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Dissimilatory nitrate reduction in *Bacillus*

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PhD, Doctor of Philosophy, before I fully understand what it means, I came here far away from home for it. This 4-years exploring journey is both long and short. It is long because it took so much effort to figure out one topic in science even not fully understood it in the end, or let's say that human beings can only take one step further in the exploring of knowledge with much more left to explore. Too short it is because I just figured out what it means to work in science and how to do it better, and 4 years has passed. Anyway in short, it has been my great great great pleasure to do my PhD in our Lab of microbiology in Ghent University. There are so much that I want to say and to thank for my 4 years life here, but here I have to make it short.

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Gent, January, 2017

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孙艺华

Gent, January 20th, 2017

List of Abbreviations

A

amtB/ AmtB	ammonium transporter gene / protein
Anammox	anaerobic ammonium oxidation
ANOVA	analysis of variance
ANI	average nucleotide identity
ATP	adenosine triphosphate

B

BCCM	Belgian Coordinated Collections of Microorganisms
BLAST	basic local alignment search tool
BOF	Bijzonder Onderzoeksfonds
bp	base pair

C

c	cytochrome
cbaBA	nitric oxide reductase Cu_ANor gene
CDS	coding sequences
cNirK	copper-dependent nitrite reductase
cnor/ cNor	nitric oxide reductase gene / enzyme accepting electrons from heme *c*
Cu_ANor	nitric oxide reductase accepting electron from cytochrome c
CO_2	carbon dioxide
codY/ CodY	GTP-sensing transcriptional pleiotropic repressor gene / protein

D

DNA	deoxyribonucleic acid
DNRA	dissimilatory nitrate/nitrite reduction to ammonium
dnrN/ DnrN	nitric oxide dependent regulator gene/ protein

F

fnr / Fnr	fumarate-nitrate reductase regulatory protein gene/ protein
FWO	Fonds Wetenschappelijk Onderzoek

G

GC	gas chromatography
gdhA / GDH	glutamate dehydrogenase gene / enzyme
glnA / GS	glutamine synthetase gene / enzyme
glnH1	ABC-type glutamine transporter, periplasmic component / domain gene
glnK/GlnK	P_{II}-type signal-transduction protein gene / protein
glnP	ABC-type glutamine transporter, permease component gene
glnQ1	ABC-type glutamine transporter, ATPase component gene
glnR / GlnR	glutamine synthetase repressor gene / protein
gltAB	glutamate synthase genes
gltT	proton/sodium-glutamate symport protein gene
GOGAT	glutamate synthase or glutamine 2-oxoglutarate aminotransferase

H

hmp/ HMP	flavohaemoglobin gene / flavohaemoglobin

I

IBM	International Business Machines Corporation
IPCC	International Panel for Climate Change

K

KEGG	Kyoto Encyclopedia of Genes and Genomes

L

LMG	Laboratory of Microbiology – Ghent University

N

NADH	nicotinamide adenine dinucleotide
nap/ NapA	periplasmic nitrate reductase gene / enzyme
nar/ NarG	membrane-bound nitrate reductase gene / enzyme
narGHJI	the *nar* operon, coding for membrane-bound nitrate reductase
narK/NarK	nitrate/nitrite antiporter gene / protein
narL	nitrate/nitrite response regulatory protein gene
narX	nitrate/nitrite sensor regulatory protein gene

nasA/ NasA	assimilatory nitrate transporter gene / enzyme
nasC/ NasC	assimilatory nitrate reductase gene / enzyme
NCBI	National Center for Biotechnology Information
NH_3	ammonia
NH_4^+	ammonium
nirB/NirB	gene/enzyme for assimilatory nitrite reductase (small subunit)
nirC/ NirC	formate/nitrite transporter gene / enzyme
nirD/NirD	gene/enzyme for assimilatory nitrite reductase (large subunit)
nirK/ NirK	periplasmic copper-dependent nitrite reductase gene / enzyme
nirS/ NirS	periplasmic cytochrome cd1-dependent nitrite reductase gene / enzyme
NtrB	nitrogen regulator II protein
NtrC	nitrogen regulator I protein
N_2	dinitrogen gas
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
N_2O	nitrous oxide
nor/ Nor	nitric oxide reductase gene / enzyme
norDQ	nitric oxide reductase activation protein genes
NorR	nitrous oxide sensing regulatory protein
norV / NorV	flavorubredoxin gene / flavorubredoxin, an oxygen-sensitive nitric oxide reductase for detoxification
norW / NorW	NADH:(flavo)rubredoxin reductase, an oxygen-sensitive nitric oxide reductase involved for detoxification
nos / NOS	nitric oxide synthase gene / enzyme
nosZ/ NosZ	periplasmic nitrous oxide reductase gene/ enzyme
nrf(A)/Nrf(A)	periplasmic cytochrome *c* nitrite reductase gene / enzyme
nsrR/ NsrR	gene / protein for a nitrite-sensitive transcriptional repressor of NO stress response

O

OD	optical density

P

(q)PCR	(quantitative) polymerase chain reaction

Q

qnor/ qNor	nitric oxide reductase gene/ enzyme accepting electrons from quinols

R

RAST	Rapid Annotation Subsystem Technology
rpm	rotations per minute
rRNA	ribosomal ribonucleic acid
RCSB	Research Collaboratory for Structural Bioinformatics

S

SPSS	Statistical Package for the Social Sciences
SOM	Soil Organic Matter

T

TCD	Thermal Conductivity Detector
tnrA/ TnrA	global nitrogen regulatory protein gene / protein
TSA	Trypticase Soy Agar
TSB	Trypticase Soy Broth

U

ureABC	the urease operon

V

v/v	volume to volume

W

w/v	weight to volume
w/w	weight to weight
WWTP	waste water treatment plant

Y

yerD	glutamate synthase large subunit-like protein gene

YNB+	yeast nitrogen base with amino acids with $(NH_4)_2SO_4$, with final concentration of 10mg/L L-histidine, 20mg/L L-methionine, or 20mg/L L-tryptophan, 5 g/L $(NH_4)_2SO_4$) in the media
YNB-	yeast nitrogen base without amino acids without $(NH_4)_2SO_4$

Table of Contents

Aims & outline

Scope and aims of this work

In the last one hundred years, the "agricultural revolution" has dramatically influenced the nitrogen cycle, leading to wide distribution of NO_3^- pollution around the world and the considerable increase of N_2O emission, a potent greenhouse gas contributing to global warming. Some 70% of the atmospheric N_2O is produced by microbial processes in soils and oceans. Denitrification (reduction of NO_3^- to N_2), dissimilatory nitrate reduction to ammonium (DNRA) and nitrification (oxidation of NH_3 to NO_3^-) are three main microbial processes that contribute to N_2O emission. Considering denitrification and DNRA can reduce NO_3^- pollution, only these two NO_3^- removal processes are studied in this work.

In the past, denitrification has been mostly studied in Gram-negative bacteria. In Gram-positive bacteria, in contrast, this process has long been underexplored because their highly divergent denitrification genes prevented this group from being detected as denitrifiers in the environment. Gram-positive bacteria possess a distinctive cell wall structure with typically a thick, multi-layered peptidoglycan layer compared with the well-studied Gram-negative bacteria. As a result, Gram-positives lack an outer membrane and periplasm, which has resulted in differences in the denitrification enzymes involved, as in Gram-negatives they are either periplasmic (periplasmic NO_3^- reductase, both NirS- and NirK-type NO_2^- reductases, and N_2O reductase) or membrane-associated with the active site in the periplasm (NO reductase) (See Chapter 1). Hence, novel features of dissimilatory nitrate reductions are expected in Gram-positive bacteria and in need of in-depth exploration.

Denitrification and DNRA have always been considered as mutually exclusive properties of bacteria, complicating comparative studies of the conditions that stimulate both processes because they could not be studied in a single organism. As denitrification was previously

thought to be the main NO_3^- reduction (and N_2O producing) process in soil, DNRA has been overlooked and less studied in the past, with the mechanism of its N_2O (byproduct) production unknown and conditions that govern NO_3^- partitioning to N_2O or NH_4^+ rarely studied. However, recent studies showed that DNRA could be responsible for 75 to 100% of NO_3^- removal in certain ecosystems (e.g. garden soil, estuary sediment), and the ratio of DNRA bacteria to denitrifiers in certain soils was found to be as high as 3:1 to 4:1. Therefore, the study of DNRA has been revived in recent years.

Five years ago, the Laboratory of Microbiology LM-UGent initiated research on the dissimilatory NO_3^- reduction in representatives of the genus *Bacillus,* the model genus of the Gram-positive phylum *Firmicutes* and a pet taxon of the research group for over twenty years. *Bacillus* are widely distributed in natural ecosystems and many of them live primarily in soil. After first demonstrating that NO_3^- reduction and N_2O production are indeed widespread and are common traits among bacilli, *Bacillus* strains representing three species, one strain each of *B. azotoformans* and *B. bataviensis* and three strains of *B. licheniformis* were selected for genome sequencing. Genome analyses of the former two strains - *B. azotoformans* LMG 9581^T and *B. bataviensis* LMG 21833^T - led to very unexpected observations on combined denitrification and DNRA as well as high redundancy for nitric oxide reductase encoding genes (including novel genes), responsible for nitrous oxide production . This dissertation is a continuation of this work on *Bacillus* with respect to denitrification and DNRA, focusing on N_2O emission, aiming to contribute towards practical approaches of estimation of N_2O emission and mitigation strategies. This research aimed at (i) evaluation of N_2O production status under different physico-chemical conditions, (ii) investigation of phenotypic evidence for genome-based hypothesized NO_3^- reduction metabolisms, e.g. denitrification and DNRA, (iii) a survey of phenotypic diversity at species level, (iv) elucidation of the N_2O production mechanism in specific DNRA bacteria, (v) identification of the physico-chemical factors

influencing NO_3^- partitioning to NH_4^+ or N_2O in DNRA (vi) identification of the physico-chemical factors promoting NO_3^- reduction by denitrification or DNRA in a single organism.

Outline of the study and overview of chapters

Chapter 1 presents an overall introduction to the nitrogen cycle, denitrification and DNRA pathways highlighting the knowledge gaps with respect to physiology, molecular detection and general importance of Gram-positive bacteria, and the new advances in the study of genus *Bacillus*. For comprehensive understanding of nitrogen metabolism and to facilitate the experimental work on nitrogen dissimilation, an introduction to nitrogen assimilation in this group of bacteria is presented at the end of this chapter.

The experimental work of this study is described in four research **Chapters (2-5).**

Overall, **Chapter 2-3** are focusing on the mechanism of N_2O production in DNRA, environmental conditions promoting NO_3^- partitioning towards DNRA strains of *Bacillus licheniformis*,While **Chapter 4-5** are focusing on assimilatory and dissimilatory nitrogen metabolism (both in denitrification and DNRA) in *B. azotoformans* LMG 9581^T. Finally, an overall discussion and future perspectives are included in **Chapter 6**.

Chapter 2: To study the phenotypic diversity at species level of *B. licheniformis*, the genomes of *B. licheniformis* LMG 6934, LMG 7559, LMG 17339 were sequenced and analyzed, followed by physiological tests. According to the genome analysis, surprisingly, these strains, which were previously believed to be denitrifiers, do not possess the key genes for denitrification, but have the genes for DNRA. Phenotypic checking of DNRA pathways was accomplished by designed growth experiments. Growth and dissimilatory NO_3^- reduction were monitored over time for LMG 6934 and end-point measurements of nitrogenous end-products were done for all three strains in both complex and mineral medium. The observation that over 65% of consumed NO_3^- was converted to NH_4^+ and only at most 35% was reduced to N_2O proved that *B. licheniformis* is not a denitrifier but rather an ammonifier

performing DNRA with high N_2O production as side-product. Strain-dependent differences in nitrogen metabolism phenotype were also revealed, confirming the phenotypic diversity at species level of *Bacillus licheniformis*. In addition, N_2O production may be a consequence of the (transient) accumulation of NO_2^-, and hypothetical pathways for N_2O production were proposed based on current studies and obtained physiological data.

Chapter 3: Since the environmental conditions influencing NO_3^- partitioning to NH_4^+ or N_2O in DNRA remain poorly understood, yet are crucial for mitigation strategies of the potent greenhouse gas N_2O, different physico-chemical factors such as NO_3^- concentration under variable or fixed C/N-NO_3^- ratios, NO_2^- concentration and NH_4^+ concentration were tested on *B. paralicheniformis* LMG 6934 (previously known as *B. licheniform*is). Their influence on NO_3^- partitioning to NH_4^+ or N_2O was compared using end-point measurements of NH_4^+, NO_3^-, NO_2^- and gaseous nitrogenous end-products. Our observations demonstrated that NO_3^- concentration under fixed C/N-NO_3^- ratios and NO_2^- concentration under variable C/N-NO_2^- ratios and NH_4^+ concentration (only the highest) significantly promoted NO_3^- partitioning to N_2O.

Chapter 4: Based on the genome analysis, uncommonly, *B. azotoformans* LMG 9581^T possesses highly redundant dissimilatory nitrogen reduction pathways but has highly reduced nitrogen assimilation pathways. To facilitate further study of dissimilatory NO_3^- reduction, we collected and analyzed phenotypic evidence for genome-based hypothesized nitrogen assimilation mechanisms. Different concentrations and combinations of organic nitrogen (yeast extract, yeast nitrogen source, amino acids) and inorganic nitrogen (NO_3^-, NH_4^+) were tested in *B. azotoformans* LMG 9581^T to clarify its specific nitrogen assimilation metabolism. *B. azotoformans* required organic nitrogen for assimilation and NH_4^+ alone could not efficiently support growth under both aerobic and anaerobic conditions. Still, NH_4^+ was

assimilated and had a concentration-dependent influence on growth rate but not on maximal cell density. The underlying mechanisms, however, remained unclear.

Chapter 5: *B. azotoformans* LMG 9581^T possesses both gene inventories for denitrification and DNRA, with a large redundancy in the former. Batch growth experiments were performed to figure out physico-chemical factors (different NO_3^- concentrations, different C/N-NO_3^- ratios) promoting either denitrification or DNRA in this organism. Gene knockout experiments were designed to make mutants of specific genes (*nirK*, *nrfA*). These conditions and comparison of certain mutants together with wild strain would be employed in chemostat experiments which provide ideal conditions (stable pH, C/N-NO_3^- ratios, carbon/nitrogen source concentration etc.) to study the transcription and physiology of both processes. However, in batch set-up, variable C/N-NO_3^- ratios under high and low NO_3^- concentration could not induce DNRA. Gene knockout experiments to make mutants of *B. azotoformans* LMG 9581^T failed because of unsuccessful electroporation. Since published reports on other DNRA strains proved them to be notably functional in chemostat rather than in batch, an anaerobic chemostat set up was designed to test the conditions influencing NO_3^- partitioning to denitrification or DNRA. While a range of conditions could be tested, unfortunately, contamination issues required an early termination of this line of investigation. Nevertheless, NO_2^- accumulation under the lowest NO_3^- concentration combined with high C/N-NO_3^- ratio indicates that DNRA may be possibly initiated under these conditions. Further confirmation is still required.

Chapter 6: Here, an overall discussion of the ecological relevance of this study in view of DNRA's contribution to N_2O emission and microdiversity in *Bacillus* are presented, and also the complexity and difficulty of applying the achievements of our work to N_2O mitigation strategies is discussed. Finally, future perspectives are proposed.

Chapter 1: Introduction

Denitrification and non-denitrifier nitrous oxide emission in Gram-positive bacteria

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YH and KH conceived and wrote the manuscript; PDV revised the manuscript.

1.1 Agriculture increases NO_3^- pollution and N_2O emission

Since the Haber-Bosch process was first introduced for the production of nitrogen fertilizer to be used in agriculture in 1914, global starvation due to limits of agricultural production was largely alleviated [1]. From 1960 to 2000, the application of nitrogen fertilizer has increased by 800% [2]. Today, the Haber-Bosch process provided half of the nitrogen found in proteins and nucleic acids of the approximately seven billion people alive [1]. However the nitrogen uptake for some main crops (wheat, rice, maize) is typically less than 40% [3]. Most applied fertilizer is either washed out of the soil by rain or watering, or lost to the atmosphere by biological processes converting ammonium (NH_4^+) or ammonia (NH_3) to nitrate (NO_3^-) by nitrification, further to nitrous oxide (N_2O) or dinitrogen gas (N_2) by denitrification and/ or anaerobic NH_4^+ oxidation (anammox). The resulted high content of NO_3^- in the soil can cause ecosystem changes like forest decline and acidification of soils leading to higher concentrations of dissolved potentially toxic metals and pollution of ground water [4]. NO_3^- can readily leach to rivers, lakes, and aquifers, resulting in coastal eutrophication which in turn has many negative effects such as decreased dissolved oxygen (DO) or induced hypoxia (<0.5 ml O_2/liter) [5], decreased water quality [4, 6], declining biodiversity [5], harmful Cyanobacterial [7] and filamentous algae blooms and intensive secondary pollution [8].

On the other hand, N_2O is produced from NO_3^- via nitrification in aerobic and denitrification in anoxic conditions [9]. N_2O is a potent greenhouse gas with 310 times the warming potential of CO_2 (per molecule) and destroys the stratospheric ozone layer. Since 1800, the atmospheric concentration of N_2O has increased from 270 ppb to more than 322 ppb i.e. almost 20% increase [10, 11]. In recent decades, N_2O has increased with about 0.25 % per year and this effect is expected to continue in the future [12]. Since N_2O is unregulated by the Montreal Protocol, it is currently the dominant Ozone-layer depleting substance and is expected to

remain it in the 21st century [13]. Human activities are responsible for anthropogenic N_2O emission that is up to 30% of global N_2O emission. Agricultural systems make up most part, i.e. about 25% of the global emission [14]. Other sources are fossil fuel combustion, industrial processes, human sewage and burning of biomass and biofuels making up the rest 5% [12]. It is clear that over the past century, the increased nitrogen input to satisfy a growing global demand for food has drastically disrupted the nitrogen cycle to such an extent that earth's boundaries have been exceeded [3, 15].

1.2 NO_3^- removal and N_2O production in microbial nitrogen cycle

Management strategies and interventions to mitigate the environmental issues resulting from excess nitrogen input require in-depth knowledge of the nitrogen cycle and the different processes involved.

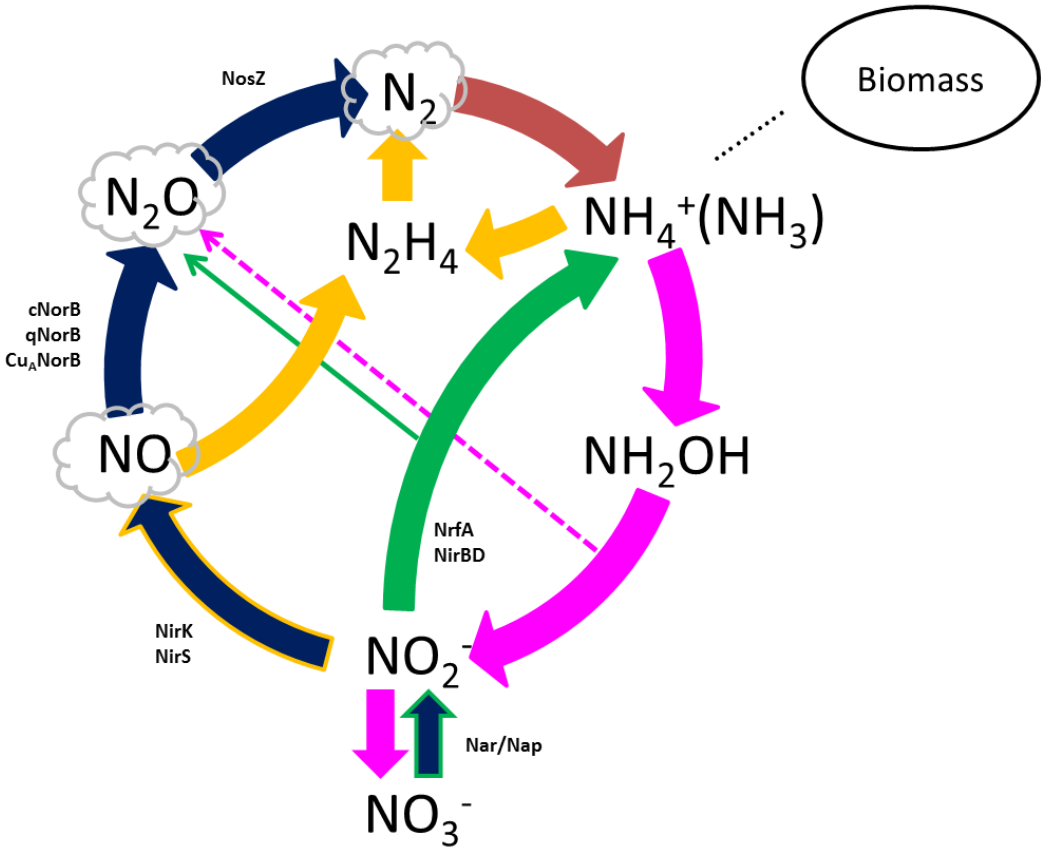

Figure 1.1 Schematic overview of nitrogen cycle. (1) Nitrogen fixation (in red), (2) Nitrification (in pink), (3) Denitrification (in blue), (4) Anaerobic ammonium oxidation (anammox) (in yellow), (5) Dissimilatory nitrate/nitrite reduction to ammonium (in green). Enzymes for each reduction in denitrification and DNRA are indicated on the arrow: respiratory nitrate reductase Nar, periplasmic nitrate reductase Nap; copper containing nitrite reductase NirK (or cNirK/CuNIR), cytochrome *cd*1 nitrite reductase NirS (*cd*1NIR);cytoplasmic NADH-dependent nitrite reductase NirBD; periplasmic pentaheme cytochrome *c* nitrite reductase NrfA; cytochrome *c*-dependent nitric oxide reductase cNor, quinol-dependent nitric oxide reductase qNor, copper A-dependent nitric oxide reductase Cu_ANor; the copper-dependent nitrous oxide reductase NosZ.

The nitrogen cycle as it is understood at this moment is illustrated in Figure 1.1. N_2 is the most abundant, albeit inert, form of nitrogen in the atmosphere, and can be fixed to ammonia

(NH_3) or NH_4^+ by a minority of prokaryotes called diazotrophs and thus become biologically available. Then, NH_4^+ or NH_3 can be oxidized to nitrite (NO_2^-) and further into NO_3^- via a two-step nitrification process under aerobic conditions by NO_2^-- and NH_4^+-oxidizing bacteria respectively. NH_4^+ oxidation to NO_2^- can also produce N_2O during intermediate hydroxylamine (NH_2OH) oxidation, but with distinct isotopomer abundance compared to denitrification [16]. The nitrogen in the produced NO_3^- can escape back to the atmosphere by a subsequent four-step anaerobic reduction over NO_2^- to gaseous nitric oxide (NO), N_2O and/or N_2, a process called denitrification. In addition, NH_4^+ can be combined with NO_2^- and get reduced to N_2 in the anammox process, i.e. the anaerobic NH_4^+ oxidation, discovered only 20 years ago [17, 18] and restricted to specific taxa of the *Planctomycetes*. In contrast, NO_3^- can also be retained in the system as fixed nitrogen via the anaerobic dissimilatory NO_3^- reduction to NH_4^+ (DNRA), with again N_2O produced as by-product.

In this dissertation, we will focus on the two competing, facultative dissimilatory NO_3^- removal pathways, denitrification and DNRA, that are both widespread traits among Prokaryotes, and limit the discussion to the currently underexplored Gram-positive bacteria. Both these processes were until quite recently thought to be mutually exclusive, not occurring in a single organism, which complicated exploration of the conditions that would favor one or the other process.

1.3 Enzymes and genes involved in denitrification and DNRA

Current studies of denitrification mostly focus on Gram-negative bacteria and the primers used to study key denitrification encoding genes are biased towards this group. The primers arewell suited for screening Gram-negative but not Gram-positive bacteria due to the high divergence of their encoding genes (See below Section 1.7). Based on these studies, the reductases involved in each reduction step of these two pathways are indicated in Figure 1.1.

Generally in denitrification, NO_3^- can be reduced to NO_2^- by a membrane-bound respiratory nitrate reductase (Nar) or a periplasmic nitrate reductase (Nap), which is encoded by the *nar* gene cluster or the *nap* gene cluster, respectively. Nar enzymes are found to be exclusively expressed under anaerobic conditions, while Nap can also be functional under aerobic conditions. Co-occurrences of Nar and Nap have been found in many denitrifers, as well as in DNRA performing strains. NO_2^- can be converted to NO by two isofunctional, evolutionary unrelated periplasmic enzymes: a copper containing nitrite reductase NirK (or cNirK/CuNIR), encoded by the *nirK* gene and (ii) a cytochrome *cd*1 nitrite reductase NirS (*cd*1NIR), encoded by the *nirS* gene. This is the key step in denitrification, converting fixed nitrogen to the first gaseous product. Next to its main product NO, NirK can also result in a small amount (3-6%) of N_2O if NO is allowed to accumulate [19]. It has long been assumed that these two Nir were mutually exclusive, however recently it was found not the case [20], although the functionality of the two enzymes within one organism still requires confirmation. The NirS denitrifiers are often assumed to be predominant in the environment and more widespread, while NirK denitrifiers comprise more diverse taxa [21]. N_2O can be formed from the very reactive radical NO by three kinds of respiratory nitric oxide reductases: (i) cNor, also known as short-chain Nor, is a cytochrome *c*-dependent nitric oxide reductase, encoded by the *cnorB* gene and accepts electrons from cytochrome *c* (ii) qNor, also known as long chain Nor, is a quinol-dependent nitric oxide reductase, encoded by the *qnorB* gene and accepts electrons from ubiquinol or menaquinol (iii) Cu_ANor, a copper A-dependent nitric oxide reductase, is encoded by *cbaA*, and accepts electrons from cytochrome c_{551} [22]. cNor are found to be unique to denitrifying bacteria, whereas qNor is present in both denitrifying and non-denitrifying bacteria [23] with especially Gram-positive bacteria hosting qNor. Cu_ANor for now has only been detected in Gram-positive bacteria. The only known enzyme for N_2O conversion to N_2 is NosZ (NOS, N_2OR), the copper-dependent nitrous oxide reductase, which

is encoded by the *nosZ* gene. It is a periplasmic enzyme in Gram-negative bacteria while it appears to be associated with the membrane by a lipid anchor while facing the periplasm "like" space in Gram-positive bacteria. At present, two phylogenetical variants of NosZ have been described, each with its distinct regulatory and functional components: (i) typical NosZ, commonly found in *Alpha-*, *Beta-* and *Gammaproteobacteria* performing complete denitrification [21] and (ii) atypical NosZ, found in taxonomically diverse bacteria including some DNRA bacteria or other non-denitrifying bacteria [24].

Although DNRA by fermentative bacteria was documented many years ago [25], denitrification has long been believed to be the main NO_3^- consuming process in the soil environment [26] and was studied extensively whereas DNRA was overlooked and underestimated. Recently, studies showed DNRA can be a significant or even dominant NO_3^- reduction process (up to 80% or higher percentage of total NO_3^- removal) in many ecosystems, such as estuary sediments, mangrove soil, brackish marsh, etc [27, 28] and this revived research into this process.

Similarly as in denitrification, in DNRA, NO_3^- reduction to NO_2^- can be catalyzed by either or both of Nar and Nap reductases [29]. Subsequently, NH_4^+ can be produced from NO_2^- by two systems, depending on the organism and growth conditions, (i) a cytoplasmic NADH-dependent nitrite reductase (NirBD), encoded by the *nirBD* operon or (ii) a periplasmic pentaheme cytochrome *c* nitrite reductase (NrfA) [30], encoded by the *nrfA* operon. *Escherichia coli* K-12 and *Bacillus vireti* LMG 21834^T were shown to harbor both types of genes which were proved to be functional [31, 32], while some DNRA organisms such as *Wollinella succinogenes* [33] and *Bacillus subtilis* [34] contain either *nrfA* or *nirB*. It has been proved in the tests with *E.coli* [19, 35] and *Salmonella enterica* serovar Typhimurium [36] that expression of both *nap* and *nrfA* genes is repressed under excess NO_3^-, and assumed to be

optimal under low to intermediate level of NO_3^-. While the expression of Nar and NirBD is induced only by high NO_3^- conditions. Unfortunately, the mechanism of N_2O production from DNRA still remains unclear at the moment: studies on the environmental conditions influencing DNRA initiation gave contradictory results while factors affecting NO_3^- partitioning to NH_4^+ or N_2O in DNRA are rarely studied, even though they are crucial for mitigation strategies of the potent greenhouse gas N_2O (See Section 1.6).

1.4 Denitrification is widespread but underexplored in Gram-positive bacteria

As mentioned above, denitrification is a facultative anaerobic process involving the step-wise dissimilatory reduction of NO_3^- over NO_2^- to the cytotoxic NO, the potent greenhouse gas N_2O and the stable, non-reactive-N_2, coupled to electron transport phosphorylation [37]. It is a highly modular process, meaning that the four reductions steps are not necessarily linked in a microorganism and that the intricate metalloproteins involved in each reduction can occur separate or in any combination possible [20, 38]. So, the presence of one or more of their encoding genes is not *per se* indicative of the capacity to denitrify. Over the last decades it has become generally accepted that not only denitrification, but also DNRA and NO_2^- detoxification can produce various nitrogen containing gasses from NO_3^- or NO_2^-, albeit as side products and not intermediate or major end-products. We adhere to the most recent definition of denitrification [37] and consider those microorganisms that can at least convert NO_2^- to N_2O in stoichiometric amounts (with at least 80% N converted) while conserving energy for growth as "true denitrifiers".

Gram-positive bacteria stain purple with the classical Gram staining because of their distinctive cell wall structure with typically a thick, multi-layered peptidoglycan lacking an outer membrane and periplasm, although a small periplasm "like" space exists between the cytoplasmic membrane and the peptidoglycan. The latter feature is of importance as most denitrification enzymes are either periplasmic (periplasmic NO_3^- reductase, both NirS- and NirK-type NO_2^- reductases, and N_2O reductase) or membrane-associated with the active site in the periplasm (NO reductase), and so this might have consequences on the organization of the denitrification proteome in Gram-positive denitrifiers (See below Section 1.5). Gram-positive bacteria are phylogenetically separated into two phyla, *Firmicutes* and *Actinobacteria*; this subdivision was historically based on their guanine + cytosine ratio in DNA with low GC (well below 50%) and high GC (well above 50%), respectively. Since the introduction of the

term denitrification by Gayon and Dupetit in 1882 and the subsequent search for its etiological agents, several Gram-positive genera have been described to contain denitrifiers, although these were always severely outnumbered by the description of their Gram-negative counterparts in the lists of denitrifying taxa compiled based on literature surveys [39-42]. In 2011, we scrutinized the described Gram-positive denitrifiers by assessing the experimental and molecular data available [43], and more recently, Shapleigh [38] did a similar exercise focusing on those *Firmicutes* and *Actinobacteria* for which whole genome sequences were available. In summary, most *Firmicute* denitrifiers belong to endospore-forming species of the genus *Bacillus* or close relatives, termed 'bacilli' in the remainder of this chapter, such as *Bacillus azotoformans* [37, 44], *Geobacillus thermodenitrificans* [45], *Virgibacillus halodenitrificans* [46, 47], and multiple *Paenibacillus* species [48, 49], while actinobacterial denitrifiers are mostly limited to the *Actinomycetales* with both spore-formers such as *Streptomyces* [50, 51] and non-spore-formers like *Corynebacterium* [52, 53]. As a general feature they seem to have a truncated denitrification pathway, most often lacking a N_2O reductase, which suggests that they might be vigorous N_2O emitters in their habitats.

Although the denitrifying capacities of mainly bacilli have been known for a very long time, denitrification-related biochemistry, physiology, regulation and environmental surveys have almost exclusively focused on Gram-negative denitrifiers. Like for other non-proteobacterial lineages such as *Bacteroidetes*, the main reasons why this trait is underexplored in Gram-positives are its strain-dependent nature, the mismatches with primers targeting denitrification genes due to their high sequence divergence and the relatively uncommon nature of the trait in its model organisms like *Bacillus subtilis*, *Bacillus cereus* or even in type strains of newly described species [43]. Nevertheless, our screening of a collection of 180 *Bacillus* strains, at that time covering half of the validly described taxonomic diversity of the genus, revealed that around 25% were able to produce stoichiometric amounts of N_2 from NO_3^- and/or NO_2^- while

supporting growth [54]. That study suggested that denitrification is potentially widespread and clearly has been underestimated in strains of the genus *Bacillus*, and by extrapolation, in Gram-positive bacteria. For the remainder of this chapter we will describe the current understanding of denitrification in *B. azotoformans*, one of the few *Bacillus* species which contains the complete denitrification pathway and for which the genome sequence is available, including details on other bacilli when relevant. We will also highlight the old and more recent insights into N_2O emission by non-denitrifying *Bacillus* strains as this might be another, highly undervalued trait in bacilli, often confused with denitrification in and we will explore the methodological issues hampering the assessment of the environmental abundance and importance of denitrifying bacilli.

1.5 Novel features in the denitrifier *Bacillus azotoformans*

1.5.1 Membrane-bound enzymes and a novel NO reductase

B. azotoformans LMG 9581^T was originally isolated from garden soil and has been recognized as a true denitrifier for decades [37, 44, 55]. It is capable of the complete dissimilatory NO_3^- reduction to N_2. In Gram-negative denitrifiers, the canonical denitrification pathways is carried out by at least two periplasmic reductases, the copper- or cd_1-dependent NO_2^- reductase (NirK or NirS respectively) and the copper-dependent N_2O reductase (NosZ). Activity of all four reduction steps were shown to be membrane-associated in *B. azotoformans* LMG 9581^T[56], which was already previously demonstrated for NirK in *V. halodenitrificans* [47]. *In silico* sequence analyses of the *nirK* and *nosZ* genes of *B. azotoformans* and other bacilli [57] indeed predicted that they encode lipoproteins, which covalently bind to the membrane, while being preceded by a Sec or Tat signal respectively for protein export, indicative of a periplasmic localization of the processed protein. So, despite Gram-positive denitrifiers only having a small periplasm 'like' space, the periplasmic reductases indeed appear to be located there but as membrane-bound variants.

In addition to the more common cytoplasmic membrane protein complex NO_3^- reductase (Nar) also a periplasmic variant (Nap) is known; both bind a molybdenum bis molybdopterin guanine dinucleotide (Mo-bis-MGD) with a 4Fe-4S cluster at the catalytic subunit for electron transfer [58]. Surprisingly, for the first time in a Gram-positive bacterium, a complete *nap* operon was found in the genome of *B. azotoformans* LMG 9581^T [57], which based on *in silico* analyses seemed to constitute a new variant in addition to the four known Nap systems [59]. This putative new organizational structure of Nap was described in detail previously [57] and is shown in Figure 1.2. The *nap* operon lacks genes for the cytoplasmic maturation factors NapF and NapL as well as the quinol-oxidizing membrane-bound NapC but contains two

gene copies for NapG as well as a gene coding for one of the enzymes involved in Mo-bis-MGD biosynthesis. In this Nap system, NapA represents the catalytic subunit, NapD is involved in the posttranslational assembly of NapA, which receives its electrons for NO_3^- reduction from the companion diheme *c* protein NapB. With NapA having a distinct Tat signal, and NapB possessing a Sec signal, it can be inferred that both of them are exported to the periplasm. As in other organism [33, 60, 61], NapH, a membrane-bound enzyme specifically oxidizing menaquinol, and NapG2, a periplasmic adaptor protein delivering electrons from menaquinol oxidation [61], are most likely forming a membrane-bound complex for transfer of electrons. As expected NapG2 has a Tat signal while NapG1 does not, so the latter might substitute for NapF found in other organisms at the cytoplasmic side. As such, *B. azotoformans* LMG 9581^T has a NapAB and a NapGH module, possibly assembled as one membrane-bound complex, for menaquinol-dependent NO_3^- reduction to NO_2^- (Figure 1.2). As is the case for other Nap systems, its topology and architecture suggests it will not contribute to the generation of a proton motive force.

