabolism, except *nirK* encoding a nitrite reductase. Its genome gives indications for alternative energy metabolism and exhibits adaptations to (high temperature) extreme environments. However, it lacks an identifiable reverse gyrase, which is found in most thermophiles with optimal growth temperatures above 65°C and apparently harbors a provirus of head-and tail structure that is not usually found at high temperatures. *Ca.* N. cavascurensis differs also in its gene sets for replication and cell division, which has implications for the function and evolution of these systems in archaea. In addition, its extensive mobilome and the defense system indicate that thermophilic AOA are in constant exchange with the environment and with neighboring organisms as discussed for other thermophiles (van Wolferen et al., 2013). This might have shaped and continues to shape the evolution of Thaumarchaeota in hot springs. The pivotal phylogenetic position of *Ca*. N. cavascurensis will enable the reconstruction of the last common ancestor of AOA and provide further insights into the evolution of this ecologically widespread group of archaea.

For our enriched strain we propose a candidate status with the following taxonomic assignment:

Nitrosocaldales order
Nitrosocaldaceae fam. and
Candidatus Nitrosocaldus cavascurensis sp. nov.
Etymology: L. adj. nitrosus, "full of natron," here intended to mean nitrous (nitrite producer); L. masc.n. caldus, hot;
cavascurensis (L.masc. gen) describes origin of sample (Terme di Cavascura, Ischia)
Locality: hot mud, outflow from hot underground spring, 77°C
Diagnosis: an ammonia oxidizing archaeon growing optimally around 68°C at neutral pH
under chemolithoautotrophic conditions with ammonia or urea,
spherically shaped with a diameter of approximately 0.6–0.8 μm, 4% sequence divergence in 16S rRNA gene from its next cultivated relative *Ca.* Nitrosocaldus yellowstonensis.

AUTHOR'S NOTE

Another report on the enrichment and genome analysis of a thermophilic Thaumarchaeota was submitted to Frontiers in Microbiology, Daebeler et al. (2018, in review), shortly before submission of this manuscript. The core genomes of both organisms share many of the above-discussed features, but it remains to be seen if the observed similarities extend to their shell genome.

AUTHOR CONTRIBUTIONS

CS conceived the study; MS did the first enrichments; MM and CR made the growth characterizations; KP did the electron microscopy; SSA assembled the genome and performed the phylogenetic and genomic analyses for annotation; MKe annotated all metabolic and information processing genes;

MKr analyzed the mobile genetic elements; CS, SSA, and MKe wrote the manuscript with contributions from MKr and MM.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00028/full#supplementary-material

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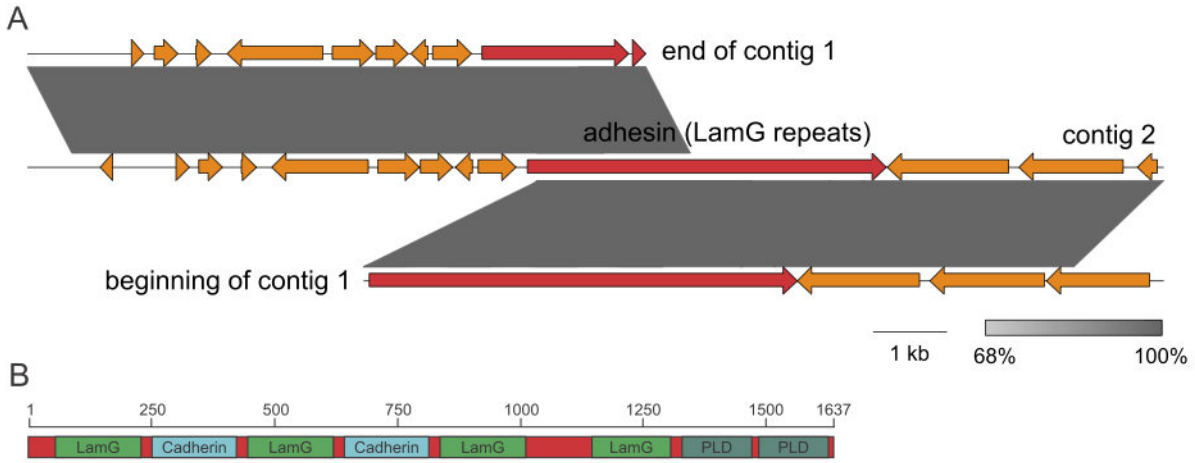
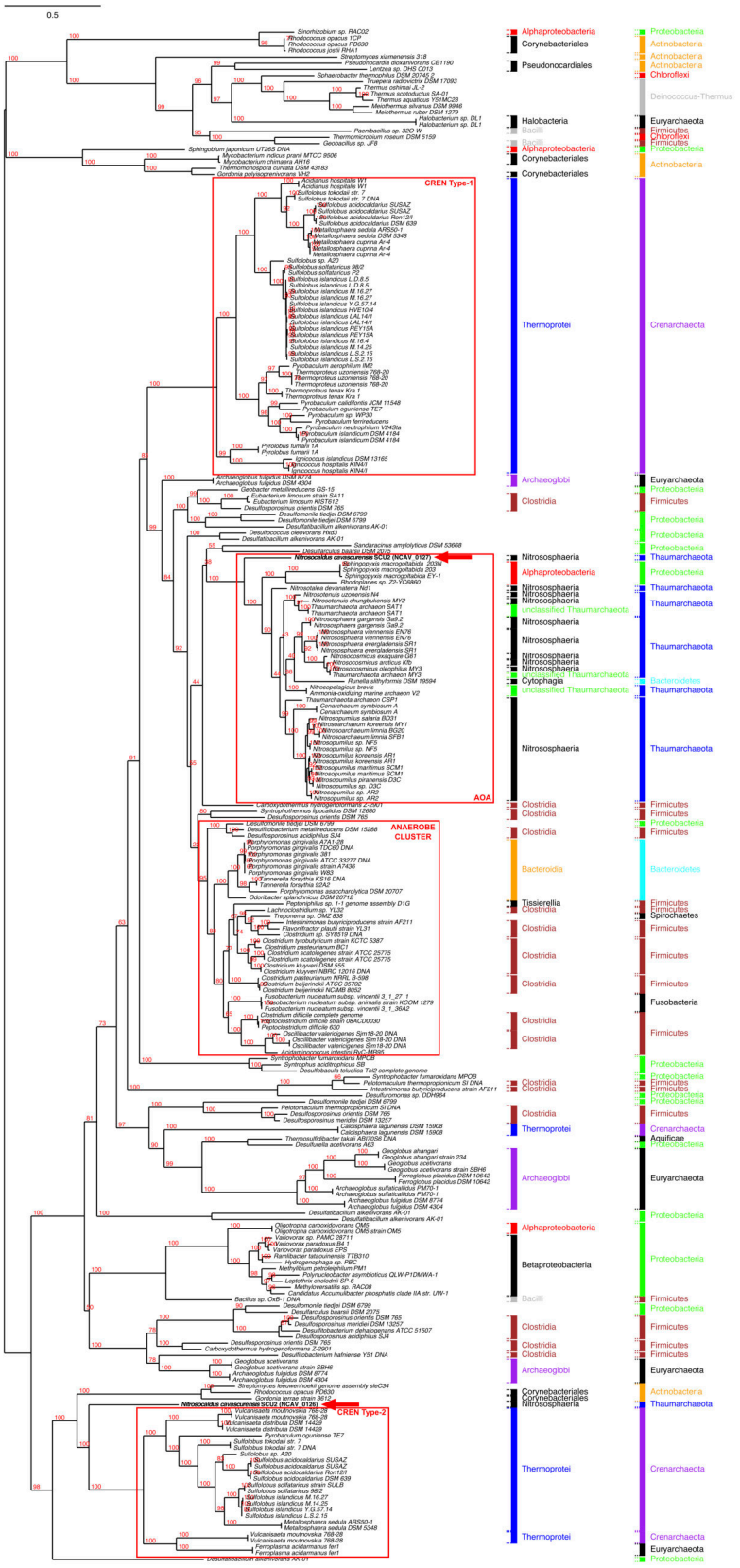
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material

Supplementary Figures



Supplementary Figure 1. Bioinformatic genome closure in a repeat-rich region encoding a hypothetical adhesin. (A) The two initially assembled contigs were aligned using MUMMER (Kurtz et al., 2004), revealing a sequence overlap with very high sequence identity (>99.5%) that could be resolved and resulted in the genome closure: contig 2 was a redundancy, and contig 1 was circularized through a long gene carrying repeated sequences (B) Analysis of this long gene showed that it encodes for an adhesin (a likely surface protein involved in adhesion). As is typical for such proteins, it contains several repeats including LamG/concavalin A domains interspersed with Ca2+-binding cadherin domains (both involved in adhesion) and terminates with two phospholipase D domains (PLD).



Supplementary Figure 2. Phylogenetic tree of the 4-hydoxybutyryl-CoA dehydratase protein family. A maximum likelihood phylogenetic tree was obtained with IQ-Tree v 1.5.5. The groups previously defined in (Konneke et al., 2014) "CREN type-1", "CREN type-2", "Anaerobe cluster", and "AOA" are indicated by red boxes. The organisms' class and phylum are indicated for each sequence along brackets on the right. The two homologs of this enzyme found in Ca. N. cavascurensis genome are indicated by a red arrow. This figure was generated using the Scriptree program (Chevenet et al., 2010), and the resulting SVG file modified using Inkscape.

Chapter III

Nitrosocaldus cavascurensis gen. nov., sp. nov., the first pure culture of a thermophilic ammonia-oxidizing archaeon

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Abstract

In hot springs the process of ammonia oxidation seems to be solely performed by archaea. While a number of metagenomic studies and three enrichment cultures have revealed some of the characteristics of these organisms, a pure culture that allows for detailed physiological investigations has not been obtained yet. Here we describe the isolation of *Nitrosocaldus cavascurensis* as axenic culture, which grows at 68°C under chemolithoautotrophic conditions at an optimal pH of 7.0. Like *Nitrosopumilus maritimus* , cells contain an S-layer of a p6 symmetry. A CO_3^{2-} buffer system was established to ensure stable growth conditions in batch cultures. With the help of low-level antibiotic treatments a pure culture was eventually obtained. Different from moderate AOA, growth of *N. cavascurensis* is not enhanced by the addition of organic acids that were found to act as ROS-scavengers. However, a positive effect of pyruvate on the viability of cryo-stocks was observed. No nitrite reductase activitiy could be detected in crude cell extracts in line with the fact, that the organism does not encode a nitrite reductase like most other AOA. However, a re-investigation of the effect of NO-scavengers on its growth confirmed inhibitory effects, which could not be alleviated by the addition of an NO donor. Therefore the role of NO and NirK for the archaeal ammonia oxidation remains still ambiguous.

Introduction

The global importance of the microbial Nitrogen cycle cannot be overstated, as it is responsible for the bioavailability and harmful consequences of reactive nitrogen forms in all terrestrial and oceanic ecosystems. Nitrogen compounds such as ammonia, nitrite and nitrate are also important intermediates in various chemolithoautotrophic metabolisms occurring in high temperature geothermal environments, responsible for the survival of an important

fraction of the microbial diversity in these habitats (Afshar et al., 1998; Cava et al., 2008; Gokce et al., 1989; Jaeschke et al., 2009; Mishima et al., 2009; Nakagawa et al., 2004; Ramírez-Arcos et al., 1998; Takai et al., 2003; Volkl et al., 1993). These compounds are generated through the processes of nitrification, nitrate reduction, denitrification and dissimilatory nitrate reduction to ammonium (DNRA), which have been measured repeatedly in a number of hot springs and sediments and geothermal soils (De La Torre et al., 2008; Hatzenpichler et al., 2008; Jiang et al., 2010; Kanokratana et al., 2004; Lebedeva et al., 2005, 2011; Pearson et al., 2004, 2008; Reigstad et al., 2008; Zhang et al., 2008). While a number of thermophilic microorganisms can perform denitrification and DNRA, the rate-limiting step of nitrification, the aerobic oxidation of ammonia to nitrite, in geothermal environments seems to be solely performed by thermophilic ammonia-oxidizing archaea (AOA) of the family “Ca. Nitrosocaldaceae”. Recent cultivation efforts have resulted in three enrichment cultures (Ca. Nitrosocaldus yellowstonensis HL72, Ca. Nitrosocaldus cavascurensis SCU2 and Ca. Nitrosocaldus islandicus 3F)(Abby et al., 2018; Daebeler et al., 2018; De La Torre et al., 2008). In addition, metagenomic surveys have revealed a number of metagenome-assembled genomes (MAGs) and an additional candidate genus with the species *Ca.* Nitrosothermus koennekii and candidate species *Ca.* Nitrosocaldus schleperae and have significantly increased our knowledge of this lineage, revealing their pivotal phylogenetic position as deep branching sister clade to all moderate AOA (Luo et al., 2021).

Enrichments of Ca. Nitrosocaldaceae were shown to be capable of chemolithoautotrophic oxidation of ammonia to nitrite with the participation of the key enzyme ammonia monooxygenase like moderate AOA, although the cultivated lineages curiously lacked an identifiable nitrite reductase family enzyme. This is particularly intriguing as Nirk is highly expressed in laboratory cultures (Hollibaugh et al., 2011; Kerou et al., 2016) as well as environmental metatranscriptomic datasets (Hollibaugh et al., 2011). Two models have been proposed for the ammonia oxidation pathway, of which both have been discussed to involve a nitrite reductase activity (or its reverse reaction). In the first model, which is similar to the revised model of ammonia oxidizing bacteria (AOB), Lancaster *et al* (2018) hypothesized that AOA oxidize NH_3 to NO_2^- via NH_2OH and NO. The last step being performed by the inverse reaction of a nitritie reductase. Alternatively, Kozlowski *et al* (2016) postulated that AOA oxidize NH_3 to NH_2OH and finally NO_2^-, whereby an unknown enzyme (Cu “P460”) accepts both NH_2OH and NO to catalyze the formation of two molecules of NO_2^-. The constant supply of NO needed for this reaction could theoretically be provided via the reduction of NO_2^- by a nitrite reductase (NirK).

While genomic data revealed the repertoire responsible for carbon fixation via the modified HP/HB cycle like moderate AOA, an additional set of genes indicated the possibility of an organotrophic metabolism of thermophilic AOA by anaerobic aminoacid fermentation (Daebeler et al., 2018), which, however, could not be experimentally confirmed so far. Additionally, all thermophilic AOA genomes encode a fascinating variety of mobile genetic elements including conjugative systems, transposons and proviruses, suggesting highly dynamic genomes and the putative role of Thaumarchaeal viruses in AOA evolution (Abby et al., 2018, 2020).

Nevertheless, the absence of a pure culture for the thermophilic AOA lineage has so far limited the in-depth investigation of this fascinating metabolism which has been shown to have one of the highest total energy yields in geothermal environments when proceeding to equilibrium (Dodsworth et al., 2012). In this study, we present the first pure culture of a thermophilic AOA, formally proposed as *Candidatus* Nitrosocaldus cavascurensis and propose to assign this species as the type species of the genus *Nitrosocaldus* gen. nov. in the family *Nitrocaldaceae* fam. nov. of the order *Nitrososphaerales* of the class *Nitrososphaeria*.

The pure culture allowed us to further characterize the thermophilic AOA and to investigate potential alternative metabolisms and nitrite reductase activity.

Materials and Methods

Cultivation of SCU2B enrichments

Similar to the cultivation described in (Abby et al., 2018), SCU2B enrichments were grown at 68 °C, shaken with 100 rpm, in 40 mL FWM amended with MTE, FeNaEDTA, 1 mmol L^{-1} NH_4Cl and 2 mmol L^{-1} $NaHCO_3$ in 120 mL serum flasks (rinsed three times with MilliQ water (MQ), autoclaved filled completely with MQ, rinsed twice with MQ, filled up to approximately 40 mL with MQ and autoclaved again) with blue butyl rubber stoppers (boiled and water exchanged for at least six times) and air as atmosphere. Cultures were inoculated with 5 % v/v and transferred every 4 days once they had at least produced 700 µmol L^{-1} NO_2^-. After preliminary pH and O_2 tests the atmosphere was changed to 10 % O2, 5 % CO2, 85 % N2 and $NaHCO_3$ was increased from 2 mmol L^{-1} to 6 mmol L^{-1} $NaHCO_3$ to obtain a pH of 7.0. The atmosphere was produced by a gas mixing module (DASGIP MX4/4, Eppendorf) and serum flasks with 40 mL medium were sparged with 0.2 µm filtered gas mix for 4 min with 20 sL h^{-1} per flask.

Cultivation of *N. cavascurensis*

The growthrate (µ) of pure cultures increased considerably, most likely due to the buffered pH, and therefore passaging intervals were reduced to 3-4 days (700 µmol L^{-1} NO_2^- produced), 5 % v/v seemed to be the most reliable inoculation volume in terms of reproducibility and high µ. After the determination of the optimal oxygen concentration, the atmosphere was changed to 5 % O_2, 5 % CO_2 and 90 % N_2. Cryo stocks worked best with cultures grown with 1 mmol L^{-1} pyruvate and flash freezing aliquotes without further additives at -70 °C. Even though *N. cavascurensis* can deal with a wide range of O_2 concentrations, O_2 concentrations of reviving cultures should be kept the same as the cryo stock they were inoculated with. Solutions that were added to cultures for experimental reasons were always adjusted to pH 7.0 to avoid effects on µ due to suboptimal pH (NaNO2, NH_4Cl, PTIO, cPTIO).

Bioreactor experiments

The optimal pH of *N. cavascurensis* was determined with enrichments and pure cultures in 2 L all-glas bioreactors (Eppendorf AG, Hamburg, Germany) filled with 1.5 L medium as described above, except CO_3^{2-} was solely supplied by the gas mix. Enrichments were grown at pH 5.0, 6.0, 6.5 and 7.0 at 400 rpm stirring speed with 10 % O_2, 5 % CO_2, rest N_2 at 20 sL L^{-1} h^{-1} gassing rate, which was turned off after 16.5 h. Pure cultures were grown at pH 5.0, 6.0, 7.0 and 8.0 at 400 rpm stirring speed with 5 % O_2, 1 % CO_2, rest N_2 at 2 sL L^{-1} h^{-1} gassing rate. The CO_2 concentration of the gas mix was reduced due to precipitation at pH 8.0. pH was controlled in both experiments with 0.5 mol L^{-1} HCl and 0.5 mol L^{-1} NaOH.

Enrichment and cultivation of *Ca.* Thermus sp. for nitrite reductase assays

Ca. Thermus sp. was enriched from SCU2B enrichment glycerol stocks (30 % glycerol) by inoculating (2.5 % v/v) 40 mL FWM containing 5 mmol L^{-1} $NaHCO_3$, 200 µmol L^{-1} NH_4Cl, MTE, vitamines, FeNaEDTA and 1 g L^{-1} peptone in 120 mL serum flasks with blue butyl rubber stoppers and air as atmosphere. The culture was incubated at 68 °C, shaken at 100 rpm and used to inoculate (2 % v/v) 50 mL FWM with 10 mmol L^{-1} HEPES pH 7.5, 2.5 g L^{-1} Tryptone, 1 g L^{-1} yeast extract, MTE, FeNaEDTA, Vitamines and 4 mmol L^{-1} $NaNO_2$ in 120 mL serum flasks with blue butyl rubber stoppers and air as atmosphere. Once NO_2^- was consumed to about 1.3 mmol L^{-1}, cultures were passaged with 2 % v/v to new medium but this time with N_2 as atmosphere. Once all NO_2^- was consumed, 4 mmol L^{-1} $NaNO_2$ were added to the cultures twice to increase biomass before cultures were harvested in 50 mL centrifugation tubes (Greiner) at 4000 rpm for 30 min at 4°C and stored at -20 °C until further usage.

Sampling

1 mL of culture were sampled and centrifuged in 1.5 mL reaction tubes at 23k rcf at 4 °C for 30 min. Supernatant was used to measure NO_2^- and NH_4^+ concentrations to follow growth as described before (Tourna et al., 2011) and the cell pellet was stored at -20 °C for possible DNA extraction.

DNA extraction

DNA was extracted from cell pellets by beat beating using SDS-buffer, phenol/chloroform/isoamylalcohol [25:24:1 (v/v/v), Fisher BioReagents] and chloroform/isoamylalcohol [24:1 (v/v)] as already described elsewhere (Abby et al., 2018; adapted from Griffiths et al., 2000; Zhou et al., 1996).

PCR

Purification of *N. cavascurensis* cultures after Carbenicillin treatment was confirmed by PCR of the bacterial 16S rRNA gene using the primers Eubac27F [5‘-AGA GTT TGA TCC TGG CTC AG-3‘ (Lane et al., 1991)] and Eubac1492R [5‘-GGT TAC CTT GTT ACG ACT T-3‘ (Lane et al., 1991)] and archaeal 16S rRNA gene using the primers A109F [5‘-ACK GCT

CAG TAA CAC GT-3‘ (Großkopf et al., 1998)] and A1492R [GYY ACC TTG TTA CGA CTT-3‘ (Nicol et al., 2008)]. PCR conditions were 95 °C for 5 min as initialization, followed by 35 cycles of 30 s denaturing at 94 °C, 30 s primer annealing at 55 °C, 2 min elongation at 72 °C, and finishing with final elongation at 72 °C for 10 min. Reactions were done in 25 µL containing 2 µL sample, 0.15 µL GoTaq Polymerase, 5 µL 5x Flexi Buffer, 2 µL MgCl2 25 mmol L^{-1}, 0.5 µL dNTP 10 mmol L^{-1}, 0.25 µL BSA 20 mg mL^{-1}, 1.25 µL Primer F 10 µmol L^{-1}, 1.25 µL Primer R 10 µmol L^{-1}, 12.6 µL nuclease free water.

Quantitative PCR

The cell number of *N. cavascurensis* was quantified by qPCR of the archaeal 16S rRNA gene using the primers Arch931F [5‘-AGG AAT TGG CGG GGG AGC A-3‘ (Jackson et al., 2001)] and Arch1100R [5‘-BGG GTC TCG CTC GTT RCC-3‘ (Ovreas et al., 1997)] in triplicate 20 µL reactions containing 10 µL qPCR Master Mix 2x (Luna Universal qPCR Master Mix, NEW ENGLAND BioLabs Inc.), 1 µmol L^{-1} of each primer, nuclease free water and 4 µL DNA sample or standard. Reactions were performed in a qPCR (BIO-RAD CFX Connect Real-Time System) with initialization at 95 °C for 2 min, 40 cycles of 30 s denaturing at 95 °C, 30 s joint annealing-extension at 60 °C, and extension with fluorescence measurement at 60 °C for 30 s. The specificity of qPCR products was confirmed by melting curve analysis. Standards were prepared by amplifying the 16S rRNA gene of *N. cavascurensis* under the conditions as described above for PCR of the archeal 16S rRNA but in a 50 µL reaction using 2 µL DNA template. PCR product was cleaned up using the Machery-Nagel DNA cleanup kit and DNA concentration was measured with QubitTM dsDNA BR Assay Kit (Thermo Fisher Scientific) before preparing serial dilutions with nuclease free water. The standard curve had an efficiency of 90.67 % and an R^2 of 0.999.

Electron microscopy

S-layer fragments were prepared according to sonication assisted extraction first described by Pfeifer *et al. (2022)* with slight modifications. Late exponential cells were harvested from 60 mL of culture by centrifugation (20000 g, 4°C, 30 min) and resuspended in 5mL Buffer A (10 mmol L−1 NaCl, 0.5% sodium lauroylsarcosine) and incubated at 37 °C and 80 rpm for 1 h. The suspension was sonicated for 30 s with 10 A (Thermo Fischer Q700, Thermo Fisher Scientific Inc., Eindhoven, The Netherlands) to shear the DNA. Subsequently, the cell ghosts were pelleted by centrifugation (20,000 g, room temperature 30 min). The resultant pellet was resuspended in 5mL buffer B (10 mmol L^{-1} NaCl, 0.5 mmol L^{-1} MgSO4, 0.5% sodium dodecylsulfate (SDS)), incubated at 40 °C for 1 h and then pelleted by centrifugation (20,000 g, room temperature 30 min). This process was repeated 3 times to completely remove lipids. Subsequently, the obtained sacculi were washed twice with MQ. 10 µL of the sacculi were absorbed onto a glow-discharged carbon-coated copper grid for 30 seconds, negatively stained with 2% Uranyl acetate for 10 sec and analyzed (Libra 120, Zeiss).

For the preparation of thin sections, late exponential cells were harvested from 60 mL of culture by centrifugation (16000 g, 4°C, 30 min) and resuspended in 2 mL of 2.5%

glutaraldehyde in medium. After incubating for two hours at room temperature, the cells were harvested through centrifugation (16000 g, room temperature, 30 min) the cells were further fixed with 0.1% osmium tetroxide in 0.02 mM sodium cacodylate overnight. The pellet of fixed cells was embedded into Low Viscosity Resin (Agar scientific; Agar Low Viscosity Resin Kit) according to the manufactures instruction and cut into 40 nm section (LEICA EM UC6). The sections were absorbed onto formvar coated copper grids, contrasted (2.,5% Gadolinium acetate, 30 min; 3% Lead citrate, 8 min) and analyzed using a Libra 120 (Zeiss).

