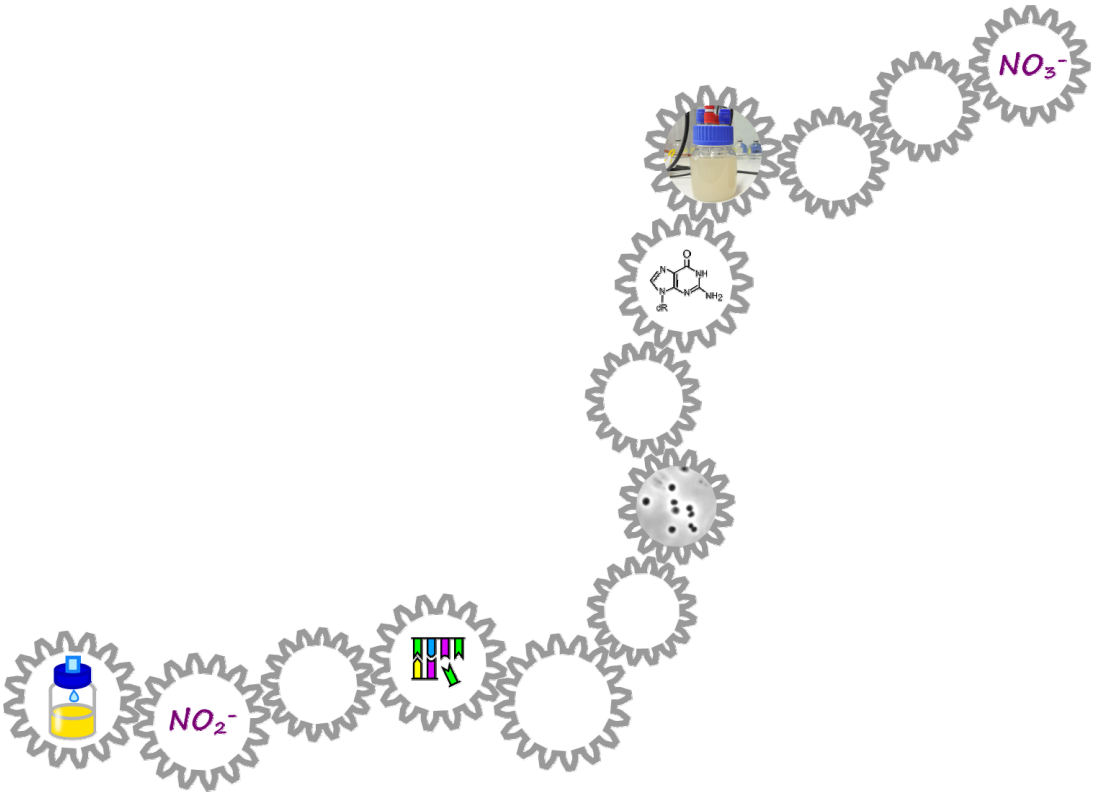
Experimental evolution of *Paracoccus denitrificans* in anoxic chemostats



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Experimental evolution of *Paracoccus denitrificans* in anoxic chemostats

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For my Family

Summary

Natural microbial communities play a central role in ecosystems and global cycles of elements. The microbial community compositions, functions as well as interactions between species and the environment have been studied with increasing effort. However, it is challenging to understand which parameters determine for the success of individual species to survive in a specific habitat. The often highly diverse microbial communities are continuously subjected to environmental stress such as biotic and abiotic fluctuations that cannot be completely tracked. To investigate the influence of different parameters on the ability of microorganisms to adapt to the environment, simple microbial communities, often single species are cultivated in the laboratory under strictly controlled conditions with reduced complexity. Such long-term experiments provide insight into the association between genetic and phenotypic alterations that evolve over hundreds or even thousands of generations.

The availability of nutrients often affects microbial growth. This thesis describes the experimental evolution of *Paracoccus denitrificans* Pd1222, a model denitrifying soil bacterium, to study the adaptation on acetate or nitrate limitation. Initially, nutrient limitation for the anaerobic growth of *P. denitrificans* was addressed with focus on trace elements (*Chapter 2*). New trace element solutions were designed based on previous reports and tested to exclude growth limitation or inhibition by these nutrients during long-term cultivation. Improved generation times of 4.4 hours were achieved with a chelated trace element solution and lower concentrations than frequently used media.

Chapter 3 describes the adaptive responses of *P. denitrificans* to acetate and nitrate limitation during experimental evolution in chemostats. In the course of at least 800 generations of *P. denitrificans* under denitrifying conditions the metabolic conversions of substrates were monitored. For deeper insights into different adaptive mechanisms of *P. denitrificans* under both conditions we investigated the transcriptomes and genome variations. Throughout the experiment the different treatments led to significantly different substrate conversion rates and transcriptomic profiles. Specifically, in nitrate limited cultures genes of the citric acid cycle and the nitrogen metabolism showed higher transcriptional activities than in acetate limited cultures. In the latter the transcription of genes encoding regulators and transporters was more pronounced. Additionally, more changes in transcriptional activities and in metabolism were observed over time than under nitrate limitation. Most notably, denitrification became more efficient resulting in the depletion of nitrite that accumulated in the culture during the first 500 generations. Although numerous mutations were detected in DNA obtained from this culture, they

could not be related to the observed phenotypic changes. In all cultures the types and numbers of genetic variations did not considerably differ.

The study indicated that *P. denitrificans* had a stronger potential to adapt to acetate limitation than to nitrate limitation and underlines the capacity of this bacterium to improve denitrification even in absence of environmental fluctuations. The possible explanation that phenotypic changes may have been independent of genetic variations is discussed in *Chapter 4*. The relevance of the insights gained in this study for natural, in particular denitrifying communities is presented and future studies towards the understanding of natural microbial community functions are suggested.

Zusammenfassung

Natürliche mikrobielle Gemeinschaften sind für Ökosysteme und globale Stoffkreisläufe von zentraler Bedeutung. Ihre Zusammensetzung, Funktion und Wechselwirkungen von Spezies mit der Umwelt werden mit zunehmendem Aufwand untersucht. Jedoch ist es schwierig herauszufinden welche Umwelteigenschaften für das Überleben einzelner Spezies in einem bestimmten Lebensraum verantwortlich sind. Die meist sehr diversen mikrobiellen Gemeinschaften unterliegen kontinuierlich zahlreichen Stresssituationen wie biotischen und abiotischen Schwankungen, an die sie sich sehr schnell anpassen müssen und können. Um die Auswirkung solcher Umwelteinflüsse auf die Anpassungsfähigkeit von Mikroorganismen zu untersuchen, werden einfache mikrobielle Gemeinschaften, meist beschränkt auf eine einzige Spezies, in streng kontrollierten, weniger komplexen Laborsystemen kultiviert. Solche Experimente werden häufig über Hunderte oder gar Tausende von Generationen dieser Organismen durchgeführt und die Zusammenhänge zwischen genetischer und phänotypischer Veränderungen untersucht.

Umweltrelevante Stresssituationen umfassen auch Nährstoffverfügbarkeit, die in der vorliegenden Arbeit untersucht wurde. Anhand experimenteller Evolution wurde die Anpassung des Bakteriums *Paracoccus denitrificans* Pd1222, einem denitrifizierenden Modellorganismus, an Acetat- und Nitratlimitierung untersucht. Zunächst wurde das anaerobe Wachstum von *P. denitrificans* in Abhängigkeit von Spurenelementen untersucht (*Kapitel 2*). Auf der Grundlage von zuvor eingesetzten Spurenelementlösungen wurden neue Spurenelementlösungen entwickelt und erprobt, um in der Langzeitstudie Einschränkungen des Wachstums durch limitierende oder inhibierende Wirkung dieser Nährstoffe auszuschließen. Die Generationszeit wurde mittels einer komplexierten Spurenelementlösung und mit niedrigeren Konzentrationen als in häufig verwendeten Medien auf 4,4 Stunden reduziert.

Kapitel 3 beschreibt die Anpassung von *P. denitrificans* an Acetat- und Nitratlimitierung während der experimentellen Evolution in Chemostaten. Im Verlauf von mindestens 800 Generationen unter denitrifizierenden Bedingungen wurde die metabolische Substratumsetzung verfolgt. Um die Anpassungsmechanismen von *P. denitrificans* näher zu untersuchen, wurden die Transkriptome und genetischen Veränderungen analysiert. Im gesamten Versuchszeitraum führten die beiden Kulturbedingungen zu unterschiedlichen Substratumsetzungsraten und Transkriptionsprofilen. Gene des Zitratzyklus und des Stickstoffmetabolismus waren unter Nitratlimitierung höher transkribiert als unter Acetatlimitierung. In den acetatlimitierten Kulturen hingegen waren mehr Regulator- und Transporter-kodierende Gene transkribiert. Zusätzlich wurden in

diesen Kulturen mehr zeitliche Änderungen in den Transkriptionsaktivitäten und im Metabolismus beobachtet. Hervorzuheben ist die Veränderung der Denitrifizierung in einer acetatlimitierten Kultur. Diese Veränderung führte zu dem vollständigen Abbau von Nitrit, einem Zwischenprodukt der Denitrifizierung, welches sich in den ersten 500 Generationen angesammelt hatte. Obwohl in der aus dieser Kultur gewonnenen DNA zahlreiche Mutationen im Vergleich zur Ausgangskultur nachgewiesen wurden, konnten diese nicht in Zusammenhang mit den beobachteten phänotypischen Änderungen gebracht werden. Die Art und Anzahl der genetischen Veränderungen unterschieden sich nicht wesentlich zu den anderen Kulturen. Die Studie bietet Hinweise, dass *P. denitrificans* ein größeres Potenzial hatte sich an Acetatlimitierung als an Nitratlimitierung anzupassen. Weiterhin hebt sie die Fähigkeit dieses Bakteriums hervor seinen Denitrifizierungsweg selbst in Abwesenheit von Umweltschwankungen anzupassen und zu verbessern.

Die Möglichkeit, dass die phänotypischen Änderungen unabhängig von genetischen Änderungen erschienen sein könnten, wird in der Gesamtdiskussion dieser Arbeit beleuchtet (*Kapitel 4*). Die Bedeutung der hier erlangten Erkenntnisse wird in einen ökologischen Zusammenhang gestellt, insbesondere für denitrifizierende Gemeinschaften. Weiterführende Untersuchungen werden vorgeschlagen, die zu einem besseren Verständnis von natürlichen mikrobiellen Gemeinschaften beitragen können.

List of abbreviations

ABC	ATP-binding cassette
anammox	anaerobic ammonium oxidation
cDNA	complementary DNA (transcriptomics)
C-limited (C-lim)	carbon limited (in this thesis: acetate limited)
CCR	crotonyl-CoA carboxylase/reductase
CoA	coenzyme A
C/N ratio	carbon over nitrogen ratio
cyt.	cytochrome
DIC	dissolved inorganic carbon
DNRA	dissimilatory nitrate/nitrite reduction to ammonium
EDTA	ethylenediamine-N,N,N',N'-tetraacetic acid
EMC	ethyl-malonyl-CoA
FMN	flavin mononucleotide or riboflavin-5'-phosphate
GOGAT	glutamine oxoglutarate aminotransferase
HEPES	3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic
HGT	horizontal gene transfer
HPLC	high-performance liquid chromatography
ICL	isocitrate lyase
indel	insertion/deletion
log FC	$\log_2$ fold change
maxOD	maximum optical density
MOPS	3-(N-morpholino)propanesulfonic acid
NADP	nicotinamide adenine dinucleotide phosphate
NADH	nicotinamide adenine dinucleotide hydride
NADH DH	NADH dehydrogenase
N-limited (N-lim)	nitrate limited
OD	optical density
OPA	orthophthalaldehyde
ORF	open reading frame
Paz	pseudoazurine
PBS	phosphate buffered saline solution
PEP	phosphoenolpyruvate
PHB	polyhydroxybutyrate

List of abbreviations (continued)

Q	quinone
RI	refractive index
ROS	reactive oxygen species
RPKM	Reads Per Kb per Million reads
rRNA	ribosomal RNA
RT	room temperature
SCCM	standard cubic centimeters per minute
SMRT	single-molecule real-time (DNA sequencing)
SNP	single nucleotide polymorphism
TCA	tricarboxylic acid
TE	trace element
TE-(1-4)-(CO,Zn,B,Ni)	different variants of TE solutions (see *Chapter 2*)
TRAP	tripartite ATP-independent periplasmic (transporter)

Contents

Chapter 1

Introduction

1.1 Historical view on experimental evolution

In 1887, William Dallinger described the first evolution experiment with three protozoans (Dallinger, 1887). He maintained these organisms in a custom-built apparatus for seven years and showed that they adapted to high temperatures after incrementally increasing temperatures from 16 ℃ to 79 ℃. The evolved protozoans were not able to survive at the initial temperature. A technical failure terminated the experiment. Only few studies followed, until in 1984 Michael Rose described a laboratory evolution experiment with *Drosophila melanogaster* (Rose, 1984). His experiment was designed to study the evolution of aging and revealed increased life times linked to early fecundity. Thereupon, researchers became increasingly interested in studying evolution of a number of organisms 'in action'. Four years later, Richard Lenski initiated the well-known long-term evolution experiment (LTEE) with *Escherichia coli* (Lenski et al., 1991). By propagation in liquid cultures every day for more than 26 years these bacteria reached more than sixty thousand generations and still continue to evolve (Lenski, 2014). Experimental evolution has opened the possibility to follow evolution in defined laboratory environments in real time using organisms with short generation times. During the last decades this approach has become the method of choice for studying the relationship between evolutionary history and phenotypic traits (Le Gac et al., 2013). Microorganisms are particularly suitable since they reproduce fast, can reach high population sizes in small spaces and are relatively easy to maintain (Lenski et al., 1991; Elena and Lenski, 2003). With low growth demands and high physiological versatility their evolution can be studied at various environmental conditions. Moreover, cryo-conservation allows direct comparisons between different evolutionary states (Lenski et al., 1991; Elena and Lenski, 2003). Certainly the vast progress in the development of molecular methods,

most notably the possibility to sequence entire genomes, has contributed to the augmenting attempts to study genotypic and phenotypic effects of adaptive evolution in detail (Barrick et al., 2009; Brockhurst et al., 2011; Dettman et al., 2012). This area of research has been accompanied by many specific terms that have been used in different contexts, some of which are listed in Table 1.1. The organisms that have been evolved in the laboratory most frequently (Hindré et al., 2012; Kawecki et al., 2012) are *Escherichia coli*, *Pseudomonas aeruginosa* (Racey et al., 2010; Hall and MacLean, 2011), *Saccharomyces cerevisiae* (Lang et al., 2013), viruses (Wichman, 1999; Bono et al., 2013; Hall et al., 2013), *Drosophila melanogaster* (Haag-Liautard et al., 2007; Condon et al., 2014) and marine and freshwater stickleback (Barrett et al., 2011). The set of organisms was extended by a digital organism – Avida, a self-replicating and mutating computer software (Wilke and Adami, 2002).

1.2 Research questions addressing experimental evolution studies

Experimental evolution has broadened our knowledge on the versatility of microbial populations in many areas, covering fundamental and applied aspects. Fundamental questions mostly address adaptive genotypic and phenotypic evolution in a specific environment with a defined property. These include for example the question if and how microorganisms increase their ability to survive and reproduce in the environment, which is commonly referred to as fitness (Table 1.1). Many researchers are interested in molecular aspects of adaptation, with increasing focus on genetic parameters (see *Section 1.3*) and the effects of mutations on a microorganism's phenotype and fitness (e.g., Eyre-Walker and Keightley, 2007; Herring et al., 2006). Adaptation is a dynamic process, in other words the fitness of an evolving organism increases at different rates (Elena and Lenski, 2003; Barrick and Lenski, 2013). One of the most important underlying reasons is epistasis, the interaction between two or more mutations within a genome (Figure 1.1). If an organism is subjected to a new environment, its fitness may be far from the optimum and appearing mutations are more likely to be beneficial. With time, the organism becomes better adapted and the potential to increase fitness will be low. Barrick et al. (2009) have shown that the rate of genome evolution is non-linearly related to fitness improvement during thousands of generations (Figure 1.1), demonstrating that evolution is constrained by epistasis (Khan et al., 2011). Although very long evolution experiments showed declining rates of fitness gains, it remains unexplored whether a limit

Table 1.1 Relevant terms used in this thesis.

Term	Definition
Experimental evolution	Study of genotypic and phenotypic changes occurring in populations during maintenance in defined and controlled environments over tens, hundreds or thousands of generations (Kawecki et al., 2012; Barrick and Lenski, 2013).
Selective force	Any kind of biological, chemical or physical parameter that acts on an organism's ability to survive and reproduce at different intensity.
Mutation	"Any alteration in the nucleotide sequence of the genome" according to Arber (2000), disregarding its origin, type and effect.
Fitness	(also: adaptive fitness, biological fitness) The ability of an organism to live and reproduce in a particular environment; given as relative fitness between two strains or species (e.g., evolved versus ancestral strain).
Adaptation	"The process of change by which an organism or species becomes better suited to this environment" (Ryall et al., 2012). Often restricted to modification in DNA sequence. Appearance of beneficial mutations that increase in frequency within a population until they reach a frequency high enough to displace the cells that do not have the mutation (Bataillon et al., 2012; Merchant and Helmann, 2012).
Chemostat	(Greek chemo, chemical; stat, control) A device to continuously grow microorganisms at constant physical, chemical and biological conditions by a steady inflow of nutrients and removal of bacterial suspension, with one nutrient being supplied at a stoichiometrically lower concentration ('controlling growth factor'; Novick and Szilard, 1950).
Divergence	Splitting of an isogenic ancestor into distinct genotypes among biological replicates.
(Adaptive) Diversification	"Splitting of an ancestral lineage into distinct descendant lineages as a consequence of natural selection acting on individuals within the population", generating coexisting and competing lineages (Tyerman et al., 2005).
Subpopulations	Two or more lineages that evolved from one common ancestor and differ in their phenotype, but not necessarily in their genotype.
Nongenetic individuality	Variable phenotype within an isogenic population (Davidson and Surette, 2008).
Clonal interference	"Competition between lineages that have different beneficial mutations in asexual populations" (Barrick and Lenski, 2013).

exists for adaptation in constant environments (Kawecki et al., 2012). The dynamics of evolution, diversification and the evolution of interspecies interactions are ecologically relevant and the increasing knowledge contributes to the understanding of microbial community structures and functions (Figure 1.2). *E. coli* was shown to diversify into cross-feeding subpopulations in glucose limited chemostats (Helling et al., 1987; Treves et al., 1998). One of these subpopulations achieved higher fitness by improved uptake and partial degradation of glucose while the other subpopulation specialized on excreted waste products. These findings motivated researchers to investigate the success of co-existing organisms by experimental coevolution with at least two organisms, which, for example, revealed costs and benefits of sociality (Xavier, 2011; Celiker and Gore, 2012). Furthermore, interspecies interactions have been shown to enhance the evolution of mutation rates in virus-host cocultures (Pal et al., 2007) and high within-population genetic diversity improved the success of a bacterial pathogen cocultured with its host (Racey et al., 2010).

Interestingly, unexpected traits or novel functions have occasionally appeared during experimental evolution that provided evidence for high evolutionary flexibility even in absence of environmental changes (Blount et al., 2012). It was proposed that this process, termed 'neo-functionalization' may typically involve three steps: First, the establishment of a genetic background that may allow the pivotal mutation to appear (potentiation). Here, epistasis likely plays an important role. Second, the mutational event that confers a new function to the organism (actualization) and third, the subsequent improvement of the function which can be considered as adaptation to a new ecological niche (refinement). By careful molecular analyses of these steps, Blount and colleagues identified the mechanism by which *E. coli* acquired the ability to use citrate in the long-term evolution experiment under oxic conditions (described in *Section 1.1*). An amplification mutation led to the formation of a new genetic module that allowed the expression of *citT* encoding a citrate transporter which is normally suppressed during aerobic growth of *E. coli* (Blount et al., 2012). In the refinement step, the most efficient improvement of growth on citrate resulted from an increase in copy number of this genetic module. Another example for functional innovation is the evolution of phage lambda to attach to an alternative bacterial outer membrane protein of *E. coli* (Meyer et al., 2012). This trait conferred an advantage to the phage in coculture with *E. coli* which became resistant to the ancestral phage within eight days.

This observation was followed by a systematic study targeting the frequency and importance of different types of genetic variation for the evolution of new functions (Blank et al., 2014). Besides mutations in existing regulatory or structural genes researchers

uncovered principles by which genes can emerge de novo, often by rearrangements of DNA fragments (see *Section 1.3*, Chen et al., 2013). Such genetic variations may enable the organism to expand to a new niche within the habitat for example by utilizing another resource, and escape the competition for a limiting resource.

An important area of experimental evolution comprises biomedical applications. The identification of mutations that confer antibiotic resistance to pathogens (Hall and

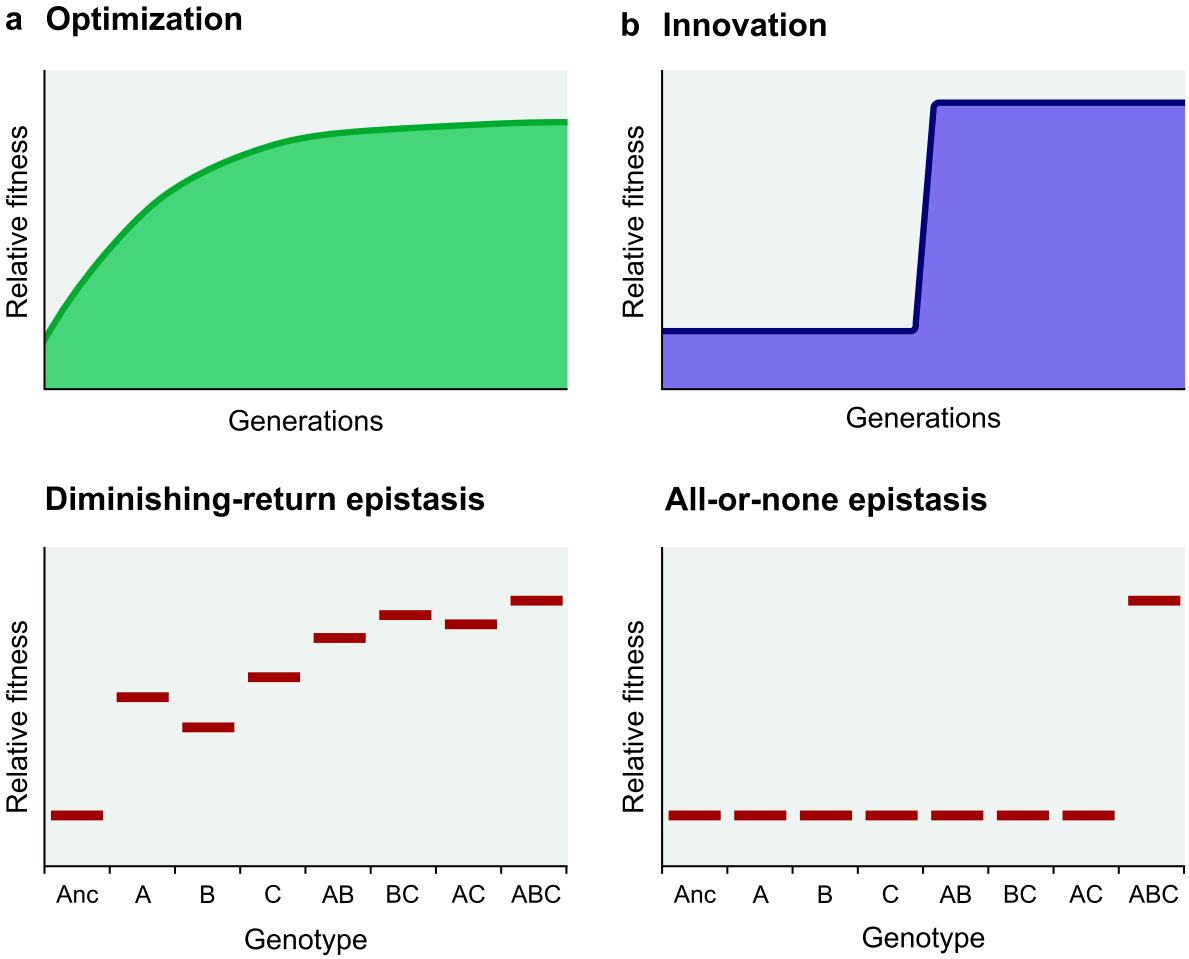

Figure 1.1 Relation between mutation and fitness during adaptation of organisms to a new environment. The fitness increase over the ancestral strain Anc depends on the effect of a mutation and the combination of mutations (genetic context). **(a)** Mutations A, B, C can appear alone, each leading to a fitness increase or in combination in a genome. The fitness increase by a mutation is often lower in combination with another mutation due to interactions between the mutations (epistasis). In presence of beneficial mutations, each additional mutation will less likely further improve fitness. **(b)** Alternatively, mutations can accumulate without showing any effect on fitness. These mutations alter the genetic background that is essential for a large effect of a subsequently appearing mutation. Adapted from Barrick et al. (2013).

MacLean, 2011) facilitates the design of new drugs that target pathogens and treatment of diseases in advance (Kawecki et al., 2012). Experimental evolution of pathogens can also be used to produce vaccines with attenuated pathogenic effects in humans (Ebert, 1998). For biotechnological processes microorganisms are experimentally evolved to enhance product formation, often in combination with genetic engineering (Dragosits and Mattanovich, 2013). This is often achieved by an improved tolerance to organic solvents and is of high relevance for biofuel production (Goodarzi et al., 2010; Dragosits and Mattanovich, 2013).

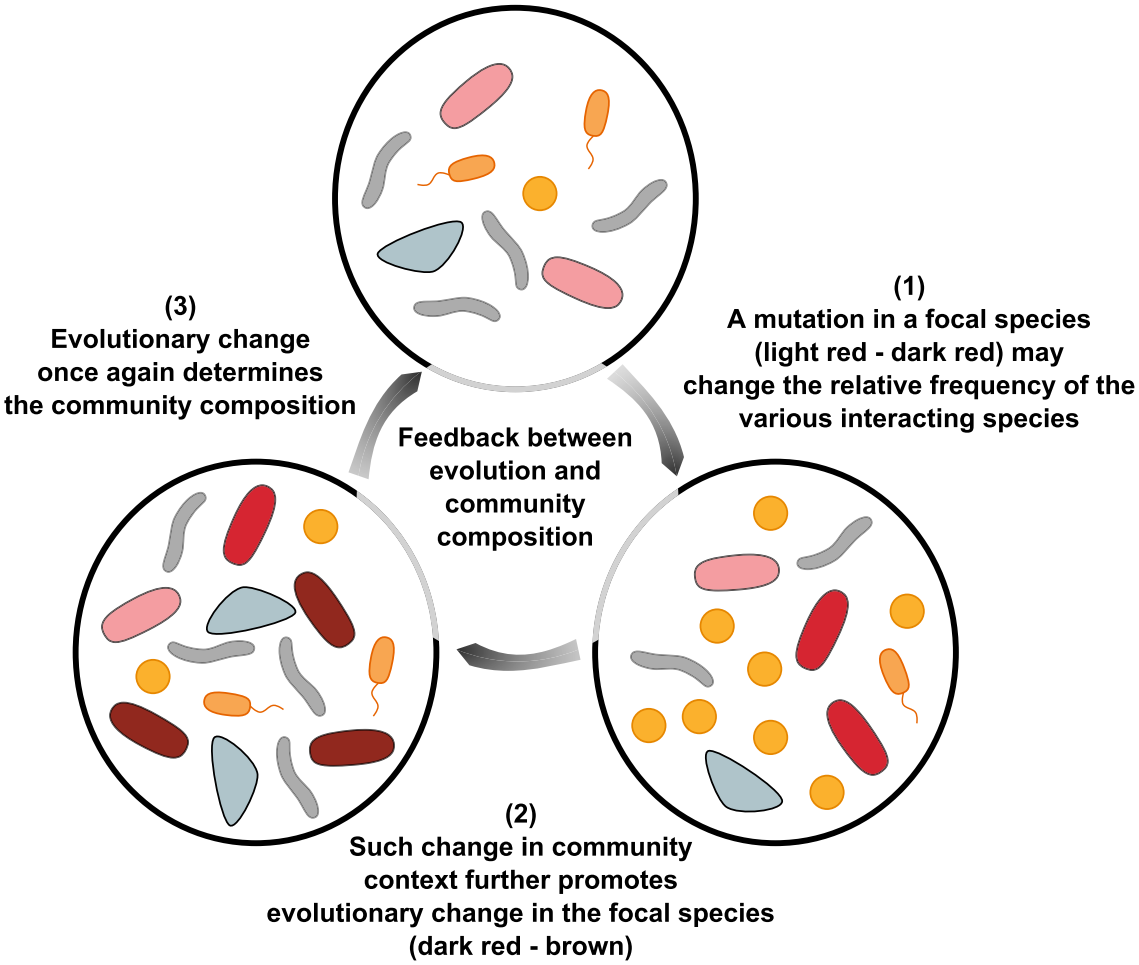

Figure 1.2 Schematic illustration of microbial evolutionary dynamics. A mutation in the focal species may affect the composition of the microbial community it interacts with. In turn, this biotic change causes further evolutionary change of the focal species. Adapted from O'Brien et al. (2013).

1.3 The genetics of microbial evolution

Genome variation plays a central role in the evolution of organisms. Whole-genome sequencing provides an important tool to reconstruct the history of evolved populations by identifying genome variants at different evolutionary states compared to their ancestor (Brockhurst et al., 2011). With this technique, numerous insights have been gained into mutation rates (Lanfear et al., 2014), interactions between mutations (e.g., epistasis, Figure 1.1) and consequences of chromosomal rearrangements (Avelar et al., 2013). Mutations can be beneficial, neutral, deleterious or lethal and are generally considered the only way to improve the ability of an organism to survive or to adapt to the spatiotemporally changing conditions of its environment. Elena et al. (1998) investigated the fitness effects of 226 *E. coli* mutants, obtained by transposon-mutagenesis of an evolved *E. coli* clone (10,000 generations), by competition experiments under the same culture conditions as used for experimental evolution. They observed that 80% of the mutations were deleterious, 20% neutral and none were beneficial. The absence of detected beneficial mutations can be explained by the low frequency of one beneficial mutation in 10^6 random mutations, as estimated by Gerrish and Lenski (1998) for the ancestral *E. coli* strain. The rate of beneficial mutations in evolved clones is therefore expected to be lower. Deleterious mutations can be compensated by mutations at other positions in the genome and thus modify the genetic background which can affect subsequent mutations (Elena and Lenski, 2003). It has been assumed that mutations occur randomly over the genome (Lederberg and Lederberg, 1952; Kimura, 1991). However, DNA is more susceptible to modifications in its single-stranded state. Thus, frequently transcribed genes receive mutations at higher rates (Singer and Kuśmierek, 1982; Williams, 1997).

DNA can be modified in many ways. In *E. coli*, single nucleotide polymorphisms (SNP, the substitution of single bases) occur most frequently (61%), while deletions (29%), insertions (7%) and transposon mediated movements (3%) occur less frequently (Conrad et al., 2011). Comparable results have been obtained for *Saccharomyces cerevisiae* (Wenger et al., 2011). Substitutions of purines by pyrimidines and vice versa (transversions) are distinguished from substitutions within purines or pyrimidines (transitions). Furthermore, single or multiple nucleotides can be inserted or removed (insertions and deletions, often termed 'indel'). This is caused by (replication) slippage, a process by which the DNA polymerase pauses and resumes the DNA replication, affecting repeated sequence elements where the formation of secondary structures or chemical lesions is likely (Michel, 2000; Viguera et al., 2001). SNPs and indels can have severe effects resulting in a malfunction of the gene product, specifically if the open reading

frame (ORF) is altered by (i) the gain or loss of start/stop codons or (ii) shifts of the ORF. Though, irrespective of the type of mutation, each modification of genetic material can potentially provide a benefit to the organism and even play a role in the evolution of new functions (Chen et al., 2013).

DNA rearrangements include a number of mechanisms such as recombination of related sequences, inversion, transposition, duplication of DNA segments, the combination of functional domains into a fusion gene or, accordingly, the splitting of one gene into shorter genes or fragments (fission) (Arber, 2000; Gordo et al., 2011; Chen et al., 2013). Fusion genes are not necessarily caused by genetic rearrangements, but can also occur by fusion of neighboring genes when one stop codon is lost. Other sources of DNA variations are retrotranspositions referring to catalytic RNAs that facilitate RNA intron splicing and insertion into another DNA locus (Chen et al., 2013). This RNA is encoded for example by the bacterial group II intron and functions as reverse transcriptase, RNA maturase and DNA endonuclease (Matsuura et al., 1997; Ichiyanagi et al., 2003). Bacteria can also obtain new DNA (e.g., plasmids) from other, distantly related bacteria by horizontal gene transfer (HGT). This mechanism often confers a new function to the recipient cell and enables it to expand to new habitats (Arber, 2000; Ochman et al., 2000).

SNPs can be caused physically (e.g., UV light), or chemically by deamination, depurination and depyrimidation of DNA bases (Singer and Kuśmierek, 1982). The most important group of chemical mutagens are reactive oxygen species (ROS). For example, nitrous acid, the protonated form of nitrite, can deaminate cytosine to form uracil and at lower rates deaminate adenine and guanine to form hypoxanthine and xanthine, respectively (Singer and Kuśmierek, 1982). Nitric oxide causes transitions of adenine:tyrosine to guanine:cytosine and guanine:cytosine to adenine:tyrosine (Routledge et al., 1993) and cytosine to tyrosine (Wink et al., 1991; Merchant and Helmann, 2012). Furthermore, in the presence of oxygen nitric oxide is able to inhibit DNA repair enzymes (Wink and Laval, 1994).

