Doctoral Thesis

The consequences of nitrite reactivity on the ecology and evolution of denitrifying microorganisms

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Publication Date:
2015

Permanent Link:
https://doi.org/10.3929/ethz-a-010598764

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The consequences of nitrite reactivity on the ecology and evolution of denitrifying microorganisms

A thesis submitted to attain the degree of
DOCTOR OF SCIENCES of ETH ZURICH

(Dr. sc. ETH Zurich)

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2015
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Chemical reactivity of metabolic intermediates is important in shaping the organization of metabolic processes within microbial cells and communities. Chemical reactivity will influence which processes can take place at the same time by the same cell and which processes must be distributed among different cells. Thus microbial cells have different solutions to separate different metabolic processes in time and space. In Chapters 3-5, I investigate the consequences of chemical reactivity using the bacterium *Pseudomonas stutzeri*, a facultative anaerobe that uses N-oxides as terminal electron acceptors under anaerobic conditions. *P. stutzeri* can reduce nitrate via nitrite, nitric oxide and nitrous oxide into di-nitrogen gas. The intermediate nitrite becomes increasingly reactive as the pH decreases, resulting in severe growth-inhibiting effects. I exploited this feature to experimentally manipulate the reactivity of this single intermediate by lowering the pH of the media. Throughout this dissertation I use pH 7.5 (low reactivity) and pH 6.5 (high reactivity) to investigate the consequences of nitrite reactivity on ecological and evolutionary processes. Furthermore, I use isogenic mutants that cross-feed nitrite in co-culture to measure the consequences of spatially segregating different metabolic processes across different cell-types. My experiments generated three key findings. First, the chemical reactivity of a metabolic intermediate determines whether substrate cross-feeding (and thus dividing metabolic labor) accelerates substrate consumption (Chapter 3). This result emerges because different steps of the denitrification pathway compete with each other for intracellular resources, resulting in the accumulation of the intermediate nitrite. Dividing metabolic labor between two nitrite cross-feeding cell-types eliminates intra-enzyme competition, thus reducing the accumulation of the intermediate and accelerating substrate consumption when the intermediate has growth-inhibiting effects. This reflects a potentially general mechanism for how the increased reactivity of a single metabolite could promote the division of metabolic labor and the evolution of metabolically specialized cell-types. Second, chemical reactivity may accelerate evolution (Chapter 4). I used experimental evolution to determine how increased nitrite reactivity affects the pace of evolution of the completely-consuming cell-type. After 700 generations of experimental evolution, clones from populations evolved at high nitrite reactivity accumulated significantly more mutations than clones from populations evolved at low nitrite reactivity, and also increased more in fitness relative to the ancestor. The increase in mutations after evolution at high nitrite reactivity were most likely due to increased selection pressure and an increased availability of mutations with large beneficial effects rather than a change in the mutation rate in response to stressful conditions. Third, I describe how the evolution of nitrite cross-feeders at high nitrite reactivity leads to different genetic changes than evolution of completely-consuming cell-types (Chapter 5). My results demonstrate that dividing metabolic labor creates novel solutions to adapt to the growth-inhibiting effects of nitrite. Together, my results illustrate how chemical reactivity can affect both ecological and evolutionary processes, and thus have profound impacts on the structure and functioning of microbial populations.
E. Zusammenfassung (Deutsch)


Insgesamt illustrieren meine Ergebnisse, wie die chemische Reaktivität von Metaboliten sowohl ökologische als auch evolutionäre Prozesse beeinflussen kann, und damit einen signifikanten Einfluss auf Struktur und Funktion mikrobieller Populationen ausübt.
1 General Introduction

1.1 The problem of chemical reactivity

A living microbial cell has to perform a variety of metabolic processes to survive and proliferate. The cell also has to adapt in response to the environment, which is not constant and changes in both space and time. Thus it must modify its metabolic processes to deal with a number of changing environmental variables with which it continuously interacts. An important challenge that the cell faces is that different metabolic processes may be incompatible with each other under certain conditions. I use the term incompatible to refer to a situation where two (or more) metabolic processes cannot be performed effectively in the same cellular space or at the same time. An example is the incompatibility between oxygenic photosynthesis and nitrogen fixation (Fay, 1992). This incompatibility emerges because the nitrogenase enzyme, which is needed to fix nitrogen, is sensitive to oxygen, which is an end-product of oxygenic photosynthesis (Burris, 1998; Berman-Frank et al., 2005). Thus, oxygenic photosynthesis produces metabolites that directly inhibit nitrogen fixation. Nitrogen-fixing microorganisms have evolved different solutions to fix nitrogen and perform photosynthesis. This includes spatially segregating the two processes by confining nitrogen fixation into heterocysts (Meeks et al., 2001; Wong and Meeks, 2001) or temporally segregating the two processes by performing photosynthesis during the day and nitrogen fixation during the night (Compaoré and Stal, 2010).

Such metabolic incompatibilities have promoted the evolution of a variety of structural or regulatory solutions to minimize the effects of reactive intermediates and side products. Reactivity typically cannot be eliminated, as reactivity is often essential for the cell’s metabolism or cannot be avoided without acquiring new metabolic pathways. Instead, reactivity must be managed by preventing the accumulation of metabolites. For example, this is the case for the essential amino acid serine (Cosloy and Mcfall, 1970; Raskó and Alföldi, 1971; Uzan and Danchin, 1976). Serine can have negative effects on microbial cells because the transamination of serine leads to formation of hydroxypyruvate which is reactive (Duggleby, 2005). Thus the accumulation of hydroxypyruvate must be prevented, and this is accomplished by serine dehydratase enzyme that diverts serine catabolism towards pyruvate (Grabowski et al., 1993). While mitigating the effects of hydroxypyruvate, however, serine dehydratase leads to the formation of 2-aminoacrylate, which also has toxic effects. The accumulation of 2-aminoacrylate must therefore also be prevented, and this is achieved by another enzyme. This example demonstrates that preventing accumulation (here by the activity of specific enzymes) can be an important method to deal with reactivity.

Other solutions to minimize the accumulation of metabolic intermediates are also possible. These solutions include substrate channeling where one enzyme
delivers the reactive metabolite to the next enzyme (Hardwick et al., 2012); scaffolding, where reactive intermediates are bound to scaffolding enzymes (Dorrestein and Kelleher, 2006; Idan and Hess, 2013); specific transport out of the cell to the external medium (Endo et al., 2007); and detoxification by detoxifying enzymes (Lambrecht et al., 2012, 2013) (Fig. 1.1, A-D). Another strategy to deal with the effects of reactive metabolites is to compartmentalize the responsible metabolic processes using physical barriers, such as confining the metabolic processes into specialized organelles. Acidocalcisomes are organelles with an acidic interior and they potentially function in osmoregulation as well as calcium homeostasis and maintenance of intracellular pH (Docampo and Moreno, 2011). Anammoxosomes are organelles surrounded by specialized lipids and detoxifies NO$_2^-$ and NH$_3$ in anaerobic ammonium oxidizing bacteria (Kartal et al., 2013). Carboxysomes store CO$_2$ and make it available for cellular metabolism (Bonacci et al., 2012). Reactions can also be separated by the cytoplasmic membrane, as is the case for avoiding mixing of D- and L-amino acids (Alvarez et al., 2014; Mattei et al., 2010). This type of separation allows otherwise incompatible processes to occur within the same cell (Fig. 1.1, E and F). In addition, specialized compartments can be used by the cell for disposing of harmful products (Lloyd-Price et al., 2012)

![Figure 1.1](image_url)

**Figure 1.1** A few examples on how cells prevent reactive metabolites from interacting with cellular targets.

A. Substrate channeling; the reactive metabolite is delivered directly to the next enzyme. B. Scaffolding; the reactive intermediate is bound to a scaffolding enzyme, thus preventing its free diffusion. C. Detoxification by detoxifying enzyme. D. Transport of the metabolite out of the cell. E. Confinement of reactive processes into specific organelles. F. Confinement of reactive processes to the periplasmic space. Orange circles represent the reactive metabolite.
1.2 Division of metabolic labor and cross-feeding interactions

One possible consequence of reactive intermediates is the division of metabolic labor and the emergence of metabolically specialized cell-types, where different cell-types specialize at performing different metabolic processes. One specific type of metabolic specialization is cross-feeding (Fig. 1.2). Cross-feeding occurs when one cell-type produces a nutrient that supports the growth of another cell-type. Cross-feeding microorganisms are frequently observed in the natural environment and control numerous ecosystem processes that are of critical importance (e.g., the recycling of fixed carbon and nitrogen). Cross-feeding may occur between different microorganisms (Cord-Ruwisch et al., 1998; Schnurer et al., 1996; Winter and Wolfe, 1979) or between microorganisms and eukaryotic hosts (Wu et al., 2006; Graber and Breznak, 2005). Microorganisms may cross-feed for example cofactors (Seth and Taga, 2014) and amino acids (McCutcheon and Von Dohlen, 2011) in mutually beneficial relationships where two or more cell-types (or species) are dependent on each other for growth. The construction of amino acid cross-feeding strains of the bacterium Escherichia coli in the laboratory has shown that often these cross-feeding cell-types can outcompete isogenic strains that biosynthesize all of their own amino acids. Thus, cross-feeding is indeed sometimes beneficial, even when the cross-fed metabolites are costly to produce (Pande et al., 2014). With regards to the example discussed above with E.coli, overproducing one amino acid thus had a cost that was less than the benefit for receiving another amino acid from another cell-type.

The examples above are mostly of mutually beneficial interactions but cross-feeding can also be commensal (where only one of the cell-types benefits from the interaction), such as in the case of degradation of glucose via acetate to methane (Winter and Wolfe, 1979). These types of commensal cross-feeding interactions are common in biodegradation, such as cellulose degradation (Leschine, 1995). However, cross-feeding within one metabolic pathway (referred to here as substrate cross-feeding) can also be considered more mutualistic if one cell-type performs a step that produces a toxic metabolite that another cell-type then uses as a substrate. The first cell-type thus benefits from the presence of the second cell-type because the second cell-type reduces toxicity from the environment. Indeed, cross-feeding of substrates where intermediates are toxic have been widely observed (De Souza et al., 1998; Drzyzga and Gottschal, 2002; Holmes et al., 2006). Interestingly, there are also metabolic pathways that are sometimes completely performed by one cell-type but are also sometimes partitioned into different substrate-cross-feeding strains. One environmentally important example of this is anaerobic denitrification (Martienssen and Schöps, 1999) where nitrate is reduced into dinitrogen gas in several ATP-yielding steps and the intermediates nitrite or nitrous oxide gas can be cross-fed. However, the environmental and ecological conditions that sometimes select for complete consumption or substrate cross-feeding within this pathway remain unclear.
Figure 1.2 Examples of different types of cross-feeding interactions.

(A) Two cell-types each produce a metabolite that the other cell-type requires to reach its maximal growth. The interaction is therefore mutualistic. (B) One cell-type (red) consumes a non-toxic waste product generated by the metabolism of another cell-type (green). The interaction is therefore commensal, where only one cell-type benefits from the presence of the other. (C) One cell-type (yellow) consumes a toxic waste product generated by the metabolism of another cell-type (purple). The interaction is therefore mutualistic, where both cell-type benefit from the presence of the other.

While substrate cross-feeding is often observed in nature, why it sometimes occurs is often not entirely clear. Why would an organism only partly degrade an energy yielding substrate, if it could gain further energy by consuming the intermediate? Why would an organism instead provide this intermediate to potential competitors? Thus, there must be costs for a cell to completely consume the substrate. These costs have been summarized in the theory of optimal pathway length (Pfeiffer and Bonhoeffer, 2004; Costa et al., 2006). For each additional step of a biochemical process a new enzyme is needed, thus the more steps involved in the metabolic process the more enzymes need to be produced. Considering that a cell has both limited resources (building blocks) to construct enzymes (Scott et al., 2010; Dekel and Alon, 2005) as well as limited cytoplasmic or periplasmic space (Beg et al., 2007) for those enzymes to function, it is clear that adding steps to a metabolic process could be costly and result in trade-offs between different metabolic processes. If additional enzymes are produced, there is less space and resources available for the other enzymes of the pathway. Other than competing for space and building blocks, enzymes may also need other intracellular resources such as cofactors or electron donors, which will also limit the total number of enzymes that can function within a cell at a given condition. Then, it follows that at certain conditions some enzymes in the same pathway may compete for a limiting resource.

In Chapter 3, I hypothesize how substrate cross-feeding might reduce the accumulation of reactive metabolic intermediates. If the first enzyme of a metabolic pathway has preferential access to a limiting resource than the next enzyme of that same pathway, then this would lead to accumulation of the metabolic intermediate. As discussed previously, metabolic intermediates may have inhibitory effects on a cell if they have certain types of reactivities, and these intermediates would have increasingly negative effects with increased accumulation. The proposed competition between enzymes occurs because the enzymes co-exist within the same cell. Thus, the competition and the accumulation of the intermediate should be eliminated (or reduced) if different
metabolic processes are divided into two (or more) cell-types, where one cell-type performs the enzymatic step(s) leading to the production of the reactive intermediate and another cell-type consumes the intermediate. This would then mean that if intra-enzyme competition leads to the accumulation of an inhibitory intermediate, then substrate cross-feeding should reduce accumulation and accelerate substrate consumption. This would then provide a mechanism that explains why substrate cross-feeding sometimes occurs. So far, there is a lack of empirical research explicitly testing how intermediate accumulation and the inhibitory effects of intermediates affect the relative benefit of substrate cross-feeding as compared to complete consumption. To address this, a direct comparison between isogenic cross-feeding cell-types and completely consuming cell-types is necessary, as well as the ability to manipulate the reactivity of the cross-fed intermediate and measure the consequences.