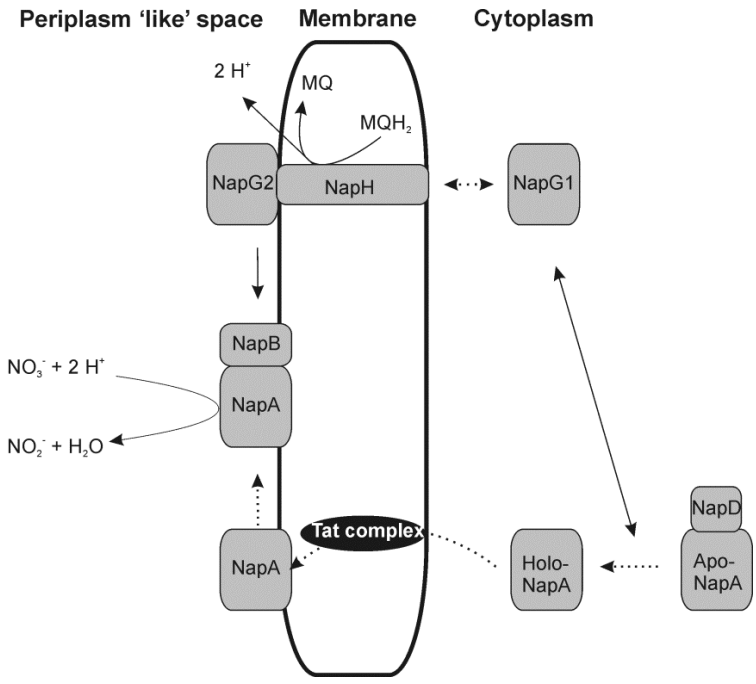

Figure 1.2 Proposed novel organization of periplasmic NO_3^- reductase in *B. azotoformans*. The scheme is based on the four Nap organizational structures described previously [59] .

All NO reductases are integral membrane proteins and three kinds have been described thus far, all belonging to the heme-copper oxidase (HCO) superfamily: cytochrome *c* oxidizing cNor, the quinol-dependent qNor and the Cu_ANor with cytochrome c_{551} as electron donor [22]. The latter Nor, encoded by the *cbaBA* operon [57], was discovered in *B. azotoformans* LMG 9581^T by the research group of Simon De Vries and previously thought to be bifunctional, able to accept electrons from both menaquinol and cytochrome *c* [56, 62, 63]. The cNor is considered not electrogenic as it takes both electrons and protons from the periplasm [64-66]. In contrast, NO reduction by qNor could be electrogenic as the crystal structure of the qNor from the Gram-positive *Geobacillus stearothermophilus* [67] revealed the presence of a putative proton transfer pathway between the cytoplasm and the binuclear metal centers. Recently, work with Cu_ANor reconstituted in closed liposomes provided the first experimental evidence of the formation of a proton electrochemical gradient across the membrane [22]. The proton electrochemical gradient is formed because protons are taken from the cytoplasm causing a decrease in cytoplasmic charge and proton concentration; actual proton pumping has yet to be determined. This means that denitrifiers with Cu_ANor could exploit NO reduction for increased cellular ATP production (6.7% higher than those with cNor or even 13% if Cu_ANor would in addition pump two protons) [22]. Thus far, all Gram-positive denitrifiers harbor either a gene for a Cu_ANor, a qNor or both, suggesting that they have the potential to conserve more energy from denitrification than their Gram-negative counterparts.

1.5.2 High genetic redundancy and potential metabolic versatility

Genome analyses of *B. azotoformans* LMG 9581^T revealed a strikingly high gene redundancy for the canonical denitrification pathway [57]. The organism encodes two Nar and one Nap for NO_3^- reduction to NO_2^-, one NirK for NO_2^- reduction to NO, two qNor and two Cu_ANor for NO reduction to N_2O and three NosZ for N_2O reduction to N_2 (Figure 1.3). Hypothetically,

thirty-six different gene combinations could result in a complete denitrification pathway. Like Jeff Cole remarked for the alternative sets of NO_3^- reductase genes in *Escherichia coli*, "either their co-existence is just an accident of evolution that arose after trivial gene duplication or acquisition event, or these sets of genes remained conserved long after they evolved because they are physiologically useful to the organism, otherwise they would have been eliminated by evolutionary selective pressures" [31]. We concur with the latter hypothesis and assume that the various enzymes for the same N conversion will become expressed in different environmental contexts and/or may have slightly different structural roles. Indeed, even copies of the same enzyme differed quite substantially in amino acid sequence identity (NarG1-NarG2: 74.9%; qNor1-qNor2: 38%; NosZ1-NosZ2-NosZ3: 76.5-83.1%). The denitrification pathway might be completely modular or it can have preferential combinations of specific gene sets due to their regulation. Also, some denitrification genes might be constitutively expressed while others might depend on specific environmental triggers, or the proteome might contain multiple enzymes for the same reduction step at the same moment. In addition, it is plausible that under some conditions (e.g., low pH), only truncated denitrification is carried out [68, 69].

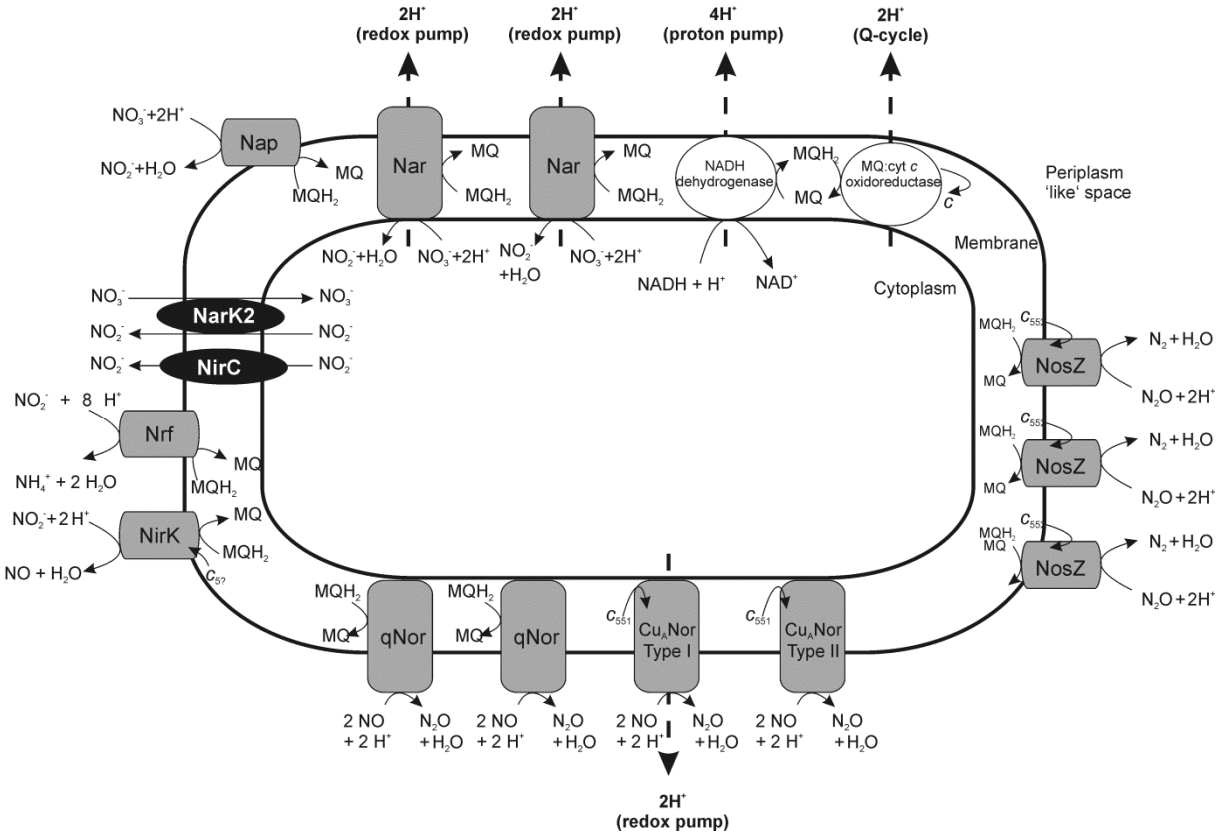

Figure 1.3 Schematic overview of gene inventory related to dissimilatory nitrate reduction in *B. azotoformans*. Menaquinol (MQH_2) donates electrons to a menaquinol: cytochrome c oxidoreductase (related to bc_1/b_6f), Nar, Nir, Nrf, qNor and NosZ. Membrane-bound c-type cytochromes subsequently donate electron to their corresponding enzymes. The locations of the substrate-binding sites are hypothesized to be similar to other bacteria (see text for further explanation). Enzymes involved in respiratory nitrate reduction are depicted in dark grey. Nitrate/nitrite transport systems are in black. Menaquinol: cytochrome c oxidoreductase (related to bc_1/b_6f) and NADH dehydrogenase are in white. Cytochrome *c* lipoproteins are in light grey. Charge displacements contributing to the proton motive force are given. Deduced from genome data or taken from Suharti & De Vries [56].

To our surprise, the genome of *B. azotoformans* LMG 9581^T also contains the gene for the periplasmic pentaheme cytochrome *c* NO_2^- reductase NrfA [70] involved in DNRA, which also is predicted to be a lipoprotein (Figure 1.3). DNRA is, like denitrification, a facultative anaerobic process involving NO_3^- reduction to NO_2^- followed by the 6-electron reduction of NO_2^- to NH_4^+ [70]. Their co-occurrence was very unexpected because, at that time, denitrification and DNRA were still considered to be mutually exclusive NO_3^- reduction pathways thought to occur in completely different microbial populations. Nevertheless, we observed the same for *Bacillus bataviensis* [57], and around the same time the co-occurrence

of both pathways was seen in several Gram-negative denitrifiers like *Opitutus terrae* strain PB90-1 (DSM 11246), *Marivirga tractuosa* DSM 4126 and *Shewanella loihica* PV-4 (DSM 17748) [24]. Since then data mining of over 250 genomes unexpectedly revealed that over 20% of NirK-type denitrifiers also encode a NrfA gene (Decleyre & Heylen, unpublished) and have the genetic potential for both pathways. This is very exciting as now the environmental drivers partitioning NO_3^- can be investigated without organism-dependent variation blurring the data, which could mean a giant leap forward for the design of predictive models of NO_3^- removal and N_2O emissions. Worth mentioning is that both *S. loihica* PV-4 and *B. azotoformans* LMG 9581^T lack the assimilatory NO_3^- and NO_2^- reductase genes. The concerted action of Nar (and/or Nap for *B. azotoformans*) and NrfA could compensate for this loss and still enable anaerobic NO_3^- assimilation, which might be an evolutionary reason for the co-occurrence of both pathways.

Actual functionality of both pathways has thus far only been demonstrated for *S. loihica* PV-4 [71, 72]. That work demonstrated that denitrification dominated at NO_3^- sufficiency and low carbon-to-nitrate (C/N-NO_3^-) and NO_2^--to- NO_3^- ratios, while high C/N-NO_3^- and NO_2^--to-NO_3^- ratios, pH above 7.0, temperature over 30°C, and NO_2^- as sole electron acceptor favored DNRA. To our knowledge, *B. azotoformans* LMG 9581^T is the first denitrifier described with such a high genetic redundancy on functional gene and pathway levels, and therefore is the ideal model organism to study redundancy and modularity of dissimilatory NO_3^- reduction applying experimental, transcriptional, regulatory and mutational work to verify the above-mentioned theoretical considerations. Interesting to note is that another *B. azotoformans* strain MEV2011 contains an identical dissimilatory NO_3^- reduction gene inventory but also appears to be an obligate microaerophilic NO_3^- reducer capable of co-denitrification [73]. We are convinced that its high genetic redundancy conveys metabolic versatility to the organism and

enables various ecological strategies in the soil matrix, in analogy to the rRNA copy numbers [74].

1.6 DNRA as a short circuit in the N cycle is overlooked and underexplored

1.6.1 N_2O emission by ammonifying bacilli

More than three decades ago, Smith and Zimmerman [75] were the first to report that nitrous oxide could also be anaerobically produced by non-denitrifiers. They found that 163 of 209 N_2O producers isolated from soil were not respiratory denitrifiers, but rather NO_2^- accumulators and NH_4^+ producers. These non-denitrifying soil isolates evolved N_2O (up to 34% of NO_3^-), though the produced amount was always less than that of NO_2^- and/or NH_4^+. Most belonged to the genus *Bacillus* or were members of the family *Enterobacteriaceae*. Further characterization of a *Bacillus* (and a *Citrobacter*) strain confirmed fermentative NO_2^- reduction to NH_4^+ and N_2O production, while NO and N_2 were not produced in detectable amounts [75]; this was quickly afterwards verified for another *Bacillus* and several other strains [76]. Added NH_4^+ did not inhibit N_2O or NH_4^+ production, indicating that these processes were not assimilatory [75, 76]. N_2O evolved slowly in batch cultures and mostly after apparent growth ceased [75, 76]. Work on *Citrobacter* isolate C48 suggested that N_2O production was unrelated from NO_2^- ammonification but probably connected to intracellular NO_2^- accumulation, and both N conversions might occur in environmental conditions with contrasting C/N-NO_3^- ratios [77]. Recently, the influence of carbon and NO_3^- availability on N_2O production from DNRA was verified and refined, again through the investigation of two fresh soil isolates affiliated to the genus *Bacillus* (and *Citrobacter*) [78]. In defined media with lower C/N-NO_3^- ratios (5- and 10-to-1: glycerol 20 mM, NO_3^- concentration varied), in which NO_2^- accumulated and no NH_4^+ was produced, up to 2.7% of NO_3^- was reduced to N_2O by *Bacillus* sp. However, at higher C/N-NO_3^- ratios (25- and 50-to-1: glycerol 20 mM, NO_3^- concentration varied) NH_4^+ was produced and only 0.1% of NO_3^- was reduced to N_2O by *Bacillus* sp. These findings were corroborated with chemostat cultures under NO_3^--sufficient

and -limited conditions [78]. Further work was performed on *Bacillus vireti* LMG 21834^T, a versatile soil bacterium capable of fermentation, DNRA, N_2O production and its further reduction to N_2 [32]. For NO_3^- concentrations equal to or higher than 15 mM, NO_2^- accumulated during NO_3^- reduction and growth (OD_{660}) ceased when NO_3^- was depleted. Only then, NH_4^+ started to accumulate and N_2O and N_2 production was observed. In contrast, for lower NO_3^- concentration, the NO_2^- peak was much lower and growth was still supported by NO_2^- reduction to NH_4^+. Also nitrogen gases started to evolve during growth, which continued during stationary phase. Confirming previous observations discussed higher, the portion of the reduced NO_3^- recovered as gases in the headspace (i.e. NO, N_2O and N_2) increased from 6% with 5 mM NO_3^- to between 49 to 55% with 20 mM or higher NO_3^- concentration. It is interesting to note is that the ratio of N_2O/N_2 in nitrogen gas end-products increased with increasing NO_3^- concentrations.

It is currently unclear if all organisms capable of DNRA can produce N_2O. For example, *B. subtilis* is the second most studied model organism next to *E. coli*, both are known to perform DNRA. But there are almost no reports on N_2O production from *B. subtilis*, and in those that exist, only very small amounts of N_2O were observed, in the nanomolar range [78] (compared to micromolar ranges for microorganisms described above). Nevertheless, this is within the same order of magnitudes as *E. coli* [78]. Because *B. subtilis* was long considered not to produce N_2O, no experimental evidence is available on the mechanisms for this process. Some studies suggested that N_2O production is probably a consequence of the (transient) accumulation of NO_2^-. Indeed it makes bioenergetic sense to maximize NO_3^- reduction to NO_2^- and minimize the use of scarce electrons to reduce NO_2^- to NH_4^+ [78]. Nevertheless, the mechanisms for N_2O formation by DNRA bacilli remains underexplored based on these current studies.

1.6.2 Environmental factors promoting DNRA

From the currently available data on *Bacillus* (and *Enterobacteriaceae*) described above and *S. loihica* PV-4 [72], it can be concluded that indeed DNRA is favored at higher C/N-NO_3^- ratios or NO_3^- limitation. This fits with the traditional C/N-NO_3^- hypothesis based on Gibbs free energy calculations that more energy can be conserved per mole NO_3^- via DNRA than via denitrification [79] and confirms findings from the soil environment [80] and enrichment cultures from a wastewater treatment plant [71] or marine environments [81]. Yin et al. [82] showed that significant DNRA occurred only when the C/N-NO_3^- ratio is above 12. While for *S. loihica* PV-4, DNRA was observed at a C/N-NO_3^- ratio above 3 [72]. And in the study of *Bacillus* and *Citrobacter* (see above [78]), NH_4^+ production was observed at a C/N-NO_3^- ratio above 25. Therefore we assume the C/N-NO_3^- ratio required for initiation of DNRA is probably strain-dependent.

In 1988, suitable organic carbon sources were pointed out as another important factor that regulates the population of DNRA bacteria [40]. Many studies showed that glucose, a carbohydrate that supports respiration as well as fermentation stimulated DNRA [75, 83]. Acetate was used as carbon source for enrichment of DNRA strains [84], glycerol [78] and lactate [72] were applied in the study of DNRA. Contradictorily, some studies concluded that glycerol, methanol and succinate did not promote DNRA [82, 85]. Addition of glucose was also shown to have no influence on DNRA in another study [86] and acetate could not support DNRA in certain organisms [87]. According to Buresh and Patrick [85] as well as Yin et al. [82] explained this by the fact that some mentioned carbon sources are poor substrates for fermentation. As there are two distinct pathways of DNRA, one fermentative (Nar and NirBD) and one respiratory (Nap and NrfA) [19], this can explain some results. In a respiratory DNRA study, Simon [88] listed formate, H_2, and sulphide as substrates, which indicates that probably many above mentioned carbon sources may not favor the respiratory pathway. This

may explain why in some cases addition of glucose results in contradictory observations. However, the reasons for this are not fully understood and deserve further investigations.

There are several studies on the effect of pH on DNRA, but these findings are also partly contradictory. Woods reported that pH 6.5 is optimal for NO_2^- reduction and 7.5 is optimal for NO_3^- reduction [25]. Higher DNRA was found associated with alkaline conditions in certain studies [89, 90]. And in the single organism test of *S. loihica* PV-4, DNRA was preferred when pH was over 7 [72]. In contrast, other studies showed a negative relationship between low pH and DNRA in soil. Waring and Gilliam reported that DNRA increased at lower pH (<4) in poorly drained soils, which was linked to the soluble carbon content [91]. Under acidic conditions, organic matter in soil breaks down slowly, resulting in a decrease of the available organic carbon for microorganisms [68]. However, given that in these conditions the C/N-NO_3^- ratio is decreased, we believe this pH effect on soil carbon availability cannot explain the unexpected DNRA increase. Further studies are needed.

Taken together, based on current studies, C/N-NO_3^- ratio appears to be the most important factor regulating DNRA and pH may have an influence in certain ecosystems since its effect is not consistent. Furthermore, correlation between DNRA and soil organic matter (SOM), moisture or soil nitrogen was also shown in other studies. However, not enough data is available in literatures for a comprehensive analysis of the importance of these factors. And further studies on the main environmental controllers of DNRA are required for an overall understanding.

In addition, NO_3^- partitioning to N_2O or NH_4^+ in DNRA has never been systematically investigated, although it may be highly relevant to the estimation of global N_2O emission or mitigation scenarios for N_2O. Only limited studies (see above Section 1.6.1) provided hints for N_2O versus NH_4^+ production in DNRA. It has been shown that N_2O is a more significant

product of DNRA at low C/N-NO_3^- ratios (note that exact ratios are dependent of type of carbon source used as electron donor and initial concentration of NO_3^-) in the study of *Bacillus* (and *Citrobacter*) [78]. NO_3^- concentration effects in DNRA were tested only in *Bacillus vireti* LMG 21834^T at NO_3^- concentrations of 5 mM and 20 mM, showing NO_3^- partitioning to N_2O is facilitated under NO_3^- sufficiency. To address this gap of knowledge in this topic, we initiated the research on the effect of environmental factors on NO_3^- partitioning in DNRA strains (Chapter 3).

1.7 Environmental relevance of bacilli and related methodological issues

1.7.1 Their ubiquitous nature

Bacilli are widely distributed in the natural environment. Their habitats range from all kinds of soils to the water columns and sediments of fresh and marine waters. They are found in the rhizosphere of various plants and crops, can be associated with sea weeds, are part of the gut microbiota of humans and other higher organisms, can be major soil-borne food contaminants, being either food-borne pathogens or causing microbial food spoilage, but also have numerous commercial and agricultural uses (e.g. production of peptide antibiotics, chemicals and proteases, mitigation of fungal pathogens). Despite their ubiquitous nature, bacilli are generally considered as soil-related microorganisms. This dates back to cultivation-based qualitative and quantitative microbial diversity studies of soils, in which their numerical dominance in isolates was assumed to reflect their *in situ* relative abundance. However, this was disproved by Peter Janssen landmark meta-data analyses of 3,240 16S rRNA gene sequences from 32 clone libraries from a variety of bulk soil samples [92]. Soil bacterial communities appeared to be dominated by *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Proteobacteria*, and *Verrucomicrobia*, while bacilli only contributed a mean of 2% (range of 0 to 8%), while in contrast, bacilli comprised up to 45% of isolates from traditional cultivation-based studies. These findings were later confirmed by many studies, the most elaborate to date was a Illumina-based 16S rRNA gene amplicon sequencing survey targeting sixteen soils selected to span a wide range of ecologically distinct biomes [93]. Bacilli were found in nearly all soils, but their relative abundances were highly variable and typically represented less than 5% of the 16S rRNA reads in any individual soil. However, molecular surveys using DNA extracted from environmental samples are confounded by the lack of information on contributions of endospores that can long persist in the environment, and

vegetative cells. In addition, it is possible that inappropriate DNA extraction protocols might cause insufficient lyses of Gram-positive vegetative cells and definitely of their endospores, making them underrepresented in DNA-based molecular surveys [94]. Besides the bias in DNA extraction, there can also be an uncoupling between specific activity and abundance of microorganisms in the environment [95, 96], albeit this has not been reported for bacilli. Nevertheless, a series of 16S rRNA studies based on ribosome isolation [97-99], later validated through fluorescent whole cell *in situ* hybridization [100], demonstrated that bacilli can predominate in the active bacterial population in a grassland soil, next to *Acidobacteria* and *Alphaproteobacteria*. In addition to soil, bacilli have been found to make up considerable part of the bacterial community of other ecosystems with important NO_3^- removal activities and high nitrous oxide emissions. Various DNA-based studies using Denaturating Gradient Gel Electrophoresis demonstrated that bacilli are abundant in the thermophilic stage of animal manure composting [101]. They are also major constituents of the microbiota in wastewater treatment systems, with for example 25% of the 16S rRNA gene sequences attributed to bacilli in the biofilm of a constructed wetland for enhanced NO_3^- removal [102] and more than 30% in a modified rotating biological contactor wastewater treatment process [103].

1.7.2 Their contribution to denitrification

Comprehensive understanding of the identities and activities of microorganisms as well as the cellular mechanisms involved in NO_3^- removal are crucial for improving models that predict fluxes of NO_3^-, NO_2^- and N_2O [3]. Denitrification is a facultative trait and thus believed to be weak selectors of the microbial community [40, 104], meaning that the occurrence of these functional guilds are mainly determined by their aerobic, mostly heterotrophic, metabolism. So the ubiquitous nature and the abundance of bacilli in specific environments can indeed be informative of their potential relevance as anaerobic NO_3^- reducers. This of course needs to be

verified through specific determination of denitrifier community composition, their abundance and activity using functional genes as biomarkers [105]. For denitrification, frequent targets are either *nirK* or *nirS* for copper- or cytochrome cd_1-dependent NO_2^- reductase to NO, as these encode the key step of converting fixed nitrogen into a gaseous form, or *nosZ* for the N_2O reductase converting the potent greenhouse gas into harmless N_2. Unfortunately, until recently, most commonly used PCR primers or molecular probes for these functional genes were designed using almost exclusively reference sequences from *Proteobacteria* [52, 106-110], and thus produced consistent negative results when tested on physiologically confirmed denitrifying members of the genera *Bacillus* (Heylen; unpublished results), *Paenibacillus* [49] or *Geobacillus* [111]. In the exceptional case that amplicons were obtained from Gram-positive denitrifiers, their sequence phylogeny was highly related to that of sequences derived from Gram-negative denitrifiers [112] suggesting horizontal gene transmission. It has now become generally accepted that widely used primers are not broad range [113]. We believe that, as a consequence, the ecological relevance of bacilli, but also of other non-targeted taxa such as *Bacteroidetes* or *Actinobacteria*, for anaerobic NO_3^- removal and N_2O emission has been minimalized over the past two decades.

In the last few years, whole genome mining and detailed functional sequence analyses have demonstrated that *Firmicutes* and other unaccounted denitrifiers and ammonifiers have very divergent biomarker sequences and form clades distinct from commonly detected *Alpha-*, *Beta-* and *Gammaproteobacteria* for *nosZ* [69, 114], *nirK* and *nirS* [115] [116]. Using newly developed primers, the *nosZ* clade II organisms were detected in a range of geographically diverse environmental samples, including various soil types, wetlands, lake sediments, and activated sludge from wastewater treatment plants (WWTP), and appeared at least as abundant as the commonly targeted clade I [114]. Given the relative coherence of the *nosZ* and 16S rRNA phylogenies [117, 118], the phylogenetic placement of environmental

sequences in combination with the quantitative PCR results indicate that organisms within the *Bacteroidetes*, *Gemmatimonadetes* and *Deltaproteobacteria* make up a significant proportion of N_2O-reducing communities in different environments. So, unfortunately, the use of improved primers for *nosZ* did not result in identification of bacilli as major component of the targeted functional guild. Indeed, of the 403 cloned *nosZ* clade II sequences, only two sequences, from activated sludge from wastewater treatment plants, were most similar to those from included *Firmicute* representatives *Geobacillus thermodenitrificans* and *Desulfitobacterium hafniense*. We again refer to the above-mentioned potential difficulties with cell lyses in DNA extraction protocols, as we noticed that the DNA of two out of four included WWTP sample were extracted with a modified protocol for Gram-positive bacteria. In addition, the validation of the *nosZ* clade II primers was least strong for the *Firmicute* strains, with weak amplification from pure cultures and non-specific amplification products of considerable size (500 bp) [114]. Of course we cannot be certain that these issues have caused an underdetection of *in situ* present bacilli within *nosZ* clade II, they might just have been absent from the investigated environmental samples or might have had truncated denitrification pathways. Another, very recent effort to target unaccounted clades of *nirS* and *nirK* again demonstrated that the previously undetected denitrifiers are highly diverse and two to six times more abundant *in situ* than the commonly targeted clades [116]. Unfortunately this study did not include any sequences from bacilli in the analyzed data set and an *in silico* analyses indeed confirmed that the newly developed primers do not target bacilli-derived *nir* genes (Decleyre & Heylen, unpublished). Because of the high divergence of denitrification genes from bacilli, attempts to design primers specifically targeting those genes were thus far either unsuccessful (Decleyre & Heylen, unpublished) or resulted in primers with a coverage limited to those sequences included for primer design [111, 119].

Without efficient molecular tools to detect denitrifying bacilli and other Gram-positives *in situ*, culture-dependent methods, albeit extremely biased, can provide some insight in their environmental relevance. Cultivation studies suggest that bacilli and other Gram-positives capable of denitrification can be found in various ecosystems, from WWTPs [116, 120], many types of soil and plant rhizospheres [51, 121, 122], mangrove roots [52] to lagoon sediments [109] and thus might actively contribute to denitrification in these systems.

Methodological issues for PCR-based community structure analyses and abundance assessment might become less relevant as shotgun sequence data analyses becomes more accessible to non-expert users, but are until then very relevant for assessing which organisms contribute to NO_3^- removal and denitrification. As has become clear for other previously not considered groups of denitrifiers, bacilli and other Gram-positive bacteria might be relevant and abundant NO_3^- removers is specific environments but we currently lack the tools to specifically detect and quantify them. In addition, we know too little of their ecological lifestyle, besides their ubiquitous nature, to predict in what kind of habitats they could thrive as denitrifiers and ammonifiers. Nevertheless, their persistence in nature through endospore formation is an important feature to resist environmental stresses and may also contribute to the metabolic resilience of denitrifying communities under perturbation. Furthermore, their high level of modularity of the dissimilatory NO_3^- reduction pathway, as outlined in this chapter both for denitrifiers and non-denitrifiers, will definitely determine their niche specialization and relevance in nature.

1.8 Introduction to nitrogen assimilation metabolism in bacilli

NH_4^+ is not only produced by DNRA but can also be produced during nitrogen assimilation and it can be consumed to support growth. Physiological experiments should take this into account and therefore this introduction also addresses nitrogen assimilation.

1.8.1 NH_4^+ assimilation

NH_4^+ is the preferred sole nitrogen source over many other nitrogen sources, such as amino acids or more complex organic nitrogen compounds and is the most commonly used nitrogen source for culture study [123, 124]. NH_4^+ taken up by the cells can either from the environments [125-127] or be self-produced from NO_3^- assimilation or DNRA processes or breakdown of the organic nitrogen.

Bacteria employ different strategies for NH_4^+ uptake in function of the external pH. When at alkaline pH, a large proportion of NH_4^+ is present as NH_3. As other lipophilic and small, uncharged compounds (CO_2, H_2O, CH_4, H_2, O_2, N_2), NH_3 can rapidly pass membranes by unspecific diffusion and be incorporated to nitrogen intermediates (glutamate & glutamine) afterwards [128]. At low pH and low NH_4^+ concentrations, although some diffusion of NH_3 must occur, it is very slow compared with specific transport. NH_4^+, which poorly permeates the membranes, predominates and another uptake strategy is required, i.e. by homotrimeric transport protein AmtB, encoded by *amtB* (*nrgA* in *B. subtilis* [129]) which together with *glnK* (*nrgB* in *B. subtilis* [129]) forms the *glnKamtB* operon. GlnK is a member of the regulatory P_{II} protein family which can sense the nitrogen status of cells and modulates NH_4^+ uptake by AmtB [130, 131]. The *glnKamtB* operon is well conserved and present in most non-pathogenic bacteria, archaea, fungi, plants, protists and lower animals.

In general, NH_4^+ taken up by bacteria will be incorporated directly only to glutamate and glutamine by three key enzymes: (i) glutamate synthase (glutamine 2-oxoglutarate

aminotransferase or GOGAT), encoded by *gltAB* operon, (ii) glutamine synthetase (GS) encoded by *glnRA* operon and glutamate dehydrogenase (GDH), encoded by *gdhA* gene, via the GS/GOGAT or the GDH pathway respectively. At high concentrations of NH_4^+, *E. coli* primarily assimilates NH_4^+ by GDH which forms glutamate by catalysing the reductive amination of 2-oxoglutarate. While at low concentrations of NH_4^+, glutamine synthetase/glutamate synthase (GS/GOGAT), which produces two molecules of glutamate by transferring the amide group from glutamine to 2-oxoglutarate, takes over. This may be because of the very low affinity of GDH for NH_4^+ [132, 133]. However, in contrary to *E.coli* and many other organisms, *B.subtilis* assimilates NH_4^+ exclusively using the GS/GOGAT cycle. This may be because the GDHs of *B.subtilis* have about sevenfold lower affinity for NH_4^+ than the *E.coli* enzyme (Figure 1.4) [134].

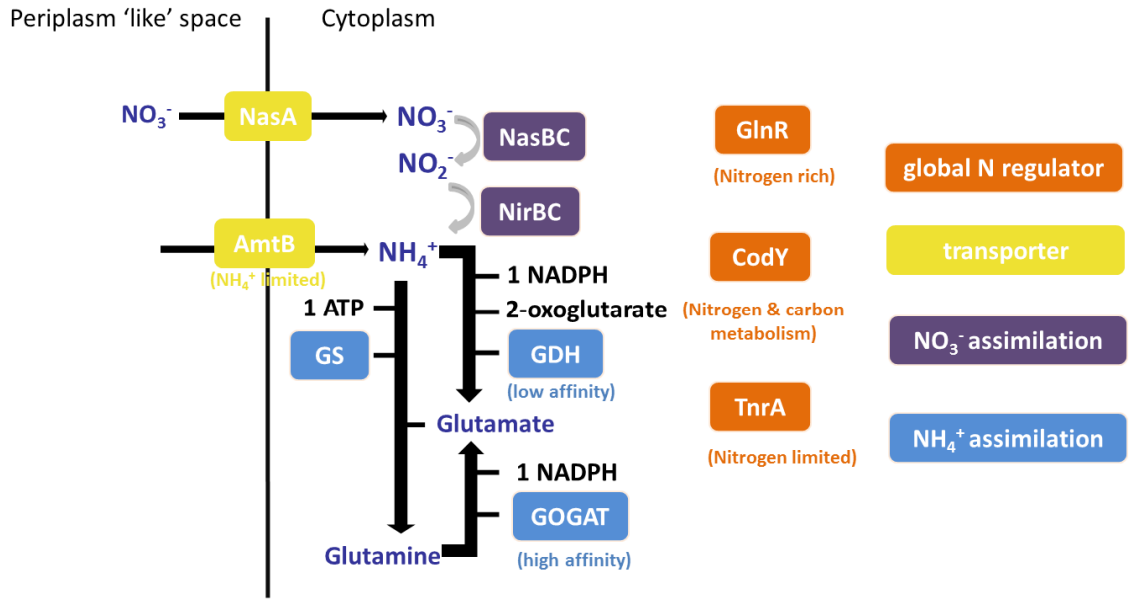

Figure 1.4 Overview of nitrogen assimilation pathways in *Bacillus*: (1) Regulation, global nitrogen regulators are indicated in orange: GlnR, TnrA, CodY (2) Transport, transporters in the membrane are in yellow (NasA, AmtB) (3) NO_3^- assimilation enzymes are indicated in purple (4) NH_4^+ assimilation enzymes are indicated in blue.

Since GS/GOGAT coding genes are highly conserved in almost every organism, glutamate and glutamine are widely used as organic nitrogen source in culture. The glutamine and glutamate assimilated or produced as intermediates function as nitrogen donors in transamination and transamidation reactions which form other nitrogen compounds necessary for life, such as amino acids, or precursors for purines and pyrimidines [135, 136]. It is estimated that, glutamate delivers 80-88% of the nitrogen that is incorporated into biomass while glutamine is involved in only few enzymatic reactions [133, 137].

1.8.2 NO_3^- assimilation

NO_3^- assimilation is carried out by many bacteria, with NO_3^- as a nitrogen source for growth. NO_3^- is taken up by the cells via high-affinity transport systems (NasA in *Bacillus subtilis*, encoded by *nasA*) [19, 138]. Some proteins, like NarK of *E.coli* are involved in NO_3^-/NO_2^- exchange but not simply in the uptake of NO_3^- or NO_2^-. The incorporated NO_3^- in the cell is further reduced to NH_4^+ via NO_2^-, by two sequential reductions using NO_3^- reductase (NasBC, encoded by *nasBC* operon) [138] and NO_2^- reductase (NirBD, encoded by *nirBD* operon [35, 139], or NasDE encoded by *nasDE* operon in *Bacillus subtilis* [34]) (Figure 1.4). The resulting NH_4^+ is further involved in GS/GOGAT pathways as mentioned above.

1.8.3 Organic nitrogen assimilation

Organic nitrogen is widely distributed in the environment, e.g. in soil, oceans and deep-sea sediments, and serves an important nitrogen source for various organisms all over the world [125, 140-144]. On average, 50% of the nitrogen contained in soil is organic nitrogen [145, 146]. This soil organic nitrogen originates from fertilization by humans, animal excreta, N_2 fixation, atmospheric deposition, and the incorporation of dead and decaying plant and microbial residues, the latter representing the main direct input of organic N to the soil [147].

Some mono-organic nitrogen, e.g. amino acids and oligomeric organic nitrogen, e.g. small peptides can be taken up directly by microorganisms via specific transport proteins or carriers[128, 148]. While larger size of organic nitrogen has to be broken down to small size by extracellular enzymes prior to uptake by certain organisms[148]. Yeast extract is an commonly used organic nitrogen of bacteriological media for a variety of microorganisms including media for enrichment, isolation and cultivation of denitrifiers [149-151], and sometimes it is essential for cultivating specific organisms in mineral media [152, 153]. Yeast extract contains a mixture of amino acids, peptides, water soluble vitamins and carbohydrates that act as growth stimulants, growth factors or nitrogen sources for bacteria [154-156].

1.8.4 Regulation of nitrogen assimilation

The regulatory mechanisms involved show an impressive diversity, resulting from specific strategies in response to the changing conditions of nitrogen supply in virtually every phylum of bacteria. For example, in the Gram-negative enteric bacteria the regulatory two-component system NtrBC controls the level of GS and other enzymes involved in nitrogen assimilation [157].