Nitrite reductase assay

A discontinuous assay was used to assess nitrite reduction activities in crude cell extracts (CCE) of *Ca.* Thermus sp., *N. cavascurensis* and *N. viennensis*. The reaction mixture consisted of a 25 mmol L^{-1} potassium phosphate buffer at pH 7, 0.5 mmol L^{-1} $NaNO_2$, 5mmol L^{-1} methyl viologen $[MV]^{2+}$ as the electron donor, crude cell extract at 10-50 µg total protein pre-incubated with 0.1 µmol L^{-1} $CuSO_4$ (Abraham et al., 1993) and was initiated by the addition of 10 mmol L^{-1} sodium dithionite. Samples were taken at different timepoints and the reaction was stopped by vigorous shaking to oxidize the remaining reduced MV. Quantification of nitrite was performed by the Grieß method. Initial reaction rates were determined as µmol NO_2^- reduced min^{-1} ng^{-1} NirK, assuming a relative NirK abundance in the cellular proteome of 2 %, based on existing shotgun proteomics data of AOA (Kerou et al., 2016). Control experiments without cellular extract were performed, and the rate of abiotic nitrite reduction was subtracted from the samples containing cellular extracts. The electron donor benzyl viologen (5 mmol L^{-1} $[BV]^{2+}$) and a system consisting of ascorbic acid as an electron donor to the electron carrier phenazine methosulfate (PMS)(10 mmol L^{-1} and 1 mmol L^{-1} respectively) were also assayed. Assays were repeated three times with fresh crude cell extract preparations, in technical triplicates.

Crude cell extracts of *Ca.* Thermus sp., *N. cavascurensis* and *N. viennensis* were prepared by resuspending cell pellets in lysis buffer (50 mM potassium phosphate pH 7.4, 0.2 mol L^{-1} NaCl, 2 mmol L^{-1} Pefabloc protease inhibitor) and sonicating them on ice for 5-10 times for 15 seconds. Protein concentration was measured by Qubit protein assay (ThermoFisher), and cell extracts were stored in aliquots at -20 °C until use.

NO-Scavanger and donor experiments

In preliminary experiments aqueous solutions of 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) or 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) were added to early exponential cultures (PTIO after 24 h, 134.1 ±6.4 µmol L^{-1} NO_2^-, cPTIO after 27 h, 172.8 ±11.1 µmol L^{-1} NO_2^-) in final concentrations of 5, 10, 20 and 40 µmol L^{-1} to find out at which concentrations growth would be reduced but not completely inhibited. To test if the inhibitory effect of PTIO and cPTIO could be alleviated by the addition of NO, early exponential cultures were exposed to 10 and 40 µmol L^{-1} aqueous PTIO or cPTIO which were incubated with the NO-donor Spermine NONOate prior to the addition to the cultures. To obtain 1.6 mmol L^{-1} stock solutions of PTIO (MW 233.35 g mol^{-1}) and Sodium cPTIO (299,28 g mol^{-1}), 3 mg and 4 mg respectively were solved in 4 mL of MQ in 15 mL PP tubes (Greiner) and 1.8 mg of Spermine NONOate (MW 262,35 g mol^{-1}) solved in

4 mL MQ were added to each of the solutions. Stock solutions contained 1.67 mmol L^{-1} PTIO, 1.61 mmol L^{-1} cPTIO and 0.858 mmol L^{-1} Spermine NONOate of which each molecule releases two molecules of NO. Spermine NONOate has a half life of 40 min at 37 °C and pH 7.4, increasing with pH. Therefore NO-scavanger-donor solutions (pH 8.5 – 9.0) were adjusted with 80 µL of 0.1 mol L^{-1} HCl to pH 7.0 (measured with pH stripes) and incubated for 4 h at 37 °C after which 1.69 mmol L^{-1} NO should have been released by the NO-donor (98.4 % after 4 h at 37 °C and pH 7.4). The solutions were sterile filtrated with 0.2 µm filters in a laminar flow hood before 1 mL of stock solution (40 µmol L^{-1} final concentration) or a 1:4 dilution (10 µmol L^{-1} final concentration) were added to early exponential cultures.

Results & Discussion

Obtaining an axenic culture of Ca. N. cavascurensis

Initial ammonia oxidizing enrichment cultures were set up from samples derived from thermal water outflow of the freshwater hot spring in Terme di Cavascura, Ischia, Italy, as described in Abby *et al.* (2018). A diverse set of strategies was applied to increase the relative abundance and specific growth rate (µ) of *Ca.* Nitrosocaldus cavascurensis. Growth temperature optimization, omitting vitamin solution, reduction of inoculum as nitrite production increased, avoiding stationary phase by maintaining actively growing cultures (4 day passaging rhythm) and selecting the best out of multiple cultures for propagation (based on µ and relative abundance determined by microscopy) resulted in enrichment levels of 70 – 92 %, based on qPCR and cell counts. The remaining microorganisms could almost exclusively be assigned to the genus Thermus based on 16S rRNA amplicon sequencing (Abby et al., 2018).

From this starting point, a variety of strategies were employed to further reduce bacterial abundance and obtain a stable anxenic culture of AOA. A pH screen done in 2 L bioreactors indicated an optimal pH for the enrichment culture between 6.5-7.0 (Fig. S1a), which resulted in an increase of AOA abundance from 75 % to 90 % without the use of antibiotics (Fig. S1). Different buffer systems were applied in order to maintain a stable pH of 7, of which a carbonate buffer system with 6 mM NaHCO3, 5% CO2 and 10% O2 was the most effective (Fig. S1b), resulting in a 97% AOA abundance in conjunction with 12.5 µg mL^{-1} Kanamycin sulfate treatment (Fig. 1). Other buffer systems at pH 7.0 such as MES, MOPS, HEPES, Citrate, Bis/Tris, Phosphate (all at 10mM) impeded AOA growth completely.

Former antibiotic treatments with Sulfamethazine, Rifampicin and Novobiocin (100 µg mL^{-1}) with and without 0.1 mmol L^{-1} α-keto-acids (pyruvate, oxalacetate, glyoxylate) killed all cells of the enrichment cultures. These results were interpreted as a dependency of *Nitrosocaldus cavascurensis* on Thermus sp. due to the lack of a proposed nitrite reductase (NirK) in the AOA genome and the presence of a nirK gene in the Thermus genome. Further only few antibiotics were tested as most antibiotics were believed to be unstable at 68 °C. However,

the observed results could have been also the result of simply using too much or the wrong antibiotics and therefore killing the AOA by unspecific side effects. The application of various antibiotics with different modes of action and in a range of concentrations was then attempted in an effort to eliminate the remaining bacterial contaminants. Crucially, the inoculum cultures used in the antibiotic treatments were not exposed to antibiotics previously to avoid adaptation effects of the bacterial community. The effects of the antibiotics novobiocin (an inhibitor of bacterial DNA gyrase/type IIA topoisomerase), vancomycin and carbenicillin (both inhibitors of bacterial peptidoglycan cell wall synthesis) were investigated at concentrations ranging from 6.25 µg mL^{-1} to 50 µg mL^{-1} (Fig. S2). The application of low concentrations of carbenicillin (6.25 µg/ml) were successful in eliminating the remaining contaminants and achieving a pure culture of the AOA after another passage.

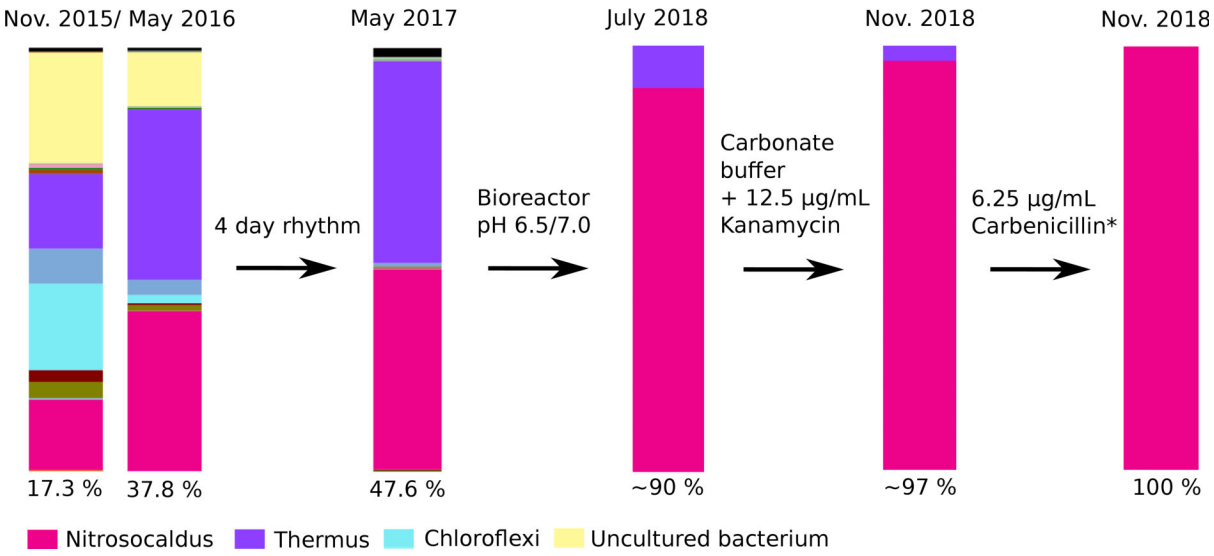

Figure 1: Enrichment strategy that led to a pure culture of *N. cavascurensis*. Establishing a four day passaging rhythm increased relative AOA abundance only slightly from 37.8 % to 47.6 % but reduced the complexity of the microbial community almost exclusively to *N. cavascurensis* and *Ca.* Thermus sp.. Stabilizing the enrichment culture at pH 6.5 or 7.0 in bioreactors increased AOA abundance up to about 90 %. Establishing a carbonate buffer system to stabilize the pH in closed batch cultures and treatment with Kanamycin increased the relative abundance further up to 97 %. Carbenicillin treatment of antibiotic unexposed enrichments (abundance of inoculum about 60 %) lead to a pure culture after another passage. Relative abundances of *N. cavascurensis* were determined by amplicon sequencing with universal 16S rRNA primers for November 2015 to May 2017 samples. Later relative abundances are based on phase contrast microscopy cell counts and the pure culture was confirmed by negative PCR with bacterial 16S rRNA primers after 35 cycles and cultivation with 1 mmol L^{-1} pyruvate and no antibiotics in the medium.

Morphology of N. cavascurensis SCU2

N. cavascurensis is spherically shaped with a diameter of 0.6 – 0.8 µm. The cells are enveloped by a S-layer lattice with p6-symmetry like N. maritimus (Qin et al., 2017) (Fig. 2), unlike the p3-symmetry observed with N. viennensis (Stieglmeier et al., 2014). Even though genes for chemotaxis and motility are present in the genome (Abby et al., 2018), mobile cells were never observed. Cells typically do not form agglomerates and can usually be observed alone or in pairs.

To be extended.

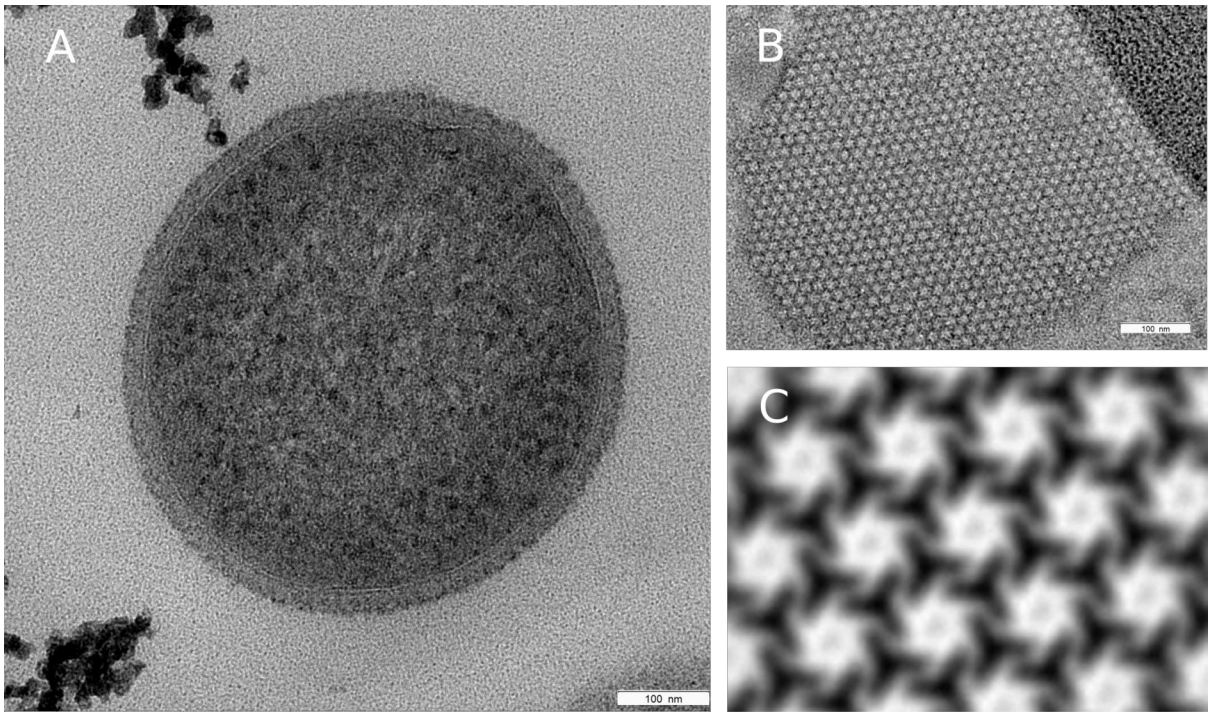

Figure 2: Morphology of *N. cavascurensis*. **(A)** Transmission electron micrograph of thin sections (contrasted with Uranyl acetate 2% and Lead citrate 2%). **(B)** High resolution transmission electron micrograph of negatively stained (2% uranyl acetate) S-layer fragments. **(C)** Computer image reconstruction (2D projection) of the p6-ordered hexagonal S-layer lattice from *N. cavascurensis*.

Physiological characterization of the strain SCU2

Strain SCU2 is a thermophilic and neutrophilic organism, growing optimally at 68 °C and pH 7.0. Energy generation is dependent on the aerobic oxidation of ammonia, resulting in the stoichiometric production of NO_2^- (Fig. 3). In serum flasks with a defined medium and carbonate buffer system SCU2B grows with a µ of 0.0442 h^{-1} based on NO_2^- production or 0.0437 h^{-1} based on 16S rRNA gene copy number, which corresponds to a generation time of 15.7 h or 15.9 h respectively. Compared to enrichment cultures (Abby et al., 2018) µ increased from 0.0301 h^{-1} to 0.0442 h^{-1} (+46.8 %) and the maximum cell number of 1 mmol L^{-1} NH_4Cl cultures increased from $1.26 \cdot 10^{-7} \pm 9.66 \cdot 10^{-5}$ cells mL^{-1} to $5.19 \cdot 10^{-7} \pm 7.08 \cdot 10^{-6}$ cells mL^{-1} (+312 %), likely as a result of pH stabilization and lack of bacterial competitors.

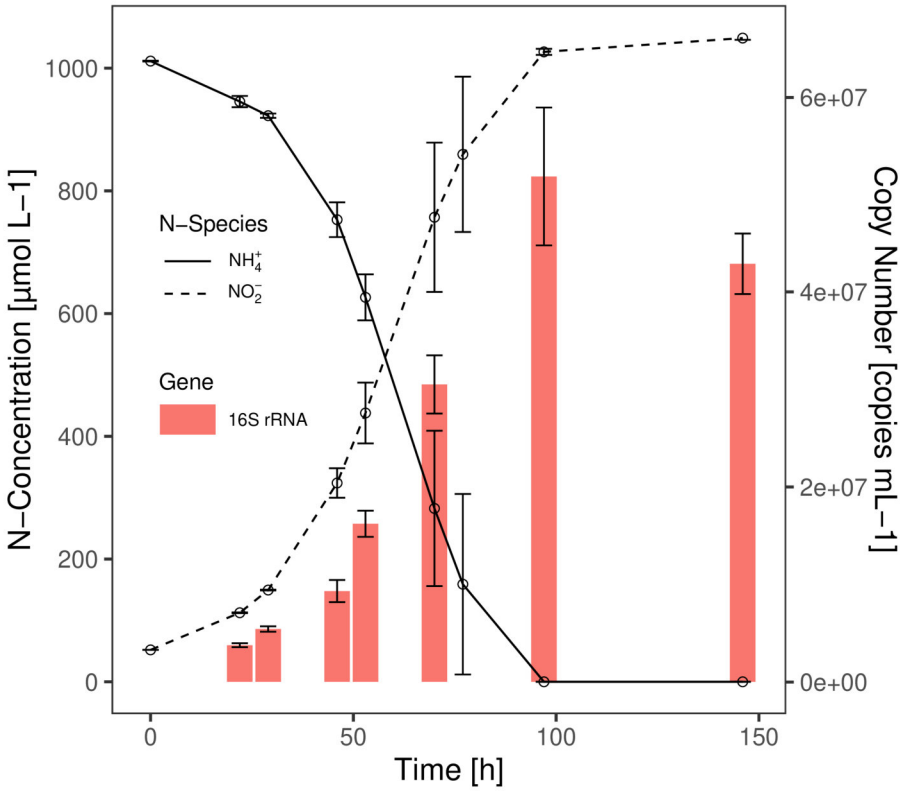

Figure 3: Growth of *N. cavascurensis* pure culture. Nearly stochiometric conversion of NH_3 to NO_2^- accompanied by an increase in cell number measured by qPCR of the 16S rRNA gene. Data points for NH_4^+ and NO_2^- show the mean of biological duplicates and technical triplicates were done for each biological duplicate to determine gene copy number. Error bars represent the standard deviation of the mean.

N. cavascurensis was grown in bioreactors with 2 sL L^{-1} h^{-1} gassing for determination of the optimal pH (see methods). As seen in the pH screen with enrichment cultures, SCU2B grows best at pH 7.0 but µ decreased considerably to 0.0187 h^{-1} compared to closed batch cultures (Fig. 4a), presumably due to the continuous removal of important metabolites.

Inhibitory effects of NH_4^+ concentration on the growth rate of the strain SCU2 started appearing after 4 mmol L^{-1} NH_4^+ and growth was observed up to 32 mmol L^{-1}, while only marginal NO_2^- production was observed at 64 mmol L-1 (Fig. 4b). NO_2^- reduced µ gradually with increasing concentrations and growth was observed up to 8 mmol L^{-1} NO_2^-, while complete inhibition occurred at 16 mmol L^{-1} NO_2^- (Fig. 4c). In order to prevent the cumulative inhibition effects of a low pH, the stock solutions of NH_4CL and $NaNO_2$ were previously adjusted to pH 7.0. The inhibition profiles indicate a rather high sensitivity of strain SCU2 to NH_4^+ and NO_2^- concentrations compared to mesophilic AOA. Kinetic analyses have shown that while the apparent substrate affinities for NH_3 for the thermophilic enrichment cultures *Ca.* Nitrosocaldus yellowstonensis HL72 and *Ca.* N. tenchongensis DRC1 are comparable to those exhibited by the mesophilic and neutrophilic AOA of genus *Nitrososphaera*, further increase of the substrate concentrations after V_{max} leads to a rapid reduction of the substrate oxidation rates (Jung et al., 2021). These analyses corroborate the increased sensitivity to reactive nitrogen species (RNS) stress observed here. While a proportion of the inhibition could be potentially attributed to the increased proportion of NH_3 as a fraction of total NHx with increasing temperatures (Emerson et al., 1975). Similar studies in *Nitrosomonas europaea* however indicated that temperature itself was affecting oxidation activity as an independent factor from substrate availability (Groeneweg et al., 1994).

Given the decreased solubility of oxygen in higher temperatures, we investigated whether thermophilic AOA can tolerate/are adapted to lower oxygen concentrations than mesophilic AOA. To this end, cultures of *N. cavascurensis* and *N. viennensis* were incubated with 2.5 %, 5 %, 10 % and 21 % O2 (*N. cavascurensis* additionally with 0.5 % and 1 %). Both strains grew with marginal variations in their growth rates up to 2.5 % for N.viennensis and 1 % for *N. cavascurensis*, indicating that they are well adapted to low oxygen concentrations (Zheng et al., 2020). The highest growth rates were achieved for both strains at an oxygen concentration of 5 %, corresponding to 44.6 µmol L^{-1} and 53.5 µmol L^{-1} dissolved O_2 at 68 °C/ 1.392 bar and 42 °C/ 1.106 bar respectively (Fig. 4d). In the light of recent research reporting oxygen production and consequently a minimal level of metabolic activity by the marine AOA strain *N. maritimus* (Kraft et al., 2022) after near-total consumption of available oxygen, it remains to be seen whether this is a general trait for AOA and whether this is observable also in the thermophilic strains faced with chronic oxygen limitations.

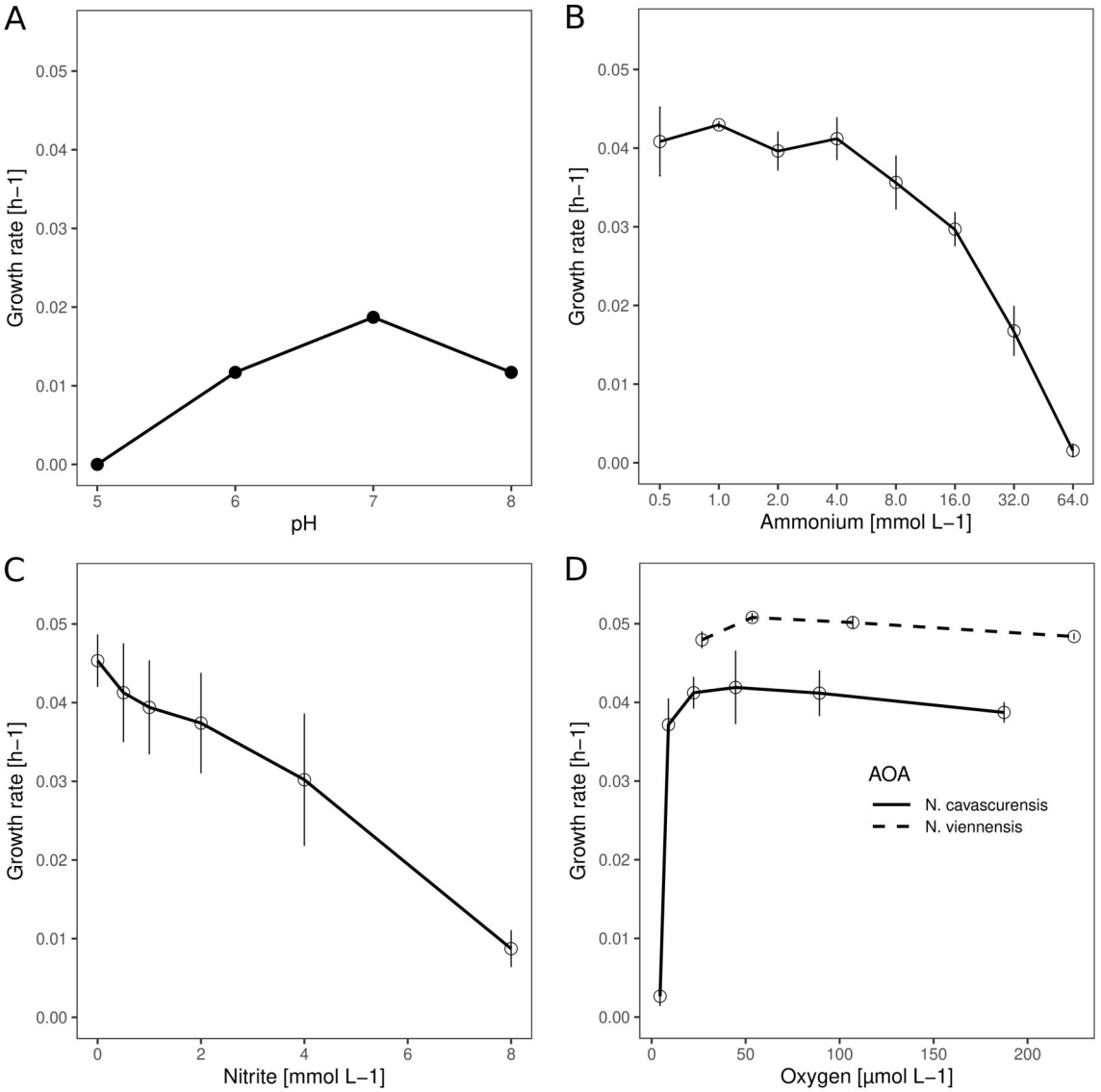

Figure 4: Physiological characterization of *N. cavascurensis.* (A) Optimal pH 7.0 determined in bioreactors. The reduced µ compared to closed batch experiments was most likely caused by the gassing of the reactors (2 sL L^{-1} h^{-1}). All following experiments were done at pH 7.0 in serum flasks. (B) Effect of initial NH_4^+ concentrations on µ. *N. cavascurensis* tolerates NH_4^+ well up to 4 mmol L^{-1} and grows up 32 mmol L^{-1} NH_4^+, at 64 mmol L^{-1} only marginal NO_2^- production was observed. Datapoints show mean values of biological triplicates and error bars indicate standard deviation of the mean. Please mind the semi-logarithmic scale. (C) Effect of initial NO_2^- concentrations on µ. NO_2^- reduces µ in a linear fashion and growth was observed up to 8 mmol L^{-1} NO_2^- (tested up to 16 mmol L^{-1}). Datapoints show mean values of biological triplicates and error bars indicate standard deviation of the mean. (D) Effect of different dO_2 concentrations on µ of *N. cavascurensis* in comparison with *N. viennensis*. Both AOA seem to slightly increase µ with decreasing dO_2 and have a maximum µ of 0.0419 ±0.0047 h^{-1} and 0.0508 ±0.005 h^{-1} at 44.6 and 53.5 µmol L^{-1} dO_2 (5 % dO_2) for *N. cavascurensis* and *N. viennensis* respectively. A steep drop of µ was observed for *N. cavascurensis* from 8.9 µmol L^{-1} dO2 (1 % dO_2) to 4.5 µmol L^{-1} (0.5 % dO_2) indicating the minimum dO_2 for effective growth (not determined for *N. viennensis*). Data points show the mean of quadruplets, except *N. cavascurensis* at 44.6 and 8.9 µmol L^{-1} show the mean of triplicates, and error bars indicate the standard deviation of the mean.