1.3 Chemical reactivity and evolution

In addition to being an important driver of cellular compartmentalization and the division of metabolic labor, chemical reactivity of metabolites may also have strong effects on evolution itself. Indeed, all of the different solutions that have evolved in response to chemical reactivity and toxicity suggest that highly reactive metabolic intermediates should lead to strong selection pressure on the cells to adapt and manage reactivity.

The rate of evolutionary adaptation depends on the mutation rate and the fitness effects of beneficial mutations. If either of those parameters changes the rate of adaptation should change as well. As mutations arise randomly, most mutations will be deleterious and have negative effects on fitness. However, evolutionary adaptation is driven by (rare) beneficial mutations. Several theoretical studies, using fitness landscape models, (Gros et al., 2009; Martin and Lenormand, 2006; Lourenço et al., 2011), as well as empirical studies (MacLean et al., 2010; Elena et al., 1998; Kassen and Bataillon, 2006; MacLean and Buckling, 2009; McDonald et al., 2011; Hietpas et al., 2011; Carrasco et al., 2007; Rozen et al., 2002; Perfeito et al., 2007; Domingo-Calap et al., 2009; Imhof and Schlotterer, 2001; Barrett, MacLean, et al., 2006) have aimed to describe the distribution of fitness effects of novel mutations. Although the predicted distribution of fitness effects differ between studies, it seems that the distribution of fitness effects of beneficial mutations depends highly on the fitness of the ancestral cell-type (Kassen and Bataillon, 2006; MacLean and Buckling, 2009; Bataillon et al., 2011; McDonald et al., 2011). That is, if ancestral fitness is low, the distribution of the fitness effects of beneficial mutations will be different than if ancestral fitness is high (Barrett, M’Gonigle, et al., 2006) (Fig. 1.3). Thus by changing the initial fitness of an ancestral organism (by manipulating its environment or its genotype), this should influence the mutations available for selection to act upon during adaptive evolution. The pace of molecular evolution and adaptation (i.e. the number of mutations fixed per generation) would thus be quicker if this change in fitness lead to more mutations being beneficial or increase the benefit of beneficial mutations.
Figure 1.3 Hypothetical fitness benefits of new (beneficial) mutations.

Consider the blue line for the distribution of fitness effects of beneficial mutations in an environment to which an organism is well-adapted. If the environment changes in a way that decreases the fitness of the organism, this could change the distribution of fitness effects of new (beneficial) mutations in some way as exemplified by the red line.

As mentioned previously, mutation rate is another important factor in determining the rate of evolutionary adaptation. Mutation rate varies between organisms and genotypes but has also been shown to sometimes increase in response to stressful conditions, such as the addition of antibiotics (Do Thi et al., 2011), starvation, oxidative shock (Bjedov et al., 2003), or UV irradiation (Cirz et al., 2007). Different hypotheses have been proposed to explain stress-induced mutagenesis (MacLean et al., 2013). First, increased mutation rate could be under selection at stressful conditions to increase the standing pool of mutations that are available for selection to act upon, and thus accelerate evolvability (Sniegowski et al., 2000; de Visser, 2002; Ram and Hadany, 2012). Second, there could be an intrinsic trade-off between stress resistance and mutation rate where the increase in mutation rate is simply a by-product of mechanisms that improve fitness under stressful conditions (Saint-Ruf et al., 2007; Miller, 1996). Third, increased genetic drift could cause an elevated mutation rate if selection on DNA repair and replication is less efficient under stress (Van Dyken and Wade, 2010). In this context, stress can be considered as any factor that decreases growth, survival or competitiveness, and therefore includes the effects of chemically reactive metabolites.

The rate of molecular evolution and adaptation can thus increase in response to chemical reactivity by either an increase in the availability of mutations with fitness benefits large enough to fix during adaptive evolution or by an increase in
the mutation rate caused by stressful conditions. Importantly, these mechanisms are not mutually exclusive. That is, chemical reactivity could potentially increase the mutation rate and change the availability of beneficial mutations simultaneously. One way to distinguish between these two mechanisms is by quantifying neutral (synonymous) mutations, as an increase in the mutation rate should elevate the number of synonymous mutations while an increased availability of beneficial mutations would not.

1.4 Evolutionary consequences of biotic interactions

Experimental evolution of microorganisms has been used in numerous laboratory experiments where replicates of initially clonal populations have been used to study their adaptation to a novel environment. A seminal example is the long term evolution experiment with *E. coli* (Lenski *et al.*, 1991), where replicate populations are evolving on glucose minimal media. This is an ongoing experiment and has recently reached over 60,000 generations (Maddamsetti *et al.*, 2015). Experimental microbial evolution has been used to investigate a large number of evolutionary hypotheses and concepts. For example: epistatic interactions between mutations (Chou *et al.*, 2011; Khan *et al.*, 2011; Woods *et al.*, 2011), evolutionary trade-offs (Bennett and Lenski, 2007; Lee *et al.*, 2009; Meyer *et al.*, 2010; Hughes *et al.*, 2007), adaptive radiation (McDonald *et al.*, 2009; Rainey *et al.*, 1998; Maharjan *et al.*, 2006) and adaptation to fluctuating environments (Quan *et al.*, 2012).

In the natural environment, however, microbial cells rarely live alone in clonal populations, but rather interact in many different ways with other microorganisms and with other species (Torsvik *et al.*, 2002; Kent and Triplett, 2002; Dykhuizen, 1998; Curtis *et al.*, 2002). Indeed, most natural microbial communities are very diverse and organize into complex networks of interactions (Williams *et al.*, 2014; Freilich *et al.*, 2010; Barberán *et al.*, 2012). However, it is less clear how such biotic interactions shape the evolution and adaptation of microorganisms. Evolution is driven by the interaction of an organism with its environment, which is defined by a number of abiotic and biotic factors. Thus, it is possible that the biotic environment of an organism, such as competitive, antagonistic and mutualistic interactions, can be a more important driver of evolution than the abiotic environment (*e.g.*, nutrient availability, the presence of toxins, acidity or temperature). In addition, adaptation to both biotic and abiotic factors can lead to trade-offs between the two (Lawrence *et al.*, 2012), demonstrating that biotic and abiotic factors interact to create selection pressures.

Indeed, interspecific competitors have been shown to influence the diversification of *Pseudomonas flourescens* (Bailey *et al.*, 2013). In addition, resource competition between bacterial species can shape the evolution of growth rate and survival when the species are evolved in co-culture (Pekkonen *et al.*, 2013), and competition can impede adaptation to the abiotic environment (Collins, 2010). The Red Queen hypothesis (Van Valen, 1973; Brockhurst *et al.*, 2014) states that negative interactions can be a stronger evolutionary force than abiotic factors, and this has been observed for antagonistic interactions between bacteria and phage. Experimental laboratory evolution of *Pseudomonas*
fluorescens with phage $\phi 2$ showed that this type of antagonistic coevolution accelerates the pace of evolution as well as increases genetic divergence between phage populations (Buckling and Rainey, 2002; Brockhurst et al., 2003; Paterson et al., 2010). Moreover, co-evolution with phages causes increased host colony morphology diversity (Vogwill et al., 2011). Here it is clear that changes in, for example, the bacteria that causes phage resistance will drive the co-evolution of changes in the phage to overcome that resistance, and so on leading to an evolutionary arms race (Fig. 1.4).

With regards to biotic interactions that are beneficial, one type of interaction that has been studied quite extensively by genome comparisons and phylogeny is the interactions of bacterial symbionts with insects. Bacterial symbionts tend to have small genomes compared to other related bacteria and have lost functions that are essential for life outside the host, creating obligate interactions (Moran, 2003; Bennett et al., 2014). In addition, older symbionts have smaller genomes than newer symbionts, and newer symbionts experience more rapid mutation and gene loss (Bennett et al., 2014) than the older symbionts indicating that symbiotic interactions may be a strong driver of evolution. Furthermore, the black queen hypothesis argues that adaptive gene loss can occur in response to leaky functions leading to essential leaky functions being performed by a minority of the population (Morris et al., 2012). This was exemplified by the prevalence of ocean bacteria such as Prochlorococcus that depend on other cyanobacteria such as Synechococcus for dealing with oxidative stress (Morris et al., 2012, 2014). Thus, when one cell type is already dependent on another for an essential function this may also drive further genes loss this cell type (Fig. 1.5) In addition, by using an experimentally imposed obligate mutualistic cross-feeding interaction it was shown that experimental evolution of these interacting microorganisms leads to rapid increase in productivity (Hillesland and Stahl, 2010) and to loss of metabolic functions and erosion of functional independence over time (Hillesland et al., 2014). However there are still few experimental investigations directly testing how beneficial biotic interactions may affect evolutionary adaptation as compared to the absence of those interactions.

![Figure 1.4 Antagonistic coevolution.](image)

(A) A bacterium (green) is susceptible to a phage (orange). (B) The bacterium adapts and is no longer susceptible. (C) The phage adapts and can now again infect the bacterium. (D) The bacterium adapts again to become resistant.
Figure 1.5 Coevolution leading to adaptive gene loss.

(A) One cell-type (blue) is dependent on another cell-type (yellow) to detoxify their shared environment. (B) The beneficiary cell-type (blue) develops further dependencies on the other cell-type for additional functions, depicted here as the leaky production of a substrate.

1.5 Summary of knowledge gaps

1) Substrate cross-feeding, where a single metabolic pathway is distributed among two or more cell-types, is prevalent in nature. However, the underlying causes of substrate cross-feeding and the consequences on ecological processes remain unclear.

2) The reactivity, and thus the negative effects, of metabolites produced during microbial metabolism have been important in shaping metabolic networks over evolutionary time. However, there is still a lack of direct experimental tests investigating how chemical reactivity itself affects the pace and trajectory of molecular evolution.

3) Biotic interactions are abundant in most environments and for most species. Antagonistic interactions can accelerate evolution. However, it is less clear how beneficial interactions (such as commensal or mutualistic interactions) will affect evolution.

1.6 Experimental system

The organism that I used for the research described throughout this dissertation is the gram-negative bacterium *Pseudomonas stutzeri* strain A1501. This strain was isolated from rice paddy soils, has a fully sequenced genome (Yan et al., 2008), and is amenable to genetic engineering. For the purpose of my research, an important characteristic of *Pseudomonas stutzeri* is that it is a facultative anaerobe and uses N-oxides as terminal electron acceptors when oxygen is absent (Allen and Grove, 1952). This process is called dissimilatory denitrification and is an ATP yielding process allowing for respiration in the absence of oxygen (Knowles, 1982). The complete denitrification pathway of *P. stutzeri* reduces nitrate (NO$_3^-$) via nitrite (NO$_2^-$), nitric oxide (NO) and nitrous oxide (N$_2$O) into di-nitrogen gas (N$_2$) (Zumft, 1997). Each step is performed by a different metalloenzyme complex (Hochstein and Tomlinson, 1988) and is encoded by a total of more than 50 genes (Vollack et al., 1998). In *P. stutzeri* A1501, the denitrification genes have been identified and are located in four
different gene clusters (**nar**, **nir**, **nor** and **nos**). The **nir** and **nor** genes are next to each other on the chromosome while the **nar** and **nos** are distally located (Yan, 2005). This arrangement of the denitrification gene makes it possible to delete distinct enzymatic steps of the pathway individually. This enabled us to construct mutants that lack a functional nitrate, nitrite or nitrous oxide reductases. We could therefore construct mutants that cannot perform certain steps of the denitrification pathway, thus enabling us to create isogenic cross-feeding mutants that we can grow together in co-cultures.

A key property of denitrification by *Pseudomonads* is the fact that nitrite (NO$_2^-$), which forms as a metabolite during the reduction of nitrate (NO$_3^-$) by nitrate reductase, can be highly reactive depending on the environmental conditions. Nitrite can form nitrous acid (HNO$_2$) (Zhou *et al.*, 2011) and acts as a proton uncoupler (Sijbesma *et al.*, 1996; Almeida, Julio, *et al.*, 1995). Nitrite can also generate nitric oxide radicals (NO) that are toxic to cells (Zumft, 1993). Importantly, the reactivity of nitrite depends highly on the pH, where more acidic conditions lead to the increased formation of both nitrous acid and nitric oxide radicals (Zhou *et al.*, 2011).

Increased nitrite (NO$_2^-$) reactivity thus has inhibitory effects on cell growth. During denitrification, nitrite forms in the cytoplasm by the membrane bound nitrate reductase but is subsequently transported to the periplasm to be further reduced by the nitrite reductase (Lalucat *et al.*, 2006). As the periplasm of gram-negative bacteria tends to equilibrate the pH of the extracellular environment (Wilks and Slonczewski, 2007), we can change the environmental pH, which will in turn change the periplasmic pH, thus allowing us to experimentally manipulate the reactivity of nitrite. Nitrite has been shown to accumulate during batch growth (Almeida, Reis, *et al.*, 1995), most likely due to competition between the nitrate and nitrite reductase for available electron donors.

**Figure 1.6 Experimental system.**

The arrows shows which part of the denitrification that each strain is able to perform.
The main power of this experimental system is that it allows us to assemble communities consisting of a single completely-consuming cell-type or communities consisting of nitrite cross-feeding cell-types. Moreover, we can change the reactivity of nitrite by simply adjusting the pH of the culture media. This gives us the means to study how the performance of nitrite cross-feeding changes depending on the reactivity of the cross-fed intermediate. We can also measure how nitrite reactivity and/or cross-feeding influences evolution. As *P. stutzeri* 1501 is fully sequenced and annotated, we can investigate both genotypic and phenotypic differences after experimental evolution.