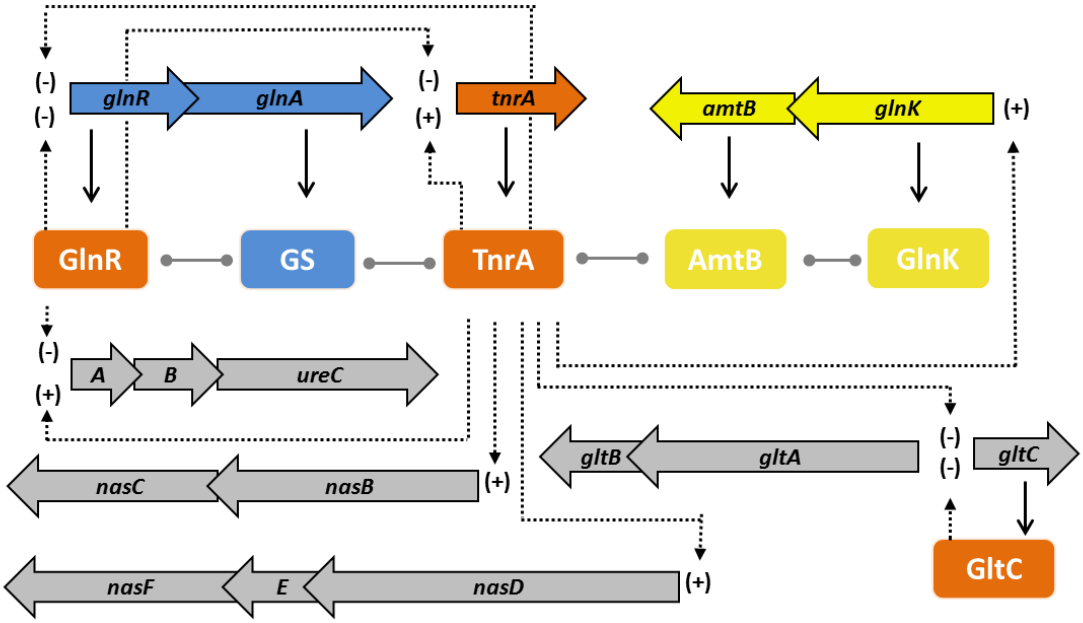

Figure 1.5 Regulatory network of nitrogen assimilation in *Bacillus subtilis*. Arrows indicate the relative length and organization of genes on the chromosome. Dotted black arrows illustrate the regulatory interactions of the respective regulator of nitrogen metabolism (orange) including its function in activating (plus) or repressing (minus) the target gene(s). Gray lines with circles indicate post-translational interactions (blue, glutamine synthetase; yellow, ammonium transporter AmtB and P_{II} protein GlnK; gray, further genes involved in nitrogen assimilation; for further details, see text.) [132].

In low-GC Gram-positive bacteria, nitrogen assimilation is generally mediated by regulatory proteins GlnR, TnrA and to a certain degree the global regulatory protein CodY (Figure 1.4). GlnR is a specific regulator of nitrogen metabolism and represses the *glnRA* operon, the urease operon *ureABC* and also the *tnrA* gene. TnrA activates the transcription of the ammonium transporter operon *glnKamtB* genes, the urease operon, and nitrate and nitrite reductase gene clusters *nasBC* and *nasDE* (*nirBD*). Furthermore, TnrA is positively autoregulated and exhibiting a negative cross-regulation for *glnRA* and repressing the *gltAB* operon, while the *gltAB* operon is additionally under control of the GltC regulator. Based on previous studies, GlnR is only active under conditions of ample nitrogen supply and TnrA is

only active under nitrogen-limited conditions [158]. CodY functions at the intersection of nitrogen and carbon metabolism, mostly by direct repression of the urease operon and control of genes involved in transport, catabolism and biosynthesis of amino acids [159]. Their distribution is not uniform: GlnR is found in almost all *Bacillus* species, TnrA is also specific for *Bacillaceae* (except *Bacillus cereus*, *Bacillus anthracis* and *Bacillus thuringiensis*) [160] and CodY is found in all *Firmicutes* based on available genome data [132].

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Chapter 2:

Nitrous oxide emission by the non-denitrifying, nitrate ammonifier *Bacillus licheniformis*

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Author's contribution:

YH performed the experiments, data analyses and wrote the paper; KH conceived the study, performed genome data analyses and wrote the paper; PDV helped with interpretation of data and revised the paper.

Summary

Background: *Firmicutes* have the capacity to remove excess NO_3^- from the environment via either denitrification, dissimilatory NO_3^- reduction to NH_4^+ or both. The recent renewed interest in their nitrogen metabolism has revealed many interesting features, the most striking being their wide variety of dissimilatory NO_3^- reduction pathways. In the present study, nitrous oxide production from *Bacillus licheniformis*, a ubiquitous Gram-positive, spore-forming species with many industrial applications, is investigated.

Results: *B. licheniformis* has long been considered a denitrifier but physiological experiments on three different strains demonstrated that nitrous oxide is not produced from NO_3^- in stoichiometric amounts, rather NH_4^+ is the most important end-product, produced during fermentation. Significant strain dependency in end-product ratios, attributed to NO_2^- and NH_4^+, and medium dependency in nitrous oxide production were also observed. Genome analyses confirmed the lack of a NO_2^- reductase to nitric oxide, the key enzyme of denitrification. Based on the gene inventory and building on knowledge from other non-denitrifying nitrous oxide emitters, hypothetical pathways for nitrous oxide production, involving NarG, NirB, qNor and Hmp, are proposed. In addition, all publically available genomes of *B. licheniformis* demonstrated similar gene inventories, with specific duplications of the *nar* operon, *narK* and *hmp* genes as well as NarG phylogeny supporting the evolutionary separation of previously described distinct BALI1 and BALI2 lineages.

Conclusions: Using physiological and genomic data we have demonstrated that the common soil bacterium *B. licheniformis* does not denitrify but is capable of fermentative dissimilatory NO_3^-/ NO_2^- reduction to NH_4^+ (DNRA) with concomitant production of N_2O. Considering its ubiquitous nature and non-fastidious growth in the lab, *B. licheniformis* is a suitable candidate for further exploration of the actual mechanism of N_2O production in DNRA bacteria and its relevance *in situ*.

2.1 Introduction

Denitrification and dissimilatory NO_3^-/ NO_2^- reduction to NH_4^+ (DNRA) are two key processes, performed by a wide range of *Bacteria* and *Archaea* as well as some *Eukaryotes* [1], responsible for removal of excess NO_3^- from the environment. Denitrification is the modular step-wise reduction of fixed nitrogen, NO_3^- or NO_2^- to a gaseous form, either nitric oxide (NO), nitrous oxide (N_2O) and/or dinitrogen gas (N_2). DNRA retains nitrogen in the environment, although N_2O, contributor to both climate change and ozone depletion in the stratosphere, can also be produced as side product. Comprehensive understanding of the identities and activities of microorganisms as well as cellular mechanisms involved in NO_3^- removal are crucial for improving models that predict fluxes of NO_3^-, NO_2^- and N_2O [2]. Although several *Firmicutes* have been known for a long time to be NO_3^- reducers and N_2O emitters [3-8], their ecological relevance has been minimalized over the past two decades based on molecular community surveys using primers not targeting their divergent denitrification [9-12] or DNRA genes [13] (note that recent primers for DNRA do indeed target *Firmicute* genes [14]). Nevertheless, *Firmicutes* and specifically *Bacillus* can be dominant in ecosystems with important NO_3^- removal activities such as soil [15], animal manure compost [16] and advanced wastewater treatments [17].

Renewed interest in *Bacillus* has revealed many interesting features like (i) the widespread occurrence of NO_3^- reduction and denitrification in the genus [18], (ii) the gene inventory for both denitrification and DNRA in one microorganism [19, 20], (iii) a novel type of copper-A-dependent, electrogenic nitric oxide reductase (Cu_ANor) [21-24], or (iv) membrane-bound denitrification [25] with a novel organization for the periplasmic NO_3^- reductase [19, 26]. The most striking observation however is the wide variety of dissimilatory NO_3^- reduction pathways in members of this genus. The model organism *Bacillus subtilis* uses the

cytoplasmic NO_3^- reductase NarGHI and NO_2^- reductase NirBD to anaerobically reduce NO_3^- to NH_4^+ [27, 28], while *Bacillus selenitireducens* produces NH_4^+ via the periplasmic NO_2^- reductase NrfA [14, 29]. *Bacillus vireti* can do the same but with concomitant N_2O production via Cu_ANor that can be converted to the harmless N_2 with a NosZ-type reductase [30]. On the other hand, *Bacillus azotoformans* and *Bacillus bataviensis* are canonical denitrifiers, the latter lacking the final reductase, but both also encode the NrfA NO_2^- reductase, making them potential NH_4^+ producers [19]. In addition, these two organisms demonstrate an unusual high level of gene redundancy, i.e. multiple genes or gene copies encoding the same function (*B. azotoformans* encodes three NO_3^-, two NO_2^-, four NO and three N_2O reductases) [19]. Considering the modularity of denitrification and DNRA, a multitude of enzyme combinations for NO_3^- reduction are imaginable, even within one microorganism.

Bacillus licheniformis, a close relative of *B. subtilis*, is widely distributed as a saprophytic organism in the environment, has numerous commercial and agricultural uses (e.g. production of peptide antibiotics, chemicals and proteases, mitigation of fungal pathogens) and some strains, with abortifacient potential or toxin production, might pose a threat to public health. Certain *B. licheniformis* isolates have been described as denitrifiers [5, 18, 28], mostly based on their ability to produce gas from NO_3^- anaerobically. Many genomes from *B. licheniformis* have been sequenced and described to date [31-36]. However, their lack of genes encoding either a copper- or a cytochrome cd_1-dependent NO_2^- reductase (NirK or NirS respectively), the key enzyme of denitrification, has gone unnoticed, probably because of limited interest in their anaerobic nitrogen metabolism. We have sequenced and analyzed the genomes of three *B. licheniformis* strains previously reported to produce N_2O [18], and confirmed the lack of *nirS* or *nirK* in their genomes. In addition, physiological data was gathered demonstrating that *B. licheniformis* does not denitrify but is capable of fermentative dissimilatory NO_3^-/ NO_2^- reduction to NH_4^+ with concomitant production of N_2O. Both types of data were combined to

propose hypothetical pathways for N_2O production, which present new alternative routes for NO_3^- reduction and N_2O production in members of the genus *Bacillus*.

2.2 Materials and methods

2.2.1 Strains and DNA extraction

B. licheniformis LMG 6934, LMG 7559 and LMG 17339 were obtained from the BCCM/LMG bacteria collection. Strains were grown aerobically in trypticase soy broth (TSB) at 37°C. Cells were harvested after overnight growth and DNA was extracted by the method of Pitcher et al [37], slightly modified as described previously [38].

2.2.2 Genome Sequencing & annotation

Library preparation and genome sequencing was performed by Baseclear B.V. For sequencing, paired-end strategy on the Illumina Genome Analyzer IIx was used yielding average read lengths of 75 bp for LMG 7759 and LMG 17339 and 50 bp for LMG 6934. Automatic trimming (based on a threshold of Q = 20 and maximum 2 ambiguous bases) and assembly was performed using CLC Genomics Workbench 6.5. The k-mer and bubble size parameters were varied to maximize the N50 and minimize the number of contigs of the resulting assembly for each genome. For the consensus sequence, conflicts were resolved by using quality scores and insertion of ambiguity codes. Functional annotation and metabolic reconstruction was performed with the Rapid Annotation Subsystem Technology (RAST) server [39, 40], using RAST gene calling and allowing frame shift correction, backfilling of gaps and automatic fixing of errors. Assigned functions were checked with pBLAST [41] and InterProScan [42]. Missing genes were searched for in the genome with PSI-BLAST using homologous amino acid sequences. The average nucleotide identity (ANI) was calculated with the ANI calculator (http://enve-omics.ce.gatech.edu/ani/) [43].

2.2.3 Growth experiments

Anaerobic growth experiments were performed in TSB and mineral medium, amended with 10 mM potassium NO_3^- as electron acceptor. Mineral medium was as described by Stanier *et al* [44], including 10 mM phosphate buffer, 2.3 mM $(NH_4)_2SO_4$, 0.4 mM $MgSO_4.7H_2O$, 0.04

mM $CaCl_2.2H_2O$, 27 µM EDTA, 25 µM $FeSO_4•7H_2O$, 10 µM $ZnSO_4•7H_2O$, 25 µM $MnSO_4•H_2O$, 3.8 µM $CuSO_4•5H_2O$, 2 µM $Co(NO_3)_2•6H_2O$, 0.196 µM $(NH_4)_6Mo_7O_{24}•24H_2O$, supplemented with 30 mM glucose as electron donor. Serum vials (120 ml) were rinsed with 1 M HCl overnight to remove growth inhibiting substances, and subsequently washed four times and rinsed with distilled water before use. Serum vials with 50 ml medium were sealed with black butyl-rubber stoppers. After autoclaving, the headspace of the serum vials was replaced via five cycles of evacuating and refilling with helium. Serum vials were inoculated (1% v/v) with a suspension of optical density OD_{600} of 1.0 ± 0.05. Each growth experiment was performed in triplicate and non-inoculated media in duplicate were included to check for potential nitrosation reactions in sterile medium. After inoculation, serum vials were incubated at 37 °C, 150 rpm, for 72h. Preliminary end-point analyses demonstrated that all three strains did not produce N_2 (later confirmed by absence of *nosZ* gene from the genomes, see further) so their dissimilatory NO_3^- reduction metabolism was explored without addition of acetylene to the headspace. Statistical differences in growth rate and yield of LMG 6934 between TSB and TSB amended with 10 mM NO_3^- were assessed using the independent t-test after Levene's test for equality of variances, and main and interaction effect of medium and strain on end-product concentration using factorial ANOVA and Least Significant Difference post-hoc testing in SPSS23.

2.2.4 Analytical procedures

Samples of 1 ml were taken from cultures through the rubber septum of serum vials with sterile syringes for growth determination and colorimetric determination of NH_4^+, NO_3^- and NO_2^-. Growth was determined by measuring the optical density OD_{600} of 100 µl sample in duplicate in microtiter plates and standardized to 1 cm path length using PathCheck Sensor of the spectrophotometer (Molecular Devices, Spectramax plus 384, USA). For colorimetrics, 500 µl from remaining sample was pretreated with 2.5ml of 2 M potassium chloride by

shaking 1h at 150 rpm and subsequent filtration (0.2 µm) to extract inorganic nitrogen and remove interfering compounds. Filtered samples were centrifuged at 13000 rpm for 2 min to remove the cells and kept frozen at -20°C until colorimetric determination. NH_4^+ concentration was determined with the salicylate-nitroprussidine method (absorption at a wavelength of 650 nm) [45], NO_2^- and NO_3^- concentrations were determined with Griess reaction [46] and Griess reaction with cadmium [47, 48] respectively. For end-point measurements, NH_4^+ production was corrected per strain for the amount of NH_4^+ assimilated based on OD_{600} values obtained. Standard curves covered ranges suitable for the tested media and were strictly linear with an R_2 of 0.99. For determination of N_2O, 1 ml sample of the headspace of serum vials was taken with sterile syringes, and was injected into the gas chromatograph (Compact GC with EZChrom Elite Software, Interscience, Netherlands, 2012) and measured by thermal conductivity detector (TCD). N_2O concentrations were corrected for pressure and solubility based on Henry's law.

2.2.5 Accession numbers

The Whole Genome Shotgun projects of *B. licheniformis* LMG 6934, LMG 7559 and LMG 17339 have been deposited at DDBJ/EMBL/GenBank under the accession numbers AZSY00000000, AZSX00000000, and AZSZ00000000 respectively. The versions described in this paper are the first versions.

2.3 Results

2.3.1 Dissimilatory NO_3^- reduction metabolism

Three genotypically distinct *B. licheniformis* strains (Coorevits, A. & De Vos, P., personal communication) from various origins were selected for determination of their dissimilatory NO_3^- reduction metabolism based on a previous study that demonstrated their capacity to produce N_2O [18]. *B. licheniformis* LMG 6934 was originally isolated from garden soil, LMG 7559 from flour and LMG 17339 from silage.

Growth of LMG 6934 (Figure 2.1A) under anaerobic headspace in TSB amended without and with NO_3^- (11 mM) commenced after a short lag phase of approximately 3h, a steep exponential phase followed, with maximal growth achieved after 8.5h, after which cells sporulated very quickly without an obvious stationary phase (Figure 2.1A). Between 6.5-7.5h, growth slowed down probably due to a depletion of the preferential carbon source in the medium with a shift to another electron donor, as this was observed both for fermentative and respiratory growth. The anaerobic growth rates were comparable with and without NO_3^- as electron acceptor ($\mu_{nitrate}$/h = 0.189 ± 0.004 h^{-1} and μ_{ferm} = 0.179 ± 0.011 h^{-1} (p=0.264)), but with significantly different maximal growth yield (OD_{600} of 1.05 ± 0.02 with NO_3^- and 0.75 ± 0.06 without NO_3^- (p = 0.002)) reflecting the different ATP yield of a respiratory and a fermentative life style. In the presence of NO_3^- (Figure 2.1B), growth was initially supported by NO_3^- reduction (μ_1 = 0.19 ± 0.004 h^{-1}), with concomitant NO_2^- production. All NO_3^- was converted to NO_2^- but the maximal NO_2^- peak was probably missed between 5 and 6.5h of incubation, which was deduced from the onset of N_2O production (0.2 mM N-N_2O at 6.5h). When NO_3^- was almost depleted (at 6.5h, 0.62 mM residual NO_3^-), NO_2^- was reduced (μ_2 = 0.16 ± 0.02 h^{-1}), which continued after maximal growth at 8.5h was achieved and sporulation had started (μ_3). This suggested that NO_2^- reduction did not support growth during the μ_2 phase, but rather fermentation was responsible for growth after NO_3^- depletion. During NO_2^-

reduction, a continuous increase in N_2O was observed, with a maximum of 1.3 ± 0.07 mM N-N_2O at the end of the incubation (accounting for 12% of all reduced NO_2^-), and 0.5 ± 0.18 mM of NO_2^- remaining in the medium (Figure 2.1B). Due to technical constraints and interference of amines from degradation of proteins in the TSB during bacterial growth, NH_4^+ was not monitored during these growth experiments.

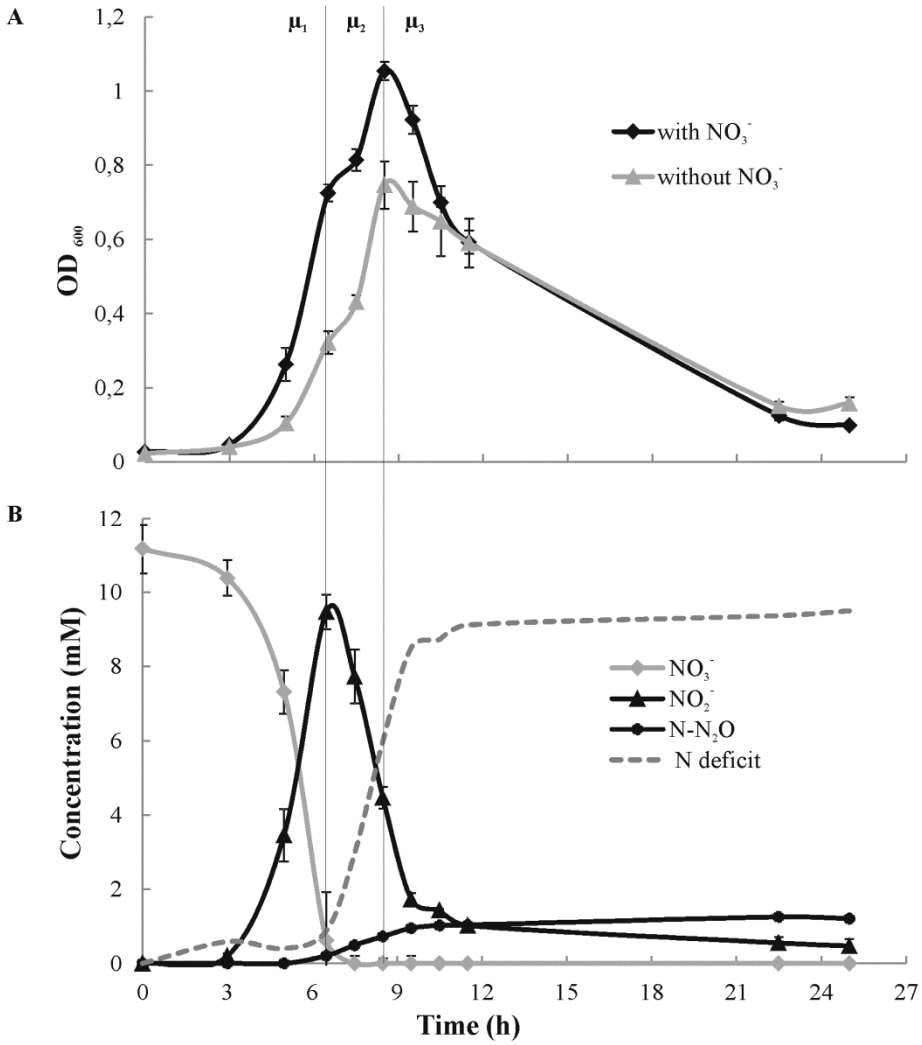

Figure 2.1 Anaerobic growth (OD_{600}) of *B. licheniformis* LMG 6934 in TSB (A) and NO_3^-, NO_2^- and N_2O concentrations (mM) (B) over time. Error bars show standard deviation (n = 3). Different growth phases based on primary metabolism in TSB amended with NO_3^- are marked: μ1, respiratory growth with NO_3^- as electron acceptor; μ2, fermentative growth after NO_3^- is depleted; μ3, sporulation. Dashed curve visualizes N deficit caused by the lack of NH_4^+ data.

To compare the dissimilatory NO_3^- reduction metabolism of LMG 6934 with those of LMG 7559 and LMG 17339 and to confirm NH_4^+ production from NO_2^-, end-point experiments after a 72h-incubation in anaerobic conditions were performed in TSB and mineral medium with 30 mM glucose, both amended with NO_3^-. Maximal growth of LMG 7559 and LMG 17339 was achieved within 11h, again immediately followed by a rapid sporulation (data not shown). In mineral media with glucose, most NO_3^- was converted to NH_4^+ (31.8 to 89.1%; Figure 2.2), confirming the NH_4^+-producing capacity of all three strains (for TSB, the nitrogen deficit was attributed to NH_4^+ production, which could not be measured). A significant strain effect on the ratios of end-products was observed ($p \leq 0.008$) (Figure 2.2), which after decomposition appeared to be mostly attributed to differences in NH_4^+ and NO_2^- concentrations. In addition, the amount of $N\text{-}N_2O$ produced from NO_3^- was substantially lower in mineral medium than in TSB for all strains (15.8-32.9% for TSB vs 10.9-24.1% in MM) ($p = 0.023$). Only for LMG 17339, the medium also had a significant effect on NH_4^+ and NO_2^- ($p \leq 0.024$). Both strain and medium effect were quite unexpected, as the three strains are closely related and the major carbon source in TSB, namely glucose, is also used in the mineral medium.

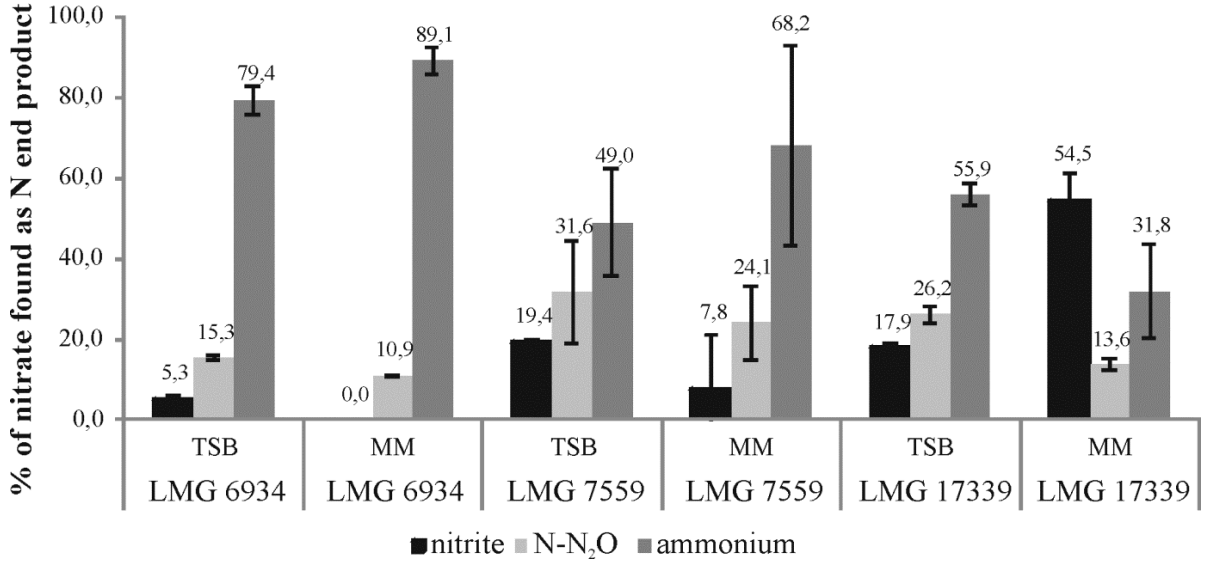

Figure 2.2 Percentages of end-products of anaerobic NO_3^- reduction by *B. licheniformis* strains in TSB and mineral medium with 30 mM glucose, amended with 10 mM NO_3^-. Error bars represent standard deviation (n=3). The larger error bars for LMG 7559 result from differences in one of the three replicates. Measured concentrations of NH_4^+ were corrected for loss through assimilation. N deficit in TSB experiments was attributed to NH_4^+ production, which could not be measured, and visualized as such for convenience of comparison.

2.3.2 Genome analyses

Draft genomes of the three *B. licheniformis* strains were obtained, the genome statistics are given in Table 2.1. The gene inventory for assimilatory and dissimilatory NO_3^- reduction and related transport and regulation was almost identical for the three genomes (Table 2.2). Details will be given for LMG 6934 and differences with LMG 7559 and LMG 17339 will be highlighted.

Table 2.1 Genome characteristics of three analyzed *B. licheniformis* genomes. Number of coding sequences is based on annotation obtained via RAST.

	LMG 6934	LMG 7559	LMG 17339
# contigs (# bp)	53 (4,138,686 bp)	69 (4,341,862 bp)	80 (4,333,151 bp)
N_{50}	654,545	123,311	102,733
av. read coverage	87.4	82.3	233.7
% G+C	45.9	45.8	46.1
RNA	1 rRNA operon 30 tRNA	1 rRNA operon 30 tRNA	1 rRNA operon 36 tRNA
# coding sequences	4,576	4,559	4,425
accession number	AZSY00000000	AZSX00000000	AZSZ00000000

The genome of LMG 6934 contained two copies of the *nar* operon (*narGHJI*) coding for the cytoplasmic, membrane-bound NO_3^- reductase (Table 2.2; Figure S1). The two NarG sequences are quite divergent, only sharing 53.7% amino acid sequence identity. The *nar1* operon (Figure 2.3) is located in a gene cluster with downstream the genes for the anaerobic regulatory protein Fnr (Fumarate- NO_3^- reductase Regulation) (*fnr1*), a NarK2-type low-affinity NO_3^-/ NO_2^- antiporter (*narK1*) (Figure S2), a second Fnr (*fnr2*), a quinol-dependent NO reductase (*qnorB*) and a NO-dependent regulator (*dnrN*). The *nar2* operon (Figure 2.3) is immediately downstream of CDS for a second NarK2-type NO_3^-/ NO_2^- antiporter (*narK2*) (Figure S2) and a flavohemoprotein (*hmp1*). The two-component NO_3^-/ NO_2^- sensor regulator system (*narXL*) is encoded downstream of the genes for a NO_2^--sensitive transcriptional repressor of NO stress response (*nsrR*) and a second flavohemoprotein (*hmp2*), while genes for a third NarK2-type NO_3^-/ NO_2^- antiporter (*narK3*), a formate/ NO_2^- transporter (*nirC*), a second *narL* copy NO reductase activation proteins (*norDQ*), and the global nitrogen regulator (*tnrA*) are found separate on the genome. The gene for NO synthase (*nos*) was also found. Two genes encoding a putative NorV, a flavorubredoxin that could be capable of detoxification of NO to N_2O [49, 50], were also found. However, no gene for the associated oxidoreductase NorW or regulator NorR was found down- and upstream respectively, suggesting that NorV is unlikely to be functional as NO reductase. Nevertheless, all features

for NO_3^- sensing, transport, reduction to NO_2^- and its regulation are found, as well as for NO reduction to N_2O. In addition, related to nitrogen assimilation, the operon for assimilatory NO_3^- and NO_2^- reduction and two genes for AmtB-type NH_4^+ transporters with each upstream the regulatory gene *glnK* are found. Notably, genes for a NirS- or NirK-type NO_2^- reductase to NO, a NosZ-type N_2O reductase, or a Nrf-type NO_2^- reductase to NH_4^+ are absent from the genome. The gene inventory and organization for LMG 7559 was identical to LMG 6934 (Table 2.2). Note that strain LMG 7559 is equivalent to ATTC 9945, for which a complete genome sequence has already been published since the start of our genome analyses [33]. For clarity, both genomes will be included in the remainder of the genome analyses. The genome of LMG 17339 only contained one *nar* operon, two NarK2-type NO_3^-/ NO_2^- antiporters and one copy of *hmp* and *narL* (associated with *narX*), but for the remainder was identical in gene content and organization to LMG 6934 (Table 2.2).

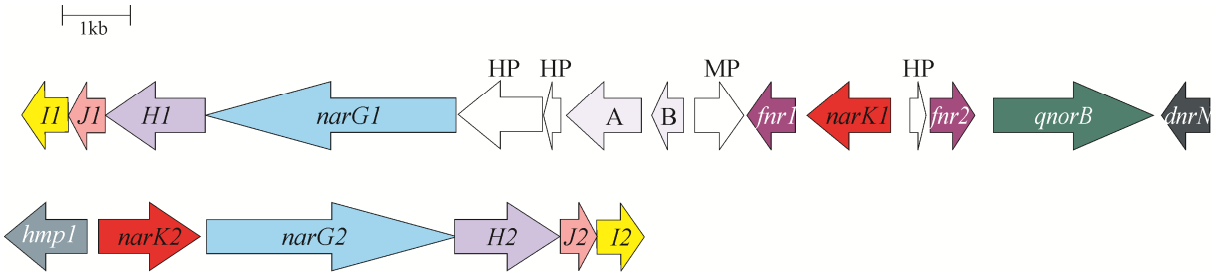

Figure 2.3 Physical map of *B. licheniformis* LMG 6934 and LMG 7559 *nar* gene clusters and their genome environment. *Arrows* show the direction of transcription. Open reading frames are drawn to scale. Homologous genes are shown in identical colors. Note that LMG 17339 only contains *nar1* gene cluster with identical genome environment except for an extra HP immediately upstream of *narG1*. HP, hypothetical protein; MP, membrane protein; A, gene for the radical SAM domain heme biosynthesis protein; B, gene for a probable transcription regulator arfM.

Whole genome clustering based on the peptidome content [51, 52], in which the amino acid sequences of a genome are converted to tryptic peptides, i.e. the tryptic peptidome, of all

publically available *B. licheniformis* genomes (dd June 2014) confirmed the two generally accepted distinct lineages within *B. licheniformis*, BALI1 and BALI2 [53] (Figure S3). Average nucleotide identities (ANI) of the genomes from the strains within BALI1 (99.70% ± 0.03) and BALI2 (98.92% ± 0.03) were well-above the arbitrary 94-95% cut-off criterion for species delineation, while between group ANI values were ambiguous (94.24% ± 0.07) [54, 55]. Interestingly, comparison of the NarG sequences of the three *B. licheniformis* genomes from this study, all other publically available *B. licheniformis* genomes and representatives of other *Bacillus* species showed two distinct clusters, each supported by high bootstrap values (Figure S1). NarG1 from LMG 6934, NarG2 from LMG 7559 and NarG from LMG 17339 grouped within the BALI1 cluster, consisting of sequences derived only from *B. licheniformis* and one *Bacillus* sp. NarG2 from LMG 6934 and NarG1 from LMG 7559 fell within cluster BALI2, which also included *B. bataviensis* and one of the two NarGs from *B. azotoformans*. In addition, all *B. licheniformis* genomes from BALI1 consistently harbored only one copy of the *nar* operon, two copies of *narK* and one copy of *hmp*, in contrast to those from BALI2 with two *nar* operons, three *narK* copies and two *hmp* copies (data not shown).

Table 2.2 Overview of gene inventory involved in nitrogen metabolism, transport and regulation of *Bacillus licheniformis*.

function	**protein**	**gene**	**locus_tag (gene coordinates)**		
			LMG 6934	**LMG 7559**	**LMG 17339**
dissimilatory nitrate reduction to nitrite	respiratory nitrate reductase subunit alpha	*narG1*	LI6934_10240 (contig17_44191_40505)	LI7559_10220 (contig17_109260_112943)	LI17339_03250 (contig01_602796_599110)
		narG2	LI6934_11815 (contig19_3766_7449)	LI7559_18375 (contig29_333921_330235)	-
	respiratory nitrate reductase subunit beta	*narH1*	LI6934_10235 (contig17_40515_39046)	LI7559_10225 (contig17_112933_114483)	LI17339_03245 (contig01_599120_597651)
		narH2	LI6934_11820 (contig19_7439_8989)	LI7559_18370 (contig29_330245_328776)	-
	respiratory nitrate reductase subunit delta	*narJ1*	LI6934_10230 (contig17_39028_38486)	LI7559_10230 (contig17_114470_115018)	LI17339_03240 (contig01_597632_597090)
		narJ2	LI6934_11825 (contig19_8976_9524)	LI7559_18365 (contig29_328758_328216)	-
	respiratory nitrate reductase subunit gamma	*narI1*	LI6934_10225 (contig17_38489_37803)	LI7559_10235 (contig17_115039_115740)	LI17339_03235 (contig01_597093_596407)
		narI2	LI6934_11830 (contig19_9545_10246)	LI7559_18360 (contig29_328219_327533)	-
assimilatory nitrate/nitrite reduction to ammonium	assimilatory nitrate reductase large subunit	*nasC*	LI6934_20135 (contig48_34712_36769)	LI7559_01055 (contig02_88956_91013)	LI17339_17560 (contig10_88671_90728)
	assimilatory nitrite reductase [NAD(P)H] large subunit	*nirB*	LI6934_20140 (contig48_36883_39303)	LI7559_01060 (contig02_91127_93547)	LI17339_17565 (contig10_90842_93262)
	assimilatory nitrite reductase (NAD(P)H) small subunit	*nirD*	LI6934_20145 (contig48_39334_39654)	LI7559_01065 (contig02_93578_93898)	LI17339_17560 (contig10_93293_93613)
transporters	ammonium transport	*amt1*	LI6934_11075 (contig18_87147_88526)	LI7559_21025 (contig40_60895_59966)	LI17339_05915 (contig03_429269_428058)
		amt2	LI6934_06945 (contig13_69958_71169)	LI7559_12245 (contig20_86027_87406)	LI17339_15165 (contig06_266544_265900)
	nitrate/nitrite transporter (NarK2-type)	*narK1*	LI6934_10275 (contig17_50582_49398)	LI7559_10215 (contig17_107711_109207)	LI17339_03285 (contig01_609186_608002)
		narK2	LI6934_11810 (contig19_2217_3713)	LI7559_18410 (contig29_340309_339125)	LI17339_16960 (contig09_16054_161849)
		narK3	LI6934_04585 (contig09_42906_44111)	LI7559_1255 (contig21_43506_44711)	-
	formate/nitrite transporter	*nirC*	LI6934_08170 (contig13_306187_306975)	LI7559_03215 (contig06_176423_177211)	LI17339_04610 (contig03_178114_177326)
NO reduction to N_2O	NO reductase large subunit-like protein	*qnorB*	LI6934_10290 (contig17_51910_54264)	LI7559_18425 (contig29_341636_343990)	LI17339_03300 (contig01_610516_612870)

function	protein	gene	locus_tag (gene coordinates)		
			LMG 6934	**LMG 7559**	**LMG 17339**
	NO reductase activation protein	*norD1*	LI6934_02700 (contig04_119995_118082)	LI7559_00105 (contig01_24308_22395)	LI17339_13375 (contig04_735870_736760)
		norD2	LI6934_02705 (contig04_120896_120006)	LI7559_00110 (contig01_25209_24319)	LI17339_13380 (contig04_736770_738683)
detoxification	flavohemoglobin	*hmp1*	pLI6934_11805 (contig19_1907_689)	LI7559_10210 (contig17_107401_106181)	LI17339_14225 (contig06_89281_88067)
		hmp2	LI6934_03825 (contig07_12629_11415)	LI7559_14100 (contig23_84124_85338)	-
	NO synthase	*nos*	LI6934_16440 (contig34_14900_15997)	LI7559_14725 (contig27_17226_16129)	LI17339_20905 (contig20_78781_79878)
regulation	P_{II}-type signal transduction protein	*glnK1*	LI6934_11070 (contig18_86749_87090)	LI7559_12240 (contig20_85629_85970)	LI17339_05910 (contig03_428039_427689)
		glnK2	LI6934_06950 (contig13_71188_71538)	LI7559_21020 (contig40_59850_59500)	LI17339_15170 (contig06_267014_266677)
	global nitrogen regulator	*tnrA*	LI6934_15560 (contig31_18775_18443)	LI7559_22170 (contig47_44732_45064)	LI17339_00475 (contig01_88311_87979)
	NO-dependent regulator DnrN or NorA	*dnrN*	LI6934_10295 (contig17_55022_54309)	LI7559_18430 (contig29_344748_344035)	LI17339_03305 (contig01_613628_612915)
	Nitrite-sensitive transcriptional repressor of NO stress response	*nsrR*	LI6934_03830 (contig07_12887_13318)	LI7559_14095 (contig23_83866_83432)	LI17339_14220 (contig06_87809_87378)
	Nitrate/nitrite sensor protein	*narX1*	LI6934_03855 (contig07_16463_15315)	LI7559_14090 (contig23_83082_83402)	LI17339_14195 (contig06_84234_85382)
	Nitrate/nitrite response regulator protein	*narL1*	LI6934_03850 (contig07_15327_14677)	LI7559_02755 (contig06_92319_91687)	LI17339_14200 (contig06_85379_86023)
		narL2	LI6934_07715 (contig_13_222155_221523)	LI7559_14070 (contig23_80295_81443)	-
	transcriptional regulator Crp/Fnr	*fnr1*	LI6934_10270 (contig17_49251_48538)	LI7559_18405 (contig29_338978_338265)	LI17339_03280 (contig01_607856_607143)
		fnr2	LI6934_10285 (contig17_51086_51754)	LI7559_18420 (contig29_340813_341487)	LI17339_03295 (contig01_609691_610359)

2.4 Discussion

2.4.1 General metabolism

Strains belonging to the species *B. licheniformis* have often been considered as denitrifiers [5, 18], based on their ability to produce gaseous end-products specifically N_2O from NO_3^-. However, our analyses demonstrated that *B. licheniformis*, like *B. subtilis* [27, 28], is capable of NO_3^- respiration and fermentative dissimilatory NO_2^- reduction to NH_4^+ rather than denitrification. All currently available *B. licheniformis* genomes lack a *nirK*- or *nirS*-type NO_2^- reductase and growth experiments with three strains confirmed that nitrogen gasses were not produced in stoichiometric amounts. *B. licheniformis* first reduced NO_3^- to NO_2^- (Figure 2.1, μ1), using the cytoplasmic NarGHI, to support growth accumulating high levels of NO_2^- before subsequently switching to fermentation after NO_3^- got depleted. During fermentation (Figure 2.1, μ2), NH_4^+ was produced from NO_2^-, probably using the NADP-dependent NO_2^- reductase NirBD (also called NasDE) that can serve for both assimilation and dissimilation. For *B. subtilis* it is still undetermined if NO_2^- reduction is coupled to energy production through proton motive force [28]. However, since NO_2^- reduction and production of N_2O continued after growth had ceased, as described before for other non-denitrifying N_2O producers [30, 56, 57], these N conversion seem unrelated to energy conservation. Nevertheless, during fermentative growth, DNRA can serve as an electron-sink allowing re-oxidation of NADH with the generation of one extra ATP by substrate level phosphorylation for each acetate formed [58, 59].