However, the DNA polymerase itself is likely the most important source of DNA modifications by introducing wrong nucleotides during replication. These modifications generate base mismatches that can be recognized and corrected by DNA repair enzymes (Shee et al., 2011) or by the proofreading subunit of certain DNA polymerases during replication. A base modification that is not corrected is retained in the genome upon DNA amplification. Mutations in DNA repair enzyme complexes and DNA polymerase themselves can be targets of natural selection often observed in populations with low mutation supply rates (Cooper et al., 2003). Disrupted proofreading and repair functions

elevate the rates at which mutations appear and result in a higher chance for beneficial mutations (Loh et al., 2010). The disruption of DNA repair is of high value for the evolution of evolvability, often seen by the emergence of mutator phenotypes with increased mutation rates (Sniegowski et al., 1997; Lenski et al., 2003).

A mutation within a coding sequence mostly alters the amino acid sequence of a protein and affects proper folding. This results in protein malfunction and aggregation (Bogumil and Dagan, 2012). The last provision that can be undertaken to keep the original protein function is during protein folding. Chaperones may mediate proper folding of proteins even with an altered amino acid. These proteins play an important role under stress conditions and their expression is indirectly induced by intracellular or environmental stress, most frequently studied for heat stress (Lindquist, 1986; Bogumil and Dagan, 2012). Stress can also be induced by limitations of bioavailable elements required for growth. The impact of such limitations on microbial adaptation is central in this thesis and will be introduced in the following section.

1.4 Microbial response to nutrient limitation

Over 4.5 billion years of earth history, the ocean and the atmosphere underwent two intense shifts in elemental composition (Dupont et al., 2010). During this evolutionary time scale, the elemental composition of organisms has been shaped by the bioavailability of elements in the environment they inhabit (Williams, 1997; Dupont et al., 2010). Also in modern ecosystems spatiotemporal variations in elemental composition can lead natural microbial populations to be subjected to nutrient limitations. The term 'nutrient' is defined as any organic or inorganic compound or ion that an organism requires to survive and grow. These compounds comprise macro elements H, C, O, N, S, P that are required for biosynthesis of cellular compounds such as nucleic acids, proteins, lipids and saccharides (Williams, 1997; Overmann, 2013). Furthermore, metal ions are essential as cofactors or part of cofactors for many proteins, most of all Mg^{2+}, K^{+}, Ca^{2+}, Fe^{2+}, Fe(III), Mn^{2+} and Zn^{2+} (Williams, 1997; Overmann, 2013). Many organisms additionally depend on enzymes that require Cu(II), Mo(VI), Ni(II), Co(II), Se(VI) and W(VI) (Williams, 1997; Overmann, 2013). Furthermore, organic or inorganic compounds serve as electron donor (energy source) and oxidized compounds as electron acceptor during energy conservation. A stoichiometric imbalance between the elemental composition of total bioavailable nutrients in habitats and of microorganisms confers a stress condition to the organisms (Overmann, 2013; Mooshammer et al., 2014). Consequently many

microorganisms cannot survive in certain environments either because they cannot compensate for this imbalance or because they are outcompeted by species that are better adapted to such environmental conditions. For example fungi have generally higher C/N ratios than bacteria and predominate in carbon rich upper layers of soil ecosystems while bacteria prevail in deeper layers (Moore et al., 2010).

Microorganisms can respond to nutrient limitation in many ways. The term 'adaptation' often refers to genetic alterations but may also include phenotypic changes that do not depend on genotypic changes. Merchant and Helmann (2012) distinguish between 'adaptation' (alterations in DNA sequence) and 'acclimation' (alterations in cellular physiology). Adaptation according to their definition rather occurs over long time scales and comprises changes that more likely affect specialized organisms within an environment with relatively constant available nutrient composition. First of all, protein sequences are optimized for example to reduce their demand for the limiting nutrient in their amino acid composition. The 'cognate bias hypothesis' states that an amino acid is present at a reduced frequency in its biosynthetic protein (Alves and Savageau, 2005). Additionally, proteins can be replaced by alternative proteins with distinct cofactor requirements (Merchant and Helmann, 2012).

Also on shorter time scales there are many possibilities. The most common response is presumed to be an improved acquisition of nutrients. Microorganisms can for example increase the expression of transporters or produce, secrete and take up scavenging molecules (siderophores) (Miethke and Marahiel, 2007; Cordero et al., 2012). Multiple transport systems for one substrate are common, characterized by different affinities and transport capacities (Harder and Dijkhuizen, 1983). Under carbon limitation, the expression of catabolic enzymes is derepressed to maximize the carbon conversion efficiency while anabolic enzymes are kept at minimal expression levels to sustain growth (Harder and Dijkhuizen, 1983). Alternatively, microorganisms can repress the synthesis of nonessential biomolecules that contain the limiting nutrient (elemental sparing; Merchant and Helmann, 2012). In the first instance, highly abundant molecules are affected and depending on the limiting nutrient, this can for example result in changes in membrane or protein composition. In some cases this mechanism can lead to the utilization of alternative pathways that do not depend on enzymes containing the limiting metal as cofactor. This mechanism is referred to as functional substitution (Merchant and Helmann, 2012). Furthermore, cellular compounds containing the limiting nutrient can be degraded and invested for the synthesis of more indispensable molecules (elemental recycling). Finally, microbes can substitute the limiting element by other elements with similar characteristics, for example a metal ion of the same valence (Rosenbusch and We-

ber, 1971) or phosphorlipids by sulfurlipids when phosphorous is rare (Van Mooy et al., 2009). Occasionally microorganisms have the capacity to retrieve alternative sources that would not be favored under conditions rich in the element, which mostly applies to pathways with high energetic costs (Mooshammer et al., 2014).

Most of these physiological responses to nutrient limitations can be improved during evolution. For example, genes with regulatory functions were frequently shown to be increasingly transcribed when they were affected by a specific mutation (Philippe et al., 2007). Experimental evolution studies addressing elemental limitations most frequently consider carbon or nitrogen limitation (Goddard and Bradford, 2003). Most cellular processes involve carbon and therefore carbon metabolic pathways likely provide the most possibilities for adaptation. Indeed, it has frequently been shown that carbon limitation leads to improved efficiency of carbon acquisition and conversion (Egli, 2010). This is often associated with diversification into coexisting subpopulations from one common ancestor (Rosenzweig et al., 1994; Rainey and Travisano, 1998). Organisms evolving in environments with a single carbon source can also diversify where one subpopulation feeds on metabolic intermediates excreted by the other subpopulation (Pfeiffer and Bonhoeffer, 2004). Though, microorganisms adapted to nutrient limitation showed trade-offs in environments with high availability of the nutrient. Wenger et al. (2011) showed that yeast evolved under glucose limitation attained higher fitness under distinct carbon limiting conditions compared to the ancestor. In contrast, the evolved organisms had reduced fitness in carbon sufficient environments.

1.5 Denitrification and specialization of microorganisms

As described above, much is known about microbial responses to carbon limitation as essential nutrient for the generation of biomass. In addition to carbon, nitrogen is required for the synthesis of biomolecules. Furthermore, in anoxic environments nitrogen oxides can serve as electron acceptors to many microbial species. Therefore, the availability of nitrogen oxides may have high implications on the composition of microbial species that are able to reduce these compounds for energy conservation. Nitrogen accounts for 78% in the atmosphere, however terrestrial and marine ecosystems constitute less than 0.1% of the global nitrogen budget (Thamdrup, 2012). Many ecosystems are limited in nitrogen (Zehr et al., 2001; Thamdrup, 2012). Therefore, nitrogen cycling is one of the most important global processes where nitrogen fixing bacteria and archaea

convert the chemically inert form of nitrogen into bioavailable ammonium. Besides the assimilation of ammonium into biomass, bacteria can oxidize this compound to nitrite which is further oxidized to nitrate. Denitrifying microorganisms, comprising bacteria (Zumft, 1997), archaea (Cabello et al., 2004), fungi (Shoun et al., 1992), foraminifera and Rhizaria (Piña Ochoa et al., 2010), close the nitrogen cycle by reducing nitrate to dinitrogen and emitting it to the atmosphere.

Denitrification involves four sequential reactions via nitrite, nitric oxide and nitrous oxide. All reactions are exergonic (at 25 °C, pH 7; Table 1.2) and are catalyzed by the four metalloenzymes nitrate reductase (Nar; Nap), nitrite reductase (cytochrome cd_1, NirS; NirK), nitric oxide reductase (Nor) and nitrous oxide reductase (Nos). Many microbial species only feature a subset of these enzymes and are therefore specialized on performing only a part of the reaction sequence (Hayatsu et al., 2008; Hashimoto et al., 2009; Green et al., 2010). The activity of partial denitrifiers can lead to the release of nitrite or nitrous oxide to the environment. In addition, complete denitrifiers contribute to the emission of these intermediates, often influenced by environmental conditions. The enzymes of the denitrifying respiratory pathway can be inhibited by external parameters such as temperature, low pH, available organic carbon or oxygen (Sears et al., 1997; Bergaust et al., 2011). It was also suggested that limitations in available metal ions may be a reason (Matsubara et al., 1982; Granger and Ward, 2003). The denitrifying enzymes require Fe(II), Mo(VI) or Cu(II) or a combination thereof as cofactors for electron transfer and large emissions of nitrite and nitrous oxide could be related to copper limitation for example as a result of strong nitrate fertilization (Felgate et al., 2012).

Nitrite is an intermediate of particular interest due to its toxic effect by binding to cellular iron atoms, for example in hemoglobin. At low pH nitrite forms nitrosonium (Simon and Klotz, 2013), which is mutagenic as described in *Section 1.2*. However, nitrite provides an important electron acceptor to microorganisms performing three different pathways: dissimilatory nitrite reduction to dinitrogen (partial denitrification), dissimilatory nitrite reduction to ammonium (DNRA) and anaerobic oxidation of ammonium (anammox). Nitric oxide is a cytotoxin and denitrifying bacteria cannot grow with a malfunctioning nitric oxide reductase (de Boer et al., 1996). Nitrous oxide is a greenhouse gas with a global warming potential approximately 300-fold higher than carbon dioxide (Dalal et al., 2003). The high flexibility of microorganisms to perform either complete or partial denitrification determined by abiotic components, suggests a complex network in which many species interact by exchanging nitrogen metabolites.

Table 1.2 Reactions of denitrification steps, involved respiratory enzymes of *P. denitrificans*, metal ions required for cofactors and Gibbs energy ($\Delta G^{0'}$ in kJ per mol e^-). Reactions are normalized to one mol acetate linked to the transfer of 8 electrons. Values of $\Delta G^{0'}$ were calculated for the transfer of one electron with ΔG_f^0 values given by (Thauer et al., 1977). NirS is a cytochrome cd_1 nitrite reductase.

Reaction		Enzyme	Metal cofactor	$\Delta G^{0'}$
$4\ NO_3^- + C_2H_3O_2^-$	$\rightarrow 4\ NO_2^- + 2\ HCO_3^- + H^+$	Nar, Nap	Fe(II), Mo(VI)	–68.5
$8\ NO_2^- + C_2H_3O_2^- + 7\ H^+$	$\rightarrow 8\ NO + 2\ HCO_3^- + 4\ H_2O$	NirS	Fe(II)	–60.5
$8\ NO + C_2H_3O_2^-$	$\rightarrow 4\ N_2O + 2\ HCO_3^- + H^+$	Nor	Fe(II)	–140.0
$4\ N_2O + C_2H_3O_2^-$	$\rightarrow 4\ N_2 + 2\ HCO_3^- + H^+$	Nos	Fe(II), Cu(II)	–157.6

1.6 *Paracoccus denitrificans* as study organism

P. denitrificans is a facultative anaerobic bacterium that was isolated from garden soil by the Dutch Microbiologist Martinus Willem Beijerinck and the Dutch Chemist Dirk Constant Jan Minkman in 1910 (Beijerinck and Minkman, 1910). It was initially named '*Micrococcus denitrificans*' and is today affiliated with the family of *Rhodobacteriaceae* of the *Alphaproteobacteria*. Strain Pd1222, a derivative of DSM 413^T (Verhoeven et al., 1954; de Vries et al., 1989; Kelly et al., 2006) has been increasingly used for enzymatic studies, as it is one out of two strains with a known genome sequence. The 5.2 Mbp large genome of *P. denitrificans* Pd1222 consists of two chromosomes and one plasmid with an overall G+C content of 66.8% (Integrated Microbial Genomes, IMG, ID 639633048, (https://img.jgi.doe.gov/cgi-bin/w/main.cgi). 91% of all bases are within coding sequences and a function has been predicted for approximately 73% of all protein coding genes. The numerous studies on genetics of aerobic and anaerobic respiration and carbon metabolism of C_1-compounds of *P. denitrificans* have been reviewed in detail (Baker et al., 1998). *P. denitrificans* has become a model organism for studies of the anaerobic and aerobic respiratory chain due to the high similarity with respiratory enzymes of mitochondria (Richardson, 2000). It has therefore been suggested to be a close relative to the ancestor of the mitochondria (John and Whatley, 1975).

The genome of *P. denitrificans* encodes the two respiratory nitrate reductases Nar (membrane-bound) and Nap (periplasmic). Both enzymes require molybdenum and iron. Nar is a molybdoenzyme of three subunits (NarGHI) and constitutes a molybdenum cofactor and five iron-sulfur clusters (Stouthamer, 1991; Baker et al., 1998). The catalytic site in NarG faces the cytoplasm and reduces nitrate to nitrite which is trans-

ported to the periplasm by a nitrate-nitrite antiporter (NarK). Nar contributes to energy conservation by translocating protons to the periplasm in contrast to Nap. The latter enzyme has been described to support nitrate reduction under aerobic conditions at low oxygen concentrations (Sears et al., 1997). The primary role of Nap is to dispose off reducing equivalents in the presence of oxygen (Richardson and Ferguson, 1992).

Cytochrome cd_1 nitrite reductase of *P. denitrificans* is located in the periplasm containing a *c*-type and a d_1-type heme. Both the nitrite reductase and the membrane-bound nitric oxide reductase NorBC consume protons from the periplasmic side of the membrane (Stouthamer, 1991). NorBC consists of heme-*b* and heme-*c* groups and non-heme iron. The *nir* gene cluster is located adjacent to the *nor* operon and their transcription is regulated by the same regulatory protein (NNR), presumably to ensure rapid reduction of the highly toxic nitric oxide (Van Spanning et al., 1995). The nitrous oxide reductase NosZ is a periplasmic multi-copper protein containing two iron-sulfur clusters and six copper ions in two copper clusters, which explains the high copper requirement of denitrifying organisms (Snyder and Hollocher, 1987; Richardson et al., 2009). In the denitrifying respiratory chain of *P. denitrificans*, electrons are transferred from NADH dehydrogenase and succinate dehydrogenase to the quinone pool. Reduced quinol donates the electrons to nitrate reductase and to the cytochrome bc_1 complex. Electron transfer to Nir, Nor and Nos is mediated from cytochrome bc_1 via cytochrome c_{550} or pseudoazurin in the periplasm (Baker et al., 1998).

P. denitrificans is also able to assimilate nitrate (Gates et al., 2011). The Nas complex is located in the cytoplasm and comprises a molybdenum-dependent nitrate reductase NasC and a sirohaem nitrite reductase NasB that reduces nitrite to ammonium. Assimilatory nitrate and nitrite reduction is coupled to NADH oxidation via NasG, a Rieske-type ferredoxin (Luque-Almagro et al., 2011). In presence of both nitrate and ammonium the assimilation of nitrate was reported to be suppressed (Gates et al., 2011). Under this condition ammonium is used to generate glutamate by glutamate dehydrogenase or to generate glutamine via the glutamine synthetase (GS)–glutamate synthase (glutamine:2-oxoglutarate aminotransferase, GOGAT) pathway (Leigh and Dodsworth, 2007).

P. denitrificans is capable of heterotrophic growth on a variety of reduced carbon compounds comprising carbohydrates, organic acids, alcohols, amino acids and other nitrogenous compounds (Nokhal and Schlegel, 1983) and autotrophic growth on molecular hydrogen (Porte and Vignais, 1980) or thiosulfate (Friedrich and Mitrenga, 1981). It has also been shown to grow autotrophically on the C_1-compounds methanol, methylamine and formate by oxidizing them to carbon dioxide which is subsequently assimilated via

the ribulose bisphosphate cycle (Cox and Quayle, 1975). For growth on C_2-compounds such as acetate *P. denitrificans* uses the glyoxylate cycle (Kornberg and Krebs, 1957; Claassen et al., 1987) and the ethylmalonyl-CoA pathway which was only recently fully understood (Alber et al., 2006; Erb et al., 2009). *P. denitrificans* has not been used for experimental evolution before, but the detailed knowledge about the genetics and enzymology of metabolism summarized above may facilitate the interpretation of observations during adaptation under denitrifying conditions.

1.7 Cultivation techniques for experimental evolution

Different types of evolution experiments using microorganisms can be performed. The fundamental idea of these experiments is to observe evolution during relatively short periods of time under defined conditions to exclude external unknown parameters that may influence microbial adaptation. For that reason all methods have in common that they establish controlled environments with defined growth environments. Adaptive evolution can be investigated by using the serial transfer approach or continuous cultures (chemostats; Barrick and Lenski, 2013). In the classical, most frequently used serial transfer experiments, microbial populations are grown in batch cultures and a fraction of the cell suspension is periodically transferred into fresh medium in the exponential or early stationary growth phase (Figure 1.3).

The advantage of serial transfer experiments is the simple equipment that allows the handling of many replicates. Batch cultures can be performed in very small volumes, even in deep well plates (Gonzalez and Bell, 2013). Automated, high-throughput techniques have further extended evolution experiments by enabling thousands of replications in parallel (Desai, 2013). Although the flasks are constantly shaken and cells homogeneously distributed, the cultures are subjected to changing conditions such as nutrient and product concentrations, pH, growth rate and cell density due to microbial growth (Dragosits and Mattanovich, 2013).

To avoid fluctuating growth conditions, chemostats are applied to completely control the above mentioned parameters. In chemostats microorganisms are constantly supplied with fresh medium from a reservoir containing nutrients and salts at higher concentrations (Novick and Szilard, 1950). The medium is diluted, mixed with the microbial culture and nutrients are consumed as soon as they enter the culture. As a result of this simultaneous addition of fresh medium and consumption of nutrients the chemical and physical conditions differ between the culture and the medium. The culture volume

remains constant as the cell suspension is removed at the same rate as the medium enters. The major characteristics of chemostats is growth kinetics: The cells are constantly kept in their exponential growth phase at constant growth rate. The growth rate equals the dilution rate, which is defined by the ratio of the flow rate and the culture volume. Thus, the growth rate can be set to a desired value below the maximum specific growth rate of the respective organism. The equipment of continuous cultures is more complex than that used for batch cultures and more susceptible for technical failure or contamination by microorganisms from external sources. Thus careful maintenance and regular inspection is indispensable.

The most important difference between serial transfer and chemostat experiments is nutrient limitation in the latter (Monod, 1949; Novick and Szilard, 1950). In contrast, in serial transfer experiments the culture subsamples are transferred to the new medium before the provided nutrients are fully depleted. Microorganisms are selected for growth rate or yield and need to pass a bottleneck the size of which depends on the size of the transferred fraction (Barrick et al., 2009). In chemostats, microorganisms are selected for their ability to acquire the limiting nutrient (Dykhuizen and Hartl, 1983). For this reason, this technique is more suitable for the study of microbial adaptation to nutrient limitation. It has been used to study the impact of growth rate on evolutionary pathways (Notley-McRobb et al., 2003) and on diversification (Maharjan et al., 2012) and uncovered evolutionary improvements of expression of regulators and transporters (Helling et al., 1987; Death et al., 1993; Notley-McRobb and Ferenci, 2000; Franchini and Egli, 2006).

A clearly distinct approach is the use of solid media for experimental evolution. By the transfer of only few isolated colonies, the populations are deliberately forced to pass a bottleneck. Thus, natural selection is minimized and mutations with little or no benefit are allowed to accumulate. Therefore these types of evolution experiments are termed 'mutation accumulation' and are performed to estimate the rate at which mutations occur (Elena and Lenski, 2003). Under natural selection mutations of little beneficial effect generally do not exceed a frequency high enough to be detected and are rather outcompeted by clones carrying mutations with higher benefits (Barrick and Lenski, 2013).

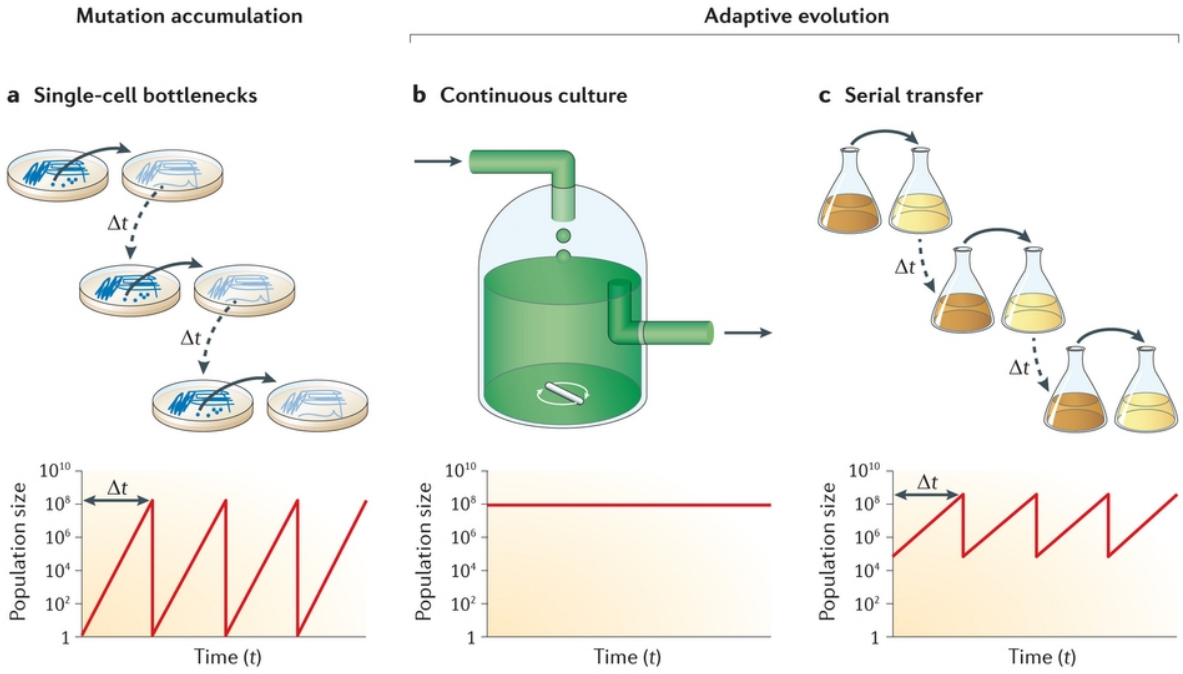

Figure 1.3 Cultivation techniques for the study of microbial experimental evolution. Populations are propagated on solid media (a), in continuous cultures (b) or in liquid batch cultures (c). The setups lead to different population dynamics as shown by the changing population sizes in batch experiments in contrast to continuous cultures. More details are described in the text. Adapted from Barrick and Lenski (2013).

1.8 Aims of this thesis

Experimental evolution with microorganisms has been used to investigate the adaptation towards higher fitness under various growth conditions. So far, only few studies considered adaptation under anoxic conditions such as the improvement of fermentative production of ethanol (Zelle et al., 2011) or C_4-dicarboxylic acids (Sonderegger and Sauer, 2003) for biotechnological applications. Wenger et al. (2011) investigated the fitness of yeast when it was subjected to anoxic environments after evolution under oxic conditions and glucose limitation. This thesis seeks to open a new field in experimental evolution that involves adaptation of *P. denitrificans* under anoxic, denitrifying conditions.

For the long-term cultivation of *P. denitrificans* it is essential to provide a medium that confers neither toxic effects nor limitation of nutrients other than carbon or nitrate. To achieve this, *P. denitrificans* was grown on different media, with special focus on new trace element compositions. The suitability for anaerobic growth was estimated by growth rate, maximum optical density and repeatability of growth curves.

Using the improved medium, *P. denitrificans* was evolved in acetate or nitrate limited chemostats under denitrifying conditions for at least five hundred generations. In the evolution experiment several research questions have been addressed. First, the metabolic conversions of carbon and nitrate compounds were monitored. This procedure answered the question whether metabolic activities changed or even improved over time and whether carbon or nitrogen conversions differed between the two selective environments. Second, transcriptomes were investigated from samples taken every hundred generations. The analyses of transcriptional activities were focused on central metabolism, carbon and nitrogen uptake and regulators and interpretations in context with physiological changes were attempted. Additionally, the global pattern of transcriptional activities comprising all genes allowed conclusions on evolutionary changes and further comparison between the treatments. Third, the experimental evolution of *P. denitrificans* was complemented with whole-genome re-sequencing to get insights into underlying causes of observed changes compared to the ancestral genome. Mutations were analyzed for their type, distributions and possible effects on the strains. To conclude on the significance of the results, the observations were discussed in an ecological context.

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Chapter 2

An improved medium for the anaerobic growth of *Paracoccus denitrificans* Pd1222

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2.1 Abstract

Paracoccus denitrificans is a well studied model organism with respect to its aerobic and anaerobic respiratory enzymes. However, until now, the growth medium for this organism has not been optimized for anaerobic growth. In particular, the requirements of *P. denitrificans* for trace elements are not well known. In the present study we aimed to improve growth rates of *P. denitrificans* Pd1222 on a defined medium under anoxic conditions. We designed media containing different combinations of trace elements at various concentrations, and tested their performance against previously reported media. Our results suggest that growth rate and yield depend on the availability and concentration of trace elements in the medium. A chelated trace element solution was more suitable than an acidified trace element solution. Highest growth rates were achieved with medium comprising the trace elements iron, manganese, molybdenum, copper and zinc ranging from 0.1 to 9 µM. On this medium, *P. denitrificans* Pd1222 grew with a generation time of 4.4 hours under anoxic conditions and 2.8 hours under oxic conditions. Diauxic growth was clearly shown with respect to nitrate and nitrite reduction under anoxic conditions.

2.2 Introduction

The first strain of *Paracoccus denitrificans* (synonym *Micrococcus denitrificans*) was isolated from soil more than one century ago by Beijerinck and Minkman (1910). It was shown to grow aerobically and anaerobically performing complete or partial denitrification. Although *P. denitrificans* is an important model organism to study the electron transfer chain and energy conservation (for review see Stouthamer, 1991), there is still a lack of detailed information about the requirements of this organism for optimal growth. However, cultivation conditions have an important influence onto the physiological phenotype of an organism. Prominent examples for physiological processes that are strongly influenced by cultivation are the aerobic oxidation of methane (Stanley et al., 1983; Prior and Dalton, 1985) and methanol, the fixation of nitrogen gas (Lehman and Roberts, 1991), and the assimilation of CO_2, which make use of different enzymes (or even pathways) depending on the composition of the growth medium (most notably the presence or absence of vitamins and trace elements). Consequently, it is important to improve cultivation conditions of an organism and prevent its growth inhibition to avoid misinterpretation of the observed phenotypes.

Several approaches have been developed for medium optimization. Approaches based on evolutionary algorithms have been used lately to develop and optimize media for the isolation of novel bacterial strains without making assumptions about the individual components of a medium (Heylen et al., 2006). In contrast, rational approaches rely on the systematic improvement of existing media by focusing on a particular subset of medium components, such as vitamin or trace element supplements. In this study, we followed a rational strategy focusing on trace element composition to improve the medium for the anaerobic growth of *P. denitrificans* Pd1222 on acetate.

P. denitrificans grows aerobically with maximum growth rate at pH 7.6 and at 36 °C and can tolerate salt concentrations of at least 3% (Nokhal and Schlegel, 1983). Whereas the suitability of different carbon sources of *P. denitrificans* has been characterized in detail (Nokhal and Schlegel, 1983; Kelly et al., 2006) its requirements for trace elements have not been investigated extensively. Different kinds of trace elements, as well as large ranges of concentrations have been previously used in different studies (an overview of common trace element solutions used for the cultivation of *P. denitrificans* and related organisms is given in Table 2.1). These solutions have been frequently used by many researchers (Meijer et al., 1979; Stouthamer and Bettenhaussen, 1980; Van Spanning et al., 1990; Moir and Ferguson, 1994; Sears et al., 1997). Trace elements are essential for the correct function of enzymes, such as those of the respiratory chain; however,

at higher concentrations they can impair growth and even be toxic. Here we present results on improvement of anaerobic growth of *P. denitrificans* Pd1222 with focus on trace element requirements.

2.3 Material and methods

Organisms

Paracoccus denitrificans Pd1222 (16S rRNA gene accession number NR_074152), a derivative of DSM 413^T (de Vries et al., 1989), was obtained from Prof. Dr. R. van Spanning, Vrije Universiteit Amsterdam, faculty of Earth and Life Sciences. The organism was maintained aerobically on solid LB medium, containing 15 g L^{-1} agar, and transferred every three months. For long-term storage the cells were frozen at -80 ℃ in 30% glycerol and revived by spreading frozen cells on LB agar plates and incubating aerobically at 30 ℃ for two to three days.

Mineral salt media

The following chemicals were purchased from AppliChem, Darmstadt, Germany: $MgSO_4 \cdot 7\,H_2O$, $CaCl_2 \cdot 2\,H_2O$, K_2HPO_4 and sodium acetate. All other chemicals were received from Carl Roth GmbH, Karlsruhe, Germany. The purity was at least 99% for most chemicals, except for $ZnCl_2$, 97%, $MnCl_2 \cdot 4\,H_2O$, 98% and $NiCl_2 \cdot 6\,H_2O$ 98%, whereas major impurities comprised sulfates or chlorides.

The experiments presented here were grouped into series 1 to 5. The mineral salt medium of series 1 was prepared after Taylor and Hoare (1969). The medium was supplemented with two different trace element solutions which are described below. For series 2 to 5, a freshwater medium modified after Widdel and Bak (1992) was used, containing (in g L^{-1}): NH_4Cl (0.5), $MgSO_4 \cdot 7\,H_2O$ (0.5), $CaCl_2 \cdot 2\,H_2O$ (0.1), KH_2PO_4 (0.04), K_2HPO_4 (0.12) and HEPES (6.0). Phosphate was added from a separately prepared and autoclaved stock solution. This medium was used to test three different trace element solutions previously described (see below).

Materials used for medium preparation were rinsed with ultra pure water (Aquintus system, membraPure, Germany) prior to usage. All media were prepared with ultra pure water and the pH was adjusted to 7.2 with 1 M HCl or 1 M NaOH if necessary. The media were supplemented with 60 mM sodium acetate as carbon and energy source and 30 mM KNO_3 as the electron acceptor were added to both anaerobic and aerobic cultures to ensure identical salt concentrations, unless otherwise stated. For anaerobic

cultures, 10 mL or 30 mL mineral salt solutions including electron donor and acceptor were filled into Hungate tubes or 50 mL serum flasks using a volumetric pipette, and capped with a butyl stopper. The headspace was exchanged by applying vacuum, supplying argon at a pressure of 1.5 bar, followed by rigorous shaking (Widdel and Bak, 1992). This procedure was repeated three times. Finally, overpressure was released through a second needle. For aerobic cultures, 50 mL medium were filled into 250 mL Erlenmeyer flasks. All media and stock solutions were autoclaved at 121 °C for 25 min.

Trace element solutions

The final trace element concentrations in the media for all growth experiments in this study are listed in Table 2.1 and Table 2.2. Five trace element (TE) stock solutions that have been previously presented, were prepared, three acidic solutions and two solutions containing a chelator (Table 2.1). Solution TE-1 was prepared according to Vishniac and Santer (1957) as described by Robertson and Kuenen (1983) and contained (in mg L^{-1}): Na_2-EDTA (5,000), $FeSO_4 \cdot 7\,H_2O$ (500), $CaCl_2 \cdot 2\,H_2O$ (728), $MnCl_2 \cdot 4\,H_2O$ (506), $CuCl_2 \cdot 2\,H_2O$ (107), $Na_2MoO_4 \cdot 2\,H_2O$ (22), $CoCl_2 \cdot 6\,H_2O$ (161) and $ZnCl_2$ (185). The acidic solution TE-2 was prepared according to Widdel (1983). The medium after Taylor and Hoare (1969) was supplemented with 2 mL L^{-1} solution TE-1, a combination which was previously used for the cultivation of *P. denitrificans* DSM 413^T (Robertson and Kuenen, 1992). Alternative growth experiments were performed with the same medium amended with 1 mL L^{-1} solution TE-2. Three more trace element solutions were prepared (Lawford, 1978; Widdel and Pfennig, 1981; Widdel and Bak, 1992) and tested with medium after Widdel and Bak (1992). The solution after Lawford was previously used for the cultivation of *P. denitrificans* ATCC 13543 (Lawford, 1978). Series 2 of the growth curves was performed with a set of trace element solutions, designed in this study (Table 2.2), with increasing Cu^{2+} concentrations that contained (in mg L^{-1}): Na_2-EDTA (7,300), $FeSO_4 \cdot 7\,H_2O$ (2,500), $MnCl_2 \cdot 4\,H_2O$ (20), $Na_2MoO_4 \cdot 2\,H_2O$ (242), $CuCl_2 \cdot 2\,H_2O$ (17; 38; 85; 128; 170; 426). Trace element solutions for the growth experiments of series 3 to series 5 (Table 2.2) comprised the components of the solutions of series 2 (with 85 mg L^{-1} $CuCl_2 \cdot 2\,H_2O$) and optional (in mg L^{-1}): $CoCl_2 \cdot 6\,H_2O$ (238), $ZnCl_2$ (340), H_3BO_3 (30), $NiCl_2 \cdot 6\,H_2O$ (24). Depending on the final trace element concentrations, the errors caused by impurities of chemicals were: 0.7 to 7.4% (iron), 0.5 to 4.6% (Co), 0.2 to 8.4% (Zn), 0.7 to 3.6% (Mn) and 0.1 to 2.6% (Mo). The impact of impurities of copper and nickel was higher; therefore the concentrations given in the results were corrected for this error. Trace element solution TE-1 and all solutions of series 2 to 5 were prepared as chelated stock solutions and the pH was adjusted to 6

Table 2.1 Compositions of frequently used trace element solutions in the literature (final concentrations in μM in the medium). TE, trace element solution, n.a., not applicable.