1.7 Objectives of the dissertation

This dissertation is organized into six chapters. Chapter 2 discusses the factors that may drive the evolution of metabolically specialized cell-types and the consequences of metabolic specialization on microbial communities. Chapter 3 experimentally and theoretically demonstrates how the inhibitory effects of a single metabolic intermediate (nitrite[NO$_2^-$]) determine whether substrate cross-feeding accelerates substrate consumption. Chapter 4 demonstrates that increasing the inhibitory effects of a single metabolic intermediate (nitrite) accelerates molecular evolution. Chapter 5 investigates how substrate cross-feeding and the inhibitory effects of the cross-fed intermediate (nitrite) affect evolution on a genetic level. Finally, Chapter 6 draws general conclusions and identifies further knowledge gaps for future investigations.
2 Metabolic specialization and the assembly of microbial communities

A modified version of this chapter was published:


2.1 Abstract

Metabolic specialization is a general biological principle that shapes the assembly of microbial communities. Individual cell types rarely metabolize a wide range of substrates within their environment. Instead, different cell types often specialize at metabolizing only subsets of the available substrates. What is the advantage of metabolizing subsets of the available substrates rather than all of them? In this perspective piece, we argue that biochemical conflicts between different metabolic processes can promote metabolic specialization and that a better understanding of these conflicts is therefore important for revealing the general principles and rules that govern the assembly of microbial communities. We first discuss three types of biochemical conflicts that could promote metabolic specialization. Next, we demonstrate how knowledge about the consequences of biochemical conflicts can be used to predict whether different metabolic processes are likely to be performed by the same cell type or by different cell types. We then discuss the major challenges in identifying and assessing biochemical conflicts between different metabolic processes and propose several approaches for their measurement. Finally, we argue that a deeper understanding of the biochemical causes of metabolic specialization could serve as a foundation for the field of synthetic ecology, where the objective would be to rationally engineer the assembly of a microbial community to perform a desired biotransformation.
2.2 Introduction

Metabolic specialization is a general biological principle that applies across every domain of cellular life. Consider a microbial cell residing within the human gut. This cell encounters a myriad of different substrates that could be metabolized to satisfy its energetic and elemental requirements (Rambaud et al., 2006). Yet, even if this cell were near starvation, it would only metabolize a subset of the available substrates (Rambaud et al., 2006). What is the advantage of metabolizing only subsets of the available substrates rather than all of them? What are the underlying causes of metabolic specialization? Can we predict which substrates are likely metabolized by the same cell type and which are likely metabolized by different cell types? Thus far, there are few general principles and rules that address these questions.

The questions posed above have relevance for one of the most perplexing enigmas in microbial ecology: why are some microbial communities so incredibly diverse? Advances in molecular ecology revealed that a single liter of seawater or gram of soil contain thousands of different microbial taxa (Curtis et al., 2002; Gans et al., 2005; Huber et al., 2007). Yet, the mechanisms that promote these levels of diversity are not fully clear (Gudelj et al., 2010). Metabolic specialization provides one plausible explanation for how diversity could be promoted, and is therefore a likely general organizing principle that shapes the assembly of microbial communities.

A substantial body of research has greatly improved our understanding of the causes of metabolic specialization (Elena and Lenski, 2003; Kassen and Rainey, 2004; Gudelj et al., 2010). An important conclusion is that metabolic specialization readily evolves in nearly every conceivable environment and can often be explained by basic ecological and evolutionary principles. This research, however, has largely focused on the ecological and genetic causes of metabolic specialization rather than the biochemical causes (Gudelj et al., 2010). It is clear that biochemical conflicts exist between different metabolic processes and likely promote the evolution of metabolic specialization. Our limited understanding about these biochemical conflicts therefore represents a significant gap in our knowledge.

In this perspective manuscript, we argue that a better understanding of the biochemical conflicts that exist between different metabolic processes could help reveal the general principles and rules that govern the evolution of metabolic specialization. We begin by discussing three types of biochemical conflicts that could promote specialization. We then demonstrate how knowledge about the consequences of biochemical conflicts can be used to predict whether different metabolic processes are performed by the same cell type or by different cell types. We conclude by discussing the major challenges in identifying and assessing biochemical conflicts and propose several approaches for their measurement.
2.3 Biochemical conflicts that could promote metabolic specialization

Conflicts resulting from competition for intracellular resources.
The synthesis and maintenance of metabolic enzymes requires the consumption of intracellular resources, including elemental building blocks (carbon and nitrogen), energy resources (ATP), mRNA synthesis machinery (RNA polymerase, sigma factors), protein synthesis machinery (amino acids, ribosomes, tRNAs, chaperones), and cellular space for housing enzymes. If one of these resources is limiting, then a cell that invests more resources in one metabolic process must invest fewer resources in other processes. Recent studies support the existence of such competitive resource conflicts (Dekel and Alon, 2005; Scott et al., 2010), but the specific intracellular resources that were under competition were not identified. Below we discuss two that might be of relevance.

Cytoplasmic solvent capacity is one intracellular resource that could lead to competitive resource conflicts. The solvent capacity determines the maximum number of enzymes and other macromolecules that can be contained within the cell (Zhou et al., 2008). If the solvent capacity is exceeded then the biochemical and biophysical properties of macromolecules can change, often with detrimental effects (Zhou et al., 2008). Recent combinations of experiments and modeling of E. coli showed that cells operate near their solvent capacity when grown with high substrate supply (Beg et al., 2007). Under these conditions, cells that are provided with mixtures of different substrates produce enzymes for metabolizing only the most productive substrate (Beg et al., 2007), thus potentially leaving the less productive substrates available for uptake by different cell types. A likely explanation for this type of specialization is that, because the solvent capacity is near saturation, cells that produce more enzymes for less productive pathways must produce fewer enzymes for the most productive pathway. Competition for solvent capacity could therefore promote metabolic specialization under specific substrate supply conditions.

RNA polymerase is another intracellular resource that could lead to competitive resource conflicts. RNA polymerase interacts with different sigma factors to regulate the transcription of different genes (Gruber and Gross, 2003). Studies with E. coli showed that the rpoS and rpoD sigma factors compete for limited RNA polymerase (Ferenci, 2005). rpoS regulates stress-response genes while rpoD regulates carbon metabolism genes that determine the metabolic versatility of E. coli. Increasing the expression of rpoS and stress-response genes must therefore coincide with decreasing the expression of rpoD and restricting the metabolic versatility of E. coli, thus resulting in metabolic specialization. Competition for limited RNA polymerase by different sigma factors could therefore promote metabolic specialization under specific stress conditions.

Conflicts resulting from inhibition.
The production of inhibitory intermediates and endproducts could also lead to biochemical conflicts between different metabolic processes (Fay, 1992; Pfeiffer and Bonhoeffer, 2004; Costa et al., 2006; MacLean and Gudelj, 2006). One
example is the antagonistic effect of oxygenic photosynthesis on nitrogen fixation. Oxygen is produced during photosynthesis but inhibits nitrogen fixation by irreversibly inactivating nitrogenase, which makes it challenging for a cell to perform both processes simultaneously (Fay, 1992). A number of strategies have evolved to overcome this conflict, such as differentiation into different cell types where one type photosynthesizes and another type fixes nitrogen (Fay, 1992).

Inhibitory conflicts between metabolic processes could also occur if intracellular intermediates are growth inhibiting (Pfeiffer and Bonhoeffer, 2004; Costa et al., 2006). Theoretical models that consider growth-inhibiting intermediates predict that cross-feeding metabolic specialists are more likely to evolve as the inhibiting effects increase (Pfeiffer and Bonhoeffer, 2004; Costa et al., 2006). The explanation for this is that performing an additional metabolic conversion step of a substrate incurs two costs: the cost of synthesizing proteins for the additional step and the cost of producing additional intracellular intermediates. If these costs exceed the energetic gain from the additional step, then it is beneficial to not perform that step and only partially consume the substrate, thus allowing the intermediates to escape and be metabolized by other cell types. Although quantitative measures of these costs are lacking, these predictions are consistent with many empirical observations. For example, consortia of partially consuming cross-feeding cell types are often responsible for degrading pollutants that produce toxic intermediates (De Souza et al., 1998; Pelz et al., 1999).

**Conflicts for enzyme specificity.**
If the same enzyme interacts with different substrates, such as occurs with some membrane transporters (Saier, 2000), then conflicts could occur for enzyme specificity. In this scenario, improving the specificity for one substrate reduces the specificity for another substrate, thus leading to metabolic specialization. While specificity conflicts are intuitive and provide an explanation for why most enzymes have only a few substrates, there is little experimental support for these conflicts. For example, artificial selection was used to select for improved utilization of alternative substrates for three different enzymes in *E. coli* (Aharoni et al., 2005). After random mutagenesis and selection, improved utilization of the alternative substrates did not result in reduced utilization of the primary substrates, suggesting that specificity conflicts were not important for these enzymes (Aharoni et al., 2005).

### 2.4 The consequences of biochemical conflicts
Even though the biochemical conflicts that exist between different metabolic processes are often unknown, knowledge about their consequences can provide important insights into the evolution of metabolic specialization. The consequence of a biochemical conflict is often antagonistic pleiotropy, which describes an outcome where a single genetic change has beneficial effects on some processes but correlated and detrimental effects on others (Cooper and Lenski, 2000). For example, if competitive resource conflicts exist between two processes, then a genetic change that increases the synthesis of one enzyme must simultaneously decrease the synthesis of other enzymes. These effects can be described graphically using constraint functions (Fig. 2.1). A constraint function is a type of trade-off function that describes how the activity levels of
different metabolic processes are connected and influenced by biochemical conflicts. Examples of constraint functions for two processes are shown in Figure 2.1. In these examples, the activity levels are plotted on independent axes for every possible phenotype. A line is then drawn that connects the maximal activities of all possible phenotypes. This line does not define all phenotypes that could be expressed by a single genotype. Instead, it delimits the set of all possible phenotypes that could evolve after long-term selection in any particular direction, and thus encompasses a wide range of genotypes.

![Figure 2.1 Constraint functions](image)

The shape of a constraint function, which is a line that connects the maximal activities of all possible phenotypes, describes the consequences of biochemical conflicts on the activity levels of two different metabolic processes. A function consisting of vertical and horizontal lines with positive intercepts describes processes that are not in conflict with each other (solid lines). Note that the maximal activities of both processes are independent of each other. Alternatively, a function consisting of vertical and horizontal lines with zero intercepts describes processes that are in complete conflict with each other and cannot occur within the same cell type (short dashed lines). Lines between these two limiting cases describe processes that are in conflict with each other but can still be performed by the same cell type. Concave functions indicate weak conflicts because the sum of activities when performing both processes is always greater than the maximum activity when performing only one (long dashed line). Convex functions indicate strong conflicts because the sum of activities when performing both processes is always less than when performing only one (medium dashed line).

What use are constraint functions for understanding the evolution of metabolic specialization? If the shape of the constraint function is known for a set of metabolic processes, then simple evolutionary models can be used to predict whether specialization is likely to evolve (Doebeli, 2002; Gudelj et al., 2007) (Fig. 2.2). For the models discussed in Figure 2.2, metabolic generalists evolve for concave constraint functions (weak conflicts between processes) while metabolic specialists evolve for convex constraint functions (strong conflicts between processes). For the latter case, the evolution of metabolic specialization occurs in two phases, under the assumption that mutations have small
pleiotropic effects. A generalist cell type first evolves that performs both processes. Selection then turns disruptive and the population splits into two co-existing cell types, where each type specializes at only one of the processes (Doebeli, 2002).

The mathematical models discussed above can thus be used to predict whether different metabolic processes are likely to be performed by the same cell type or by different cell types. These predictions are general in that they are independent of the kinetic parameters of the model (Doebeli, 2002), and are therefore applicable to any pair of processes. In the simple scenario depicted in Fig. 2.2, the outcome of the model corresponds to what one might expect based on simple considerations: two processes will segregate into different cell types if they are in biochemical conflict. However, if more than two processes are involved (Doebeli and Ispolatov, 2010) or if ecological or genetic aspects are considered (Gudelj et al., 2007), then verbal arguments are insufficient, and mathematical modeling offers a rigorous and objective way to predict evolutionary outcomes.
The shape of a constraint function can be used to predict whether metabolic specialization is likely to occur. Consider the concave and convex functions shown in Panel A. We used the modeling approach described by Doebeli (2002) to predict the evolutionary fate of the glucose and ribose metabolic pathways (Panel B). The model predicts that the same cell type performs glucose and ribose metabolism if the function is concave, but that these pathways segregate into different cell types over evolutionary time if the function is convex (Panel B). This result is not unique to parallel pathways and also occurs within a single pathway (Panel C). Using the reduction of tetrachloroethylene (PCE) to ethene as an example, our model predicts that the same cell type performs the complete pathway if the function is concave, but segregates into different cell types over evolutionary time if the function is convex (Panel C). For panels B and C, phenotypes are shown that are present in the population at frequencies greater than 5%.

Figure 2.2 Predictions of metabolic specialization from constraint functions
2.5 Generating hypotheses about biochemical conflicts
We have demonstrated that qualitative information about the consequences of biochemical conflicts (e.g. the shapes of constraint functions) enable specific predictions about whether different metabolic processes are likely to be performed by the same cell type or different cell types (Fig. 2.2). This raises the question about how such constraints can be measured. In the following section, we discuss two main approaches for obtaining such information.