2.4.2 Hypothesis for NO and N_2O formation

To our knowledge, NO or N_2O production and associated cellular mechanisms have never been described for the model organism *B. subtilis*. Therefore, we built on the knowledge from other model organisms to deduce plausible hypotheses to explain our observations (Figure

2.4). NO_2^- conversion to NO in *E. coli* was shown to occur only after NO_3^- was depleted, in presence of molybdate - the cofactor of NarGHI -, continued in *nirB*-mutants [60] but was absent in *narG*-mutants [61], suggesting that the NarGHI had a double function and converted NO_2^- to NO. Also in *Salmonella enterica* serovar *Typhimurium*, NarGHI was unequivocally responsible for NO generation from NO_2^-, which was completely eliminated in a *narGHI* mutant [62]. Later mutagenesis experiments in *E. coli* could not confirm the involvement of the cytoplasmic NO_3^- reductase in NO evolution, probably because the experiments were conducted in the absence of NO_3^- and thus lacked NO_2^- formed from NarGHI activity during growth [63]. Rather NirB and NrfA, besides their primary role converting NO_2^- to NH_4^+, appeared to be involved in NO production [63], with their relative importance dependent on the NO_2^- concentration [64, 65]. But, in contrast to *B. vireti* capable of DNRA in combination with NosZ-mediated N_2O reduction [30] but similar to *B. subtilis*, *B. licheniformis* lacks a *nrfA* gene. Furthermore, the activity of NO synthase (NOS), which produces NO from arginine as a defense mechanism against oxidative stress under aerobic conditions in *B. subtilis* [66], is unlikely under our hypoxic test conditions, making the involvement of NarGHI or NirB in the generation of NO from NO_2^- most plausible (Figure 2.4).

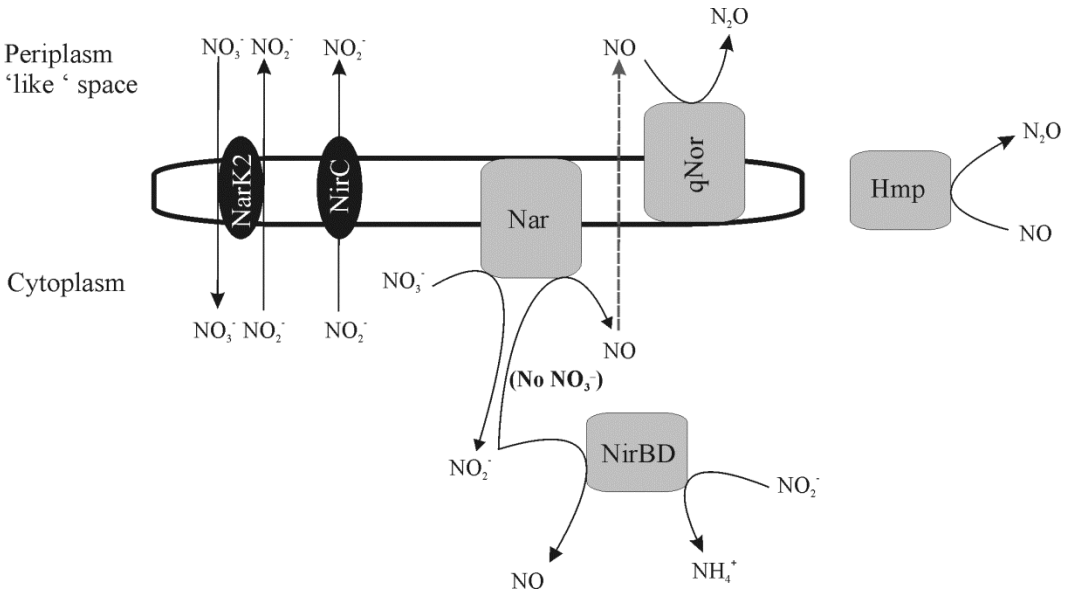

Figure 2.4 Proposed hypothetical pathways for anaerobic nitrogen reduction in *B. licheniformis*. Schematic representations of enzymes are given in grey, transporters in black. Diffusion of gaseous NO through cytoplasmic membrane is indicated by the dashed arrow. Soluble Hmp can be located in the cytoplasm and periplasm.

Next, NO homeostasis is crucial to limit the toxicity of NO, which is a reactive nitrogen species capable of damaging bacterial proteins, lipids and DNA, and binding to metal centers. NO can freely diffuse through the membrane and can be converted to N_2O in the periplasmic-like space by the quinol-dependent NO reductase qNor (Figure 2.4). This reductase is known to be present both in denitrifiers and non-denitrifiers [67-70], including pathogenic bacteria where it is part of their defense mechanism against nitrosative stress. In addition, *B. licheniformis* genomes also encode the flavohemoglobin Hmp. Hmp, found both in cytoplasm and periplasm [71], is known to convert NO to NO_3^- aerobically and to N_2O anaerobically [72, 73]. However, as the latter conversion is at greatly reduced activity [74], it is unsure whether this enzyme is relevant for NO detoxification in *B. licheniformis* with qNorB; indeed *hmp* appeared not to be upregulated in anaerobic conditions at high levels of NO_2^- in *B. vireti* that

contained a copper-dependent NO reductase type 1 [30]. The periplasmic NrfA [75, 76] and the cytoplasmic flavorubredoxin NorV and its associated oxidoreductase NorW [49, 50] that can both anaerobically reduce NO to NH_4^+ and/or N_2O, are not found in *B. licheniformis*. Notably, the gene inventory for anaerobic NO_3^- and NO_2^- metabolism in *B. licheniformis* and *B. subtilis* only seems to differ in the presence of a *qnorB* gene in the former organism, making it likely that *B. subtilis* is capable of NO production. This was hinted at by micromolar range N_2O production by *B. subtilis* 1A01 [77] for which the genome is unfortunately not available. Mutagenic and transcriptomic studies are necessary to confirm our hypothetic pathways for NO and N_2O production in *B. licheniformis*.

2.4.3 N end-products: environmental significance and microdiversity

Non-denitrifying NO_3^- reducers, mostly belonging to *Enterobacteriaceae* or *Bacillaceae*, have been reported to reduce typically about 5 to 10% of NO_3^- to N_2O, with sometimes high quantities up to 35%, which evolved mostly after growth has ceased [30, 56, 57, 65, 77]. For *B. licheniformis*, measured N_2O production from NO_3^- was within these ranges but was nevertheless quite substantial, with up to one-third of all NO_3^- converted to N_2O (Figure 2.2). How environmentally relevant these N_2O emissions from non-denitrifiers are remains difficult to establish without ways to differentiate them from denitrification; in isotope pairing experiments, non-denitrifiers will also produce ^{15}N-N_2O. In addition, even with mechanistic understanding, deducing specific target genes for molecular surveys will be nearly impossible, as different mechanisms have already been described for a single microorganisms like *E. coli* [78], *S. thyphimurium* [62] and *B. vireti* [30] and the genes involved have dual functions, e.g. NarG, NirB and NrfA.

Despite high N_2O emission, most NO_2^- was indeed converted to NH_4^+ (12.2 – 51.0%), which is in agreement with previous observations for pure cultures under NO_3^- limitation (valid for both growth conditions applied here as growth continued via fermentation after NO_3^-

depletion) [30, 56, 77]. Interestingly, the ratio of end-products from NO_3^- varied quite substantially between all three *B. licheniformis* strains. However, as differences were also apparent between LMG 6934 and LMG 7559, this phenotypic heterogeneity could not be linked the specific gene duplications in BALI2 genomes. An alternative explanation might be distinct regulatory motifs in the promotor regions of the genes involved, although the same regulatory genes were encoded in the three genomes with identical relative genome locations (Table 2.2). It is long been accepted that closely related bacteria do not necessarily share the capacity to denitrify, and even when they do, can have different denitrifying phenotypes. However, our data suggest that phenotypic heterogeneity or niche differentiation between closely related strains, which has recently been reported for N_2O production in *Bacillus* [79] , *Thauera* [80] and *Methylomonas* [81], might not always be linked to genetic variation.

2.5 Conclusions

Using physiological and genomic data we have demonstrated that the common soil bacterium *B. licheniformis* does not denitrify but is capable of fermentative dissimilatory NO_3^-/ NO_2^- reduction to NH_4^+ with concomitant production of N_2O. Based on the genomic inventory, alternative routes for N_2O production, similar to those in *Enterobacteriaceae* and thus far unreported in bacilli, were proposed. Significant strain-dependent differences were found between three closely related strains that could not be linked to genetic features. Considering its ubiquitous nature and non-fastidious growth in the lab, *B. licheniformis* is a suitable candidate for further exploration of the uncertainty of the mechanism of N_2O production in DNRA bacteria and its relevance *in situ*.

PS: After publication of this work, BALI2 strains were reclassified as *Bacillus paralicheniformis* by Dunap et al in 2015 [82], and therefore LMG 6934 and LMG 7559 are now renamed as *Bacillus paralicheniformis*, while LMG 17339 remains *Bacillus licheniformis*. But this does not interfere with our interpretation above.

2.6 Acknowledgement

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2.7 Supplementary information

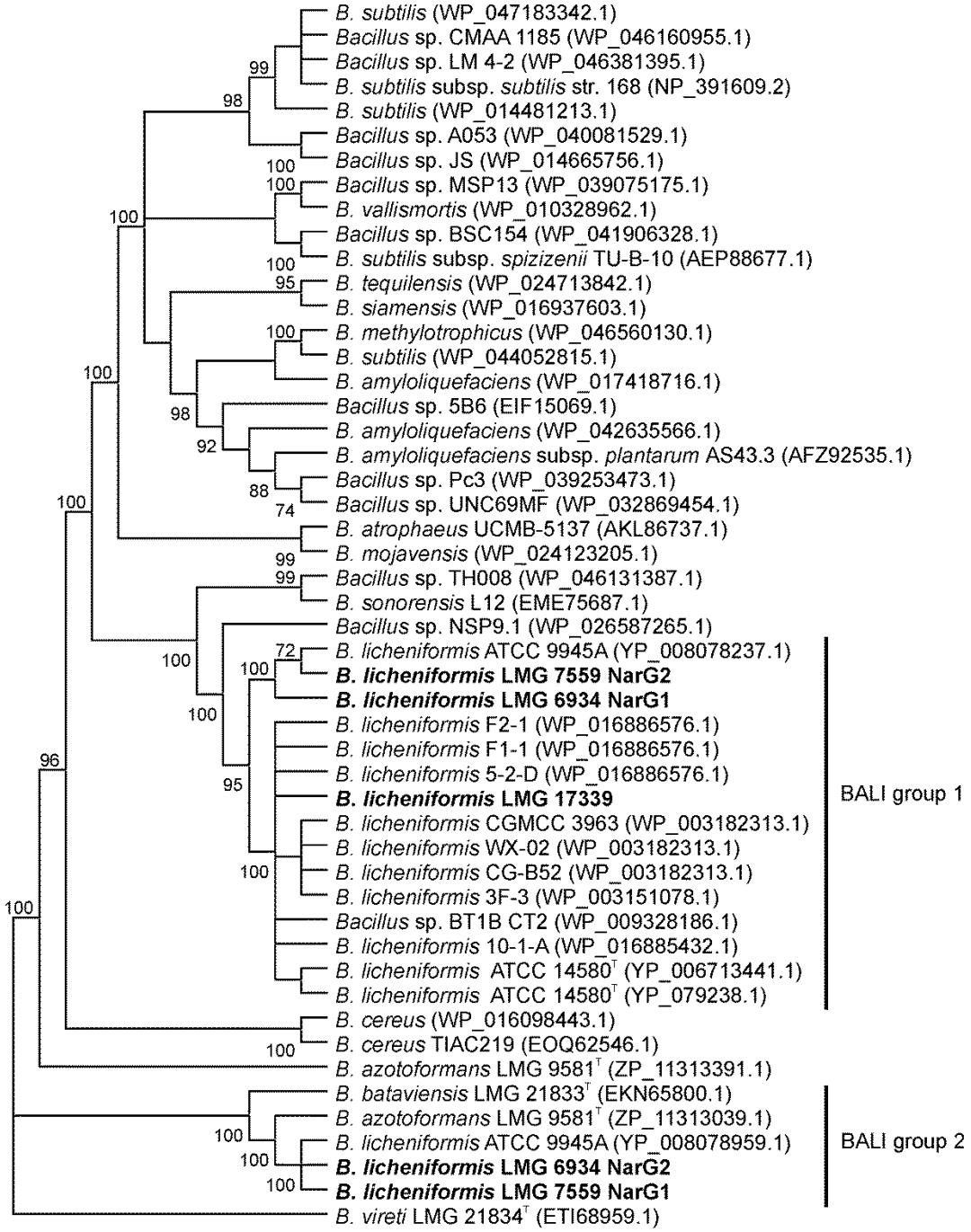

Figure S1 Phylogenetic tree of full-length NarG. Sequences were taken from genomes included in the manuscript as well as all publically available *B. licheniformis* genomes (dd June 2014) and genomes from other *Bacillus* species, protein ID or locus tag is given between brackets. The evolutionary history was inferred using the Neighbor-Joining method [83]. The optimal tree with the sum of branch length = 1,68972248 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches [84]. The evolutionary distances were computed using the Poisson correction method [85] and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 1094 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [86].

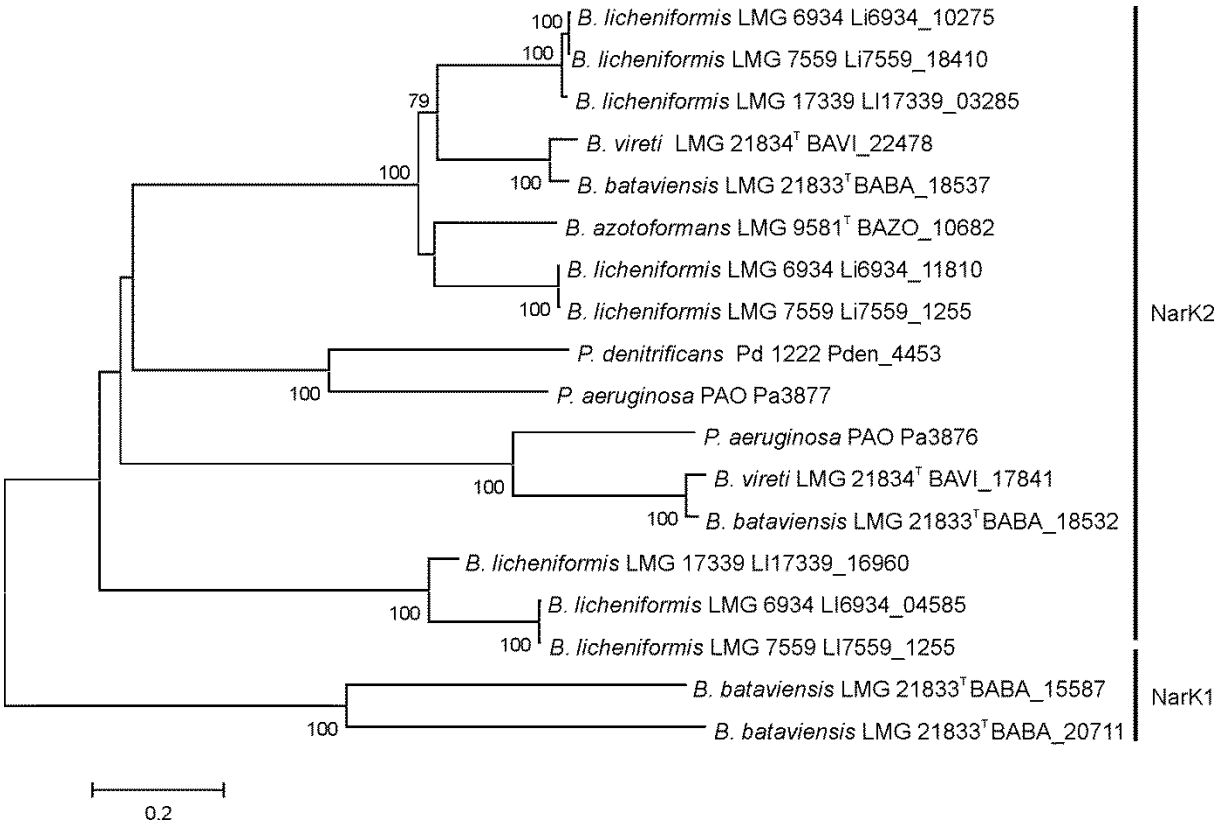

Figure S2 Phylogenetic tree of full-length NarK. Sequences were taken from several *Bacillus* species, as well as reference genomes, locus tag is given between brackets. The evolutionary history was inferred using the Neighbor-Joining method [83]. The optimal tree with the sum of branch length = 6,03046656 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches [84]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method [85] and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 358 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [86].

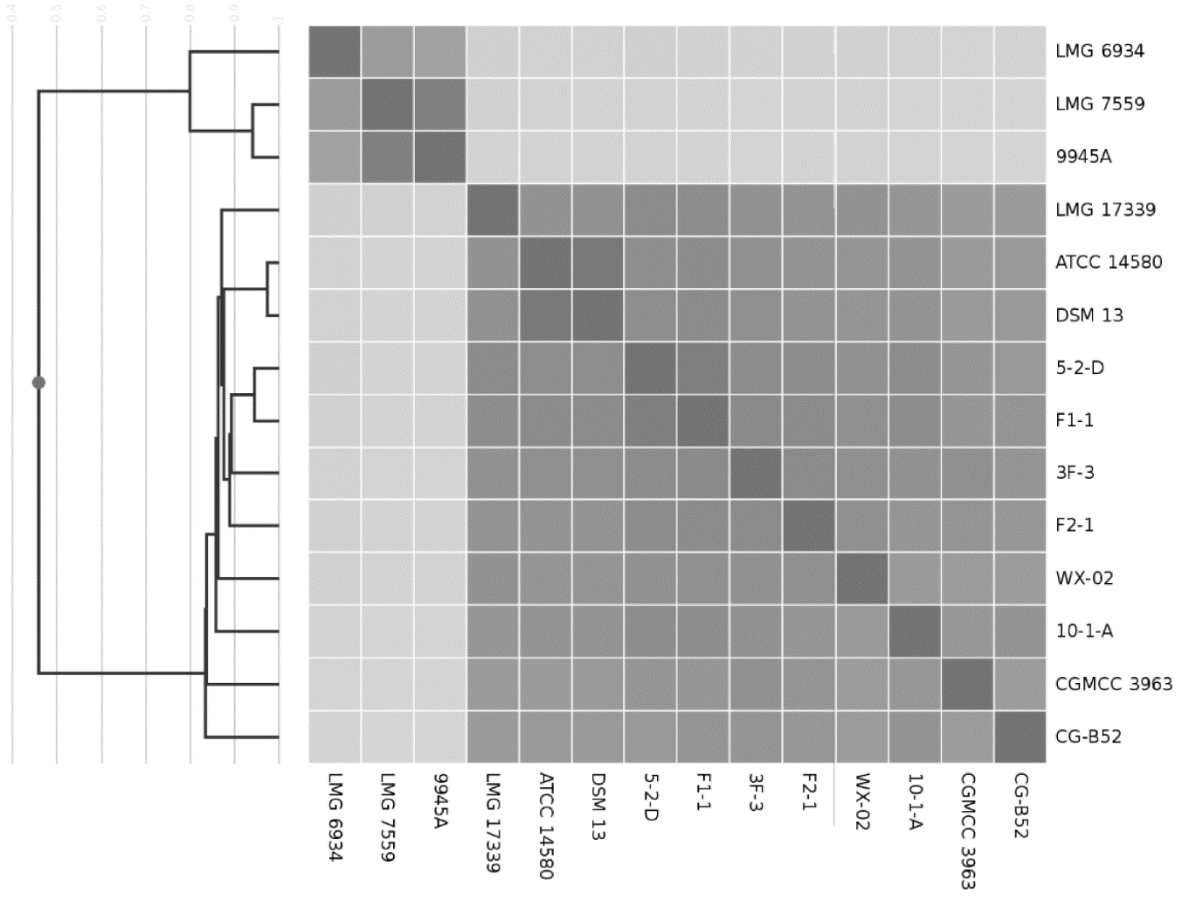

Figure S3 Whole genome clustering based on a similarity matrix using the peptidome content, in which amino acid sequences of a genome are converted to tryptic peptides, i.e. the tryptic peptidome. All publically available *B. licheniformis* genomes (dd June 2014) were included and are designated by their strain number: 9945A (accession number NC_021362) [33], ATCC 14580 (accession number NC_006270) [34], DSM 13 (=ATCC 14580; accession number NC_006322) [35], 5-2-D (accession number NZ_AJLW01000000) [87], F1-1 (accession number NZ_AZSL01000000) [31], 3F-3 (accession number NZ_JFYM01000000) [87], F2-1 (accession number NZ_AZSM01000000) [31], WX-02 (accession number NZ_JH636050) [88], 10-1-A (accession number NZ_AJLV01000001) [32], CGMCC 3963 (accession number NZ_AMWQ01000000) [36], CG-B52 (accession number NZ_AVEZ01000000). Analyses were performed using the Peptidome tool (http://unipept.ugent.be/) [51].

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Chapter 3:

Influence of nitrate and nitrite concentration on N_2O production via DNRA in *Bacillus licheniformis* LMG 6934

Redrafted from:

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Authors' contribution:

YH performed experiments, analyzed data and wrote the paper; KH conceived the study, analyzed data and wrote the paper. PDV revised the manuscript.

Summary

Until now, the exact mechanisms for N_2O production in DNRA remain underexplored. Following our observation in Chapter 2 that N_2O production may be correlated to high residual NO_2^-, we further studied the influence of different physico-chemical factors on NO_3^- partitioning and N_2O production in DNRA to shed light on the possible mechanisms of N_2O production. The effects of NO_3^- concentrations under variable or fixed C/N-NO_3^- ratios, NO_2^- concentrations under variable C/N-NO_3^- ratios and NH_4^+ concentrations under fixed C/N-NO_3^- ratios were tested during anaerobic incubation of *B. paralicheniformis* LMG 6934 (previously known as *B. licheniform*is). End-point measurements of growth, NO_3^-, NO_2^-, NH_4^+ concentration and N_2O production revealed that NO_3^- as well as NO_2^- concentration had a linear correlation with N_2O production which is a direct result of NO_2^- detoxification. Increased NO_3^- concentration under fixed C/N-NO_3^- ratios, NO_2^- concentration and NH_4^+ concentration had a significant positive effect on NO_3^- partitioning ([N-NH_4^+]/[N-N_2O]) towards N_2O, which may be a consequence of the (transient) accumulation of NO_2^-.

3.1 Introduction

Nowadays, there is an increasing concern about the year-by-year rising emissions of the N_2O production from soil, since it is a potent greenhouse gas and ozone layer destructor [1-3]. Field surveys [4-6] and research with pure cultures [7-11] suggested that NO_3^--ammonifying bacteria could be a significant source of N_2O in soil. NO_3^- ammonification or dissimilatory NO_3^- reduction to NH_4^+ (DNRA) is the reduction of NO_3^- to NH_4^+, via NO_2^- [12-14], with non-stoichiometric amounts of N_2O produced concomitantly, around 3-36 % of consumed NO_3^- [7, 8, 11]. DNRA can follow different scenarios, with respiratory membrane-bound NarG, cytoplasmic NasBC or periplasmic NO_3^- reductase NapA for NO_3^- reduction for NO_3^- reduction to NO_2^-, followed by NO_2^- reduction to NH_4^+ via cytoplasmic nitrite reductase NirB or a periplasmic nitrite reductase NrfA [15]. The exact mechanisms for N_2O production remain underexplored, might differ between ammonifiers and most likely depend on the enzymes involved in the DNRA process. Based on current insights, several mechanisms of the N_2O production are possible, with firstly NO_2^- reduction to NO, involving Nar [16-18] or NirB or NrfA [19], with NirB induced under high NO_3^- conditions and NrfA induced under low NO_3^- conditions [20, 21]. The produced NO can be subsequently converted to N_2O via dedicated NO reductases [22-25], flavohemoglobin Hmp [26, 27], periplasmic NrfA [13, 28] or cytoplasmic flavorubredoxin NorV together with its associated oxidoreductase NorW [29, 30].

It is well known that DNRA is favored over denitrification at higher C/N-NO_3^- ratios or NO_3^- limitation [31, 32], higher pH [31, 33], higher temperature [34, 35], certain NO_2^-/NO_3^- ratios [33, 34], etc. However, the influence of these environmental drivers on NO_3^- partitioning to NH_4^+ and N_2O in DNRA remains underexplored and might help unravel the underlying mechanisms and regulation of N_2O production accordingly. Early work by Smith showed that higher C/ NO_3^- ratios favored NO_3^- partitioning to N_2O in *Citrobacter* sp. with glucose as

energy source and constant NO_3^- concentration [16] and suggested that N_2O production was induced by (transient) accumulation of NO_2^-. However, recently it was found, both in batch and continuous incubation of *Citrobacter* sp. and *Bacillus* sp. that low C/N-NO_3^- (C limitation, N sufficiency) ratios resulted in higher NO_2^- accumulation accompanied by higher N_2O production compared to high C/N-NO_3^- but with constant glycerol as carbon source and variable NO_3^- concentration [9].

It has been generally known that NH_4^+ inhibits assimilatory NO_3^- reduction (general N control) [36, 37], but does not repress dissimilatory NO_3^- reduction [38]. In *B. licheniformis*, NO_3^- reductase activity increased with initial concentrations of NH_4^+, but with an upper limit, suggesting that the activity is not for NO_3^- assimilation but for other physiological functions containing a dissimilatory NO_3^- reduction [38]. In our previous study, strains of *B. licheniform*is and *B. paralicheniformis* (previously known as *B. licheniform*is) possessing assimilatory NO_3^-/ NO_2^- reduction genes (*nar, nasC*, *nirBD*) proved to be non-denitrifying nitrous oxide emitters performing DNRA [11]. Following our observation of N_2O production being correlated to high residual NO_2^- [11], we here continued our study on DNRA with soil bacterium *B. paralicheniformis* LMG 6934, selected for its high nitrite tolerance and efficient nitrite reduction ability, to study in detail the influence of NO_3^-, NO_2^- and NH_4^+ concentrations on N_2O produced via DNRA.

3.2 Materials and methods

3.2.1 Strains

B. paralicheniformis LMG 6934 was obtained from the BCCM/LMG bacteria collection. It was grown aerobically at 37°C on TSA for two days, followed by two subcultivations on TSA before use in growth experiments in mineral media.

3.2.2 Growth experiments

Anaerobic growth experiments were performed in mineral medium (containing 4.6 mM NH_4^+) supplemented with 10 mM potassium NO_3^- as electron acceptor, 30 mM glucose as electron donor unless stated otherwise. Mineral medium was as described by Stanier *et al* [39], including 10 mM phosphate buffer (pH 6.92 ± 0.05), 2.3 mM $(NH_4)_2SO_4$, 0.4 mM $MgSO_4.7H_2O$, 0.04 mM $CaCl_2.2H_2O$, 27 µM EDTA, 25 µM $FeSO_4•7H_2O$, 10 µM $ZnSO_4•7H_2O$, 25 µM $MnSO_4•H_2O$, 3.8 µM $CuSO_4•5H_2O$, 2 µM $Co(NO_3)_2•6H_2O$, 0.196 µM $(NH_4)_6Mo_7O_{24}•24H_2O$, supplemented with 30 mM glucose as electron donor. Serum vials (120 ml) were rinsed with 1 M HCl overnight to remove growth inhibiting substances, and subsequently washed four times and rinsed with distilled water before use. Serum vials with 50 ml medium were sealed with black butyl-rubber stoppers. After autoclaving, the headspace of the serum vials was replaced via five cycles of evacuating and refilling with helium. Serum vials were inoculated (1% v/v) with a suspension of optical density OD_{600} of 1.0 ± 0.05. Each growth experiment was performed in triplicate and non-inoculated media in duplicate were included to check for potential nitrosation reactions in sterile medium. After inoculation, serum vials were incubated at 37 °C, 150 rpm, for 72h.

3.2.3 Environmental drivers

Mineral media with different supplements were designed and tested to study the effect of different environmental factors on NO_3^- partitioning to NH_4^+ and N_2O: different NO_3^- concentrations (5 mM, 10 mM, 15 mM) and 30 mM glucose resulting in variable C/N-NO_3^-

ratios of 36, 18 and 12; different NO_3^- concentrations (5 mM, 10 mM, 15 mM) under identical C/N-NO_3^- ratio of 12 (glucose 10 mM, 20 mM, 30 mM respectively); different NO_2^- concentrations (1 mM, 5 mM, 10 mM) and 30 mM glucose resulting in variable C/N-]NO_3^- ratios of 180, 36, 18; different NH_4^+ concentrations (0 mM, 1mM, 4.6 mM, 10mM) and 10 mM NO_3^-, 30 mM glucose resulting a fixed C/N-NO_3^- ratios of 18.

3.2.4 Analytical procedures

Samples of 1 ml were taken from cultures through the rubber septum of serum vials with sterile syringes for growth determination and colorimetric determination of NH_4^+, NO_3^- and NO_2^-. Growth was determined by measuring the optical density OD_{600} of 100 µl sample in duplicate in microtiter plates and standardized to 1 cm path length using PathCheck Sensor of the spectrophotometer (Molecular Devices, Spectramax plus 384, USA). Samples left were centrifuged at 17949 g rpm for 2 min to remove the cells and kept frozen at -20°C until colorimetric determination. NH_4^+ concentration was determined with the salicylate-nitroprussidine method (absorption at a wavelength of 650 nm) [40] [41], NO_2^- and NO_3^- concentrations were determined with Griess reaction [42] and Griess reaction with cadmium [43, 44] respectively. For end-point measurements, NH_4^+ production was corrected per strain for the amount of NH_4^+ assimilated based on OD_{600} values obtained. Standard curves covered ranges suitable for the tested media and were strictly linear with an R_2 of 0.99. For determination of N_2O, 1 ml sample of the headspace of serum vials was taken with sterile syringes, and was injected into the gas chromatograph (Compact GC with EZChrom Elite Software, Interscience, Netherlands, 2012). N_2O concentrations were corrected for pressure and solubility based on Henry's law.

Statistical differences of end-product concentration (OD_{600}, NO_3^-/NO_2^-/NH_4^+ concentration, N_2O production) and ratios of N-NH_4^+ production to N-N_2O production (indicating NO_3^- partitioning to NH_4^+ and N_2O) in the tests of different environmental drivers were processed

using factorial ANOVA and Least Significant Difference post-hoc testing in IBM SPSS23 or nonparametric tests-Kruskal-Wallis H testing accordingly.

3.3 Results and Discussion

3.3.1 NO_2^- reduction ability

Already three decades ago, it was suggested that N_2O production during DNRA originates from detoxification of accumulated NO_2^- [8, 17]. Our previous study demonstrated that LMG 6934 had a high nitrite tolerance and could efficiently perform DNRA [11]. Here, NO_2^- reduction was anaerobically tested at concentrations of 1 mM, 5 mM, 10 mM under variable C/N-NO_2^- ratios of 180, 36 and 18. After 72h incubation, growth was observed under all NO_2^- concentrations tested, with all NO_2^- converted to NH_4^+ or N_2O, thus confirming its high tolerance to NO_2^- (Table 3.1; Figure 3.1). Indeed, compared with other DNRA strains [11] such as *Bacillus* sp. and *Citrobacter* sp. [9], *B. licheniformis* [38] and *Pseudomonas stutzeri* D6 [45], LMG 6934 showed a high NO_2^- reduction ability, with up to initial 10 mM NO_2^- consumed, up to 15 mM NO_3^- converted to NH_4^+ and N_2O with none NO_2^- left in the end. In the study of *E. coli* [20], NirB was demonstrated to be induced by NO_2^- and is optimally synthesised when the amount of NO_3^- or NO_2^- excess the consumption capacity of the cells. Therefore, the vigorous high NO_2^- reduction ability observed in our tests might mainly be due to the functioning of NirB and the elevated NirB by the induction of high NO_3^- or NO_2^- induction concentration also explained the relatively low amount of NO_3- partitioned to N_2O (<15%) compared to other strains [11, 21].