The addition of organic compounds such as the α-keto-acids pyruvate, oxaloacetate, glyoxylate at concentrations of 0.5 mmol L^{-1} – 1 mmol L^{-1} resulted in only minor stimulation of growth of the strain SCU2 (Fig. S3). As mentioned previously, *N. cavascurensis* SCU2 does not encode a catalase, but we can speculate that, as in other AOA that lack this enzyme, the existing peroxiredoxins are capable of dealing with ROS-induced damage, especially in the low O_2 concentrations encountered at these temperatures compared to temperate environments. An auxiliary effect in handling ROS damage cannot be ruled out, however, since we observed that pyruvate had a positive effect in the recovery of cryo-preserved cultures. Namely, cultures grown in the presence of pyruvate prior to flash-freezing in liquid nitrogen (see Methods) were able to recover and reach optimal growth rate faster than cultures grown in the absence of pyruvate (Fig. 5). More extensive experiments on cryo-preservation included addition of DMSO and glycerol. To our surprise, cryo-stocks were considerably more viable when an aliquot of the grown culture was directly flash-frozen in liquid nitrogen and stored at -70°C without any treatment or additives (although pyruvate increased viability, see above, Fig. 5).

Tests for alternative energy metabolisms

With the pure culture of *N. cavascurensis* at hand it became possible to test experimentally alternative energy metabolisms that were predicted from the genome content of *N. cavascurensis* and *Ca.* N. islandicus, respectively. Those included besides a type 3b (NiFe)-hydrogenase (Abby et al., 2018; Daebeler et al., 2018; Luo et al., 2021) proteins for the fermentation of aromatic aminoacids, in particular three transaminases (tyrosine, phenylalanine and aspartate) and an indolepyruvate oxidoreductase (Daebeler et al., 2018; Luo et al., 2021). Experiments with added hydrogen (+/- thiosulfate as electron acceptor) to growing cultures did not show any effect on growth or was rather inhibitory (Fig. S4). *N. cavascurensis* was also anaerobically incubated with different combinations of aromatic aminoacids with and without the addition of yeast extract, peptone, elemental sulfur or iron oxide (Table S1). However, no growth was observed based on microscopic observations. Therefore, the ability of the strain to ferment aromatic amino acids remains ambiguous.

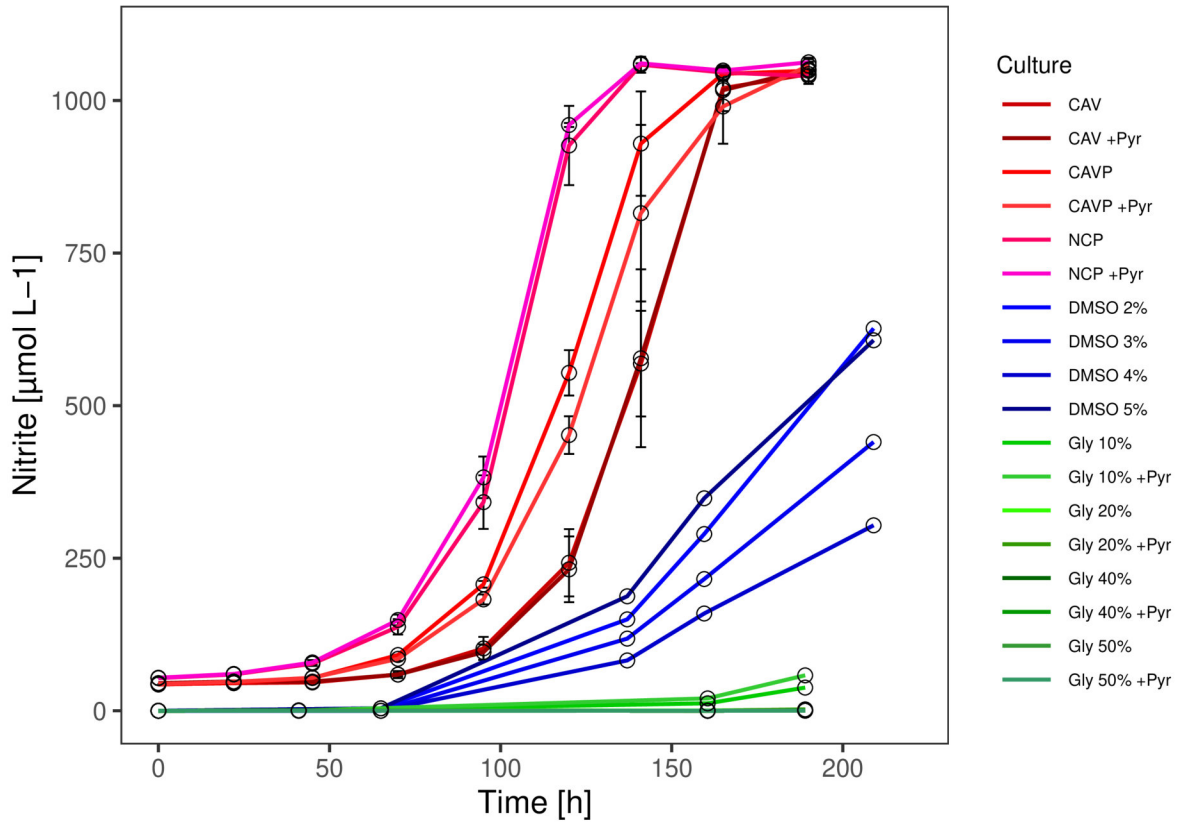

Figure 5: Methods for cryopreservation of *N. cavascurensis*. Initial tests with the cryo protectant DMSO showed moderate results with a lag phase of at least 65 h. Glycerol in concentrations of 10 to 50 % v/v had a detrimental effect and only cultures grown from cryo stocks with 10 % glycerol showed marginal growth after 160 h, however control cultures without any additives showed slightly better growth than DMSO cultures (data not shown). A follow up experiment investigated the performance of cryo stocks without any additives (CAV), 1 mmol L^{-1} pyruvate added before freezing (CAVP) and cryo stocks from cultures grown with 1 mmol L^{-1} pyruvate (NCP). Cultures indicated with "+Pyr" contained 1 mmol L^{-1} pyruvate in the medium. Best results were obtained with cryo stocks from cultures grown in the presence of pyruvate (22 h lag phase), adding pyruvate before freezing reduces the lag phase but the presence of pyruvate in the medium of the revived cultures does not seem to have any effect on the lag phase or growth rate. Cryo stocks with DMSO or glycerol were centrifuged at 23k g for 30 min at 4 °C and supernatant was removed prior to inoculation to reduce the detrimental effect of the cryo protectant on the growth which otherwise would be completely inhibited. However this harsh treatment might have very well damaged the cells. NO_2^- values of DMSO and Gly cultures are from single cultures and values of CAV, CAVP and NCP cultures represent the mean of duplicates and error bars indicate the standard deviation of the mean.

Phylogenetic placement of *N. cavascurensis*

Phylogenetic studies based on 16S rRNA, amoA and ribosomal proteins all suggest a thermophilic origin of the class *Nitrososphaeria* to which all AOA belong to (Abby et al., 2018, 2020; Daebeler et al., 2018; De La Torre et al., 2008; Luo et al., 2021). Members of the family Nitrosocaldaceae, to which *N. cavascurensis* belongs to, are all thermophiles and form a sister clade to all other AOA. Interestingly NirK is absent in all Nitrosocaldaceae except for members of the recently proposed genus *Ca.* Nitrosothermus (Luo et al., 2021), while it is present in all genomes of moderate AOA, except for the genome of *Ca.* Cenarchaeum symbiosum (Fig. 6).

To be extended.

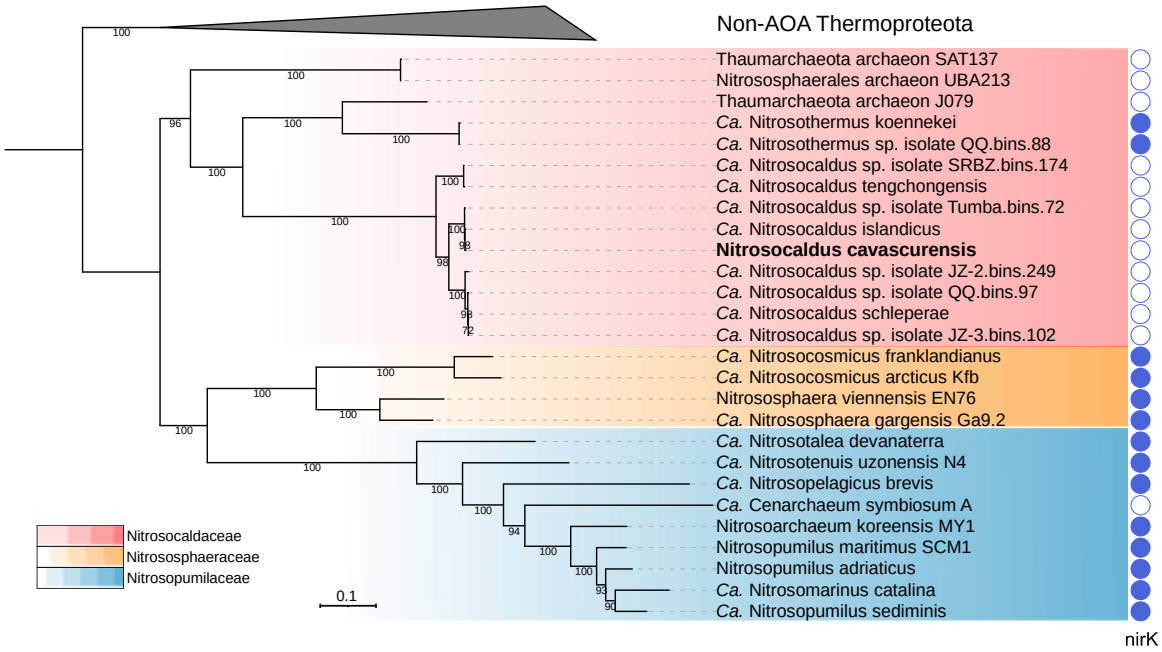

Figure 6: Phylogenetic tree of AOA based on ribosomal proteins with presence and absence of NirK in AOA genomes. *N. cavascurensis* is a member of the family *Nitrosocaldaceae* of which all known members are thermophiles. It is a sister clade to all other more moderate AOA that belong to the families *Nitrososphearaceae* and *Nitrosopumilaceae*. While NirK is present in all but one genome in moderate AOA (sponge symbiont Ca. Cenarchaeum symbiosum A), it is absent in all Nitrosocaldaceae except for members of the genus Ca. Nitrosothermus.

Investigation of the effect of NO in the metabolism of strain SCU2 and searching for nitrite reductase activity

While neither a canonical dissimilatory copper-containing nitrite reductase family enzyme, nor other types of nitrite reductases (NirS, ccNiR), were identified in the genome of *N. cavascurensis* SCU2, the central role of NO in AOA physiology compels us to consider the effects of this reactive compound in the physiology of the thermophilic strain, as well as other possible routes for NO production. Direct measurement of NO production above 40 ºC has so far not been possible, due to inherent temperature limitations in the appropriate instruments (e.g. temperature limitation in Clark-type electrodes), or the observed transient appearance of the compound in mesophilic AOA cultures (Kozlowski et al., 2016) which disqualifies end-point measurements.

Effects of NO-scavengers and NO-donors

Another method for testing the central role of NO in the metabolism of AOA is the application of the NO-scavangers PTIO (Abby et al., 2018) or cPTIO. In enrichment cultures *N. cavascurensis* showed a high sensitivity to PTIO (<20 µmol L-1), which was further investigated together with cPTIO to find the concentration range at which NO_2^- production would be reduced but not completely inhibited. PTIO caused strong reduction of NO2- production with 10 µmol L-1 and complete inhibition with 20 µmol L-1, while cPTIO had a less severe effect on the NO2- production, still showing growth with 20 µmol L-1 and complete inhibition at 40 µmol L-1 (Fig. S5). To test if the inhibitory effect of NO-scavangers truly comes from removing an important metabolite or by unspecific toxicity, the NO-donor Spermine NONOate was added to PTIO/ cPTIO solutions prior to the addition to early exponential cultures (Fig. 7). Even though NO was supplied in slight excess and solutions were incubated as described in materials and methods, no alleviation of the inhibitory effect of PTIO and cPTIO was observed. Therefore the observed inhibitory effect of the tested NO-scavangers can not solely be accounted for by the removal of NO but unspecific toxicity of the NO-scavangers has to be taken into consideration as well.

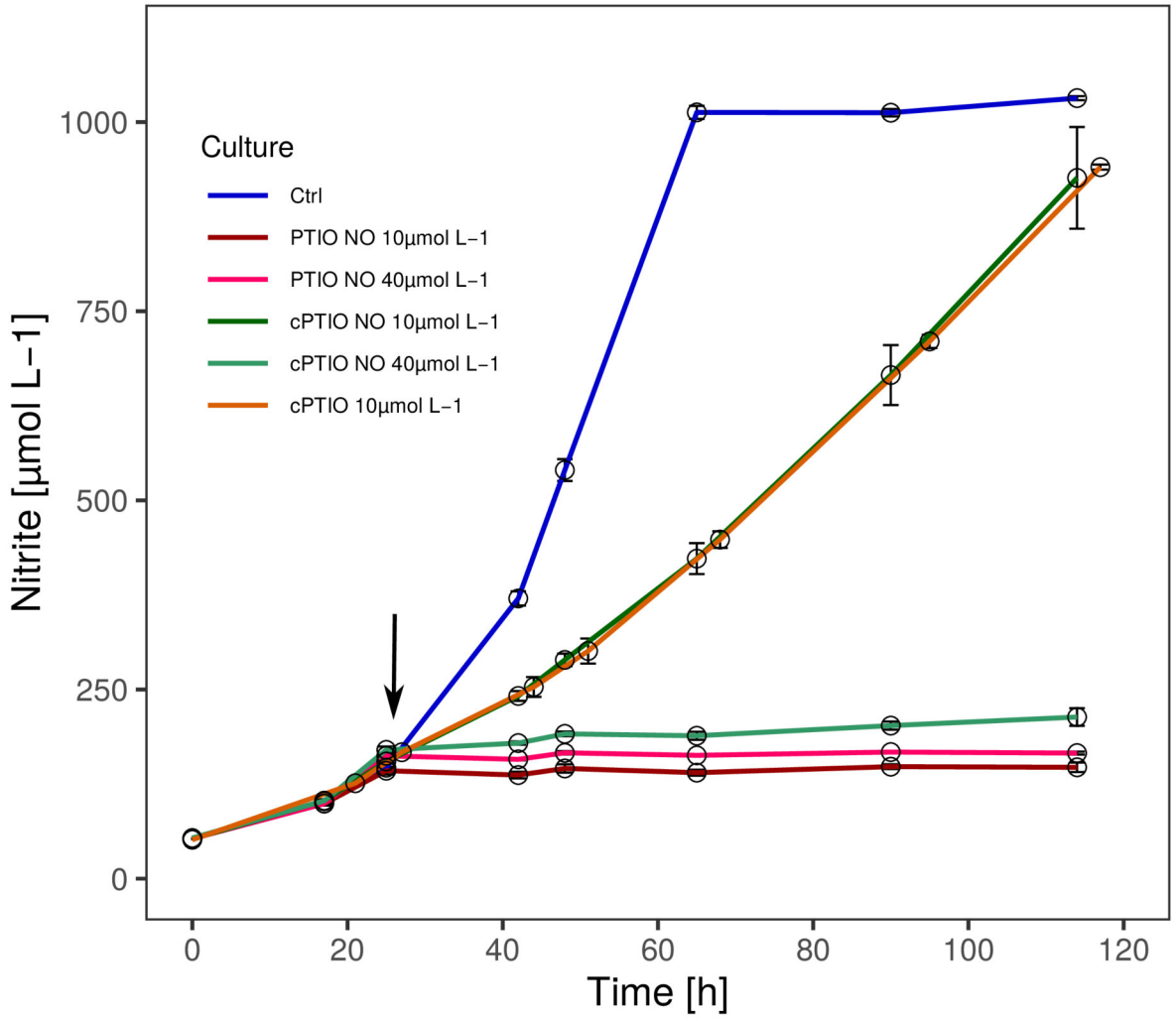

Figure 7: Effect of the NO-scavangers PTIO and cPTIO together with the NO-donor Spermine NONOate on growth of *N. cavascurensis*. To test if the inhibitory effect of NO-scavangers on the growth of SCU2B could be alleviated, the NO-donor spermine NONOate was added to the stock solutions of PTIO and cPTIO in slight surplus of NO and incubated for 4 h at 37 °C at pH 7.0 prior to the addition to the cultures. No change of the inhibitory effect of both NO-scavangers was observed, particularly evident by the comparison of cultures with 10 µmol L^{-1} cPTIO and 10 µmol L^{-1} cPTIO together with spermine NONOate. It is therfore questionable, if the inhibitory effect of the NO-scavangers comes solely from the uptake of NO ,a hypothesized intermediate and cosubstrate of the archaeal ammonia oxidation, or if there are unspecific toxic side effects of the NO-scavangers that inhibit growth of SCU2B. The addition of NO-scavanger/ NO-donor and 10 µmol L^{-1} cPTIO solutions is marked by the arrow. Control cultures (Ctrl) were grown in quadruplets, PTIO and cPTIO with spermine NONOate (PTIO NO, cPTIO NO) in triplicates and cPTIO without spermine NONOate (cPTIO) in duplicates. Nitrite values represent the mean and error bars indicate the standard deviation of the mean.

Nitrite reductase activity assays with crude cell extracts

We investigated the possibility of a nitrite reduction reaction being performed by an unidentified enzyme by screening crude cell extract preparations of *N. cavascurensis* for nitrite consumption using a modified assay by Kakutani et al. (1981). The assay relies on the presence of an electron donor such as the dication methyl viologen $[MV]^{2+}$ which has a midpoint redox potential of −446mV and is membrane impermeable, which under anaerobic conditions enables catalysis by the putative nitrate reductase of the one-electron reduction of NO_2^- to NO (see Methods). Crude cell extracts of *N. cavascurensis* were assayed for the presence of a nitrite reduction activity at temperatures 55ºC -68ºC, under anaerobic conditions, using MV reduced with an excess of dithionite as the electron donor. As a positive control, the remaining contaminant bacterium of the initial enrichment culture, affiliated with the genus Thermus, was also purified and was shown to be able to grow in the presence of nitrite. A putative copper-containing nitrite reductase gene had previously been identified on a contig affiliated with the lineage Deinococcus/Thermus in the metagenomic analysis of the enrichment culture presented in Abby et al. (Abby et al., 2018). As a control for nitrite reductase activity of an AOA we used crude cell extracts of *Nitrososphaera viennensis*.

Nitrite consumption rates in assays containing crude cell extract of the strain SCU2 were similar to the non-enzymatic control incubations, indicating that under these assay conditions, there was no detectable nitrite reductase reaction performed by the strain (Fig. 8, Table 1). In contrast, crude cell extract preparations of *Ca.* Thermus sp. exhibited nitrite reductase activity reproducibly at all temperatures tested (55-68ºC), with the optimal activity observed at 60ºC (Fig. 8, Table 1) and initial rates of $6.21 \cdot 10^{-5}$ µmol of nitrite reduced/min/ng of NirK, assuming a relative abundance of NirK of 2%, (based on available shotgun proteomics data for *N. viennensis*). Nitrite reductase activity was also observed with crude cell extracts of *N. viennensis* done at 42 °C with MV as electron donor (Fig. 8). The observed minimal nitrite reduction rates in *N. cavascurensis* samples can be attributed to the fact that a crude cell extract preparation was used which naturally contains reactive compounds able to react with nitrite, an extremely reactive molecule itself.

Alternative electron donors were also considered: benzyl viologen $[BV]^{2+}$, which has a midpoint redox potential of −350mV, is membrane-permeable in its radical form (Jones & Garland, 1977) and is also reduced by sodium dithionite; and a system consisting of ascorbic acid as an electron donor to the electron carrier phenazine methosulfate (PMS), with a higher redox potential of +80mV (Zumft & Vega, 1979). While both alternative electron donor systems were able to reduce the putative nitrite reductase of the positive control *Ca.* Thermus sp., and result in initial reaction rates of $4.71 \cdot 10^{-5}$ and $2.98 \cdot 10^{-5}$ µmol of nitrite reduced/min/ng NirK for BV and PMS respectively (Table 1), no activity was observed for crude cell extracts of strain SCU2 (Fig 8, Table 1). Additionally, mixing CCEs of *Ca.* Thermus and *N. cavascurensis* resulted in assay rates similar to *Ca.* Thermus, indicating that the N. cavascurensis CCE does not contain any inhibitory compounds.

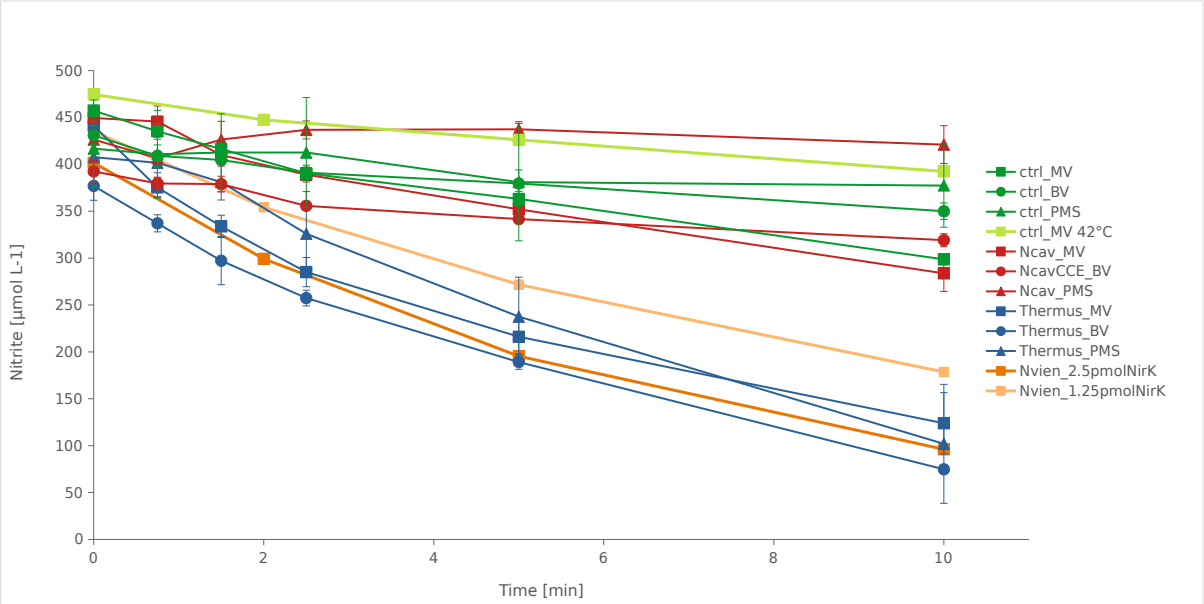

Figure 8: Nitrite reductase assay with crude cell extract preparations from *Ca.* Thermus, *N. cavascurensis*, *N. viennensis* and non-enzymatic controls with different electron donors: methyl viologen (MV, ■), benzyl viologen (BV, ●) and phenazine methosulfate (PMS, ▲). Samples were incubated anaerobically at 60 °C (*Ca.* Thermus, *N. cavascurensis*) in technical triplicates or 42 °C (*N. viennensis*) in technical duplicates.

Tabel 1: Nitrite reduction rates of crude cell extracts of *Ca.* Thermus sp. and *N. cavascurensis*. CCE were incubated anaerobically at different temperatures with the electron donors/ carriers methyl viologen (MV), benzyl viologen (BV), ascorbic acid/ phenazine methosulfate (A/PMS).

Initial rates (µmol NO2/min/ng NirK) *					
electron donor	**Temp (ºC)**	**Ca. Thermus CCE**	**N.cav CCE**	**Therm+Ncav CCE 1:1**	**notes**
MV	60	6.21E-05	4.97E-06		average of 3 biological assay replicates
MV	68	4.50E-05	9.66E-06	4.73E-05	average of 3 biological assay replicates
MV	55	6.92E-05	0		
BV	60	4.71E-05	0		
A/PMS	60	2.98E-05	0		

*assuming NirK is 2% of the cellular proteome

Our inability to detect a bona-fide nitrite reductase activity in crude cell extracts of strain SCU2 does not preclude the possibility that NO is produced by another reaction, such as the oxidation from hydroxylamine (NH_2OH), as described for the hydroxylamine oxidoreductase (HAO) from AOB (Caranto & Lancaster, 2017; Kostera et al., 2010). This possibility exists in general for AOA, considering that the role of the nitrite reductase in mesophilic AOA remains unclear and the second step enzyme remains uncharacterized.

Strain description

Here we describe the first thermophilic strain of ammonia oxidizing archaea in pure culture.
Nitrosocaldaceae fam. and
Nitrosocaldus cavascurensis spec. nov.
Etymology: L. adj. nitrosus, “full of natron,” here intended to mean nitrous (nitrite producer); L. masc.n. caldus, hot;
cavascurensis (L.masc. gen) describes origin of sample (Terme di Cavascura, Ischia)
Locality: outflow from hot underground spring, muddy, 77 °C
Diagnosis: an ammonia oxidizing archaeon growing optimally around 68 °C at neutral pH under chemolithoautotrophic conditions with ammonia or urea, spherically shaped with a diameter of approximately 0.6–0.8 µm, 4% sequence divergence in 16S rRNA gene from its next cultivated relative *Ca.* Nitrosocaldus yellowstonensis, does not contain nirK gene but is sensitive to PTIO and c-PTIO.