Property and composition of solution	Reference							
	Robertson and Kuenen (1983)	Strohm et al. (2007)	Lawford (1978)	Widdel and Pfennig (1981)	Widdel and Bak (1992)	Chang and Morris (1962)	Nokhal and Schlegel (1983)	Harms (1985)
Metal dissolution	EDTA	acidic	acidic	acidic	EDTA	iron citrate	iron citrate	EDTA, citric acid
Na_2-EDTA	342.2				14.0			
Fe(II)	36.0[a]	7.5[b]		7.5[b]	7.5[a]	19.8[a]		20.0[a]
Fe(III)			90.0[b]				4.6[c]	
Mn(II)	51.1[b]	0.5[b]	50.0[b]	0.5[b]	0.5[b]	4.5[a]	0.2[b]	13.2[a]
Cu(II)	12.6[a]	0.01[b]	5.0[b]	0.1[b]	0.01[b]		0.1[b]	
Mo(VI)	1.8[d]	0.2[e]	10.0[e]	0.2[e]	0.2[e]	728.4[e]	0.1[e]	599.0[e]
Co(II)[b]	13.5	0.8	10.0	0.8	0.8		0.8	
Zn(II)	27.3[a]	0.5[b]	25.0[b]	0.5[b]	0.5[a]		0.4[a]	
B(III)[f]		0.1		1.0	0.5		4.9	
Ni(II)[b]		0.1		0.1	0.1		0.1	
Relevant organism studied	*P. denitrificans* DSM 413^T	*P. pantotrophus* DSM 65^T	*P. denitrificans* ATCC 13543	*Desulfobacter postgatei*	sulfate-reducing bacteria	*Micrococcus denitrificans*	*P. denitrificans* DSM 413^T and other strains	*P. denitrificans*, various strains
Comments	modified after Vishniac and Santer (1957)	according to Widdel (1983)	modified after Light and Garland (1971)				modified after Pfennig (1974)	
Reproduced in this study	TE-1, Fig. 1A	TE-2, Fig. 1B	not shown	not shown	Fig. 2A and 2B	n.a.	n.a.	n.a.

[a] Elements supplied as sulfates
[b] Elements supplied as chlorides
[c] Iron supplied as Fe(III)NH_4-citrate
[d] Molybdenum supplied as $(NH_4)_6Mo_7O_{24}$
[e] Molybdenum supplied as Na_2MoO_4
[f] Boron supplied as H_3BO_3

with 5 M NaOH. Stock solutions were autoclaved at 121 ℃ for 25 min and added to the media with syringes. To reduce the volumetric error, the trace element stock solutions were diluted ten or one hundred times, allowing the addition of a larger volume to the mineral media in Hungate tubes.

Table 2.2 Compositions of trace element solutions tested in different series of growth experiments in this study (final concentrations in µM in the medium).

Compound	Series 1		Series 2[a]	Series 3[b]						Series 4[c]	Series 5[d]
	TE-1[e]	TE-2[f]		TE-3	TE-3-Co	TE-3-Zn	TE-3-B	TE-3-Ni	TE-4	TE-3-Zn	TE-3-Zn
Na_2-EDTA	342.2		19.6	19.6	19.6	19.6	19.6	19.6	19.6	19.6	19.6
Fe(II)	36.0	7.5	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0
Mn(II)	51.1	0.5	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Cu(II)	12.6	0.01	0.4–2.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Mo(VI)	1.8	0.2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Co(II)	13.5	0.8			1.0				1.0		
Zn(II)	27.3	0.5				2.5			2.5	2.5	2.5
B(III)		0.1					0.5		0.5		
Ni(II)		0.1						0.07	0.07		
pH	6	1	6	6	6	6	6	6	6	6	6

[a] Test of the impact of copper on growth
[b] Test of the impact of other trace elements on growth (also tested at ten times higher concentrations)
[c] Aerobic growth on he improved medium
[d] Temperature dependence of anaerobic growth on the improved medium
[e] Trace element solution after Vishniac and Santer (1957)
[f] Trace element solution after Widdel (1983)

Anaerobic and aerobic growth experiment

Depending on the medium to be tested, the inoculum was prepared in different ways: (1) several colonies from solid LB medium were suspended in 1 mL of the respective mineral salt solution described above. (2) Cells were grown aerobically on the medium to be tested to a final optical density (OD_{600}) of 0.2. Both aerobic and anaerobic cultures were inoculated with 1% cell suspension. The cultures were incubated at 30 °C. Aerobic cultures were shaken constantly at 120 rpm. Anoxic cultivation at different temperatures (11 °C to 45 °C) was performed in Hungate tubes in a temperature gradient metal incubator.

Sample withdrawing and analyses

From cultures with an initial volume of 30 mL or more, samples of 1 to 1.5 mL were taken aseptically in different time intervals depending on medium and growth conditions. Bacterial growth was followed by measuring the OD_{600} at 600 nm (cuvette path length 1 cm) with a spectrophotometer (Genesis 10S UV-VIS, Thermo Scientific). Three replicate growth curves were additionally followed by protein determination (Lowry et al., 1951), revealing a linear relationship between OD_{600} and protein concentration. Subsequently, the sample was centrifuged at 14,500× g at 4 °C for 5 min. The supernatant was removed and stored at -20 °C for nitrate, nitrite and acetate determination. The cultures in Hungate tubes were not sampled. Instead, the optical density was measured

at 600 nm directly in the culture tube with a spectrophotometer (UV-1201V, Shimadzu) in different time intervals depending on medium and growth conditions. The OD_{600} values were corrected corresponding to measurements in cuvettes with 1 cm path length to allow comparison between all measured OD_{600} values.

Nitrate was determined colorimetrically after reaction with salicylic acid (Cataldo et al., 1975). Nitrite was determined colorimetrically using the Griess-Romijn reagent (Griess-Romijn van Eck, 1966). Acetate was determined with an HPLC system (Sykam GmbH) at a retention time of 13.8 min and a detection limit of 25 µM acetate. With 5 mM H_2SO_4 as eluent and a flow rate of 0.6 mL min^{-1}, acetate was detected using a refractive index (RI) or UV detector (210 nm).

2.4 Results

Although *Paracoccus denitrificans* is a model organism for denitrification, we found that the growth media and trace element solutions described for this organism enabled only very poor anaerobic growth of *P. denitrificans* Pd1222. This phenomenon was most apparent when growing this strain on medium (Taylor and Hoare, 1969) that was amended with either trace element solution TE-1 (Vishniac and Santer, 1957; Robertson and Kuenen, 1983) or TE-2 (Widdel, 1983; Strohm et al., 2007). Both trace element solutions have been used before for the cultivation of *P. denitrificans* or *P. pantotrophus* and are listed as final concentrations in the medium in Table 2.1. TE-1 is characterized by high metal concentrations between 1.8 and 36 µM and contains ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA) as a chelator to keep the metals dissolved, while metals in TE-2 are 4 to more than 1,000 times lower concentrated and metal dissolution is achieved by lowering the pH instead of adding EDTA. Under anoxic, denitrifying conditions, very slow growth was detected with a generation time of 12.3 hours in medium supplemented with TE-1 and 9.3 hours in medium supplemented with TE-2 (Figure 2.1; for specific growth rates, please also refer to Table 2.3). Thus, we sought for a defined medium which was more suitable for the cultivation of *P. denitrificans* Pd1222 under anoxic, denitrifying conditions.

Anaerobic growth of *P. denitrificans* Pd1222 was investigated with a different freshwater medium that was modified after Widdel and Bak (Widdel and Bak, 1992) and three different trace element compositions as previously described (Lawford, 1978; Widdel and Pfennig, 1981; Widdel and Bak, 1992). The acidic trace element solution after Lawford (Table 2.1) contained up to 100 times higher metal concentrations than the two other

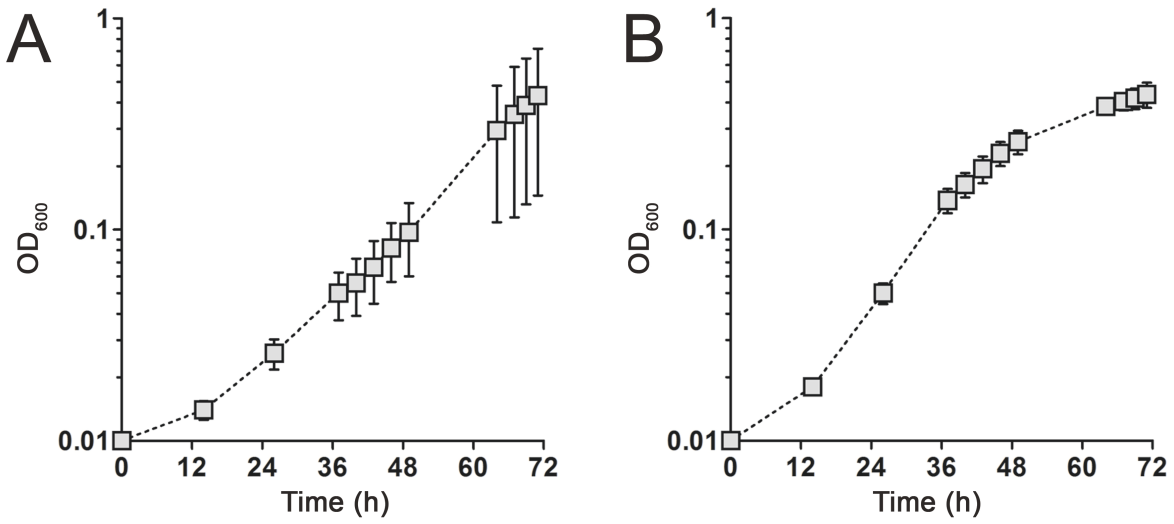

Figure 2.1 Anaerobic growth of *P. denitrificans* Pd1222 on published media. Growth was performed on two media previously described for *P. denitrificans* or *P. pantotrophus* with trace element concentrations as given in Table 2.1. (A) Growth with chelated trace element solution TE-1 after Robertson and Kuenen (1983). (B) Growth with acidic trace element solution TE-2 after Strohm et al. (2007). The graphs represent three replicates; error bars indicate standard deviation and may be smaller than the symbols.

trace element solutions (Table 2.1) that were prepared either as an acidic or a chelated (EDTA containing) solution. All media supplemented with acidic trace element solutions supported only limited growth under anoxic, denitrifying conditions after one week of incubation. In contrast, good growth was observed in the medium supplemented with chelated trace element solution according to Widdel and Bak (1992), with a generation time of 8.1 hours (Figure 2.2A and Table 2.3, at 10 mM acetate). This indicated that at least one essential compound was insufficiently available to the cells in the absence of a chelator.

Based on these results all subsequent growth experiments were performed with trace element solutions that contained EDTA for dissolution. The suitability of media subsequently tested, was estimated based on growth rates that are summarized in Table 2.3, and described in more detail below.

Trace metals such as iron and copper are required for the biosynthesis of essential enzymes, such as the membrane bound protein complexes of the aerobic and anaerobic respiratory chain. Thus we investigated whether the absence of an essential trace element might have been the reason for growth limitation. For this purpose, we cultured *P. denitrificans* Pd1222 with increasing acetate and nitrate concentrations in the medium. In the absence of any (trace) element limitation, higher substrate concentra-

Table 2.3 Growth characteristics of *Paracoccus denitrificans* Pd1222, grown in batch cultures under anoxic, denitrifying conditions unless otherwise stated.

Growth experiment	Parameter	Growth rate μ (h^{-1})	Generation time (h)
	Trace element solution		
Medium according to the literature (Figure 2.1)	TE-1	0.056 ± 0.013	12.3
	TE-2	0.075 ± 0.002	9.3
	Acetate concentration (mM)		
Trace elements according to Widdel and Bak (1992); increasing substrate concentrations (Figure 2.2)	10	0.086 ± 0.002	8.1
	20	0.092 ± 0.011	7.6
	40	0.093 ± 0.001	7.4
	60	0.095 ± 0.004	7.1
	100	0.097 ± 0.006	7.6
	Cu^{2+} concentration (μM)		
Increasing Cu^{2+} concentrations, anaerobic	0.4	0.123 ± 0.004	5.7
	0.5	0.134 ± 0.026	5.2
	0.8	0.133 ± 0.012	5.2
	1.0	0.129 ± 0.044	5.4
	1.3	0.157 ± 0.003	4.4
	2.8	0.144 ± 0.010	4.8
Increasing Cu^{2+} concentrations, aerobic	0.5	0.159 ± 0.008	4.4
	0.8	0.089 ± 0.008	7.8
	1.0	0.054 ± 0.002	12.8
	1.3	0.029 ± 0.003	18.6
	2.8	0.021 ± 0.007	32.4
	Trace element solution		
Different trace element compositions (Figure 2.3A)	TE-3	0.129 ± 0.002	5.4
	TE-3-Co	0.154 ± 0.011	5.2
	TE-3-Zn	0.159 ± 0.002	4.4
	TE-3-B	0.135 ± 0.004	5.1
	TE-3-Ni	0.141 ± 0.017	4.9
	TE-4	0.168 ± 0.001	4.7
10x concentrated trace elements (Figure 2.3B)	TE-3	No growth (<0.008)	No growth (>84 h)
	TE-3-Co	0.104 ± 0.007	6.7
	TE-3-Zn	0.125 ± 0.033	5.5
	TE-3-B	No growth (<0.008)	No growth (>84 h)
	TE-3-Ni	No growth (<0.008)	No growth (>84 h)
	TE-4	0.129 ± 0.035	5.4
Aerobic growth (Figure 2.4)	TE-3-Zn	0.251 ± 0.032	2.8
	Temperature (°C)		
Increasing temperature (Figure 2.5)	11.2	0.010 ± 0.001	67.7
	16.1	0.023 ± 0.001	30.3
	19.3	0.044 ± 0.001	15.9
	21.0	0.056 ± 0.002	12.5
	22.5	0.063 ± 0.003	11.0
	24.2	0.066 ± 0.004	10.4
	25.9	0.061 ± 0.008	11.3
	27.4	0.084 ± 0.006	8.3
	29.0	0.093 ± 0.009	7.5
	30.5	0.104 ± 0.004	6.7
	32.1	0.152 ± 0.004	4.6
	33.7	0.188 ± 0.007	3.7
	35.3	0.201 ± 0.008	3.4
	36.9	0.214 ± 0.015	3.3
	40.0	0.161 ± 0.008	4.3

tions should lead to proportionally higher growth yields. Figure 2.2B shows the growth curves of cultures supplied with 10 to 100 mM acetate (5 to 50 mM nitrate). With 10 mM acetate and 5 mM nitrate the maximum optical density ($maxOD_{600}$) reached 0.23, whereas at 20 mM acetate and 10 mM nitrate the $maxOD_{600}$ reached 0.37, which is only a 1.6-fold increase instead of the expected 2-fold increase in $maxOD_{600}$. At ten times higher acetate and nitrate concentrations, the effect was even more pronounced, as the $maxOD_{600}$ was 0.96, which corresponded only a 4-fold increase instead of the 10-fold $maxOD_{600}$ which would be expected in the absence of any (trace) element limitation. In short, the increase in OD_{600} was never proportional to the increase of substrate concentration. We may explain this observation in two ways. First, the limited availability of at least one trace element might have limited growth. Second, non-optimal growth conditions might have promoted the accumulation of toxic metabolic intermediates that inhibit growth. In this case, the organism would require additional energy as biochemical response to the stress conditions at the cost of growth yield. The latter could also be caused by an inappropriate trace element composition, leading to the accumulation

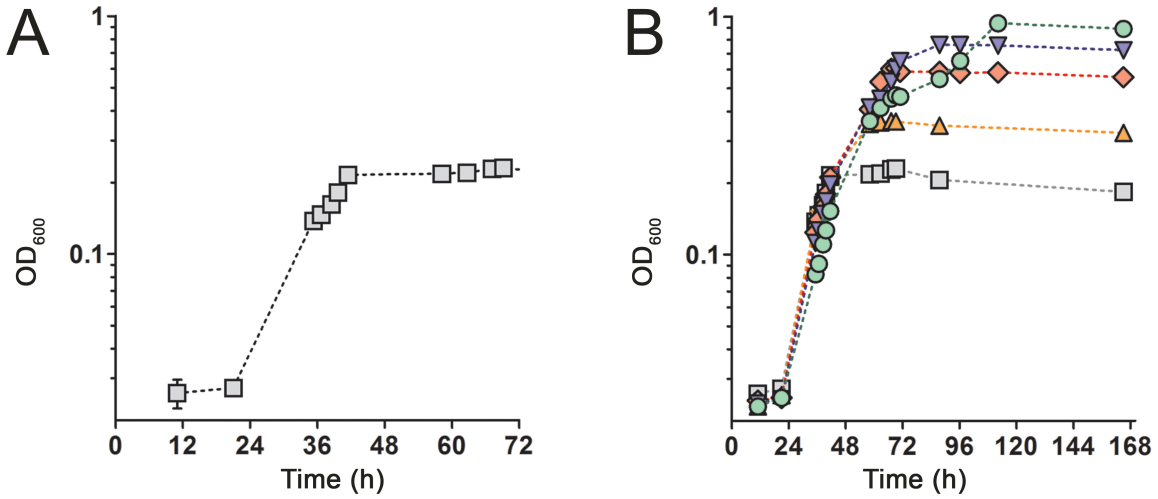

Figure 2.2 Growth curves of anaerobically grown *P. denitrificans* Pd1222 on freshwater medium supplemented with a chelated trace element solution (Table 2.1) after Widdel and Bak (1992). (A) Growth with 5 mM nitrate and 10 mM acetate. (B) Growth with different concentrations of electron donor (acetate) and acceptor (nitrate) to investigate whether a component of the medium is limiting. Concentrations were 5 mM nitrate, 10 mM acetate (gray squares), 10 mM nitrate, 20 mM acetate (orange up triangles), 20 mM nitrate, 40 mM acetate (red diamonds), 30 mM nitrate, 60 mM acetate (blue down triangles), 50 mM nitrate, 100 mM acetate (green circles). The graphs represent three replicates; error bars indicate standard deviation and may be smaller than the symbols.

of toxic intermediates (Sullivan et al., 2013). Thus, we examined whether growth rates could be improved by providing different concentrations of trace elements.

Copper was the first trace element that was investigated in more detail. It was supplied to different batch cultures in concentrations from 0.4 to 2.8 µM. In this series we used a trace element solution containing (besides copper) only iron, manganese and molybdenum, as these elements were sufficient to sustain aerobic and anaerobic growth of *P. denitrificans* (Chang and Morris, 1962). Our results did not suggest any trend in generation times with increasing copper concentration (between 4.4 and 5.7 hours, Table 2.3). However the reproducibility was better at lower copper concentrations; at higher copper concentrations (1.0 µM and higher) at least one out of three replicate cultures did not grow. Overall a copper concentration of 0.8 µM was shown to be high enough for growth and low enough to prevent observed negative effects on growth. For comparison, we grew *P. denitrificans* Pd1222 with increasing copper concentrations under oxic conditions (Table 2.3). In this case, generation times increased with increasing copper concentrations, with a minimum of 4.4 hours in medium supplemented with 0.4 µM copper and a generation time of 7.8 hours at 0.8 µM copper. Growth was severely affected above 1.0 µM copper (generation time 12.8 hours). Thus, a copper concentration of 0.8 µM was provided in the minimal trace element solution TE-3 (Table 2.2) that was used for subsequent cultivation of *P. denitrificans* Pd1222 under anoxic conditions.

Next, we investigated the effect of other trace elements on growth. For that purpose, trace element solution TE-3 that contained only the four essential elements as described above, was amended with one of four additional trace elements (cobalt, zinc, boron and nickel) to obtain four modified TE-3 solutions as listed in Table 2.2. Solution TE-4 was obtained by adding all four trace elements to solution TE-3. Growth of *P. denitrificans* Pd1222 was examined at two different concentrations of each modified TE-3 solution and TE-4. *P. denitrificans* Pd1222 grew in all cultures containing low concentrations of trace elements (Table 2.2 and Table 2.3; Figure 2.3A). In medium supplied with TE-3 the cultures grew at a generation time of 5.4 hours. The shortest generation time of 4.4 hours was achieved with the addition of TE-3-Zn, indicating that zinc positively affected growth of *P. denitrificans* Pd1222. Cultures supplemented with TE-3-Co and TE-3-B resulted in growth with a prolonged lag phase of approximately 22 hours (10 hours longer than in all other treatments). When combining all individual tested trace elements into one trace element solution TE-4, medium complemented with TE-4 enabled growth at a generation time of 4.7 hours and did not show any improvement over the medium complemented with TE-3-Zn.

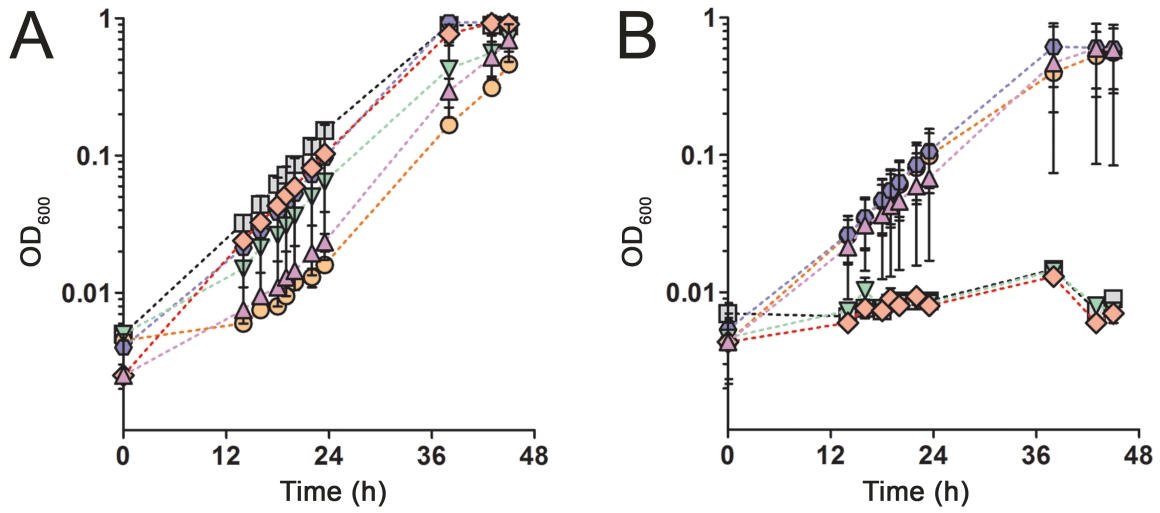

Figure 2.3 Anaerobic growth of *P. denitrificans* with respect to different trace element compositions and concentrations. The influence of a set of trace elements on growth was studied by growing *P. denitrificans* with six different trace element compositions, as listed in Table 2.2 (series 3). (A) Solution TE-3 (purple up triangles); TE-3-Co, solution TE-3 with additional cobalt (orange circles); TE-3-Zn, solution TE-3 with additional zinc (blue hexagons), TE-3-B, solution TE-3 with additional borate (green down triangles); TE-3-Ni, solution TE-3 with additional nickel (red diamonds); TE-4, solution TE-3 containing all additional elements (gray squares). (B), The same trace element solutions as described in (A) were supplied at ten times higher concentrations. Growth curves depicted in all panels represent at least two duplicates; error bars indicate standard deviation and may be smaller than the symbols.

In cultures supplied with ten times higher concentrations of TE-3, modified TE-3 solutions and TE-4 solution growth was impaired as shown in Figure 2.3B (note that most of the final metal concentrations in these media correspond to concentrations that were reached with the trace element solution after Lawford). No growth was observed in cultures supplied with ten times concentrated TE-3, TE-3-B or TE-3-Ni after one week of incubation, and only two out of three replicate cultures grew on medium supplied with ten times concentrated TE-3-Co, TE-3-Zn or TE-4. In this case, generation times were generally higher than in medium containing low concentrations of trace elements as given in Table 2.2.

In conclusion, trace element solution TE-3-Zn was the most suitable trace element solution among all solutions tested in this study for the anaerobic, denitrifying growth of *P. denitrificans* Pd1222. With this solution, a highly reproducible and short generation time of 4.4 h was achieved. Compared to aerobic growth with a generation time of 2.8 h Figure 2.4), the generation time was only 1.6 times longer (Table 2.3).

Besides trace elements, temperature has a substantial influence on growth (Table 2.3). Anaerobic growth of *P. denitrificans* Pd1222 in medium containing trace element solution TE-3-Zn was observed between 11 ℃ and 40 ℃ (Figure 2.5, with maximum growth rates at 37 ℃, similar to what was reported by Nokhal and Schlegel (1983). The generation time was 3.2 hours. As compared to previous experiments in this study, these were the highest growth rates observed for anaerobic denitrifying growth of *P. denitrificans* Pd1222.

To study the performance of *P. denitrificans* Pd1222 during growth on the improved medium in more detail, the conversion of substrates and production of intermediates were analyzed (Figure 2.6). Nitrate was completely converted to nitrite (the first intermediate in denitrification) in the first half of the exponential growth and nitrite reached a maximum concentration of 62 mM, which corresponded to the initial nitrate concentration. At this point, growth was slowed down, which can be explained by the time required by *P. denitrificans* Pd1222 to induce the expression of nitrite reductase. After approximately two hours, the growth rate was recovered and nitrite was reduced within 21 hours. Accordingly, acetate was consumed with a lower rate during the transition from nitrate to nitrite respiration. This diauxic growth was previously observed (Blaszczyk, 1993; Sáez et al., 2003), but lower concentrations of nitrite were accumulated and consumed than in this study. The depletion of both nitrate and nitrite confirms that the electron acceptor limited growth, implying that trace and macro element concentrations were sufficiently high.

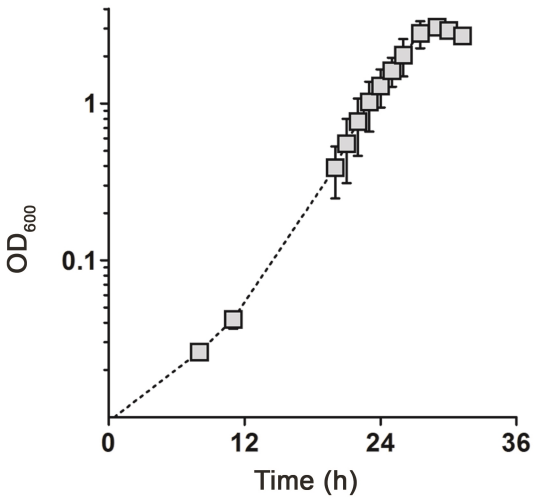

Figure 2.4 Aerobic growth of *P. denitrificans* Pd1222 on the improved medium. Growth was performed with trace element solution TE-3-Zn with concentrations as given in Table 2.2 (series 4). The graphs represent three replicates; error bars indicate standard deviation and may be smaller than the symbols.

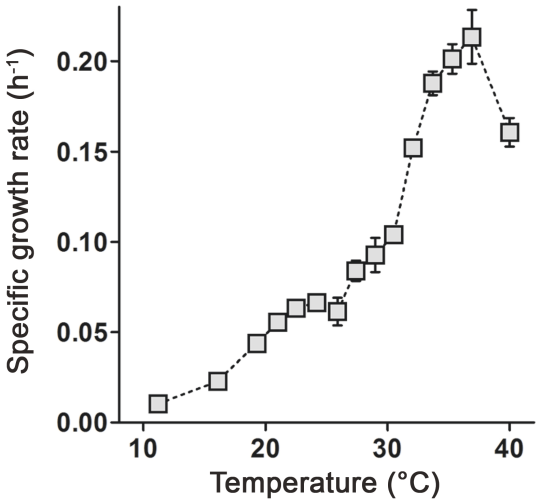

Figure 2.5 Dependence of anaerobic growth rates of *P. denitrificans* Pd1222 on temperature. The growth rates were determined from the early exponential growth phases of duplicate or triplicate growth curve measurements on the improved medium, supplemented with trace element solution TE-3-Zn (Table 2.2, series 5). Error bars indicate standard deviation and may be smaller than the symbols.

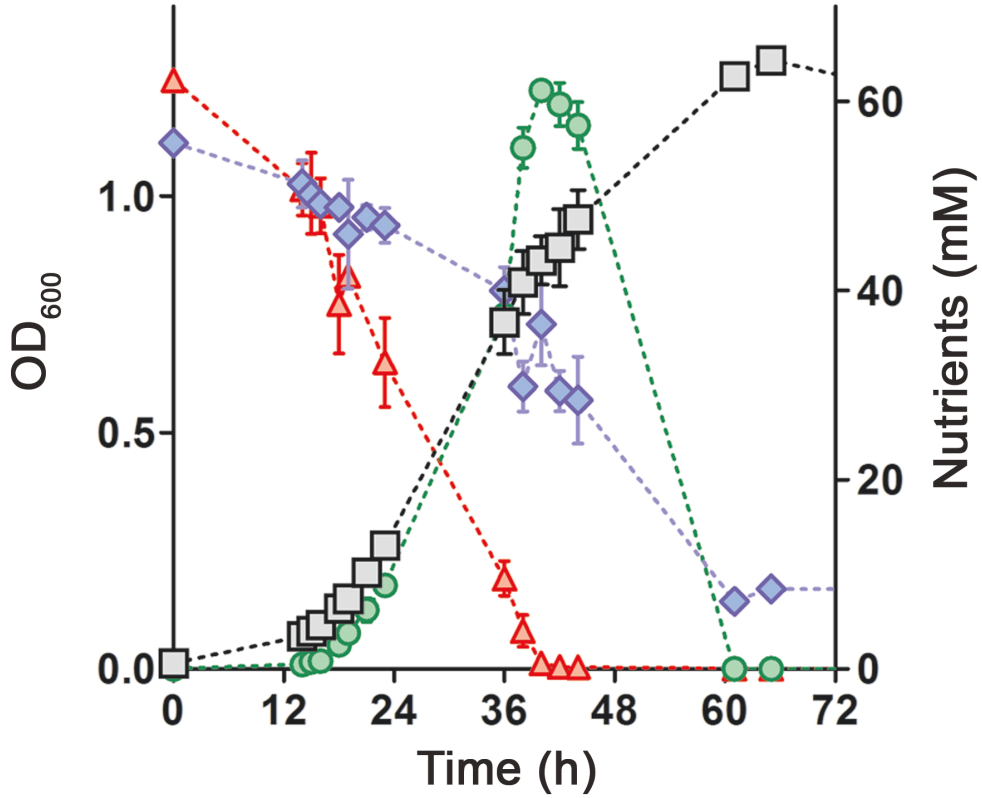

Figure 2.6 Growth curve and nutrient analyses of anaerobically grown *P. denitrificans* Pd1222 on the improved medium, supplemented with trace element solution TE-3-Zn (Table 2.2). Growth was followed by OD_{600} measurements (gray squares). Consumption of acetate (blue diamonds) and nitrate (red up triangles), as well as production and consumption of nitrite (green circles) were quantified. All data represent triplicates; error bars indicate standard deviation and may be smaller than the symbols.

2.5 Discussion

This study aimed at improving cultivation conditions for anaerobic, denitrifying growth of *P. denitrificans* Pd1222, to achieve high growth rates on a minimal medium that are comparable to growth rates in the presence of oxygen. Although *P. denitrificans* has been a long-standing model organism for the study of respiratory enzymes and energy conservation, it was surprising that *P. denitrificans* Pd1222 grew poorly on published media. Thus we sought to test and improve different media that had been used in the past to cultivate this and closely related organisms (Lawford, 1978; Robertson and Kuenen, 1983; Strohm et al., 2007).

Supplementation of media with trace elements (and vitamins) in suitable concentrations is known to have an important influence on the successful cultivation of microorganisms (Widdel, 1980; Peters et al., 2004; Pol et al., 2014). Consequently, this study focused on improving the composition of the trace element solution. Our experiments suggested that for anaerobic growth of *P. denitrificans* Pd1222, chelator-based trace element solutions are superior to solutions in which trace metals are solubilized and kept in their redox state by lowering the pH. Media that were used by many researchers to study *P. denitrificans* contain citrate as chelator (Chang and Morris, 1962; Lawford, 1978; Nokhal and Schlegel, 1983). Since *P. denitrificans* DSM 413^{T} has been reported to be able to utilize citrate under oxic, but not under anoxic conditions (Davis et al., 1970; Robertson and Kuenen, 1983), we note that citrate might become a potential carbon source under anoxic conditions, particularly during long-time cultivations. Similar observations have been recently made with *E. coli* (Blount et al., 2008, 2012). Therefore, we used EDTA instead of citrate as the chelator. One previously published medium for *P. denitrificans* DSM 413^{T} (Robertson and Kuenen, 1983) also featured EDTA, but in this medium the concentrations of trace elements ranged between 13 and 51 µM, notably higher than the 0.1 to 1 µM which are usually used for trace elements (Overmann, 2010, 2013). Higher concentrations of trace elements are known to exert toxic effects (Overmann, 2010), and our experiments indeed indicated growth-inhibiting effects of higher concentrations of trace elements.