3.3.2 Influence of NO_3^- and NO_2^- concentration on N_2O production

Anaerobic growth experiments with 5, 10 and 15 mM NO_3^- under variable C/N-NO_3^- ratios of 36, 18 and 12 after 72 h incubation revealed that NO_3^- or NO_2^- were completely converted to N_2O or NH_4^+ as product, without any residual NO_2^- left for all conditions tested. Growth ceased due to NO_3^- limitation. Growth (OD_{600}), consumption of NO_3^-, production of NO_2^- and NH_4^+ were summarized in Table 3.1. Percentages of NO_3^- recovery as NO_2^- or N_2O or NH_4^+

under different NO_3^- concentration are shown in Figure 3.1. Percentage of NO_3^- recovery as N_2O and growth (OD_{600}) under 10 mM NO_3^- condition agreed with previous observations [11]. Under variable C/N-NO_3^- ratios of 36, 18 and 12, NO_3^- concentration had an influence on N_2O production (p=0.0018) and NH_4^+ production (p=0.000027), with higher NO_3^- concentration leading to production of more NH_4^+ and more N_2O (Table 3.1; Figure 3.2). However, growth did not significantly increase with NO_3^- concentration (p= 0.287), and different NO_3^- concentrations had no significant influence on NO_3^- partitioning ([N-NH_4^+]/ [N-N_2O]) (p= 0.417) (Figure 3.3). As mentioned above, higher C/N-NO_3^- ratios with constant NO_3^- concentration were shown to favor NO_3^- partitioning to N_2O [16]. Therefore we speculated that the NO_3^- concentration probably also favored NO_3^- partitioning to N_2O, and higher NO_3^- concentration combined with lower C/N-NO_3^- ratios in our test concealed their influence in the observations.

To exclude the influence of C/N-NO_3^- ratio, which might be strain-dependent [9], in the test above, the same experiment was repeated under fixed C/N-NO_3^- ratio of 12. Again, after 72 h anaerobic incubation, all NO_3^- or NO_2^- were completely converted to N_2O or NH_4^+ without any residual NO_2^- left for all conditions tested. Surprisingly, NO_3^- concentration showed a positive effect on growth (p = 0.000128), in addition to NH_4^+ production (p = 0.000101) and N_2O production (p = 4.95×10^{-9}). Increase of NO_3^- under fixed C/N-NO_3^- ratio increased NH_4^+ and N_2O produced, as well as cell density (Table 3.1; Figure 3.2). In addition, increased NO_3^- concentration from 5 to 10 mM promoted NO_3^- partitioning to N_2O and negatively impacted NH_4^+ production (p= 0.008) (Figure 3.3), but this effect leveled off at 15 mM NO_3^- (p = 0.155).

In contrast to NO_3^- under variable C/N-NO_2^- ratios, NO_2^- concentration under variable C/N-NO_2^- ratios did show a positive effect on growth (p = 0.000017), NH_4^+ production (p = 0.027) and N_2O production (p = 0.034). As expected, with more NO_2^- consumed in the media, more

NH_4^+ and N_2O were produced, resulting in more growth (Table 3.1; Figure 3.2). In addition, NO_2^- concentration had a significant influence on NO_2^- partitioning to NH_4^+ and N_2O but the significance was only shown between 1 mM and 10 mM NO_2^- (p = 0.00028) (Figure 3.3), which is also the case for N_2O production (Table 3.1).

A linear but non-stoichiometric correlation was observed between NO_3^- or NO_2^- concentration and N_2O production (Fig 3.2). In addition, NO_3^- concentration under fixed C/N-NO_3^- ratio but not under variable C/N-NO_3^- ratios, and NO_2^- concentration under variable C/N-NO_2^- ratios significantly influenced NO_3^- partitioning to NH_4^+ and N_2O in *B. paralicheniformis* LMG 6934. This might result from that combined effect of C/N-NO_3^- ratio and NO_3^- concentration in the NO_3^- concentration tests, while NO_2^- might have a direct effect, probably by action of NirB. Higher NO_3^- concentration under fixed C/N-NO_3^- ratio promotes NO_3^- partitioning to N_2O, this agrees with physiological data of a previous study [16], and makes sense as higher NO_3^- concentration, more NO_2^- transiently accumulates and therefore needs to be detoxified, leading to a higher proportion of nitrate to N_2O.

Table 3.1 Overview of growth (OD_{600}), electron acceptors (NO_3^- or NO_2^-) consumption and NH_4^+ production (Measured concentrations of NH_4^+ corrected with loss through assimilation), N_2O production of *B. paralicheniformis* LMG 6934 after 72h incubation under different media composition. All NO_3^- added was consumed by the end of the experiment. Standard deviations are given between brackets (n=3 if not stated otherwise). Statistics were determined via one-way ANOVA or nonparametric tests accordingly. Significant differences ($p < 0.05$) of each parameter (OD_{600}, NO_3^- or NO_2^- consumption, NH_4^+, N_2O production) within the same experiment (four experiments: (i) NO_3^- concentration test under variable C/N- NO_3^- ratio, (ii) NO_3^- concentration test under fixed C/N- NO_3^- ratio, (iii) NO_2^- concentration test, (iv) NH_4^+ concentration test (with initial 10 mM NO_3^-)) are displayed as different lowercase letters (combined lower letters are used to indicate non-significance for multiple variables). Significant differences of each parameter between four different experiments when 5 mM NO_3^-/NO_2^- or 10 mM NO_3^-/NO_2^- supplied are displayed as capital letters.

Media	C/N-NO_x^-	OD_{600}	Concentration (mM)		
			NO_3^- or NO_2^- consumption	NH_4^+ production	N_2O production
5 mM NO_3^-	36	0.60 ^{aA} (0.10)	5.23 ^{aA} (0.15)	4.80 ^{aA} (0.27)	0.33 ^{aA} (0.12) *
10 mM NO_3^- #	18	0.71 ^{aAB} (0.20)	9.87 ^{bA} (0.43)	8.69 A^{b} (0.36)	0.59 ^{bA} (0.03)
15 mM NO_3^- ##	12	0.76 ^{a} (0.09)	14.67 ^{c} (1.13)	12.94 ^{c} (1.15)	0.87 ^{c} (0.02)
5 mM NO_3^-	12	0.22 ^{aB} (0.03)	4.91 ^{aA} (0.21)	4.50 ^{aA} (0.23)	0.20 ^{aA} (0.01)
10 mM NO_3^-	12	0.50 ^{bA} (0.05)	9.55 ^{bA} (1.13)	8.57 ^{bA} (1.11)	0.49 ^{bB} (0.01)
15 mM NO_3^- ##	12	0.76 ^{c} (0.09)	14.67 ^{c} (1.13)	12.94 ^{c} (1.15)	0.87 ^{c} (0.02)
1 mM NO_2^-	180	0.35^{a} (0.02)	1.17 ^{a} (0.01)	1.17 ^{a} (0.01)	0 ^{a} (0.00)
5 mM NO_2^-	36	0.51^{bA}(0.02)	6.19 ^{bB} (0.17)	5.71 ^{bB} (0.15)	0.19^{abA} (0.16)
10 mM NO_2^-	18	0.66^{cA} (0.03)	13.76 ^{cB} (0.97)	12.99 ^{cB} (0.99)	0.39^{bC} (0.01)
0 mM NH_4^+	18	0.67 $^{a\ AB}$ (0.08)	10.32 ^{aAB} (1.34)	9.16 ^{aA} (1.26)	0.58 ^{aA} (0.04)
1 mM NH_4^+	18	0.82 ^{aB} (0.02)	10.95 ^{aAB} (0.18)	9.71 ^{aA} (0.20)	0.62 ^{aA} (0.02)
4.6mM NH_4^+ #	18	0.71 $^{a\ AB}$ (0.20)	9.87 ^{aA} (0.43)	8.69 ^{aA} (0.36)	0.59 ^{aA} (0.03)
10 mM NH_4^+	18	0.87 ^{aB} (0.03)	8.99 ^{aA} (0.99)	7.68 ^{aA} (0.91)	0.65 ^{aA} (0.04)

* n=2;

or ## indicates data from the same test analyzed twice in different experiment interpretation.

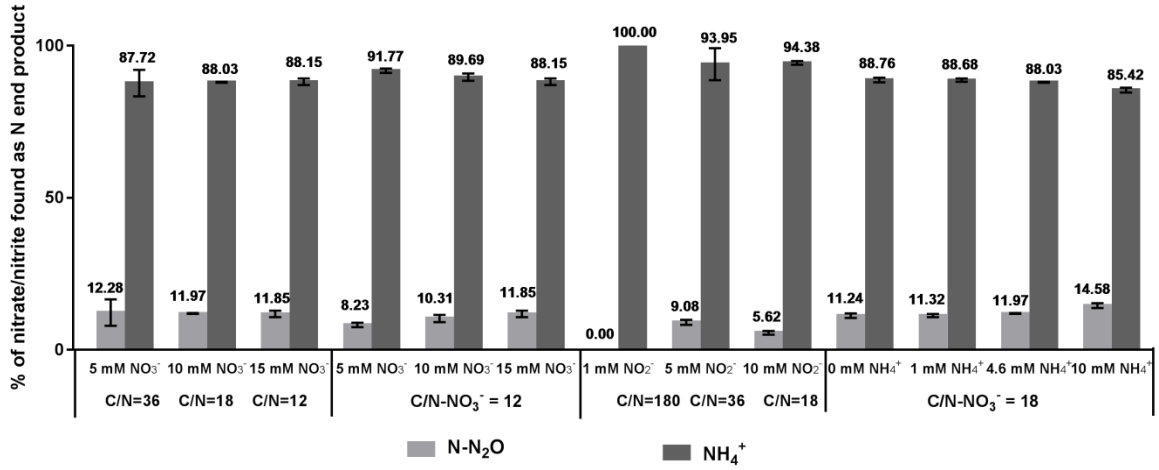

Figure 3.1 Percentages of end-products of anaerobic NO_3^-/ NO_2^- reduction by *B. paralicheniformis* LMG 6934 in mineral medium with increasing NO_3^- concentration under variable C/N-NO_3^- ratio (n=2 for C/N ratio of 36); with increasing NO_3^- concentration under fixed C/N-NO_3^- ratio of 12 (for 15 mM NO_3^-, it is the same experiment as above, same data used twice for analysis); with NO_2^- concentration under variable C/N-NO_3^- ratio; NH_4^+ concentration under fixed C/N-NO_3^- ratio of 18. Error bars represent standard deviation (n = 3 if not stated otherwise). Measured concentrations of NH_4^+ were corrected for loss through assimilation.

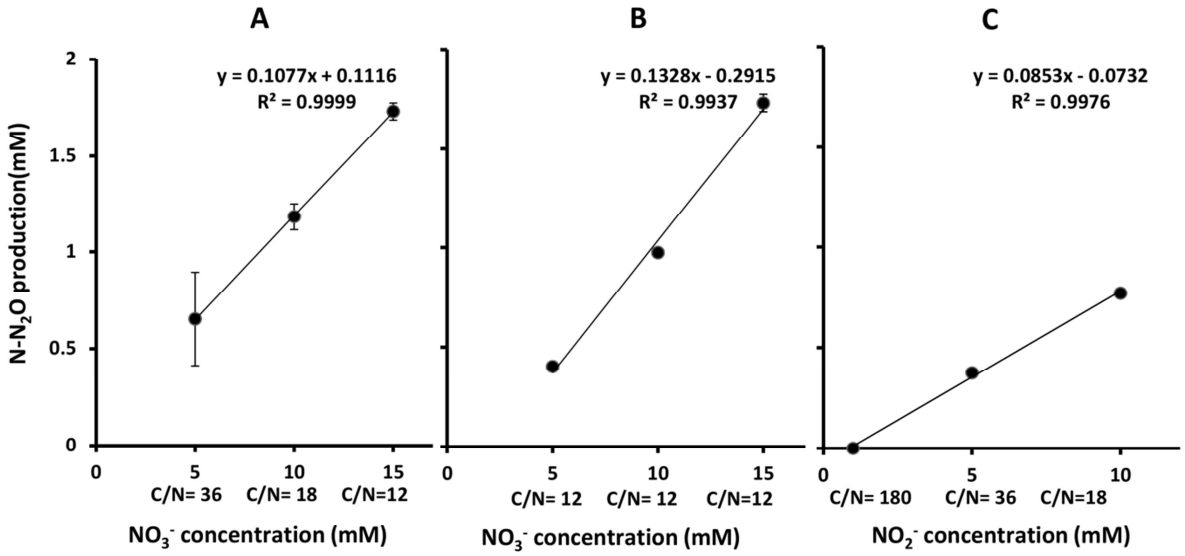

Figure 3.2 N_2O production by *B. paralicheniformis* LMG 6934 in mineral medium with: (A) increased NO_3^- concentration under variable C/N-NO_3^- ratio of 36 (n=2), 18, 12; (B) increased NO_3^- concentration under fixed C/N-NO_3^- ratio of 12; (C) increased NO_2^- concentration under variable C/N-NO_3^- ratio of 180, 36, 18. Error bars represent standard deviation (n = 3 if not stated otherwise). Trend line equations and R-squared value are given.

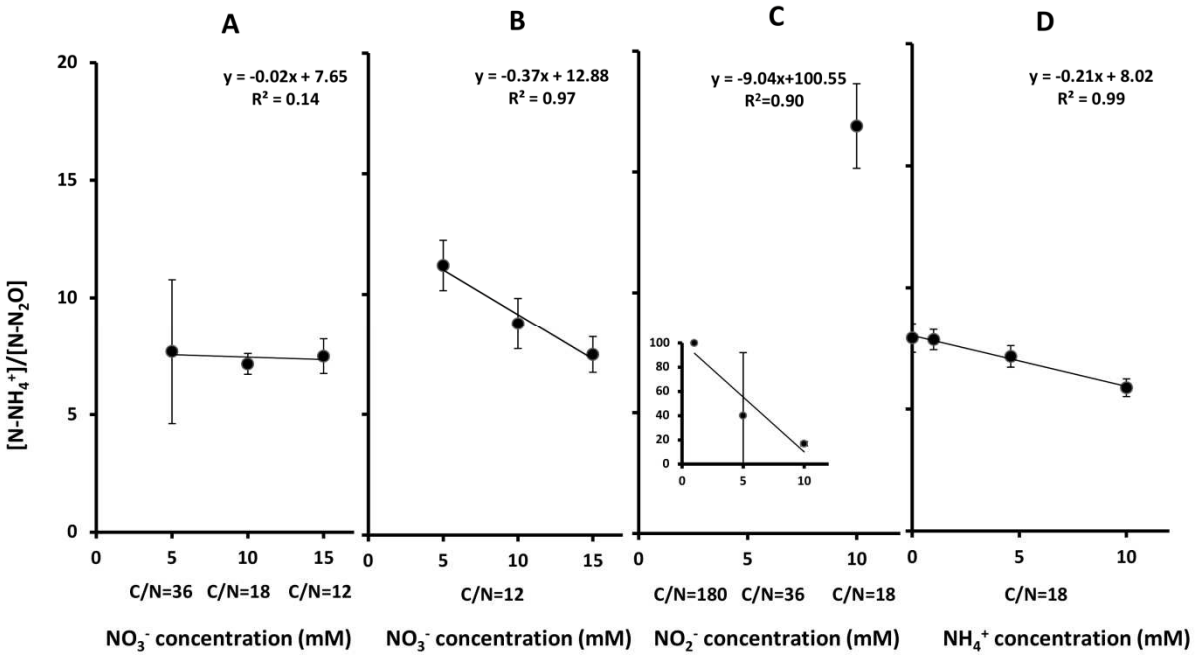

Figure 3.3 Ratios of N-NH_4^+ production to N-N_2O production by *B. paralicheniformis* LMG 6934 in mineral medium with: (A) increased NO_3^- concentration under variable C/N- NO_3^- ratio of 36 (n=2), 18, 12; (B) increased NO_3^- concentration under fixed C/N-NO_3^- ratio of 12; (C) increased NO_2^- concentration under variable C/N-NO_3^- ratio of 180, 36 , 18; (D) increased NH_4^+ concentration under fixed C/N- NO_3^- ratio of 18. Error bars represent standard deviation (n = 3 if not stated otherwise). The inserted figure in panel C is the complete figure of this NO_2^- concentration test with a [N-NH_4^+]/[N-N_2O] range from 0 to 100. Trend line equations and R-squared value are given.

3.3.3 Influence of NH_4^+ concentration on N_2O production

As mentioned above, NH_4^+ can repress assimilatory nitrate causing NO_2^- to accumulate but cannot inhibit nitrate reduction for dissimilation. In our tests, with 10 mM NO_3^- under fixed C/N-NO_3^- ratio of 18, the ammonium concentration varied over 0, 1, 4.6 (standard) and 10 mM. After 72 h incubation, growth was obtained under all NH_4^+ concentrations, even without NH_4^+ added (Table 3.1; Figure 3.1). All NO_3^- converted to NH_4^+ or N_2O, with some samples reaching up to appr. 10 mM NH_4^+ produced (Table 3.1). Unexpectedly, there is no statistically significant effect of NH_4^+ concentration on either growth (p = 0.12) or NH_4^+ production (p = 0. 12) or N_2O production (p = 0.11), again confirming that LMG 6934 is a vigorous ammonifier able to produce and take up sufficient NH_4^+ for growth. However, there is a significant effect of NH_4^+ on NO_3^- partitioning to N_2O but only in medium with the highest NH_4^+ concentration

(10 mM) compared with media with lower NH_4^+ concentration (p = 0. 000932). However this observation requires further confirmation with higher NH_4^+ concentrations.

Thus, the anaerobic growth was not repressed by NH_4^+ (as high as 18.47 ± 0.10 mM measured after incubation, with 10mM initial NH_4^+ and 8.47 mM produced from NO_3^-) (data not shown), which is in agreement with previous studies on *Bacillus* sp. and *Citrobacter* sp. [7]. Almost no difference in growth was obtained under different NH_4^+ concentrations. Similar observations were described with *B. licheniformis* No. 40-2, a strain isolated from hot spring but under aerobic conditions [38].

Here, we demonstrated that indeed NO_3^- as well as NO_2^- concentration shows a linear correlation with N_2O production and increased concentrations lead to more partitioning to N_2O which is a direct result of NO_2^- detoxification. Furthermore, high NH_4^+ concentration also leads to more NO_3^- partitioning to N_2O. These observations were obtained with high concentrations of NO_3^- or NO_2^-, however, they are still relevant as comparable concentration can exist in the environment [46, 47], for example during fertilization event of agricultural land [48]. We realize that the N_2O production during ammonification might be considered negligible compared to that during canonical denitrification, especially when considering LMG 6934 is highly tolerant to NO_2^-. Nevertheless, ammonifiers are widely distributed in the environment and DNRA is considered the preferred NO_3^- reduction process in agricultural soils as it retains N in the system [49]. Therefore, future N_2O mitigation strategies promoting DNRA need to consider the potential concomitant N_2O production.

3.4 Acknowledgement

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Chapter 4:

Nitrogen assimilation in denitrifier *Bacillus azotoformans* LMG 9581^T

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YH performed experiments, analyzed data and wrote the paper; KH conceived the study, analyzed data and wrote the paper. RVS and PDV helped with interpretation of data and revised the paper.

Summary

Genome analysis of *Bacillus azotoformans* LMG 9581^T showed a remarkable redundancy of dissimilatory nitrogen reduction, with multiple copies of each denitrification gene as well as DNRA genes *nrfAH*, but demonstrated a reduced capacity for nitrogen assimilation, with no *nas* operon nor *amtB* gene. Here, nitrogen assimilation was explored using growth experiments in media with different organic and inorganic nitrogen sources at different concentrations. Monitoring of growth, NO_3^- NO_2^-, NH_4^+ concentration and N_2O production revealed that *B. azotoformans* LMG 9581^T could not grow with NH_4^+ as sole nitrogen source. NH_4^+ could however be assimilated and contributed up to 50% of biomass but only if yeast extract was also provided. NH_4^+ also had a significant but concentration-dependent influence on growth rate. The mechanisms behind these observations remain to be resolved but hypotheses for this deficiency in nitrogen assimilation are discussed.

4.1 Introduction

Nitrogen, an essential element for synthesis of nucleic acids and proteins, is important for all forms of life [1]. Microorganisms can take up both inorganic nitrogen such as NH_4^+ and NO_3^- and organic nitrogen. However, NH_4^+ is the key compound involved in nitrogen assimilation and is the preferred sole nitrogen source over amino acids or more complex organic nitrogen compounds for most microorganisms in culture media or in soil [2, 3]. NH_4^+ can be taken up from the environments [4-6] or be self-produced by NO_3^- or NO_2^- dissimilation processes or breakdown of nitrogen-containing organic compounds.

As mentioned earlier (Chapter 1), microorganisms can demonstrate different strategies for NH_4^+ uptake, either by diffusion of NH_3 into the cell at alkaline pH and high NH_4^+/NH_3 concentration, or by active transport through the membrane via its transporter AmtB at low pH and low NH_4^+/NH_3 concentration. AmtB works together with GlnK, which modulates the NH_4^+ uptake by AmtB at low NH_4^+ concentration [7, 8]. While at high NH_4^+ concentration, AmtB binds to GlnK and is not functional for the NH_4^+ uptake [9]. This *glnKamtB* operon is well conserved in prokaryotes, except pathogenic bacteria such as *Borrelia burgorferi* and *Mycoplasma genitalium* [10] which probably rely on their hosts for nitrogen provision and thus have lost the operon due to reductive evolution within their hosts.

After uptake by the cell, NH_4^+ will be incorporated via the GS/GOGAT or the GDH pathway into glutamine and glutamate which are further involved as an amino donor in the generation of other amino acids necessary for life.The regulatory mechanisms for nitrogen assimilation are very diverse, and specific strategies are employed in response to the changing conditions of nitrogen supply in virtually every phylum of bacteria. In the genus *Bacillus*, global regulatory proteins GlnR, TnrA and CodY are responsible for mediating the nitrogen assimilation via transcriptional control of the enzymes involved (See Section 1.8.4).

We previously reported that genome analysis of the denitrifier *Bacillus azotoformans* LMG 9581^T [11], showed a high level of redundancy for dissimilatory nitrate reduction process including *nrfAH* genes encoding periplasmic NrfAH for NO_2^- reduction to NH_4^+ (DNRA). We also noticed that *B. azotoformans* LMG 9581^T had a reduced nitrogen assimilation gene inventory, lacking *nasC* and *nirBD* genes encoding assimilatory NO_3^- and NO_2^- reductases as well as *glnKamtB* operon. So, it remains to be determined if a functional NrfAH would assist nitrogen assimilation or dissimilation and if passively diffused NH_4^+ (diffuse in the form of NH_3) provided or possibly produced is sufficient to support growth. In addition, *B. azotoformans* has been reported to require yeast extract for growth [12, 13]. Yeast extract is a commonly used organic nitrogen source in many bacteriological media including media for enrichment, isolation and cultivation of denitrifiers [14-16], and is essential for cultivating certain organisms in mineral media [17, 18]. It contains a mixture of amino acids, peptides, water soluble vitamins and carbohydrates and can function as not only nitrogen source but also provides growth stimulants or growth factors for certain bacteria [19, 20]. Therefore, here we performed a genome analysis of nitrogen assimilation pathways of *Bacillus azotoformans* LMG 9581^T and initiated experimental tests to obtain more insight in the role of NH_4^+ and yeast extract in the nitrogen assimilation of *B. azotoformans* LMG 9581^T.

4.2 Materials and methods

4.2.1 Growth conditions

B. azotoformans LMG 9581^T was obtained from the BCCM/LMG Bacteria Collection. It was grown aerobically at 28 °C for 3 days on Tryptone Soy Agar (TSA, Oxoid) before use in growth experiments in mineral medium. Mineral medium as described by Stanier *et al*. [15], including 10 mM phosphate buffer (pH 6.8), 0.4 mM $MgSO_4.7H_2O$, 0.04 mM $CaCl_2.2H_2O$, 2.3 mM $(NH_4)_2SO_4$, and 0.027 mM EDTA, 0.025 mM $FeSO_4 \cdot 7H_2O$, 0.01 mM $ZnSO_4 \cdot 7H_2O$, 25 µM $MnSO_4 \cdot H_2O$, 3.8 µM $CuSO_4 \cdot 5H_2O$, 2 µM $Co(NO_3)_2 \cdot 6H_2O$, 0.196 µM $(NH_4)_6Mo_7O_{24} \cdot 24H_2O$, was used supplemented with 30 mM sodium acetate as electron donor and carbon source and 100 mg/L yeast extract, unless stated otherwise. For anaerobic growth, 10 mM KNO_3 was added as electron acceptor, unless stated otherwise. Serum vials (120 ml) were soaked overnight with 1 M HCl to remove growth inhibiting substances, and subsequently washed four times and rinsed with distilled water before use. Serum vials with 50 ml medium were sealed with grey or black butyl-rubber stoppers for aerobic and anaerobic experiments, respectively. After autoclaving, the headspace of the serum vials for anaerobic experiments was replaced via five cycles of evacuating and refilling with helium. Acetylene was added (10% v/v) after the same volume of helium was removed from the headspace, to stop the last step of denitrification, i.e. the reduction of N_2O to N_2. Serum vials were inoculated (1%) with 0.5 ml suspension of optical density OD_{600} of 1.00 ± 0.05. Each growth experiment was performed in triplicate, unless stated otherwise, and non-inoculated media in duplicate were always included as a reference. After inoculation, serum vials were incubated at 28 °C and shaken at 150 rpm.

4.2.2 Nitrogen assimilation experiments

In previous exprements, growth of *B. azotoformans* LMG 9581^T in mineral medium required addition of yeast extract [12, 13, 21-25]. In order to find a defined substitute for this organic

compound to use in further dissimilatory nitrate reduction studies of *B. azotoformans* LMG 9581^T, we set up nitrogen assimilation tests to find out the function of yeast extract. Therefore different concentrations of yeast extract and different composition of substances replacing yeast extract were tested in mineral media (Table S1, with final concentrations of the components): a) different concentrations of yeast extract (Oxoid) (0 mg/L, 100 mg/L, 500 mg/L, 1000 mg/L), b) casein enzymatic hydrolysate (114 mg/L, 1.14 g/L) (N-Z-Amine AS, Sigma), c) mixed vitamin B solutions of: 100 µg/L nicotinic acid, 20 µg/L D-(+)-biotin, 40 µg/L 4-aminobenzoic acid, 50 µg/L calcium D(+) pantothenate, 150 µg/L pyridoxine hydrochloride, 40 µg/L folic acid, 10 µg/L lipoic acid, 10 µM NaH_2PO_4 pH 7.1; 200 µg/L thiamine hydrochloride; 100 µg/L cyanocobalamin; 50 µg/L riboflavin, d) 761 mg/L L-glutamate sodium or 658 mg/L L-glutamine with mixed vitamin B solutions described above. e) 6.7g/L yeast nitrogen base with amino acids (YNB+, Sigma, descriptions see below) , f) 6.7g/L yeast nitrogen base without amino acids without $(NH_4)_2SO_4$ (YNB-, Sigma), g) 6.7g/L YNB- supplemented with single amino acid (L-histidine, L-methionine, or L-tryptophan with final concentrations of 10mg/L, 20mg/L, 20mg/L in the media), h) 6.7g/L YNB- supplemented with 5g/L $(NH_4)_2SO_4$, i) 6.7g/L YNB- supplemented with 5g/L $(NH_4)_2SO_4$ and single amino acid (L-histidine, L-methionine, or L-tryptophan with final concentrations of 10mg/L, 20mg/L, 20mg/L in the media), j) 5g/L $(NH_4)_2SO_4$, k) mixture of three amino acids (L-histidine, L-methionine, and L-tryptophan with final concentrations of 10mg/L, 20mg/L, 20mg/L in the media), l) 5g/L $(NH_4)_2SO_4$ and mixture of three amino acids (L-histidine, L-methionine, and L-tryptophan with final concentrations of 10mg/L, 20mg/L, 20mg/L in the media). According to the manufacturers the composition of the complex nitrogen sources is as follows: yeast extract contains 10-12.5 % total nitrogen, 5.1 % amino nitrogen, water soluble B vitamins, 0.3 % sodium chloride (%, w/w; casein enzymatic hydrolysate contains 6.5 % amino nitrogen, 13.0 % total nitrogen, 52.0 % free amino acids, 5.00 % ash, 0.44 % calcium,

0.02 % magnesium, 2.49 % sodium, 0.99 % chloride, 2.85 % phosphate, 0.06 % sulfate, 0.08 % potassium (%, w/w); 6.7 g/L yeast nitrogen base in the media contains 5g/L $(NH_4)_2SO_4$, vitamins (2 µg/L Biotin, 400 µg/L calcium pantothenate, 2 µg/L folic acid, 2000 µg/L inositol, 400 µg/L niacin, 200 µg/L p-aminobenzoic acid, 400 µg/L pyridoxine hydrochloride, 200 µg/L riboflavin, 400 µg/L thiamine hydrochloride), amino acids (L-histidine 10mg/L, DL-methionine 20mg/L, DL-tryptophan 20mg/L), trace elements (i.e. boric acid, copper sulfate, potassium iodide, ferric chloride, manganese sulfate, sodium molybdate, zinc sulfate), and salt (potassium phosphate monobasic 1 g/L, ammonium sulfate 5 g/L, magnesium sulfate 0.5 g/L, sodium chloride 0.1 g/L, calcium chloride 0.1 g/L).

Four different combinations of NO_3^- and NH_4^+ under both aerobic and anaerobic conditions were tested in mineral media with 100 mg/L yeast extract: a) 2.3 mM $(NH_4)_2SO_4$ and 10 mM KNO_3, b) 2.3 mM $(NH_4)_2SO_4$, c) 10 mM KNO_3, d) no $(NH_4)_2SO_4$ no KNO_3. Effects of NH_4^+ concentration and SO_4^- on growth were tested in mineral media with four different concentrations of NH_4^+ and replacement of NH_4^+ with Na^+ under aerobic and anaerobic conditions: a) 2.3 mM $(NH_4)_2SO_4$, b) 0.5 mM $(NH_4)_2SO_4$, c) 0.05 mM $(NH_4)_2SO_4$, d) 0 mM $(NH_4)_2SO_4$, e) 2.3 mM Na_2SO_4. Growth, NO_3^- NO_2^-, NH_4^+ concentration, N_2O production (when grown anaerobically) of the cultures was followed overtime to check the metabolism in detail.

4.2.3 Analytical procedures

Samples of 1 ml were taken from cultures through the rubber septum of serum vials with sterile syringes for growth determination and colorimetric determination of NH_4^+, NO_2^-, and NO_3^-. Growth was determined by measuring OD_{600} of 100 µl sample in duplicate in microtiter plates and standardized to 1 cm path length using PathCheck Sensor of the spectrophotometer (Molecular Devices, Spectramax plus 384, USA). Growth rate was determined by linear fit through the data points.

For colorimetrics, 500 µl sample was pretreated with 2.5 ml of 2 mM potassium chloride (KCl) by shaking 1h at 150 rpm and subsequent filtration (0.2 µm) to extract inorganic nitrogen and remove interfering compounds. After centrifugation at 18000 g for 2 min to remove the cells, samples were kept frozen at -20 °C. NH_4^+ concentration was determined with the salicylate-nitroprussidine method [26], NO_2^- and NO_3^- concentrations were determined with Griess reaction [27] and Griess reaction with cadmium [28, 29], respectively. Standard curves covered concentration ranges corresponding to those of the media and were strictly linear with an R^2 of 0.99. For analysis of N_2O, 1 ml samples of the headspace of serum vials were taken through the rubber by sterile syringes, and were injected into gas chromatograph (Compact GC with EZChrom Elite Software, Interscience, NL). N_2O production was corrected for pressure and solubility based on Henry's law, using Henry's law constant of 0.025M/atm [30] and was calculated to concentration in each vial.

Statistical differences in growth rate, growth, NH_4^+ consumption, and N_2O production under different experimental conditions were assessed using paired t-test, independent t-test after Levene's test for equality of variances, One-Way ANOVA after Levene's test for equality of variances, post-hoc testing or nonparametric test-Kruskal-Wallis test in IBM SPSS23.

4.2.4 Genome analysis

The draft genome of *B. azotoformans* LMG 9581^T was previously published [11] and is publically available (accession number AJLR00000000). Functional annotation of genes involved in NH_4^+ assimilation pathways were verified with pBLAST [31] and InterProScan [32] and compared with reference sequences from RSCB Protein Data Bank, when possible from closely related *Bacillus* strains. Missing genes were searched for in the genome with the BLAST function in RAST [33, 34] also using these reference sequences.

4.3 Results

4.3.1 Genomic inventory for nitrogen assimilation

The genes involved in nitrogen assimilation, regulation and transport found in the genome of *B. azotoformans* LMG 9581^T are listed in Table 4.1. *B. azotoformans* LMG 9581^T possesses the two main routes for NH_4^+ assimilation for biosynthesis: GDH type 1 encoding *gdhA* and the operon encoding GS type 1 (*glnA*) with upstream its repressor (*glnR*) are present. No functional GOGAT genes can be found, although its transcription activator *gltC* was found upstream and in opposite direction of *gdhA*. Instead, two gene copies of GOGAT domain 2 like protein (*yerD*) with the three conserved cysteine residues for iron-sulfur binding motifs were found but without a gene for the glutaminase domain, making its functionality highly unlikely. Transporters for glutamate (*gltT*) and glutamine (*glnQHPP*) were also encoded in the genome. Probable scenarios to obtain NH_4^+ for biosynthesis of glutamate and glutamine are provided by specific genes encoding GDH, asparagine synthetase, L-asparaginase, tryptophanase, asparatate ammonia-lyase and cystathionine beta-lyase (Table 4.1). As reported previously [11], the gene encoding an Amt or AmtB-type NH_4^+ transporter is absent, as well as the signal transduction protein P_{II} encoding *glnK*. Also, the genes for the global nitrogen regulatory protein TnrA, the urease operon (*ureABC*) and assimilatory NO_3^- and NO_2^- reductases are missing. Although genes coding for global nitrogen regulators GlnR and CodY are present in the genome, it still remains unanswered whether in the absence of TnrA, these other main regulatory proteins will take over its role. So, *B. azotoformans* LMG 9581^T appears to have a reductive nitrogen assimilation pathway dependent on amino acids and di- and oligo-peptides, for which indeed many specific transporters were found in the genome (in Table S2).

Table 4.1 Overview of gene inventory involved in nitrogen assimilation, regulation and transport in *B. azotoformans* LMG 9581^T

Function	Gene	Protein	Gene coordinates (locus tag)	pBLAST best hit	
				Genbank Identifier	% ID
Assimilatory nitrate reduction	*nasC*	Assimilatory nitrate reductase	Not found		
	nirB	Assimilatory nitrite reductase, small subunit	Not found		
	nirD	Assimilatory nitrite reductase, large subunit	Not found		
Glutamine/glutamate biosynthesis	*glnA*	glutamine synthetase (GS) type 1	contig42_29414_30751 (BAZO_05934)	KEF39969.1 *Bacillus azotoformans* MEV2011	99
	gltC	transcription activator of glutamate synthase operon	contig57_26840_25938 (BAZO_10453)	WP_017753919.1 *Bacillus* sp. ZYK	78
	gdhA	NADP-specific glutamate dehydrogenase (GDH)	contig57_27013_28398 (BAZO_10458)	KEF39039.1 *Bacillus azotoformans* MEV2011	99
	yerD	glutamate synthase large subunit-like protein	contig05_18926_20527 (BAZO_00525)	WP_026772506.1 *Sediminibacillus halophilus*	79
			contig55_11168_12841 (BAZO_10306)	WP_015594105.1 *Bacillus* sp. 1NLA3E	65
Glutamate assimilation	*rocC*	Regulatory protein	contig43_1860_442 (BAZO_06529)	WP_034263318.1 *Bacillus* sp. J33	78
	rocD	ornithine aminotransferase	contig43_2122_3327 (BAZO_06534)	WP_026582174.1 *Bacillus* sp. J33	89
	rocG	NAD-specific glutamate dehydrogenase	contig43_3339_4622 (BAZO_06539)	WP_034296254.1 *Bacillus* sp. 2_A_57_CT2	86
			contig48_114604_113333 (BAZO_09471)	WP_017755611.1 *Bacillus* sp. ZYK	92
Transport	*amtB*	Ammonium transporter	Not found		
	narK	Nitrate transporter	contig69_15531_17030 (BAZO_10682)	P_041967323.1 *Bacillus selenatarsenatis*	73
	nirC	Nitrite transporter	contig05_16758_15988 (BAZO_00505)	WP_044157570.1 *Salinibacillus aidingensis*	70
			contig107_<1_505 (BAZO_11644)	WP_032949972.1 *Lactococcus lactis*	97
	gltT	proton/sodium-glutamate symport protein	contig44_80221_78941 (BAZO_06959)	WP_018707959.1 *Bacillus fordii*	81
	glnQ1	ABC-type glutamine transporter, ATPase component	contig41_90277_91005 (BAZO_05760)	WP_041073717.1 *Bacillus* sp. OxB-1	90

	glnH1	ABC-type glutamine transporter, periplasmic component/domain	contig41_91029_91868 (BAZO_05765)	WP_041073719.1 *Bacillus* sp. OxB-1	70
	glnP1	ABC-type glutamine transporter, permease component	contig41_91936_92589 (BAZO_05770)	WP_041073723.1 *Bacillus* sp. OxB-1	81
	glnP2	ABC-type glutamine transporter, permease component	contig41_92601_93251 (BAZO_05775)	WP_041073721.1 *Bacillus* sp. OxB-1	83
Regulation	*tnrA*	Global nitrogen regulatory protein	Not found		
	glnK	P_{II}-type signal-transduction protein	Not found		
	glnR	glutamine synthetase repressor	contig42_28968_29363 (BAZO_05929)	WP_035193715.1 *Bacillus azotoformans* MEV2011	95
	codY	GTP-sensing transcriptional pleiotropic repressor	contig125_23153_23932 (BAZO_15489)	WP_017753747.1 Bacillus sp. ZYK	91
Nitrogen assimilation	*asnB*	Asparagine synthetase (glutamine-hydrolyzing)	contig39_90542_88653 (BAZO_04370)	WP_017753563.1 *Bacillus* sp. ZYK	76
	ansZ	L-asparaginase	contig48_112218_111253 (BAZO_09461)	WP_035193034.1 *Bacillus massilioanorexius*	70
	tnaA	tryptophanase	contig123_26513_27961 (BAZO_14849)	WP_017754447.1 *Bacillus* sp. ZYK	86
	aspA	aspartate ammonia-lyase	contig147_116872_118338 (BAZO_18491)	WP_017756012.1 *Bacillus* sp. ZYK	81
	metC	Cystathionine beta-lyase	contig147_120163_118976 (BAZO_18506)	WP_009335479.1 *Bacillus* sp. 2_A_57_CT2	77
			contig148_61270_62400 (BAZO_18858)	WP_041071496.1 *Bacillus* sp. OxB-1	77

Genes are ordered according to their location in the operon when applicable

4.3.2 Organic nitrogen is required for growth

To find out whether yeast extract is required for growth of *B. azotoformans* LMG 9581^T, aerobic growth with 4.6 mM NH_4^+ and different concentrations of yeast extract (0 mg/L, 100 mg/L, 500 mg/L, 1000 mg/L) was monitored. No growth was observed when yeast extract was absent, although sufficient NH_4^+ was provided. The maximum growth (OD_{600}) and growth rates appeared concentration-dependent (Figure 4.1, Table 4.3). And higher concentration of yeast extract (500 mg/L, 1000 mg/L vs 100 mg/L) resulted in a higher growth (p= 0.0001) and higher growth rate (p= 0.001), but growth obtain in media containing 500 mg/L and 1000 mg/L yeast extract were comparable (p= 0.126). This indicated that yeast extract is required and promotes growth of *B. azotoformans* LMG 9581^T.