Description of *Nitrosocaldaceae* fam. nov.
Nitrosocaldaceae (Ni.tro.so.cald.ace.ae. N.L. fem. n. *Nitrosocaldus* type genus of the family; L. suff. -aceae ending to denote a family; N.L. fem. pl. n. *Nitrosocaldaceae* the family of the genus *Nitrosocaldus*).

The description is the same as for the genus *Nitrosocaldus.*
The type genus is *Nitrososcaldus.*

Conclusion

With this study we present the first pure culture of an extremely thermophilic AOA of the familiy *Nitrosocaldaceae*. It grows optimal at 68 °C, pH 7.0 under reduced O2 concentrations and is rather sensitive to NH_3 and NO_2^-. Unlike with moderate AOA the addition of organics does not increase growth, but a positive effect of pyruvate was visible with cryo stocks. Alternative energy metabolisms like aromatic amino acid fermentation or hydrogenase activity that were predicted from its genome could not be confirmed experimentally. The role of NO in the archaeal ammonia oxidation remains ambiguous, because no nitrite reductase activity could be observed with CCE. NO-scavenger experiments with live cells showed inhibition, but due to possible toxic side effects of the substance the interpretation of this results is unclear. Future studies will hopefully share more light on the topic of alternative energy metabolisms as well as the role of NO in the archaeal ammonia oxidation.

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Supplementary Materials

Supplementary Figures

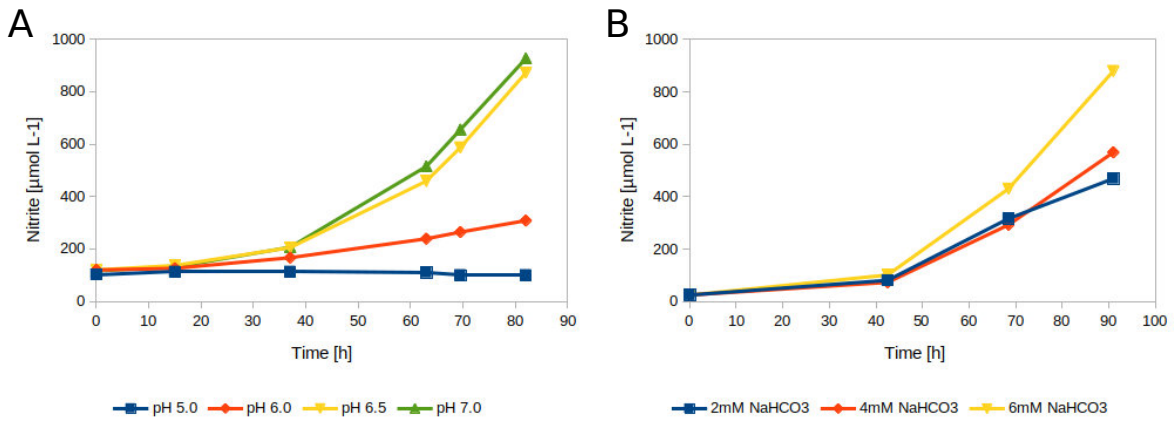

Figure S1: Optimization of pH and carbonate buffer with SCU2B enrichment cultures. **(A)** The optimal pH was determined in 2 L all-glas bioreactors filled with 1.5 L medium and an atmosphere of 10 % O_2, 5 % CO_2, rest N_2 (no gassing). The pH was controlled at pH 5.0, 6.0, 6.5 and 7.0 by titration with 0.5 mol L^{-1} HCl and 0.5 mol L^{-1} NaOH. Cultures grown at pH 6.5 and 7.0 showed highest µ together with a strong increase of the relative abundance of N. cavascurensis from about 75 % of the inoculum to 90 % (determined by microscopy). **(B)** Optimization of the carbonate buffer system. Cultures were grown in serum flasks with an atmosphere of 10 % O_2, 5 % CO_2, rest N_2 and 2, 4 and 6 mmol L^{-1} $NaHCO_3$ to buffer the pH between pH 6.5 and 7.0. The buffer system with 6 mmol L^{-1} $NaHCO_3$ and 5 % CO_2 showed the best results in terms of µ and relative abundance. All data points show NO_2^- concentrations of single cultures.

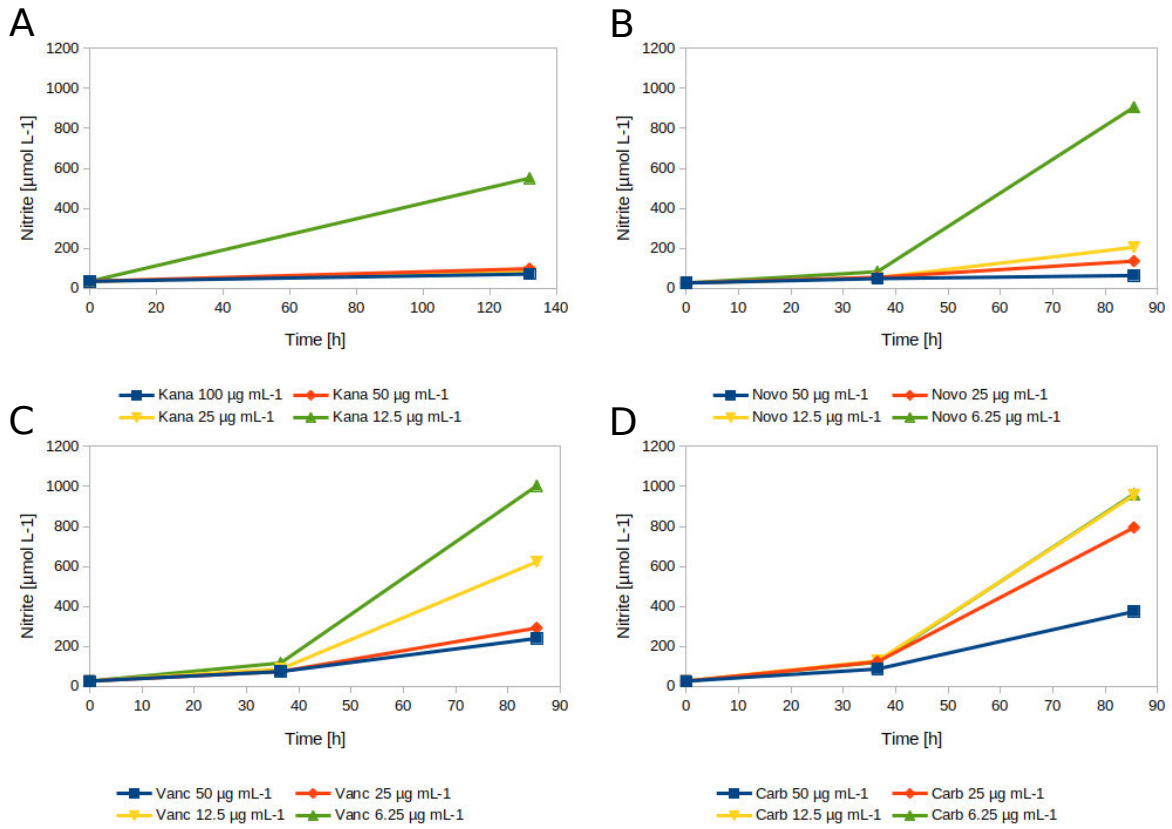

Figure S2: Antibiotic treatment of SCU2B enrichment cultures. Enrichment cultures were treated with Kanamycin **(A)**, Novobiocin **(B)**, Vancomycin **(C)** and Carbenicillin **(D)** with concentrations ranging from 12.5 to 100 µg mL^{-1} for Kanamycin or 6.25 µg mL^{-1} for the other antibiotics. Only Carbenicillin treatment with 6.25 µg mL^{-1} produced a culture were no bacterial cells could be detected by microscopy. Later verification with PCR using bacterial 16S rRNA primers showed that a pure culture was obtained after a second treatment with 6.25 µg mL^{-1} Carbenicillin.

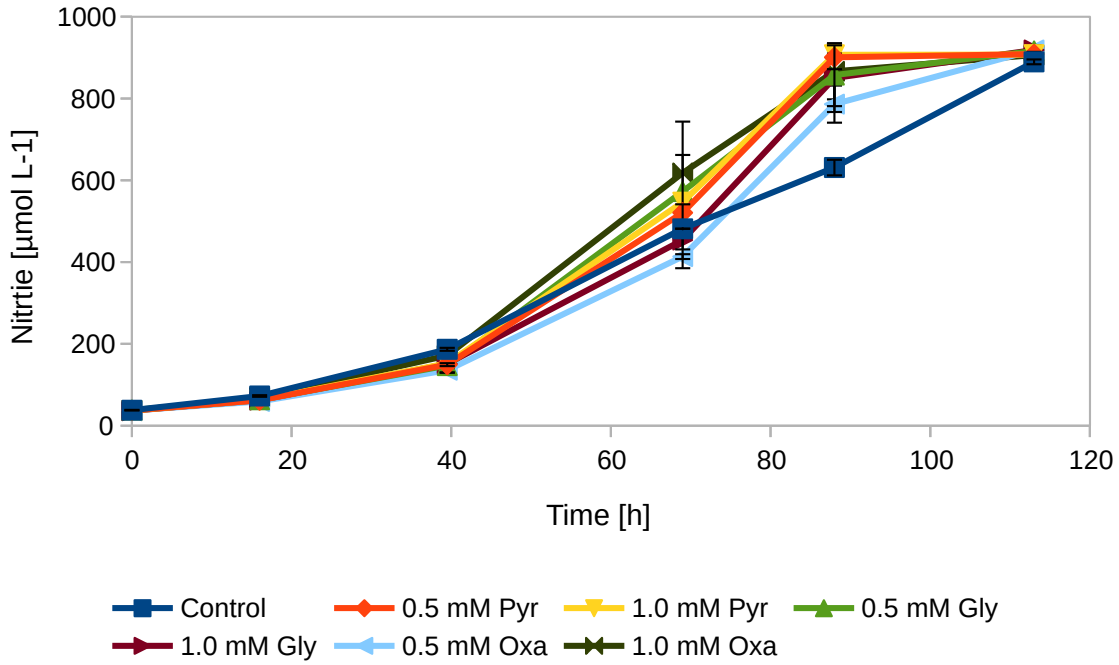

Figure S3: Effect of ROS-scavanging organics on the growth of *N. cavascurensis*. Pyruvate (Pyr), Glyoxylate (Gly) and Oxaloacetate (Oxa) were added in concentrations of 0.5 and 1.0 mmol L^{-1} to test for the growth enhancing effect of α-keto acids as observed with other AOA. Only a very limited effect was observed at mid to late exponential growth phase compared to the cultures without organics (Control). Unfortunately Control cultures of this experiment exhibited an unusual decrease of µ in the late exponential growth phase, therefore over examplifying the effect of organics on µ of N. cavascurensis. NO_2^- values represent the mean of duplicates and error bars indicate the standard deviation of the mean.

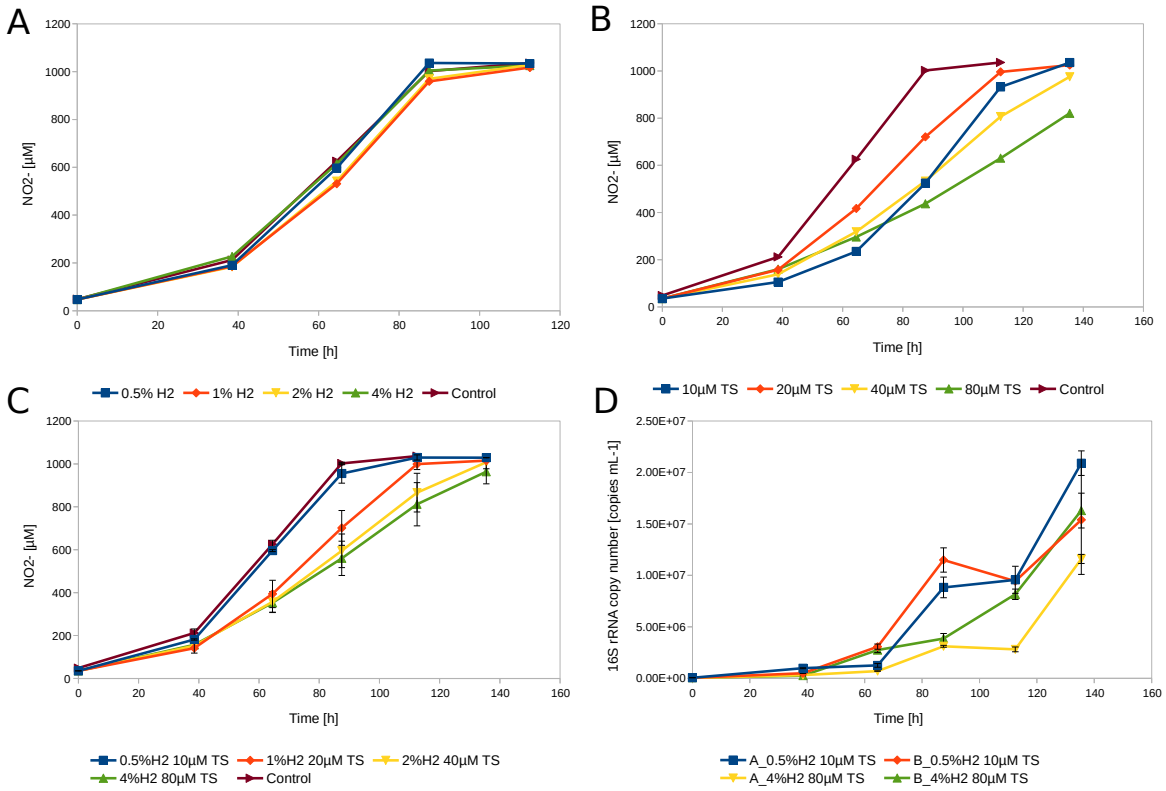

Figure S4: Hydrogenase cultivation experiment. **(A)** Cultures grown with different concentrations of H_2 in the atmosphere with no effect on nitrite production observed. **(B)** Cultures grown with different concentrations of thiosulfate which seemed to have a rather detrimental effect on nitrite production. **(C)** Cultures grown with different concentrations of H_2 and thiosulfate confirm the detrimental effect of thiosulfate on nitrite production. Nitrite values show the mean of biological duplicates and error bars indicate the standard deviation of the mean. **(D)** Determination of copy number by qPCR of 16S rRNA gene of cultures grown with 0.5 % H_2 and 10 µmol L^{-1} thiosulfate and cultures grown with 4 % H2 and 80 µmol L^{-1} thiosulfate. The reduced nitrite productivity could have been the result of H_2 beeing used as alternative energy source, but copy number decreased with higher H_2/ thiosulfate concentrations. However the reduced copy number could have also been the result of toxic effects of thiosulfate. Copy number values show the mean of technical triplicates and the error bars indicate the standard deviation of the mean.

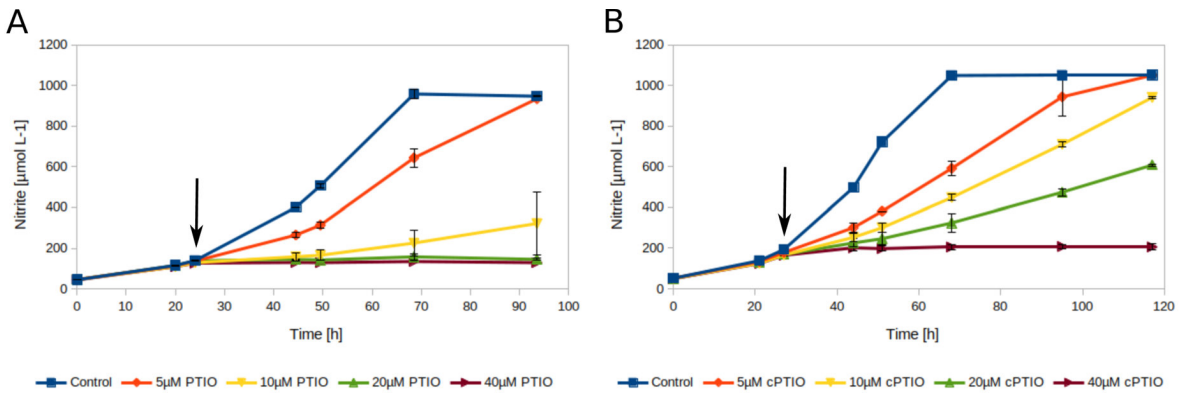

Figure S5: Inhibitory effect of PTIO and cPTIO on N. cavascurensis. The NO-scavangers PTIO **(A)** and cPTIO **(B)** were added in concentrations of 5, 10, 20 and 40 µmol L^{-1} to cultures in early exponential growth phase as indicated by the arrows. PTIO completely inhibited growth at concentrations as low as 20 µmol L^{-1} whereas cPTIO showed a more gradual inhibitory effect with increasing concentration and completely prevented growth at 40 µmol L^{-1}. Nitrite values show the mean of duplicates and error bars indicate the standard deviation of the mean.

Supplementary Tables

Table S1: Fermentation of aromatic amino acids. All setups had an 95% N2 5% CO2 atmosphere, with 100 µmol L^{-1} NH4Cl, 6 mmol L^{-1} NaHCO3, Trace elements, 7.5 µmol L^{-1} FeNaEDTA in fresh water medium. No growth was observed under any condition.

Tyrosine [0.5 mM]	Phenylalanine [0.5 mM]	Tryptophan [0.5 mM]	Yeast extract [0.5 mM]	Peptone [0.5 mM]	Sulfur [1 spartula]	Fe(OH)3 [5 mM]
X	X	X	X	X	X	X
	X	X	X	X	X	X
	X	X			X	
	X	X				
	X	X		X		X
	X	X				X

Chapter IV

Analysis of biomass productivity and physiology of *Nitrososphaera viennensis* grown in continuous culture

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Analysis of biomass productivity and physiology of *Nitrososphaera viennensis* grown in continuous culture

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Abstract

Microbial ammonia oxidation is the first and usually rate limiting step in nitrification and is therefore an important step in the global nitrogen cycle. Ammonia-oxidizing archaea (AOA) play an important role in nitrification. Here, we report a comprehensive analysis of biomass productivity and the physiological response of *Nitrososphaera viennensis* to different ammonium and carbon dioxide (CO_2) concentrations aiming to understand the interplay between ammonia oxidation and CO_2 fixation of *N. viennensis*. The experiments were performed in closed batch in serum bottles as well as in batch, fed-batch, and continuous culture in bioreactors.

A reduced specific growth rate (μ) of *N. viennensis* was observed in batch systems in bioreactors. By increasing CO_2 gassing μ could be increased to rates comparable to that of closed batch systems. Furthermore, at a high dilution rate (D) in continuous culture (≥ 0.7 of μ_{max}) the biomass to ammonium yield ($Y_{(X/NH3)}$) increased up to 81.7% compared to batch cultures. In continuous culture, biofilm formation at higher D prevented the determination of D_{crit}. Due to changes in $Y_{(X/NH3)}$ and due to biofilm, nitrite concentration becomes an unreliable proxy for the cell number in continuous cultures at D towards μ_{max}. Furthermore, the obscure nature of the archaeal ammonia oxidation prevents an interpretation in the context of Monod kinetics and thus the determination of K_S. Our findings indicate that the physiological response of *N. viennensis* might be regulated with different enzymatic make-ups, according to the ammonium catalysis rate.

We reveal novel insights into the physiology of *N. viennensis* that are important for biomass production and the biomass yield of AOA. Moreover, our study has implications to the field of archaeal biology and microbial ecology by showing that bioprocess technology and quantitative analysis can be applied to decipher environmental factors affecting the physiology and productivity of AOA.

Keywords

Archaea, bioreactor, closed batch, batch, fed-batch, chemostat, carbon dioxide, biofilm

Introduction

Nitrification is the oxidation of ammonia (NH_3) to nitrate (NO_3^-) via the intermediate nitrite (NO_2^-). Ammonia oxidation is the first and usually rate limiting step in nitrification and is therefore

important for the global nitrogen cycle. For over a century ammonia oxidation was thought to be performed by the few bacterial genera *Nitrosomonas*, *Nitrosococcus* and *Nitrosospira*, until 20 years ago when evidence started to accumulate that archaea might play an important role in this process as well (Könneke et al., 2005; Treusch et al., 2005; Venter et al., 2004). *Nitrosopumilus maritimus* was the first isolate from a marine aquarium (Könneke et al., 2005; Qin et al., 2017) followed by *Nitrososphaera viennensis* (Stieglmeier et al., 2014; Tourna et al., 2011) from garden soil. Since then multiple isolates and enrichments of ammonia oxidizing archaea (AOA) have been established and characterized to further improve our understanding of these ubiquitously abundant organisms (Abby et al., 2018; Alves et al., 2019; Bayer et al., 2019; Blainey et al., 2011; Daebeler et al., 2018; De La Torre et al., 2008; Jung et al., 2014; Lebedeva et al., 2013; Lehtovirta-Morley et al., 2016, 2011; Nakagawa et al., 2021; Qin et al., 2017; Santoro et al., 2015; Sauder et al., 2018, 2017; Zhalnina et al., 2014). Viable habitats include oceanic crust (Jørgensen and Zhao, 2016; Nunoura et al., 2013; Zhao et al., 2020) deep sea sediments (Francis et al., 2005; Kerou et al., 2021; Nunoura et al., 2018; Park et al., 2008; Vuillemin et al., 2019; Zhao et al., 2019), marine water column (Bayer et al., 2019; Qin et al., 2017; Santoro, 2019), oxygen minimum zones (Bristow et al., 2016), various kinds of soils (Alves et al., 2019; Jung et al., 2014; Lehtovirta-Morley et al., 2016, 2011; Tourna et al., 2011; Zhalnina et al., 2014), fresh water ecosystems (French et al., 2021, 2012; Sauder et al., 2018), waste water treatment plants (Mußmann et al., 2011; Sauder et al., 2017), terrestrial hot springs (Abby et al., 2018; Daebeler et al., 2018; De La Torre et al., 2008; Dodsworth et al., 2011; Luo et al., 2020; Reigstad et al., 2008) and human skin (Moissl-Eichinger et al., 2017; Probst et al., 2013). AOA outnumber their bacterial counterparts, ammonia oxidizing bacteria (AOB), by orders of magnitude in many habitats (Adair and Schwartz, 2008; Hollibaugh et al., 2011; Karner et al., 2001; Leininger et al., 2006; Nicol et al., 2008), but their contribution to the nitrification process is still not completely resolved. While all known AOA and AOB are confined to oxidize NH_3 to NO_2^-, another group of bacteria was recently identified which are able to perform the complete oxidation of NH_3 to NO_3^-, thus given the name Comammox (Daims et al., 2015; van Kessel et al., 2015).

In bacteria, NH_3 is oxidized to hydroxylamine (NH_2OH) by the enzyme ammonia monooxygenase (AMO) (Hollocher et al., 1981; Hyman and Wood, 1985) which is then further oxidized to nitric oxide (NO) by the hydroxylamine oxidoreductase (HAO) (Caranto and Lancaster, 2017; Hooper and Terry, 1979). The enzyme responsible for the oxidation of NO to NO_2^- is still unknown. Unlike the bacterial NH_3 oxidation pathway, the archaeal one is still very poorly characterized. Only the oxidation of NH_3 to NH_2OH is inferred to be catalyzed by an AMO (Vajrala et al., 2013) which is very distantly related to bacterial AMO and all other enzymes of the copper membrane monooxygenase superfamily (CuMMO) (Alves et al., 2018). However, a counterpart to the bacterial HAO is still missing in archaea. Two models are currently proposed, one that mimics the bacterial pathway (Lancaster et al., 2018) and another one where ammonia is oxidized to hydroxylamine which would then be further oxidized to NO_2^- with NO as a co-substrate (Kozlowski et al., 2016). NO would be produced by the reduction of NO_2^- by a proposed nitrite reductase (NirK), which is highly expressed in most AOA, but whose role is still ambiguous as AOA do not perform nitrifier denitrification, unlike AOB (Wrage-Mönnig et al., 2018).

One important factor in niche differentiation of organisms is their substrate affinity, which is described either as reaction rate (v) based on the K_m value (Eq. 1) or as specific growth rate (μ) based on the K_S value (Eq. 2). In a steady state the residual substrate concentration (S) remains constant over time.

$$v = \frac{v_{max} \cdot [S]}{K_m + [S]} \quad \textbf{Eq. 1}$$

$$\mu = \frac{\mu_{max} \cdot [S]}{K_S + [S]} \quad \textbf{Eq. 2}$$

AOA are notoriously difficult to grow and produce only very little biomass and therefore most information about AOA is provided in the form of the apparent substrate affinity ($K_{m(app)}$), which is based on whole cell activity measurements of molecular oxygen (O_2) consumption in micro-respiratory chambers (Jung et al., 2022; Kits et al., 2017). Most AOA are considered oligotrophs and their $K_{m(app)}$ values range from 0.1 – 1 µmol L^{-1} NH_3 and NH_4^+ for marine strains, 1 – 10 µmol L^{-1} for soil or thermophilic strains to 0.1 – 10 mmol L^{-1} for the soil clade Nitrosocosmicus. Comammox have $K_{m(app)}$ in the range of 0.1 – 10 µmol L^{-1} NH_3 and NH_4^+ and are thus considered also oligotrophs while AOB are rather considered eutrophs with $K_{m(app)}$ between 0.1 and 100 mmol L^{-1} NH_3 and NH_4^+ (Jung et al., 2022). In an attempt to measure the K_S value of a fresh water AOA enrichment in batch cultures, no effect of the initial substrate concentration on µ was observed, suggesting that the K_S is much lower than the lowest tested concentration of about 15 µmol L^{-1} NH_4^+ (French et al., 2021). In a chemostat experiment, *N. maritimus* was grown with 150 µmol L^{-1} NH_4^+ and different dilution rates (D) of 0.010, 0.023 and 0.032 h^{-1} (μ_{max} is 0.036 h^{-1}) to investigate the influence of µ on the lipid composition of the organism. NO_2^- concentration only varied by a maximum of 7% (Hurley et al., 2016) but no information was given about the residual substrate concentration.