We achieved good growth of *P. denitrificans* Pd1222 with the trace elements molybdenum, manganese and copper in concentrations between 0.1 and 1 µM, zinc at 2.5 µM and iron(II) at 9 µM. Three of these elements represent metals required as cofactors by the enzymes of the denitrification pathway of *P. denitrificans* (Stouthamer, 1991). Iron is present in all four respiratory enzymes as iron-sulfur clusters or as part of a cytochrome domain. The membrane-bound nitrate reductase (Nar) additionally requires molybde-

num (Stouthamer, 1991) and the nitrous oxide reductase requires copper (Snyder and Hollocher, 1987). Zinc is not essential for the biosynthesis of the enzymes involved in denitrification, but the increased growth rates in presence of zinc might indicate that it stimulates anaerobic growth in a different way.

In summary, we provide a minimal medium that allows anaerobic growth of *P. denitrificans* Pd1222 at a minimum generation time of 4.4 hours at 30 ℃ without the need of vitamin addition. The generation times under anoxic conditions were only 1.6 times longer than under oxic conditions. This may suggest that a more intense study of concentration ranges of all trace elements would only result in minor improvements of growth. Our medium is easy to prepare and allows physiological studies of the model organism *P. denitrificans* Pd1222 under anoxic conditions. With this medium, we show that *P. denitrificans* Pd1222 strictly grows diauxically with respect to nitrate and nitrite respiration. Nitrate was completely converted to nitrite before nitrite was reduced to dinitrogen.

We expect that our findings are also relevant to other strains of *P. denitrificans* since previous physiological studies of various strains of *P. denitrificans* showed identical physiological characteristics and similar carbon utilization patterns among the tested strains (Nokhal and Schlegel, 1983). These features specifically relate to major metabolic pathways that constitute the most trace metal dependent enzymes. Therefore, it is likely that the trace element composition suggested here represents a suitable composition for more strains of *P. denitrificans*.

2.6 Acknowledgments

We thank Maike Kortmann and Patrick de Jonge for help with monitoring growth curves and for nutrient analyses. We are grateful to Prof. Dr. Jens Harder, Prof. Dr. Friedrich Widdel and Prof. Dr. Rob van Spanning for the scientific discussions and advice on cultivation. We thank Prof. Dr. Rob van Spanning for providing the bacteria. This research was funded by the ERC grant "MASEM" (242635, granted to M.S.), the German Federal State of Nordrhein-Westfalen, and the Swiss National Science Foundation (SNF-Ambizione grant PZ00P3_136828/1, granted to T.J.E.).

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Chapter 3

Adaptive evolution of *Paracoccus denitrificans* in acetate and nitrate limited anoxic chemostats

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Contributions to the manuscript:
S.M.H. and M.S. conceived the project and designed the experiments, S.M.H. performed the experiments and data analyses. H.E.T. and S.M.H. performed genome variant analyses and sequenced transcriptomes. S.M.H. performed statistical analyses. P.M. and T.J.E. analyzed enzyme activities, S.M.H. and M.S. wrote and edited the manuscript.

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3.1 Abstract

So far, the experimental evolution of bacteria has mainly addressed adaptation toward improved aerobic conversion of carbon substrates. In this study we investigated potential driving forces for adaptation of an anaerobic denitrifying bacterium under anoxic, nutrient limiting conditions. We evolved four denitrifying cultures of *Paracoccus denitrificans* Pd1222 in chemostats at either acetate (carbon and energy source) or nitrate (electron acceptor) limitation for more than 800 generations. We combined metabolic analyses of substrates and intermediates with identification of genome variants by genome sequencing and differentiation of transcriptional activities by transcriptomics. Transcriptional activities differed significantly between the two treatments and showed a stronger response to acetate than to nitrate limitation. In acetate limited cultures significantly more genes encoding transporters, regulators and enzymes of the central carbon metabolism were up- or down-regulated than in nitrate limited cultures. Increasing efficiency in denitrification under acetate limitation was shown by changes in substrate conversion rates and agreed with the changing transcriptional activities of denitrification genes. Under nitrate limitation transcription of denitrification genes was more pronounced as well as genes encoding citric acid cycle enzymes and enzymes of glutamate synthesis pathways. Numerous mutations were detected in all cultures, most of which did not persist. The majority of beneficial mutations appeared under carbon limitation, consistent with the strong physiological responses induced by this condition.

3.2 Introduction

Experimental evolution studies are a promising tool to investigate adaptive processes in evolution. They have opened the prospect to answer a vast number of research questions by studying evolution in controlled environments with a defined selective force. The numerous insights gained by these studies underline the huge phenotypic flexibility of microorganisms and their enormous potential for rapid adaptation (Kussell, 2013). Different selective forces have been addressed, most notably nutrient limitation or adaptation to alternative substrates, promoting selection for high affinity for the limiting nutrient or diversification into cross-feeding subpopulations (Helling et al., 1987; Rozen and Lenski, 2000; Pfeiffer and Bonhoeffer, 2004). Other studies have revealed enhancements in the high-affinity glucose uptake system under glucose limitation (Notley-McRobb et al., 2003), or in improved kinetics of glycerol metabolizing enzymes under glycerol limitation (Herring et al., 2006). Mutations enabled cells to secrete substances providing improved access to the limiting nutrient (Kim et al., 2014). During competition for nutrients, novel metabolic pathways evolved to better exploit the available sources (Blount et al., 2012).

However, so far anaerobic metabolism has been less studied in experimental evolution (Sonderegger and Sauer, 2003; Wenger et al., 2011; Zelle et al., 2011). In the absence of oxygen, nitrate can serve as electron acceptor to many different microorganisms belonging to numerous phylogenetic groups. Denitrification, the sequential reduction of nitrate to dinitrogen via nitrite, nitric oxide and nitrous oxide, may offer a great potential for microbial adaptation. First, the pathway can be performed by one single organism or shared between different populations. Second, previous studies showed that denitrification is often ineffective and may lead to the (transient) accumulation of the intermediates nitrite, nitric oxide and nitrous oxide (Baumann et al., 1997; Bergaust et al., 2010, 2011; Felgate et al., 2012). This might provide a driving force in experimental evolution experiments. The denitrifying respiratory chain has been well studied in *Paracoccus denitrificans*, a facultative anaerobic bacterium capable of performing the complete pathway (Stouthamer, 1991). Recently we showed that this organism accumulates and tolerates up to 60 mM nitrite in batch cultures, which it subsequently reduces to dinitrogen (Hahnke et al., 2014). Another recently studied characteristic of *P. denitrificans* is its ability to use two distinct pathways to assimilate acetate, the glyoxylate cycle and the ethyl-malonyl-CoA (EMC) pathway (Claassen et al., 1987; Erb et al., 2009). These anaplerotic pathways provide malate and succinyl-CoA to replenish the intermediates of the tricarboxylic acid (TCA) cycle during acetate assimilation.

While the glyoxylate cycle constitutes a shortcut of the TCA cycle and avoids the two decarboxylation steps, the EMC pathway holds two carbon fixation reactions (Alber et al., 2006; Erb et al., 2007). Both pathways are active in cells of *P. denitrificans* growing anaerobically (Tobias Erb, Philipp Moosmann, personal communication). Experimental evolution of *P. denitrificans* provides a unique opportunity to study the selective force that acts on these two pathways. Conceivably the involved enzymes differ in their affinity to their carbon substrates, which would result in the selection for subpopulations that express the pathway with high affinity enzymes at carbon limiting conditions (Harder and Dijkhuizen, 1983).

In the present study we investigated the adaptive mechanisms of *P. denitrificans* Pd1222 with respect to denitrification and carbon metabolic pathways. To compare the effect of carbon and nitrate limitation under strictly controlled conditions, we experimentally evolved this strain in anoxic chemostats for more than 800 generations. Potential evolution was monitored with whole-genome re-sequencing, metabolic analyses of nutrients and intermediates, and transcriptomic analyses, to reveal the impact of culture conditions on the genetic basis of adaptation, and the consequences for the metabolism of the strain.

3.3 Material and methods

Organism and media

P. denitrificans Pd1222 (16S rRNA gene sequence, NR_074152) was kindly provided by Prof. Dr. R. van Spanning, Vrije Universiteit Amsterdam, faculty of Earth and Life Sciences. This organism was experimentally evolved in four anoxic chemostats, two with carbon limitation (C-limited cultures 1 and 2) and two with nitrate limitation (N-limited cultures 1 and 2). Acetate was used as sole carbon and energy source and nitrate as sole electron acceptor. Media were commonly prepared in charges of 18 L. All solutions were prepared with ultra pure water (Aquintus system, membraPure, Germany). The mineral salt media contained (in g L^{-1}): NH_4Cl (0.5), $MgSO_4 \cdot 7\,H_2O$ (0.5), $CaCl_2 \cdot 2\,H_2O$ (0.1), and HEPES (6.0). Media for carbon limited cultures were supplemented with 62 mM sodium acetate and 70 mM KNO_3, and media for carbon limited cultures with 70 mM sodium acetate and 60 mM KNO_3. The pH was adjusted to 4.5 to 4.6 using 20% HCl. After autoclaving at 121 °C for 20 min, the media were supplemented with 1 mL L^{-1} 1 M phosphate stock solution (40 g L^{-1} KH_2PO_4 and 120 g L^{-1} K_2HPO_4, autoclaved) and 2 mL L^{-1} autoclaved trace element solution TE-3-Zn (Hahnke et al., 2014).

Chemostat setup, maintenance and sampling

All cultures were treated identically, except for acetate and nitrate concentrations and the medium supply rate during startup (see below). Media were continuously pumped into the culture vessels at 60 mL h^{-1}, corresponding to a dilution rate of 0.1 h^{-1}, and a generation time of 6.9 h. With this growth rate, we obtained 920 generations after 270 days of cultivation. Chemostat vessels (supplementary Figure 3.S1 were built from 500-mL duran bottles and tightly closed with multiport screw caps to connect them to medium reservoirs, effluent containers, and gas lines and to install pH electrodes (InPro 3030/120, Mettler Toledo, Giessen, Germany). The cultures were mixed with magnetic stirrers at 400 rpm. To provide anoxic conditions, argon was sparged into the culture after passing a 0.2 µm pore size filter at a rate of 5 $\pm$ 0.2 mL min^{-1}, dosed with a mass flow controller (0 to 100 SCCM, Alicat Scientific, Tucson, AZ, USA). The effluent tube inside the vessel touched the liquid surface to allow removal of both liquid and gas. Thus, constant volumes of 0.6 L were achieved by removing culture liquid through an overpressure of 10 to 30 mbar. The temperature was set at 30 ℃ and pH at 7.5, which was maintained solely by the low pH of the medium. Argon was additionally amended to medium vessels to keep the oxygen concentrations minimal.

Before inoculation, the chemostat vessels were autoclaved at 121 ℃ for 30 min, including medium, gas tight viton tubes and pH electrodes. A revived cryoculture of strain Pd1222 was pre-grown as batch culture and served as inoculum (60 mL per chemostat), which we refer to as ancestor. After three days of batch growth, the dilution rate was gradually increased to allow time for the organism to adapt to continuous growth conditions. The final dilution rate was reached after 10 days in N-limited cultures and after 40 days in C-limited cultures due to temporary elevated nitrite accumulation after increasing the rate. Samples were withdrawn aseptically in three different ways. First, liquid samples up to 5 mL for quick inspection of nitrate and nitrite concentrations, optical densities, purity, and for dissolved inorganic carbon (DIC) measurements, were taken with a sterile syringe through an ethanol/heat-sterilized rubber stopper fixed to a T connector in the effluent line. Second, liquid samples of up to 80 mL were collected in autoclaved 100 mL duran bottles connected to the effluent line via another T connection. The bottles were flushed with argon through a 0.2 µm pore size filter before sampling, when samples were destined for enzyme activity assays or transcriptome analyses. Third, gas was sampled directly from the culture headspace by connecting a gas tight peek tube with a 0.2 µm pore size filter to a mass spectrometer.

Analytical procedures

The samples were checked for purity by phase contrast microscopy and examining colony morphology after plating cell suspension on solid LB medium and incubating at 30 ℃ for several days. Vital sample backups were frozen in 30% glycerol at -80 ℃. Optical densities were measured at 600 nm. Nutrients were determined from media and from supernatants of culture samples obtained after centrifugation at 4,700× g and 4 ℃ for 20 min. Nitrate, nitrite, ammonium and protein were determined colorimetrically. Nitrate was determined after reaction with salicylic acid (Cataldo et al., 1975). Nitrite was determined using the Griess-Romijn reagent (Griess-Romijn van Eck, 1966). Ammonium was determined using the OPA method (Taylor et al., 1974). Acetate was determined by HPLC (Sykam GmbH) at a retention time of 13.8 min and a detection limit of 25 µM acetate. For protein determination, 0.1 and 0.2 mL culture liquid were centrifuged at 15,000× g and 4 ℃ for 10 min and supernatants were carefully removed. Proteins were extracted in 1 mL NaOH (1 M) at 99 ℃ for 10 min, cooled to room temperature and assayed according to Lowry using bovine serum albumin as a standard (Lowry et al., 1951). Assimilated carbon and nitrogen were measured by gas chromatography after complete oxidation of a known amount of cell material (washed three times with 1 mL PBS and freeze-dried) to CO_2 and N_2 using a CNS analyzer (Carlo Erba Instruments Elemental Analyzer NA 1200) at an operating temperature of 1,050 ℃ and helium as carrier gas. DIC was converted to CO_2 by acidifying 200 or 500 µL culture liquid with 0.2 M sodium acetate buffer (pH 4.0) in an exetainer® (Labco Limited, Lampeter, UK). After equilibration for several hours and vigorous mixing, 500 µL of the gas phase were injected into the inlet port of a quadrupole mass spectrometer (GAM 400 QMG 422, InProcess Instruments) with helium as carrier gas. To measure the gas composition of the culture headspace, the gas was carried to a mass spectrometer via a peek tube (inner diameter 0.03 inch) and its composition measured for several hours after the signal had stabilized. An N_2/CO_2 (80/20) mixture was used as reference gas. All data were used to calculate mass balances for all cultures to get insights into the substrate conversions. Therefore, the assimilation of acetate and nitrogen was determined based on carbon and nitrogen content in freeze-dried biomass, protein content and the assumption that 50% of dry weight consisted of protein.

Isocitrate lyase and crotonyl-CoA carboxylase/reductase activity assays

Biomass from 50 mL culture was frozen at -80 ℃ for examinations of ICL and CCR activities immediately after sampling and centrifugation at 4,700× g and 4 ℃ for 20 min. Crude cell extracts were prepared after thawing the biomass pellets at room

temperature and resuspending them in 700 µL MOPS/NaOH buffer (100 mM, pH 7.2). Suspended cells were lysed by sonication (4 to 8 times for 30 s at 5 microns, depending on the turbidity of the suspension) and centrifuged at 20,000× g and 4 ℃ for 12 min. Protein concentration of the lysate was determined according to Bradford (1976). Isocitrate lyase activity was determined according to Alber (2006). The CCR activity measurement was modified after Erb (2007). Crude cell extract was mixed with 100 µL of 100 mM MOPS/NaOH buffer (pH 7.2), 33 mM $NaHCO_3$, and 1 mM NADPH. The reaction was started by adding 1 mM crotonyl-CoA. The consumption of NADPH was followed at 340 nm ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$). All reactions were followed with a Cary 50/60 (Varian) spectrophotometer at 30 ℃.

RNA extraction and illumina sequencing

Total RNA from 2 mL pelleted culture (centrifuged at 15,000× g and 4 ℃ for 5 min and stored in RNA later solution, at -80 ℃) was extracted as follows: The pellet was re-suspended in 1 mL of TRI Reagent solution® (Applied Biosystems). The suspension was transferred to a bead beater tube containing 0.25 mL sterile glass beads (0.1 mm diameter) for bead beating at 6.5 m s^{-1} for 45 s. After incubation at room temperature (RT) for 5 min, the tube was centrifuged for 5 min at 12,000× g and 4 ℃, and the supernatant was transferred to a fresh tube. 200 µL chloroform were added followed by vigorous shaking by hand for 15 s, incubation at RT for 10 min, and centrifugation at 12,000× g and 4 ℃ for 15 min. The upper phase was transferred to a fresh tube, 500 µL ice-cold isopropanol was added and the tube was inverted several times, followed by incubation on ice for at least 30 min for RNA precipitation. After centrifugation at 20,000× g and 4 ℃ for 25 min, the pellet was washed three times with 1 mL ice-cold ethanol (10 min centrifugation at 20,000× g, 4 ℃ between washing steps) and air dried at RT for approximately 10 min. The pellet was re-suspended in sterile TE buffer (pH 8.0) and incubated on ice for approximately 30 min for complete dissolving. The extracted RNA was treated with DNase (Promega) and purified using RNeasy MinElute spin columns (Qiagen). Ribosomal RNA was depleted from 5 µg total RNA using the Ribo-ZeroTM rRNA Removal Kit (Bacteria) (epicentre). Barcoded illumina cDNA libraries were prepared from approximately 100 ng of the rRNA depleted total RNA according to the TruSeq RNA Sample Preparation Kit v2 manual. The libraries were sequenced on a MiSeq instrument in a 2× 250 bp paired end run.

DNA extraction and illumina sequencing

DNA from 2 mL pelleted culture (stored at -20 ℃) was extracted according to Zhou et al. (1996) after incubation for 30 min at 37 ℃ with 3 mg mL^{-1} lysozyme and 0.1 mg mL^{-1} RNAse. Barcoded illumina libraries were prepared from 50 ng extracted DNA, according to the Nextera® DNA Sample Preparation Kit manual. The libraries were sequenced on a MiSeq instrument in a 2× 250 bp paired end run.

Sequence data analysis

The sequenced reads from the generated DNA and cDNA libraries were quality trimmed using the trimmomatic tool (v. 0.30) (Bolger et al., 2014), with the following settings: DNA – removal of first 10 bases, removal of trailing bases below a Phred score of 20, sliding window option 2:19, minimum length of 50 bases; RNA – removal of trailing bases below a Phred score of 20, sliding window option 3:20, minimum length of 35 bases. The quality trimmed DNA reads were mapped to the *Paracoccus denitrificans* Pd1222 reference genome (two chromosomes and one plasmid, GenBank accessions: CP000489.1 CP000490.1 and CP000491.1) and SNP calling was done according to the pipeline developed by (Dettman et al., 2012). Genome variation between the *P. denitrificans* Pd1222 genome and the ancestor that persisted over time were excluded from data interpretation. The detected mutations were visualized using BRIG (Alikhan et al., 2011). The quality trimmed cDNA reads were mapped to the *P. denitrificans* Pd1222 reference genome using bowtie2 (v2. 0.0-beta6), and processed using the edgeR package (v. 3.4.2; Robinson and Oshlack, 2010) in R (v. 3.0.2; R Core Team, 2014) (supplementary R script). Genes with less than 10 cDNA reads in all samples were excluded from analysis. cDNA reads were upper quartile (0.75) normalized and Reads Per Kb per Million reads (RPKM) values (Mortazavi et al., 2008) were calculated using the function rpkm based on normalized library sizes. Differences in expression profiles between the samples were visualized with heatmap.2 of the package gplots (v. 2.12.1; Warnes et al., 2014). The dendrogram was calculated based on Manhattan distance measure, Ward's method for hierarchical clustering and 1,000 subsamplings using pvclust (v. 1.2-3; Suzuki and Shimodaira, 2011) and vegan (v. 2.0-10; Dixon, 2003). The significance of different transcriptional activities between C-limited and N-limited cultures and the significance of up- or down-regulation of genes were calculated with Fisher's exact test of the R package edgeR (v. 3.4.2; Robinson and Oshlack, 2010).

3.4 Results

Four *Paracoccus denitrificans* cultures were experimentally evolved in anoxic chemostats to investigate driving forces for adaptation under denitrifying conditions. All cultures were continuously supplied with nitrate as electron acceptor and acetate as carbon and energy source. Acetate and nitrate were supplied at different ratios to provide either carbon limiting (C-limiting, 2 replicates) or nitrate limiting (N-limiting, 2 replicates) conditions. Ammonium was supplied as the nitrogen source to all cultures. During up to 900 generations (270 days) we monitored the conversion of carbon and nitrogen compounds to investigate metabolic consequences of evolution (Table 3.1).

Acetate was continuously supplied as carbon and energy source to C-limited cultures at a rate of 60.5 ± 1.2 μmol min^{-1} and to N-limited cultures at a rate of 68.4 ± 1.5 μmol min^{-1}. Despite the different rates of substrate supply, all cultures constantly consumed acetate at approximately the same rates (Table 3.1, supplementary Figure 3.S2). The biomass yield, determined based on protein content, was very similar in all four cultures: 33.3 to 34.6% of acetate was assimilated (Table 3.1, supplementary Figure 3.S3) and also the nitrogen and protein contents of the biomass were similar at both conditions. The acetate dissimilation rates were determined from dissolved inorganic carbon in the culture liquid and CO_2 in the headspace between 170 and 550 generations (Table 3.1, supplementary Figure 3.S3). These rates were higher in C-limited (43.7 ± 2.1 μmol min^{-1}) than in N-limited cultures (39.0 ± 0.8 μmol min^{-1}, $p = 0.002$). The sum of dissimilated and assimilated carbon was almost equal to the consumed acetate in N-limited cultures (94.3 to 109.0% mol carbon), but was mostly higher than 100% in C-limited cultures (97.3 to 113.5%, supplementary Figure 3.S4).

Nitrate was continuously supplied at a rate of 59.0 ± 1.7 μmol min^{-1} to the N-limited cultures, and was completely reduced to dinitrogen. Nitrite was below the detection limit throughout the experiment. C-limited cultures were supplied with nitrate at a rate of 68.5 ± 2.7 μmol min^{-1}. In this case, nitrate was largely reduced to dinitrogen, but a part was only reduced to nitrite (between 1.6 and 3.2 μmol min^{-1}, accumulation of 1.6 to 3.2 mM, Table 3.1). In all cultures nitric oxide and nitrous oxide concentrations remained below the detection limit. Except for one peak of nitrate accumulation and simultaneous depletion of nitrite in C-limited culture 1 after about 200 generations, the conversions of nitrate were stable throughout the first 430 generations. Interestingly, C-limited culture 1 showed a transition between 490 and 630 generations (Figure 3.1A), in which both nitrate and nitrite accumulated. After this transition, nitrite was no longer detected and instead, nitrate accumulated in this culture at a rate of 0.5 μmol min^{-1}

Table 3.1 Carbon and nitrogen mass balance during experimental evolution of *P. denitrificans* under acetate limitation (C-lim) and nitrate limitation (N-lim). P-values were calculated with the two-sided t-test by comparing rates among C-lim or N-lim culture replicates (1 and 2) or between all C-lim culture replicates and all N-lim culture replicates. r_m, mean rate; n, number of measurements in the course of 800 generations.

Condition		Rate				Rate			p-value		
		C-lim		N-lim		C-lim	N-lim	All	C-lim 1 vs. C-lim 2	N-lim 1 vs. N-lim 2	C-lim vs. N-lim
		1	2	1	2	1,2	1,2				
Carbon metabolism											
Acetate comsumption	r_m	60.0	60.3	59.3	59.5	60.1	59.4	59.7			
(µmol C min^{-1})	±	1.3	0.5	1.4	1.7	1.1	1.5	1.4	0.404	0.757	0.037
	n	18	9	15	14	27	29	56			
Bicarbonate production	r_m	44.9	41.8	39.1	38.9	43.7	39.0				
(µmol C min^{-1})	±	1.5	0.9	1.0	0.6	2.1	0.8		0.285	0.519	0.002
	n	3	2	4	4	5	8				
Bicarbonate production	r_m	74.0	68.0	65.6	65.4	71.6	65.5				
(% acetate consumed)	±	3.9	0.7	2.4	2.8	4.3	2.4		0.302	0.698	0.006
	n	3	2	4	4	5	8				
Biomass production	r_m	20.7	20.6	20.7	20.7	20.6	20.7	20.7			
(µmol C min^{-1})	±	1.7	1.9	1.3	1.0	1.7	1.1	1.4	0.253	0.673	0.088
	n	10	6	10	9	16	19	35			
Biomass production	r_m	33.9	33.3	34.6	34.2	33.7	34.5	34.1			
(% of acetate consumed)	±	2.9	3.1	2.7	2.1	2.9	2.4	2.6	0.127	0.613	0.774
	n	9	5	7	6	14	13	27			
Nitrogen metabolism											
N_2 production[a]	r_m	67.1	65.9	59.4	59.0	66.6	59.2				
(µmol N min^{-1})	±	3.2	3.2	1.6	1.5	3.2	1.5		0.369	0.013	< 0.001
	n	27	18	27	27	45	54				
N_2 production[b]	r_m	67.1	61.7	61.1	61.3	65.1	61.2				
(µmol N min^{-1})	±	4.2	8.7	2.7	4.8	6.3	3.7		0.520	0.962	0.357
	n	5	3	6	6	8	12				
Ammonium consumption	r_m	6.8	5.9	9.3	9.2	6.5	9.3				
(µmol N min^{-1})	±	0.6	0.9	0.4	0.4	0.8	0.4		0.524	0.488	< 0.001
	n	6	3	6	6	9	12				
Biomass production	r_m	9.1	8.6	9.0	9.1	8.9	9.1				
(µmol N min^{-1})	±	0.6	0.6	0.8	0.5	0.6	0.6		0.061	0.699	0.844
	n	5	3	5	5	8	10				

[a] determined from NO_3^- and NO_2^- in media and cultures

[b] determined from N_2 measurements in the culture headspace

(0.5 mM). The ammonium consumption (9.3 ± 0.4 µmol min^{-1}) and the nitrogen assimilation (9.1 ± 0.6 µmol min^{-1}) were balanced in N-limited cultures (Figure 3.1 D and E; Table 3.1). However, in both C-limited cultures, ammonium consumption rates were significantly lower than nitrogen assimilation rates (6.5 ± 0.8 µmol min^{-1} versus 8.9 ± 0.6 µmol min^{-1}, $p < 0.0001$; Figure 3.1B and C; Table 3.1). This suggested that besides ammonium, some nitrate was assimilated under carbon limitation. As the culture evolved, the amount of nitrate assimilated appeared to decrease from approximately 3 µmol min^{-1} to 1 µmol min^{-1}. Overall, in none of the cultures significant changes in the carbon metabolism were observed over time. Interestingly, all cultures consumed similar amounts of acetate and reached similar biomass yields, although the use of the nitrogen sources differed considerably. Compared to the C-limited cultures, the N-limited cultures wasted acetate as they converted about 4 mM more acetate than expected for the reduction of 60 mM nitrate. Under carbon limitation, the conversion of acetate and nitrate was near the expected ratio.

To infer specific responses and adaptation to acetate and nitrate limitation that could not be detected directly by chemical and physiological examination transcriptomes were analyzed for all cultures at different time points. cDNA obtained from samples taken every 100 generations between 30 and 500 generations was sequenced with Illumina technology (supplementary Table 3.S1). Transcriptional activities of functional genes with at least 10 cDNA reads were determined by upper quartile normalization and converting the number of reads mapped to each gene to Reads Per Kb per Million reads (RPKM). Hierarchical clustering and ANOSIM revealed a clear separation ($r = 1.0$, $p = 0.001$) of the transcription profiles of C-limited and N-limited cultures (Figure 3.2). Overall, the transcriptional activities of 589 genes (11.6% of all 5,077 genes) differed significantly between the culture conditions throughout the experiment (with p-values $< 10^{-5}$). Furthermore, the samples grouped into early (generations 30 and 100) and later (generations 200, 400 and 500) stages of the experiment (Figure 3.2). In the course of the experiment, the changes in the transcriptional profiles of the C-limited cultures were more pronounced ($r = 0.97$, $p = 0.02$) than in the N-limited cultures ($r = 0.72$, $p = 0.02$). 333 genes in C-limited and 98 genes in N-limited cultures were significantly up- or down-regulated (p-values $< 10^{-5}$).

All genes encoding enzymes involved in denitrification were highly transcribed under both conditions, with the highest transcriptional activity observed for the gene encoding cytochrome cd_1 nitrite reductase (*nirS*) in N-limited cultures (RPKM up to > 9,000; Table 3.2, Figure 3.3). Regulatory genes were generally transcribed at lower levels. The transcription of *narK* encoding the nitrate/nitrite antiporter was significantly higher in

N-limited cultures than in C-limited cultures throughout the experiment. The *narGHJI* genes encoding nitrate reductase were initially significantly higher transcribed under carbon limitation and later decreased to similar levels as observed in N-limited cultures. Similar transcriptional activities were found for the genes of the two *nar* regulators NarR and FnrP. In contrast to *narGHJI*, *nirS* was significantly higher transcribed in N-limited than in C-limited cultures throughout the experiment which is consistent with the nitrite accumulation observed in C-limited cultures. The transcriptional regulator of *nir* and *nor* (NNR) is encoded by the *nnr* gene located upstream of the *nir* and *nor* operons (van Spanning et al., 1997). This gene was increasingly transcribed in C-limited cultures and after 200 generations, RPKM values were significantly higher than in N-limited cultures. The transcription of *nirS* was not affected, whereas the *nor* operon showed similar transcription patterns as *nnr*. The transcription of *norQBC* increased 1.8-, 2.4- and 3.2-fold, respectively. After about 200 generations, the transcription levels were similar in both culture conditions. The transcription of the *nos* operon did not significantly differ, although the genes of its regulators NosR and FnrP (van Spanning et al., 1997; Bouchal et al., 2010) were significantly more active in N-limited than in C-limited cultures. This may be explained by co-regulation with NNR (Bouchal et al., 2010). Furthermore, the transcriptional activities of the genes encoding cytochrome bc_1, cytochrome c_{550} and pseudoazurin were significantly higher under N-limitation throughout the experiment.

For growth on acetate as sole carbon source, *P. denitrificans* has the potential to perform the glyoxylate cycle, a shortcut of the TCA cycle and the EMC pathway to replenish the pools of TCA cycle intermediates during acetate assimilation. All genes involved in both pathways were highly transcribed under both conditions (Table 3.3, Figure 3.4). The transcriptional activities of genes encoding the acetate transporter (*actP*) and the acetyl-CoA synthetase even increased over time. The low transcription of genes encoding 2-oxoglutarate dehydrogenase is in accordance with a previous report (Amarasingham and Davis, 1965). The highest transcription (RPKM up to $>$ 20,000) was observed for genes encoding enzymes that constitute branching points between the TCA cycle and other carbon and nitrogen assimilatory pathways. Isocitrate lyase (ICL), encoded by *icl*, withdraws isocitrate from the TCA cycle as entry to the glyoxylate cycle. Oxaloacetate, synthesized by malate dehydrogenase (Pden_0561, EC 1.1.1.37) serves as an intermediate for gluconeogenesis via phosphoenolpyruvate (PEP), and as a substrate for citrate synthase as entry point for acetyl-CoA into the TCA cycle. Isocitrate dehydrogenase provides 2-oxoglutarate, branching to amino acid biosynthesis.

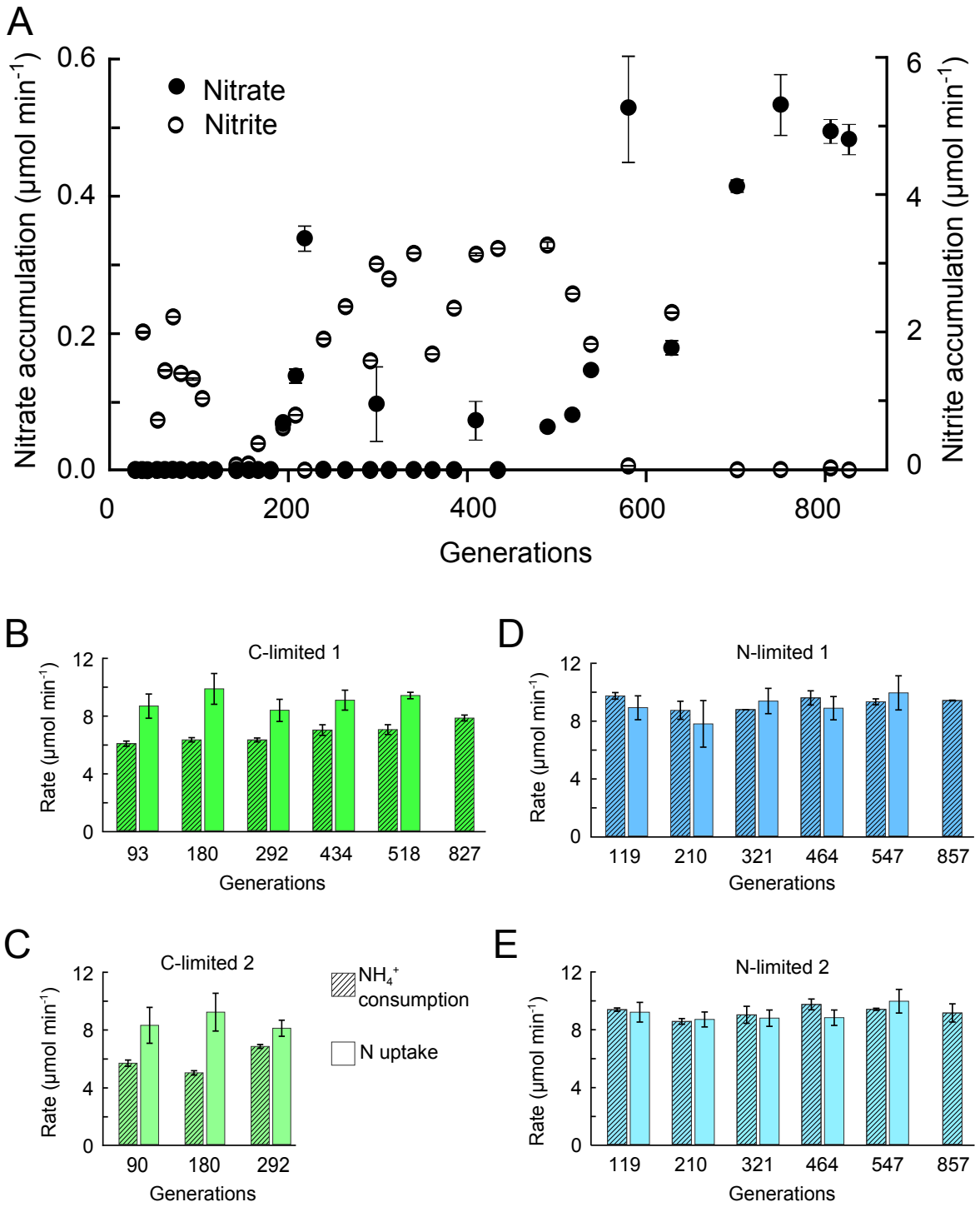

Figure 3.1 Metabolic conversion of nitrogen compounds in chemostat cultures of *P. denitrificans*. A, nitrate and nitrite accumulation rates in C-limited culture 1. The rates were similar in C-limited culture 2 up to 300 generations (not shown). Error bars represent standard deviations and may be smaller than the symbols; B-E, ammonium consumption rates and nitrogen assimilation rates in C-limited cultures 1 and 2 (panels B and C) differed from those in N-limited cultures 1 and 2 (panels D and E).