Genome comparisons across organisms
A first approach that generates hypotheses about possible biochemical conflicts is to analyze the distributions of metabolic processes across microbial genomes. The complete sequences of several thousand microbial genomes are currently available and automated gene annotation (Markowitz et al., 2006; Aziz et al., 2008) enables the rapid assessment of co-occurrence patterns of different processes across different genomes. One could thus identify combinations of processes that are more often found in the same cell type or in different cell types. The latter are candidates for processes that are in biochemical conflict.

One main advantage of this approach is that it considers information from a large number of species and strains, of which an increasing number are not experimental model systems and cannot be grown in the laboratory. It thus allows rapid and comprehensive assessment of co-occurrence patterns of a large number of metabolic processes. Although promising, this approach has several major caveats. First, the approach assumes that automated genome annotation is sufficient to predict the complete set of metabolic pathways of microorganisms. Although this is increasingly possible for model microorganisms, this remains challenging for non-model microorganisms that contain unusual and poorly characterized enzymes and pathways. Second, this approach does not consider gene expression. Cells could maintain incompatible pathways by expressing them at different times or in different environments. Finally, this approach does not consider the environment in which microorganisms live. The absence of co-occurrence of two processes might result from the absence of their substrates in the same environment rather than a biochemical conflict. The influence of the latter two confounding factors could be lessened by analyzing groups of microorganisms that live in similar environments. For example, one could analyze metagenome information collected from a single environmental sample, provided that genes within the metagenome can be accurately assigned to different cell types (Eisen, 2011).

Experimental evolution
A second and more direct approach to measure biochemical conflicts is through experimental evolution in the laboratory. If one is interested in measuring the constraint function between two metabolic processes, replicated populations can be selected to maximize the first process in isolation, the second process in isolation, or both processes simultaneously. This would provide a measure of multiple points on the constraint function (Fig. 2.1), which could then be used to estimate its shape.

Experimental evolution has, to the best of our knowledge, not been systematically used to investigate biochemical conflicts. Yet, we believe this
method has potential. First, it offers experimental control. Confounding factors can be excluded by comparing different genotypes that have evolved under well-controlled conditions (Elena and Lenski, 2003; Jessup et al., 2004). Experiments can also be conducted such that the only target of selection is the rate at which a strain grows on one particular substrate or combination of substrates (Ibarra et al., 2002). Second, whole-genome resequencing and metabolic analyses can reveal detailed insights about the genetic changes that occurred over the course of experimental evolution (Herring et al., 2006; Barrick et al., 2009), and can thus help generate hypotheses about the molecular basis of biochemical conflicts. While the timescale of laboratory evolution experiments does not extend to the timescales of evolutionary processes in natural environments, such experiments can nevertheless give insights into the initial phase of metabolic specialization. This initial phase can determine further evolutionary processes (Travisano et al., 1995; Le Gac and Doebeli, 2010; Khan et al., 2011), and studying this phase experimentally might thus provide information about how biochemical conflicts promote metabolic specialization.

2.6 Challenges with measuring biochemical conflicts

Conflicts likely affect many metabolic processes

Our discussion of biochemical conflicts thus far has focused on constraining relationships between two metabolic processes (Figures 2.1 and 2.2). In reality, constraining relationships likely involve more than two processes (Pease and Bull, 1988) (Fig. 2.3). To illustrate this, consider again one of the examples discussed above. We argued that RNA polymerase could be a limiting intracellular resource for which different sigma factors compete, and that this might lead to a conflict between stress response and metabolic versatility (Ferenci, 2005). One obvious resolution to this conflict is to produce more RNA polymerase. However, the production of RNA polymerase, and gene expression in general, is metabolically costly (Dekel and Alon, 2005), and this resolution might therefore have detrimental effects on other processes. A more realistic model would include a large number of metabolic processes and other cellular traits that are connected through complex interactions, both suppressive and facilitative.

One consequence of the involvement of multiple metabolic processes in constraining relationships is that constraints between two metabolic processes are not absolute; simultaneous improvements in both are possible at the cost of others (Fig. 2.3). The combinations of processes that are observed in a particular cell type represent evolutionary compromises across many different selection pressures. If the environment changes and the strength of selection on some processes increases, then one would expect that it is possible to simultaneously improve these at the cost of other processes that are subject to weaker selection.

Another consequence of the involvement of multiple metabolic processes is that complex interactions might generally promote metabolic specialization. A recent theoretical study showed that the probability of diversification increases as constraining relationships involve more traits (Doebeli and Ispolatov, 2010). While complex interactions between different metabolic processes are more
difficult to experimentally measure, they might generally promote the diversification processes depicted in Fig. 2.3

Figure 2.3 Constraints between several metabolic processes.

Constraining relations are likely to involve more than two metabolic processes. For example, consider a situation where three processes are connected in a constraining relationship, but where an experimenter only measures two of them (process 1 and process 2); the experimenter thus only observes the projections of the constraint functions in the horizontal plane (dashed lines). If the activity of process 3 is kept constant during the experiment, the experimenter observes a concave constraint function between process 1 and process 2 (green dashed line). Changes in process 3 during the experiment can lead to the observation of qualitatively different constraint functions (red dashed line). Also, simultaneous improvements of process 1 and process 2 beyond their constraint function are possible at the cost of process 3 (orange).

Dependence on the environmental conditions
A second challenge with measuring constraining relationships is their dependence on environmental conditions. Constraints are ultimately based on resource conflicts and interactions between different metabolic processes; the
environment will affect both of these aspects, and is thus expected to influence constraining relationships both quantitatively and qualitatively. Given the wide range of natural environments, how can we make progress in determining constraining relationships and understanding how they promote metabolic specialization? In our opinion, the main goal is to establish basic principles about how different environmental factors affect biochemical conflicts and influence the main constraining relationships between different processes.

The emerging concept is thus a dynamic view on biochemical conflicts between metabolic processes, on how these conflicts are influenced by environmental factors, and on how they promote the evolution of metabolic specialization and shape the assembly of microbial communities. Each process in a cell is connected to a large number of other processes, and many of these connections will depend on the environmental conditions. Mutations that increase the activity of one process are likely to affect other processes. There might be combinations of mutations that would improve single processes without substantial impairments of other processes, but these genotypes might take a long time to evolve, or they might not be easily accessible by consecutive mutational steps that are all individually beneficial (Weinreich et al., 2006). Such a situation can promote the emergence of mutants that specialize at metabolizing certain substrates. Genetic and ecological interactions might then lead to the consolidation of these differences and the evolution of specialized cell types that consume only subsets of the available substrates within their environment.

2.7 The foundation for synthetic ecology?
What is the broader importance of understanding the biochemical causes of metabolic specialization? A deeper understanding of these causes could serve as a foundation for establishing a field of synthetic ecology (Dunham, 2007). In our view, the objective of synthetic ecology would be to elucidate basic design principles that enable the rational engineering of the assembly of microbial communities to perform desired biotransformations. For example, consider a biological process where a microbial population is used to transform a substrate into an intermediate and then into a value-added end product, such as a pharmaceutical or bioenergy source. One strategy would be to engineer a single cell type that catalyzes the complete pathway (Ro et al., 2006). An alternative strategy would be to engineer a community of specialized cell types, where one type transforms the substrate into the intermediate and another type then transforms the intermediate into the desired end product (Kato et al., 2005). We currently lack basic design principles that predict which strategy maximizes the performance of such a process. A better understanding of the causes of metabolic specialization will likely help elucidate such engineering design principles and contribute towards establishing a discipline of synthetic ecology.
3 Distributing metabolic processes among microbial cells accelerates the consumption of substrates that produce growth-inhibiting intermediates

A modified version of this chapter is in review:

Lilja EE, Johnson DR. Distributing metabolic processes among microbial cells accelerates the consumption of substrates that produce growth-inhibiting intermediates.

3.1 Abstract

Different metabolic processes are often distributed among different microbial cells. A canonical example is substrate cross-feeding, where one cell-type partially consumes a primary substrate into a metabolic intermediate and another cell-type subsequently consumes the intermediate. While substrate cross-feeding is widely observed, its consequence on the rate of substrate consumption is often unclear. Here, we hypothesize that collections of substrate cross-feeding cell-types consume substrates more rapidly than a single completely-consuming cell-type when the following three conditions are met. First, the enzyme that transforms the primary substrate competes with the enzyme that transforms the intermediate for the same finite pool of intracellular resources, and this competition results in the accumulation of the intermediate. Second, intra-enzyme competition is eliminated when the enzymes are segregated into cross-feeding cell-types, and this consequently reduces the accumulation of the intermediate. Third, the intermediate has growth-inhibiting effects. Taken together, we expect substrate cross-feeding to accelerate substrate consumption by preventing the accumulation of the growth-inhibiting intermediate. We experimentally tested this hypothesis using three isogenic mutant strains of the bacterium *Pseudomonas stutzeri*. One strain completely consumes nitrate (NO$_3^-$) to di-nitrogen gas (N$_2$), the second strain consumes nitrate (NO$_3^-$) to nitrite (NO$_2^-$), and the third strain consumes nitrite (NO$_2^-$) to di-nitrogen gas (N$_2$). We grew the first strain alone or the latter two strains together in nitrite cross-feeding co-cultures. We demonstrate that nitrite cross-feeding eliminates intra-enzyme competition and reduces the accumulation of nitrite. We further demonstrate that nitrite cross-feeding accelerates nitrogen oxide consumption, but only when nitrite has growth-inhibiting effects. Knowledge about intra-enzyme competition and the growth-inhibiting effects of intermediates could therefore be useful for deciding how best to distribute different metabolic processes across different cells to optimize a desired biotransformation.
3.2 Introduction
Metabolic specialization is a general principle that shapes the structure and functioning of nearly every microbial community (Costa et al., 2006; Johnson et al., 2012; Zeleznjak et al., 2015). A canonical example is substrate cross-feeding. Substrate cross-feeding occurs when one cell-type partially consumes a primary substrate into a metabolic intermediate and another cell-type then further consumes the intermediate. Substrate cross-feeding controls numerous environmentally and economically important microbial processes, including the mineralization of organic carbon (Schink, 1997; McInerney et al., 2009), the degradation of environmental contaminants (De Souza et al., 1998; Møller et al., 1998; Pelz et al., 1999; Drzyzga and Gottschal, 2002; Holmes et al., 2006), and the recycling of fixed nitrogen into di-nitrogen gas (N₂) (Costa et al., 2006; Martienssen and Schöps, 1999; Van de Pas-Schoonen et al., 2005). While substrate cross-feeding is frequently observed within natural and engineered microbial communities, it is often not clear why it occurs (Costa et al., 2006; Johnson et al., 2012; Doebeli, 2002; Pfeiffer and Bonhoeffer, 2004). Why would a cell partially consume a primary substrate and release an intermediate that could further support its own growth?

We propose a hypothesis about how substrate cross-feeding could accelerate the consumption of substrates, thus providing an explanation for why substrate cross-feeding might occur. Our hypothesis is based on the following main assumption: the enzyme that transforms the primary substrate competes with the enzyme that transforms the intermediate for the same finite pool of intracellular resources. Different enzymes may compete for elemental building blocks required for enzyme biosynthesis (e.g., carbon, nitrogen, or phosphorous building blocks), for energy transfer molecules or co-factors required for enzyme activity (e.g., ATP, NADH), or for cytosolic or periplasmic space for enzymes to occupy (Johnson et al., 2012). If the enzyme that transforms the primary substrate has preferential access to those intracellular resources (e.g. via higher affinity, regulatory mechanisms, etc.), then the rate of transformation of the parent substrate would be greater than that for the intermediate when the parent substrate is available (Fig. 3.1A; early time). The consequence is the accumulation of the intermediate (Fig. 3.1B; note that we use batch culture for illustrative purposes, but intra-enzyme competition would also result in the accumulation of the intermediate in chemostat culture). After the parent substrate is sufficiently depleted, intracellular resources are then increasingly available to the enzyme that transforms the intermediate and the rate of transformation of the intermediate would increase (Fig. 3.1A; late time). Indeed, experiments and mathematical simulations support intra-enzyme competition as an explanation for the accumulation of intermediates within some microbial populations (Thomsen et al., 1994; Almeida, Reis, et al., 1995).

An important and intrinsic property of intra-enzyme competition, then, is that competition is eliminated when the enzymes that transform the parent substrate and the intermediate are segregated into different cells (Fig. 3.1C). The enzyme that transforms the intermediate then has increased access to intracellular resources that would otherwise be diverted to the enzyme that transforms the parent substrate. The consequence is that the rate of transformation of the
intermediate would increase, thus reducing the accumulation of the intermediate (Fig. 3.1D).

The assumption of intra-enzyme competition then leads to the following prediction: substrate cross-feeding should accelerate substrate consumption as the growth-inhibiting effects of the intermediate increase. This is because substrate cross-feeding reduces the accumulation of the intermediate (Fig. 3.1B and D), thus decreasing its growth-inhibiting effects. We note that this prediction does not state that substrate cross-feeding results in faster substrate consumption than complete consumption, as this transition point depends on a variety of environmental and biological factors. Instead, it simply states that substrate cross-feeding improves in performance relative to complete consumption. Empirical evidence to support this prediction, however, remains anecdotal. For example, while substrate cross-feeding is often observed for the consumption of environmental pollutants that produce toxic and growth-inhibiting intermediates (Costa et al., 2006; De Souza et al., 1998; Møller et al., 1998; Pelz et al., 1999; Drzyzga and Gottschal, 2002; Holmes et al., 2006), there is no empirical evidence that substrate cross-feeding itself accelerates the consumption of those pollutants.