Nitrosospera viennensis was isolated from garden soil and grows optimal at 42 °C (Stieglmeier et al., 2014) with the addition of pyruvate to scavenge reactive oxygen species (ROS) that are endogenously produced (Kim et al., 2016). Cell concentrations and µ are usually approximated by NO_2^- concentrations, as they have been shown to correlate well (Stieglmeier et al., 2014; Tourna et al., 2011). However, *N. viennensis* produces far too little biomass to measure optical density or dry cell weight.

As the different forms of cultivation systems are rarely discussed in the field of nitrification (except for waste water treatment plants), a short overview of the most commonly used systems shall be given here. In general, systems can be distinguished by the level of which they allow energy and matter to be transferred through them. Closed systems (transfer of energy but not matter) and open systems (transfer of energy and matter) are the extreme cases of reality and the isolated system (no transfer of energy or matter) serves as an important theoretical construct that can only be asymptotically approached by closed systems. Cultivation systems are characterized by the level of transfer of matter and in analogy, closed batch (e.g., serum flask with rubber stopper) is a closed system (Mauerhofer et al., 2019; Taubner and Rittmann, 2016), continuous culture (e.g., bioreactor with gassing and in- and outflow of medium) is an open system, with the openness of the system depending on the transfer rates. Open batch (e.g., Erlenmeyer flask with cotton plug, bioreactor with gassing) and fed-batch (e.g., bioreactor with gassing and inflow of medium) are open system intermediates in between the two extremes (Mauerhofer et al., 2019).

Batch systems are probably the most common cultivation systems used in microbiology, because they are very easy to set up and require little technological infrastructure. Closed batch is usually only used if an atmosphere different from air is required (Hanišáková et al., 2022; Mauerhofer et al.,

2019). A major disadvantage of batch systems are the changing substrate concentrations that lead to a very heterogeneous biomass which can complicate analysis. Fed-batch systems consist of a shorter batch phase followed by a feed phase, where usually a concentrated feed medium is used to increase the biomass concentration but at the same time avoid substrate inhibition. By using an exponential feeding strategy μ can be kept constant and a relatively homogeneous biomass can be produced, as long as no product inhibition or other limitations hamper growth. In continuous culture systems a stable flow of medium is maintained after an initial batch phase and by changing the dilution rate (D, Eq. 3), different steady states can be established by changing the flow rate (Q) or the volume of the culture (V).

$$D = \frac{Q}{V} \quad \textbf{Eq. 3}$$

A system is usually considered to be in a steady state after five volume exchanges (99.3% of medium exchanged) while all parameters are kept constant. As a result, the produced biomass is very homogeneous, because in a steady state μ is equal to D. Technically speaking, productivities of organisms that are grown in continuous culture are up to tenfold higher compared to batch systems (Herbert et al., 1956), because the system can be stably operated near μ_{max} and downtime for disassembly, sterilization and reassembly of the bioreactor becomes increasingly negligible with longer operation times. Continuous cultures are also excellent tools to study the physiology of microorganisms due to the high level of control and the extended periods of time a steady state can be maintained. A typical application is the determination of the K_S value by varying D and measuring the residual substrate concentration (S), thereby relating μ to S and thus allowing to calculate K_S when assuming Monod kinetics. However, biofilm formation, genetic adaptation or other factors might flaw the determination of such values with extended process runtimes.

In stirred tank reactors gas is usually supplied by a fumigation tube at the bottom of the reactor. The gas transfer rate, which is often a limiting factor for fast growing organisms, can be increased by e.g., the gassing rate, stirrer speed and operating pressure (Pappenreiter et al., 2019; Rittmann et al., 2018). Due to the low μ_{max} and biomass concentration (X) of AOA the gas transfer rate is not a limiting factor but rather needs to be considered because of the possibility to strip important metabolic intermediates (like NO) from the system.

All cultivated AOA are chemolithoautotrophs and fix CO_2 by a modified version of the hydroxypropionate/hydroxybutyrate cycle (Könneke et al., 2014). For the cultivation of AOA and AOB in bioreactors CO_2 is usually supplied by air and $NaHCO_3$ that is used to titrate the pH and act as C-source (French et al., 2021; Hurley et al., 2016; Sedlacek et al., 2020). Given the fast reaction kinetics of the aqueous carbonate system, the liquid phase in a gassed reactor will tend to be in equilibrium with the supplied gas mix independently of $NaHCO_3$ titration (Wang et al., 2010).

The aim of this study was to investigate the growth behavior of *N. viennensis* in continuous culture systems to develop a biomass production process that would enable biochemical studies of the organism to eventually elucidate the energy metabolism of AOA. Growth conditions were optimized to ensure that only NH_3 acts as a limiting substrate while characterizing process parameters such as K_S, biomass to substrate yield ($Y_{(X/NH3)}$) and the critical dilution rate (D_{crit}) at which the organism will be washed out, for the bioprocess development. To further increase the biomass productivity per reactor volume we also established a continuous culture at a higher substrate concentration.

Materials and Methods

Cultivation of *Nitrososphera viennensis*

Nitrososphera viennensis was routinely grown as batch cultures in 30 mL Quickstart universal containers (STERILIN) at 42 °C in freshwater medium (FWM) (De La Torre et al., 2008; Tourna et al., 2011) amended with non-chelated trace element solution (MTE) (Könneke et al., 2005), 7.5 µmol L^{-1} FeNaEDTA, 2 mmol L^{-1} NH_4Cl, 2 mmol L^{-1} $NaHCO_3$, 1 mmol L^{-1} sodium pyruvate and 10 mmol L^{-1} HEPES buffer (final pH 7.5). Cultures used as inoculum for bioreactors were inoculated with 1% (v/v), but for regular culture maintenance 1:10^6 (v/v) inoculum was used. Long term storage works best by aliquoting late exponential cultures without any additives and storing them at -70 °C. For reviving the culture 5% (v/v) inoculum should be used and a lag phase of one to two days need to be expected. Growth was followed by measuring NO_2^- production and NH_3 consumption as described before (Tourna et al., 2011).

Batch

To elucidate the effect of gassing on µ, *N. viennensis* was grown in 2 L all-glass bioreactors (Eppendorf AG, Hamburg, Germany) filled with 1.5 L. The medium composition and conditions were the same as for batch cultures, except for omitting HEPES buffer, and the inoculum was 5% (v/v). The pH was controlled at pH 7.50 (± 0.05 deadband) using 0.5 mol L^{-1} HCl and 0.5 mol L^{-1} NaOH for titration. Dissolved molecular oxygen concentration (dO_2) was measured with optodes (Hamilton) and recorded but not controlled. Cultures were continuously stirred at a rate of 400 rpm and depending on the experiment not gassed (in- and off-gas clamped off), gassed with 2 sL L^{-1} h^{-1} air, gassed with 1 sL L^{-1} h^{-1} air or 1 sL L^{-1} h^{-1} air/N_2 mix (12.6 Vol.-% O_2). In the last batch experiment cultures were gassed with 2 sL L^{-1} h^{-1} air for 63 h, in- and off-gas clamped off and NO-donor 2,2'-(2-Hydroxy-2-nitrosohydrazinylidene)bis-ethanamine (DETA NONOate) added via a septum in different concentrations (0.15, 0.6 and 2.4 µmol L^{-1}, sterile H_2O as control). After 25 h one reactor was gassed with 2 sL L^{-1} h^{-1} air, one with 2 sL L^{-1} h^{-1} 99% air 1% CO_2 mixture, one was kept clamped off and one clamped off and 2 mmol L^{-1} $NaHCO_3$ were added via a septum.

Chemostat

Chemostat cultures were grown like batch cultures but without $NaHCO_3$ in the medium, instead the cultures were gassed with an air/CO_2 mix of 0.5, 1 or 2 Vol.-% CO_2 with 2 sL L^{-1} h^{-1} gassing rate. The culture volume was 1.5 L but had to be reduced to 1.1 L at the highest D of 0.07 h^{-1} to not exceed the maximum pump rate of 100 mL h^{-1}. The chemostats were started in late exponential growth phase of the batch and due to the relatively low µ of the organism, the minimum volume exchanges for steady states, after every applied or observed change, were reduced from the usual five (99.3% (v/v) exchanged) to three exchanges (95.0% (v/v) exchanged) to speed up the experiment.

Peristaltic pumps were calibrated by weighing feed- and harvest bottles every day to avoid drifting over time and the reactor volume was corrected with every sampling. Deviations of the reactor volume were usually between 1% and 3%. Outliers of BR1 and BR2 at 1271 h and 1295 h were caused by technical issues of the outflow pumps after the reduction of the working volume to 1.1 L, causing a culture volume increase to about 2 L over night and thus a decrease in residual NH_4^+.

Outliers of BR2 at 1343 h and 1346 h were due to a ripped inflow pump tube, causing a temporary reduction of culture volume to about 0.9 L, a decrease in residual NH_4^+ and a contamination with small rods that was treated with kanamycin.

Washing experiment

To estimate the activity of the biofilm on the reactor walls, the planktonic cells were removed by draining the reactors and refilling them with fresh and sterile medium twice. At a working volume of 1.5 L and 7.5 mL remaining in the bioreactors, a dilution of the planktonic culture of 1:40000 was achieved (43 nmol L^{-1} NO_2^- remaining). After that the continuous cultures were started again with a dilution rate of 0.065 h^{-1}. During the washing procedure, precipitated carbonate detached from the acid/base/medium inlet in BR2 and started to scrape off the biofilm from the reactor wall, thereby creating a constant loss of biofilm to the system.

Fed-batch

The fed-batch experiment started with two batch cultures of 1.1 L gassed with an air/ 0.5 Vol.-% CO_2 mix of 2 sL L^{-1} h^{-1}. The 400 mL feed medium contained 32 mmol L^{-1} NH_4Cl (10 mmol L^{-1} final), 4.75 mmol L^{-1} pyruvate (2 mmol L^{-1} final), 4.75x MTE (2x final), 4.75x FeNaEDTA (2x final) and was exponentially fed in the beginning to maintain a µ of 0.02 h^{-1}. Due to unreliable pump rates the feed mode was changed to linear with a feed rate of 2 mL h^{-1}. The pyruvate concentration in the feed medium for the continuous culture was increased to 5 mmol L^{-1} to avoid pyruvate depletion which resulted in stagnating cell concentration during the fed-batch.

Sampling

Samples were taken from the bioreactors after the first 2 mL were discarded to remove old culture from the tubing. 1 mL aliquots were pipetted into 1.5 mL reaction tubes and centrifuged at 4 °C for 30 min at 23,000 g in a benchtop centrifuge (Eppendorf Centrifuge 5424 R). Supernatant was removed and used directly for NO_2^- and NH_3 measurements while the cell pellets were stored at -20 °C for DNA extraction.

Exchanging feed and harvest bottles

Basic FWM was autoclaved in 5 L Schott bottles and supplements were added in a laminar flow hood after room temperature was reached. The GL45 caps with tubing and 0.2 µm filter were always transferred from one bottle to the next, while keeping the inlet and outlet tubes clamped off. After attaching the bottles back to the reactor tubes via luer locks, the connections were put into beakers with boiling water for at least 10 min to reduce the contamination risk, before opening the clamps and restarting the pumps. While BR2 was slightly contaminated due to ripped feed pump tubing, BR1 remained clean over the whole run (103 days with 36 bottle exchanges). The contamination of BR2 occurred at 1343 h and lasted at most up to 2041 h.

DNA-Extraction

DNA from cell pellets was extracted by beat beating using SDS-buffer, phenol/chloroform/isoamylalcohol [25:24:1 (v/v/v), Fisher BioReagents] and

chloroform/isoamylalcohol [24:1 (v/v)] as already described elsewhere (Abby et al., 2018; Griffiths et al., 2000; Rittmann and Holubar, 2014; Zhou et al., 1996).

PCR

DNA samples were checked for bacterial contamination using the 16S rRNA primers Eubac27F 5‘-AGA GTT TGA TCC TGG CTC AG-3‘ and Eubac1492R 5‘-GGT TAC CTT GTT ACG ACT T-3‘. PCR conditions were 95 °C for 5 min as initialization, followed by 35 cycles of 30 s denaturing at 94 °C, 30 s primer annealing at 55 °C, 2 min elongation at 72 °C, and finishing with final elongation at 72 °C for 10 min. Reactions were done in 25 µL containing 2 µL sample, 0.15 µL GoTaq Polymerase, 5 µL 5x Flexi Buffer, 2 µL MgCl2 25 mmol L^{-1}, 0.5 µL dNTP 10 mmol L^{-1}, 0.25 µL BSA 20 mg mL^{-1}, 1.25 µL Primer F 10 µmol L^{-1}, 1.25 µL Primer R 10 µmol L^{-1}, 12.6 µL nuclease free water.

Quantitative PCR

The archaeal 16S rRNA gene was quantified to estimate the cell number using the primers Arch931F 5‘-AGG AAT TGG CGG GGG AGC A-3‘ (Jackson et al., 2001) and Arch1100R 5‘-BGG GTC TCG CTC GTT RCC-3‘ (Øvreås et al., 1997) in triplicate 20 µL reactions containing 10 µL qPCR Master Mix 2x (Luna Universal qPCR Master Mix, NEW ENGLAND BioLabs Inc.), 1 µmol L^{-1} of each primer, nuclease free water and 4 µL DNA sample (diluted to have approximately 10 ng per reaction) or standard. Reactions were performed in a qPCR (BIO-RAD CFX Connect Real-Time System) with the following conditions: initialization at 95 °C for 2 min, 40 cycles of 30 s denaturing at 95 °C, 30 s joint annealing-extension at 60 °C, and extension with fluorescence measurement at 60 °C for 30 s. The specificity of qPCR products was confirmed by melting curve analysis. Standards were prepared by amplifying the 16S rRNA gene of *N. viennensis* under the conditions as described above in a 50 µL reaction using 2 µL DNA template and the archaeal 16S rRNA primers A109F 5‘-ACK GCT CAG TAA CAC GT-3‘ (Großkopf et al., 1998) and A1492R GYY ACC TTG TTA CGA CTT-3‘ (Nicol et al., 2008). PCR product was cleaned up using the Machery-Nagel DNA cleanup kit and DNA concentration was measured with Qubit™ dsDNA BR Assay Kit (Thermo Fisher Scientific) before preparing serial dilutions with nuclease free water. The standard curve had an efficiency of 93.38% and an R^2 of 0.999.

Extrapolating cell number and biomass from DNA concentration

DNA concentration of extracted samples was measured with Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific) and several samples with concentrations ranging from 3.58 to 35.5 ng μL^{-1} were quantified by qPCR as described above. Using a linear regression model the mass of DNA was correlated to the cell number, assuming that each cell contained a single copy of the 16S rRNA gene. Using this model one copy of 16S rRNA correlates to 3.42 fg which is 25.6% more than the calculated mass of a *N. viennensis* genome of 2.72 fg (2.52 Mbp genome size). Assuming a cell diameter of 0.75 µm (0.6 – 0.9 µm cell diameter) and a density of 1.1 g cm^{-3} a theoretical *N. viennensis* cell would weight $2.43 \cdot 10^{-13}$ g or 243 fg.

Harvesting of biomass

The outflow of the continuous cultures was collected in sterile 5 L bottles and usually harvested once a week by concentrating the biomass using a tubular centrifuge (CEPA) at 40,000 rpm and subsequent centrifugation of the cell concentrate in a bucket centrifuge (Thermo SCIENTIFIC Sorvall LYNX 4000 Centrifuge) at 4 °C, 24,470 g for 40 min. The pellets were resuspended in a small amount of supernatant and split up into pre-weighted 2 mL reaction tubes which were centrifuged in a benchtop centrifuge (Eppendorf Centrifuge 5424 R) at 4 °C for 30 min at 23,000 g. Supernatant was removed, reaction tubes weighted and stored at -70 °C.

Equations

Active biofilm estimation

To estimate the amount of biofilm biomass contributing to nitrification, biofilm BR1 was modeled as a continuously stirred tank reactor (CSTR) at the final time point taken . BR1 was chosen based off of its relative stability compared to BR2. The change of substrate with relation to time can be described using the following equation:

$$V\frac{dS}{dt} = Q S_i - QS + r_f A_f L_f + r_S V \quad \textbf{Eq. 4}$$

Where V is the volume of the reactor, Q is the flow rate, S_i is the concentration of ammonium in the inflow, S is the concentration of ammonium in the outflow, r_s represents the reaction of ammonium to nitrite by the planktonic cells, r_f represents the reaction of ammonium to nitrite by biofilm cells, A_f is the surface area of the reactor covered by biofilm, and L_f is the height of the biofilm. The volume of the biofilm is assumed to be negligible when compared to the planktonic volume (and therefore total volume) of the reactor system.

The reaction of ammonium to nitrite (mmol L^{-1} h^{-1}) can be represented by:

$$r = \frac{-1}{Y_{XS}} \mu X \quad \textbf{Eq. 5}$$

Where $Y_{(X/NH3)}$ represents the yield of *N. viennensis* biomass from ammonia oxidation (mg biomass/mmol ammonium), μ is the specific growth rate (h^{-1}), and X represents the respective biomass concentration (mg/L; planktonic or biofilm).

Substituting the reaction rate into the substrate mass balance:

$$V\frac{dS}{dt} = Q(S_i - S) + \left(\frac{-1}{Y_{XS}} \mu_f X_f A_f L_f\right) + \left(\frac{-1}{Y_{XS}} \mu XV\right) \quad \textbf{Eq. 6}$$

Assuming steady state (dS/dt=0), a μ that is the same for both biofilm and planktonic cells ($\mu_f=\mu$), and the volume of the biofilm to be surface area multiplied by height ($A_fL_f=V_f$), Equation 3 simplifies to :

$$0 = Q(S_i - S) + \left(\frac{-1}{Y_{XS}} \mu X_f V_f\right) + \left(\frac{-1}{Y_{XS}} \mu XV\right)$$ **Eq. 7**

The exact volume of the biofilm is unknown due to the unknown height. Assuming a homogenous biofilm and equilibrium between the biofilm and planktonic biomass, the total amount of biomass (mg) contributing to nitrification from the biofilm can be determined by solving Equation 4 can for X_fV_f:

$$X_f V_f = \frac{-Y_{XS}}{\mu} Q(S - S_i) - XV$$ **Eq. 8**

The left side of the equation is now represented by known variables when using BR1: $Y_{(X/NH3)}$= 15 mg biomass/mmol ammonia (calculated from batch cultures); μ=0.048 h^{-1}; Q=0.0975 L h^{-1} (set parameter); S_i=2 mmol ammonium/L (set parameter); S=0.3295 mmol ammonium/L (measured in effluent); X=13.3 mg biomass/L (measured in effluent); V=1.5 L (set parameter).

Results

Effect of gassing rate and ingas flow composition in batch and closed batch experiments

Initial experiments of *N. viennensis* grown as batch cultures in 2 L bioreactors showed that gassing with air had a detrimental effect on μ even at low gassing rates such as 2 sL L^{-1} h^{-1} (0.0276 h^{-1}) when compared to cultures without gassing (0.0445 h^{-1}, Figure S1). In non-gassed cultures dO_2 decreased to about 50% of its initial concentration. Therefore, growth under decreased gassing rate and reduced dO_2 was tested. As shown in Figure S2 reducing the gassing rate to 1 sL $L^{-1}h^{-1}$ did not increase μ (0.0267 h^{-1}) and reducing dO_2 to about 60% did decrease μ to 0.0205 h^{-1}, which contradicted the results of the first experiment. Due to the hypothesized importance of NO for the archaeal ammonia oxidation pathway (Kozlowski et al., 2016) we decided to test if the reduction of μ is a result of stripping NO from the system by gassing. As shown in Figure 1, addition of the NO-donor 2,2'-(2-Hydroxy-2-nitrosohydrazinylidene)bis-ethanamine (DETA NONOate) to early exponential phase cultures (300 μmol L^{-1} NO_2^- produced) did not show any effect on recovering μ. However, adding 1 Vol.-% CO_2 to the ingas or 2 mmol L^{-1} $NaHCO_3$ to the culture medium, and keeping the bioreactor closed did increase μ from 0.0259 ± 0.0002 h^{-1} to 0.0338 h^{-1} and 0.0395 h^{-1}, respectively. Gassing with air or keeping the reactor closed did not change μ (0.0260 h^{-1}) or even reduced it (0.0234 h^{-1}), respectively. Hence, the above results showed that the organism was carbon limited likely due to the stripping of CO_2 from the supplied gassing.

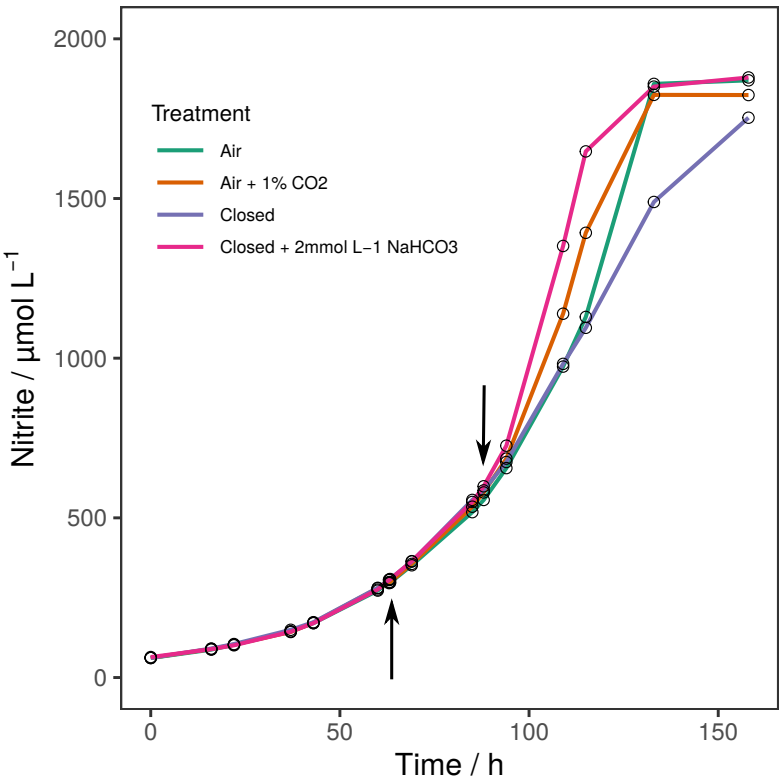

Figure 1: Effect of NO-donor and CO_2 on µ. Four 1.5 L batch cultures grown in 2 L bioreactors gassed with 2 sL L^{-1} h^{-1} air. Upward arrow indicates the addition of different amounts of the NO-donor 2,2'-(2-Hydroxy-2-nitrosohydrazinylidene)bis-ethanamine (DETA NONOate) to early exponential cultures, after which gassing was stopped and reactors clamped off to prevent gas exchange. As no effect on the nitrite production was observed, cultures were tested for CO_2 limitation indicated by the downward arrow. One culture was gassed again with air, one with air plus 1 % CO_2, one was kept closed and one was kept closed but with the addition of 2 mmol L-1 $NaHCO_3$. Addition of CO_2 to the ingas or $NaHCO_3$ to the culture medium increased µ, while gassing with air or keeping the reactor closed did not change or reduce µ respectively.

To determine μ_{max} in closed batch systems, *N. viennensis* was grown in serum flasks with 0.5% CO_2 and different dO_2 concentrations (Figure S3). A slight increase of µ was observed with decreasing dO_2 from 0.0484 ± 0.0004 h^{-1} to 0.0508 ± 0.0004 h^{-1}at 21 Vol.-% to 5 Vol.-% O_2, respectively (224.7 to 53.5 µmol L^{-1} dO_2 considering a pressure of 1106 hPa and an incubation temperature of 42 °C). Due to the minor effect of dO_2 on µ, 21 Vol.-% O_2 were used to gas the following cultures and a μ_{max} of 0.0484 h^{-1} is therefore considered. Moreover, by using an inoculum of 1:10^6 (v/v), µ was increased from 0.024 h^{-1} (Stieglmeier et al., 2014) to 0.048 h^{-1} (data not shown).

Continuous culture experiments at high dilution rates

To determine the K_S and D_{crit} of *N. viennensis* eight different D (0.035, 0.038, 0.042, 0.046, 0.050, 0.060, 0.065 and 0.070 h^{-1}) were applied in two bioreactors. As shown in Figure 2, at D 0.035 h^{-1} S stabilized at an unexpectedly high level around 750 µmol L^{-1} NH_4^+ and increasing D further only had a marginal effect on S. In section A (D 0.035 to 0.050 h^{-1}, 382 to 990 h) S stabilized at 801.9 ±43.2 µmol L^{-1} NH_4^+. Activity of both cultures spontaneously increased 58 h after D was set to 0.050 h^{-1} (94.5% of medium exchanged) and S decreased to 575.2 ±21.2 µmol L^{-1} NH_4^+. The increase of D to 0.060 h^{-1} was responsible for the abrupt increase of S to 690.1 ±29.9 µmol L^{-1} NH_4^+ which gradually decreased, despite negative outliers, to 456.2 ±7.9 µmol L^{-1} NH_4^+. Overall S stabilized at 541.6 ±68.6 µmol L^{-1} NH_4^+ in section B (D = 0.050 to 0.070 h^{-1}, 1032 to 1654 h) and negative outliers at 1271, 1295, 1343 and 1346 h were due to technical issues (see Materials and Methods). Another spontaneous increase of activity occurred in both cultures 11 days after D was set to 0.070 h^{-1} (18 working volume exchanges), which first led to a complete consumption of NH_4^+ in both cultures but subsequently two different S stabilized. As shown in section C of Figure 2 (D = 0.070 and 0.065 h^{-1}, 1796 to 2035 h), S of the BR1 culture stabilized at 262.2 ±19.9 µmol L^{-1} NH_4^+ and at 7.4 ±11.1 µmol L^{-1} NH_4^+ for the BR2 culture. From section C to D (D 0.065 h^{-1}, 2062 to 2402 h) the reactor volume was increased to minimize the effect of sampling larger volumes which caused S of BR1 to increase from 258 to 356 µmol L^{-1} NH_4^+ (ratio of concentrations matches the ratio of volumes). S of BR2 gradually increased from 4.1 µmol L^{-1} NH_4^+ to 375.0 µmol L^{-1} NH_4^+ probably as a result of the disturbances caused by the repeated sampling process.