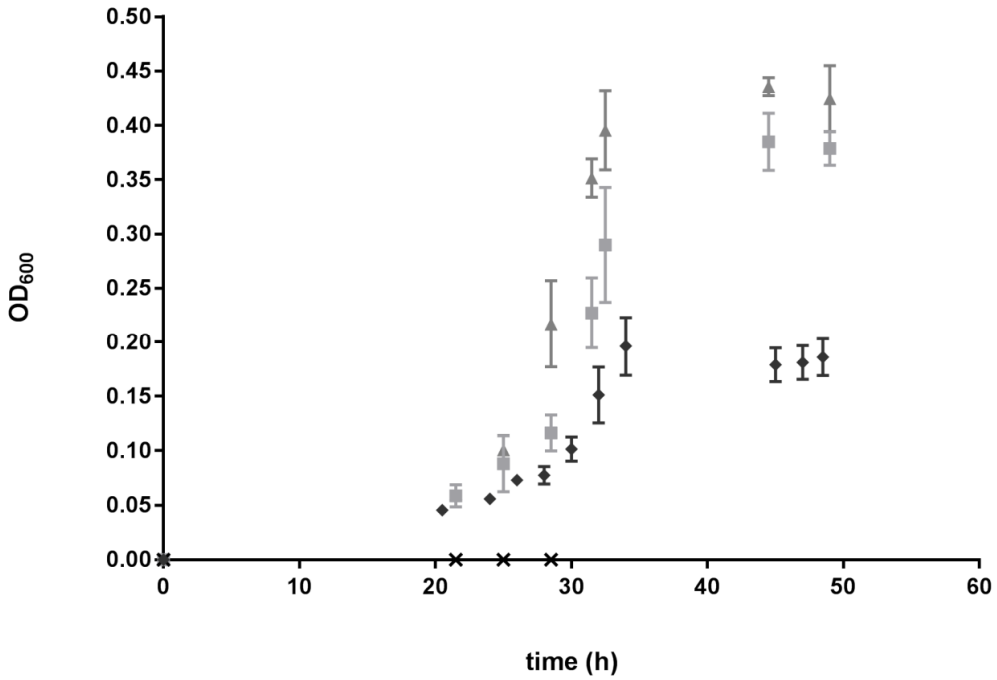

Figure 4.1 Growth of *B. azotoformans* LMG 9581^T in mineral media with NH_4^+ (4.6 mM) and different concentrations of yeast extract as nitrogen source. Aerobic growth (OD_{600}) at the following concentrations of yeast extract (mg/l) is plotted: 0 (cross), 100 (diamond), 500 (square, n=2), 1000 (triangle), n=3.

To find out more specifically the function of components that are (supposed or known) to be part of the yeast extract shown to be indispensable for growth, different substitutes were tested and resulted in data reflecting the different growth as observed in aerobic conditions. (Table S1). Growth was supported by casein enzymatic hydrolysate, defined YNB+, YNB- supplemented with $(NH_4)_2SO_4$ and at least one amino acid (either L-methionine or L-tryptophan), $(NH_4)_2SO_4$ and mixture of three amino acids (L-histidine, L-methionine, or L-tryptophan), suggesting that it is indeed nitrogen in yeast extract, not other compounds, such as vitamins or inorganic constituents, that supported growth of *B. azotoformans* LMG 9581^T. In addition, we found the combination of $(NH_4)_2SO_4$ 5g/L, together with amino acids L-methionine 20mg/L and L-tryptophan 20mg/L can be used as defined nitrogen source for assimilation by *B. azotoformans* 9581^T during further quantitative studies of this strain. Furthermore, our study showed that *B. azotoformans* 9581^T did not grow as expected in media with either NH_4^+, L-histidine, L-methionine, L-tryptophan, L-glutamate sodium or L-glutamine, albeit transporters for the two latter compounds are encoded in the genome of *B. azotoformans* 9581^T (Table S2).

4.3.3 NO_3^- is only utilized for energy generation

Different combinations of inorganic nitrogen source (NO_3^-, NH_4^+) under both aerobic and anaerobic conditions were tested and detailed growth data were analyzed to investigate to what extent NO_3^- was partitioned towards DNRA for assimilation or denitrification for energy generation. In general, under aerobic conditions growth was supported irrespective of addition, of NO_3^- or NH_4^+. Under anaerobic conditions, as expected growth was only observed when NO_3^- was provided, irrespective of addition of NH_4^+ (data not shown).

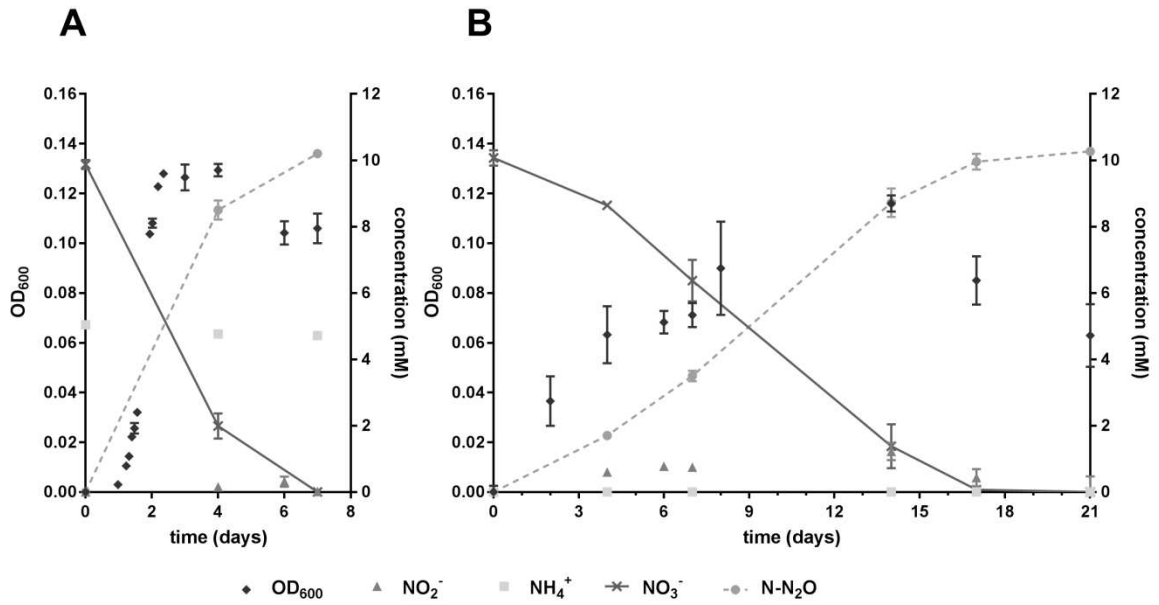

Figure 4.2 Assimilation of inorganic nitrogen during anaerobic growth (diamond OD_{600}) of *B. azotoformans* LMG 9581^T in 10 mM NO_3^- and (A) with or (B) without 4.6 mM NH_4^+.Growth (diamond OD_{600}) plotted as well as concentrations of NH_4^+ (square), NO_2^- (triangle), NO_3^- (cross), and N-N_2O (dot) (n>=2). Decline of NO_3^- and increase of N-N_2O are given as full line and dashed line, respectively, to clarify the same concentration changes in the two tests after different incubation time.

With 10 mM NO_3^- and 4.6 mM NH_4^+ supplied, after 1 day anaerobic incubation, OD_{600} increased exponentially and reached a plateau after 3 days (Figure 4.2A). All NO_3^- was gradually consumed, concomitant with NO_2^- production and followed by consumption (maximum 0.31 ± 0.15 mM), finally yielding 5.10 ± 0.07 mM N_2O indicative of a denitrification phenotype. This confirmed the previously reported anaerobic respiratory metabolism of *B. azotoformans* LMG 9581^T with NO_3^- as electron acceptor. No production of NH_4^+ was observed, with a limited amount of NH_4^+ consumed (Figure 4.2A; Table 4.3) suggesting that DNRA did not take place. In the same growth conditions without NH_4^+, *B. azotoformans* LMG 9581^T showed a lower growth rate but reaching a comparable maximum cell density (p= 0.296) after 14 days (Figure 4.2B, Table 4.3). Similarly, all NO_3^- was gradually consumed, concomitant with NO_2^- production (maximum 1.22 ± 0.26 mM) followed by consumption yielding 5.13 ± 0.05 mM N_2O in the end, with no NH_4^+ production

measured. Again, DNRA was not performed when NH_4^+ was absent in the media. In addition, these results suggest that addition of NH_4^+ had an effect on the growth rate but not on the maximal cell density, despite the uptake of 0.35 ± 0.04 mM of NH_4^+ (Table 4.3).

4.3.4 Influence of NH_4^+ concentration on the growth rate

Since addition of NH_4^+ did not affect on the maximal cell density during anaerobic growth, but clearly influenced the growth rate (Figure 4.2), the effect of different concentrations of NH_4^+ (0 mM 0.1 mM, 1 mM, 4.6 mM) on anaerobic growth tests of *B. azotoformans* LMG 9581^T was tested. Similar as in former growth tests, the initially supplied NO_3^- (10 mM) was gradually consumed under all conditions with limited transient NO_2^- observed and subsequently consumed and converted to equivalent N_2O as end product (data not shown), demonstrating a denitrification phenotype. Growth rates, but not maximal cell density, positively correlated with the NH_4^+ concentration (p= 0.000029; Table 4.3). And, 0.35 ± 0.04 mM and 0.26 ± 0.04 mM NH_4^+ were consumed respectively when 4.6 mM and 1 mM NH_4^+ were supplied, all NH_4^+ was consumed when 0.1 mM NH_4^+ (actually 0.08 ± 0.04 mM) was added. NH_4^+ concentration remained as 0 mM when it had not been added the medium (Table 4.3).

So under anaerobic conditions, NO_3^- was not assimilated but used as electron acceptor for denitrification generating energy and growth was limited by this electron acceptor, not by the nitrogen source NH_4^+. Therefore, identical experiments were performed under aerobic conditions, in which the main nitrogen source, i.e. yeast extract (100 mg/L) would limit growth, as observed above (Figure 4.1). Again higher growth rates were observed with higher NH_4^+ concentrations (p= 0.000029). Similar to anaerobic conditions, limited amounts of NH_4^+ were consumed when available (Table 4.3). NH_4^+ remained undetected in the medium if not added (Table 4.3). However, maximal OD_{600} values obtained were comparable under NH_4^+ concentration of 4.6 mM, 1 mM, 0.1 mM (p= 0.574), but was significantly lower with no

NH_4^+ added (0.151 ± 0.012, n=3, *p*= 0.005). As expected, anaerobic and aerobic growth in the media with 4.6 mM NH_4^+ were significantly different (*p*= 0.024), but the NH_4^+ consumption under these two conditions was not. This indicated that although a limited amount of NH_4^+ was consumed, it did contribute to growth. The lack of a clear correlation between the difference in growth, and the NH_4^+ consumption may due to limitations of our measurement procedures. The theoretical nitrogen in biomass resulting from consumed NH_4^+ was calculated and listed as indication (Table 4.3). Afterwards, identical observations were made when 0 mM NH_4^+ or 2.3 mM Na_2SO_4 (data not shown) was supplied under both aerobic and anaerobic tests, excluding that the NH_4^+ concentration effect on growth rate was due to a salt effect.

Table 4.3 Overview of growth rates, growth and NH_4^+ consumption of *B. azotoformans* LMG 9581^T during anaerobic incubation under different NH_4^+ concentrations and yeast extract concentrations (n>=2)

NH_4^+ (mM)	**Yeast extract (mg/l)**	**Electron acceptor**	**Growth rate (/ h)**	**Growth (maximal OD_{600})**	**NH_4^+ consumption (mM)**	**% N in biomass from NH_4^+ [a]**
4.6	0	O_2	-	-	0	
4.6	100	O_2	0.0044 ± 0.0008	0.196 ± 0.025	0.39 ± 0.02	42.64
4.6	500	O_2	0.0199 ± 0.0034	0.385 ± 0.0224	ND	-
4.6	1000	O_2	0.0314 ± 0.0027	0.436 ± 0.0081	ND	-
4.6	100	NO_3^-	0.0016 ± 5.7735E-05	0.156 ± 0.014	0.35 ± 0.04	48.08
1.0	100	O_2	0.0039 ± 0.0003	0.207 ± 0.014	0.25 ± 0.018	25.88
1.0	100	NO_3^-	0.0015 ± 0.0002	0.136 ± 0.008	0.26 ± 0.04	40.97
0.1	100	O_2	0.0025 ± 0.0004	0.195 ± 0.014	0.08 ±0.003	8.79
0.1	100	NO_3^-	0.0008 ± 5.7735E-05	0.169 ± 0.008	0.08 ± 0.04	10.14
0	100	O_2	0.0022 ± 0.0002	0.151 ± 0.012	0	-
0	100	NO_3^-	0.0007 ± 0	0.161 ± 0.020	0	-

[a], The percentage of nitrogen in biomass derived from NH_4^+ was calculated using the assumptions that (i) OD_{600} of 0.15 correlates with 70 mg/l cell dry weight and (ii) 14% of cell dry weight is nitrogen

4.4 Discussion

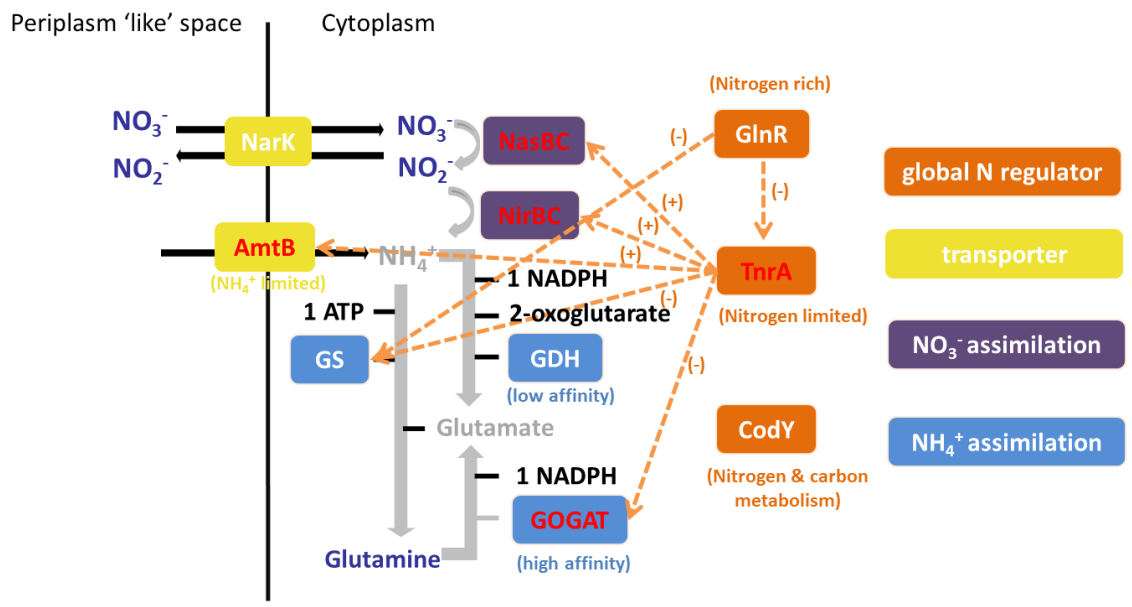

Figure 4.3 Scheme overview of nitrogen assimilation pathways in *B. azotoformans* LMG 9581^T: (1) Regulation, global nitrogen regulators are indicated in orange: GlnR, TnrA, CodY (2) Transport, NH_4^+ transporter and NO_3^- / NO_2^- transporter (NarK) on the membrane are in yellow (3) NO_3^- assimilation, enzymes involved (NasBC, NirBD) are indicated in purple (4) NH_4^+ assimilation, enzymes involved are indicated in blue. Proteins/enzymes with red font indicate their genes are absent. Dotted orange arrows illustrated the regulatory interactions of the respective regulator including its function in activating (plus) or repressing (minus) the target gene(s) of certain enzymes.

NH_4^+ assimilation is controlled by a complex and hierarchical regulatory network involving transport, signalling, metabolism, posttranslational modification, and transcription [35]. The genome analysis of *B. azotoformans* LMG 9581^T showed a degenerated nitrogen assimilation pathway of both NO_3^- and NH_4^+ assimilation (Figure 4.3). With *glnKamtB* and *tnrA* lacking, the strain may not take up NH_4^+ efficiently at low levels. Here, we demonstrated that in *B. azotoformans* LMG 9581^T NH_4^+ can be assimilated, probably via a high-affinity-possessing GS (Km= 0.1mM in *Escherichia coli* [35]) or a low-affinity-possessing GDH (Km= 1mM in *E. coli* [35] and Km sevenfold higher in *B.subtilis* than in *E. coli* [36]) after uptake via diffusion under different NH_4^+ concentration (even at 0.1 mM), and can contribute up to 50%

of N biomass. However, organic nitrogen is required for growth, indicating a deficiency of NH_4^+ assimilation. A possible reason may be in the lack of GOGAT in the subsequent NH_4^+ assimilation pathways. In other studies, *Sporosarcina pasteurii* (previously known as *B. pasteurii*) and *S. ureae*, both with no detectable GS activity and GOGAT activity only present in the latter, required high NH_4^+ concentration (40 mM) and alkaline pH for growth [37, 38], conditions under which conditions the NH_4^+ diffusion (in the form of NH_3) is enhanced. However, in our further tests in media without yeast extract with 30 mM, 50 mM or 100 mM NH_4^+ at pH 8.6, no growth was observed, confirming the deficiency of NH_4^+ assimilation unrelated to impaired NH_4^+ uptake ability (*glnKamtB* and *tnrA* lacking) and the necessity of organic nitrogen (data not shown). It is known that defective GOGAT genes can influence the glutamate synthesis, which contributes up to 80-88% of the nitrogen to biomass [39, 40]. Indeed, GOGAT mutants have difficulty growing on NH_4^+ at low concentration (too dilute for efficient fixation by GDH, exact concentration differs for strains depending on carbon sources supplied [41, 42]), glutamine and a variety of nitrogen sources that can be catabolized to NH_4^+, glutamate or both [41, 43]. This may because the glutamine pool that results from the ratio between glutamine synthesis and glutamine degradation is too high to allow derepression of nitrogen regulatory system and too low to support growth [43], thus resulting in growth rate limitation. This deduction is supported by previous observations that growth rate of a GOGAT-deficient mutant is much lower than that of a mutant that lacks both GS-GOGAT deficient mutants (3-4 h doubling time vs 98min doubling time) [44]. This may in part explain our partial observations that L-glutamine as sole nitrogen source could not support growth in *B. azotoformans* LMG 9581^T. And consequently BIOLOG Phenotype MicroArrays tests with a range of over 472 nitrogen compounds (data not shown), also failed to identify sole nitrogen sources for assimilation, except when some of these organic nitrogen supplied together with NH_4^+. This indicated both NH_4^+ and simple organic nitrogen (amino acid, peptide nitrogen)

are necessary for growth of *B. azotoformans* LMG 9581^T, while complex organic nitrogen such as yeast extract and casein enzymatic hydrolysate can support growth without NH_4^+, which may because both NH_4^+ and simple organic nitrogen, e.g. amino acids can be provided after their hydrolyzation. Unfortunately, this fails to explain the inability to utilize NH_4^+ at high concentration and L-glutamate as sole nitrogen source in the presence of encoded GS and GDH pathways and thus the underlying mechanism of the deficiency of nitrogen assimilation in *B. azotoformans* LMG 9581^T still remains unclear.

Surprisingly, a significant concentration-dependent influence of NH_4^+ on growth rate of *B. azotoformans* LMG 9581^T was observed. Previously GOGAT mutants of *S. typhimurium* and wild type strains were reported to show pronounced differences in growth rate when growing under different NH_4^+ concentrations [45]. In GOGAT mutants of *E. coli* and *Klebsiella aerogenes*, similar NH_4^+-mediated effects were observed with added NH_4^+ increasing the growth rate in GOGAT deficient strain but not in both GOGAT and GDH deficient strains [43]. The specific underlying regulatory mechanisms in *B. azotoformans* LMG 9581^T require further investigations of GDH and the global nitrogen regulators of GlnR and CodY.

As mentioned above, *B. azotoformans* LMG 9581^T is a denitrifier that also contains DNRA encoding genes, thus making it a promising strain for study of nitrate partitioning towards both dissimilatory nitrate reduction pathways. Similarly, *Shewanella loihica* strain PV-4, a Gram-negative bacterium possesses both pathways that were both proven functional [46, 47], in contrast to in *B. azotoformans* LMG 9581^T. Thus far, in our tests without added NH_4^+, DNRA was not triggered, although it is still possible that under similar conditions as in *Shewanella loihica* and other organisms DNRA can be promoted in *B. azotoformans* LMG 9581^T [48-52]. The knowledge gathered here on nitrogen assimilation can be applied to design experiments to 1) confirm the functionality of DNRA and 2) study nitrate partitioning towards DNRA and denitrification in *B. azotoformans* LMG 9581^T.

4.5 Acknowledgement

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4.6 Supplementary information

Table S1 Overview of growth observations of *B. azotoformans* LMG 9581^T under different combinations of nitrogen sources

Nitrogen source	Growth[a]
4.6 mM NH_4^+	-
100 mg/L Yeast extract , 4.6 mM NH_4^+	+
500 mg/L Yeast extract, 4.6 mM NH_4^+	+
1000mg/L Yeast extract, 4.6 mM NH_4^+	+
114 mg/L casein enzymatic hydrolysate	+
1.14 g/L casein enzymatic hydrolysate	+
mixed vitamin B solutions	-
761 mg/L L-glutamate sodium or 658 mg/L L-glutamine with mixed vitamin B solutions	-
6.7 g/L YNB+ [b]	+
6.7 g/L YNB-[c]	-
6.7 g/L YNB-, 10 mg/L L-histidine	-
6.7 g/L YNB-, 20 mg/L L-methionine	-
6.7 g/L YNB-, 20 mg/L L-tryptophan	-
6.7 g/L YNB- supplemented with 5 g/L $(NH_4)_2SO_4$	-
6.7 g/L YNB- supplemented with 5 g/L $(NH_4)_2SO_4$, 10 mg/L L-histidine	-
6.7 g/L YNB- supplemented with 5 g/L $(NH_4)_2SO_4$, 20 mg/L L-methionine	+
6.7 g/L YNB- supplemented with 5 g/L $(NH_4)_2SO_4$, 20 mg/L L-tryptophan	+
5 g/L $(NH_4)_2SO_4$	-
10 mg/L L-histidine, 20 mg/L L-methionine, 20 mg/L L-tryptophan	-
5 g/L $(NH_4)_2SO_4$, 10 mg/L L-histidine, 20 mg/L L-methionine, 20 mg/L L-tryptophan	+

[a] Growth '+' indicates that growth (OD_{600} increase $\geq$ 0.03) was observed during maximum 15 days incubation; Growth '-' indicates that growth (OD_{600} increase $\geq$ 0.03) was not observed during maximum 15 days incubation

[b] '√' means mixed vitamin B solutions added, see '**materials and methods**' for specific composition

[c] 'YNB+' yeast nitrogen base with amino acids with $(NH_4)_2SO_4$ (10mg/L L-histidine, 20mg/L L-methionine, or 20mg/L L-tryptophan, 5 g/L $(NH_4)_2SO_4$)

[d] 'YNB-' yeast nitrogen base without amino acids without $(NH_4)_2SO_4$

Table S2 Overview of amino acid, di- and oligopeptide ABC-type transporters in the genome of in *B. azotoformans* LMG 9581^{T}

Function	Locus tags
Cysteine transport	BAZO_01572-01582
L-proline glycine betaine transport	BAZO_18331-18346
Glycine betaine transport	BAZO_05685-05695
Methionine transport	BAZO_01177-01187
	BAZO_18541-18551
	BAZO_08084-08094
Dipeptide transport	BAZO_01422-BAZO_01442-BAZO_01452
Oligopeptide transport	BAZO_16969-16989
Amino acid transport	BAZO_06654-06664
	BAZO_06944-06954
	BAZO_19203-19213
Branched amino acid transport	BAZO_00590-00610
	BAZO_09056-09076
	BAZO_10276-10296
	BAZO_13069-13089
	BAZO_16524-16539
	BAZO_17734-17754
	BAZO_19618-19638

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Chapter 5:

Investigation of functionality of DNRA in denitrifier *Bacillus azotoformans* LMG 9581^T

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YH performed experiments, analyzed data and wrote the paper; KH conceived the study and revised the paper. PDV performed experiments, analyzed data and revised the paper.

Summary

Until recently, it was not generally known that bacteria can contain the gene inventory for both denitrification and dissimilatory NO_3^-/ NO_2^- reduction to NH_4^+ ammonium (DNRA). Detailed studies of such microorganisms may shed light on the differentiating environmental drivers of both processes without interference of organism-specific variation. Genome analysis of *B. azotoformans* LMG 9581^T showed a remarkable redundancy of dissimilatory nitrogen reduction, with multiple copies of each denitrification gene as well as DNRA genes *nrfAH*, but indicated a reduced capacity for nitrogen assimilation, with no *nas* operon nor *amtB* gene. In the previous chapter we verified the degenerated nitrogen assimilation in *B. azotoformans* LMG 9581^T and only denitrification not DNRA was observed under the anaerobic conditions tested. Here we continue our study on the functionality of DNRA in *B. azotoformans* LMG 9581^T by growing the strain under different conditions in both batch tests and chemostat tests. A gene deletion system for *B. azotoformans* LMG 9581^T was designed and tested. Unfortunately, this experiment failed to yield mutants lacking the *nrfH* gene due to the unsuccessful electroporation of pNW33N into *B. azotoformans* LMG 9581^T. In batch experiments, variable C/N-NO_3^- ratios, NO_2^- concentrations which are supposed to influence DNRA expression, were investigated but failed to promote DNRA. Therefore, an anaerobic chemostat was set up and employed to promote DNRA, under NO_3^- concentrations of 1 mM, 2 mM, 10 mM, with variable C/N-NO_3^- ratio of 15, 7.5, 1.5 respectively, but due to contamination problems not all conditions could be explored.

5.1 Introduction

Application of the Haber-Bosch process increased the global active nitrogen compounds over the last hundred years. This human impact on the global nitrogen cycle has exceeded the limit of the planet's capacity by a factor of four [1]. Besides the excess of reactive nitrogen in the environment, the major consequence is the increasing emission of nitrous oxide (N_2O), a potent greenhouse gas with a 310 times greater global warming potential than carbon dioxide and contributor to the ozone layer destruction [2]. Nitrate (NO_3^-) can be removed from a system via denitrification, a modular process involving four consecutive steps in which NO_3^- is reduced to nitrite (NO_2^-), nitric oxide (NO), N_2O and/or dinitrogen gas (N_2). Alternatively, NO_3^- can be retained in a system as biologically available ammonium (NH_4^+) via dissimilatory reduction of NO_3^-/NO_2^- to NH_4^+ (DNRA; also sometimes termed NO_3^-/NO_2^- ammonification), with NO_2^- as intermediate and small amounts of N_2O produced as by-product [3-8]. However, DNRA was, until recently, often overlooked as relevant NO_3^- removal process in terrestrial [9] and marine ecosystems [5, 10]. Understanding how NO_3^- is partitioned between both processes is pivotal in the development of predictive models of the global nitrogen cycle and N_2O emissions and subsequent mitigation practices.

Based on the Gibbs free energy calculation in previous studies, energy yield per electron and per NO_3^- in the two NO_3^- reduction pathways were calculated, with glucose representing biomass, as shown in the following equations.

Denitrification: $5\ C_6H_{12}O_6 + 24\ NO_3^- + 24\ H^+ \rightarrow 30\ CO_2 + 12\ N_2 + 42\ H_2O$

($\Delta G°' = -2670$ kJ /mol glucose; $\Delta G°' = -111.3$ kJ /mol electron; $\Delta G°' = -556$ kJ /mol NO_3^-)

DNRA: $C_6H_{12}O_6 + 3\ NO_3^- + 6\ H^+ \rightarrow 6\ CO_2 + 3\ NH_4^+ + 3\ H_2O$

($\Delta G°' = -1870$ kJ /mol glucose; $\Delta G°' = -77.9$ kJ / mol electron; $\Delta G°' = -623$ kJ / mol NO_3^-)

Therefore, theoretically DNRA generates more energy per NO_3^- and less energy per electron compared with denitrification. This indicates that high C/N-NO_3^- ratio (e.g. electron donor to electron acceptor ratio) or NO_3^- limitation favors DNRA, which indeed has been confirmed in current studies, especially via well-defined continuous culture [6, 11, 12]. Yin et al. [13]showed that significant DNRA occurred in soil samples only when the C/N-NO_3^- ratio is above 12. While in pure culture study with *Bacillus*, *Citrobacter* [14] or *Shewanella loihica* PV-4 [11], NH_4^+ production was observed at different C/N-NO_3^- ratios above 25, 25 or 3. Therefore C/N-NO_3^- ratio required for initiation of DNRA is probably varies from strain to strain. Another important factor that regulates DNRA/denitrification in populations of bacteria is the available carbon sources [6, 15, 16]. Also pH [11, 17], temperature [18, 19], NO_2^- versus NO_3^- concentration [17, 20, 21], soil sand content [17], and sulfide concentration [10, 22, 23] have been reported as potential environmental controls of both processes. Overall, C/N-NO_3^- ratio appears to be the most important regulating factor. However, because denitrification and DNRA were thought to be mutually exclusive, they were either studied separately in single organisms or together in mixed communities or environments, clouding the differentiation between organism-specific and process-specific observations.

In the last few years whole genome sequencing projects of pure cultures have revealed that the co-occurrence of both NO_3^- removal pathways in a single organism is not uncommon [24-26]. However, thus far, only for *Shewanella loihica* PV-4 functionality of both pathways has been demonstrated. Avoiding the interference of organism-specific metabolic variation or complex interactions between members of a mixed community, the study of *S. loihica* PV-4 has indeed confirmed the determining roles of high C/N-NO_3^- ratios (over 3), high pH (above 7.0), high temperature (over 30°C), NO_2^- as sole electron acceptor and high NO_2^-/ NO_3^- ratios in partitioning NO_3^- towards DNRA with more convincing results in chemostat set-up than in batch set-up [11, 21].

We previously reported the genome analyses of the denitrifier *Bacillus azotoformans* LMG 9581^T [25] encodes genes for DNRA, in addition to showing a high level of redundancy for genes involved in the denitrification process: it contains one *nap* for a periplasmic and two *narG* for cytoplasmic NO_3^- reductases, one *nirK* for copper-containing NO_2^- reductase, two *qnorB* for quinol-dependent and two *cbaA* for two types of copper A-containing NO reductases, and three *nosZ* genes for N_2O reductases. Interestingly, like *S. loihica* PV-4, *B. azotoformans* LMG 9581^T lacks *nasC* and *nirBD* genes encoding assimilatory NO_3^- and NO_2^- reductases respectively, which might be compensated for by combined action of NapA and NrfAH in the periplasmic space and could be a plausible reason for the co-occurrence of both NO_3^- removal pathways. However, different from *S. loihica* PV-4, *B. azotoformans* LMG 9581^T also lacks genes for a dedicated NH_4^+ transporter, protein GlnK for nitrogen sensor, global nitrogen regulatory protein TnrA and glutamate synthase (GOGAT). From the previous study (Chapter 4) we know that *B. azotoformans* has a degenerated nitrogen assimilation pathway, NH_4^+ can only partially support growth and needs to be supplemented with organic nitrogen. Using these insights and based on the state-of-the-art, a stepwise approach was designed (Figure 5.1) to decipher the environmental drivers of NO_3^- partitioning to denitrification or DNRA, including subsequent batch experiments and gene knock-out experiments and finally chemostat experiments.

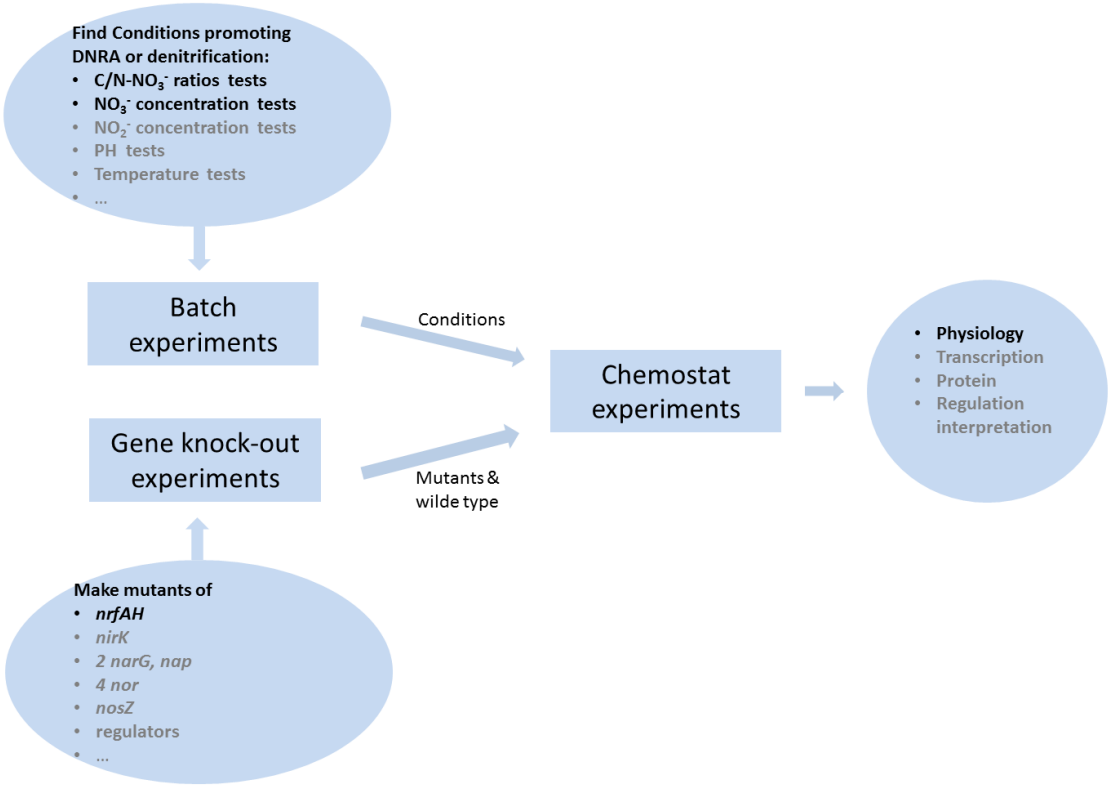

Figure 5.1 Schematic drawing of experimental design for studies of *B. azotoformans* LMG 9581^T. Experiments listed in black were planned and performed; experiments listed in grey were planned but not performed.