Figure 3.1 Hypothesis for how distributing metabolic processes among different cell-types would accelerate substrate consumption.

Consider a metabolic pathway where a primary substrate (S) is transformed by an enzyme (E_S) into an intermediate (I). The intermediate is then further transformed by another enzyme (E_I) into an end-product (P). (A) If E_S and E_I compete for the same pool of an intracellular resource and E_S has preferential access to those resources, then I will accumulate at early times and not be transformed until later times. (B) The consequence is the accumulation of the intermediate. (C) Conversely, if E_S and E_I are segregated into different cell-types, then competition is eliminated and E_I has increased access to those resources. (D) The consequence is the reduced accumulation of the intermediate.
Our objective was to experimentally test this prediction, and thus test a principle that could explain why some metabolic processes are distributed among different microbial cells. To accomplish this, we genetically engineered a novel substrate cross-feeding system based on the denitrification pathway of the gram-negative bacterium *Pseudomonas stutzeri* A1501 (Yan *et al.*, 2008) (Fig. 3.2). In the absence of oxygen, *P. stutzeri* can support its growth by sequentially reducing nitrate (NO$_3^-$) to nitrite (NO$_2^-$), nitric oxide (NO), nitrous oxide (N$_2$O), and finally to di-nitrogen gas (N$_2$), where the nitrogen oxides serve as terminal electron acceptors (Laluca* et al.*, 2006). We constructed three isogenic mutant strains of *P. stutzeri* A1501 that differ in their ability to reduce nitrate and nitrite (note that ammonia is used as the nitrogen source for biosynthesis) (Fig. 3.2 and Table S3.1). One strain completely reduces nitrate to di-nitrogen gas (strain A1601; designated as the completely-consuming strain), a second strain contains a loss-of-function deletion in the *nar* gene cluster that encodes nitrate reductase and partially reduces nitrite to di-nitrogen gas (strain A1602; designated as the nitrite-consuming strain), and a third strain contains a loss-of-function deletion in the *nir* gene cluster that encodes nitrite reductase and partially reduces nitrite to nitrite (strain A1603; designated as the nitrite-producing strain) (Fig. 3.2 and Table S3.1). We then grew the nitrite-producing and -consuming strains together in nitrite cross-feeding co-cultures or the completely-consuming strain alone and measured how rapidly nitrogen oxides were consumed.

![Figure 3.2](https://example.com/figure3.2.png)

**Figure 3.2** Experimental system established for this study.

We constructed three isogenic mutant strains of *P. stutzeri* A1501 that differ in their ability to consume nitrate (NO$_3^-$) and nitrite (NO$_2^-$) as terminal electron acceptors. Strain A1601 completely consumes nitrate to di-nitrogen gas (N$_2$), strain A1602 has a loss-of-function deletion in the *nar* gene cluster and cannot consume nitrate, and strain A1603 has a loss-of-function deletion in the *nir* gene cluster and cannot consume nitrite. Definitions: Nar, nitrate reductase encoded by the *nar* gene cluster; Nir, nitrite reductase encoded by the *nir* gene cluster; Nor, nitric oxide (NO) reductase encoded by the *nor* gene cluster; and Nos, nitrous oxide (N$_2$O) reductase encoded by the *nos* gene cluster. Arrows indicate the metabolic steps that each strain can perform.
There are two critically important features of our experimental system. First, for some denitrifying microorganisms, the nitrate (NO\textsubscript{3}\textsuperscript{-}) and nitrite (NO\textsubscript{2}\textsuperscript{-}) reductases are thought to compete for the same finite pool of intracellular electron donors, but the nitrate reductase has preferential access to this intracellular resource (Thomsen \textit{et al.}, 1994; Almeida, Reis, \textit{et al.}, 1995). Second, we can manipulate the growth-inhibiting effects of the cross-fed metabolic intermediate – nitrite – and measure the consequences on substrate consumption. Nitrite accumulates within the periplasm during denitrification (Laluca\textit{t et al.}, 2006; Klueglein \textit{et al.}, 2014) and can inhibit growth by at least two different pH-dependent mechanisms, both of which act indirectly. First, as the pH decreases, nitrite increasingly protonates to nitrous acid (HNO\textsubscript{2}) and uncouples proton translocation (Sijbesma \textit{et al.}, 1996; Zhou \textit{et al.}, 2011). Second, as the pH decreases, nitrite increasingly and spontaneously generates nitric oxide radicals (NO) that interact with enzymes to form metal-nitrosyl complexes (Zumft, 1993). Nitrite inhibition is typically negligible at pH > 7.5 but severely reduces growth at pH < 6.8 (Sijbesma \textit{et al.}, 1996; Zhou \textit{et al.}, 2011; Almeida, Julio, \textit{et al.}, 1995; Baumann \textit{et al.}, 1997). We exploited this pH-dependence to experimentally manipulate the magnitude of nitrite inhibition by adjusting the pH of the culture medium. This approach is effective because the pH of the culture medium is approximately equal to the pH of the periplasm for gram-negative bacteria (Wilks and Slonczewski, 2007). We then measured how the magnitude of nitrite inhibition affects the speed at which completely-consuming cells and co-cultures of nitrite cross-feeding cells consume nitrogen oxides, where we expect that nitrite cross-feeding increasingly accelerates the consumption of nitrogen oxides as the growth-inhibiting effects of nitrite increase.
3.3 Materials and methods

Bacterial strains, genetic manipulations, and growth conditions
We obtained wild-type *P. stutzeri* A1501 from the Biological Resource Center of Institut Pasteur (www.crbip.pasteur.fr) and used this strain to construct all of the isogenic mutant strains used in this study. *P. stutzeri* A1501 was originally isolated from a rice patty soil and its genome has been fully sequenced and annotated (Yan *et al.*, 2008). The genetic modifications of all mutant strains are summarized in Table S1. In brief, we deleted the *narG* gene to prevent nitrate (NO$_3^-$) reduction and the *nirS* gene to prevent nitrite (NO$_2^-$) reduction. We additionally deleted the *comA* gene from all strains to prevent the internalization of extracellular DNA (Meier *et al.*, 2002), thus reducing the probability that nitrite cross-feeding strains would recombine with each other via natural transformation when grown together in co-cultures. Detailed descriptions of the methods used for gene deletion and phenotype validation are provided in the Supplementary Methods.

We cultivated all *P. stutzeri* strains under aerobic conditions with a completely defined asparagine-citrate synthetic medium (ACS medium) (*Coyle et al.*, 1985) in 1-ml mixed batch reactors. We cultivated all *P. stutzeri* strains under anaerobic conditions with di-nitrogen gas (N$_2$) sparged ACS medium in 25-ml serum bottles fitted with gas-tight stoppers. We provide a detailed description of methods to prepare and inoculate anaerobic ACS medium in the Supplementary Methods.

pH dependence of nitrite inhibition
We streaked the completely-consuming strain (strain A1601) onto a lysogeny broth (LB) agar plate, inoculated one colony into a different test-tube containing 1 ml of aerobic ACS medium buffered to pH 7.5 or 6.5, and incubated the test-tubes for 24 hours at 30°C with continuous shaking (220 rpm). We then transferred the cultures at a dilution of 1:100 (vol:vol) into 16 wells of a 96-well microtitre plate containing fresh ACS medium to achieve a final volume of 200 µl per well. In total, we prepared 4 wells each containing ACS medium buffered to pH 7.5, ACS medium buffered to pH 6.5, ACS medium buffered to pH 7.5 and amended with 10 mM of sodium nitrite (NaNO$_2$), and ACS medium buffered to pH 6.5 and amended with 10 mM of sodium nitrite. After inoculation, we incubated the microtitre plate at 30°C with continuous shaking (220 rpm) and measured the OD$_{600}$ every 10 minutes for 1440 minutes with an Eon plate reader (BioTek Instruments, Luzern, Switzerland). We quantified the maximum growth rate for each well by fitting a zero-order growth model to ten consecutive data points that coincide with the most rapid period of growth.

Time to stationary-phase experiments
We streaked the completely-consuming strain (strain A1601), the nitrite (NO$_2^-$)-consuming strain (strain A1602), and the nitrite-producing strain (strain A1603) onto LB agar plates, inoculated one colony of each strain into a different test-tube containing 1 ml of aerobic ACS medium set to pH 7.5 or 6.5, and incubated the test-tubes for 24 hours at 30°C with continuous shaking (220 rpm). We then
inoculated the aerobic cultures (the completely-consuming strain alone or co-cultures of nitrite cross-feeding strains together at a 50:50 vol:vol mixture) at a dilution of 1:25 into serum bottles containing anaerobic ACS medium containing 10 mM of sodium nitrate (NaNO$_3$) and set to pH 7.5 or 6.5 to achieve a final volume of 20 ml. These cultures then served as precultures for the following experiment. In total, four anaerobic precultures were prepared: the completely-consuming strain at each pH and consortia of nitrite cross-feeding strains together at each pH. Once the anaerobic precultures had reached stationary phase, we used them to inoculate bottles containing fresh anaerobic ACS medium containing 10 mM of sodium nitrate set to pH to 7.5 or 6.5 (1:100 dilution in 20 mL) and measured the time for the cultures to reach stationary-phase. In total, we prepared three serum bottles each for the completely-consuming strain and the co-cultures of nitrite cross-feeding strains at pH 7.5, and six bottles each at pH 6.5. We increased the replication at pH 6.5 because we knew from preliminary experiments that the cultures would occasionally stop growing prematurely at this pH. After inoculation, we incubated the serum bottles at 30°C with continuous shaking (220 rpm) and measured the OD$_{600}$ by transferring aliquots to a 96-well microtitre plate and analyzing the plate with a Synergy Mx plate reader (BioTek). We continued the experiment until there was no further increase in the OD$_{600}$.

**Nitrate and nitrite measurements**

We streaked the completely-consuming strain (strain A1601), the nitrite (NO$_2^-$)-consuming strain (strain A1602), and the nitrite-producing strain (strain A1603) onto LB agar plates, inoculated one colony of each strain into a different test-tube containing 1 ml of aerobic ACS medium and 10 mM of sodium nitrate (NaNO$_3$) set to pH 7.5 and incubated the test-tubes for 24 hours at 30°C with continuous shaking (220 rpm). We then inoculated the aerobic cultures (the completely-consuming strain alone or co-cultures of nitrite cross-feeding strains together at a 50:50 vol:vol mixture) at a dilution of 1:100 into 3 serum bottles each containing anaerobic ACS medium amended with 10 mM of sodium nitrate to achieve a final volume of 20 ml. After 24 hours when the cultures had reached stationary-phase, we amended the cultures with 5 mM of additional sodium nitrate and sampled small volumes (approximately 200 μl) from the cultures every 20 minutes while they were growing at 30°C with continuous shaking (220 rpm). We then removed the cells by centrifugation and stored the supernatants at -20°C until chemical analysis. We provide detailed descriptions of the chemical analysis methods in the Supplementary Methods.

**Mathematical model**

We developed a mathematical model to predict the kinetics of cell growth, nitrate (NO$_3^-$) consumption, and nitrite (NO$_2^-$) production and consumption. The model includes a term that imposes competition between the nitrate and nitrite reductases for reduced electron carriers (see Equation 7 in the Supplementary Methods) (Almeida, Julio, et al., 1995). The model also explicitly accounts for mass transfer between the periplasm and the culture medium and for the pH-dependent inhibitory effects of nitrite. We provide a complete description of the model and its parameters in the Supplementary Methods.
3.4 Results

Competition between the nitrate and nitrite reductases
We first tested for intra-enzyme competition between the nitrate (NO$_3^-$) and nitrite (NO$_2^-$) reductases. To test this, we exploited a well-known feature of denitrifying microorganisms growing in batch culture: nitrate and nitrite are consumed sequentially, resulting in the transient accumulation of the intermediate nitrite (Betlach and Tiedje, 1981; Carlson and Ingraham, 1983). Previous experimental and theoretical studies hypothesized that the nitrate reductase has a stronger affinity for those reduced electron carriers and out-competes the nitrite reductase when nitrate is present, resulting in the preferential consumption of nitrate and the transient accumulation of the intermediate nitrite (Thomsen et al., 1994; Almeida, Reis, et al., 1995). We reasoned that if intra-enzyme competition is biologically significant, then segregating the nitrate and nitrite reductases into different nitrite cross-feeding cells should eliminate intra-enzyme competition. The consequence would be the simultaneous consumption of nitrate and nitrite and the reduced accumulation of the intermediate nitrite (Fig. 3.1 D).

We experimentally tested this by measuring the accumulation of the intermediate nitrite (NO$_2^{-}$) in batch cultures containing completely-consuming cells (strain A1601) or co-cultures of nitrite cross-feeding cells (strains A1602 and A1603). We set the pH of the culture medium to 7.5 because we found that nitrite does not cause growth inhibition under those conditions (see results below), thus allowing us to separate the effects of nitrite inhibition from the effects of competition between the nitrate and nitrite reductases on nitrite accumulation. We first grew completely-consuming cells alone or co-cultures of nitrite cross-feeding cells together with 10 mM of nitrate until they reached stationary phase. This allowed each nitrite cross-feeding strain to achieve a relative frequency that reflects its realized yield from its particular substrates. We then amended the stationary-phase cultures with an additional 5 mM of nitrate and measured the concentrations of nitrate and nitrite over time.