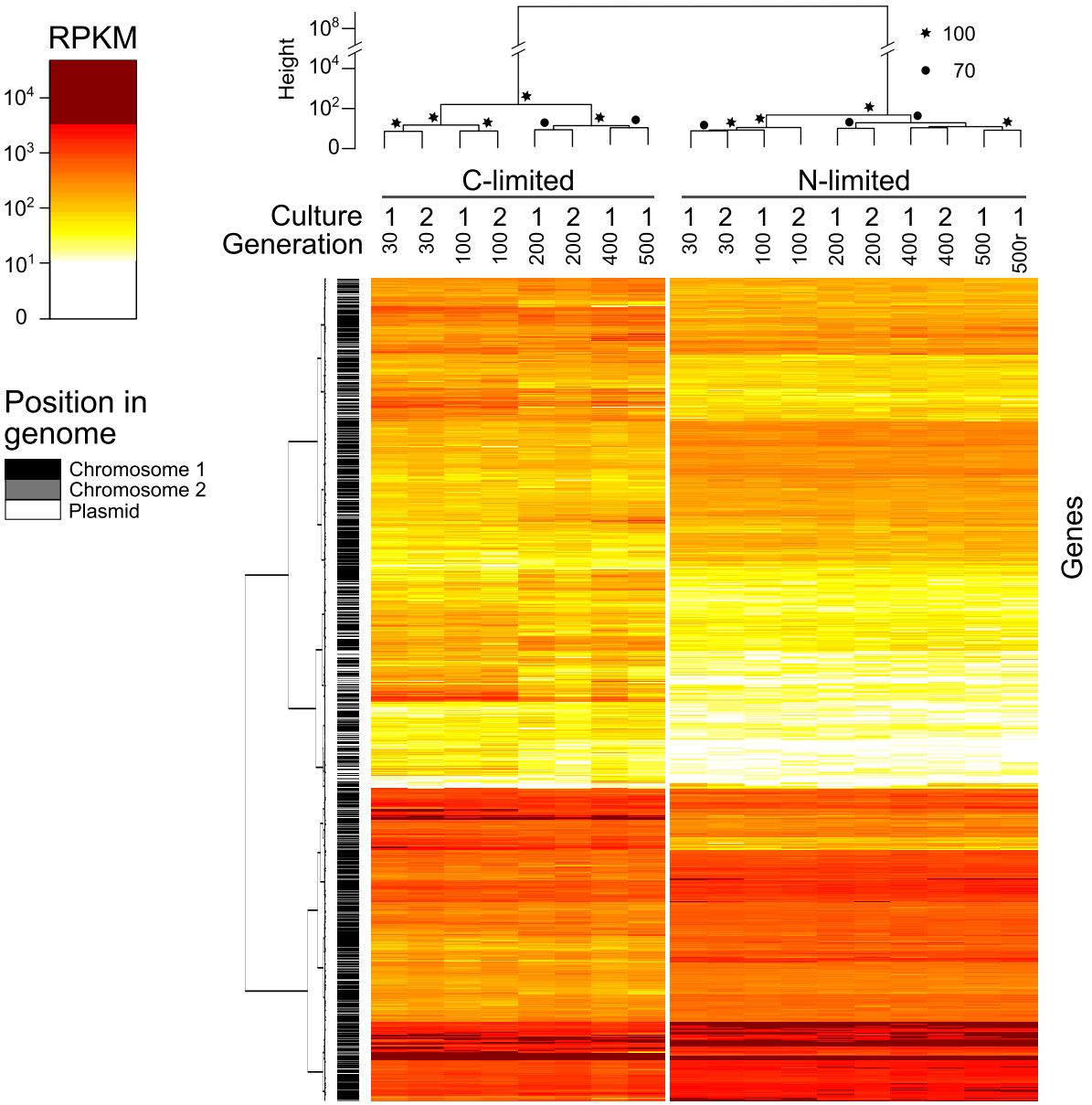

Figure 3.2 Heatmap of gene expression data (converted to RPKM) obtained by RNA-sequencing of cDNA from C-limited and N-limited *P. denitrificans* chemostat cultures within 30 to 500 generations. The dendrogram shows a separation of samples from both culture conditions. Bootstrap values are indicated by stars (100%) or circles ($>$ 70%). The samples grouped into early (generations 30 and 100) and later stages (generations 200, 400 and 500). After comparison of groups (early versus late and C-limited versus N-limited), only genes with significantly different RPKM (p-values $< 10^{-5}$) between at least two groups were included in the visualization (1,305 genes).

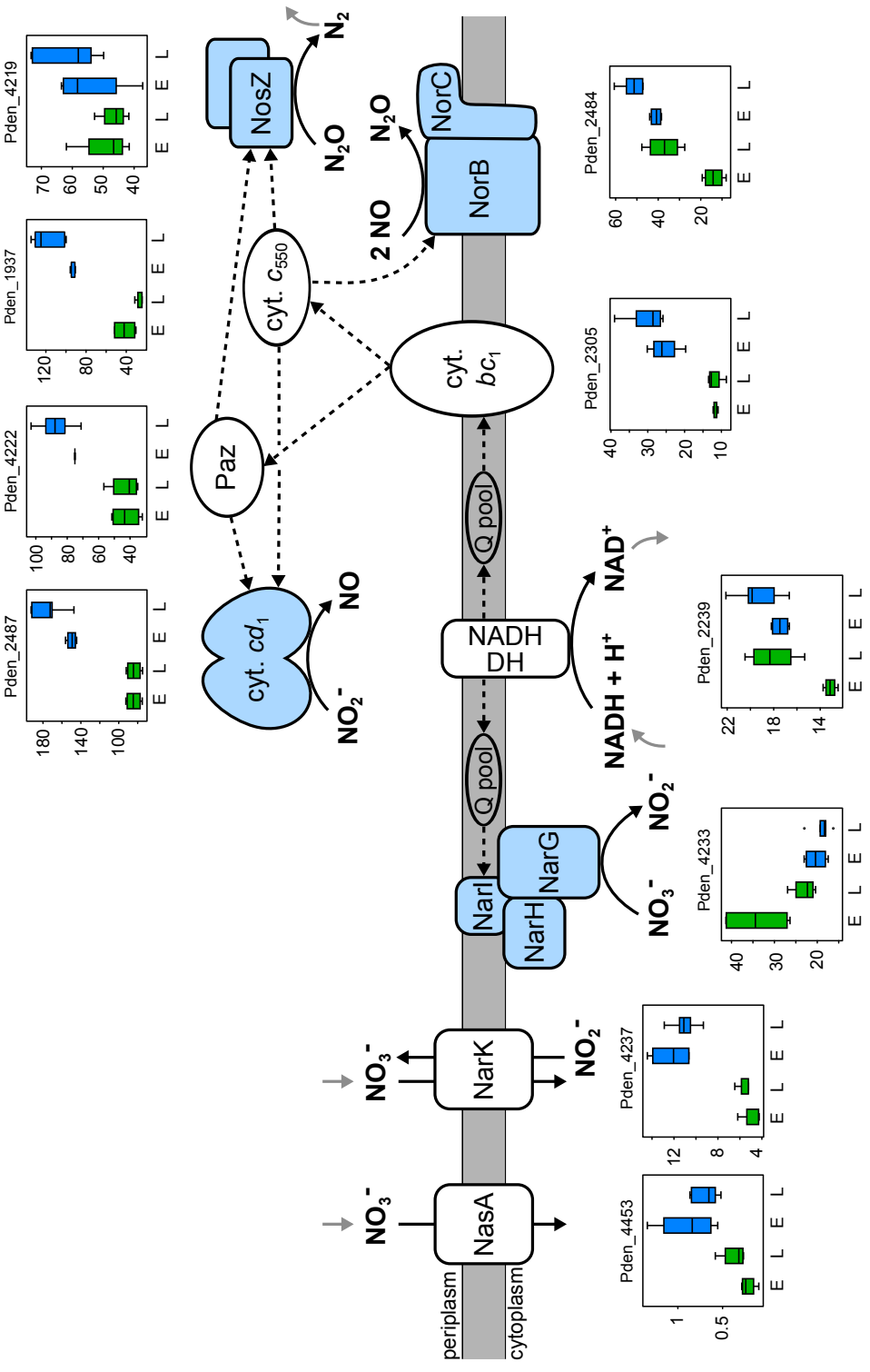

Figure 3.3 Metabolic pathways for respiratory nitrate reduction and transcriptomic profiles of *P. denitrificans* during experimental evolution *P. denitrificans* under C-limitation (green box plots) and N-limitation (blue box plots). Box plots present RPKM values divided by 100 for grouped samples from 30 and 100 generations (early stages, E), and grouped samples from 200, 400 and 500 generations (late stages, L). Dashed lines indicate electron transfer, gray lines indicate entry or exit of compounds into or from the respiratory chain. Paz, pseudoazurin; cyt., cytochrome; NADH DH, NADH dehydrogenase; Q, quinone. More details are stated in Table 3.2.

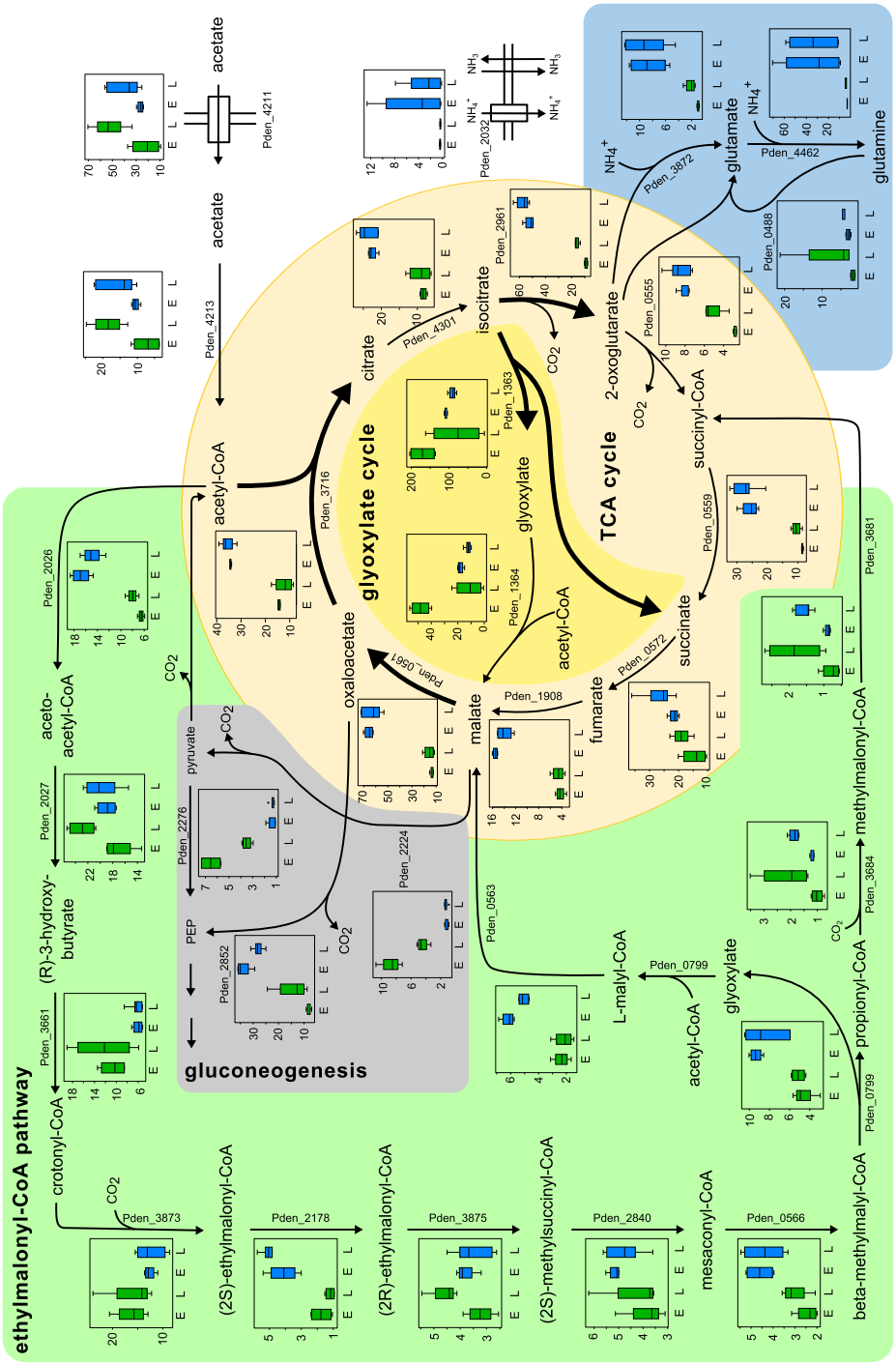

Figure 3.4 Metabolic pathways for carbon assimilation and transcriptomic profiles of *P. denitrificans* during experimental evolution of *P. denitrificans* under C-limitation (green box plots) and N-limitation (blue box plots). Box plots present RPKM values divided by 100 for grouped samples from 30 and 100 generations (early stages, E), and grouped samples from 200, 400 and 500 generations (late stages, L). Bold arrows highlight the importance of genes at branching points with markedly high expression particularly in N-limited cultures (RPKM 4,000 to 7,000). PEP, phosphoenolpyruvate. More details are stated in Table 3.3.

Table 3.2 Transcriptional activities of genes involved in anaerobic nitrogen metabolism during experimental evolution of *P. denitrificans*. Log fold changes (log FC) are given for the comparison between acetate and nitrate limitation (C-lim vs. N-lim) and among early and late stages of the experiment (early vs. late). A positive log FC represents higher transcriptional activity under N-limitation, a negative log FC represents higher transcriptional activity under C-limitation. Colored shading indicates significantly higher transcriptional activities ($p < 10^{-5}$) in either C-limited (green) or N-limited (blue) cultures. Gray shading indicates significant up- or down-regulation ($p < 10^{-5}$) over time. max RPKM indicates the maximum RPKM value (upper quartile normalized) of the respective gene among all samples. Annotations were verified with KEGG, Pfam, UniProt, microbesOnline, String or MIST. Stars indicate FMN binding sites.

Gene name	Gene tag (Pden_)	Function	max RPKM	log FC: C-lim vs. N-lim early	log FC: C-lim vs. N-lim late	log FC: early vs. late C-lim	log FC: early vs. late N-lim
Transporters							
narK	4237	nitrate/nitrite transporter	1,443	1.3	1.0	0.3	-0.1
nasH	4450 [a]	formate/nitrite transporter	14	0.5	-1.8	1.1	-1.3
nasA	4453 [a]	nitrate transporter	135	2.1	0.7	1.0	-0.4
amtB2	2032	ammonium transporter	1,261	3.7	3.5	-0.1	-0.2
Nitrate reductase Nar							
	4231	hypothetical protein	3,246	-1.0	-0.1	-1.1	-0.2
	4232	PpiC-type peptidyl-prolyl cis-trans isomerase	2,556	-0.7	-0.1	-0.9	-0.3
narI	4233	respiratory nitrate reductase subunit gamma	4,133	-0.8	-0.3	-0.5	0.0
narJ	4234	nitrate reductase cofactor assembly chaperone	3,356	-0.7	-0.4	-0.4	-0.1
narH	4235*	nitrate reductase subunit beta	4,127	-0.9	-0.8	0.0	0.0
narG	4236	nitrate reductase subunit alpha	3,183	-0.5	-0.6	0.0	0.0
Nitrite reductase cytochrome *cd*$_1$ [b]							
nirX	2485	ApbE family lipoprotein, regulation of nir gene expression	315	0.2	0.4	-0.2	0.0
nirI	2486 [c]	nitrite reductase transcriptional regulator	290	1.5	1.1	0.4	0.1
nirS	2487*	nitrite reductase transcriptional regulator	19,258	0.8	1.0	0.0	0.2
nirE	2488	uroporphyrinogen-III C-methyltransferase	1,330	0.4	0.7	-0.2	0.2
nirC	2489	cytochrome *c* (c_{55X}); monoheme c-type cytochrome	743	1.0	0.9	0.2	0.1
nirF	2490	cytochrome d_1, heme region	739	0.8	0.5	0.7	0.4
nirD	2491	nitrite reductase heme biosynthesis D/L protein	440	0.3	0.2	0.1	0.0
nirL	2492	nitrite reductase heme biosynthesis G	565	1.0	0.3	0.7	0.1
nirG	2493	nitrite reductase heme biosynthesis H protein	738	0.6	0.1	0.8	0.3
nirH	2494	nitrite reductase heme biosynthesis J protein	813	0.8	0.4	0.5	0.2
nirJ	2495	cytochrome d_1, heme region	780	0.8	0.4	0.9	0.4
nirN	2496	putative denitrification protein	249	0.1	-1.2	0.9	-0.4
Nitric oxide reductase Nor							
norF	2479* [d]	putative denitrification protein	720	-0.1	-0.5	0.4	0.0
norE	2480	accessory protein for nitric oxide reductase	724	-0.2	-1.0	0.6	-0.2
norD	2481	nitric oxide reductase	2,386	-0.1	-0.4	0.1	-0.1
norQ	2482	nitric oxide reductase, ATPase	3,079	0.8	0.1	0.8	0.2
norB	2483	nitric oxide reductase, subunit B (large subunit)	5,053	1.3	0.2	1.3	0.1

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Table 3.2 (continued)

Gene name	Gene tag (Pden_)	Function	max RPKM	log FC C-lim vs. N-lim early	log FC C-lim vs. N-lim late	log FC early vs. late C-lim	log FC early vs. late N-lim
norC	2484	nitric oxide reductase subunit C (small subunit)	6,064	1.6	0.3	1.7	0.4
Nitrous oxide reductase Nos							
nosX	2414	TAT (twin-arginine translocation) pathway signal sequence	430	-0.4	0.0	-0.3	0.1
nosL	2415	Cu(I) lipoprotein involved in nitrous oxide reduction	797	-0.3	-0.3	0.5	0.5
nosY	2416	nitrous oxide maturation protein	535	-0.4	-0.3	-0.2	-0.2
nosF	2417	ABC transporter	383	-0.5	-0.3	0.3	0.5
nosD	2418	nitrous oxidase accessory protein, periplasmic copper-binding	2,138	-0.4	-0.2	0.1	0.2
nosZ	2419	nitrous-oxide reductase, regulated by FnrP	7,334	0.1	0.4	0.0	0.3
nosR	2420*	FMN-binding domain protein	897	1.4	0.7	0.8	0.1
nosC	2421	hypothetical protein	761	2.0	0.4	0.9	-0.7
Glutamine synthetase							
glnA	1397	glutamine synthetase, glutamate-ammonia ligase	230	1.1	0.4	0.9	0.2
glnA	3702	glutamine synthetase, glutamate-ammonia ligase	184	0.6	0.2	0.4	0.1
glnA	4462	glutamine synthetase, glutamate-ammonia ligase	7,051	3.5	3.6	0.3	0.4
glnA	4547	glutamine synthetase, glutamate-ammonia ligase	42	0.0	-0.9	0.8	-0.1
Glutamate synthase							
	3872	glutamate dehydrogenase	1,229	3.1	2.2	0.7	-0.2
	0488	glutamate synthase, large subunit (GOGAT)	2,118	0.8	-1.7	2.9	0.4
	0490	glutamate synthase, small subunit (GOGAT)	1,235	-0.4	-1.2	1.6	0.8
Assimilatory nitrate reduction							
nasT	4455 [a]	nitrate/nitrite sensor	131	3.4	1.9	1.2	0.3
nasS	4454 [a]	regulator – transcription anti-terminator	56	2.2	2.3	-1.2	1.1
nasA	4453 [a]	nitrate transporter	135	2.1	0.7	1.0	0.4
nasB	4452 [a]	nitrite reductase large subunit	11	-0.6	-1.0	-0.6	1.0
nasG	4451 [a]	nitrite reductase small subunit, Rieske-type ferredoxin	11	0.0	-2.2	0.7	1.5
nasH	4450 [a]	formate/nitrite transporter	14	0.5	-1.8	1.1	1.3
nasC	4449 [a]	assimilatory nitrate reductase catalytic subunit	20	-1.4	-1.8	-0.6	1.0
	0655	nitrite/sulfite reductase hemoprotein beta subunit	1,706	2.1	1.8	0.3	0.1
	0658	NADPH-ferredoxin reductase	3,204	0.6	1.1	-0.2	0.2

[a] (Luque-Almagro et al., 2011) [b] (Bueno et al., 2012) [c] (Baker et al., 1998) [d] (van Spanning et al., 1997)

Table 3.3 Transcriptional activities of genes involved in acetate metabolism during experimental evolution of *Paracoccus denitrificans* Pd1222. Further information is provided in the description of Table 3.2.

Gene name	Gene tag (Pden_)	Function	max RPKM	log FC C-lim vs. N-lim early	log FC C-lim vs. N-lim late	log FC early vs. late C-lim	log FC early vs. late N-lim
Acetate uptake							
	4210	response regulator receiver protein	1,819	0.3	-0.8	1.5	0.4
actP	4211	acetate transporter	7,062	0.3	-0.4	1.5	0.8
	4212	hypothetical protein	8,777	0.6	-0.3	1.7	0.8
TCA cycle							
	3716	citrate synthase I	4,775	1.3	2.1	-0.7	0.1
acnA	3071	aconitate hydratase	43	0.6	0.9	-0.1	0.2
	4301	aconitate hydratase	2,717	1.4	1.9	-0.3	0.2
	4567	aconitate hydratase	3,286	0.7	2.0	-0.7	0.6
	2961	isocitrate dehydrogenase	6,459	2.5	1.9	0.8	0.2
sucA	0555	2-oxoglutarate dehydrogenase E1 component	1,040	1.5	0.9	0.7	0.1
	0554	2-oxoglutarate dehydrogenase E2 component	998	0.1	0.1	-0.1	-0.1
	1603	2-oxoglutarate dehydrogenase E2 component	6	-1.2	-3.0	0.3	-1.5
	0551	dihydrolipoamide dehydrogenase	458	0.6	1.9	-0.1	0.0
	0611	dihydrolipoamide dehydrogenase	985	-1.3	-0.1	-0.8	0.5
	4760	dihydrolipoamide dehydrogenase	131	-0.5	-0.3	-0.1	0.0
	0558	succinyl-CoA synthetase subunit alpha	3,268	1.0	1.0	0.2	0.1
sucC	0559	succinyl-CoA synthetase subunit beta	3,234	1.6	1.5	0.3	0.2
	0567	succinate dehydrogenase cytochrome b_{556} subunit	2,040	2.8	2.8	0.2	0.3
	0568	succinate dehydrogenase membrane anchor	1,834	3.0	2.9	0.2	0.2
sdhA	0569	succinate dehydrogenase flavoprotein	1,964	2.0	1.5	0.7	0.1
sdhB	0572	succinate dehydrogenase iron-sulfur	3,611	0.5	0.6	0.2	0.3
	3020	succinate dehydrogenase, membrane anchor	121	-0.5	-0.7	-0.7	-0.9
	3021	succinate dehydrogenase cytochrome b_{556} subunit	134	0.3	-0.9	0.4	-0.8
	3022	succinate dehydrogenase iron-sulfur	126	0.3	-1.4	0.7	-1.0
fumC	1908	fumarate hydratase, class II	1,604	1.9	1.9	-0.2	-0.1
	4119	fumarate hydratase, class I	182	-1.8	-2.5	1.9	1.1
	0561	malate dehydrogenase	7,217	2.3	2.3	-0.1	-0.2
Glyoxylate cycle							
	3716	citrate synthase I	4,775	1.3	2.1	-0.7	0.1
acnA	3071	aconitate hydratase	43	0.6	0.9	-0.1	0.2
	4301	aconitate hydratase	2,717	1.4	1.9	-0.3	0.2
	4567	aconitate hydratase	3,286	0.7	2.0	-0.7	0.6
icl	1363	isocitrate lyase	20,410	-0.7	2.1	-3.0	-0.3
	1364	malate synthase	5,615	-1.4	2.3	-4.2	-0.5
	4051	malate synthase G	299	0.7	1.3	-0.6	0.0
	0561	malate dehydrogenase	7,217	2.3	2.3	-0.1	-0.2
	1365	potential regulator for glyoxylate cycle	386	-1.7	-0.3	-2.0	-0.5
iclR	0169	regulator	438	0.2	1.8	-1.1	0.5
Ethyl-malonyl-CoA-pathway							
phaA	2026	acetyl-CoA acetyltransferase	1,864	1.4	1.1	0.2	-0.1
	2663	acetyl-CoA acetyltransferase, β-ketothiolase	353	1.0	0.6	0.1	-0.2
	2870	acetyl-CoA acetyltransferase	108	0.1	-0.1	0.0	-0.2

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Table 3.3 (continued)

Gene name	Gene tag (Pden_)	Function	max RPKM	log FC C-lim vs. N-lim early	log FC C-lim vs. N-lim late	log FC early vs. late C-lim	log FC early vs. late N-lim
	2907	acetyl-CoA acetyltransferase	61	-0.1	-1.2	0.4	-0.6
	4811	acetyl-CoA acetyltransferase, β-ketoadipyl CoA thiolase	339	-2.1	-2.8	0.7	0.0
	4819	acetyl-CoA acetyltransferase	137	-0.7	-1.8	0.7	-0.4
	5022	acetyl-CoA acetyltransferase, β-ketoadipyl CoA thiolase	161	-1.8	-0.9	-1.3	-0.4
phaB	2027	acetoacetyl-CoA reductase	2,537	0.2	-0.1	0.3	0.0
	3661	3-hydroxybutyryl-CoA dehydratase	1,901	-0.8	-0.4	-0.5	0.0
ccr	3873	crotonyl-CoA carboxylase/reductase	2,392	-0.4	-0.2	-0.3	-0.1
epi	2178	ethylmalonyl-CoA/methylmalonyl-CoA epimerase	576	1.1	2.2	-0.7	0.4
ecm	3875 [a]	ethylmalonyl-CoA mutase	546	0.2	-0.4	0.4	-0.3
mcd	2840 [b]	methylsuccinyl-CoA dehydrogenase	622	0.4	0.3	-0.1	-0.2
mch	0566 [a]	mesaconyl-CoA hydratase	546	0.9	0.4	0.3	-0.2
mcl-1	0799 [a]	beta-methylmalyl-CoA/L-malyl-CoA lyase	1046	1.0	0.8	-0.1	-0.3
mcl-2	0563	citrate lyase beta chain	686	1.3	1.6	-0.6	-0.3
pccA	3684	propionyl-CoA carboxylase, alpha subunit	351	0.3	-0.7	1.6	0.6
pccB	3688	propionyl-CoA carboxylase, beta subunit	372	-1.4	-0.6	0.0	0.8
mcm	3681	methylmalonyl-CoA mutase	270	0.2	-0.7	1.7	0.9
	2028	hypothetical protein	11,918	-0.3	-0.8	0.9	0.4

[a] (Erb et al., 2007) [b] (Erb et al., 2009)

In N-limited cultures, the transcription of most genes relevant to the TCA cycle was significantly higher than in C-limited cultures, and did not change significantly over time. Notably, the transcriptional activity of the gene encoding isocitrate dehydrogenase was up to 5.8-fold higher and the transcriptional activities of the genes encoding glutamate dehydrogenase and glutamine synthetase were up to 8.5- and 12-fold higher under nitrate limitation, respectively. The latter and its regulator gene *glnB* were among the most highly active genes in N-limited cultures (RPKM up to $>$ 7,000) and contributed strongly to the differentiation between culture conditions. These results suggest that under nitrate limitation *P. denitrificans* prioritized nitrogen uptake and assimilation. Another intermediate of the TCA cycle, oxaloacetate, seemed to be withdrawn for gluconeogenesis via PEP. In turn, two anaplerotic pathways, the glyoxylate cycle and the EMC pathway, replenished the TCA cycle with succinate and malate, as indicated by the transcription of relevant genes. Enzyme activity measurements of ICL and crotonyl-CoA carboxylase/reductase (CCR), the key enzymes of the glyoxylate cycle and the EMC pathway, respectively, confirmed the activity of both pathways under both carbon and nitrate limited conditions (supplementary Figure 3.S5).

Whereas the transcriptional activities of the two genes unique for the glyoxylate cycle remained nearly constant under N-limitation, under C-limitation this pathway played an important role for the synthesis of malate and succinate only in the early stage of cultivation. The transcriptional activities of *icl* were among the highest of all genes (RPKM up to $>$ 20,000). However, the transcription of glutamate synthase (GOGAT) indicated increasing importance of glutamate conversions, withdrawing 2-oxoglutarate from the TCA cycle. Consequently, the gene encoding isocitrate dehydrogenase was increasingly transcribed to replenish the 2-oxoglutarate pool, consistent with decreasing transcription of the genes unique in the glyoxylate cycle. The withdrawal of 2-oxoglutarate from the TCA cycle may have caused a lack of succinate supply to the TCA cycle, which was obviously compensated by the increased transcription of 2-oxoglutarate dehydrogenase and genes involved in the EMC pathway, providing a source of succinyl-CoA to the TCA cycle. In contrast to N-limitation, under C-limitation both malate and oxaloacetate were used to fuel gluconeogenesis in the early stage, as indicated by the high transcription of genes encoding malate dehydrogenase (Pden_2224, EC 1.1.1.40), pyruvate kinase and PEP carboxylase. However, the transcription of the former two genes decreased over time. The observation that pathways withdrawing isocitrate and malate from the TCA cycle were down-regulated and anaplerotic pathways were up-regulated may support the hypothesis that nitrite was assimilated in the early stage of the experiment, while at later stages (between 400 and 500 generations), the assimilation of ammonium supplied with the medium, increased, which was seen in the metabolic analyses (Figure 3.1B). However, the genes *nasCHGBA*, encoding assimilatory nitrate and nitrite reductases, nitrate and nitrite transporters (Luque-Almagro et al., 2011) were only transcribed at low levels at both culture conditions (RPKM $<$ 20). The first two reactions of the EMC pathway are in common with the initial steps of polyhydroxybutyrate (PHB) synthesis, but additional genes involved in the synthesis of this storage compound were not transcribed in any culture. Instead, in all cultures, three genes were transcribed that are relevant to PHB degradation (*phaZ*) or to inhibition of PHB formation (*phaP*, *phaR*, not shown). The transcriptional activity of *phaP*, encoding a phasin, was initially among the highest under C-limitation, and decreased to the level of N-limited cultures with time.

In summary, under N-limitation, the TCA cycle had a central function in ammonium assimilation. In contrast, for C-limitation the main observations were (i) the drastic decrease in the activity of genes involved in the glyoxylate cycle linked to (ii) increasing importance of glutamate metabolism and (iii) the generally lower transcriptional activities of genes of the central metabolic pathways (carbon and nitrogen). Interestingly, the two culture conditions differed in numbers of genes with significantly higher tran-

scriptional activities among the regulators and transporters (supplementary Table 3.S3). These included 72 to 83 regulatory genes in C-limited cultures and 19 to 25 regulatory genes in N-limited cultures. Among ATP-binding cassette (ABC) and tripartite ATP-independent periplasmic (TRAP) transporters higher transcriptional activities were observed for 14 to 42 genes in C-limited cultures and for 1 to 14 genes in N-limited cultures. This suggests that under C-limitation transport and regulation seemed to be more important than under N-limitation, which is also seen in up- and down-regulation of genes encoding regulators (17 to 20 in C-limited and 1 to 5 in N-limited cultures) and transporters (10 to 26 in C-limited and 1 to 2 in N-limited) during the experiment.