5.2 Materials and methods

5.2.1 Strain and media

B. azotoformans LMG 9581^T was obtained from the BCCM/LMG bacteria collection. It was grown aerobically at 28 °C for 3 days on Tryptone Soy Agar (TSA, Oxoid) before inoculation in batch tests, grown aerobically at 28 °C for 2 days in 50 ml Tryptone Soy Broth (TSB, Oxoid) before inoculation in chemostat tests. Mineral medium was as described by Stanier et al [27], including 10 mM phosphate buffer (pH 6.8), 0.4 mM $MgSO_4.7H_2O$, 0.04 mM $CaCl_2.2H_2O$, 2.3 mM $(NH_4)_2SO_4$, and 0.027 mM EDTA, 0.025 mM $FeSO_4 \cdot 7H_2O$, 0.01 mM $ZnSO_4 \cdot 7H_2O$, 25 µM $MnSO_4 \cdot H_2O$, 3.8 µM $CuSO_4 \cdot 5H_2O$, 2 µM $Co(NO_3)_2 \cdot 6H_2O$, 0.196 µM $(NH_4)_6Mo_7O_{24} \cdot 24H_2O$, supplemented with 100 mg/L yeast extract, sodium acetate as electron donor and carbon source, KNO_3 as electron acceptor .

5.2.2 Batch tests

Two sets of experiments were performed, either with variable or fixed NO_3^- concentrations. Six different NO_3^- concentrations (0.2 mM, 0.5 mM, 1 mM, 2 mM, 4 mM and 10 mM) and 30 mM sodium acetate resulting in variable C/N-NO_3^- ratios of 300, 120, 60, 30, 15, 6), and six different C/N-NO_3^- ratio (1.5, 3, 7.5, 15, 30, 150) with either 1 or 0.2 mM KNO_3 with adjusted concentration of sodium acetate and with or without 2.3 mM $(NH_4)_2SO_4$ were tested in mineral media, respectively. Fifty milliliter of each media were added in 120 ml serum vials in triplicate for growth tests afterwards, together with a duplicate non-inoculated blank. The serum vials were cleaned beforehand by soaking overnight with 1 M HCl to remove growth inhibiting substances, and subsequently washing four times and rinsing with distilled water before use. After being filled with media, they were sealed with black butyl-rubber stoppers for anaerobic conditions. After autoclaving, the headspace of the serum vials for anaerobic experiments was replaced via five cycles of evacuating and refilling with helium. Acetylene was added (10%v/v) after removing the same volume of helium to stop the last step

of denitrification, i.e. the reduction of N_2O to N_2. Serum vials were inoculated (1%) with 0.5 ml suspension of optical density OD_{600} of 1.00 ± 0.05. After inoculation, they were incubated at 28 °C and shaken at 150 rpm. Growth (OD_{600}), NO_3^-, NO_2^-, NH_4^+ concentration and N_2O production of the culture was evaluated at start and end-point when all NO_3^- added converted to NH_4^+ or N_2O.

5.2.3 Gene knock-out design

Since *B. azotoformans* LMG 9581^T is a non-model organism lacking suitable tools for genetic manipulation, the group II intron targeting technology with the commercial kit TargeTron® Gene Knockout System (Sigma-Aldrich) was employed together with the designed vectors that permit gene inactivation through group II intron insertion based on other *Bacillus* studies [28, 29]. The schematic overview of principle adapted for knock-out of DNRA gene *nrfH* (or *nrfAH*) is shown in Figure 5.2.

At first, a preliminary electroporation tests of pNW33N (*Bacillus* Genetic Stock Center, Columbus) into competent cells of *B. azotoformans* LMG 9581^T was performed with Gene Pulser apparatus (Bio-Rad) (2mm cuvette and maximum 2.5 kV/cm) (methods adapted from Zarschler et al., 2009 [28]) and further checked on TSA plate containing chloramphenicol 25µg/ml with different amounts of *B. azotoformans* LMG 9581^T competent cells and pNW33N to check the efficiency of this electroporation method. Competent cells were prepared from exponential phase of *B. azotoformans* LMG 9581^T (OD_{600} =0.4-0.5), as describe by Zarschler et al., 2009. Because the electroporation failed, the knock-out experiments were abandoned, however, below we provide the detailed experimental protocol planned.

Plasmid pNW33N would be first digested with HindIII, and the 5' overhangs would be filled in to form blunt ends by a large (Klenow) fragment of DNA polymerase I. The modified plasmid is self-ligated resulting in pNW33NΔHindIII (Figure 5.2A). The promoter of *nrfH* would be amplified from genomic DNA of *B. azotoformans* LMG 9581^T by PCR using primers P(NrfH)_*Sph*I_for and P(NrfH)_*Hind*III_rev. The resulting fragment would be digested with SphI and HindIII, cloned into SphI/ HindIII-linearized and dephosphorylated plasmid pJIR750ai (TargeTron® vector, Sigma-Aldrich), resulting in plasmid pJIR750ai_P(NrfH) (Figure 5.2B). Purified plasmid DNA of pJIR750ai_P(NrfH) would be used as a template for PCR with primers Targe_*Sph*I_for and Targe_*Sph*I_rev. The resulting fragment containing P(NrfH), the Ll.LtrA ORF, and the *plc* targetron would be digested with SphI, cloned into SphI-linearized and dephosphorylated pNW33NΔHindIII plasmid, resulting in plasmid pTT_*plc* (Figure 5.2C). The Ll.LtrB targetron would be retargeted to be inserted into the *nrfH* of *B. azotoformans* LMG 9581^T by using a computer algorithm that identifies potential insertion sites and directly designs PCR primers for modifying the intron RNA to base pair with these sites (TargeTron; Sigma-Aldrich). Modifications of intron RNA sequences to base pair with the *nrfH* target site sequences would be introduced via PCR by primer-mediated mutation with the designed primer sets (TargeTron; Sigma-Aldrich, online primer design tool). The amplified fragment would be subsequently digested with HindIII and BsrGI and ligated into pTT_*plc* vector digested with the same restriction enzymes, resulting in plasmid pTT_nrfH (Figure 5.2D). The primers would be designed based on the genome of *B. azotoformans* LMG 9581^T and/ or TargeTron kit. In short, the modified plasmid pJIR750ai carrying a promoter for *nrf* would be integrated in the modified *Geobacillus-Bacillus-Escherichia coli* shuttle vector pNW33N. The resulting new plasmid would be digested and ligated with *nrfH* targetron, a PCR fragment made from TargeTron Gene Knockout system. After electroporation of the new vector pTT_nrfH into *B. azotoformans* LMG 9581^T and

incubation of cells afterwards, the intron would be expressed and inserted to the target gene *nrfH* in into *B. azotoformans* LMG 9581^{T}. After plating the cells on 25µg/ml Chloramphenicol-containing TSA medium (Based on genome analysis, *B. azotoformans* LMG 9581^{T} possess no resistance to Ampicillin, Kanamycin and Chloramphenicol, and this was confirmed by resistance tests on TSA plate containing Ampicillin 50µg/ml, Kanamycin 50µg/ml or Chloramphenicol 25µg/ml), the cells with *nrfH* disruption would be selected for further physiological tests in the assembled anaerobic chemostat (Figure 5.1). The gene insertion can also be confirmed by colony PCR.

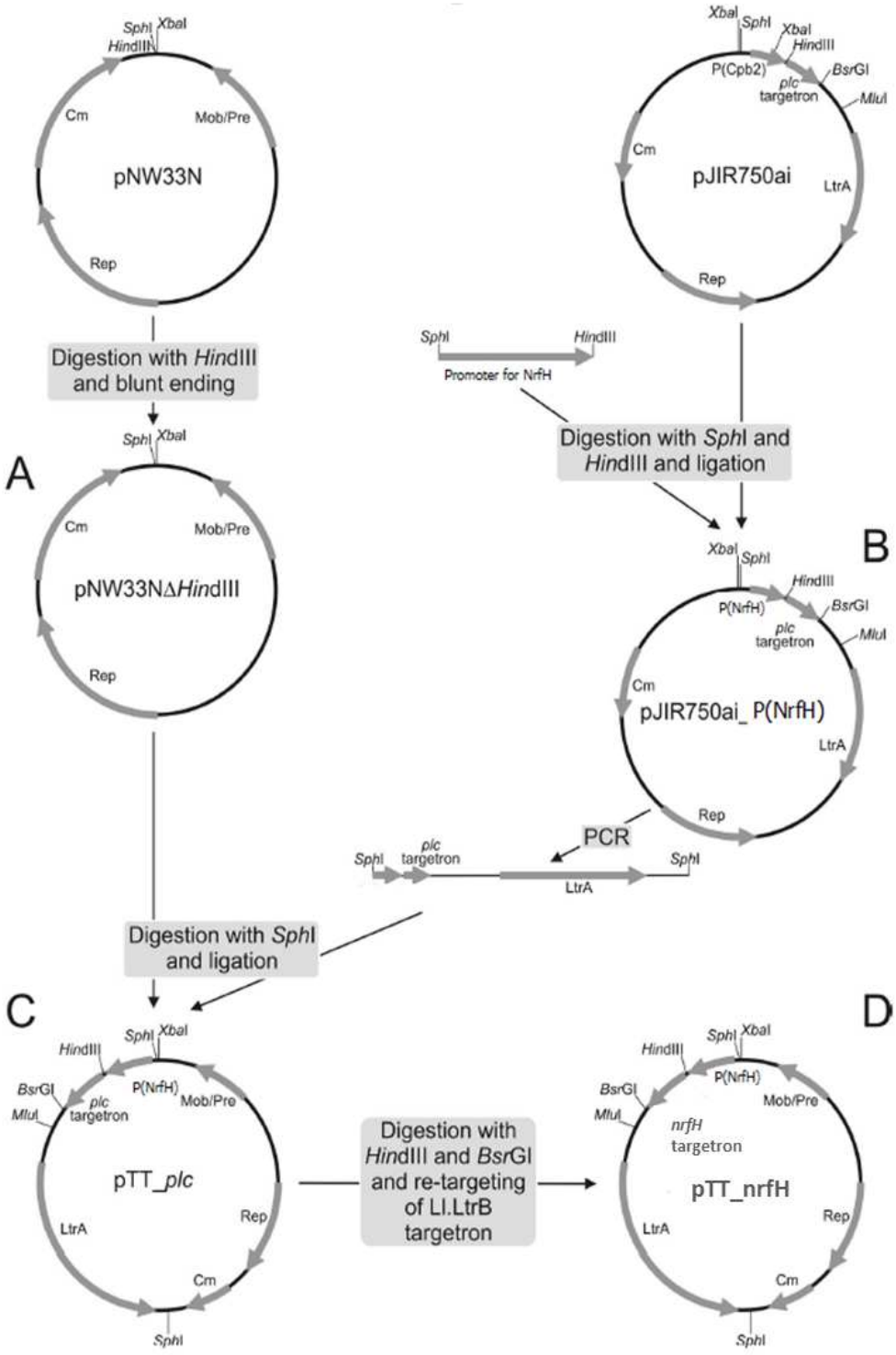

Figure 5.2 Schematic drawing of the construction of the shuttle plasmid pTT_*nrfH*, containing the *nrfH* targetron. Adapted from figure in Zarschler et al.,2009 [28].

5.2.4 Chemostat tests

A double-jacket glass bioreactor (3L, Applikon, Delft, Netherlands) with a working volume of 1.5 L medium, operated as an open continuously stirred reactor was set up for cultivation. The scheme of the chemostat set up is shown in Figure 5.1. The Bioreactor filled with 1.5 L mineral medium and a 10 L vessel with 7 L mineral medium or 20 L vessel with 12 L mineral medium and appropriate tubing were autoclaved and connected under sterile conditions after cooling to room temperature. The medium in the reactor was inoculated (1%) with 15 ml culture suspension with an OD_{600} of 1.00 ± 0.05. The inoculum was made by resuspending under sterile conditions, cells collected from a growing TSB culture after centrifugation at 8000 g for 10 min in the appropriate amount of sterile physiological water (0.85% NaCl) to obtain the desired OD. After inoculation the whole system was assembled. Helium (Air Liquide, Belgium) was flushed into the reactor and medium vessel via sterile filter (0.2 µm Gelman Sciences Corp.) using sterile tubing (Iso-Versinic®1, I/O 5mm x 8mm, Charny, France) for 2 hours to remove oxygen (O_2). The tubing from the headspace of the reactor was via an air filter submerged in a washing bottle filled with distilled water for overpressure compensation. The medium vessel headspace was connected to a 9 L calibrated inverted glass cylinder filled with helium under acid water for pressure compensation due to pumping of medium from the medium reservoir. The outlet of the reactor submerged in the culture was via sterile tubing and pumping devices (Watson-Marlow 502S, United Kingdom) connected with a waste vessel. The reactor culture was stirred by a magnetically coupled stirring device set at 250 rpm. The temperature was controlled at 28 °C via the water jacket connected to a warm water bath. The pH of the reactor culture liquid was monitored by a pH electrode (AppliSens, Delft, Netherlands) and was maintained at 6.92 ± 0.05 by pH pump (Watson-Marlow 502S, United Kingdom) and pH controller (Hanna Instruments Blankstone BL931700, Belgium) using filter-sterilized 0.1 M HCl. The tubing (Masterflex, C-Flex (50A), L/S 14,

Belgium) for all liquid medium and culture fluids as well as for the pH buffer were autoclaved and were compatible with acids and bases. Tubing (Iso-Versinic®1, I/O 5mm x 8mm, Charny, France) for gas flow were also resistant to high temperature and chemicals such as acids, oxidative agents. After assembling, the reactor was started up as a batch culture; once growth increased exponentially, the pumps for in and out flows of media and culture liquid were switched on and adjusted to a pumping speed of 40 ml/ h, resulting a dilution rate of 0.0267/ h. Filter-sterilised acetylene was added (10%, v/v) by injection via a stoppered port every three days to inhibit N_2O reduction to N_2. Growth (OD_{600}), NO_3^-, NO_2^-, NH_4^+ concentration and N_2O production of the culture was monitored over time to reveal steady state under the experimental conditions. Each measurement was done in triplicate unless stated otherwise.

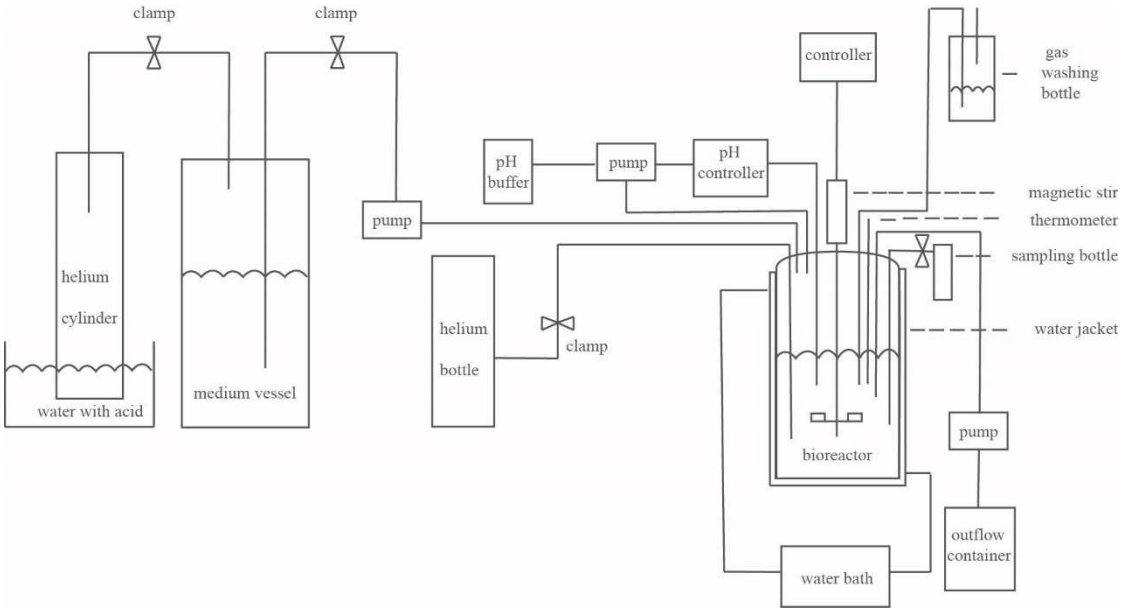

Figure 5.3 Diagram of anaerobic chemostat set up.

NO_3^- concentrations of 1 mM, 2 mM and 10 mM with variable C/N-NO_3^- ratio of 15, 7.5, 1.5, respectively, were tested. After each test condition the medium vessel was replaced with an appropriate medium for the next test condition. After 3 culture replacements in the reactor,

sampling started in order to check the new steady state level. Therefore, growth (OD_{600}), NO_3^-, NO_2^-, NH_4^+ concentrations and N_2O production of the culture were followed overtime, until at least three consecutive stable measurements were observed.

5.2.5 Analytical procedures

For batch tests, samples of 1 ml were taken from cultures through the rubber septum of serum vials with sterile syringes. For chemostat tests, cultures were taken via sampling vial device of the reactor vessel. Cultures were checked for purity by plating on TSA, incubated aerobically at 28°C for several days and checked under phase contrast light microscopy. Colorimetric determination of NH_4^+, NO_2^-, and NO_3^- was performed. Finally, growth was always determined by measuring OD_{600} of a 100 µl sample in duplicate in microtiter plates and standardized to 1 cm path length using PathCheck Sensor of the spectrophotometer (Molecular Devices, Spectramax plus 384, USA).

For colorimetrics of the samples from batch tests, 500 µl sample was pre-treated with 2.5 ml of 2 mM potassium chloride (KCl) by shaking 1h at 150 rpm and subsequent filtration (0.2 µm) to extract inorganic nitrogen and remove interfering compounds. After centrifugation at 18000 g for 2 min to remove the cells, samples were kept frozen at -20 °C. Samples of the chemostat, after addition of two drops of chloroform in order to stop biological activity, were kept frozen at -20 °C. NH_4^+ concentration was determined with the salicylate-nitroprussidine method [30], NO_2^- and NO_3^- concentrations were determined with Griess reaction [31] and Griess reaction with cadmium [32, 33], respectively. Standard curves covered concentration ranges corresponding to those of the media and were strictly linear with an R^2 of 0.99. For analysis of N_2O, 1 ml samples of the headspace of serum vials or chemostat were taken via the rubber stoppers with sterile syringes, and injected into gas chromatograph (Compact GC with EZChrom Elite Software, Interscience, the Netherlands). N_2O production was corrected

for pressure and solubility based on Henry’s law, using Henry's law constant of 0.025M/atm [34] and calculated to concentrations in the culture in each vial.

Statistical differences in the parameters analysed were assessed using paired t-test, independent t-test after Levene’s test for equality of variances, One-Way ANOVA after Levene’s test for equality of variances, post-hoc testing or nonparametric test-Kruskal-Wallis test in IBM SPSS23.

5.3 Result and Discussion

5.3.1 Gene knock out tests were unsuccessful

Unfortunately, in the preliminary electroporation tests, pNW33N could not be successfully electroporated into *B. azotoformans* LMG 9581^T under conditions tested, most probably due to (i) the limitation of the parameters setting of the pulse controller (Bio-Rad) with only 2mm cuvette and maximum 2.5 kV/cm that can be tested, or (ii) electroporation method was not effective for the slow grower *B. azotoformans* LMG 9581^T. Since many closely related bacteria such as *Paenibacillus alvei* CCM 2051^T [28], *Bacillus anthracis* [29, 35], *Bacillus cereus* ATCC14579 [36] were successfully transformed with external DNA under strain-dependent preferable electroporation setting of 17.5kV, 2.5 kV and 20 kV, we still believe it is highly possible that *B. azotoformans* LMG 9581^T could be successfully transformed with external plasmid under suitable electroporation parameters. Unfortunately, it was not possible to allocate more time for further exploring electroporation thus this line of experiments was abandoned.

5.3.2 NO_3^- concentrations and molar C/N-NO_3^- ratios under low NO_3^- concentration do not trigger DNRA in batch tests

In the tests of six different NO_3^- concentrations (0.2 mM, 0.5 mM, 1 mM, 2 mM, 4 mM and 10 mM), *B. azotoformans* LMG 9581^T only demonstrated a denitrification phenotype, with NO_3^- gradually consumed and the N_2O produced accounting for all initially provided NO_3^- in all conditions tested (Figure 5.2). More N_2O was produced with increased NO_3^- concentrations ($p< 0.002$) (Figure 5.2). Production of NH_4^+ was absent under all conditions tested ($p> 0.05$), but NH_4^+ was assimilated only with 10 mM NO_3^- condition. Maximal cell density (maximal OD_{600}) correlated with the NO_3^- concentration as expected, being higher with NO_3^- concentrations of or above 1 mM ($p < 2.02\times 10^{-8}$) but with no significant

difference when NO_3^- concentration was 0.2 mM or 0.5 mM (p= 0.257) which may due to measurement limitation of the spectrophotometer or maintenance of energy in the cells.

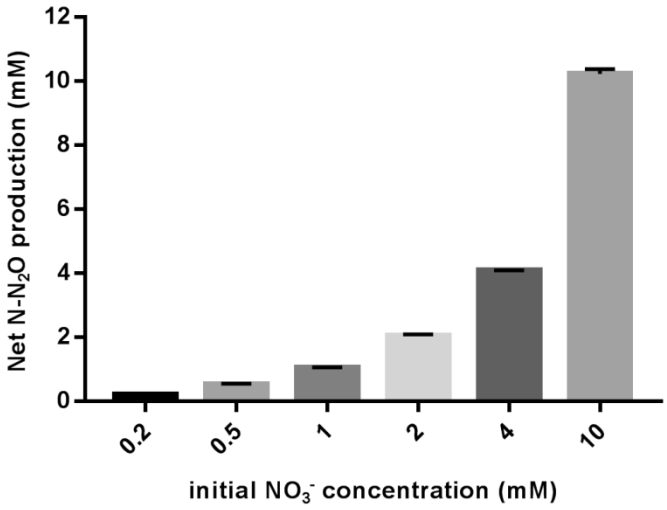

Figure 5.4 Effect of NO_3^- concentration on NO_3^- partitioning in *B. azotoformans* LMG 9581^T. Net N-N_2O production (mM) in media with different NO_3^- concentrations (n = 3).

Next, low concentrations of NO_3^- (0.2 mM and 1 mM), which supposedly favour DNRA over denitrification [11], were applied at different C/N-NO_3^- ratios (1.5, 3, 7.5, 15, 30, and 150) with and without 4.6 mM NH_4^+. Again, all NO_3^- was gradually consumed and the N_2O produced accounted for all initially provided NO_3^- (Figure 5.3). For a C/N-NO_3^- ratio of 150, especially with 1 mM NO_3^- and 4.6 mM NH_4^+, growth rate decreased drastically from 0.0011 ± 0.0002 / h to 0.0006 ± 5.7735×10^{-5} / h (p < 0.01), reaching stationary phase after six instead of three days, with all NO_3^- converted to N_2O (a repeat of this experiment is shown in Figure S1). In a control experiment *Paracoccus denitrificans* LMG 4049, a canonical denitrifier lacking the genetic ability to perform DNRA, in similar C/N-NO_3^- ratio tests also showed a slower growth under C/N-NO_3^- ratio of 150. This indicated that the observed decreased growth rate was not linked to a potential induction of DNRA but rather resulted from the excess of carbon in the medium (Figure S2).

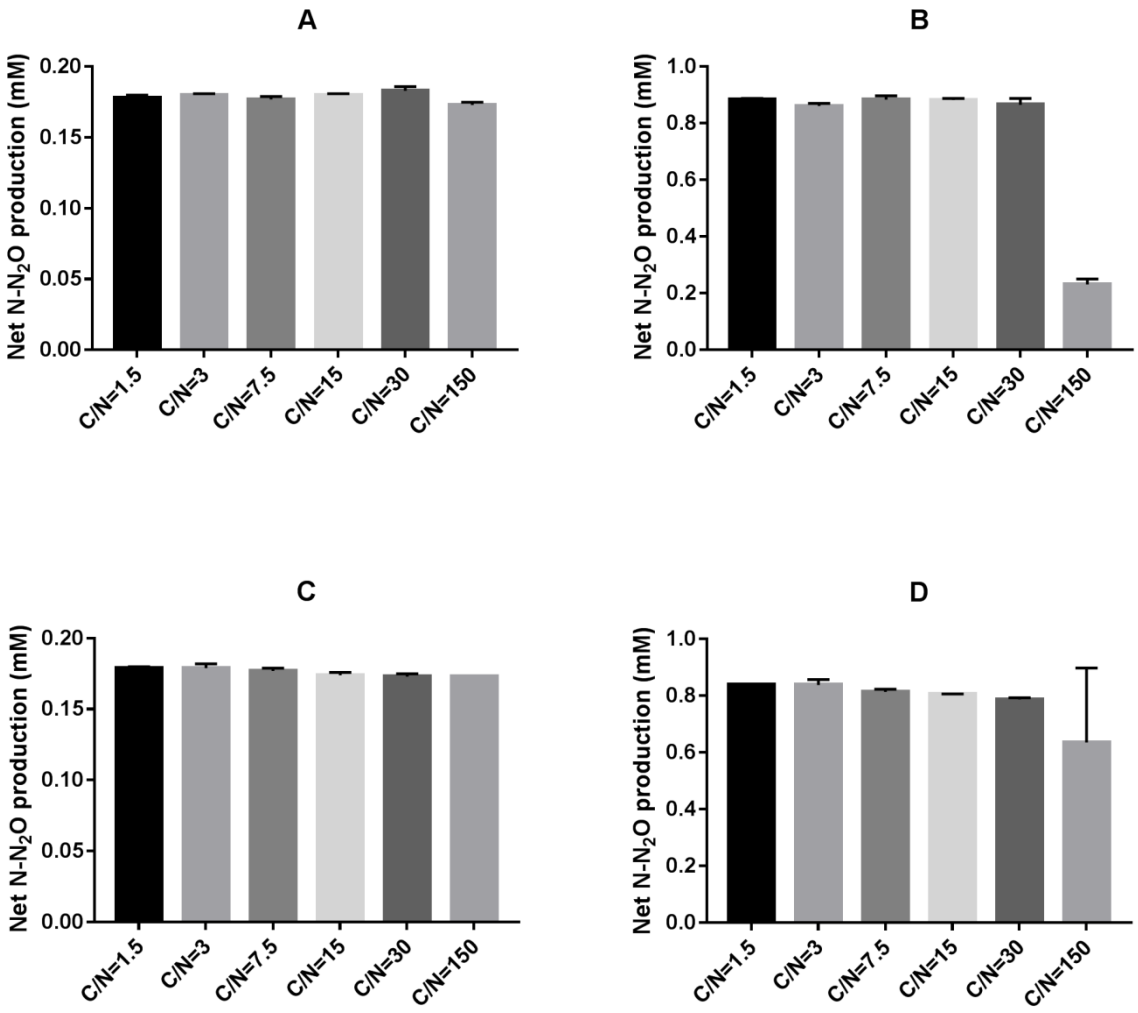

Figure 5.5 Effect of C/N-NO_3^- ratios under NO_3^- concentration of 0.2 mM or 1 mM on NO_3^- partitioning in *B. azotoformans* LMG 9581^T. Net N-N_2O production (mM) after three days anaerobic incubation in mineral media with different C/N-NO_3^- ratios (1.5, 3, 7.5, 15, 30, 150, n = 3): with 4.6 mM initial NH_4^+ with 0.2 mM initial NO_3^- concentrations (A), with 4.6 mM initial NH_4^+ with 1 mM initial NO_3^- concentrations (B), without initial NH_4^+ with 0.2 mM initial NO_3^- concentrations (C), without initial NH_4^+ with 1 mM initial NO_3^- concentration (D).

Unexpectedly, neither low NO_3^- concentrations nor high C/N ratios, both already described to regulate partitioning of NO_3^- to denitrification and DNRA in *S. loihica* PV-4 [11], seemed to stimulate dissimilatory NO_3^- reduction to NH_4^+, instead, all NO_3^- was denitrified in our tests. This may be due to distinct strain-specific features or regulation features. The strain studied here is a Gram-positive bacterium originating from soil, while *S. loihica* PV-4 is Gram-

negative isolated from marine environment, which indicates different physico-chemical environmental preferences. Another more plausible explanation is that nutrients and other medium parameters were continuously changing during bacterial growth in batch cultures, which can result in unstable and unexpected physico-chemical factors that not positively promote the DNRA process. This was clearly exemplified by *S. loihica* PV-4 [11] that when incubated under high C/N-NO_3^- ratios (favouring DNRA) in batch, carried out both denitrification and DNRA, while under the same C/N-NO_3^- ratios combined with other stable physico-chemical factors in chemostat, performed only DNRA. Although other conditions such as high pH [11, 17], high temperature [18, 19], high NO_2^-/ NO_3^- ratio [17, 18, 20, 21, 37], other carbon sources such as sodium lactate [11] instead of sodium acetate that was used in our study [16], are plausible inducers of DNRA over denitrification, due to time constrains we did not test these in batch experiments but switched to the continuous incubation set up that was proved more efficient for enrichment of DNRA strains [12] and activation of DNRA pathways [7, 11].

5.3.3 High C/N- NO_3^- ratio with low NO_3^- concentration shows incomplete denitrification in chemostat tests

A chemostat was set up and employed to check the functionality of DNRA in *B. azotoformans* LMG 9581^T, with three kinds of mineral media containing 1 mM, 2 mM or 10 mM NO_3^-, under variable C/N-NO_3^- ratio of 15, 7.5, 1.5, respectively (Figure 5.4). With 1 mM NO_3^- and C/N-NO_3^- ratio of 15, NO_3^- was completely consumed after 1 day incubation, with constant NO_2^- accumulated in the media (0.70 ± 0.04 mM, n=4), N_2O gradually accumulated after NO_3^- was consumed. Growth (using CO_2 production as proxy) reached steady states after 3 days incubation (Figure 5.4). There was 0.16 mM ± 0.005 mM NH_4^+ increase after 2 days incubation, but followed by a 0.23± 0.10 mM decrease afterwards. However, this variation is

within the error range of NH_4^+ measurements (4.52 ± 0.10 mM, n=5). Using 2 mM NO_3^- and C/N-NO_3^- ratio of 7.5, similar as the test above, NO_3^- was completely consumed after 1 day incubation, and growth reached steady state after 3 days. In contrast, NO_2^- concentration increased and subsequently decreased, with no measurable NO_2^- left after 8 days incubation. More N_2O was produced and accumulated in the media and reached steady state when NO_2^- was depleted. NH_4^+ concentration fluctuated (+/-0.08 mM) during incubation, but in the end showed an obvious decrease of 0.22 ± 0.01 mM. Comparing these two tests, the initial NO_3^- of 1 mM and 2 mM was similarly consumed after 1 day incubation and no NH_4^+ was produced, indicating DNRA was not functional under both conditions. However , surprisingly the NO_2^- concentration remained steady after 1 day incubation in the former test, while with more NO_2^- produced in the latter test, this was completely consumed and remained 0 mM, indicating a different physiological strategy involved.

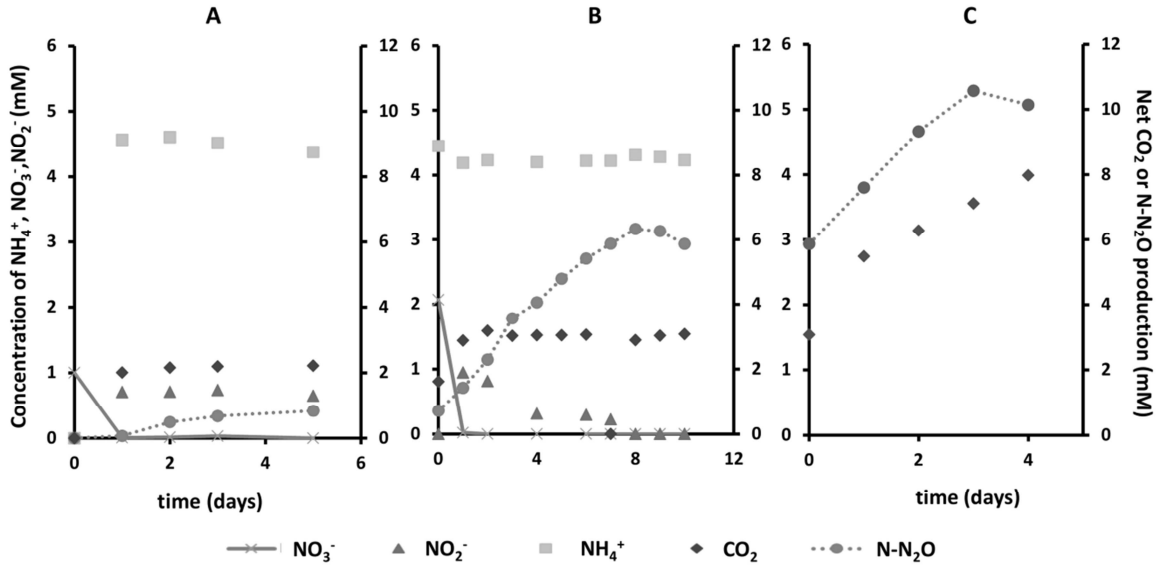

Figure 5.4 Effect of continuous incubation on NO_3^- partitioning in *B. azotoformans* LMG 9581^T. Concentration of NH_4^+, NO_3^-, NO_2^- (mM) in the chemostat and CO_2 (proxy for growth) or N_2O production from 1.5 L culture (mM) over time during anaerobic incubation in mineral media with different composition: with 1 mM NO_3^- concentration under C/N- NO_3^- ratio of 15 (A), with 2 mM NO_3^- concentration under C/N- NO_3^- ratio of 7.5 (B), with 10 mM NO_3^- concentration under C/N- NO_3^- ratio of 1.5 (C). Only averages of data at each time points (n ≥2) are shown, error bars are not indicated.

Unfortunately, in the test with 10 mM NO_3^- and C/N- NO_3^- ratio of 1.5, an unexpected production of CH_4 ((9.93 ± 0.07) × 10^{-2} mM) was observed. After checking previous chemostat incubations, CH_4 production was found constant during all incubations, but not at the start of incubation nor from non-inoculated media. In addition, no contamination was found on the daily purity check with the culture growing aerobically on TSA plate or by visual observation under the light microscope (Figure 5.5). Therefore, we speculated that there was contamination of anaerobic methanogenic Archaea throughout the chemostat. Experiments were stopped to take measures to eliminate the contamination. However, after rinsing all glassware of the bioreactor overnight with 1M HCl and two consecutive autoclave cycles of the whole system under 120 °C 20 min (2 days in total for the program for bioreactor set up), the contaminating methanogen(s) could not be eradicated. Additional tests again demonstrated steady CH_4 production in all conditions tested. Due to time constraints and this persistent contamination, chemostat tests were terminated.

Figure 5.5 Images of microscope-purity check of *B. azotoformans* LMG 9581^T culture from the chemostat tests. The culture seemed pure based on the cell morphology observed under microscope.

Irrespective of the contamination issue and early abortion of the chemostat experiments, one very promising observation was made. As mentioned above, only in chemostat more NO_2^- accumulated under lower NO_3^- concentration of 1 mM and higher C/N-NO_3^- ratio of 15, compared with higher NO_3^- concentration and lower C/N- NO_3^- ratios. We interpreted this raised NO_2^- accumulation as indicative for a shift or initiation of another metabolism, i.e.

DNRA that was expected to take place under those conditions based on the bacteria energy strategies [38]. Although we cannot provide solid data to support this hypothesis, as steady state was not continued for longer than three days for all conditions (as little was known about incubation time required for activation of DNRA in *B. azotoformans* LMG 9581^{T}, we opted to completely refresh at least twice with same medium after growth reached steady state, but indeed longer incubation times may make a difference), this condition in chemostat set-up should be revisited to confirm activation and functionality of DNRA in *B. azotoformans* LMG 9581^{T}.