The cell concentrations declined gradually with D from $7.67 \cdot 10^7$ ±$2.11 \cdot 10^7$ cells mL^{-1} at D = 0.035 h^{-1} to $4.36 \cdot 10^7$ ±$2.18 \cdot 10^6$ cells mL^{-1} at D = 0.046 h^{-1} (549 to 932 h). µ calculated from measured cell concentrations as shown in Figure S4 gradually increased with increasing D from 0.0362 ±0.0011 h^{-1} at D = 0.035 h^{-1} to 0.0437 ±0.0007 h^{-1} at D = 0.046 and after the activity increase at D 0.050 h^{-1} µ increased further to 0.0505 ±0.0003 h^{-1} (1107 h). Cell concentrations remained surprisingly stable from D = 0.050 to 0.060 h^{-1} at $4.13 \cdot 10^7$ ± $5.30 \cdot 10^6$ cells mL^{-1} (1032 to 1419 h) and declined to $3.08 \cdot 10^7$ ±$7.06 \cdot 10^5$ cells mL^{-1} at D = 0.070 h^{-1} (1654 h) before the second activity increase, after which cell concentrations started to alternate in both reactors. Average NH_4^+ and cell concentrations of sections A to D are shown in Figure S5. K_S and D_{crit} could not be determined due to the unusual growth behavior and biofilm formation, respectively.

Ammonia oxidizing activity of biofilm

Over the course of the experiment a biofilm had formed gradually on the reactor walls. To estimate its contribution to the gross activity, the medium was aseptically removed from the reactors, the reactors were washed once with sterile medium and then refilled with fresh medium to restart the continuous culture at D 0.065 h^{-1}. Only a residual NO_2^- concentration of 43 nmol L^{-1} should have been present at the restart of the continuous culture but instead a starting NO_2^- concentration of about 100 µmol L^{-1} was measured in both reactors. As shown in Figure 3 within two days the same steady state as before the washing step was recovered in both reactors.

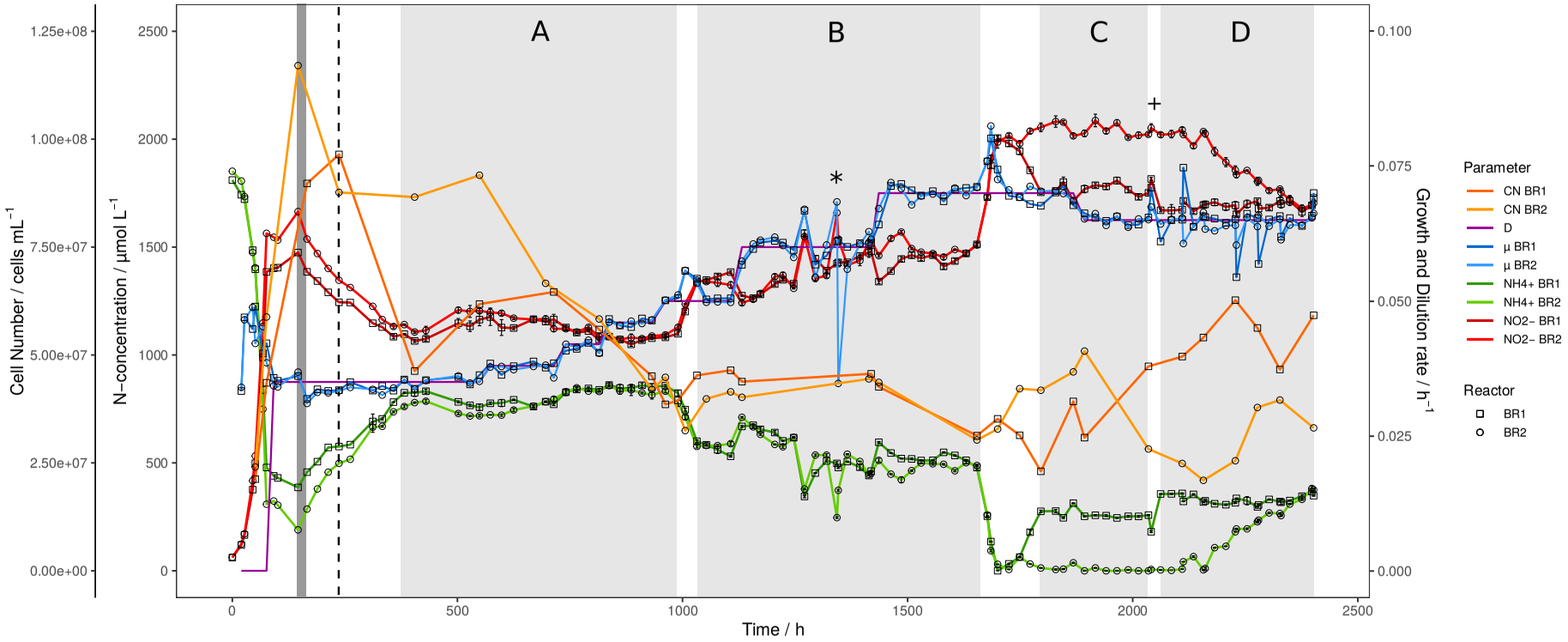

Figure 2: Continuous culture of N. viennensis at high D. Two continuous cultures with D ranging from 0.035 h^{-1} to 0.070 h^{-1} and the development of cell number (CN), NO_2^- derived µ, NH_4^+ and NO_2^- concentrations of the cultures grown in the corresponding reactors BR1 and BR2. Cultures were gassed with 1 % CO2 enriched air at 2 sL L^{-1} h^{-1} gassing rate, except for the period marked in dark grey where the gassing rate was temporarily increased to 10 sL L^{-1} h^{-1}. The dashed line indicates the increase to 2 % CO_2 in the ingas. Sections A to D signify regions where NH_4^+ concentrations stabilized, even though D was increased. Spontaneous increases of activity happened between section A and B and section B and C without external influences. Increase of NH_4^+ of BR1 from section C to D was due to an increase of reactor working volume and the increase of NH_4^+ of BR2 in section D was probably induced by taking larger sampling volumes. The asterisk indicates the point of contamination of BR2 due to a ripped pump tube and the plus the point at which the contamination was no longer detectable by PCR off the bacterial 16S rRNA gene. NH_4^+ and NO_2^- curves show mean values of technical triplicates and error bars represent the standard deviation of the mean.

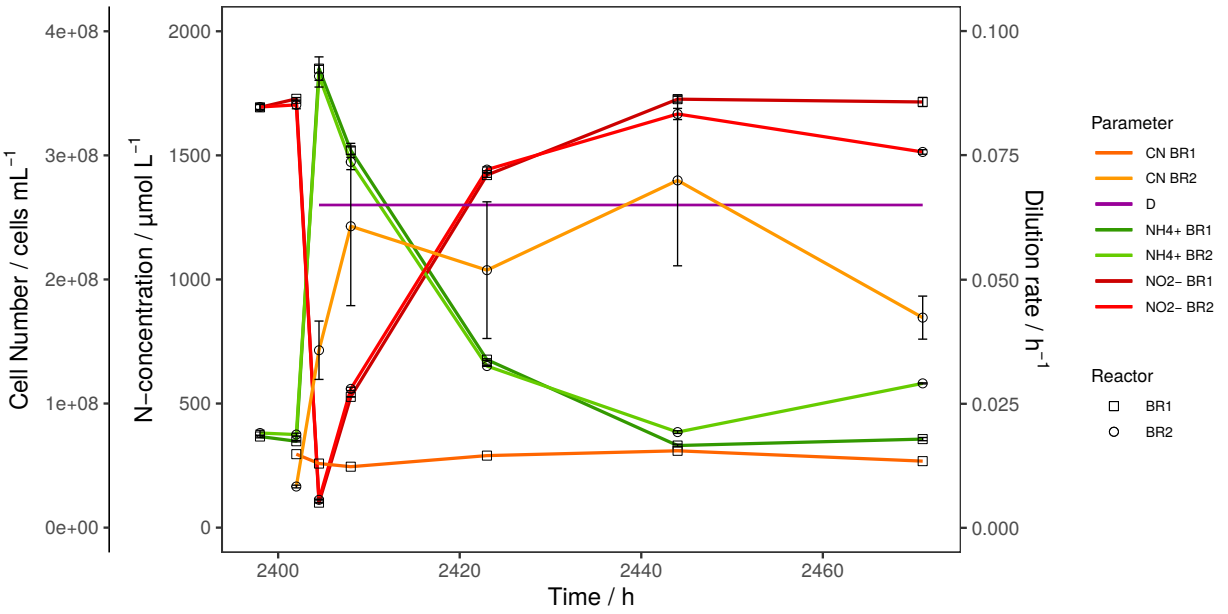

Figure 3: Ammonia oxidizing activity of biofilm. Bioreactors of continuous cultures grown at high D were washed, refilled with sterile medium and operated at D 0.065 h^{-1} to estimate the activity of the biofilm which had formed on the reactor walls due to the prolonged cultivation time. NH_3 oxidation started right away and within two days the same steady state as before the washing step was recovered in both reactors. Cell number in BR1 was almost unaffected by the washing step, indicating that most planktonic cells were seeded by the biofilm. The steep increase of the cell number in BR2 was due to biomass being scrapped off from the reactor wall by precipitate that had formed at the inlet and was washed off during the washing step. Both reactors exhibited very comparable NH_3 oxidizing activities despite the fact that BR2 lost a considerable amount of its biofilm biomass as it was washed out with the effluent. NH_4^+, NO_2^- and cell number of BR2 curves show mean values of technical triplicates and error bars represent the standard deviation of the mean.

Almost no change in planktonic cell concentration of BR1 was observed after the washing step and the concentration remained stable afterwards between $4.91 \cdot 10^7$ and $6.19 \cdot 10^7$ cells mL^{-1}. In BR2 the cell concentration increased after the washing step from $3.31 \cdot 10^7 \pm 1.09 \cdot 10^6$ to $1.43 \cdot 10^8 \pm 2.35 \cdot 10^7$ cells mL^{-1} and increased further to $2.43 \cdot 10^8 \pm 6.40 \cdot 10^7$ cells mL^{-1} within the next 3 h as a result of precipitate being washed off of the inlet and subsequently scraping off biofilm from the reactor wall. The cell concentration remained rather stable from there for 36 h up to a concentration of $2.80 \cdot 10^8 \pm 6.88 \cdot 10^7$ cells mL^{-1} and decreased to $1.69 \cdot 10^8 \pm 1.73 \cdot 10^7$ cells mL^{-1} within the following 27 h.

Thus the biofilm was not only highly active but also contributed considerably to the planktonic cell concentration in the bioreactors by seeding cells. Even though BR2 lost substantial biofilm biomass it still had comparable NH_3 oxidizing activity to BR1 in which the biofilm was retained.

Estimation of active biofilm biomass in BR1

Based on a substrate mass balance and assuming a steady state at time point 2402 h (Figure 3) , 30.9 mg of *N. viennensis* biomass is contributing to nitrification in the biofilm (see Materials and Methods). This represents approximately 1.55 times as much biomass as would be found in the planktonic phase of the reactor. While this number represents the amount of actively nitrifying

biomass, it is plausible that the biofilm itself is much more in terms of weight that could be represented by extrapolymer substances (EPS), precipitated bicarbonate, and cells that are potentially inactive due to substrate limitation. While these calculations demonstrate a significant portion (over half) of the nitrification activity is coming from the biofilm, this number could change if it is determined that certain growth parameters, such as the $Y_{(X/NH3)}$ and μ, are differing between the planktonic and biofilm phases. However, within the presented model, the biofilm is affecting the amount of NH_4^+ oxidized to NO_2^- and could help explain the NO_2^- productivity within these bioreactors. A large portion of activity from the biofilm is also expected due to NO_2^- being produced even though D is higher than the predicted μ_{max}. The combined ammonium oxidation of planktonic cells and biofilm, and planktonic cells being seeded from the biofilm, could explain the absence of an expected cellular washout of the reactor.

Estimation of biofilm biomass in BR2

An estimate for the total biofilm biomass was obtained from BR2 under the assumption that all biofilm from the bioreactor wall had been scraped off during the biofilm activity experiment. By summing up all cells washed out and subtracting the cells produced during the time of the experiment, the biofilm biomass at the start can be estimated. To assess the amount of cells produced, the total amount of substrate consumed was calculated and then multiplied by the estimated $Y_{(X/NH3)}$. Total cells washed out was calculated as the area under the curve from 2402 hours to 2471 hours (Figure 3) after converting time to volume using the set flow rate. Total ammonia consumed was calculated by subtracting residual ammonia (area under the curve) from total supplied ammonia during the given time span. Using a $Y_{(X/NH3)}$ of 15 mg/mmol NH_4^+ and an estimated mass per cell of $2.3x10^{-13}$ g/cell (see materials and methods) the amount of cells produced during the time span was calculated to be 167.77 mg. The mass of total cells washed out was calculated to be 378.16. Subtracting produced biomass from total washed out biomass gives an estimate biofilm mass of 210.49 mg. Assuming the amount of biofilm in BR2 from the washout experiment to be representative of the amount of biofilm in BR1 at the end of the chemostat experiment (Figure 2), this data can be combined with the active biofilm estimation (30.9 mg; BR1) calculation to determine the percentage of active biomass in the biofilm. According to these estimations and under the assumption that BR1 and BR2 produced similar amounts of biofilm, only about 14.67% of the cells in the biofilm would have been active.

This percentage should be taken as a conservative estimate as it would be lower if not all biofilm was removed from the reactor during this time period. Biofilm that was observed at the takedown or BR6 would indicate this to be a possibility barring the production of new biofilm during the washout experiment.

Continuous culture experiments at low dilution rates

To get the full picture of how D effects S, a new continuous culture run was set up with 0.5 Vol.-% CO_2 enriched air to reduce the precipitation of CO_3^{2-} both at the inlets and in the medium. Initial D was set at 0.01 h^{-1} and all substrate was consumed. As shown in Figure 4 an increase in D to 0.020 h^{-1} resulted in a temporary increase of S to 11 and 44 μmol L^{-1} for BR3 and BR4 respectively, after which S returned to 0 μmol L^{-1}. Then CO_2 was increased to 2 Vol.-% at 96 h after D was increased, which surprisingly induced an increase of S in BR3 with a 55.5 h delay. S in BR3 slowly started toincrease of S in BR3 with a 55.5 h delay. S in BR3 slowly started to increase from 17 to finally 192 μmol L^{-1} whereas in BR4 S concentrations increased up to 95 μmol L^{-1} but only 216 h after the

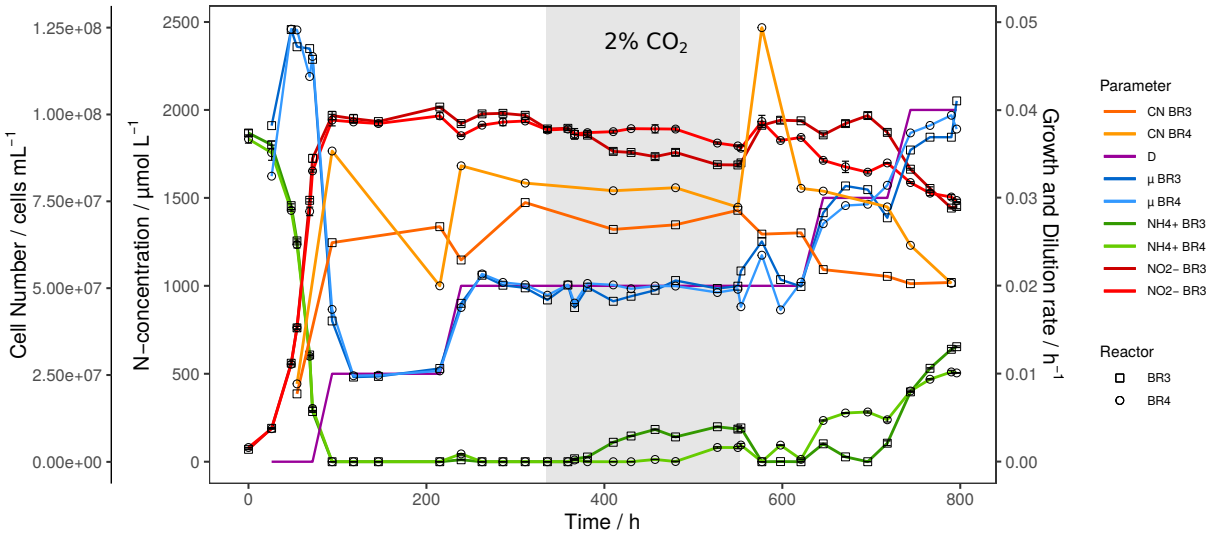

Figure 4: Continuous cultures of N. viennensis at low D. Two continuous cultures operated at D 0.010 h^{-1} to 0.040 h^{-1} and the development of cell number (CN), NO_2^- derived µ, NH_4^+ and NO_2^- concentrations of the cultures grown in the corresponding reactors BR3 and BR4. Cultures were gassed with 2 sL L^{-1} h^{-1} 0.5 % CO_2 enriched air, except for the gray area where CO_2 was increased to 2 %. The elevated CO_2 concentration induced an increase of NH4+ with a delay of 55.5 h and 216 h in BR3 and BR4 respectively. NH_4^+ was consumed again right after decreasing CO_2 back to 0.5 % which coincided with a temporary increase of the cell number in BR4. NH_4^+ and NO_2^- curves show mean values of technical triplicates and error bars represent the standard deviation of the mean.

CO_2 was increased. The reduction of CO_2 to 0.5 Vol.-% caused an immediate consumption of all NH_4^+ in both reactors (553.5 to 577 h). Increasing D to 0.030 h^{-1} increased S in both reactors, but at this time BR4 showed higher S than BR3. A further increase of D to 0.040 h^{-1} induced an extended increase of S similar to the behavior seen at D 0.035 h^{-1}.

Cell concentrations remained rather stable over the whole run from D of 0.010 h^{-1} to 0.030 h^{-1} at $6.94 \cdot 10^7 \pm 1.02 \cdot 10^7$ cells mL^{-1}. Only one strong increase from $7.24 \cdot 10^7$ cells mL^{-1} to $1.23 \cdot 10^8$ cells mL^{-1} was observed in BR4 directly after decreasing the CO_2 concentration of the ingas from 2 Vol.-% to 0.5 Vol.-%. However, the cell concentration decreased again to $7.77 \cdot 10^7$ cells mL^{-1} before increasing D from 0.020 h^{-1} to 0.030 h^{-1} (Figure 4). The amount of CO_2 in the gas phase seems to affect S. Depending on the CO_2 concentration of 0.5 Vol.-% and 2 Vol.-% in the gas phase S starts to accumulate at D of 0.030 h^{-1} or 0.020 h^{-1}, respectively.

Continuous cultures at high ammonia concentrations

Before starting a continuous culture with higher substrate concentrations, the inhibitory effect of NO_2^- was determined by batch cultures in 30 mL polystyrene tubes with varying NO_2^- starting concentrations of 0 to 19 mmol L^{-1} and 1 mmol L^{-1} NH_4^+. NO_2^- showed only a weak linear inhibitory effect on µ with 0.0342 to 0.0411 h^{-1} for 19 and 0 mmol L^{-1} NO_2^- starting concentrations, respectively (Figure S6).

The bioreactor experiment commenced with a 2 mmol L^{-1} NH_4^+ batch, followed by a fed-batch to increase NO_2^- up to 10 mmol L^{-1} and then switching into continuous mode to see the effect of D on cell number and S. The NH_4^+ to pyruvate ratio was changed in the feed from 2 to 5 to avoid excessive pyruvate concentrations that would promote bacterial growth in case of a contamination.

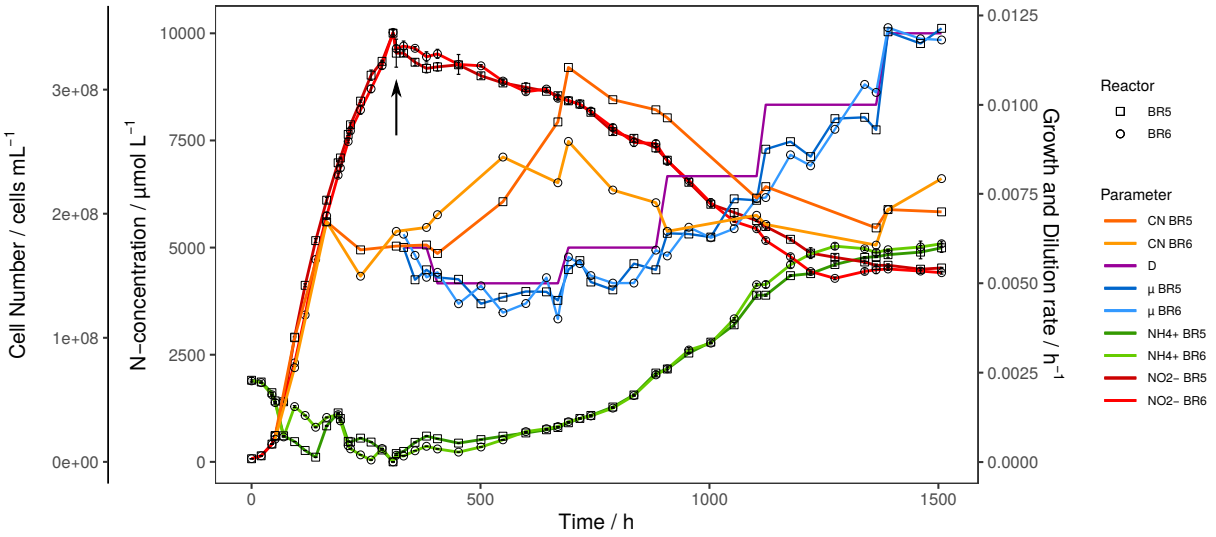

Figure 5: Continuous cultures of N. viennensis at high ammonia concentrations. Two continuous cultures gassed with 2 sL L^{-1} h^{-1} 0.5 % CO_2 enriched air, operated at D 0.005 h^{-1} to 0.012 h^{-1} and the development of cell number (CN), NO_2^- derived µ, NH_4^+ and NO_2^- concentrations of the cultures grown in the corresponding reactors BR5 and BR6. Batch cultures were grown with 2 mmol L^{-1} NH_4Cl and 1 mmol L^{-1} pyruvate and then fed with medium containing 32 mmol L^{-1} NH_4Cl and 4.75 mmol L^{-1} pyruvate to reach a final concentrations of 10 mmol L^{-1} NH_4Cl and 2 mmol L^{-1} pyruvate. The relative reduction of pyruvate to NH_4Cl caused a stagnation of the cell number, for which reason pyruvate was added to the cultures at the end of the feed phase (indicated by the arrow) to increase the concentration to 5 mmol L^{-1}. Cell number increased as a result of ROS detoxification by pyruvate, but started to decrease again as NH_4^+ increased. Once NH_4^+ stabilized at D 0.010 h^{-1} increasing D further did not result in an increase of NH_4^+. NH_4^+ and NO_2^- curves show mean values of technical triplicates and error bars represent the standard deviation of the mean.

For up to 6 mmol L^{-1} NO_2^- the cell number correlated very well with the NO_2^- concentration but then the cell number stagnated in both reactors even though NH_3 was still oxidized. Pyruvate was added to the reactors at the end of the fed-batch to increase the concentration from 2 mmol L^{-1} to 5 mmol L^{-1} and the feed media for the continuous cultures were also adjusted to 10 mmol L^{-1} NH_4Cl and 5 mmol L^{-1} pyruvate. Initial D was 0.006 h^{-1} to allow the cultures to recover but was further decreased to 0.005 h^{-1} to reduce the increase of S. In both cultures S appeared to be drifting, which means that µ (based on NO_2^- concentration) was always lower than D but still increased with increasing D (Figure 5). With such low D the residence time would have been far too long to wait for three volume exchanges to establish steady states (25 d at D = 0.005 h^{-1}). D was increased incrementally after at least 200 h from 0.005 to 0.006, 0.008, 0.010 and 0.012 h^{-1}. S eventually stabilized around 5 mmol L^{-1} at D 0.01 h^{-1} and an increase of D to 0.012 h^{-1} did not further increase S. The cell number increased in both reactors from $1.76 \cdot 10^8$ cells mL^{-1} and $1.88 \cdot 10^8$ cells mL^{-1} at the beginning of the continuous phase up to $3.22 \cdot 10^8$ cells mL^{-1} and $2.62 \cdot 10^8$ cells mL^{-1} for BR5 and BR6 respectively after D was increased to 0.06 h^{-1} and from there gradually decreased to about $2.00 \cdot 10^8$ at D = 0.01 h^{-1} (Figure 5).

Even though very low D were used in this continuous culture experiments, surprisingly high NH_4^+ concentrations were measured. Once S stabilized, increases of D did not result in an increase of S as observed before with 2 mmol L^{-1} cultures. The NH_4Cl to pyruvate ratio should remain 2:1 to ensure ROS detoxification.