To investigate whether the observed physiological changes can be attributed to genomic variations, we sequenced DNA extracted from the cultures at every one hundred generations. Comparison of all re-sequenced genomes including the ancestral strain and the original genome of *Paracoccus denitrificans* Pd1222 revealed four variants in the ancestral chromosome 1; three of them were retained in all cultures throughout the experiment. Furthermore, we identified the appearance and type of all mutations (Figure 3.5), and their effects on the amino acid sequence (supplementary Table 3.S4). Among a total of 211 mutations in all cultures, we detected 179 SNPs, 12 insertions and 20 deletions (indels) between 1 and 45 nucleotides long, 13 of which caused shifts in open reading frames (ORFs). The number and characteristics of mutations was similar between the culture conditions (Figure 3.5D, supplementary Table 3.S6). Transversions from cytosine:guanine to adenine:thymine, and transitions from cytosine:guanine to thymine:adenine predominated under both conditions. However, in C-limited culture 1, mutations appeared at different frequencies over time. Before 500 generations, only half as many mutations were detected as compared to the other cultures, while most mutations appeared between 500 and 800 generations and reached 60 mutations (but note that C-limited culture 2 accumulated 51 mutations already after 300 generations). Intergenic regions were affected by 38 mutations, 7 of which appeared in C-limited culture 1 between 500 and 800 generations.

Most mutants went extinct after they had been detected once. This was the case for example regarding 7 genes encoding transcriptional regulators and 3 genes of regulators with proteins as target. To gain insight whether the phenotypic observations can be explained by sequence variations of functional genes, we predicted the effects of the mutations on protein function by the type and position of the mutations within the ORFs (supplementary Table 3.S4). We assumed that variable regions are insensitive to amino acid alterations and would keep their original function. This may not always be the case, as shown by the loss of antibiotic resistance by a transition of adenine:thymine to

guanine:cytosine within the *rpoB* gene (Pden_0747), which we found in all cultures, and appeared to confer an advantage to clones under N-limitation. Many mutations caused alterations of the amino acid sequence within functional sites. We inferred severe effects on protein function if mutations led to intolerated amino acids in conserved regions or in case of frame shifts, large insertions/deletions, and introduction or loss of stop codons. At least 28 ORFs were affected by mutations that altered protein function (Figure 3.5), 8 of which persisted for at least 100 generations. Most of these persistent mutations emerged in C-limited culture 1 after 500 generations, suggesting that adaptive processes occurred increasingly in the later stages of the experiment. Most notable was the rarely occurring cytosine:guanine to guanine:cytosine transversion causing an amino acid substitution in the catalytic site of the proofreading subunit of DNA polymerase III (Pden_2815). Another mutation caused an amino acid change in a highly conserved region of hydantoinase B/oxoprolinase (Pden_4262). The latter two mutations appeared in C-limited culture 1 after 630 and 500 generations, respectively, and persisted. These clones replaced clones with three mutations in *pgi* (Pden_1950), encoding glucose-6-phosphate isomerase. Here, an amino acid substitution in a highly conserved dimer interface was followed by the introduction of a stop codon and a deletion causing a frame shift. These mutations persisted during at least 100 generations and went extinct simultaneously, suggesting that these mutations were present in a single subpopulation. Further, one mutation in the gene encoding peptidoglycan glycosyltransferase in C-limited culture 1 and two mutations in the gene encoding phosphatidylethanolamine N-methyltransferase in both N-limited cultures appeared to provide a selective advantage. Four mutations were identical in all cultures most of which persisted. They affected one position in an intergenic region and three coding sequences. Besides a mutation in *rpoB* (described above), a deletion of 15 amino acids affecting the function of ribokinase (Pden_2872) was likely beneficial in all cultures. A transition of cytosine:guanine to thymine:adenine in a variable region of a gene coding for a resolvase (Pden_1202) persisted in all cultures except for N-limited culture 2, where it was detected at only one time point.

In summary, most mutations disappeared after their detection at one time point, whereas few mutations likely conferred benefits to the populations that are of interest for future analyses. Perhaps most surprisingly, the observed mutations did not explain the observed strong differences in transcriptional adaptations between the treatments.

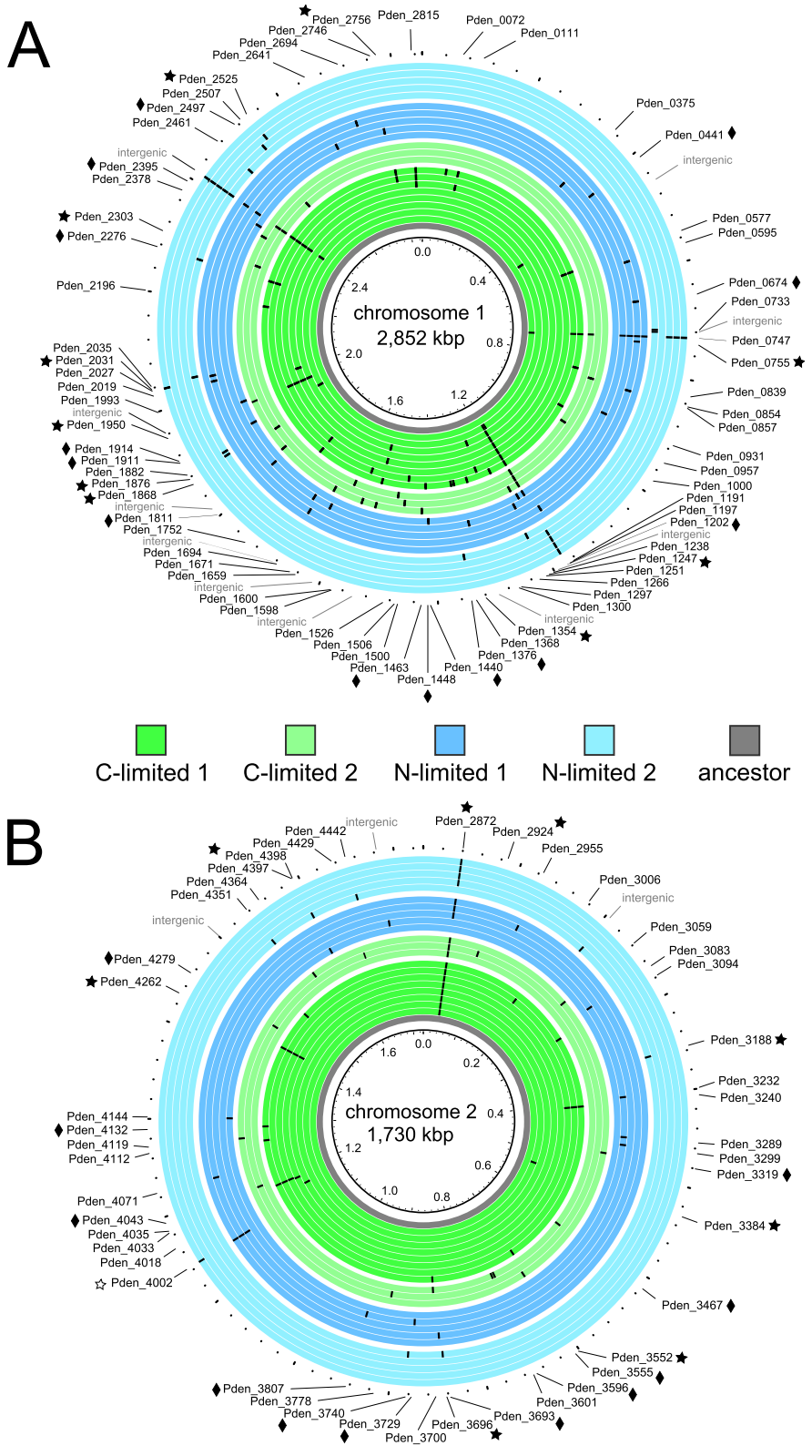
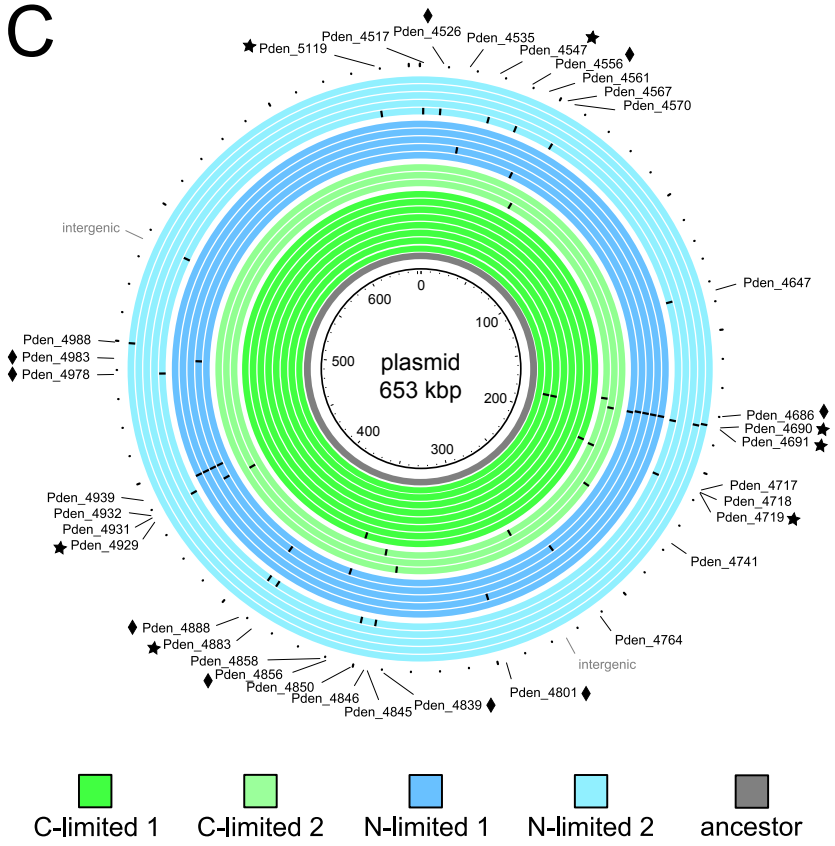


Figure 3.5 (Caption next page.)



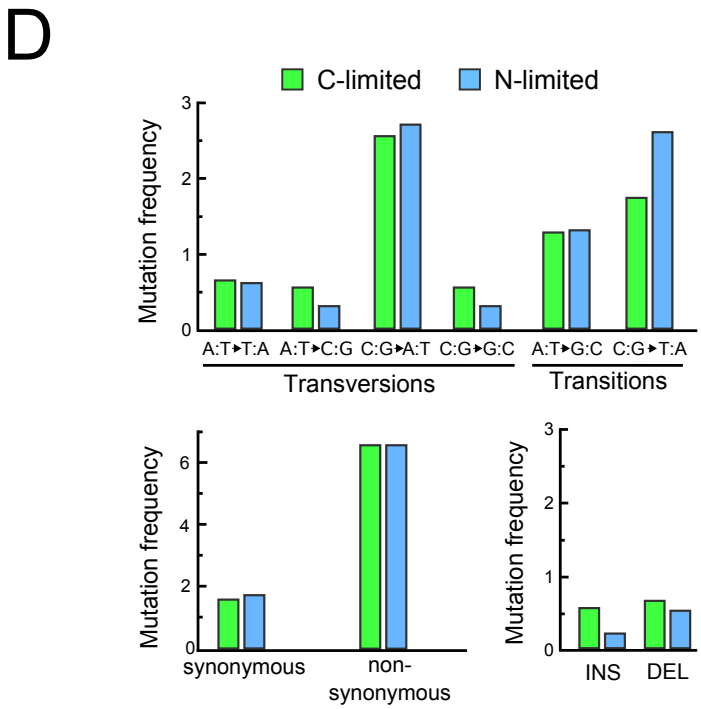


Figure 3.5 (Caption next page.)

Figure 3.5 (Previous page.) Mutations detected by whole-genome re-sequencing of every 100 generations of experimentally evolved *P. denitrificans* compared to the reference genome position in megabase pairs. Mutations were distributed over chromosome 1 (A), chromosome 2 (B) and the plasmid (C). Mutations are marked as black strokes in each circle, representing the genome sequences of: ancestor (gray), C-limited culture 1 (dark green), C-limited culture 2 (light green), N-limited culture 1 (dark blue) and N-limited culture 2 (light blue). For each culture, the innermost ring represents the genomes after 100 generations and the outermost represents the genomes after 800 or 300 generations (C-limited cultures 1 and 2, respectively) or after 500 generations (both N-limited cultures). Star, mutations with strong effect on the amino acid sequence; diamond, synonymous mutations without effect on amino acid sequence; open star, strong effect of the mutation in N-limited culture 2 and an unknown effect in N-limited culture 1 (different mutations). Mutations comprise SNPs, insertions and deletions (D). Mutation frequencies per 100 generations are shown for transversions, transitions, insertions and deletions. More details on the appearance, types and consequences of mutations are given in supplementary Tables 3.S4 and 3.S6. The predicted function of the affected gene products is presented in supplementary Table 3.S5.

3.5 Discussion

Here we presented the experimental evolution of the facultative anaerobic bacterium *P. denitrificans* Pd1222. To our knowledge, this is the first evolution study to investigate adaptive mechanisms in a denitrifying microbial culture, extending the numerous evolution studies that focus on improved carbon utilization linked to aerobic respiration. We investigated the genetic, transcriptional and metabolic changes and their possible effects on the physiology of the strain during up to more than 800 generations.

The results demonstrated that stable selective pressures in the form of acetate or nitrate limitation led to distinct utilization of dissimilatory and assimilatory pathways of carbon and nitrogen. Based on the metabolic substrate conversions we suggest that *Paracoccus denitrificans* applied different strategies to cope with the different substrate limitations. Carbon limitation conceivably caused a cascade of responses in nitrate respiration and nitrogen assimilation. Nitrate limitation resulted in a significantly higher ammonium uptake than carbon limitation. Further, the competition for nitrate resulted in transcriptional up-regulation of the genes of the respiratory chain. Under carbon limitation the strong competition for acetate led to transcriptional up-regulation of genes involved in carbon uptake, metabolic pathways and regulation. Generally, *Paracoccus denitrificans* showed significantly stronger adaptive responses to C-limitation compared to N-limitation. This observation is supported by a previous study that showed a

higher fitness increase of Saccharomyces paradoxus under carbon limitation compared to nitrogen limitation (Goddard and Bradford, 2003). Similar to this, the stronger adaptive response under C-limitation indicated that the ancestral strain of *Paracoccus denitrificans* Pd1222 was better adapted to nitrate limitation than to acetate limitation. The up-regulation of genes encoding transporters might be explained by the emergence of different subpopulations specializing on different carbon intermediates secreted after incomplete degradation by other subpopulations. Diversification into subpopulations is a frequently described process in evolution, where the fitness (the ability to reproduce) of bacteria growing in a nutrient limiting environment increases by partial substrate utilization and cross-feeding of metabolites (Helling et al., 1987; Rosenzweig et al., 1994; Treves et al., 1998). The exchange of carbon intermediates would not be surprising since *Paracoccus denitrificans* is a very versatile organism with respect to the spectrum of carbon sources it can metabolize (Nokhal and Schlegel, 1983). Alternatively, diversification of carbon uptake might not have been functional but might simply be a generic response to carbon limitation that was previously described for *E. coli* (Ihssen and Egli, 2005).

The emergence of subpopulations may also explain the observed changes in the denitrification pathway in C-limited culture 1. In the beginning, the complete pathway might have been performed by one single population with a lower reduction rate of nitrite than of nitrate (Betlach and Tiedje, 1981). After the transition after about 630 generations, one subpopulation could have reduced nitrate to nitrite and another could have reduced nitrite to dinitrogen while both subpopulations may have competed for acetate. We are not aware of any previous laboratory evolution studies that considered the possibility that bacteria diverge in their utilization of electron acceptors. However, within the nitrogen cycle, the partitioning of the respiratory reactions among several microbial species is not unique. A number of environmentally important microorganisms are known to perform partial denitrification (Zumft, 1997; Green et al., 2010).

The effect of prolonged exposure to nitrite on growth of *Paracoccus denitrificans* has not been studied so far. Our results indicate that prolonged exposure to millimolar concentrations of nitrite as a result of incomplete nitrate reduction under C-limitation was a potential driving force for adaptation in the nitrogen metabolism. Indeed, nitrite accumulation stopped after about 500 generations under C-limitation, consistent with a decreasing transcriptional activity of the *nar* operon. Conceivably the evolved population(s) gained a fitness advantage over the ancestral population, potentially by preventing the accumulation of (toxic) nitrite. Although we did not identify any mutational event that could be assigned to respiratory genes or their transcriptional regulators, a mutation in another genetic context might be responsible for the phenotypic change.

Alternatively, transcriptional activities could be regulated at a non-genetic level, for example by epigenetics (Lim and van Oudenaarden, 2007).

Unexpectedly, the C-limited cultures consumed less ammonium than the N-limited cultures, although all cultures reached similar biomass yields and carbon/nitrogen ratios. This indicates that the C-limited cultures might have assimilated both nitrate and ammonium. Nitrate assimilation was described to occur at low ammonium concentrations, but not when ammonium is sufficiently present, because it is energetically more costly than ammonium assimilation (Gates et al., 2011; Luque-Almagro et al., 2011). Possibly, under C-limitation, the assimilation of nitrate confers a benefit to anaerobically growing *Paracoccus denitrificans* by decreasing the accumulation of nitrite and associated toxic effects (Gates et al., 2011). This hypothesis may be supported by the decrease in nitrite accumulation in C-limited culture 1. At lower nitrite concentrations, the costs of nitrate assimilation would be higher than the benefits, which could explain the increase in ammonium consumption during evolution. Similar observations have been reported on the degradation and assimilation of ethanol to circumvent toxic effects at increasing concentrations (Goodarzi et al., 2010).

The assimilation of ammonium appeared to play a central role in the carbon metabolism of *Paracoccus denitrificans* under both conditions. Especially the transcriptomic data obtained from C-limited cultures showed drastic changes in one pathway that were compensated by other pathways. We inferred that the removal of 2-oxoglutarate from the TCA cycle for glutamate synthesis controlled the activity of the enzymes of the TCA cycle, the glyoxylate cycle, and the EMC pathway. Both these anaplerotic pathways were active in all cultures, demonstrating that C- or N-limitation does not select between them.

Mutations causing the changes in transcriptional activities commonly occur in genes encoding transcriptional regulators such as RpoS and SpoT (Notley-McRobb et al., 2002; Cooper et al., 2003). Alternatively, mutations in intergenic regions can modify the binding sites for sigma factors (Blank et al., 2014). However, such mutations appearing in our study did not persist. Apparently, they did not provide a selective advantage and did certainly not explain the observed changes in transcriptional activities. The emergence of persistent, apparently beneficial mutations in coding sequences after 500 generations under carbon limitation did provide several targets to investigate how genetic and metabolic variations were linked. Most interesting was a mutation in the gene encoding hydantoinase B/oxoprolinase after 500 generations, affecting a highly conserved region. This enzyme catalyzes the glutamate synthesis from prolin (Seddon and Meister, 1986), and constitutes another linkage between carbon and nitrogen metabolism. Further

investigation of the effect of this mutation may show whether it contributed to the observed changes of phenotype and transcriptomes. It is well possible that the high mutation frequency after 500 generations resulted from an amino acid alteration in the catalytic centre of the proofreading subunit of DNA polymerase III. Such a mutation can cause elevated mutation rates due to a disrupted proofreading function (Loh et al., 2010). This has been reported to occur under stress conditions and was found to trigger rapid evolution (Gonzalez et al., 2008). However, at large population sizes, the adaptation rate is not limited by the mutation supply rate but rather by the fixation rate of beneficial mutations (de Visser et al., 1999). The latter rate decreases with increasing population size because of increased clonal interference, the competition between lineages carrying different beneficial mutations. The highly dynamic appearance and extinction of mutants observed in all cultures can most likely be explained by clonal interference (Fogle et al., 2008).

3.6 Conclusions

Evolution of *Paracoccus denitrificans* under carbon limitation led to more effective denitrification (complete conversion of nitrate to dinitrogen). The phenotype with accumulation of the intermediate nitrite was lost. Thus, besides carbon limitation, the presence of nitrite likely selected for improved phenotypes. Carbon limitation additionally led to significantly more up- or down-regulated genes relevant for carbon uptake and regulation, caused by competition for acetate and carbon intermediates. This agrees with numerous findings previously reported for aerobic cultures. Accordingly, nitrate limitation caused up-regulated transcription of genes involved in denitrification, resulting from competition for nitrate and intermediates. Overall, more changes in transcriptional activities were observed under carbon limitation, indicating that the ancestral strain was better adapted to nitrate than to acetate limitation. The phenotypic changes could not be clearly linked to the numerous mutations detected. Further investigations would be required to understand the effect of observed mutations and to analyze the cultures for potential subpopulations.

3.7 Acknowledgments

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3.8 Supplementary information

Adaptive evolution of *Paracoccus denitrificans* in acetate and nitrate limited anoxic chemostats

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Supplementary Tables

Table 3.S1	Simplified number of generations at time points when samples were taken from *P. denitrificans* experimental evolution cultures.
Table 3.S2	RPKM values of *P. denitrificans* Pd1222 genes during experimental evolution. (electronic version, upon request)
Table 3.S3	Number of *P. denitrificans* Pd1222 genes with significantly different transcriptional activities.
Table 3.S4	Genome variations and predicted effects during continuous cultivation of *P. denitrificans* Pd1222 under acetate and nitrate limitation.
Table 3.S5	Mutated genes identified during experimental evolution of *P. denitrificans* Pd1222 and their predicted function.
Table 3.S6	Overview of mutations in C-limited and N-limited cultures of *P. denitrificans* Pd1222 during experimental evolution.

Supplementary Figures

Figure 3.S1	Anoxic chemostats for experimental evolution of *P. denitrificans* Pd1222.
Figure 3.S2	Acetate consumption rates during experimental evolution of *P. denitrificans* Pd1222 in anoxic C-limited and N-limited chemostat cultures.
Figure 3.S3	Absolute carbon mass balances during experimental evolution of *P. denitrificans* in C-limited and N-limited chemostat cultures.
Figure 3.S4	Relative carbon mass balances during experimental evolution of *P. denitrificans* in C-limited and N-limited chemostat cultures.
Figure 3.S5	Enzyme activities of isocitrate lyase (ICL) and crotonyl-CoA carboxylase/reductase (CCR).
R script	Analysis and statistics of transcriptional profiles. (electronic version, upon request)

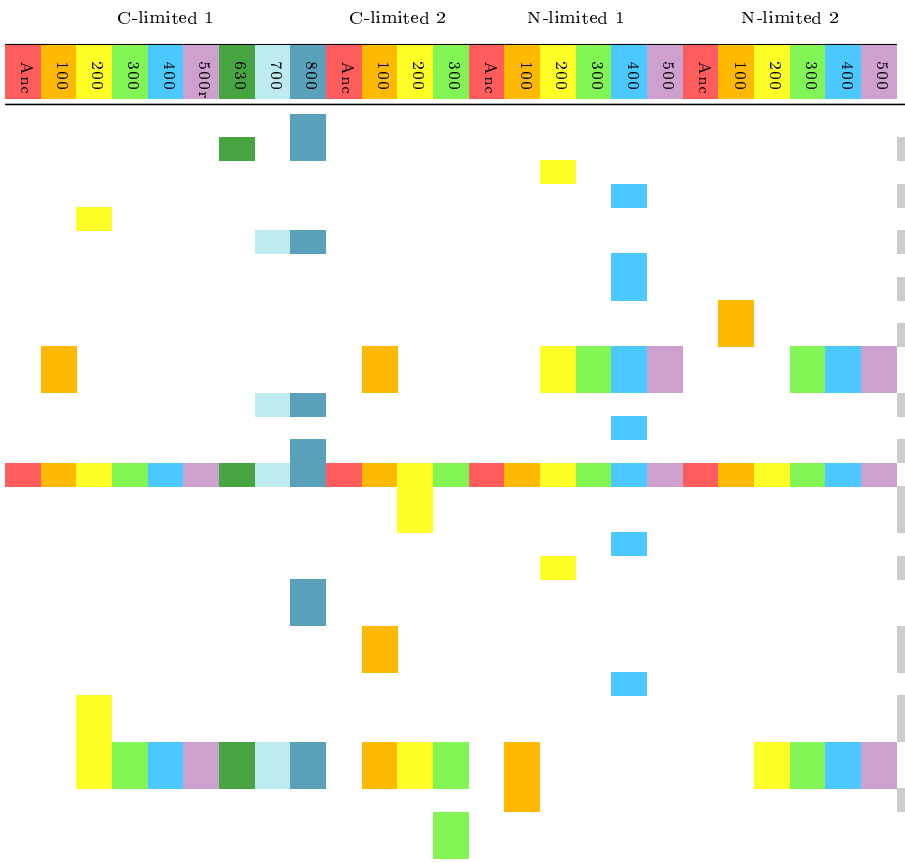
Table 3.S1 Number of generations at time points when samples where taken from *P. denitrificans* experimental evolution cultures for genome re-sequencing (DNA) and transcriptomics (RNA). For better readability, the actual number of generations was rounded to a simplified number of generations.

Simplified numer of generations		30	100	200	300	400	500	600	700	800
Actual number of generations	**DNA**									
	C-limited 1		104	208	312	410	518	629	702	827
	C-limited 2		100	208	313					
	N-limited 1		119	210	321	415	519			
	N-limited 2		119	210	321	415	519			
	RNA									
	C-limited 1	30	104	215	316	410	518			
	C-limited 2	26	100	215	316					
	N-limited 1	43	105	220	321	415	519			
	N-limited 2	43	105	220	321	415	519			

Table 3.S3 Number of *P. denitrificans* Pd1222 genes with significantly different transcriptional activities ($p < 10^{-5}$). Transcriptional activities of genes were compared between acetate-limited (C-lim) and nitrate-limited (N-lim) culture conditions at the early stage (generations 30 – 100) and late stage (generations 200 – 500) of experimental evolution. Significant up- or down-regulation of transcriptional activities were determined within either the C-limited or the N-limited cultures. The significance was calculated with Fisher's exact test in the R package edgeR.

	Number of genes with significantly different transcriptional activities between C-lim and N-lim cultures		Number of genes that significantly changed their transcriptional activities within the respective culture condition	
	Generations 30 – 100	Generations 200 – 500	Up-regulated	Down-regulated
ABC-type transporters				
C-lim	38	42	15	11
N-lim	14	8	1	1
TRAP transporters				
C-lim	14	15	6	4
N-lim	2	1	1	0
Other transporters				
C-lim	23	26	12	4
N-lim	17	15	1	2
ABC-type transporters for inorganic compounds				
C-lim	6	5	0	5
N-lim	6	5	0	3
Regulators				
C-lim	83	72	20	17
N-lim	25	19	5	1
$\sum$ C-lim	164	160	53	41
$\sum$ N-lim	64	48	8	7

Table 3.S4 Single nucleotide polymorphisms (SNPs), insertions (Ins), deletions (Del) identified during experimental evolution of *P. denitrificans* Pd1222. Colored boxes represent detected mutations over all time points analyzed, given in numbers of generations. For SNPs the original amino acid, the position in the amino acid sequence and the new amino acid are given. Stars in place of an amino acid indicate that the affected nucleotide is part of a stop codon. Stars in the right column indicate strong effects of mutations on protein function. Anc, ancestor; aa, amino acid; Chr, chromosome; P, plasmid. Gene annotations are given in Table 3.S5.



Chr	Position	Gene tag (Pden_)	Characterization of mutation	Codon change	aa change	
1	68112	0072	outside of CHAD domain	aTc/aCc	I145T	
1	103167	0111	unknown	Tac/Cac	Y146H	
1	350709	0375	unknown	Gcc/Acc	A111T	
1	410591	0441	synonymous coding	gtC/gtT	V120	
1	455678		intergenic			
1	549332	0577	unknown	tAc/tGc	Y25C	
1	569785	0595	outside of functional domains	gaC/gaA	D76E	
1	656951	0674	synonymous coding	ctG/ctT	L181	
1	715976	0733	in variable aa position	gTc/gCc	V251A	
1	720304		intergenic			
1	729499	0747	variable region near RPB1 interaction site, G not tolerated	gAc/gGc	D540G	
1	731742	0747	flexible loop for DNA-binding site	Agc/Cgc	S1288R	
1	741245	0755	STOP at aa 362 of total 707 aa	Aag/Tag	K362*	*
1	827023	0839	ACPS domain	gAc/gTc	D185V	
1	839288	0850	conserved position of PRK00142 domain	ctG/ctA	L34	
1	844281	0854	highly conserved region in PLN03185 domain	ggG/ggC	G321	
1	846582	0857	variable end of surface alpha-helix	gAa/gGa	E86G	
1	912214	0931	variable aa position	atG/atT	M197I	
1	943942	0957	PHB_depo_C domain, PHB de-polymerase, variable aa position	aaG/aaT	K281N	
1	977256	1000	variable aa position of TIGR02675 domain, V,A tolerated	gTc/gCc	V165A	
1	1163296	1191	synonymous coding	Cgg/Agg	R453	
1	1170261	1197	variable region, but near active site, in OYE-like-3-FMN domain	Ctg/Atg	L167M	
1	1176417	1202	variable loop, dimer interface, H tolerated	cGc/cAc	R63H	
1	1192743		intergenic		Del-3	
1	1201265	1238	conserved aa position in pfam05951, S not tolerated	Cgc/Agc	R182S	

Continued on next page

Table 3.S4 (continued)

Chr	Position	Gene tag (Pden_)	Characterization of mutation	Codon change	aa change	
1	1209396	1247	highly conserved region, but F,W in other sequences, R not tolerated	Tgg/Cgg	W375R	*
1	1214935	1251	variable aa position in ATP binding site, V,A tolerated	gTc/gCc	V200A	
1	1227408	1266	hypervariable region	Ctg/Atg	L291M	
1	1259922	1297	hypervariable region, V also tolerated	Ctg/Gtg	L29V	
1	1263155	1300	synonymous coding	atC/atA	I112	
1	1297629		intergenic			
1	1320388	1354	highly conserved, but L,I in other sequences, P not tolerated	cTc/cCc	L109P	*
1	1335561	1368	variable region in beta sheet	Ctg/Atg	L554M	
1	1344124	1376	synonymous coding	ctC/ctA	L159	
1	1344364	1376	synonymous coding	atC/atA	I79	
1	1412246	1440	synonymous coding	gtG/gtT	V36	
1	1416841	1448	synonymous coding	cgG/cgT	R180	
1	1427347	1463	synonymous coding	cgG/cgT	R257	
1	1466424	1500	unknown	tCt/tAt	S119Y	
1	1470949	1506	variable aa position, Q,E,R,P tolerated	Cag/Gag	Q642E	
1	1490110	1526	outside of DEXDc or HELICc domain	Gcc/Tcc	A825S	
1	1542506		intergenic			
1	1542527		intergenic			
1	1542544		intergenic			
1	1577297	1598	unknown	Ctt/Ttt	L11F	
1	1579491	1600	hypervariable region	caG/caT	Q281H	
1	1612114		intergenic		Del-2	
1	1642156	1659	variable aa position, V tolerated, outside of functional site	gCg/gTg	A176V	
1	1652500	1671	hypervariable region, outside of binding site	gaG/gaT	E276D	
1	1677024	1694	variable region	tCg/tAg	S286*	
1	1684852		intergenic		Ins+1	
1	1740280	1752	variable region	gaG/gaT	E42D	
1	1805860	1811	synonymous coding	ggC/ggG	G511	
1	1816591		intergenic			
1	1867277	1868	conserved aa position, only R tolerated	aGg/aAg	R131K	*
1	1877304	1876	frame shift at aa position 204, part of putative active site	-117	Del-2	*
1	1885210	1882	unknown	Ccc/Tcc	P170S	
1	1908354	1911	synonymous coding	ccG/ccA	P31	
1	1912112	1914	synonymous coding	tcC/tcT	S12	
1	1952767	1950	STOP in variable region of SIS_PGI_2 domain	Cag/Tag	Q442*	*

Continued on next page

Table 3.S4 (continued)

Chr	Position	Gene tag (Pden_)	Characterization of mutation	Codon change	aa change	
1	1952958	1950	highly conserved dimer interface, P not tolerated, SIS_PGI_2 domain	cAg/cCg	Q378P	*
1	1952997	1950	conserved aa position, G,F,N tolerated	gGc/gAc	G365D	*
1	1953313	1950	frame shift at aa 258	-258	Del-1	*
1	1963011		intergenic			
1	1999168	1993	unknown	cgtcgc/cgc	Del-3	
1	2027753	2019	variable aa position, L,W,I,R tolerated	cGg/cTg	R76L	
1	2035451	2027	variable region in homotetramer interface	cAg/cGg	Q238R	
1	2037081	2031	substrate binding pocket, conserved, only H,Q tolerated	cAt/cTt	H86L	*
1	2043147	2035	variable aa position	gGg/gTg	G61V	
1	2200891	2196	conserved aa position near catalytic site and NAPD binding sites	cTg/cAg	L244Q	
1	2275009	2276	synonymous coding	atC/atA	I114	
1	2302431	2303	in substrate binding site, D,E,H,N tolerated	gAt/gGt	D240G	*
1	2376295	2378	variable aa position in THUMP_AdoMetMT domain	Aag/Cag	K166Q	
1	2392701	2395	synonymous coding	ggT/ggG	G55	
1	2414843		intergenic		Ins+18	
1	2414843		intergenic		Ins+6	
1	2414849		intergenic		Ins+6	
1	2415209		intergenic			
1	2415221		intergenic			
1	2415227		intergenic			
1	2415230		intergenic			
1	2416121		intergenic			
1	2416124		intergenic			
1	2416127		intergenic		Ins+18	
1	2416127		intergenic		Ins+6	
1	2416334		intergenic		Ins+6	
1	2474457	2461	unknown	Ggc/Tgc	G569C	
1	2514617	2497	synonymous coding	gcG/gcT	A457	
1	2526569	2507	variable aa position	gcggtg/gtg	Del-3	
1	2540883	2525	fusion protein with Pden_2526 possible with intergenic spacer of 23 aa	Tga/Gga	*233G	*
1	2652917	2641	unknown	Tgt/Cgt	C22R	
1	2711187	2694	variable aa position	Ggc/Agc	G84S	
1	2766109	2746	variable region	cGc/cAc	R385H	
1	2775667	2756	mutation in dimer interface, but not conserved position, E not tolerated	gTg/gAg	V166E	*