Our results are consistent with intra-enzyme competition between the nitrate (NO$_3^-$) and nitrite (NO$_2^-$) reductases for the same finite pool of intracellular resources. The intermediate nitrite accumulated to relatively high concentrations for the completely-consuming cells (Fig. 3.3A, solid blue line) but accumulated to nearly undetectable concentrations for the co-cultures of nitrite cross-feeding cells (Fig. 3A, solid red line). Overall, the maximum observed nitrite concentration over the time-course of the experiment was 8-fold lower for the co-cultures of nitrite cross-feeding cells than for the completely-consuming cells (two-sample Welch test, two-sided P < 0.05, n$_1$ = n$_2$ = 3) (Fig. 3.3A). Thus, when the nitrate and nitrite reductases were present together within completely-consuming cells, nitrate and nitrite were consumed largely sequentially, which would be expected if the nitrate reductase had preferential access to reduced electron carriers (Thomsen et al., 1994; Almeida, Reis, et al., 1995). However, when the nitrate and nitrite reductases were segregated into different nitrite cross-feeding cells and grown together, nitrate and nitrite were consumed simultaneously, which would be expected if competition between the
two reductases were eliminated. Our results are therefore consistent with intra-
zyme competition between the nitrate and nitrite reductases and preferential
access by the nitrate reductase for intracellular resources, thus providing
experimental support for the main assumption of our hypothesis.

We complemented our experimental results by simulating the dynamics of
nitrite (NO$_2$) accumulation for batch cultures. The mathematical model includes
a term that results in intra-enzyme competition between the nitrate and nitrite
reductases when they are present together within completely-consuming cells,
where the rate of nitrate (NO$_3$) consumption directly represses the rate of nitrite
consumption (see Eq. 7 in the Supplementary Methods) (Almeida, Reis, et al.,
1995). The mathematical model revealed an important insight: when the nitrate
and nitrite reductases are present together within completely-consuming cells,
the competition effect is large immediately after nitrate is added to the batch
cultures. Nitrate consumption consequently represses nitrite consumption and
the intermediate nitrite accumulates to high concentrations (Fig. 3.3B, solid blue
line). However, when the nitrate and nitrite reductases are segregated into
different nitrite cross-feeding cells and grown together, the competition effect is
zero. Nitrate consumption does not reduce nitrite consumption and the
intermediate nitrite accumulates to low concentrations (Fig. 3.3B, solid red line).
These simulations are in remarkable qualitative agreement with our
experimental results, where co-cultures of nitrite cross-feeding cells accumulated substantially less nitrite than completely-consuming cells (Fig. 3.3A
and 3.3B). Thus, the inclusion of competition between the nitrate and nitrite
reductases was sufficient to simulate the main qualitative feature of our
experimental observations. We note here that we did not fit our data to the
model but instead used literature reported kinetic parameters for our
simulations (Almeida, Reis, et al., 1995; Almeida, Julio, et al., 1995; Bryan et al.,
1985), thus demonstrating the generality of the outcome. Indeed, the qualitative
outcome (i.e., that co-cultures of nitrite cross-feeding cells accumulate less nitrite
than completely-consuming cells) is valid across a biologically relevant
parameter space.

There are alternative explanations other than intra-enzyme competition for the
transient accumulation of the intermediate nitrite (NO$_2$) by completely-
consuming cells. One often-invoked explanation is that the rate of nitrate (NO$_3$)
consumption is simply greater than the rate of nitrite consumption, which would
also result in the transient accumulation of nitrite without requiring intra-
enzyme competition (Betlach and Tiedje, 1981). To test the validity of this
explanation, we again simulated the dynamics of nitrite accumulation in batch
culture, but we removed competition between the nitrate and nitrite reductases
and set the maximum rate of nitrate consumption to twice that of nitrite
consumption. The model generated a qualitatively different outcome. When the
nitrate and nitrite reductases are present together within completely-consuming
cells, nitrate is consumed more rapidly than nitrite and the intermediate nitrite
accumulates to moderately high concentrations (Fig. 3.3C, solid blue line). However, when the nitrate and nitrite reductases are segregated into different
nitrite cross-feeding cells and grown together, the intermediate nitrite
accumulates to even higher concentrations (Fig. 3.3C, solid red line), which is
opposite of our experimental observations (Fig. 3.3A). The qualitatively opposite outcome is a consequence of the slower growth of the nitrite-consuming cells in the model simulations. Because of the lower rate of nitrite consumption, the nitrite-consuming cells are present at relatively low cell numbers after all the nitrate is consumed, thus increasing nitrite accumulation. In contrast, the completely-consuming cells are present at higher cell numbers after all the nitrate is consumed, thus decreasing nitrite accumulation. Thus, the inability of this alternative explanation to simulate the main qualitative feature of our experimentally observed dynamics provides further support that the nitrate and nitrite reductases do indeed compete with each other for the same finite pool of intracellular resources.

Another alternative explanation for the accumulation of nitrite (NO$_2^-$) by completely consuming cells is that nitrate (NO$_3^-$) represses the transcriptional or translational expression of nitrite reductase. A functional nitrite reductase might therefore not be immediately present after cultures are amended with nitrate, thus creating a lag before nitrite consumption begins. To address this possibility, we performed an additional experiment where we allowed completely-consuming cells to consume 2 mM of nitrate and enter stationary phase. We then amended the cultures with 200 uM of nitrate or nitrite. The stationary-phase cultures immediately consumed both nitrate and nitrite with no observable lag (Fig. S3.3). Thus, a functional nitrite reductase was indeed present, and the transcriptional or translational repression of nitrite reductase cannot therefore explain the transient accumulation of nitrite by completely-consuming cells.

Figure 3.3 Nitrate and nitrite consumption.

Nitrate (NO$_3^-$) and nitrite (NO$_2^-$) consumption dynamics for completely-consuming cells (strain A1601) or co-cultures of nitrite cross-feeding cells (strains A1602 and A1603). (A) We experimentally measured nitrate and nitrite concentrations over time after adding 5 mM of nitrate to stationary-phase cultures. Error bars are one standard deviation (n = 3). We additionally simulated the dynamics of nitrate and nitrite concentrations when (B) imposing competition between the nitrate and nitrite reductases, or (C) when setting the maximum rate of nitrate consumption to twice that of nitrite consumption. Definitions: CC, completely-consuming cells (strain A1601); CF, co-cultures of nitrite cross-feeding cells (strains A1602 and A1603).
**pH-dependence of nitrite inhibition**

We next tested whether the accumulation of the intermediate nitrite (NO$_2^-$) imposes growth-inhibiting effects under our experimental conditions, which is required to test our main prediction. To accomplish this, we exploited the pH-dependent inhibitory effects of nitrite. We set the pH of the culture medium to 7.5 or 6.5 and grew completely-consuming cells (strain A1601) alone in the presence or absence of 10 mM of exogenous nitrite. We incubated the cells under fully aerobic conditions because oxygen represses nitrite reductase activity (Vollack et al., 1999), thus preventing nitrite reduction – and consequently detoxification – during the experiment. We measured cell density over time (measured as OD$_{600}$) (Fig. 3.4A and B) and quantified the maximum specific growth rate ($u_{\text{max}}$ [min$^{-1}$]) for each culture (Fig. 3.4C).

We observed three important outcomes. First, decreasing the pH of the culture medium from 7.5 to 6.5 in the absence of nitrite (NO$_2^-$) had no significant effect on the maximum specific growth rate of completely-consuming cells (two-sample Welch test, two-sided $P > 0.05$, $n_1 = n_2 = 4$) (Fig. 3.4C). We independently repeated this experiment with additional replication and observed a qualitatively identical outcome, namely that reducing the pH from 7.5 to 6.5 in the absence of nitrite did not cause observable growth inhibition (two-sample Welch test, one-sided $P > 0.8$, $n_1 = n_2 = 8$) (see the Supplementary Results and Fig. S3.1). Second, the presence of nitrite at pH 7.5 also had no observable effect on the maximum specific growth rate of completely-consuming cells (two-sample Welch test, two-sided $P > 0.05$, $n_1 = n_2 = 4$) (Fig. 3.4A and C). Nitrite therefore did not cause observable growth inhibition at pH 7.5. Third, the presence of nitrite at pH 6.5 completely eliminated the growth of completely-consuming cells (two-sample Welch test, two-sided $P < 10^{-6}$, $n_1 = n_2 = 4$) (Fig. 3.4B and C). Nitrite therefore imposed severe growth inhibition at pH 6.5. We observed a qualitatively consistent result when we performed similar experiments in the absence of oxygen (see the Supplementary Results). Together, our results demonstrate that i) nitrite does indeed impose pH-dependent growth inhibition under our experimental conditions, and ii) that the pH of the culture medium can be used to manipulate the magnitude of nitrite inhibition without generating substantial confounding factors caused by differences in the pH itself.
Figure 3.4 pH dependence of nitrite (NO₂⁻) inhibition.

We grew completely-consuming cells (strain A1601) (A) at pH 7.5 with or without 10 mM of nitrite, or (B) at pH 6.5 with or without 10 mM of nitrite under aerobic conditions and measured the OD₆₀₀ values over time. We only plotted every fourth data point to facilitate visualization. (C) We measured the zero-order maximum specific growth rate (μ_max) from ten consecutive data points that coincide with the most rapid period of growth for each individual culture. The data are presented as Tukey box plots. The P-values are for pair-wise comparisons using the two-sample Welch test (n = 4).

The magnitude of nitrite inhibition determines whether nitrite cross-feeding accelerates substrate consumption

We next experimentally tested the main prediction of our hypothesis: nitrite (NO₂⁻) cross-feeding increasingly accelerates substrate consumption as the growth-inhibiting effects of nitrite increase. To accomplish this, we set the pH of the culture medium to 7.5 or 6.5 and grew completely-consuming cells alone (strain A1601) or co-cultures of nitrite cross-feeding cells together (strains A1602 and A1603) with 10 mM of nitrate (NO₃⁻). We measured cell density over time (OD₆₀₀) (Fig. S3.2) and quantified the time required for completely-consuming cells (t_stat,CC) and co-cultures of nitrite cross-feeding cells (t_stat,CF) to reach stationary phase. We then calculated the ratio t_stat,CF/t_stat,CC where a value > 1 indicates that completely-consuming cells consumed all of the nitrogen oxides more rapidly and a value < 1 indicates that co-cultures of nitrite cross-feeding cells consumed all of the nitrogen oxides more rapidly.

We observed two important outcomes. First, at pH 7.5 when nitrite (NO₂⁻) does not inhibit growth (Fig. 3.4C), the value of t_stat,CF/t_stat,CC was statistically indistinguishable from a value of 1 (one-sample Welch test, two-sided P > 0.4, n = 3) (Fig. 3.5). Thus, when nitrite had no observable negative effects on growth, completely-consuming cells and co-cultures of nitrite cross-feeding cells consumed all of the nitrogen oxides equally rapidly. Second, at pH 6.5 when nitrite severely inhibits growth (Fig. 3.4C), the value of t_stat,CF/t_stat,CC was smaller than 1 (one-sample Welch test, two-sided P < 0.002, n = 3) (Fig. 3.5) and smaller than that at pH 7.5 (two-sample Welch test, two-sided P < 0.001, n₁ = n₂ = 3) (Fig. 3.5). Thus, when nitrite had severe growth-inhibiting effects, co-cultures of nitrite cross-feeding cells consumed nitrogen oxides more rapidly than completely-consuming cells. Our data therefore provide evidence that nitrite...
cross-feeding does indeed accelerate substrate consumption as the growth-inhibiting effects of nitrite increase.

We measured the time for completely-consuming cells $t_{\text{stat,CC}}$ or co-cultures of nitrite cross-feeding cells $t_{\text{stat,CF}}$ to reach stationary phase. We reported the ratio $t_{\text{stat,CF}}/t_{\text{stat,CC}}$, where a value $> 1$ indicates that the completely-consuming cells (strain A1601) consumed all of the nitrogen oxides more rapidly and a value $< 1$ indicates that the co-cultures of nitrite cross-feeding cells (strains A1602 and A1603) consumed all of the nitrogen oxides more rapidly. The data are presented as Tukey box plots. The $P$-value is for a pair-wise comparison using the two-sample two-sided Welch test ($n = 3$).

We finally asked whether our mathematical modeling approach could simulate the main qualitative feature of our experiment without fitting the model to our data. More specifically, we asked whether a magnitude of nitrite ($\text{NO}_2^-$) inhibition exists when nitrite cross-feeding accelerates substrate consumption relative to complete consumption. The model revealed a second important insight. If mass transfer of nitrite between the periplasm and the culture medium is much faster than the maximum reaction rate of the nitrate and nitrite reductases, then the concentrations of nitrite within the periplasm and the culture medium are necessarily identical for all cells, regardless of whether a cell produces or consumes nitrite. Under these conditions, we found that completely-consuming cells always consume all of the nitrogen oxides more rapidly than co-cultures of nitrite cross-feeding cells, regardless of the magnitude of nitrite inhibition (i.e., $t_{\text{stat,CF}}/t_{\text{stat,CC}}$ is always greater than 1 and increases monotonically as nitrite...
inhibition increases) (Fig. 3.6; solid line). However, if mass transfer of nitrite between the periplasm and the culture medium is slower than the maximum reaction rate of the nitrate and nitrite reductases, which is supported by experimental evidence (19), then the concentrations of nitrite within the periplasm of the nitrite-consuming cells are necessarily less than in the culture medium (i.e., nitrite is consumed faster than it enters the cell). This reduces nitrite inhibition of nitrite-consuming cells and consequently accelerates nitrite consumption, thus reducing the accumulation of nitrite. Under these conditions, we found a threshold of nitrite inhibition where co-cultures of nitrite cross-feeding cells consume all of the nitrogen oxides more rapidly than completely consuming cells (i.e., a region where $t_{\text{stat,CF}}/t_{\text{stat,CC}}$ is less than 1) (Fig. 3.6). More generally, we found that the value of $t_{\text{stat,CF}}/t_{\text{stat,CC}}$ monotonically decreases as nitrite inhibition increases (i.e., nitrite cross-feeding increasingly accelerates the consumption of nitrogen oxides as nitrite inhibition increases) (Fig. 3.6), which is consistent with our main prediction and our experimental observations (Fig. 3.5). Thus, the lower concentration of nitrite within the periplasm of nitrite-consuming cells is essential to simulate the main qualitative feature of our results.