Quantitative physiological analysis of growth parameters

For the following analysis only steady states below D 0.050 h^{-1} were considered, as we expect the results at and beyond this D to be heavily influenced by biofilm formation. Data points at D 0.0124 h^{-1} and 0.0146 h^{-1} are from three different bioreactors that were used for lab scale biomass production (data not shown).

As shown in Figure 6a the cell number of *N. viennensis* increased slightly with D from $5.84 \cdot 10^7$ $\pm 1.19 \cdot 10^7$ cells mL^{-1} at D = 0.010 h^{-1} up to $7.67 \cdot 10^7$ $\pm 2.11 \cdot 10^7$ cells mL^{-1} at D = 0.035 h^{-1} from where it decreased linearly with D to $4.36 \cdot 10^7$ $\pm 2.18 \cdot 10^6$ cells mL^{-1} at D = 0.046 h^{-1}. Up to D = 0.020 h^{-1} all substrate was consumed by cultures gassed with 0.5 Vol.-% CO_2. For 2 Vol.-% CO_2 gassed cultures S started to accumulate at D = 0.020 h^{-1} to 138 ±58.9 µmol L^{-1} and increased steeply up to 745.5 ±27.6 µmol L^{-1} NH_4^+ at D = 0.035 h^{-1} where it began to stagnate and reach a maximum of 850.0 ±11.6 µmol L^{-1} at D = 0.046 h^{-1}. Despite the early onset of increasing S, the cell concentration remained surprisingly stable up to D = 0.035 h^{-1}. Controversially, while the cell number was decreasing with D from 0.035 to 0.046 h^{-1}, S increased only marginally, thus changes in S appeared to be not reflected in the cell number and vice versa.

NO_2^- and cell productivities (P_{NO2}, P_C) are shown in Figure 6b. P_{NO2} increases logarithmically from D of 0.010 to 0.020 h^{-1} and from there on in a linear way up to 49.6 ±0.3 µmol L^{-1} h^{-1} at D = 0.046 h^{-1}. P_C increases linearly from $5.84 \cdot 10^5$ $\pm 1.19 \cdot 10^5$ cells mL^{-1} h^{-1} at D = 0.010 h^{-1} up to $2.69 \cdot 10^6$ $\pm 7.39 \cdot 10^5$ cells mL^{-1} h^{-1} at D = 0.035 h^{-1} from which point it started to decline to $2.00 \cdot 10^6$ $\pm 1.00 \cdot 10^5$ cells mL^{-1} h^{-1} at D = 0.046 h^{-1}. As a result of a variable $Y_{(X/NH3)}$ (see below), P_{NO2} and P_C do not correlate in a fixed 1:1 ratio as would be expected. For comparison P_{NO2} and P_C of 10 mmol L^{-1} cultures at D = 0.012 h^{-1} are also shown in Figure 6c with 53.6 ± 0.9 µmol L^{-1} h^{-1} and $2.61 \cdot 10^6$ $\pm 2.30 \cdot 10^5$ cells mL^{-1} h^{-1}, respectively. The highest P_C was reached at D = 0.035 h^{-1} which is therefore the optimal D for biomass production for *N. viennensis*.

As shown in Figure 6c an average $Y_{(X/NH3)}$ of $3.36 \cdot 10^7$ $\pm 5.77 \cdot 10^6$ cells μmol^{-1} L^{-1} was determined from the batch cultures before changing into continuous mode. $Y_{(X/NH3)}$ from steady states at D of 0.010 to 0.030 h^{-1} or 10 mmol L^{-1} NH_4Cl cultures (D from 0.005 to 0.012 h^{-1}) were well within this range, but from D = 0.035 h^{-1}, $Y_{(X/NH3)}$ were highly elevated with $6.12 \cdot 10^7$ $\pm 1.68 \cdot 10^7$ cells μmol^{-1} L^{-1} and decreased to $3.79 \cdot 10^7$ $\pm 1.90 \cdot 10^6$ cells μmol^{-1} L^{-1} at D = 0.046 h^{-1}. While $Y_{(X/NH3)}$ of steady states at low D are comparable to batch cultures, at higher D, $Y_{(X/NH3)}$ increased up to 81.7% (D = 0.035 h^{-1}) compared to batch cultures.

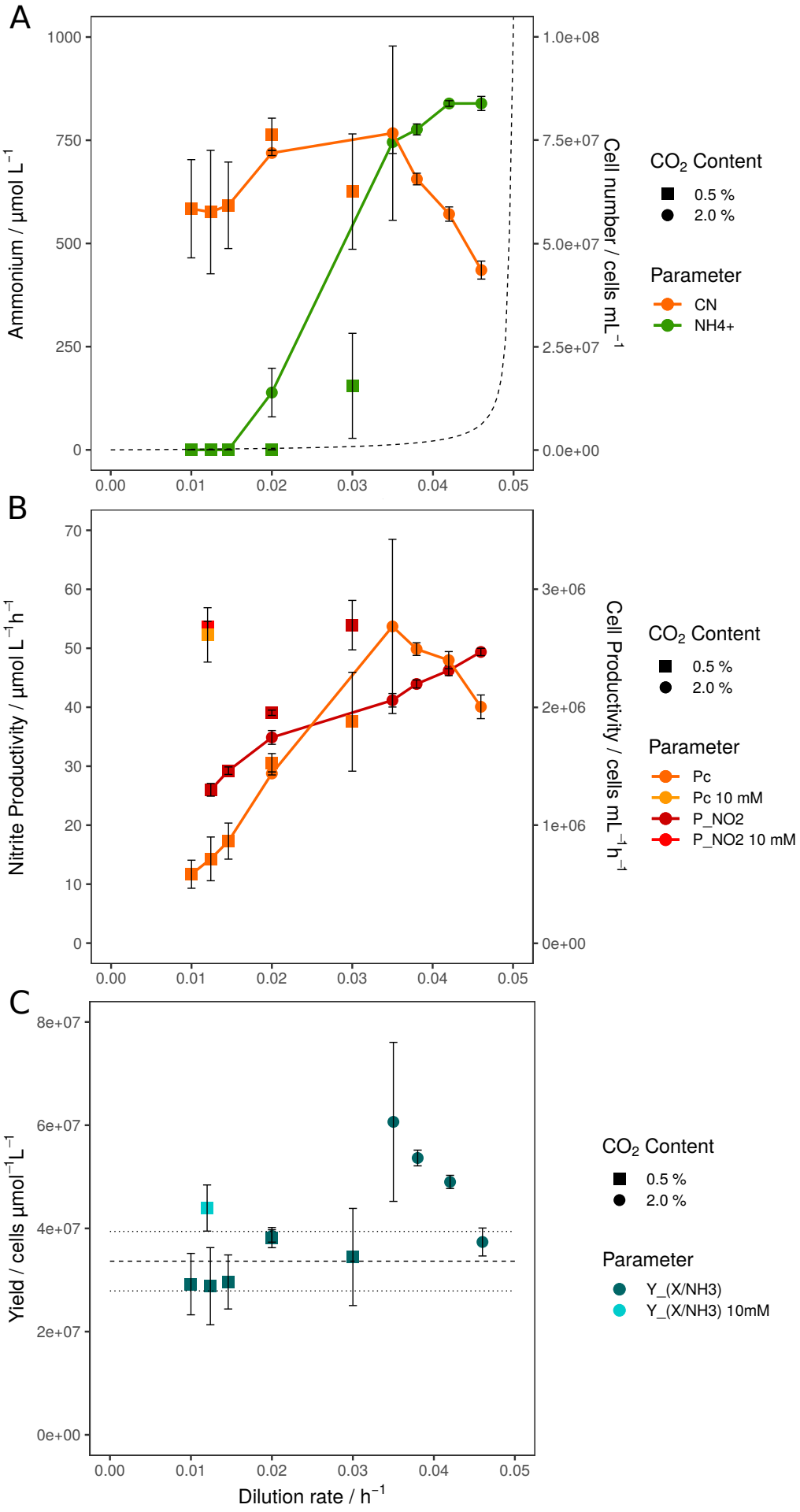
A
Ammonium / µmol L^{-1}
Cell number / cells mL^{-1}
1000
750
500
250
0
1.0e+08
7.5e+07
5.0e+07
2.5e+07
0.0e+00
0.00
0.01
0.02
0.03
0.04
0.05
CO$_2$ Content
0.5 %
2.0 %
Parameter
CN
NH4+
B
Nitrite Productivity / µmol L^{-1}h^{-1}
Cell Productivity / cells mL^{-1}h^{-1}
70
60
50
40
30
20
10
0
3e+06
2e+06
1e+06
0e+00
CO$_2$ Content
0.5 %
2.0 %
Parameter
Pc
Pc 10 mM
P_NO2
P_NO2 10 mM
C
Yield / cells µmol^{-1}L^{-1}
8e+07
6e+07
4e+07
2e+07
0e+00
Dilution rate / h^{-1}
CO$_2$ Content
0.5 %
2.0 %
Parameter
Y_(X/NH3)
Y_(X/NH3) 10mM

Figure 6: Quantitative analysis of physiological growth parameters. **(A)** Cell number and S in response to different D and CO_2 concentrations. Cell number increased slightly with D up to $7.67 \cdot 10^7 \pm 2.11 \cdot 10^7$ cells mL^{-1} at D 0.035 h^{-1} and from there decreased linearly to $4.36 \cdot 10^7 \pm 2.18 \cdot 10^6$ cells mL^{-1} at D 0.046 h^{-1}. NH_4^+ was completely consumed up to D 0.020 h^{-1} by cultures gassed with 0.5 % CO_2 but accumulated with 2 % CO_2 gassed cultures at D 0.020 h^{-1} and increased steeply up to 745.5 ±27.6 µmol L^{-1} NH_4^+ at D 0.035 h^{-1} where it began to stagnate and reach a maximum of 850.0 ±11.6 µmol L^{-1} at D 0.046 h^{-1}. The dashed line shows the theoretical development of S with increasing D assuming Monod kinetics, K_S equal to $K_{m(app)}$ of 5.4 µmol L^{-1} and μ_{max} of 0.048 h^{-1}, illustrating the difference to the observed growth behavior of *N. viennensis*. NH_4^+ and cell number curves show the mean values of biological duplicates, of which each NH_4^+ value consists of technical triplicates (samples taken at different time points), and error bars represent the standard deviation of the mean. **(B)** Nitrite (P_{NO2}) and cell productivity (P_C) of 2 and 10 mmol L^{-1} NH_4Cl continuous cultures at different D and CO_2 concentrations. P_{NO2} increased logarithmic from D 0.010 to 0.020 h^{-1} and from there on in a linear way up to 49.6 ±0.3 µmol L^{-1} h^{-1} at D 0.046 h^{-1}. P_C increased in a linear fashion from $5.84 \cdot 10^5 \pm 1.19 \cdot 10^5$ cells mL^{-1} h^{-1} at D 0.010 h^{-1} up to $2.69 \cdot 10^6 \pm 7.39 \cdot 10^5$ cells mL^{-1} h^{-1} at D 0.035 h^{-1} from which point it started to decline to $2.00 \cdot 10^6 \pm 1.00 \cdot 10^5$ cells mL^{-1} h^{-1} at D 0.046 h^{-1}. P_{NO2} and P_C of 10 mmol L^{-1} cultures at D 0.012 h^{-1} with 53.6 ±0.9 µmol L^{-1} h^{-1} and $2.61 \cdot 10^6 \pm 2.30 \cdot 10^5$ cells mL^{-1} h^{-1} respectively are shown for comparison. P_{NO2} and P_C neither correlate in a fixed 1:1 ratio as would be expected for 0.5 % nor 2 % CO_2 gassed cultures, indicating a variable $Y_{(X/NH3)}$. P_{NO2} and P_C points show mean values of biological duplicates, triplicates for D 0.0124 h^{-1} and 0.0146 h^{-1}, and error bars represent the standard deviation of the mean. **(C)** Biomass to ammonia yield in response to different D and CO_2 concentrations. The dashed line indicates the mean $Y_{(X/NH3)}$ of batch cultures, before changing into continuous mode, of $3.36 \cdot 10^7 \pm 5.77 \cdot 10^6$ cells μmol^{-1} L^{-1} and dotted lines represent the standard deviation of the mean. $Y_{(X/NH3)}$ from steady states D 0.010 to 0.030 h^{-1} and 10 mmol L^{-1} NH_4Cl cultures (D 0.005 to 0.012 h^{-1}, only D 0.012 h^{-1} shown) are quite comparable to $Y_{(X/NH3)}$ calculated from batch cultures, but $Y_{(X/NH3)}$ at D 0.035 h^{-1} was highly elevated at $6.12 \cdot 10^7 \pm 1.68 \cdot 10^7$ cells μmol^{-1} L^{-1} from where it successively decreased with D to $3.79 \cdot 10^7 \pm 1.90 \cdot 10^6$ cells μmol^{-1} L^{-1} at D 0.046 h^{-1}. Cultures at D 0.020 h^{-1} gassed with 0.5 % and 2 % CO_2 had very much the same $Y_{(X/NH3)}$ with $3.82 \cdot 10^7 \pm 1.96 \cdot 10^6$ cells μmol^{-1} L^{-1} and $3.86 \cdot 10^7 \pm 3.34 \cdot 10^5$ cells μmol^{-1} L^{-1} respectively. $Y_{(X/NH3)}$ points show mean values of biological duplicates, triplicates for D 0.0124 h^{-1} and 0.0146 h^{-1}, and error bars represent the standard deviation of the mean.

Discussion

The substrate affinity, together with μ, are very important parameters for understanding the ecological strategy of an organism (r- and k-strategists). Due to their very high substrate affinity and low μ, AOA are regarded as typical k-strategists but the growth behavior of *N. viennensis* observed in this study can not sufficiently be described with Monod kinetics or more sophisticated models like the Briggs-Haldane model. The strong increase of S at D = 0.035 h^{-1} would indicate that D was already close to μ_{max}, but then S should have increased right after the start of the continuous culture and further increases of D should have resulted in even stronger increases of S. Instead S plateaued around 800 μmol L^{-1} NH_4^+ while the cell number decreased with D increasing up to 0.046 h^{-1}. An explanation for this behavior would be the formation of a biofilm that would retain cells in the reactor, but at the same time decrease planktonic cell concentrations. Thus the activity of the whole system would increase while at the same time the measured cell concentrations would decrease, which describes the results. The stable S concentrations and spontaneous but isochronal activity increases are difficult to integrate into this explanation. Only very strong increases of D like from 0.050 h^{-1} to 0.060 h^{-1} and further to 0.070 h^{-1} caused S to increase temporarily while cell concentrations remained surprisingly stable – considering a μ_{max} of 0.048 h^{-1} for *N. viennensis*. At D > μ_{max} cells should usually be washed out over time, but due to the seeding of cells by the biofilm this phenomenon was not observed. The linear decrease of S at D = 0.060 h^{-1} might be the result of an increase in active biofilm biomass, that reaches its limits at D = 0.070 h^{-1}. This would explain the very stable concentrations of S after the spontaneous activity increase. These increases of activity might be induced by quorum sensing and be dependent on cell density in the biofilm, which should gradually increase with time until a maximum is reached. This would provide a robust principle which could explain the synchronistic nature of this very unusual physiological phenomenon. The mechanistic principle of how the cells are able to increase their substrate affinity abruptly could be explained by the expression of multiple amoC genes, of which the genome of *N. viennensis* contains six, which is likely to be the subunit that contains the catalytic center of the AMO protein complex. This assumption is based on recent findings of a bacterial particulate methane monooxygenase (Ross et al., 2019).

In addition, there seems to be another regulatory element in the growth behavior of *N. viennensis* that was observed with 2 mmol L^{-1} and 10 mmol L^{-1} NH_4^+ continuous cultures. At a certain D the organism consumes NH_3 at a slightly lower rate than provided, thus slowly increasing S until it finally stabilizes at roughly 50% of S_i. Once stabilized, S only marginally increases with D unless very strong changes are induced. This phenomenon appears to be linked to an increase in $Y_{(X/NH3)}$, which seems to be highly elevated at D > 0.030 h^{-1}. However, we must state that we analyze the biomass productivity in relation to the energy metabolism, assuming that energy is limiting the biomass formation and not carbon, which was provided in excess to *N. viennensis* during chemostat and biofilm formation experiments.

In a steady state $Y_{(X/NH3)}$ might be increased because the enzymatic machinery and metabolic networks can be fine tuned to a stable environmental condition. A batch culture needs to adapt constantly to the changing environment which requires energy. However, this phenomenon occurs in every organism and can not describe the vast increase of $Y_{(X/NH3)}$ in continuous cultures of up to 81.7% compared to batch cultures. This might only be explained by taking into account the unique nature of archaeal ammonia oxidation, which produces significant amounts of ROS that can destroy the cells if not taken care of by the environment (e.g., by catalases, alpha keto-acids). Thus, there is

a strong selection pressure on the organism to regulate its activity in accordance to its environment. Given the wide distribution of AOA, it seems that these organisms might have evolved elaborate metabolic regulations to enable them to thrive on this planet. The production of ROS by AOA might thus be key to understand their ecological success, because it could also be used by the organisms to generate substrates from the environment by oxidative decarboxylation (Kim et al., 2016) or oxidative deamination (Akagawa et al., 2002) of organic matter. This would supply the organism with both CO_2 and NH_3 and could explain why some AOA, like the members of the Nitrosocosmicus clade, are highly abundant in very organic rich soils. This kind of metabolism however requires a high degree of metabolic regulation which might be enabled by the transcription apparatus of archaea, which is usually summarized as a simplified version of the eukaryotic machinery, even though this observed simplicity is under constant revision as new insights are accumulated (Gehring et al., 2016). The unique nature of archaea and their gene regulation could therefore be responsible for this form of NH_3 oxidation. It appears that the guiding principle behind the growth dynamics of *N. viennensis*, and probably other AOA, is not substrate affinity but the maximization of $Y_{(X/NH3)}$ and therefore optimal utilization of a usually very limited substrate.

These insights into the regulation of the energy metabolism of *N. viennensis* have important implications for further bioprocess development to optimize biomass productivity. Due to the increase of $Y_{(X/NH3)}$ with higher D, biomass productivity also increases despite high S concentrations. CO_2 concentration in the ingas plays a crucial role in the way that it does not effect $Y_{(X/NH3)}$ but μ_{max} and S. An optimal CO_2 concentration should not reduce μ_{max} but at the same time minimize S and therefore maximize the cell concentration at a given D and S_i. For higher concentrated feed medium it is probably worth in terms of biomass productivity to accept higher S concentrations as long as D positively affects $Y_{(X/NH3)}$ and biomass productivity. It is important to note that the NH_4Cl to pyruvate ratio should always be at least in a 2:1 to prevent self toxification by endogenously produced ROS. Depending on the scientific question, higher NH_4^+ concentrations might not be desirable as it certainty influences biomass composition and gene expression. For compounds of interest that can not be recombinantly produced, higher substrate concentrations might very well be the method of choice as it looks promising to obtain good biomass productivities if further improved.

For the production of biomass, biofilm formation is not favourable as it reduces the amount of cells that can be harvested and thus the effective $Y_{(X/NH3)}$. Elevated temperatures are known to increase biofilm formation (Qureshi et al., 2005), therefore lowering the temperature might be a solution but at the cost of reducing μ_{max}. Reducing the CO_2 concentration will also likely reduce biofilm formation, because carbonate precipitate will be reduced and therefore also available surface that can induce biofilm formation. On the other hand, to understand the ecological function and behavior of *N. viennensis*, it would be very important to study the organism in biofilms, as this is more likely to be its prevalent form in soils. Gene abundances based on 16S rRNA or *amo*A are often used to infer NH_3 oxidizing activity of AOA in soils, but discrepancies with activity measurements of soil incubations are known. Estimates from this study show that only 14.67% of the cells in the biofilm would have been active at their maximum capacity. These results were obtained from two calculations with different assumptions in two different reactors in two different experiments. However, independent of the discrepancy of the results, this high ratio of inactive cells could also explain earlier findings of inactive cells that started the hypothesis of mixotrophic AOA together with the growth enhancing effect of alpha keto acids (Mußmann et al., 2011; Tourna et al., 2011). As biofilms have the potential for very complex cell interactions, it might also be that “inactive”

cells simply perform different tasks in the biofilm beside NH_3 oxidation. Regardless, careful validation and/or correction of these results with future experiments would be needed to have a more accurate picture of the behavior of AOA biofilms.

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Supplementary Figures

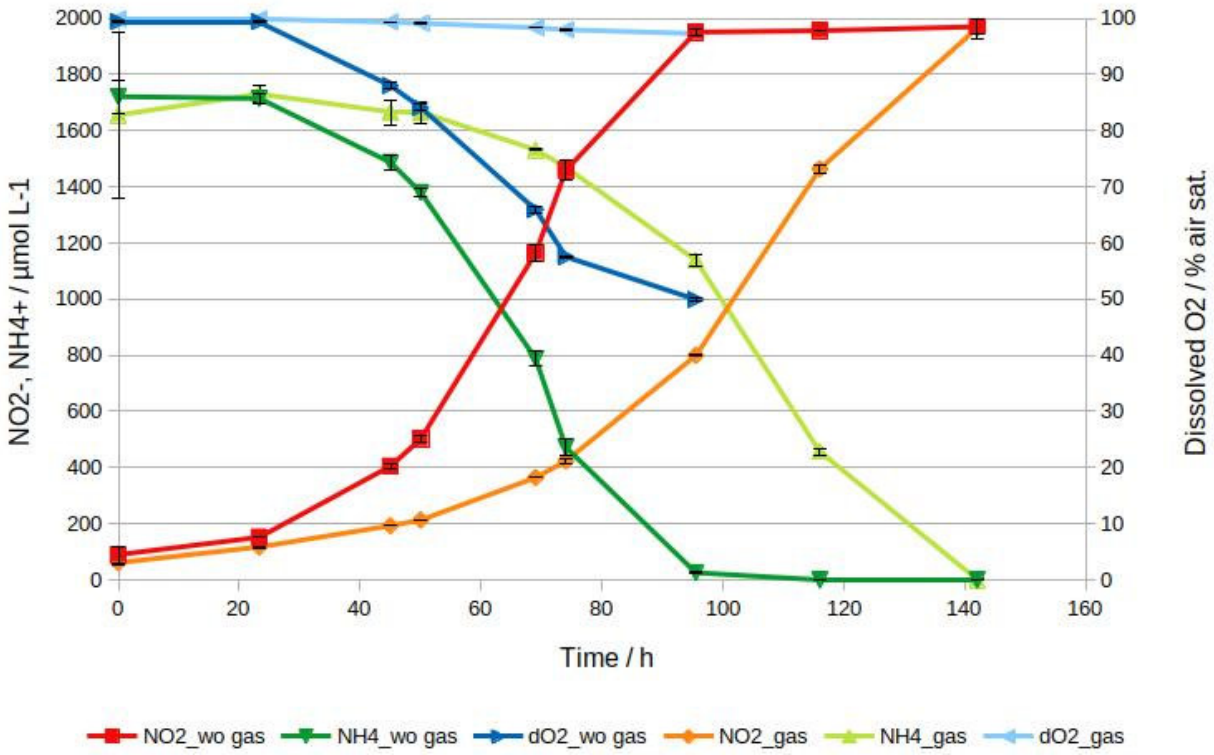

Figure S1: Effect of gassing on batch cultures. NH_4^+, NO_2^- and dO_2 concentrations of open batch cultures gassed with 2 sL L^{-1} h^{-1} air (gas) or closed batch cultures (wo gas) all containing 2 mmol L^{-1} $NaHCO_3$ as C-source. Cultures gassed with air had decreased µ of 0.0276 ±0.0001 h^{-1} compared to closed batch cultures with µ 0.0445 ±0.0004 h^{-1}. NH_4^+, NO_2^- and dO_2 curves show mean values of biological duplicates and error bars represent the standard deviation of the mean.