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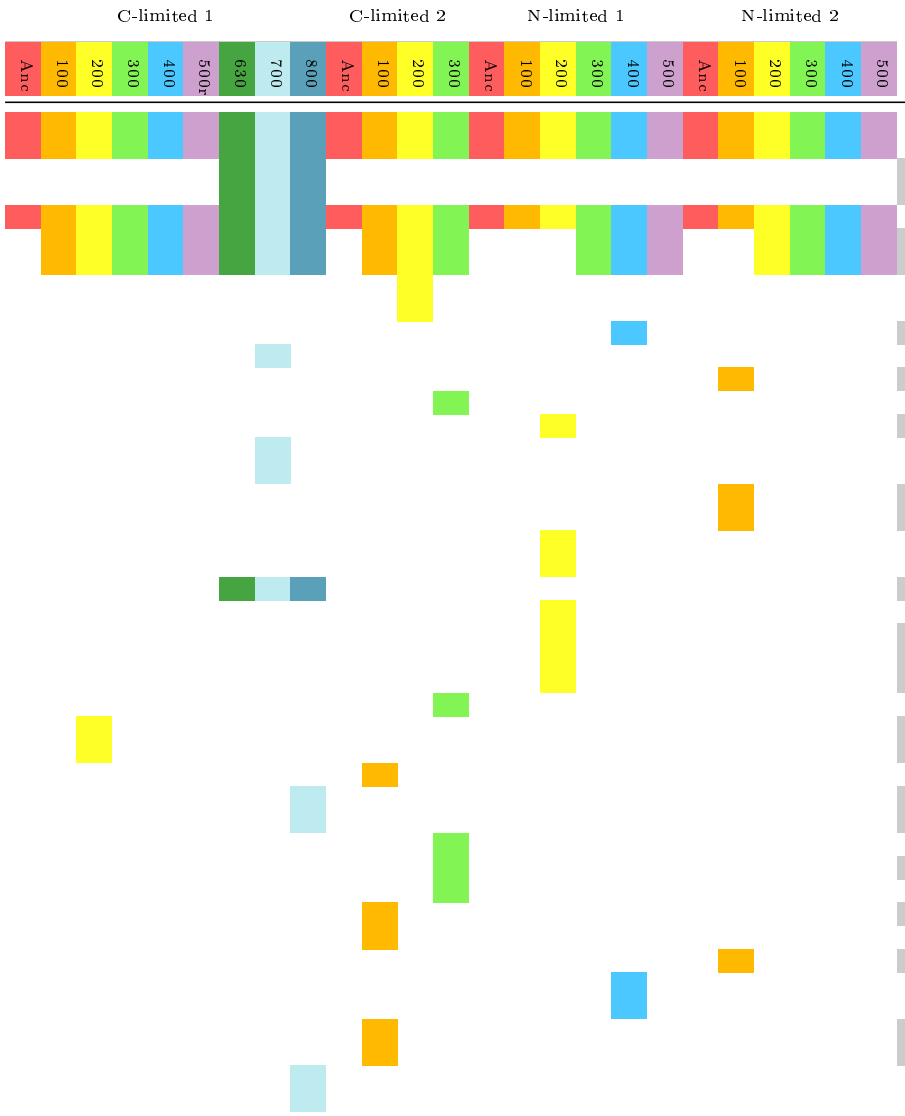
Table 3.S4 (continued)



Chr	Position	Gene tag (Pden_)	Characterization of mutation	Codon change	aa change	
1	2783017	2763	loss of 12 aa, no frame shift, highly variable region	-36	Del-36	*
1	2833009	2815	aa position for complexing two Mn^{2+} and the DNA, catalytic center	gaC/gaG	D7E	*
1	2852233		intergenic	Aaa/Caa	K224Q	
2	40074	2872	Deletion of 15 aa, partially conserved, within substrate binding site	Del-45		*
2	86057	2924	Del at aa position 171 in DUF1556 or COG1556	-171	Del-2	*
2	124015	2955	variable region	cCg/cTg	P279L	
2	177983	3006	INDel in the middle of the CDS	-73	Del-17	
2	204397		intergenic			
2	239885	3059	variable region	Att/Gtt	I412V	
2	267780	3083	variable region	Gcc/Acc	A143T	
2	278839	3094	conserved position, but not in oxidoruductase_q6 domain	Ctg/Atg	L186M	
2	355377	3188	in variable region of Trag_VirD4 domain, ATP-binding site	tGg/tAg	W220*	*
2	399179	3232	in HATPase_c-domain, but variable aa position	Gag/Aag	E209K	
2	406203	3240	unknown	Ctg/Atg	L196M	
2	454139	3289	variable region, I not tolerated	aGc/aTc	S70I	
2	464659	3299	in ATP-binding cassette domain, conserved aa position next to M-loop/switch region	aCc/aAc	T207N	
2	480383	3319	synonymous coding	ctG/ctT	L217	
2	529435	3384	highly conserved, but A,V in most sequences	Gcc/Tcc	A347S	*
2	612256	3467	synonymous coding	acC/acT	T98	
2	700898	3552	conserved, in ATP-grasp domain, but F,S,W in other sequences	Ctt/Att	L161I	*
2	704747	3555	unknown	taT/taC	Y874	
2	706277	3555	synonymous coding	tcC/tcT	S364	
2	706278	3555	unknown	tCc/tTc	S364F	
2	747061	3596	synonymous coding	gcG/gcA	A457	
2	750139	3601	unknown	Ttc/Ctc	F178L	
2	842320	3693	synonymous coding	ctG/ctA	L78	
2	845343	3696	highly conserved region, ATP-binding domain	Ggg/Tgg	G257W	*
2	849135	3700	hypervariable region, outside of Zn binding or active site	gaG/gaT	E73D	
2	850018	3700	highly conserved, active site, D,E tolerated	Gat/Tat	D368Y	

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Table 3.S4 (continued)

Chr	Position	Gene tag (Pden_)	Characterization of mutation	Codon change	aa change	
2	877299	3729	synonymous coding	gcC/gcA	A12	
2	883117	3740	unknown	Ggc/Tgc	G49C	
2	916134	3778	synonymous coding	tcG/tcT	S710	
2	943885	3807	synonymous coding	gaA/gaG	E124	
2	1144444	4002	loss of 738 aa of total 818 aa	tGg/tTg	W166L	
2	1144450	4002	818 aa, variable aa position, H,Y,L,S tolerated	Cag/Tag	Q80*	
2	1165653	4018	in periplasmatic substrate binding domain (not tolerated)	Cat/Tat	H78Y	
2	1182774	4033	STOP at aa 93	cTg/cGg	L103R	*
2	1185487	4035	highly conserved, but V,T,E in other sequences	gTg/gAg	V511E	
2	1193570	4043	synonymous coding	ccG/ccA	P104	
2	1224733	4071	variable aa position, G not tolerated	gAg/gGg	E66G	
2	1265823	4112	variable aa position	gTg/gAg	V34E	
2	1272425	4119	conserved aa position in Fumarase C-terminus, L,F,M,I tolerated	Ctt/Att	L460I	
2	1288722	4132	synonymous coding	acC/acT	T20	
2	1300981	4144	variable loop in ATP binding domain, K not tolerated	aTg/aAg	M281K	
2	1430433	4262	highly conserved	gaG/gaT	E484D	*
2	1454057	4279	synonymous coding	Ttg/Ctg	L182	
2	1500305		intergenic		Ins+9	
2	1537807	4351	unknown	tGg/tTg	W474L	
2	1553491	4364	outsite of domain	Gac/Aac	D368N	
2	1593309	4397	frame shift at aa position 122	-122	Ins+2	*
2	1594031	4398	hypervariable aa position, synonymous coding	gcC/gcT	A260	
2	1637310	4429	variable aa position near the active site, but variable aa position, M,I,L,A tolerated	Atg/Ctg	M71L	
2	1651096	4442	hypervariable aa position	Acc/Tcc	T91S	
2	1682957		intergenic			
P	1179	4517	variable aa position	caG/caT	Q64H	
P	7829	4526	synonymous coding, outside of AdoMet_Mtase domain, outside of SAM-binding site	ggG/ggT	G217	
P	16475	4535	conserved aa position, outside of plug-domain and channel	Acc/Tcc	T143S	
P	27507	4547	STOP in variable region, loss of 300 aa	Aag/Tag	K153*	*
P	39270	4556	synonymous coding	ctG/ctT	L319	
P	45557	4561	conserved aa position, L,M,I tolerated	atG/atA	M230I	

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Table 3.S4 (continued)

Chr	Position	Gene tag (Pden_)	Characterization of mutation	Codon change	aa change	
P	51753	4567	conserved aa position in aconitate hydratase B domain, D,N,G tolerated	gAc/gGc	D722G	
P	55031	4570	unknown	agC/agA	S188R	
P	135883	4647	variable aa position in putative substrate translocation pore, F,L tolerated	Ttt/Ctt	F59L	
P	178921	4686	synonymous coding	cgC/cgG	R196	
P	178927	4686	synonymous coding	cgC/cgT	R198	
P	178948	4686	synonymous coding	gtG/gtC	V205	
P	183574	4690	Deletion at aa position 109 with frame shift	-109	Del-1	*
P	183694	4690	STOP in hypervariable aa position, near SAM binding site	Cag/Tag	Q70*	*
P	183942	4691	STOP in hypervariable region, loss of 11 aa	tGg/tAg	W238*	*
P	183991	4691	Del at aa 221	-221	Del-1	*
P	183996	4691	Del at aa 220	tGg/tAg	W220*	*
P	184089	4691	STOP at aa 189	tGg/tAg	W189*	*
P	184174	4691	insertion with frame shift	11	Ins+11	*
P	184230	4691	STOP in conserved aa position, loss of 105 aa	cCc/cTc	P142L	*
P	205925	4717	STOP at the variable end	gCc/gTc	A288V	
P	206550	4718	unknown	tGg/tAg	W16*	
P	206930	4719	STOP in conserved region, loss of 10 aa	Cag/Tag	Q49*	*
P	227141	4741	outside of HisKA or HATPase_c domains	Ttc/Ctc	F34L	
P	261578	4764	variable aa position, D,E,S,N tolerated	gAc/gGc	D192G	
P	275012		intergenic			
P	297230	4801	synonymous coding	ctG/ctC	L262	
P	338872	4839	synonymous coding	ggC/ggT	G211	
P	344905	4845	variable aa position, N tolerated	Gac/Aac	D207N	
P	346102	4846	variable aa position, F,H,Y,I,L,M,C,V tolerated	tTc/tAc	F185Y	
P	350066	4850	variable aa position, outside of ATP binding site	Gcc/Tcc	A496S	
P	358978	4856	synonymous coding	atC/atA	I310	
P	361591	4858	variable aa position	Tcg/Ccg	S76P	
P	387844	4883	Del at aa 173, part of ATP binding site and H-loop/switch	-95	Del-2	*
P	391384	4888	hypervariable region at N-terminus	atC/atG	I4M	
P	391846	4888	synonymous coding	ggC/ggA	G158	
P	435476	4929	Del at aa position 36, N-terminal	-36	Del-5	*
P	437814	4931	variable aa position, A,S,P,Q tolerated	Gcc/Tcc	A270S	

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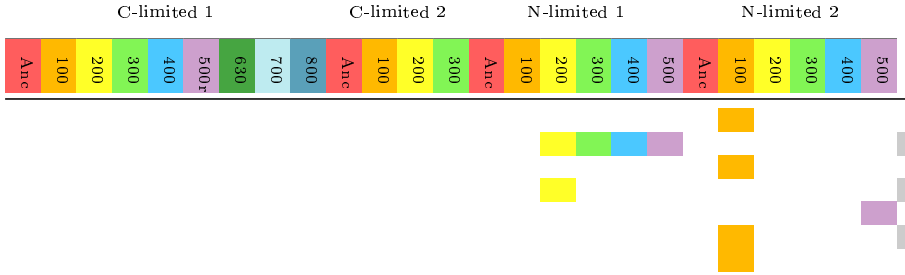
Table 3.S4 (continued)



Chr	Position	Gene tag (Pden_)	Characterization of mutation	Codon change	aa change	
P	438661	4932	H not tolerated, M,P tolerated	caG/caT	Q215H	
P	444543	4939	variable aa position	Gcc/Acc	A101T	
P	488626	4978	synonymous coding	gtG/gtA	V253	
P	493746	4983	synonymous coding	ctG/ctA	L260	
P	499186	4988	unknown	gTt/gGt	V284G	
P	536093		intergenic			
P	638223	5119	conserved aa position, S,T,A, tolerated	aGc/aAc	S84N	*

Table 3.S5 Mutated genes identified during experimental evolution of *P. denitrificans* Pd1222 and their predicted function. Annotations were retrieved from GenBank, Pfam, TIGRFAM, SMART and KEGG.

Gene tag (Pden_)	Function
0072	CHAD domain containing protein
0111	hypothetical protein
0375	hypothetical protein
0441	hypothetical protein
0577	hypothetical protein
0595	multi-sensor hybrid histidine kinase
0674	permease
0733	hypothetical protein
0747	DNA-directed RNA polymerase subunit beta
0755	elongation factor G
0839	4'-phosphopantetheinyl transferase
0850	rhodanese domain-containing protein
0854	MORN repeat-containing protein
0857	enoyl-ACP reductase
0931	extracellular solute-binding protein
0957	polyhydroxyalkanoate depolymerase
1000	phage tape measure protein
1191	FAD dependent oxidoreductase
1197	NADH:flavin oxidoreductase
1202	resolvase domain-containing protein
1238	hypothetical protein
1247	glutathionylspermidine synthase
1251	ABC transporter
1266	ATP-dependent protease ATP-binding subunit ClpX
1297	NAD-dependent epimerase/dehydratase
1300	hypothetical protein
1354	hypothetical protein
1368	TonB-dependent receptor
1376	lipoyl synthase
1440	hypothetical protein
1448	PhzF family phenazine biosynthesis protein
1463	LysR family transcriptional regulator

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Table 3.S5 (continued)

Gene tag (Pden_)	Function
1500	helix-turn-helix domain-containing protein
1506	conjugal transfer coupling protein TraG
1526	helicase
1598	hypothetical protein
1600	LysR family transcriptional regulator
1659	histidine kinase
1671	extracellular solute-binding protein
1694	LysR family transcriptional regulator
1752	coproporphyrinogen III oxidase
1811	malonyl CoA-ACP transacylase
1868	beta-N-acetylhexosaminidase
1876	fructose 1,6-bisphosphatase II
1882	hypothetical protein
1911	hypothetical protein
1914	hypothetical protein
1950	glucose-6-phosphate isomerase
1993	hypothetical protein
2019	hypothetical protein
2027	acetoacetyl-CoA reductase
2031	trans-hexaprenyltranstransferase
2035	VacJ family lipoprotein
2196	6-phosphogluconate dehydrogenase
2276	pyruvate kinase
2303	molecular chaperone DnaJ
2378	RNA methylase
2395	hypothetical protein
2461	outer membrane protein
2497	catalase/peroxidase HPI
2507	peptidase M23B
2525	cobalt transporter subunit CbtA
2641	hypothetical protein
2694	paraquat-inducible protein A
2746	ABC transporter
2756	peroxidase

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Table 3.S5 (continued)

Gene tag (Pden_)	Function
2763	porin
2815	DNA polymerase III subunit epsilon
2872	ribokinase-like domain-containing protein
2924	hypothetical protein
2955	saccharopine dehydrogenase
3006	hypothetical protein
3059	amidase
3083	outer membrane protein
3094	NADH ubiquinone oxidoreductase, 20 kDa subunit
3188	conjugal transfer coupling protein TraG
3232	histidine kinase
3240	hypothetical protein
3289	hypothetical protein
3299	ABC transporter
3319	hypothetical protein
3384	TRAP dicarboxylate transporter, DctM subunit
3467	hydroxyproline-2-epimerase
3552	phosphoribosylglycinamide synthetase
3555	hypothetical protein
3596	phage tape measure protein
3601	fibronectin, type III domain-containing protein
3693	hypothetical protein
3696	GMP synthase
3700	carboxypeptidase Taq metallopeptidase
3729	hypothetical protein
3740	hypothetical protein
3778	metallophosphoesterase
3807	transglycosylase
4002	peptidase
4018	inner-membrane translocator
4033	rod shape-determining protein MreC
4035	peptidoglycan glycosyltransferase
4043	ErfK/YbiS/YcfS/YnhG family protein
4071	NADPH-dependent FMN reductase

Continued on next page

Table 3.S5 (continued)

Gene tag (Pden_)	Function
4112	L-carnitine dehydratase/bile acid-inducible protein F
4119	tartrate/fumarate subfamily Fe-S type hydro-lyase subunit alpha
4132	L-serine dehydratase 1
4144	pyruvate carboxylase
4262	hydantoinase B/oxoprolinase
4279	major facilitator transporter
4351	cytosine/purines uracil thiamine allantoin permease
4364	ThiJ/PfpI domain-containing protein
4397	hypothetical protein
4398	FAD linked oxidase domain-containing protein
4429	glycogen/starch/alpha-glucan phosphorylase
4442	extracellular solute-binding protein
4517	MarR family transcriptional regulator
4526	methyltransferase type 11
4535	TonB-dependent siderophore receptor
4547	glutamate–ammonia ligase
4556	DNA polymerase III, epsilon subunit
4561	glycerate kinase
4567	bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase
4570	hypothetical protein
4647	major facilitator transporter
4686	ABC transporter related
4690	phosphatidylethanolamine N-methyltransferase
4691	CDP-diacylglycerol–serine O-phosphatidyltransferase
4717	TRAP dicarboxylate transporter, DctM subunit
4718	hypothetical protein
4719	periplasmic nitrate reductase NapE
4741	histidine kinase
4764	histidine kinase
4801	phenylacetate-CoA ligase
4839	HAD family hydrolase
4845	extracellular solute-binding protein
4846	periplasmic binding protein/LacI transcriptional regulator
4850	ABC transporter related

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Table 3.S5 (continued)

Gene tag (Pden_)	Function
4856	TRAP dicarboxylate transporter, DctP subunit
4858	tripartite ATP-independent periplasmic transporter DctQ
4883	ABC transporter related
4888	substrate-binding region of ABC-type glycine betaine transport system
4929	polar amino acid ABC transporter, inner membrane subunit
4931	alcohol dehydrogenase
4932	short-chain dehydrogenase/reductase SDR
4939	UbiD family decarboxylase
4978	hypothetical protein
4983	branched-chain alpha-keto acid dehydrogenase subunit E2
4988	hypothetical protein
5119	NADPH-dependent FMN reductase

Table 3.S6 Overview of mutations in C-limited and N-limited cultures of *P. denitrificans* during experimental evolution. SNP, single nucleotide polymorphism; Indel, insertion or deletion.

Property/Number of generations	All cultures	C-lim 1 100 – 500	C-lim 1 100 – 800	C-lim 2 100 – 300	N-lim 1 100 – 500	N-lim 2 100 – 500
Persistence						
Total number of mutations (including mutations that did not persist)	211	25	60	51	53	47
Number of mutations that persisted at least 100 generations (2 samples)	27	3	17	2	5	3
Number of mutations that persisted at least 200 generations (3 samples)	18	3	8	2	5	3
Number of mutations that persisted until the last sample	21		11	2	5	3
Type of mutation						
SNPs	179	18	48	44	47	40
Insertions	12	3	4	1	3	1
Deletions	20	4	1	6	3	6
Frame shift caused	13					
SNP/Indel ratio	5.6	2.6	9.6	6.3	7.8	5.7
Non-synonymous SNP/Indel ratio	5.4					
Transitions						
A:T → G:C	33	3	8	9	10	6
C:G → T:A	51	6	8	13	16	14
Transversions						
A:T → T:A	13	0	6	1	4	2
A:T → C:G	11	3	4	4	1	2
C:G → A:T	62	5	20	13	13	16
C:G → G:C	9	1	2	4	3	0
Effect of mutations						
Non-synonymous SNPs		172	53	38	42	39
Synonymous SNPs		39	7	13	11	8
Mutations in open reading frames		173	44	42	47	40
Mutations in intergenic regions		38	16	9	6	7
Non-synonymous SNPs in open reading frames		134				
Synonymous SNPs in open reading frames		39				
Repetitive mutations						
Number of mutations detected in > 1 culture	12					
Number of mutations detected in all cultures	4					
Different open reading frames affected in > 1 culture	11					
Different open reading frames affected by at least one mutation	144					
Per 100 generations			C-lim		N-lim	
Open reading frames			7.2		7.8	
Intergenic			1.3		0.7	
Synonymous			1.7		1.8	
Non-synonymous			6.7		6.7	

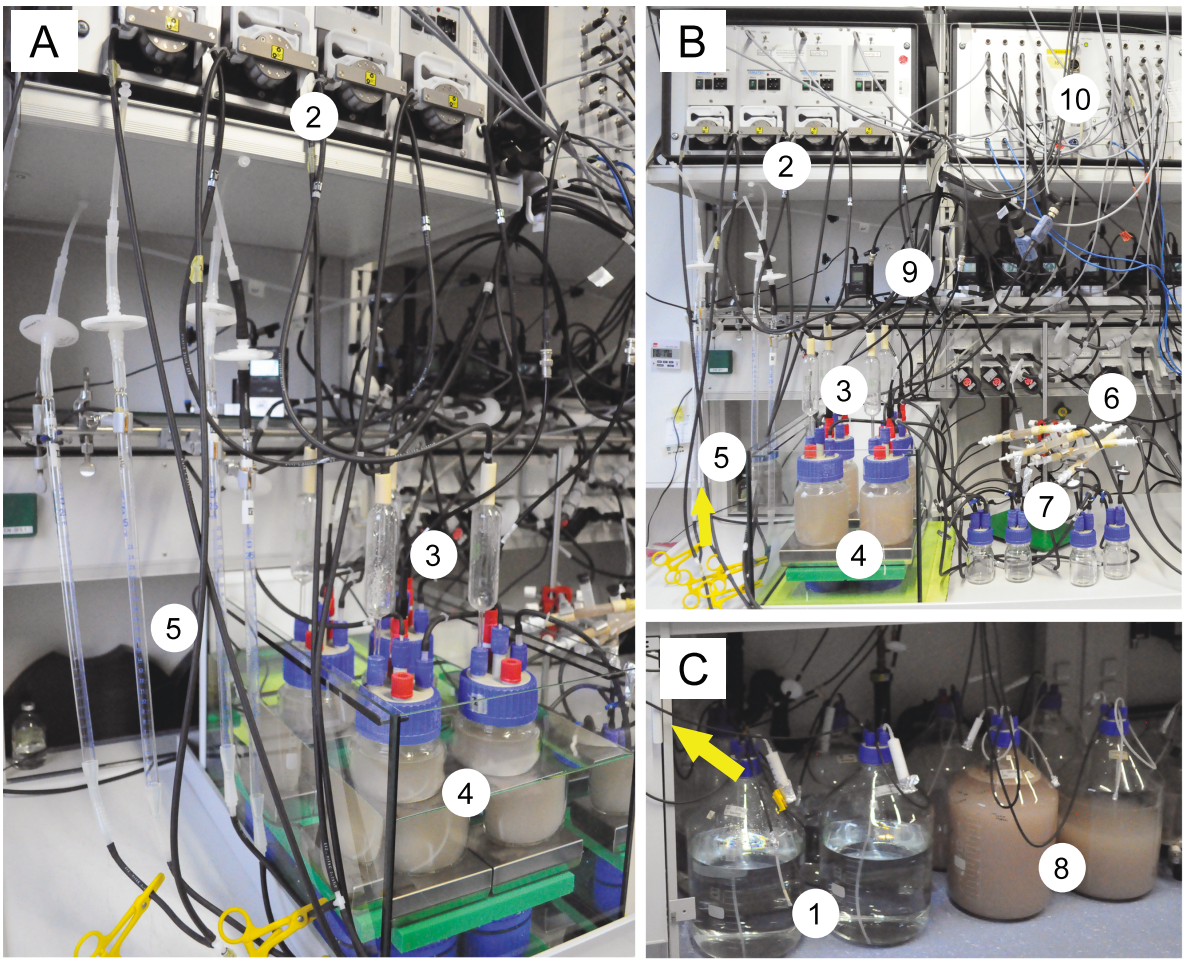

Figure 3.S1 Chemostats used for the experimental evolution of *P. denitrificans* under denitrifying conditions. Yellow arrows indicate the flow of fresh media, orange arrows indicate outflow of cell suspensions and gas. Medium reservoirs (1) were flushed with sterile argon, and were connected to the chemostat cultures with gas tight tubes via peristaltic pumps (2) and sterile traps (3) that prevented bacterial growth into the medium tubes. Chemostat vessels (4) were placed on magnetic stirrers in a water bath maintained at 30 °C. To prevent variations in medium flow sterile glass pipettes were connected to the medium tubes that allowed regular calibration of pump rates (5). Two sampling possibilities were installed in the effluent tubes, per culture one T connection for sampling with a sterile syringe (6) and one 100 mL bottles for taking large samples (7). The effluent cell suspensions were removed from the cultures by overpressure and collected in sterile reservoirs (8). Reservoirs were filled and emptied by carefully pumping through sterile connections with mobile, sterile bottles. Chemostat cultures were continuously sparged with sterile filtered argon at rates controlled by mass flow controllers (9) to provide anoxia. Medium pumps, temperature, pH and argon supply were controlled by a separate control unit (10).

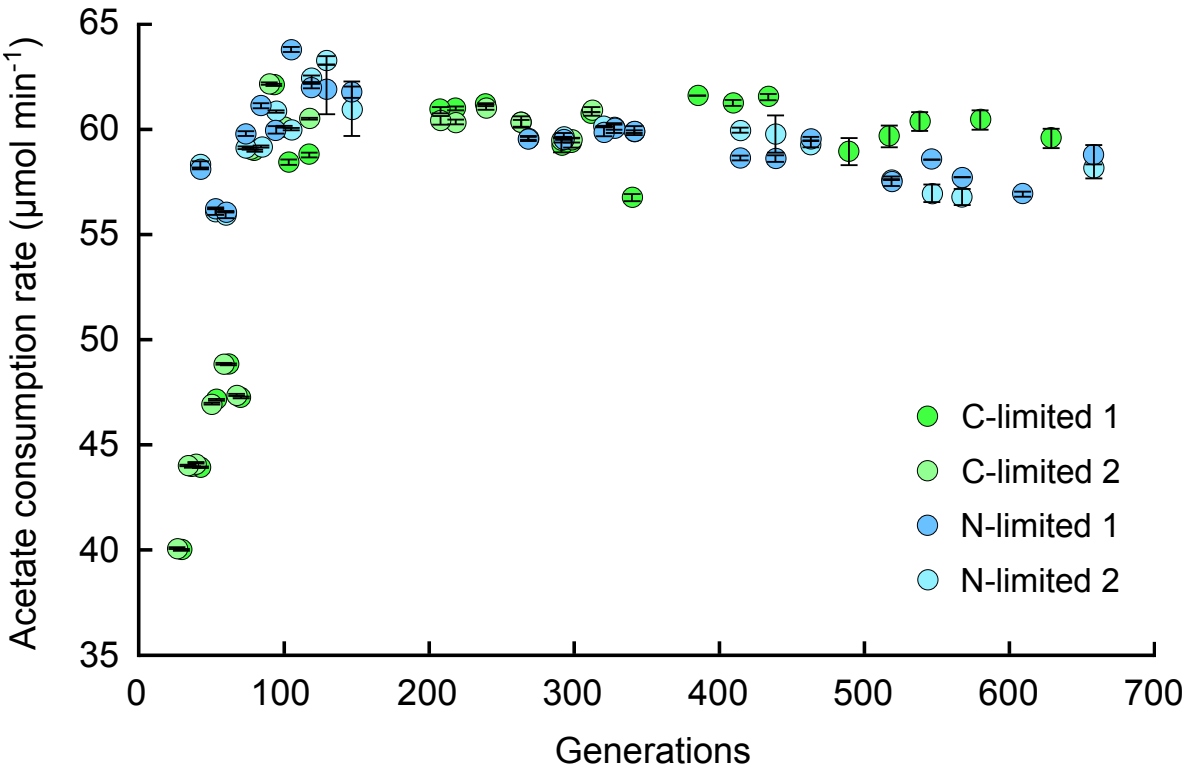

Figure 3.S2 Acetate consumption rates in the course of experimental evolution of *P. denitrificans* in anoxic C-limited and N-limited chemostats. C-limited cultures were supplied with 60.5 ± 1.2 µmol min^{-1} acetate, N-limited cultures were supplied with 68.4 ± 1.5 µmol min^{-1} acetate. Summarized data on carbon conversions are presented in Table 3.1. Error bars represent standard deviations and are smaller than the symbols.

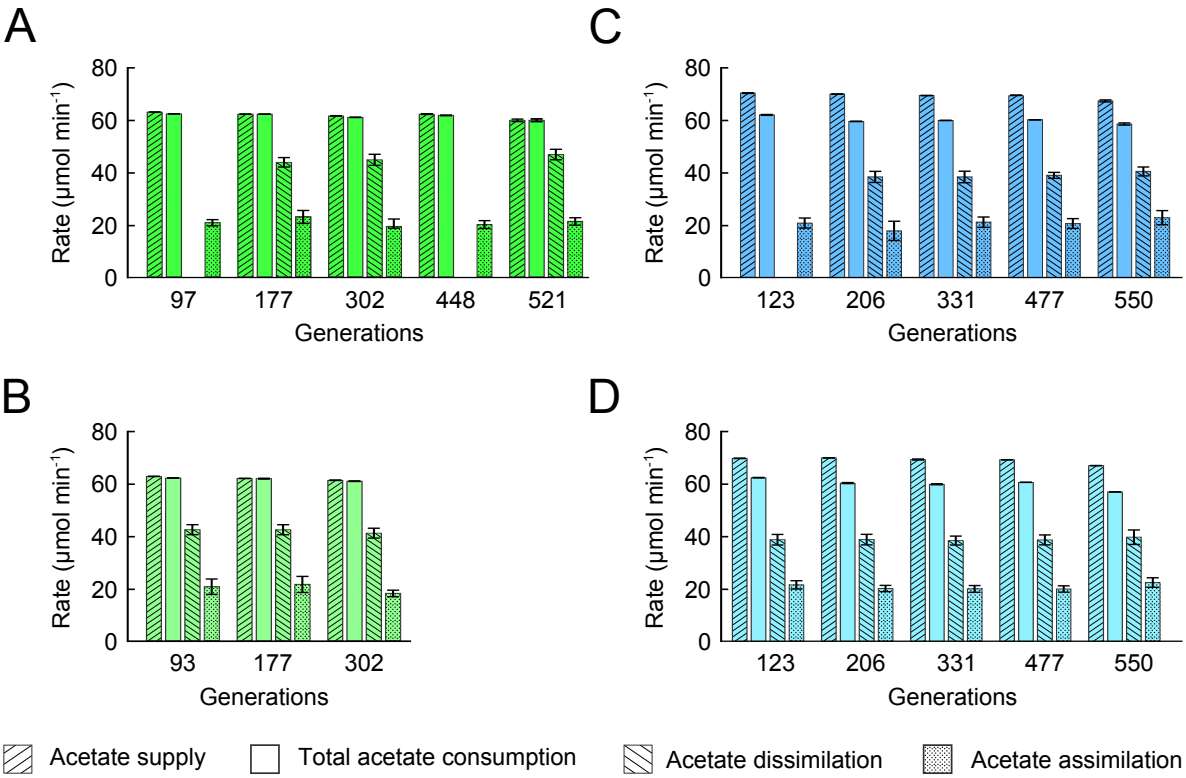

Figure 3.S3 Carbon mass balances for selected time points of experimental evolution of *P. denitrificans* in C-limited (A and B) and N-limited (C and D) chemostat cultures, expressed in absolute values of conversion rates. Approximately one third of the consumed acetate was assimilated (determined based on protein concentration and biological carbon content) and two thirds were dissimilated (determined based on inorganic carbon determinations in culture liquid and headspace). Summarized data on carbon conversions are presented in Table 3.1. Error bars represent standard deviations.