![Figure 3.6](image)

Figure 3.6 Simulations of the effect of nitrite ($\text{NO}_2^-$) inhibition on substrate consumption for completely consuming cells or co-cultures of nitrite cross-feeding cells.

We calculated the time for completely-consuming cells ($t_{\text{stat,CC}}$) or co-cultures of nitrite cross-feeding cells ($t_{\text{stat,CF}}$) to reach stationary phase. We reported the ratio $t_{\text{stat,CF}}/t_{\text{stat,CC}}$, where a value > 1 indicates that the completely-consuming cells consumed all of the nitrogen oxides more rapidly and a value < 1 indicates that the co-cultures of nitrite cross-feeding cells consumed all of the nitrogen oxides more rapidly. The solid line is for simulations where mass transfer across the periplasm was set to infinity. The dashed line is for simulations where mass transfer across the periplasm was set to one-half of the maximum rate of nitrite consumption. $K^{-1}_I$ is the inverse of the Andrews inhibition coefficient for nitrite.
3.5 Discussion

Our experiments provide evidence for a potentially general principle: The negative effects of accumulating metabolic intermediates determine whether distributing different metabolic processes among different cell-types promotes more rapid substrate consumption. For our experimental system, distributing metabolic processes eliminates intra-enzyme competition and reduces the accumulation of intermediates, thus accelerating the consumption of substrates that produce growth-inhibiting intermediates. While this principle is supported by anecdotal evidence (De Souza et al., 1998; Møller et al., 1998; Pelz et al., 1999; Drzyzga and Gottschal, 2002; Holmes et al., 2006), direct experimental evidence has so far been lacking, thus reflecting an important gap in our knowledge about the causes of metabolic specialization and the consequences on microbial processes.

Our results could be of relevance for a broad range of engineered microbial processes. Consider that the objective of nearly every engineered microbial process is to convert a substrate via one or more metabolic intermediates into a desired end-product. For example, one might want to convert a low-cost organic chemical into a pharmaceutical or convert a pollutant into an innocuous end-product. One strategy to achieve a particular engineering objective would be to engineer a single cell-type that completely consumes the substrate into the desired end-product. An alternative strategy would be to engineer and assemble together different cell-types, where each cell-type specializes at performing a different step of the pathway (i.e. a microbial [dis]assembly line). We currently lack basic engineering design principles that predict when such a microbial (dis)assembly line is likely to be advantageous, or how to best distribute different metabolic processes across different cell-types to achieve a particular objective. Based on our results, knowledge about intra-enzyme competition and the inhibitory effects of metabolic intermediates could contribute towards enabling such predictions.

Our results also illustrate a mechanism that could help address a long-standing enigma in microbial ecology: How is biodiversity promoted and maintained within microbial communities? Consider that many natural and engineered environments contain many thousands of different microbial strains (Curtis et al., 2002). What prevents a few strains from increasing in frequency and displacing the others? Our results suggest that the production of inhibitory metabolic intermediates is one factor that could promote and maintain biodiversity. Under certain conditions, competition between different metabolic processes and the production of intermediates cause different metabolic processes to segregate into different strains over evolutionary time (Johnson et al., 2012; Doebeli, 2002). The consequence is the evolution of a collection of metabolically specialized strains, each of which consumes only a subset of the available resources within a particular environment and co-exists with the others (Johnson et al., 2012; Doebeli, 2002). Given the enormous numbers of substrates and intermediates that are likely to exist in many natural environments, we argue that this mechanism could be an important factor that contributes towards the promotion and maintenance of microbial diversity.
Our study has several important advantages and perceived limitations. In our view, the main advantage and novel aspect is that we could manipulate the inhibitory effects of a single metabolic intermediate and measure the consequences on substrate consumption. A second important advantage is that all three of the investigated strains are isogenic mutants. We were therefore able to exclude confounding factors that might be caused by genomic differences at loci other than the nar and nir gene clusters. One perceived limitation is that all of our experiments and simulations were conducted in batch culture. The main qualitative prediction of our hypothesis, however, is the same for both batch and chemostat culture. Intra-enzyme competition and preferential access by the enzyme that transforms the primary substrate will result in the accumulation of intermediates, regardless of reactor configuration. Moreover, eliminating intra-enzyme competition via substrate cross-feeding will reduce the accumulation of intermediates, again regardless of reactor configuration. Nevertheless, chemostat operation would result in lower concentrations of intermediates because a fraction of the intermediates would be removed via dilution. We therefore expect a smaller parameter space where substrate cross-feeding results in faster substrate consumption than complete consumption. However, the general outcome, that substrate cross-feeding increasingly accelerates substrate consumption as the negative effects of intermediates increase, should remain valid.

We emphasize that our results do not address the evolution of substrate cross-feeding itself, which has been the focus of previous studies (Costa et al., 2006; Van de Pas-Schoonen et al., 2005; Doebeli, 2002; Turner et al., 1996; Treves et al., 1998). While we identified conditions where substrate cross-feeding accelerates substrate consumption, this is insufficient to predict whether substrate cross-feeding itself would evolve via natural selection from complete consumption under the same conditions. Other evolutionary outcomes are plausible (Pfeiffer and Bonhoeffer, 2004). For our specific experimental system, it is plausible that substrate cross-feeding cells may never displace completely-consuming cells, or that intermediate-consuming cells may co-exist with completely-consuming cells. The evolutionary outcome ultimately depends on the magnitude of intra-enzyme competition, the growth-inhibiting effects of intermediates, and the availability of regulatory solutions to minimize the accumulation of intermediates. In batch culture where the diffusion of intermediates away from intermediate-producing cells is rapid, the negative effects of accumulating intermediates might be small. The evolution of substrate cross-feeding might therefore require long periods of time or, as occurred in this study, require relatively high concentrations of growth-inhibiting intermediates (we note here, however, that the nitrite concentrations observed in our study are not atypical for some ecosystems (Fernández-Nava et al., 2008)). In contrast, in a spatially structured environment where the transport of intermediates away from cells is slowed, the negative effects of intermediates might be much larger. This is because the intermediates would be retained close to the intermediate-producing cells, thus increasing their inhibitory effects and promoting the rapid evolution of substrate cross-feeding.
Finally, our results have potentially immediate impacts on our understanding of an ecosystem service that is critical for maintaining the quality of water supplies: the consumption of nitrogen oxides. Both natural and engineered microbial communities are known to sometimes accumulate the intermediate nitrite (NO\textsubscript{2}\textsuperscript{-}), with potentially deleterious effects on environmental quality. The underlying causes of nitrite accumulation, however, remain debated (Almeida, Julio, et al., 1995; Betlach and Tiedje, 1981). Our data indicate that, for \textit{P. stutzeri}, competition between the nitrate (NO\textsubscript{3}\textsuperscript{-}) and nitrite reductases is one factor that causes nitrite accumulation, and that segregating the nitrate and nitrite reductases into different cell-types prevents nitrite accumulation. A further analysis of the cellular compartmentalization of the nitrate and nitrite reductases could therefore help elucidate the underlying causes of nitrite accumulation in natural and engineered environments.
3.6 Supplementary material

Supplementary methods

Preparation and inoculation of anaerobic ACS medium
To prepare di-nitrogen gas (N\textsubscript{2})-sparged ACS medium, we boiled the medium for 10 minutes and then cooled it to room temperature while under a constant stream of di-nitrogen gas. After reaching room temperature, we decanted 20 ml of the medium into 25-ml serum bottles and sealed the serum bottles with gas-tight stoppers, leaving a headspace of approximately 5 ml of di-nitrogen gas. We inoculated the di-nitrogen gas-sparged ACS medium with overnight cultures of \textit{P. stutzeri} strains using aseptic syringes. We used a pH electrode to verify that the pH of the culture medium remained unchanged over the time-course of the experiments.

Chemical analysis of nitrate and nitrite concentrations
We thawed and diluted cell-free liquid samples into deionized water at a dilution of 1:100 (vol:vol). We constructed standard curves for nitrate (NO\textsubscript{3}\textsuperscript{-}) and nitrite (NO\textsubscript{2}\textsuperscript{-}) by dissolving the required mass into aerobic ACS medium (0, 2, 4, 6, 8 and 10 mM for each) and then diluting the standards into deionized water at a dilution of 1:100 (vol:vol). The standards and samples for nitrate and nitrite were analyzed using the Metrohm 930 Compact IC Flex system and the Metrohm 887 Professional UV/Vis Detector. The Metrohm 889 IC Sample Center, the Metrohm Metrosep A Supp 16; 5x4 mm guard column, the Metrohm Metrosep A Supp 16; 250x4 mm analytical column with a column temperature of 45°C, an eluent consisting of 7.5 mM Na\textsubscript{2}CO\textsubscript{3}/0.75 mM NaOH and a flow rate of 0.8 ml/min was used.

Biosensor experiments to measure nitrate and nitrite directly during denitrification
We used NO\textsubscript{x}\textsuperscript{-} and NO\textsubscript{2}\textsuperscript{-} biosensors from Unisense (Aarhus, Denmark) to measure nitrate and nitrite concentrations during denitrification. The biosensors consists of an electrochemical N\textsubscript{2}O transducer and a biochamber which contains bacteria that reduce both NO\textsubscript{3}\textsuperscript{-} and NO\textsubscript{2}\textsuperscript{-} (NO\textsubscript{x}\textsuperscript{-} sensor) or only NO\textsubscript{2}\textsuperscript{-} (NO\textsubscript{2}\textsuperscript{-} sensor) to N\textsubscript{2}O, which is then detected by this electrochemical transducers. Thus, by using both sensors, real-time concentrations of nitrate and nitrite are measured. We picked a single colony of the completely-consuming strain (strain A1601), let it grow overnight in aerobic ACS media, and then inoculated 1 ml of this aerobic culture into a 2L batch of aerobic ACS medium amended with 2 mM sodium nitrate (NaNO\textsubscript{2}). The cultures contained no headspace, thus preventing oxygen from entering the medium. Once the culture had reached stationary-phase, we amended the cultures with 200 μM nitrate or nitrite sequentially. The biosensors were first calibrated by measuring nitrate and nitrite additions to a salt solution with the same pH and temperature as the bacterial culture. The biosensors were attached to a microsensor multimeter that was connected to a PC with the Unisense software and measurements were logged every 0.5 seconds.
Gene deletion methods

We deleted the narG, nirS, and comA genes from P. stutzeri 1501 using derivatives of the sacB-containing conditionally replicative pAW19 plasmid (Metcalf et al., 1995; White and Metcalf, 2004). The derivative plasmids contain deletions in the narG, nirS, or comA genes and are designated pAW19-ΔnarG, pAW19-ΔnirS, and pAW19-ΔcomA, respectively. To construct these derivative plasmids, we first introduced pAW19 into E. coli DH5α/λpir (Miller and Mekalanos, 1988) via electroporation and purified pAW19 from an overnight culture. We next amplified 1 kbp regions immediately upstream and downstream of the translational start and stop sites of the narG, nirS, and comA genes using Taq DNA polymerase and the amplification primers listed in Table S3.1. These amplification primers contain the SpeI, NotI, and SacI sites that we used to clone the amplification products into pAW19. We then digested the PCR amplification products and pAW19 with the appropriate restriction enzymes and sequentially ligated the upstream and downstream amplification products into pAW19. Finally, we introduced each of the assembled derivative plasmids into E. coli BW20767 (Metcalf et al., 1995) via electroporation.

We delivered the pAW19-ΔnarG, pAW19-ΔnirS, or pAW19-ΔcomA plasmid into P. stutzeri A1501 by conjugative mating with E. coli BW20767 as the plasmid donor (Metcalf et al., 1995). We selected P. stutzeri exconjugants by plating on MOPS agar plates containing 0.2% citrate and 50 µg kanamycin ml⁻¹ (White and Metcalf, 2004). We selected P. stutzeri plasmid segregant mutants by plating on TYE agar plates containing 5% sucrose (Metcalf et al., 1995). We verified plasmid segregation by testing for kanamycin sensitivity on LB agar plates containing 50 µg kanamycin ml⁻¹. We identified deletion segregant mutants by analyzing the size of PCR products generated with the forward amplification primer for the upstream 1 kbp region and the reverse amplification primer for the downstream 1 kbp region (Table S3.1).

Phenotype validation

We experimentally verified that the narG and nirS deletions caused the desired phenotypes by testing whether the P. stutzeri mutant strains could grow in dinitrogen gas-sparged ACS liquid medium amended with 10 mM of sodium nitrate (NaNO₃) or sodium nitrite (NaNO₂). The ΔnirS mutant strain (strain A1603) grew in medium amended with 10 mM of nitrate but did not grow in medium amended with 10 mM of nitrite. Conversely, the ΔnarG mutant strains (strain A1602) did not grow in medium amended with 10 mM of nitrate but grew in medium amended with 10 mM of nitrite. Mixtures of the ΔnirS and ΔnarG mutant strains (strains A1602 and A1603) could grow together in medium amended with only 10 mM of nitrate, thus confirming the establishment of a nitrite cross-feeding co-culture. We further used PCR amplification to verify that the correct deletions are present in each strain.