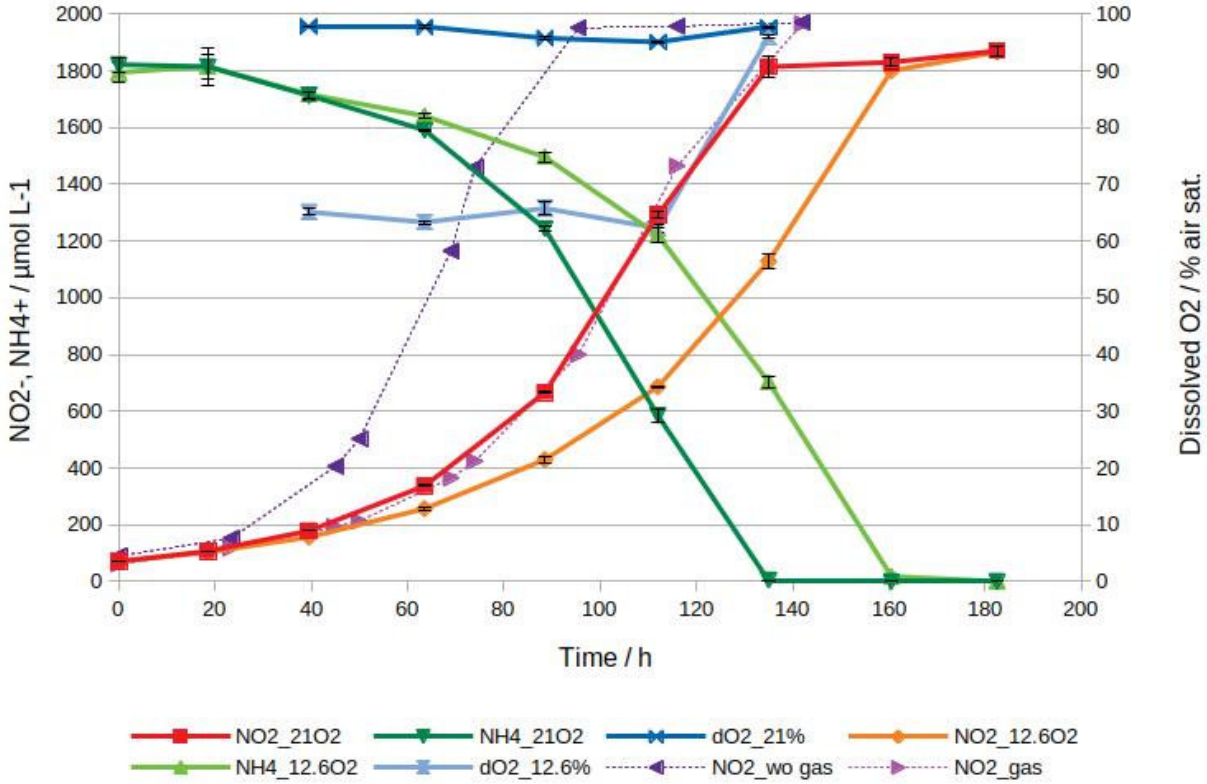

Figure S2: Effect of gassing rate and ingas flow composition on batch cultures. NH_4^+, NO_2^- and dO_2 concentrations of open batch cultures gassed with 1 sL L^{-1} h^{-1} air (21O2) or air/N_2 mix (12.6O2) to reduce the dO_2 concentration. NO_2^- curves of closed batch (wo gas) and open batch cultures gassed with 2 sL L^{-1} h^{-1} air (gas) are shown as comparison. Reducing the gassing rate did only marginally effect µ (0.0268 ±0.0001 h^{-1}) while decreasing dO_2 concentration did decrease µ to 0.0206 ±0.0001 h^{-1}. The increase of dO_2 at 135 h was due to technical issues. NH_4^+, NO_2^- and dO_2 curves show mean values of biological duplicates and error bars represent the standard deviation of the mean.

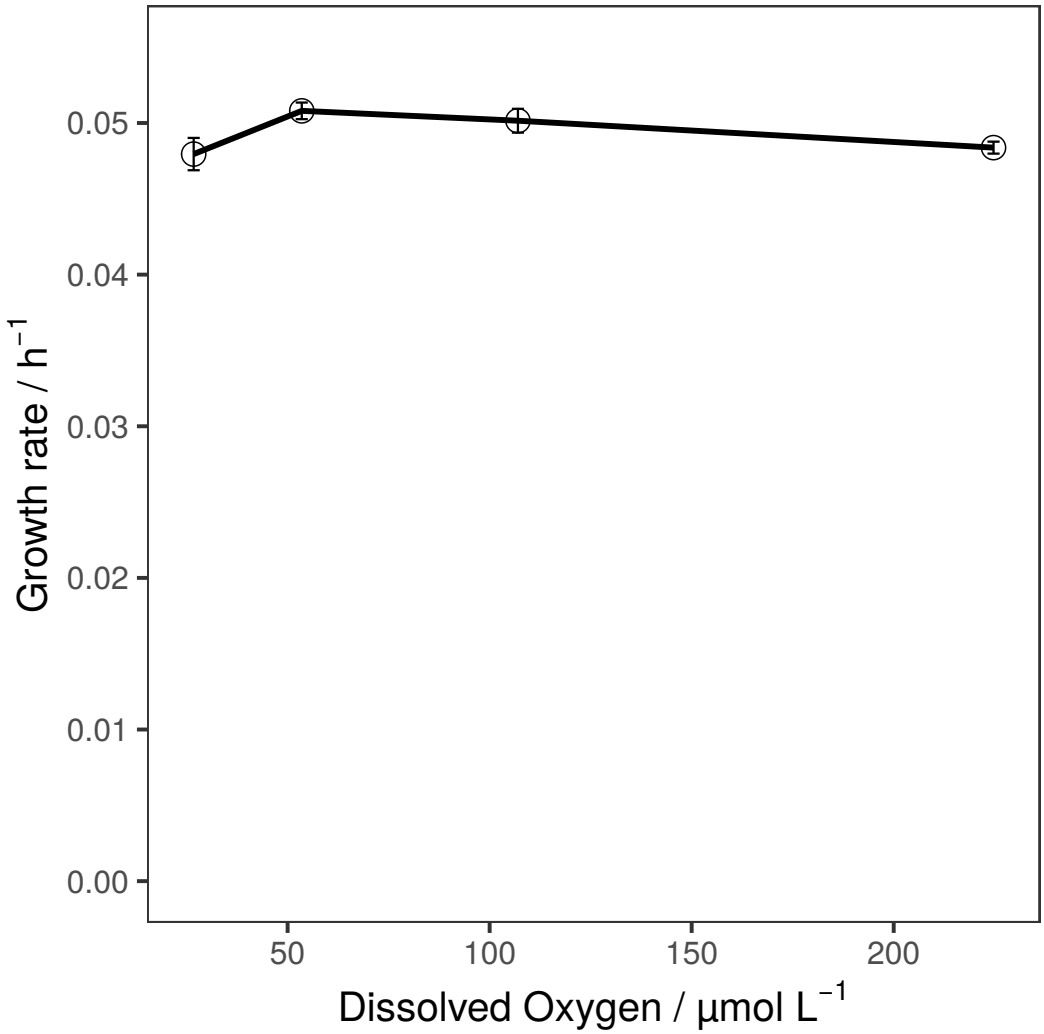

Figure S3: Maximum growth rate of N. viennensis. µ of closed batch cultures grown at different dO_2 concentrations in serum flasks with 0.5 % CO_2 in the atmosphere. Highest µ of 0.0508 ±0.0005 h^{-1} was achieved at 53.5 µmol L^{-1} dO_2 (5% O_2 in the gas phase) compared to 0.0484 ±0.0004 h^{-1} at 224.7 µmol L^{-1} (21% O_2 in the gas phase). Due to the remarkable little effect of dO_2 on µ, 21% O_2 were used for continuous cultures and a µmax of 0.0484 h-1 was therefore assumed. µ values show the mean of quadruplets and error bars represent the standard deviation of the mean.

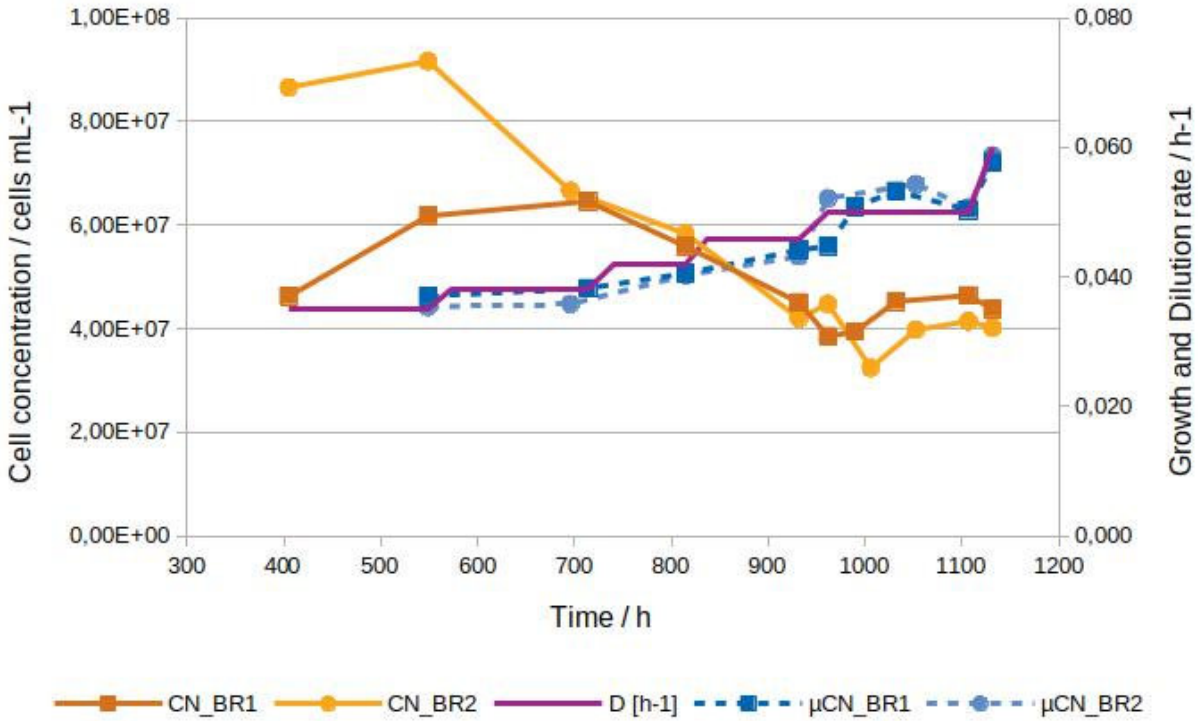

Figure S4: Increase of cell number based growth rate in continuous cultures. µ of BR1 and BR2 based on cell concentration from D 0.035 h^{-1} to 0.060 h^{-1}. Even though the cell concentration decreased from D 0.035 h^{-1} to D 0.050 h^{-1}, µ increased with D but remained slightly below the set value until D 0.050 h^{-1} where it started to surpass D leading to an increase in cell concentration.

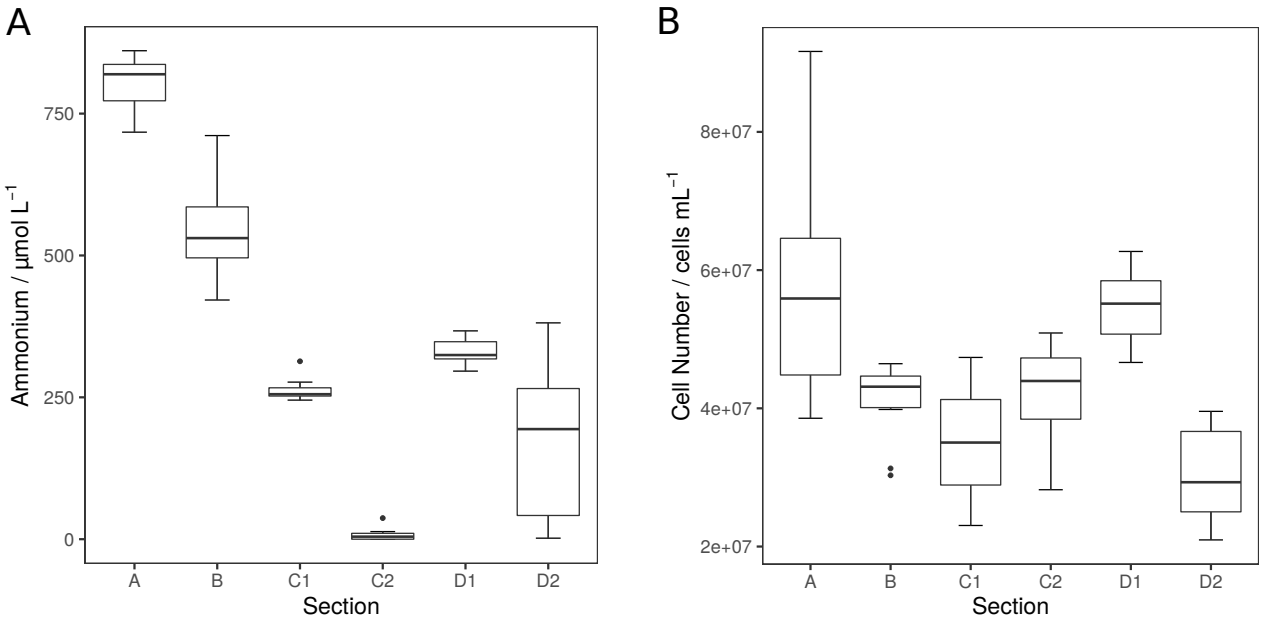

Figure S5: Boxplots of NH_4^+ and cell number of continuous cultures at high dilution rates. (A) NH_4^+ concentrations of sections A (382 to 990 h), B (1032 to 1654 h), C (1796 to 2035 h) and D (2062 to 2402 h). Different steady states of BR1 and BR2 in sections C and D are signified by the corresponding number. Outliers known to be caused by technical issues (1271, 1295, 1343 and 1346 h) were removed from the dataset. (B) Cell number of BR1 and BR2 of sections A to D, the different steady states of BR1 and BR2 in sections C and D are signified by the corresponding number.

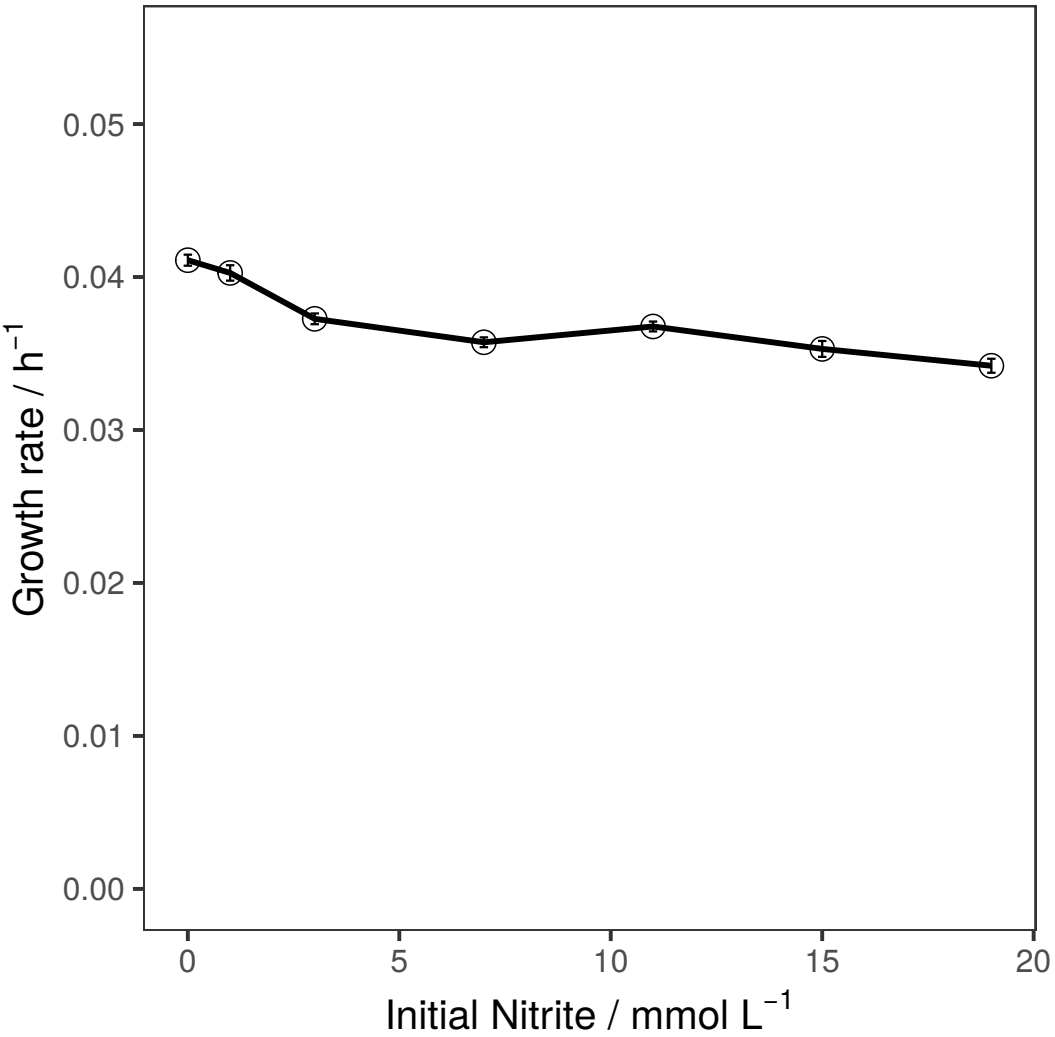

Figure S6: Inhibitory effect of nitrite on the growth rate. 20 mL batch cultures grown in 30 mL polystyrene tubes with 1 mmol L-1 NH4+ and 0 to 19 mmol L-1 NO2- starting concentrations. NO2- exhibited only a weak linear inhibitory effect on µ with 0.0342 ±0.0004 h-1 to 0.0411 ±0.0005 h-1 for 19 and 0 mmol L-1 NO2- starting concentrations respectively. µ values show the mean of triplicates and error bars represent the standard deviation of the mean.

Chapter V

Discussion

This thesis contributes to the field of nitrification and archaea research as it provides the first pure culture of an extremely thermophilic ammonia oxidizing microorganism, as no bacteria are known to perform this reaction at that high temperature. *Nitrosocaldus cavascurensis* is not only physiologically very interesting but its particular phylogenetic position make it ideal to study ancestral features of ammonia oxidizing archaea. Even though evolution does not stop, certain niches preserve genetic features of organisms like for example the genes for the AMO complex of *N. cavascurensis*, while other genetic elements such as transposons of prophages can still be highly dynamic parts of a genome. Due to the difficulty of handling and interpreting enrichment cultures, the isolation of this organism in pure culture opens up many possibilities for further research.

The application of continuous culture systems with *N. viennensis* has not only resulted in the generation of biomass that made biochemical research and structural characterization of the AMO complex possible (Hodgskiss, Melcher et al. 2022), but also revealed a unique growth behavior of the organism that might be common among other AOA as well. Although this study only provided a rough overview of this phenomenon, interesting features such as the stabilization of residual NH_4^+ concentrations (S) and spontaneous increases in NH_3 oxidizing activity were repeatedly observed. Further ongoing research in the laboratory based on RNA-Seq data from the biofilm and planktonic cells from the same reactor will hopefully shine more light on this bizarre phenomenon.

Unexpected observations in *N. cavascurensis* cultures

While *N. cavascurensis* appears to have the typical physiological features of an AOA, regarding pH range, NH_3, NO_2^- and O_2 tolerance, it also demonstrated some surprising behavior. First of all its ability to perform NH_3 oxidation in the absence of a nitrite reductase, which is believed to serve a critical function in the archaeal NH_3 oxidation, was very surprising. Though it might be possible that its genome contains a yet unknown NO_2^- reductase, activity assays with crude cell extracts from *N. cavascurensis*, *N. viennensis* and Thermus sp. rather suggest that the organism is simply not capable of performing NO_2^- reduction while *N. viennensis* can. This of course is connected to the role NO plays in the NH_3 oxidation of *N. cavascurensis* as well as other AOA. The high sensitivity of *N. cavascurensis* (and other AOA) to NO-scavengers like PTIO might also simply derive from toxic side effects on the organism, because “neutralization” of NO-scavengers with an NO-donor simply showed no effect at all. This of course raises another question about the applicability of NO-scavengers such as PTIO and cPTIO in AOA research and nitrification, as those are commonly used substances in the field. Yet, not a single study has done any effort to show that the growth inhibition of AOA actually derives from scavenging NO and not from other toxic side effects. Another intriguing result was the absence of an effect of α-keto acids on the growth behavior of *N. cavascurensis*. Even *Ca*. Nitrosocosmicus exaquare, which contains multiple enzymes to deal with oxygen derived stress, shows enhanced growth in the presence of α-keto acids. On the other hand pyruvate had a positive effect for the cryo-preservation of *N. cavascurensis*, especially cultures grown with pyruvate show very fast recovery times after inoculation.

The question about mixotrophy and alternative energy metabolisms of *N. cavascurensis*, unfortunately, remains open, as all efforts to induce growth without NH_3 oxidation were futile. This does not mean per se that *N. cavascurensis* is not able to do so, but rather that far more effort would have to go into this direction if meaningful answers were to be found. For a start it would be helpful to get a transcriptome from an NH_3 oxidizing culture to see the transcriptional level of the enzymes that are proposed to be responsible for aromatic ammino acid fermentation under normal conditions. The problem with genomic hypotheses is that only because an organism has a certain set of genes

that might enable it to perform a specific metabolism, doesn't mean it can actually do so. Yet the information provided by the genomic data can be so convincing that it can be hard to change ones mind to see things from another perspective. A perfect example for this phenomenon was the hypothesis that *N. cavascurensis* would depend on Thermus sp. due to its lack of NirK and the presence of the gene in the bacterial genome. The hypothesis made sense and fitted superficial observations, since filtration and antibiotic treatments sterilized cultures or had a detrimental effect on AOA growth and abundance. Only after the observation of dozens if not hundreds of cultures under the microscope did this picture start to shake as no clear pattern between bacterial abundance and AOA growth could be observed. Finally after the preliminary experiment to determine the pH under controlled conditions it became clear that *N. cavascurensis* can grow surprisingly well with very few bacteria if the pH is kept in its optimal range of pH 6.5 to 7.0.

Isolating a pure culture of a challenging AOA

Obtaining *N. cavascurensis* in stable and then pure axenic culture was a long journey. During the application of antibiotics one has to take into consideration that beside their main function (e.g. polymerase inhibitor) they can have also unspecific side effects and change the conditions of the medium like any other substance if added in higher concentrations. Therefore it was important to test different concentrations with each antibiotic to find the concentration range where the right antibiotic shows highest effect against the bacterial contaminants but at the same time no or only little detrimental effect on *N. cavascurensis*. Another important factor in achieving a pure culture was the state of the microbial community and the well beeing of *N. cavascurensis*. Chances to succeed with an antibiotic treatment are lowest if microbial diversity is high and the organism is already suffering without drugs because its growth conditions are not yet optimized. For the isolation of *N. cavascurensis* conditions had to be already well optimized (68 °C, pH 7.0 with CO_3^{2-} buffer to stabilize the pH, reduced oxic stress with 10 % O_2 atmosphere), AOA were healthy (good growth rate, cell walls intact, high cell number, few lysed cells) and microbial diversity was very low due to an optimized passaging rhythm that prevented cultures to become stationary.

Similar to the side effects of antibiotics, PTIO and cPTIO might very well be toxic for AOA regardless of their NO-scavenging function and the difference of those compounds observed between AOA and AOB arise out of the fact that they possess very different membranes and S-layer or gram negative cell walls respectively. One way to check for toxicity of the compound might be to add PTIO to early stationary cultures and compare the decay rate of the cells to normal stationary cultures (cell number measured by cell counts, DNA concentration or qPCR). If there are no toxic side effects of PTIO the decay rate should be the same for both cultures. In general *N. cavascurensis* seems to be a very sensitive organism, which might be a result of the high growth temperature which increases the reactivity of substances and thus amplify their negative effects.

Complex growth regulation and biofilm formation in continuous cultures of *N. viennensis*

The continuous culture study of *N. viennensis* provided fascinating observations about a very complex growth behavior for the first time. Grown at higher dilution rates (D), substrate concentration (S) starts to increase, as would be expected if D is near μ_{max}, but then stabilizes even if D is further increased. This behavior seems to be influenced by the availability of CO_2 as S increased with a delay of several days after CO_2 concentrations in the gas mix were increased. What might have been even more puzzling were the spontaneous activity increases in both reactors twice at the same time without contemporary external cause. Therefore the rationale was considered to be

intrinsic to the system and due to the nature of a steady state system it could only be related to the cell number of the forming biofilm. Apparently the organism can change its K_S by a yet unknown mechanism, but we suspect the modular architecture of the AMO enzyme complex to be responsible for it, in particular the multiple amoC variants of *N. viennensis*. Even more surprising was the fact that at D 0.065 h^{-1} two different “steady states” stabilized, but due to perturbation of the system caused by the sampling process for RNA Seq samples, one reactor started to increase S over several days until both reactors merged in the same S.

The biofilm activity experiment was no less of a surprise. First it is astonishing that after exchanging the medium in the reactors twice, the exact same steady state as before the washing step was reproduced by both cultures. This was even more puzzling as one reactor was constantly loosing biofilm biomass, because it was scraped off the reactor walls by precipitate that was washed off the inlet during the medium exchange. Either the activity of this culture should have been much higher as more active biofilm surface would be available due to free swimming flocks, or the activity should be less because of decreasing biomass. But in fact for the first two days the activity of both reactors was exactly the same, and only later did the activity of the culture decrease as more biomass was washed out of the reactor. This is clear evidence that the NH_3 oxidation activity does not need to correlate with cell number in steady state systems, which was also observed in WWTPs (Mußmann, Brito et al. 2011), another steady state system.

At the core of all this observations is the variability of the biomass to substrate yield ($Y_{(X/NH3)}$). While $Y_{(X/NH3)}$ was rather stable in batch cultures and continuous cultures with low D, at higher D $Y_{(X/NH3)}$ increased up to 81.7 %. This is most likely linked to the endogenous production of ROS and the availability of organic substances in the environment to prevent cell damage. An aspect of ROS production of AOA that is often overlooked is the generation of both major substrates, CO_2 which is produced by oxidative decarboxylation (Kim, Park et al. 2016) and NH_3 produced by oxidative deamination (Akagawa, Sasaki et al. 2002) of organic matter. This might help to explain the high abundance of certain AOA, like the Nitrosocosmicus clade, in very organic rich soils.

In summary, this thesis has provided the first pure culture of an extremely thermophilic NH_3 oxidizer to the field of AOA research and nitrification and a preliminary production process for the generation of AOA biomass to enable further biochemical research of these intriguing organisms. While typical physiological parameters like pH, temperature, NH_4^+, NO_2^- and O_2 concentration have been characterized for *N. cavascurensis*, other questions like the role of NO and alternative metabolisms could not be fully resolved. The cultivation of *N. viennensis* in continuous culture not only provided biomass for important biochemical studies but also revealed a unique growth behavior that can not easily be explained by classical models like Monod kinetics. To understand the ecological function of AOA and how they affect the N-cycle, this behavior will have to be studied in more depth, as substrate affinity and specific growth rate are clearly insufficient to describe this level of complexity. With time, effort and patience hopefully further physiological and transcriptomic data will be generated to understand these organisms in a more holistic way.

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