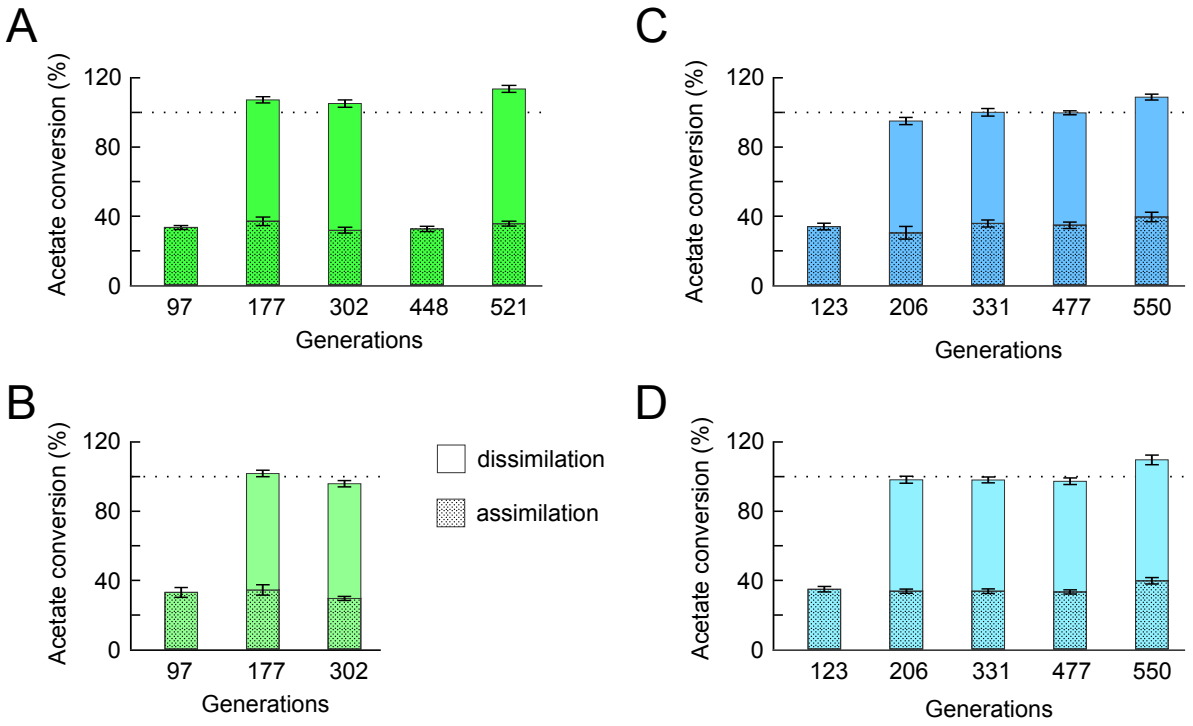

Figure 3.S4 Carbon mass balances for selected time points of experimental evolution of *P. denitrificans* in C-limited (A and B) and N-limited (C and D) chemostat cultures, expressed in percentage of total acetate consumption (indicated by the dotted line). Summarized data on carbon conversions are presented in Table 3.1. Error bars represent standard deviations.

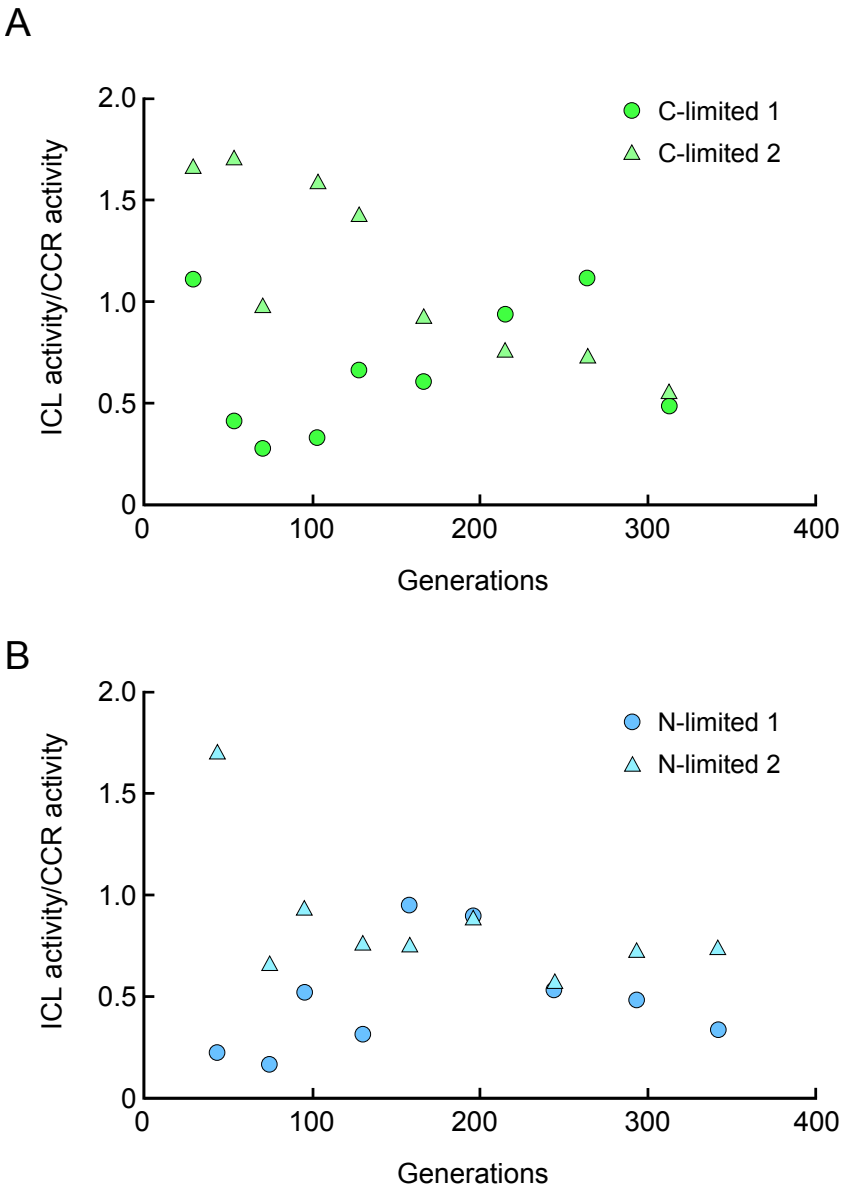

Figure 3.S5 Enzyme activities of isocitrate lyase (ICL) and crotonyl-CoA carboxylase/reductase (CCR), shown as ratios of ICL/CCR in C-limited cultures (A) and N-limited cultures (B). Activities of ICL ranged from 34 to 270 mU mg^{-1} in C-limited cultures and from 45 to 162 mU mg^{-1} in N-limited cultures. Activities of CCR ranged from 55 to 242 mU mg^{-1} in C-limited cultures and 53 to 272 mU mg^{-1} in N-limited cultures.

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Chapter 4

Conclusion and Discussion of the present work

This thesis contributes to the understanding of adaptive processes of the denitrifying bacterium *Paracoccus denitrificans* Pd1222 in constant, acetate or nitrate limited environments. Denitrification is one of the best understood processes among the natural biological nitrogen conversions. Due to the four reaction steps this pathway is composed of, five nitrogen compounds are involved that can be exchanged between denitrifying organisms and their environments. Different environmentally important parameters can affect certain steps of denitrification and promote the excretion of the intermediates nitrite, nitric oxide and nitrous oxide such as pH, availability and/or fluctuating concentrations of oxygen and nitrogen oxides (Baumann et al., 1996, 1997; Bergaust et al., 2010, 2011). In particular the reduction of nitrous oxide (the final step of denitrification) is impaired by low pH, demonstrating the impact of pH in soils and agro-ecosystems on the release of the greenhouse gas (Thomsen et al., 1994; Šimek and Cooper, 2002). Apart from studies on these environmental constraints, many researchers have cultivated *P. denitrificans* primarily for genetic and enzymatic investigations. To exclude growth inhibition or unwanted limitations of nutrients other than acetate or nitrate during long-term cultivation, a set of trace element solutions was tested for their suitability for anaerobic growth of *P. denitrificans* (*Chapter 2*). These experiments revealed the advantage of a chelated trace element solution over an acidified solution and showed better growth at concentrations lower than in frequently used media of published studies. In denitrifying batch cultures *P. denitrificans* grew diauxically where nitrate was completely reduced to nitrite before nitrite reduction was initiated.

The study presented in *Chapter 3* of this thesis provides knowledge on adaptive improvements in the denitrification pathway over a long time scale. During approximately 500 generations of evolution in an acetate limited chemostat *P. denitrificans* improved its ability to completely reduce nitrate to dinitrogen in contrast to the non-adapted bacteria that converted a part of the nitrate only to nitrite. Furthermore, the study showed significantly different phenotypes between the two treatments, including physiology, metabolic nitrogen conversions and transcriptional activities of diverse groups of genes. Most notably, carbon limitation resulted in significantly more phenotypic changes over time than nitrate limitation. In the following sections I will reflect on the observations of the presented studies in context with natural denitrifying communities and discuss the challenges that we face when integrating laboratory experiments with isolated microorganisms into complex systems.

4.1 Further considerations of evolutionary genetics

The present thesis describes the first laboratory evolution experiment carried out with a denitrifying organism. Furthermore, it is one of the few experimental evolution studies that have been performed in combination with genome, transcriptome and metabolic analyses. A detailed study of the denitrifying bacterium "Aromatoleum aromaticum" EbN1 has been performed in chemostats to study physiological and metabolic responses to different dilution rates, reflecting different levels of stress acting on the organism (Trautwein et al., 2012). These cultures were maintained in the chemostats for a relatively short time to investigate responses that were not associated with genetic evolution. An evolution experiment with *E. coli* was combined with gene expression analysis and tracking of carbon and oxygen conversion (Fong et al., 2005). *Chapter 3* presents the evolution of *P. denitrificans* and the comparison between two different conditions, acetate or nitrate limitation, based on the integration of the methods mentioned above. Numerous mutations have been identified during evolution of up to 800 generations. Here I address remaining questions on genetic modifications that have not been specifically discussed so far, but might have contributed to a fitness increase of *P. denitrificans*.

Importance of indels

The distribution of transitions, transversions and indels in *P. denitrificans* populations was similar to the pattern found in previously evolved *E. coli* populations (Maharjan et al., 2012). Interestingly, the SNP-to-indel ratios (5.6 in the present study and 1.2 to 2.1 in the study of Maharjan and colleagues) were lower when compared to a SNP-to-indel ratio of 19.6 previously determined for bacteria (Chen et al., 2009). Maharjan et al. (2012) suggested that nutrient limitation may explain this deviation, which agrees with the findings in the present study.

Importance of mutations without obvious effect on protein function

The discussion in *Chapter 3* focused on persisting mutations that were predicted to strongly affect protein function. These mutations were dominated by either SNPs that led to alternative amino acids in conserved regions or indels. However, also mutations of minor effects in protein function can result in a large fitness increase. A SNP in bla_{TEM} in *Enterobacteriaceae*, encoding beta-lactamase increased the fitness of the phenotype resistant to a spectrum of antibiotics and enabled the gene variant to become widespread among *Enterobacteriaceae* world-wide (Mroczkowska and Barlow, 2008). Moreover, a small increase in kinetics of an antibiotics resistance protein could confer high increase in growth rate and resistance of a mutant (Walkiewicz et al., 2012). This implies that a larger set of mutations than what was discussed in this thesis might have the potential to significantly improve the fitness of *P. denitrificans* in both culture conditions.

Genetic hitchhiking

Genetic hitchhiking describes a process by which a neutral or deleterious mutation is maintained in a population by the presence and selection of a beneficial mutation in the same genome (Elena and Lenski, 2003). In large population sizes more mutations appear than in small populations and thus beneficial mutations are more frequent (Elena and Lenski, 2003). Consequently, large populations are characterized by higher genetic variation that facilitates genetic hitchhiking. However, as many mutants co-occur, beneficial mutations are fixed more randomly because of high competition. The large number of mutations identified in this thesis might therefore include neutral or even deleterious mutations.

Limited information on hypothetical proteins

Mutations affected at least 18 genes without information and numerous genes with only limited information on the functions or structures of the proteins they encode. Furthermore, 150 coding sequences with unknown products had significantly higher transcriptional activities in either treatment. Six of these coding sequences had RPKM values of at least 10,000 (e.g., Pden_4136), some were significantly up- or down-regulated (Pden_0959, 4-fold down-regulated) or showed significantly different transcriptional activities between the culture conditions (Pden_5123, 32-fold higher transcriptional activities in acetate limited cultures). Hence, the effect of these mutations on the gene products could not be predicted and some important information might have been missed. It is further unknown whether a highly transcribed gene may have affected the subsequent appearance and benefits of mutations. More intense bioinformatic and laboratory work would be needed to reveal the effect of mutations and high transcription of genes with insufficient information on the phenotypes of *P. denitrificans* (see *Section 4.5*).

4.2 Mutation independent adaptation

Phenotypic changes over time can be independent of genotypic variation even under constant conditions. Although genome independent evolution was rarely considered in previous reports, phenotypic variability (plasticity) within microbial populations might play an important role for evolutionary trajectories that evolving populations follow.

The idea that evolution may not exclusively be caused by mutations is based on the option that phenotypic plasticity of an organism can have high implications on its fitness. In turn, different phenotypic states of a cell may lead to slightly different selective advantages of appearing mutations. Phenotypes can vary among individual cells as a result of micro-scale heterogeneity in a seemingly constant environment in which they evolve. On the other hand, individual cells within a population may differ in their phenotype due to different cellular compositions that can for example arise from a heterogeneous distribution of cellular components during cell division (Davidson and Surette, 2008; Tyagi, 2010). Protein concentrations can vary between individual cells due to stochastic fluctuations in transcription and translation (Smits et al., 2006). It has been discussed that heterogeneous phenotypes between individuals of an isogenic population may confer advantages to the population when for example subjected to fluctuating or heterogeneous (environmental) conditions (Raj and Oudenaarden, 2008).

Furthermore, isogenic populations can expose variations in epigenetic patterns like

DNA methylation. The DNA methylation state in cis-regulatory elements determines whether or not a gene is expressed (Lim and van Oudenaarden, 2007). This mechanism potentially contributes to divergence of populations since DNA methylation is irreversible and either results in unmethylated or hemimethylated DNA during replication or is inherited for many generations (Lim and van Oudenaarden, 2007). Increasing variation in phenotypes based on epigenetic DNA modifications within a population of *E. coli* was observed upon stress induction and was suggested to confer different fitness effects to the individuals within a population (Ni et al., 2012). It would be worthwhile to include these considerations in experimental evolution studies by using single-molecule real-time (SMRT) DNA sequencing (Fang et al., 2012). Besides the individualism of evolving microorganisms caused by cellular heterogeneity, phenotypic plasticity in turn may alter the selective advantages of appearing mutations.

4.3 Repeatability of evolution

Experiments with identical conditions can lead to diverging phenotypes of replicate cultures (divergence) based on differences in mutational events, even when evolution experiments are initialized with an isogenic population. The most striking example is the observation in the long-term evolution experiment with *E. coli*, where only one out of twelve populations evolved the ability to use citrate under oxic conditions (Blount et al., 2012). Divergence can be explained in several ways. First, mutations can appear in a random order and can be retained in a population depending on the strength of the selective force. Second, minor environmental fluctuations that cannot be avoided even in controlled environments can cause subtle genotypic variations affecting the selection of mutants (Woods et al., 2006; Ostrowski et al., 2008; Kawecki et al., 2012). Third, minor genetic polymorphisms that may not be detectable are subjected to random drift that may lead to loss of mutants from the population (Kawecki et al., 2012). These processes can either constrain or enhance subsequent evolution because mutational effects are often contingent on earlier genetic alterations (epistasis) and mutations may be beneficial only in a specific genetic context (Figure 1.1 in *Chapter 1*; Blount et al., 2008). Consequently, the replicate populations can follow different evolutionary trajectories (Woods et al., 2006; Ostrowski et al., 2008) and different mutations can be selected reaching high frequency within the populations.

In contrast, different genotypes can lead to the same or similar phenotypes in replicate experiments (Tenaillon et al., 2012; Payne and Wagner, 2014). This was the case in the

evolving cultures of *P. denitrificans* where all cultures constituted distinct co-occurring mutations. Here, similar phenotypes were observed in the replicate treatments (physiology, metabolism and transcriptional activities). Similar phenotypic observations among replicate populations are often caused by a large population size, which increases the predictability of the evolutionary process (Lang et al., 2013). Another explanation might be genotype-independent alterations as described in *Section 4.2*.

4.4 Ecological relevance of the present study

Microorganisms are the key players of global conversions of elements. To understand natural cycles of elements, it is important to know microbial responses to nutrient limitations and the impact of adaptation on community composition and function. However, the characteristics of natural environments, defined by a highly complex chemical composition, micro- and macroorganisms and spatiotemporal changes makes this to a challenging task. For example, one cubic centimeter of soil features 10^4 species (Torsvik et al., 2002). A broad range of approaches have been used to predict community compositions, metabolic capacities and possible interactions, such as *in situ* measurements, examination of isolates, metagenomics, –transcriptomics and –proteomics and mathematical and ecosystem models (*Appendix Chapter*). Although these approaches reveal the impact of microorganisms on environmental nutrient cycles, they either provide a snapshot of the environment or consider environmental conditions in a restricted way. Field studies of long time scales enable us to correlate physicochemical properties (temperature, salinity) with the diversity and dynamics of natural microbial communities (Kobayashi et al., 1995; Hashimoto and Niimi, 2001; Gerdts et al., 2004). The oldest ecological long-term experiment, known as 'The Park Grass Experiment' was initiated in 1856 and links ecology of flora and fauna with evolution (Silvertown et al., 2006). The experiment revealed stable dynamics of plant communities subjected to fluctuating nutrient availability and climate perturbation.

However, to reveal the selective forces that define microbial community composition and function, we need to exclude unknown and irrepressible factors that may have a high influence on the microbial performance. Long-term incubations of natural communities under controlled laboratory conditions allow tracking the populations' histories and considerably contribute to our understanding of selective forces, the success of competing populations and the dynamic abundance of species (Kraft et al., 2014). Though, it is complicated to trace whether changes in microbial community composition result from

ecological or evolutionary shifts due to the rapid evolution of microorganisms (O'Brien et al., 2013). Evolutionary variation strongly affects environmental communities that cause ecological shifts and vice versa, an effect termed eco-evolutionary dynamics (Fussmann et al., 2007; Schoener, 2011). The complexity of long-term experiments can be further scaled down to a single or a few microbial species, thus providing detailed information on the relation between environmental conditions, genotypic and phenotypic variation. Fluctuating environments, simulated in controlled laboratory experiments with initialy isogenic *E. coli* populations, promoted divergence of replicate cultures with high variations in fitness, whereas replicate populations showed similar fitness increases when evolved in constant environments (Cooper and Lenski, 2010). The experimental evolution of the model denitrifying bacterium *P. denitrificans* presented in this thesis showed that even in absence of external fluctuations or interaction with other species, microbes can evolve different phenotypes with improved metabolism over long time scales. It was suggested that the formation of subpopulations may have played a role in the adaptive process that enabled *P. denitrificans* to denitrify more efficiently under carbon limitation (*Chapter 3*). The formation of subpopulations in a pure culture of *P. denitrificans* was hypothesized before, however, as response to a harsh switch between oxia and anoxia (Bergaust et al., 2011).

Denitrification is an ecologically relevant process in soil (Schlesinger, 2009) and marine ecosystems (Arrigo, 2005), where it is performed by a broad diversity of bacterial denitrifiers comprising *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Epsilonproteobacteria*, *Firmicutes*, *Bacteroidetes* (Heylen et al., 2006a,b) and eukaryotes as outlined in *Chapter 1*. Consequently, multiple interactions among denitrifiers can be favored. More generally, the competition with nitrogen and carbon consuming organisms generates a complex network that allows many possibilities for adaptation. This thesis complements our understanding of the potential of microorganisms to improve the activity of the denitrification pathway depending on abiotic factors. Obviously, evolutionary effects in nitrogen and carbon metabolism of *P. denitrificans* were controlled by environmental conditions (acetate or nitrate limitation). Based on these observations I support the suggestion to integrate phenotypic responses and biotic and abiotic environmental parameters to understand evolutionary processes in ecosystems (Matthews et al., 2011).

In marine ecosystems nitrate and nitrite availability affects the denitrifier community composition and diversity (Wallenstein et al., 2006). In contrast, in soil ecosystems nitrate availability controls the kinetics of denitrification rather than the denitrifier community composition (Wallenstein et al., 2006). This agrees with the observation that

denitrification has improved under carbon limitation presented in this thesis. However, it is unknown how *P. denitrificans* adapts to different nitrate availabilities in natural environments. To address this question, many different environmental parameters would need to be considered, ideally including chemical, physical and biological constraints (Figure 4.1 in *Chapter 1*; Konopka, 2009). Initial attempts have been performed for an improved characterization of denitrifying microbial species and their responses to relevant environmental traits, most importantly transient oxic–anoxic conditions or variations of pH in soils (Bergaust et al., 2011). O'Brien and colleagues (2013) have summarized studies showing that in natural microbial communities species interact and adapt more rapidly to biotic components than to environmental parameters, particularly in competing populations. Many insights have been gained by coevolution of two antagonistic species in defined laboratory environments (Hibbing et al., 2010; Brockhurst and Koskella, 2013), but only few studies exist showing mutualism, the association between two organisms that confers a benefit to both partners (Little et al., 2008; Hillesland and Stahl, 2010). Biotic-abiotic interactions have been observed in a simple laboratory ecosystem with competing and cross-feeding bacteria and viruses affecting carbon, nitrogen and phosphorous cycling (Lennon and Martiny, 2008). A more complex community of five bacterial species was studied by Lawrence and coworkers (2012). They showed improved resource utilization of these species grown in coculture by feeding on waste products and trade-offs when grown in absence of the coevolved species. These examples underline the advantage of defined conditions in laboratory evolution experiments for studying interspecies interactions that determine microbial community structures and similar coevolution studies should be part of future research on denitrifying communities.

Although it has been proposed that biotic interactions have a higher impact on the shaping of microbial community compositions, horizontal gene transfer (HGT) may enhance abiotic adaptation in complex communities (O'Brien et al., 2013). By this mechanism the acquisition of beneficial traits can be shared among different organisms and new niches can be explored when metabolic functions are transferred (Ochman et al., 2000). During evolution, HGT apparently has affected about 83% of genes in genomes across *Alphaproteobacteria*, as shown by modular network analysis of genes shared among 181 genomes (Dagan et al., 2008). The genome of *P. denitrificans* includes a complete gene cluster that confers the capability of HGT (Lang and Beatty, 2007). The ecological and evolutionary role of HGT by *P. denitrificans* may be studied in coculture with microorganisms capable of performing this mechanism.

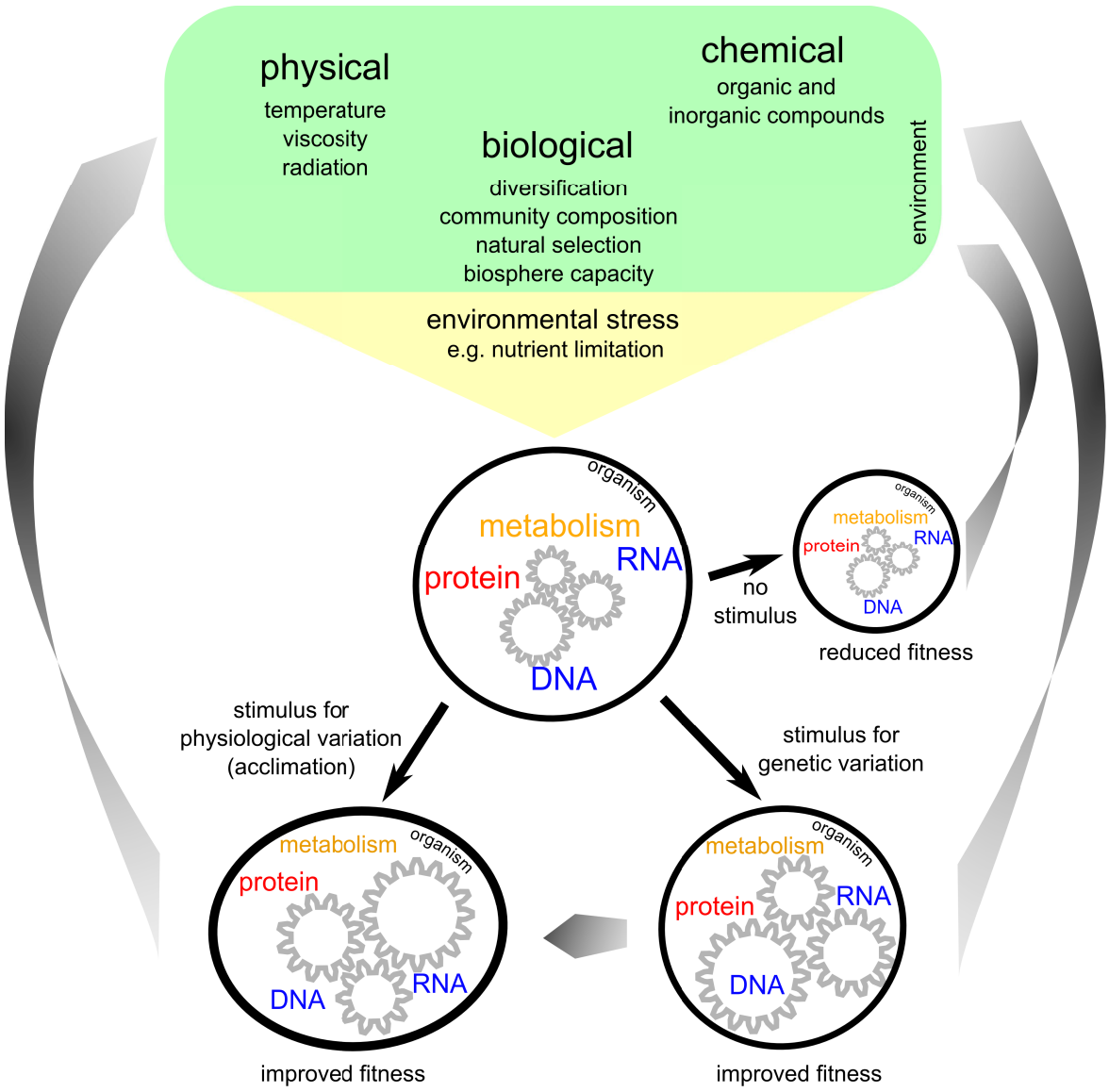

Figure 4.1 Scheme of biotic and abiotic interactions in natural microbial communities subjected to environmental stress. The fitness of a microbial species is affected by environmental components, most importantly biological parameters. Cellular responses include genetic and physiological variation that can either increase or decrease fitness of the organism. In the absence of microbial response the organism receives a disadvantage over organism with improved fitness. These responses provide feedback to the environmental components and may influence community composition. Interactions with biological components comprise interactions with living organisms that often result in cooperating phenotypes. Recent evolution studies have addressed these interactions under controlled laboratory conditions as described in the text.

4.5 Perspectives

Several observations indicated the emergence of at least two subpopulations in acetate limited *P. denitrificans* cultures during evolution. In *Chapter 3* it was speculated that the different rates of nitrate reduction to nitrite and nitrite reduction to nitric oxide may have driven specialization and diversification into subpopulations with improved kinetics of nitrite reduction. Alternatively one subpopulation may have increased the expression of nitrite reductase, while reducing the expression of nitrate reductase. The coexistence of different subpopulations is often linked to cross-feeding, which could in this case be projected to nitrite excretion by one and uptake by another subpopulation. This hypothesis might be supported by the transcriptomes that showed a drastic re-organization of transporters for organic substrates under carbon limitation. Since transcriptomic data have been obtained for the entire population, they do not provide information on variation in transcriptional activities within the population. However, as the carbon source was supplied at constant rates and concentrations, the synthesis of different transporters likely facilitates the uptake of carbon metabolites.

Further indications for the formation of subpopulations were found in the DNA sequence reads. Occasionally a certain position in the ancestral genome was covered by reads that were identical to the reference sequence along with reads that differed. A more detailed analysis of the emergence of genetically different subpopulations is commonly performed by plating and incubating a sample from the culture to obtain isolated clones. The genomes of a statistically representing number of clones is sequenced and analyzed for genetic variations among these clones (Herron and Doebeli, 2013). Alternatively, the information of genes affected by mutations can be used to restrict the sequencing on exclusively these genes. This approach gives insights into the relative abundance of clones carrying this mutation as a higher number of clones can be analyzed.

The present study included the identification of SNPs, insertions and deletions. Possibly additional DNA modifications were present that have not been detected by our pipeline. Future analyses will cover genetic modifications such as large structural variants (e.g., introduced by transposases, resolvases) in the genomes of evolved *P. denitrificans* populations. Valuable insights into the effects of the detected mutations on the growth characteristics of *P. denitrificans* will be gained by the systematic reconstruction of a mutation within a known genetic background, such as the ancestral strain. By this method, epistatic effects (see Figure 1.1 in *Chapter 1*) and the influence of epigenetic patterns on growth characteristics can be excluded (e.g., Cooper et al., 2003; Herring et al., 2006).

Besides the analysis of different genotypes, isolated clones from an evolved microbial population can be studied for differences in their metabolic activities. The simplest way is to investigate isolated clones for nitrate and nitrite reduction rates, for conversion rates of diverse carbon substrates and for their transcriptomic profiles (Le Gac et al., 2012). In case of the presence of subpopulations that are specialized on either substrate we would expect improved growth rates on one substrate and trade-offs in medium with another substrate.

Future studies remain open to understand whether *P. denitrificans* selects for the glyoxylate cycle and the ethylmalonyl-CoA pathway. The long-term cultivation revealed that both pathways were simultaneously used by the populations under both acetate and nitrate limitation. It would be interesting to know whether the cells have specialized in one pathway (by two subpopulations) or whether both pathways were simultaneously used by individual cells. This question could be addressed by growing isolated evolved clones and analyzing ICL and CCR activities, the key enzymes of these two pathways. By comparing the enzyme activities with those measured in the common ancestor under the same growth conditions, this approach could give insights into changes of pathway utilization and differences between potential subpopulations. Independent from the experimentally evolved clones are experiments with knockout mutants of *P. denitrificans* in either *ccr* or *icl*. Growth tests of such mutants could feature different physiological characteristics such as growth rate or yield.

Enzyme activity assays would also be highly recommended to get insight into the assimilation of nitrate with Nas, which in this thesis was hypothesized to be active in acetate limited cultures. Furthermore, enzyme activities of nitrate reductase and nitrite reductase could provide supporting information of the transcriptional profiles of the genes encoding denitrification enzymes.

It has long been known that adaptive evolution can be linked to increasing energy conservation due to loss of functions as long as the ancestral or evolving strains are still far away from their optimum fitness (Dykhuizen, 1978). Based on this observation, we would expect an increase in biomass yield in *P. denitrificans* as response to stress conditions. Several analytical methods to study thermodynamic efficiency in a combination with evolution experiments may be interesting. A very accurate estimate of biomass yield could be achieved by flow cytometry (Trautwein et al., 2012). Changes in biomass yield over time could answer the question whether evolutionary improvements are linked to increasing efficiencies of substrate utilization for the generation of biomass.

Generally, the evolved strains could be investigated for their fitness to find out whether carbon limitation has led to improved growth under this condition compared to the

ancestor. Further, *P. denitrificans* can be grown in various environmental conditions to study whether it has received a benefit or a disadvantage for growth under conditions that differ from the environment the bacterium had evolved in. Finally, the fitness of strains adapted to nitrate limitation will be compared with the fitness of those evolved under carbon limitation by growing them in the same environments (carbon or nitrate limited).

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Appendix Chapter

Continuous cultivation and thermodynamic aspects of niche definition in the nitrogen cycle

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Contributions to the chapter:
S.M. and M.S. performed calculations, wrote and edited this book chapter.

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Abstract

The study of model organisms in pure culture has provided detailed information about the physiology and biochemistry of nitrification and related processes. Metagenomic sequencing of environmental samples is providing information to what extent this understanding also applies to natural microbial communities. Here, we outline a conceptual and experimental strategy that links these two approaches. It consists of the mathematical modeling of nitrification and related processes. The model predictions are subsequently validated experimentally by the study of natural microbial communities in continuous cultures under precisely defined environmental conditions. Combined with calorimetry and metagenomic monitoring this form of "experimental metagenomics" enables the answering of current questions in the ecology of the nitrogen cycle.

Curriculum vitae

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Publications

In press

Hahnke, S. M., Moosmann, P., Erb, T. J. and Strous, M. (2014). An improved medium for the anaerobic growth of *Paracoccus denitrificans* Pd1222. *Front. Microbiol.* **5**, 18.

Müller, S. and Strous, M. (2011). Continuous cultivation and thermodynamic aspects of niche definition in the nitrogen cycle. In *Methods in Enzymology* (ed. M. G. Klotz), volume 486, chapter 2, pp. 33–52. Academic Press.

In preparation

Hahnke, S. M., Tegetmeyer, H. E., Moosmann, P., Erb, T. J. and Strous, M. (2015). Adaptive evolution of *Paracoccus denitrificans* in acetate and nitrate limited anoxic chemostats. *in prep.*

Scientific Meetings and Conferences

Jun 2013 Talk	**Society for the Study of Evolution**, Snowbird, USA. 'Acetate- versus nitrate-limitation: Evolution of *P. denitrificans* Pd1222 in anoxic chemostats'
Mar 2013 Poster	**VAAM Annual Meeting**, Bremen, Germany. '*In vitro* evolution of *P. denitrificans* Pd1222'
Jul 2011 Talk	**European N cycle meeting**, Nijmegen, The Netherlands. 'Approaching microbial competition in the nitrogen network by mathematical modeling'
Jan 2011 Poster	**WE-Heraeus-Seminar on Biothermodxnamics of Metabolic and Ecological Networks**, Bad Honnef, Germany. 'High resolution calorimetry – unveiling the energetics of microbial communities'
Sep 2010 Poster	**European N cycle meeting**, Birmingham, UK. 'A model of competing microbial populations involved in the nitrogen network'

Erklärung der selbstständigen Erarbeitung

Erklärung gemäß §6 Abs. 5 der Promotionsordnung der Universität Bremen für die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche

Hiermit versichere ich, dass ich die vorliegende Dissertation mit dem Titel "Experimental evolution of *Paracoccus denitrificans* in anoxic chemostats"

1. ohne unerlaubte fremde Hilfe angefertigt habe
2. keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe und
3. die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Bremen, 06. August 2014 _____

(Stefanie Müller)

Bremen, 2014