5.4 Acknowledgement

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5.5 Supplementary information

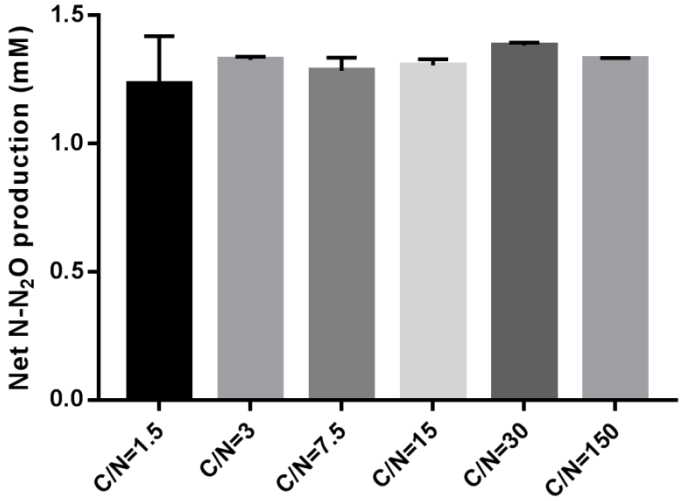

Figure S1 Effect of C/N-NO_3^- on NO_3^- reduction of *B. azotoformans* LMG 9581^T. Net N-N_2O production (mM) in media with 1mM NO_3^-, 4.6mM NH_4^+ under different C/N-NO_3^- ratio (1.5, 3, 7.5, 15, 30, 150, n = 3) after 6 days anaerobic incubation. Comparable N-N_2O production was obtained under different C/N-NO_3^- ratio.

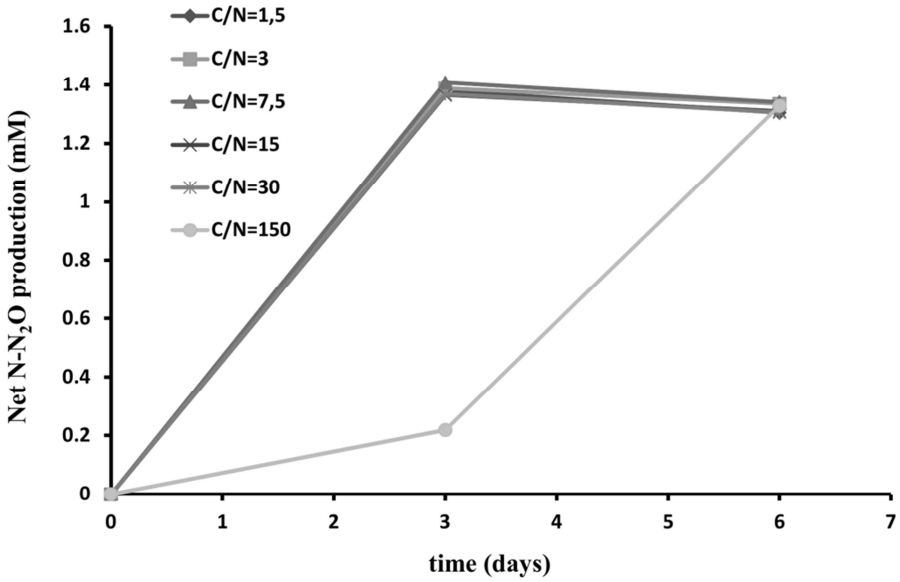

Figure S2 Effect of C/N-NO_3^- on NO_3^- reduction of *P. denitrificans* LMG 4049**.** Net N-N_2O production in media with 1.4 mM NO_3^-, 4.6mM NH_4^+ under different C/N-NO_3^- ratio (1.5, 3, 7.5, 15, 30, 150, n = 3) during 6 days anaerobic incubation. Comparable N-N_2O production was obtained in the end but N-N_2O production in the medium with C/N-NO_3^- ratio of 150 was slower than the others under lower C/N-NO_3^- ratio.

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Chapter 6:

General discussion, conclusions, and perspectives

6.1 Ecological relevance

6.1.1 Contribution of DNRA to N_2O emission

The anthropogenic activities during the past centuries such as agriculture and industrial practices have contributed to a major global nitrogen imbalance with a series of consequences including the increase of the greenhouse gas N_2O. As the major source of N_2O, soils account for an estimated 69% of its anthropogenic emissions [1]. However, soil management to mitigate N_2O emissions is limited due to poor understanding of the underlying mechanisms leading to N_2O production such as nitrification, denitrification and DNRA and related basic controls of enzyme regulations. Similar to anammox, which was long disguised as denitrification in estimates on global anaerobic NO_3^- removal [2], N_2O emitted via DNRA may often have been wrongly attributed to denitrification [3-5] and thus not considered to be a significant contributor. Indeed, in the chapters 2 and 3, I demonstrated that *B. paralicheniformis* and *B. licheniformis*, previously described as denitrifiers, actually perform DNRA. I confirmed the potentially substantial contribution of DNRA to N_2O emission, with up to one third of all initial NO_3^- being converted to N_2O. In addition, the concentration-dependent effect of NO_3^- and NO_2^- on N_2O production was demonstrated, which contributes to the currently scare knowledge of DNRA influencing environmental factors, with only C/N-NO_3^- ratios influencing NO_3^- partitioning to N_2O in DNRA investigated [6]. I acknowledge that the NO_3^- concentrations (up to 15 mM) tested in this dissertation were quite high. Nevertheless, NO_3^- concentrations are very variable in soil, both spatially and temporally [7], with a general concentration range from a few hundred µM to around 20 mM, the highest up to 70 mM. Based on my results, I can speculate that in soils with high concentrations of NO_3^-, for example shortly after fertilization events, DNRA activity can significantly contribute to N_2O emission. Field studies need to confirm these speculations.

Unfortunately, as (i) several mechanisms can be responsible for the N_2O production in DNRA, (ii) most genes encoding N_2O producing enzymes have dual functions (see Chapter 1 and 2), and (iii) those genes may not always be functional for N_2O production under fluctuating environmental conditions, it is (still) impossible to evaluate the N_2O production from DNRA *in situ* via molecular approaches. Batch incubations of environmental samples with addition of $^{15}N\text{-}NO_3^-$ using isotope pairing will also provide little conclusive evidence for DNRA, as it will render $^{15}N\text{-}N_2O$, similar to denitrification. A more promising and high-tech approach might be the use of isotopic composition analysis [8, 9] to differentiate between different N_2O production pathways. For example, intramolecular distribution of ^{15}N [10] or ^{18}O [9] has the potential to assist the identification and quantification of different sources and sinks of N_2O in the atmosphere, even between denitrification and nitrification [11, 12], on the basis of different enzymes and substrates involved. Although this isotopic signature has been widely employed in quantifying N_2O produced by ammonia-oxidizing archaea [13, 14] and ammonia-oxidizing bacteria [15], there are no reports yet on differentiation of N_2O produced from denitrification or DNRA. However following the same principle, it is theoretically possible that the intramolecular ^{15}N-site preference (SP) in the asymmetric N_2O molecule [10, 16] may offer the potential for estimating N_2O produced from NO_3^- ammonifiers *in situ* in the near future.

6.1.2 Microdiversity

Nowadays widely applied tools for microbial community assessment are sequence-based approaches, such as house-keeping gene sequencing (16S rRNA gene or *gyrB*) or functional gene sequencing. In general, some of these tools, for example rRNA sequencing are not adequate to type large numbers of strains [17] or closely related strains, and PCR-dependent surveys for specific metabolism also lead to underestimation of the diversity due to primer

coverage limitations [18, 19]. Furthermore, wide metabolic versatility within one genus or even species has been described previously, such as for *Escherichia coli* [20-22], *Prochlorococcus* [23, 24] or *Vibrio* [25, 26], exemplifying that distinct taxa do not necessarily demonstrate a specific primary/secondary metabolism. As we know from denitrification studies, identical denitrifying ability can be found within distantly related bacteria [27], and on the other hand closely related bacteria do not necessarily share identical physiologic capacity to denitrify [28]. The study on *Bacillus* at LM-UGent [29-31] so far has also demonstrated a variety of dissimilatory NO_3^- reduction pathways and capacities within this genus. The observations support a molecular approach focusing on functional genes rather than taxonomic markers to monitor microbial diversity contributing to ecosystem functions, most preferably via shotgun sequencing to avoid primer bias.

However, even when closely related bacteria share the same (nitrogen) metabolism and contain an identical functional gene inventory, they can have different capacities to express those genes. Work from LM-UGent on strains from the methanotrophic genera *Methylomonas* [32] and *Methyloceanibacter* [33] has recently elegantly shown that intragenus or intraspecies metabolic versatility is not always linked to genetic variation revealed by sequencing. Furthermore, work of Liu et al. [34] shows that strains in genus *Thauera* exhibit remarkably different denitrification phenotypes which are not in correlation with phylogeny analysis based on 16S rRNA and functional genes. In Chapter 2, I showed that the phenotypic heterogeneity between *B. paralicheniformis* LMG 6934 and LMG 7559 could not be linked to their DNRA genes duplication. In contrast, *B. paralicheniformis* LMG 7559 and *B. licheniformis* LMG 17339 shared a similar DNRA phenotype although the former had two copies of the *nar* operon while the latter only one. The reason for this apparent observed *ex situ* inconsistency between phenotypic heterogeneity and genotypic heterogeneity most probably lies in the distinct genetic regulations involved. Although regulation genes encoded

in the three *Bacillus* strains studies are identical and have the same relative genome locations, they may be activated under different physico-chemical conditions. Unfortunately, denitrification work on model organisms has already demonstrated that its regulation is very strain-dependent [35-39] and this is most likely also the case for DNRA, for which little to no studies on its regulation are available. It is clear that further investigation on DNRA in *Bacillus* should focus on the elucidation of differences in regulation mechanisms and links to observed phenotypes, with a thorough analysis of the regulatory motifs of TnrA, GlnK, Fnr, DnrN, NarX, NarL, NsrR [31, 40] as well as knockout studies.

6.1.3 Unknown niche differentiation hinders estimation of N_2O production from ammonifiers and denitrifiers

Thanks to their resistant endospores, *Bacillus* are among the most robust bacteria on earth that have been found in soil, sediments, air, marine, human systems and extreme ecosystems, [41]. Generally soil is thought to be the primary habitat of many *Bacillus*. Remarkably, *Bacillus* strains were isolated from 70% of 1115 different soil samples from all over the world [42], encompassed 1-2% of soil bacterial 16S rRNA gene sequences in a meta-analysis of 32 soil cone libraries [43] and comprised 5-45% of isolates from cultivation studies of soil, showing their wide existence and important status in soil ecosystems. Actively growing *Bacillus* are assumed to be associated with soil organic matter or the plant rhizosphere where the carbon and nitrogen are not strongly limited but physico-chemical status (SOM, C/N-NO_3^- ratios, oxygen content) may vary considerably even at micrometer distances, which further leads to different microniches and diverse physiological behaviors *in situ*. Because of this niche differentiation, *Bacillus* strains can demonstrate differences in various traits and functionalities, concerning morphology, metabolism, etc. (P. Stefanic, M. Crnigoj, and I. Mandic-Mulec, unpublished data). However, fine soil chemistry parameters *in situ* are rarely

recorded during isolation campaigns and therefore this information is not available for *ex situ* studies for which mimicking the natural ecologic conditions is the condition *sine qua non* for better understanding of organismal functionalities. Therefore, further estimations of phenotype and metabolism in the field, based on data gained from our study with pure cultures and observation of DNRA in soil *Bacillus*, may be hindered at present. Future *in situ* surveys of soil properties (and their seasonal change) (e.g. with improved soil probes) and microbial studies (molecular techniques, extended cultural campaigns) should be combined and on this basis, estimation of N_2O emission from DNRA/denitrification or application of relevant N_2O mitigation strategies will be more plausible.

In soil, interactions between different microorganism within the microbial community or with plants or metazoa can strongly affect the soil properties *in situ* and one step further, physiological behavior of microorganisms [41]. Here we only address the general interplay between plants and *Bacillus* as an example. Some *Bacillus* are considered to be plant-beneficial rhizobacteria. They influence plant health, growth and development by direct or indirect actions affecting soil chemical properties at the root (by nitrogen fixation [44], phytohormone production, phosphorus [45-47]/zinc solubilization [48]). Specific *B. subtilis* strains can influence the outcome of infection on plants significantly, by triggering induced systemic resistance or changing transcriptional response in plants [49, 50]. In turn, plants influence the distribution, diversity and activities of *Bacillus* in many different aspects. As mentioned above, *Bacillus* (since most are aerobic heterotrophic saprophytes) may prosper in environments where carbon and nitrogen are not very limited. In soil, plant litter or root exudates provide ample organic carbon, making vegetative bacilli predominate therein [51]. To a certain degree, this plant litter and its degradation products determine the soil chemical properties (e.g., carbon, pH). So, characteristics of the soil niches will influence the phenotype of *Bacillus* [52, 53] and most likely induce a specific physiological adaptation in the cells [54].

This may be the explanation for the need of organic nitrogen for the incubation of *B. azotoformans* LMG 9581^T and its high degeneracy of nitrogen assimilation pathways in the genome.

6.1.4 Contribution to N_2O mitigation strategies

Although DNRA has been widely recognized for at least 35 years, the global N_2O emission from DNRA is yet not known. The question can be raised about the cause of this knowledge gap. Certainly it is partially due to its non-stoichiometric relation with the metabolized nitrate and consequently appearance as a limited byproduct. Another, more technical reason is that it was not possible to differentiate between N_2O produced via DNRA and via denitrification. All N_2O was thought to result from denitrification, which together with nitrification contributes to more than two third of the total N_2O emission on earth. In 1988, Cole et al. has suggested typically 1% of NO_3^- or NO_2^- reduction by DNRA performing microorganisms goes to N_2O production [55]. As denitrification only accounts for less than half of the total NO_3^- removal in soils and aquatic sediments based on direct assays e.g. acetylene block techniques [56], we can assume if DNRA accounts for the rest of NO_3^- removal, that means up to one sixth of NO_3^- removal may go to N_2O emission (if anammox and other less known nitrate reduction pathways are not taken into account). Furthermore, DNRA could account for up to 75 % [57] to 100% of N_2O produced in forest soil, mangrove soil or estuary sediments, indicating a large N_2O emission that should not be neglected.

Nowadays, the N_2O emission has attracted much attention and many strategies for mitigation of N_2O emission have been suggested and applied in agriculture recently, such as controlled release fertilizers [58], systematic crop rotation system [59], liming [60], manure addition, commercial nitrification inhibitors [61, 62], etc. In addition, based on current knowledge of the two dissimilatory NO_3^- reduction pathways accompanied with N_2O emission, especially

with our work experience with culturing these N_2O producers and achievements of this study, alternative approaches can be suggested. Firstly environmental conditions could be altered so as to stimulate the least N_2O producing process in that environment. DNRA is generally favored in high C/N-NO_3^- ratios, high pH and high temperature and is capable of converting up to 32% of NO_3^- to N_2O; conversely, denitrification is favored in low C/N-NO_3^- ratios, low pH, low temperature, with an equivalent amount of NO_3^- converted to N_2O as an intermediate or as end product when NosZ is lacking or less functional at low pH [38]. From my study, it is clear that high NO_3^- concentration, high NO_2^- concentration, and high NH_4^+ concentration promotes NO_3^- partitioning to N_2O in DNRA. The C/N ratios in marine sources are usually in the range of 4-10:1, whereas higher ratios are more likely from a terrestrial source [63]. Thus generally it is suggested that DNRA may be more favored in terrestrial systems than in the ocean. However combined with other parameters such as pH, temperature and microbial communities, especially with the unknown N_2O production ability in ammonifiers and the unknown portion of *nosZ*-containing bacteria among denitrifies, it is hard to estimate how much N_2O is produced from DNRA versus denitrification when only based on these general findings. This further hinders the exploration of an overall approach for mitigating N_2O emission. Nevertheless, for certain ecosystems, where the physico-chemistry *in situ* is known and estimation of N_2O emissions from either DNRA or denitrification are available by using the promising approach-isotopic composition analysis (see above), it may be possible to adjust the relative ratio of the dissimilatory processes by controlling the environmental conditions by, for example, adding more organic matter and liming, to mediate the N_2O emission. In addition, based on the environmental NO_3^-/NO_2^-/NH_4^+ concentration *in situ* and the observations of pure culture study in this work, future estimation of N_2O production of those bacteria in the field becomes possible.

Secondly, in agricultural settings it may be beneficial to promote DNRA over denitrification based on the parameters outlined above, because the ammonium produced will increase the efficiency of fertilizer use by plants and thus allow a reduction in fertilizer application.

Finally, since the recently discovered atypical NosZ reductases were proven functional (i.e. producing N_2 from N_2O) in non-DNRA and non-denitrifying bacteria and are believed potentially to act as an important N_2O sink, I think these N_2O producers could potentially be very valuable in the mitigation of N_2O emission through their application in N_2O producing ecosystems. As most of the N_2O emission increase results from agricultural activities, application of these bacteria in soil environments especially agriculture fields, may be the most efficient as N_2O sink [40, 64]. Undoubtedly, this approach may change the microbial community structure to a certain degree, thus it is quite risky and should be seriously considered before testing in the field. For example, system-based predictive models [65-67] that can simulate ecosystem management practices and perturbations to predict community fluctuations could be used for risk assessment.

6.2 Future perspectives

6.2.1 New insights in NO_3^- partitioning by *Bacillus azotoformans* and future research

At the very start of this PhD work, early 2013, my main research objective was to show for the first time the functionality of both DNRA and denitrification in a single organism, i.e. *B. azotoformans* LMG 9581^T. In 2012, Sanford et al [64] already mentioned the co-occurrence of both pathways in a single organism based on whole genome sequence analyses and in 2012 at LM-UGent the same was observed for *B. azotoformans* LMG 9581^T [30]. Unfortunately, our attempts to confirm the functionality of DNRA in denitrifier *B. azotoformans* LMG 9581^T failed. None of the different C/N-NO_3^- ratios or different NO_3^- concentrations tested in batch set-up promoted DNRA and only a denitrification phenotype was observed (Chapter 5). This was different from results published in the meanwhile on *Shewanella loihica* PV-4 [68], a Gram-negative marine bacterium that also contains both denitrification and DNRA and did demonstrate DNRA in batch under high C/N-NO_3^- ratios in combination with low NO_3^- concentration, pH above 7.0 and temperature over 30 °C. The reason why DNRA was not favoured in our strain may be (i) a taxon-dependent feature, a Gram-positive *Bacillus* and a Gram-negative *Shewanella* are evolutionary not closely related, (ii) an environmental-related feature, with soil (*Bacillus*) and seawater (*Shewanella*) being completely different physico-chemical environments with different spatio-temporal variabilities, (iii) a regulatory feature, knowing that regulation can be very distinct even between rather closely related denitrifiers (e.g. *Agrobacterium* versus *Bradyrhizobium* [35]). Unfortunately, subsequent preliminary chemostat experiments were unable to resolve the question whether DNRA can be functional in *B. azotoformans*. Under low NO_3^- concentration (1 mM) only incomplete denitrification was observed, with accumulation of NO_2^-, which may suggest a regulatory switch to DNRA. While it is possible that DNRA was not yet activated because of the short incubation time of 5 days in our set up for the relatively slow grower *B. azotoformans* LMG 9581^T compared with

S. loihica PV-4, we have no data to support this hypothesis, again due to time constraints and repeated contamination with a methane-producer that could not be eradicated with successive autoclavation (Chapter 5).

Despite the many problems and mostly negative results, I am still convinced that my initial experimental design to demonstrate the functionality of DNRA and understand the underlying regulatory mechanisms of NO_3^- partitioning via mutant strains in chemostat experiments, is the correct and most suited approach, even with the current state-of-the-art four years later. However, in hindsight, the project was too ambitious to tackle outside a research consortium grouping all the necessary expertise and equipment. Nevertheless, I hope by describing my ideas, progress and failures here that cumulative research by others could re-visit this approach, both with LMG 9581^T as well as the recently published microaerotolerant obligate denitrifier *B. azotoformans* MEV2011[69]. My specific suggestions for continuing this study on identification of the physico-chemical factors promoting NO_3^- reduction by denitrification or DNRA are again (i) physiological tests in batch and/or chemostat under other physico-chemical conditions possibly promoting DNRA, such as other suitable carbon sources (lactate, succinate, glucose, etc.), higher pH (over 7), different NO_3^- to NO_2^- ratio, and (ii) gene knock-out tests with other feasible electroporation strategies (Figure 5.1). An alternative objective is the regulation mechanism of the redundant denitrification pathway under different physico-chemical factors and its ecological relevance, which requires much less time-consuming work.

6.2.2 Future DNRA study

The mechanism of N_2O production in DNRA requires further confirmation with the hypothesis of cooperative action of Nar, NirB, Hmp or qNor (Chapter 2). Since Nar also can reduce NO_3^- to NO_2^-, gene knock out together with physiological tests under variable concentration of NO_3^- or NO_2^- can certainly confirm its dual function. Since certain *Bacillus*

strains possess two variant copies of Nar, knock out mutants of each copy respectively will complete the understanding of the function of Nar. Similarly, the role of other reductase in N_2O production can also be verified by gene knock out experiments followed by physiological tests. In addition, transcript abundance analysis (qPCR) can also be an alternative approach although not as efficient as the former. Since regulation of DNRA is still underexplored and remains as urgent research question, it should be concerned in future studies as well. A further interesting topic of *Bacillus* is the niche differentiation and interactions of *Bacillus* strains within the community structure *in situ*. By connecting pure culture study in the lab with filed study, this future research will certainly add more value to the application of the achievements of this study into practical mitigation strategies of N_2O emission.

To conclude, with varying success I have described in this dissertation the study of four *Bacillus* strains. However, the BCCM/LMG bacteria collection and the LMG-UGent Research Collection hold many more *Bacillus* strains that can be subjected to a physiology survey. This would definitely uncover more of the genus' already broad versatility in dissimilatory NO_3^- reduction and provide more interesting examples of microdiversity, as such further refining our knowledge of the ecological niche *Bacillus* can occupy in soil and other environments. Luckily today more studies are initiated into the underexplored dissimilatory NO_3^- reductions in *Bacillus* [6, 40] and more genome data [69, 70] are available to assist the interpretation of physiologic observations, enzymatic pathways and mechanism regulations involved in *Bacillus*. It is my scientific hope that *Bacillus* can become one of the model groups for denitrification and DNRA in the near future.

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Summary

N_2O is a potent greenhouse gas, having a 300 times higher warming potential than CO_2, and a contributor to ozone layer destruction. What's more, the anthropogenic activities during the past century such as agriculture and industrial practices have contributed to major global imbalances in the nitrogen cycle including the increase of atmospheric N_2O. Without a proper understanding of N_2O emissions, the enzymatic production pathways and contributing environmental factors, it is not possible to put forward and organize efficient mitigation strategies for N_2O emission. Hence, it is of great importance to gain more knowledge of the N_2O producing processes. There are three such microbial processes that have been studied thus far: denitrificaion, dissimilatory nitrate reduction to ammonium (DNRA), and nitrification, with DNRA the least known. This PhD research studied the former two pathways in *Bacillus* with a focus on the underexplored N_2O production from DNRA, aiming at finding phenotypic evidence for genome-based hypothesized NO_3^- reduction metabolisms for *Bacillus*.

Firstly, the NO_3^- reduction metabolism was investigated in closely related *B. paralicheniformis* and *B. licheniformis* strains. The physiological data from anaerobic growth experiments proved that the strains studied are not denitrifiers but rather ammonifiers performing DNRA with high N_2O production as side-product. Strain-dependent phenotypic differences in nitrogen metabolism were revealed and hypothetical pathways for N_2O production were proposed based on our physiological observations. The influence of different physico-chemical factors on NO_3^- partitioning to NH_4^+ or N_2O was investigated, demonstrating that both NO_3^- and NO_2^- concentration had a significant effect on NO_3^- partitioning, which may be a consequence of the (transient) accumulation of NO_2^-. The NH_4^+ concentration under fixed C/N- NO_3^- ratios also has influence on NO_3^- partitioning.

Secondly, since denitrification and DNRA were previously thought to be mutually exclusive, the presence of these two pathways, with a remarkable redundancy of dissimilatory nitrogen reduction genes in *B. azotoformans* LMG 9581^T, allowed us to set up experiments to explore nitrate partitioning towards both pathways in this promising bacterium. Based on the gene repertoire in its genome, the nitrogen assimilation metabolism of *B. azotoformans* LMG 9581^T was studied, leading to the conclusion that organic nitrogen was required for assimilation and NH_4^+ alone could not efficiently support growth under both aerobic and anaerobic conditions. However, NH_4^+ was indeed assimilated and had a concentration-dependent influence on growth rate but not on maximal cell density. The underlying mechanisms however remained unclear.

Next, different physico-chemical conditions to demonstrate functionality of DNRA in *B. azotoformans* LMG 9581^T were investigated. In batch set-up, variable C/N-NO_3^- ratios under high and low NO_3^- concentration could not induce DNRA. Since other DNRA strains had been shown to be notably functional in chemostat rather than batch, a chemostat set up was designed as an alternative to the batch tests. Unfortunately, persistent contamination issues required an early termination of this line of investigation. However, with the limited physiological data from the chemostat tests, a less efficient denitrification and accumulation of NO_2^- were observed, which could not be explained by the difference in NO_2^- reduction capability but may be due to a shift or initiation of another metabolic process, possibly DNRA. However, confirmation is required through future studies.

In conclusion, this PhD dissertation explored and extended current knowledge on the dissimilatory nitrate reduction processes of denitrification and DNRA in *Bacillus*. It contributed new physiological and genomic data on the less well studied DNRA process in this genus. Various environmental drivers on denitrification and DNRA were elaborately studied in *Bacillus*, although more research remains required. Thus we highlight the

requirement of future studies on *Bacillus* strains, and especially *B. azotoformans* LMG 9581^{T} which possesses genes for the two dissimilatory nitrate reduction pathways. Although so far our chemostat and gene knockout experiments in this bacterium failed, it still provides unique opportunities to obtain valuable understanding of the regulatory mechanisms in nitrate partitioning.

Samenvatting

Met een warmte absorberend vermogen dat 300 maal groter is dan dat van CO_2, is N_2O is een krachtig broeikasgas dat ook bijdraagt aan de vernietiging van de ozonlaag. Bovendien hebben menselijke activiteiten zoals landbouw en industriële processen gedurende de laatste eeuw bijgedragen tot enorme globale verschuivingen in de stikstofcyclus waaronder een belangrijke toename aan atmosferisch N_2O. Zonder een goed begrip van N_2O vorming, de onderliggende enzymatische reacties en omgevingsparameters de processen drijven, is het onmogelijk om maatregelen voor te stellen of te implementeren om de N_2O uitstoot te beperken. Het is daarom van groot belang om meer kennis te verzamelen over N_2O producerende processen. Er zijn drie dergelijk microbiële processen tot dusver bestudeerd: denitrificatie, dissimilatorische nitraatreductie tot ammonia (DNRA) en nitrificatie. Hiervan is DNRA het minst goed gekend. In dit doctoraatsonderzoek werden de eerste twee van deze processen bestudeerd in *Bacillus* met bijzondere aandacht voor het tot nu toe weinig onderzochte DNRA proces, om zo fenotypisch bewijs te bekomen voor genoom-gebaseerde hypothesen over het nitraatreductie metabolisme van *Bacillus.*

Ten eerste werd het nitraatreductie metabolisme onderzocht in dicht verwante *B. paralicheniformis* en *B. licheniformis* stammen. Fysiologische gegevens van anaerobe groei experimenten toonden aan de onderzochte stammen geen denitrificeerders zijn, maar ammonifieerders die DNRA uitvoeren waarbij veel N_2O als bijproduct wordt gevormd. Stam-specifieke fenotypische verschillen in het stikstofmetabolisme werden gevonden en hypothetische reactieketens voor N_2O productie werden voorgesteld op basis van onze fysiologische waarnemingen. Het effect van verschillende fysicochemische factoren op de verdeling van NO_3^- naar NH_4^+ of N_2O werd onderzocht en dit toonde aan dat NO_3^- en NO_2^- concentratie een significant effect hadden op NO_3^- verdeling, mogelijk als gevolg van de

(tijdelijke) accumulatie van NO_2^-. Onder vaste C/N-NO_3^- ratio's had de NH_4^+ concentratie effect op de NO_3^- verdeling.

Ten tweede, aangezien denitrificatie en DNRA vroeger als nooit samen in één organisme voorkomende processen werden beschouwd, bood de aanwezigheid van beide processen, met een merkwaardige redundantie aan dissimilatorische stikstof reducerende genen in *B. azotoformans* LMG 9581^T, de kans om experimenten op te zetten om nitraat verdeling naar beide processen in dit unieke organisme te onderzoeken. Op basis van de gen-inhoud in zijn genoom, werd het stikstof assimilatie metabolisme van *B. azotoformans* LMG 9581^T bestudeerd, wat leidde tot het besluit dat organische stikstof vereist was voor assimilatie en NH_4^+ alleen niet volstond voor groei, zowel in aerobe als anaerobe condities. Echter, NH_4^+ werd wel degelijk geassimileerd en had een concentratie-afhankelijk effect op de groeisnelheid, maar niet op de maximale celdensiteit. De onderliggende mechanismen bleven echter onverklaard.

Vervolgens werden verschillende fysicochemische condities uitgetest om de functionaliteit van DNRA in *B. azotoformans* LMG 9581^T aan te tonen. In batch-cultuur konden diverse C/N-NO_3^- ratio's bij hoge en lage NO_3^- concentratie DNRA niet induceren. Omdat bij andere DNRA stammen de functionaliteit was aangetoond in chemostat-culturen i.p.v. batch-culturen, werd een chemostat systeem ontworpen als alternatief voor de batch-experimenten. Spijtig genoeg leidden aanhoudende contaminaties tot een vroegtijdige stopzetting van deze onderzoekslijn. Desondanks kon uit de beperkte verzamelde fysiologische gegevens uit de chemostat worden afgeleid dat denitrificatie minder efficiënt werd en NO_2^- begon op te bouwen, wat niet kon verklaard worden door een verschil in vermogen tot NO_2^- reductie maar mogelijk zou kunnen verklaard worden door een verschuiving of initiëring van een ander metabolisch proces zoals DNRA. Hiervoor zijn echter bevestigende studies nodig.

Tot besluit, in deze doctoraatsthesis werd de grenzen van de huidige kennis over de dissimilatorische processen van denitrificatie en DNRA in *Bacillus* onderzocht en verder verlegd. Dit werk bracht nieuwe fysiologische en genomische gegevens aan over het weinig bestudeerde DNRA proces in dit genus. Diverse omgevingsparameters die denitrifcatie en DNRA aansturen, werden uitgebreid bestudeerd in *Bacillus*, maar verder onderzoek blijft nodig. We benadrukken de nood aan toekomstige studies van *Bacillus* stammen, en in het bijzonder *B. azotoformans* LMG 9581^{T} die de genen voor de beide dissimilatorische nitraatreductie processen bevat. Hoewel onze chemostat en gen knockout experimenten niet gelukt zijn, biedt dit organisme nog steeds unieke mogelijkheden om waardevolle inzichten over de regulerende mechanismen van nitraatverdeling te verzamelen.

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Educations

- Ph.D. Biotechnology 2013, Feb- present
  Ghent University, Laboratory of Microbiology

  Topic: Dissimilatory nitrate reduction in *Bacillus*

- M.Sc. Marine Biology Sep, 2010 - Jan, 2013
  Ocean University of China

  Dissertation: Phylogenetic diversity analysis and evaluating of novel glycosides biosynthetic potential of Mangrove *Actinomycetes*

- B.Sc. Biotechnology Sep, 2006 - Jan, 2010
  Ocean University of China
  Dissertation: Research of a microwave-based method for genomic DNA extraction from *Actinomycetes*

Skills

Lab Skills:

- ✓ Experimental Design
- ✓ Microbial culture, Batch & Continuous incubation (bioreactor), aerobic & anaerobic culture
- ✓ Colorimetric Determination of NO_3^-, NO_2^-, NH_4^+, Gas Chromatograph, HPLC
- ✓ Molecular Biology: Gene knockout, DNA and RNA Extraction, PCR, qPCR

Software

- ✓ MS Words, MS PowerPoint, MS Excel
- ✓ CorelDraw, GraphPad Prism 7, Adobe Illustrator, PaintTool Sai
- ✓ SPSS, R, C Language
- ✓ MEGA 7, BioEditor, Primer3

Awards

Travel grant of 16th International Symposium on Microbial Ecology (ISME), Montreal, Canada 2016
Travel funding of Faculty of Sciences (FCWO) in Ghent University 2016
Best poster award in Belgium Society for Microbiology Meeting (BSM), Brussels, Belgium 2015
Scholarship from Special Research Fund (BOF) in Ghent University 2013-2017
Scholarship from China Scholarship Council for Ph.D study aboard 2013-2017
Excellence Studying Scholarship 2006-2013
Outstanding Graduate of Ocean University of China 2010
Outstanding volunteer of Olympic Sailing and Paralympic Sailing 2008

Research Activities

Local Organizer of 19th ENC meeting, Gent, Belgium 2014
Assistant in the 'Day of Science', Gent, Belgium 2014
Summer School: 'Let's Talk Science', Vrije Universiteit Brussel 2015

Symposium participation:

'How to Reach Excellence in the Life Sciences', Hasselt 2015
'Microbial Evolution: theory, simulation and experiment', Leuven 2015

Student Training Assistant

Thesis student:

Assimilation and dissimilatory nitrate reduction in genus *Bacillus* 2015

Master project:

Confirmation of two mutually exclusive nitrate reduction pathways in one *Bacillus* strain
Screening of nitrous oxide production in *Bacillus licheniformis* LMG 6934 2014
Aerobic assimilation and anaerobic dissimilation of nitrogen in *Bacillus azotoformans* 2014

Bachelor project:

Environmental influences on dissimilatory NO_3^-/NO_2^- reduction to ammonium in *Bacillus licheniformis* 2016
Nitrate partitioning between denitrification and dissimilatory nitrate reduction to ammonium in *Bacillus azotoformans* 2015
Identification of drivers from different dissimilatory nitrogen reduction pathways 2014

Publications

Sun Y, De Vos P, Heylen K. **Nitrous oxide emission by the non-denitrifying, nitrate ammonifier *Bacillus licheniformis***. BMC Genomics, 2016, 17(1):68

Sun Y, Feng G, Wang C, Xu S, Li J. **Phylogenetic diversity analysis and novel glycosides biosynthetic potential evaluation of Mangrove *Actinomycetes***. Chinese Journal of marine drugs, 2013, 32(1): 46-54

Sun Y, De Vos P, Heylen K: **Denitrification And Non-Denitrifier Nitrous Oxide Emission In Gram-Positive Bacteria**. Metalloenzymes in Denitrification: Applications and Environmental Impacts. 2016, 9: 349-367

Published Abstracts:

Sun Y, De Vos P, Heylen K, Willems A. Dissimilatory nitrate reductions in N_2O producing *Bacillus azotoformans* LMG 9581^T. 16th ISME, 2016, Montreal, Canada. **Poster Presentation.**

Sun Y, De Vos P, Heylen K. DNRA for nitrogen assimilation or energy conservation in *Bacillus azotoformans* LMG 9581^T. 20th European Nitrogen Cycle Meeting (ENC), 2015, Aberdeen, UK. **Oral Presentation.**

Sun Y, De Vos P, Heylen K. Dissimilatory nitrate reductions in N_2O producing *Bacillus azotoformans* LMG 9581^T. Belgium Society for Microbiology Meeting (BSM) 2015: Microbes and the global change, Brussels, Belgium. **Poster Presentation.**

Sun Y, De Vos P, Heylen K. Dissimilatory nitrate reduction processes in *Bacillus azotoformans* LMG 9581^T. BSM Meeting 2014: Cell Signaling in Host-Microbe Interactions, Brussels, Belgium. **Poster Presentation.**

Sun Y, De Vos P, Heylen K. Dissimilatory nitrate reduction processes in *Bacillus azotoformans* LMG 9581^T. 19th ENC meeting 2014, Gent, Belgium. **Poster Presentation.**

Sun Y, De Vos P, Heylen K. Hypotheses on dissimilatory nitrate reduction pathways in *Bacillus azotoformans*. BSM meeting 2013: Microbial Diversity for Science and Industry, Brussels, Belgium. **Poster Presentation.**

Sun Y, De Vos P, Heylen K. Hypotheses on dissimilatory nitrate reduction pathways in *Bacillus azotoformans*. 18th ENC meeting 2013, Darmstadt, Germany. **Poster Presentation.**