Mathematical model

We used the NDSolve function of Mathematica software (version 8.0.1.0) and the kinetic parameters reported by Bryan and colleagues (Bryan et al., 1985) and Almeida and colleagues (Almeida, Julio, et al., 1995; Almeida, Reis, et al., 1995) to predict the kinetics of cell growth, nitrate (NO₃⁻) consumption, and nitrite (NO₂⁻)
production and consumption. We predicted the kinetics for a completely consuming strain using Eqs. 1-5, where $X_{\text{complete}}$ is the biomass concentration of the completely consuming strain [g L$^{-1}$], $Y_i$ is the yield coefficient for biomass production [g mmol$^{-1}$], $v_i$ is the specific substrate consumption rate [mmol g$^{-1}$ min$^{-1}$], $S_{i,\text{in}}$ is the substrate concentration within the periplasm of the cell [mM], $S_{i,\text{out}}$ is the substrate concentration of the bulk medium [mM], $K_{\text{perm}}$ is the permeability coefficient for mass transfer between the periplasm and the bulk medium [min$^{-1}$ V$^{-1}$ cell], and $V_{\text{cell}}$ is the volume of cells per g cells.

\[
\frac{dX_{\text{complete}}}{dt} = Y_{N_2O_3^-} \cdot v_{N_2O_3^-} + Y_{N_2O_2^-} \cdot v_{N_2O_2^-}
\]

(1)

\[
\frac{dS_{N_2O_3^-}^{\text{in}}}{dt} = -v_{N_2O_3^-} + K_{\text{perm}} \cdot (S_{N_2O_3^-}^{\text{out}} - S_{N_2O_3^-}^{\text{in}})
\]

(2)

\[
\frac{dS_{N_2O_2^-}^{\text{in}}}{dt} = v_{N_2O_3^-} - v_{N_2O_2^-} + K_{\text{perm}} \cdot (S_{N_2O_2^-}^{\text{out}} - S_{N_2O_2^-}^{\text{in}})
\]

(3)

\[
\frac{dS_{N_2O_2^-}^{\text{out}}}{dt} = -X_{\text{complete}} \cdot K_{\text{perm}} \cdot (S_{N_2O_2^-}^{\text{out}} - S_{N_2O_2^-}^{\text{in}})
\]

(4)

\[
\frac{dS_{N_2O_2^-}^{\text{out}}}{dt} = -X_{\text{complete}} \cdot K_{\text{perm}} \cdot (S_{N_2O_2^-}^{\text{out}} - S_{N_2O_2^-}^{\text{in}})
\]

(5)

We calculated the specific substrate consumption rates using the following two equations, where $v_{\text{max}}$ is the maximum substrate consumption rate [mmol g$^{-1}$ min$^{-1}$], $K_m$ is the Michaelis-Menten coefficient for substrate limitation [mM], and $K_i$ is the inhibition coefficient for nitrite [mM].

\[
v_{N_2O_3^-} = \frac{v_{\text{max}} S_{N_2O_3^-}^{\text{in}}}{K_m + S_{N_2O_3^-}^{\text{in}} + \frac{S_{N_2O_3^-}^{\text{in}}}{K_i}}
\]

(6)

\[
v_{N_2O_2^-} = \frac{v_{\text{max}} S_{N_2O_2^-}^{\text{in}}}{K_m + S_{N_2O_2^-}^{\text{in}} + \frac{S_{N_2O_2^-}^{\text{in}}}{K_i}}
\]

(7)

There are three notable features of this system of equations. First, we explicitly accounted for mass transfer across the cell. Second, the term $-\left(\frac{v_{N_2O_3^-} v_{\text{max}} S_{N_2O_3^-}^{\text{in}}}{v_{\text{max}} S_{N_2O_3^-}^{\text{in}}}\right)$ in the numerator of Eq. 7 accounts for competition between the nitrate and nitrite reductases for reduced electron carriers as described by Almeida and colleagues (6). The consequence of this term is that the rate of nitrate consumption represses the rate of nitrite consumption. Third, the term $\left(S_{N_2O_3^-}^{\text{in}}\right)^2 / K_i$ in Eqs. 6 and 7 is the Andrews inhibition term and accounts for the pH-dependent inhibitory effects of nitrite as proposed by Wang and colleagues (Wang et al., 1995).

For the nitrite ($NO_2^-$) cross-feeding strains, we predicted the kinetics of cell growth, nitrate ($NO_3^-$) consumption, and nitrite production and consumption using Eqs. 8-15, where $X_{\text{prod}}$ is the biomass concentration of the nitrite producing strain [g L$^{-1}$], $X_{\text{cons}}$ is the biomass concentration of the nitrite consuming strain [g L$^{-1}$], $S_{i,\text{in,prod}}$ is the substrate concentration within the periplasm of the nitrite producing strain [mM], $S_{i,\text{in,cons}}$ is the substrate...
concentration within the periplasm of the nitrite consuming strain [mM], and all other parameters are identical to those described for Eqs. 1-5.

\[
\begin{align*}
\frac{dx_{\text{prod}}}{dt} &= Y_{N\text{O}_3^-} \cdot v_{N\text{O}_3^-} \\
\frac{dx_{\text{cons}}}{dt} &= Y_{N\text{O}_2^-} \cdot v_{N\text{O}_2^-} \\
\frac{ds_{N\text{O}_3^- \text{in,prod}}}{dt} &= v_{\text{cell}} = -v_{N\text{O}_3^-} + K_{\text{perm}} \cdot (S_{N\text{O}_3^- \text{out}} - S_{N\text{O}_3^- \text{in,prod}}) \\
\frac{ds_{N\text{O}_3^- \text{in,cons}}}{dt} &= v_{\text{cell}} = K_{\text{perm}} \cdot (S_{N\text{O}_3^- \text{out}} - S_{N\text{O}_3^- \text{in,cons}}) \\
\frac{ds_{N\text{O}_2^- \text{in,prod}}}{dt} &= v_{\text{cell}} = v_{N\text{O}_2^-} + K_{\text{perm}} \cdot (S_{N\text{O}_2^- \text{out}} - S_{N\text{O}_2^- \text{in,prod}}) \\
\frac{ds_{N\text{O}_2^- \text{in,cons}}}{dt} &= v_{\text{cell}} = -v_{N\text{O}_2^-} + K_{\text{perm}} \cdot (S_{N\text{O}_2^- \text{out}} - S_{N\text{O}_2^- \text{in,cons}}) \\
\frac{ds_{N\text{O}_3^- \text{out}}}{dt} &= -X_{\text{prod}} \cdot K_{\text{perm}} \cdot (S_{N\text{O}_3^- \text{out}} - S_{N\text{O}_3^- \text{in,prod}}) - X_{\text{cons}} \cdot K_{\text{perm}} \cdot (S_{N\text{O}_3^- \text{out}} - S_{N\text{O}_3^- \text{in,cons}}) \\
\frac{ds_{N\text{O}_2^- \text{out}}}{dt} &= -X_{\text{prod}} \cdot K_{\text{perm}} \cdot (S_{N\text{O}_2^- \text{out}} - S_{N\text{O}_2^- \text{in,prod}}) - X_{\text{cons}} \cdot K_{\text{perm}} \cdot (S_{N\text{O}_2^- \text{out}} - S_{N\text{O}_2^- \text{in,cons}})
\end{align*}
\]

We calculated the specific substrate consumption rates using the following two equations, where \( v_{\text{max}} \) is the maximum substrate consumption rate [mmol g\(^{-1}\) min\(^{-1}\)], \( K_{\text{m}} \) is the Michaelis-Menton coefficient for substrate limitation [mM], and \( K_{\text{l},N\text{O}_2^-} \) is the inhibition coefficient for nitrite [mM].

\[
\begin{align*}
v_{N\text{O}_3^-} &= \frac{v_{\text{max},N\text{O}_3^-} \cdot S_{N\text{O}_3^- \text{in,prod}}} {K_{\text{m},N\text{O}_3^-} + S_{N\text{O}_3^- \text{in,prod}} + \frac{S_{N\text{O}_3^- \text{in,prod}}}{K_{\text{l},N\text{O}_2^-}}} \\
v_{N\text{O}_2^-} &= \frac{v_{\text{max},N\text{O}_2^-} \cdot S_{N\text{O}_2^- \text{in,cons}}} {K_{\text{m},N\text{O}_2^-} + S_{N\text{O}_2^- \text{in,cons}} + \frac{S_{N\text{O}_2^- \text{in,cons}}}{K_{\text{l},N\text{O}_2^-}}}
\end{align*}
\]

The main difference between this system of equations and the system of equations for the completely denitrifying cell-type is that the competition term \( -\left(\frac{v_{N\text{O}_2^-} \cdot v_{\text{max},N\text{O}_2^-}}{v_{\text{max},N\text{O}_3^-}}\right) \) in the numerator of Eq. 7 is absent from Eq. 17. This is because the nitrate and nitrite reductases are segregated into different cells and, consequently, the specific and maximum nitrate consumption rates are zero.
Supplementary results

**Independent repetition of the effect of pH on growth**

We repeated the test of whether differences in pH itself cause differences in the maximum specific growth rate \( u_{\text{max}} \ [\text{min}^{-1}] \) of completely-consuming cells (strain A1601). We set the pH of the culture medium to 7.5 or 6.5 and grew completely-consuming cells under aerobic conditions. We measured the zero-order maximum specific growth rate \( u_{\text{max}} \) from ten consecutive data points that coincide with the most rapid period of growth. The data are plotted in Figure S1. We found that reducing the pH from 7.5 to 6.5 had no observable negative effect on the maximum specific growth rate (two-sample Welch test, two-sided \( P > 0.8, n = 8 \)).

**pH-dependence of nitrite inhibition in the absence of oxygen**

We tested whether nitrite (NO\(_2\)) causes pH-dependent growth inhibition in the absence of oxygen. We set the pH of the culture medium to 7.5 or 6.5 and grew completely-consuming cells (strain A1601) under anaerobic conditions. After 72 hours of incubation, cells inoculated into medium at pH 7.5 with 10 mM of exogenous nitrite had reached stationary phase. In contrast, cells inoculated into medium at pH 6.5 with 10 mM of exogenous nitrite had no observable growth. Thus, nitrite has pH-dependent effects on growth under anaerobic conditions.

Supplementary figures

![Figure S 3.1](image)

**Figure S 3.1** Repeated measurements of the effect of pH on growth.

We grew completely-consuming cells (strain A1601) at pH 7.5 (black squares) or pH 6.5 (white squares) under aerobic conditions and in the absence of nitrite (NO\(_2\)) and measured the OD\(_{600}\) values over time. We only plotted every fourth data point to facilitate visualization. Data points are the average measurements among eight independent biological replicates for each pH. The zero-order maximum growth rates are not significantly different from each other (two-sample Welch test, two-sided \( P > 0.8, n_1 = n_2 = 8 \)).
Figure S 3.2 Effect of pH on growth.

We grew completely-consuming cells (lines CC) or nitrite (NO$_2$) cross-feeding cells (lines CF) at pH 7.5 (left panel) or pH 6.5 (right panel) with 10 mM of nitrate (NO$_3$) under anaerobic conditions. We measured the OD$_{600}$ values over time. The data are plotted for $n = 3$ (pH 7.5) or $n = 6$ (pH 6.5) independent replicates.

Figure S 3.3 Nitrate and nitrite consumption dynamics.

We amended anaerobic stationary-phase cultures with 200 μM of (A) nitrate (NO$_3$) or (B) nitrite (NO$_2$) and measured nitrate and nitrite concentrations with biosensors over time. The consumption of nitrate and nitrite began immediately without an apparent lag.
**Supplementary tables**

**Table S 3.1 Bacterial strains and plasmids used for this study.**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>P. stutzeri strain</strong></td>
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</tr>
<tr>
<td>A1501</td>
<td>wild-type strain</td>
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<td>A1601</td>
<td>A1501 with ΔcomA; defective in natural transformation</td>
<td>This study</td>
</tr>
<tr>
<td>A1602</td>
<td>A1501 with ΔcomA and ΔnarG; defective in natural transformation and nitrate reduction</td>
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<td><strong>E. coli strain</strong></td>
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<td>DH5α/λpir</td>
<td>Used for replication of pAW19 derivatives; λpir80ΔlacZΔM15 Δ(lacZYA-argF)U169 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA</td>
<td>(Miller and Mekalanos, 1988)</td>
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<tr>
<td>BW20767</td>
<td>Used for conjugal transfer of pAW19 derivatives; RP4-2-Tc::Mu-1 Kan::Tn7 integrant leu-63::IS10 recA1 zbf-5 creB510 hsdR17 endA1 thi-1 uidA (ΔM1ul)::pir</td>
<td>(Metcalf et al., 1995)</td>
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<tr>
<td><strong>Plasmid</strong></td>
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<td>pAW19</td>
<td>sacB-containing conditionally replicative delivery plasmid; Ap&lt;sup&gt;R&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Metcalf et al., 1995)</td>
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<td>pAW19-ΔnarG</td>
<td>pAW19 with ΔnarG; Ap&lt;sup&gt;R&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pAW19-ΔnirS</td>
<td>pAW19 with ΔnirS; Ap&lt;sup&gt;R&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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**Table S 3.2 Primer sequences**

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<th>Genome position</th>
<th><em>Primer sequence (5'-3')</em></th>
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4 Increased chemical reactivity of a metabolic intermediate accelerates molecular evolution of microbial populations

4.1 Abstract

Over the course of evolution, the chemical reactivity of metabolites has shaped the spatial and temporal arrangement of different metabolic processes within the microbial cell. Microbial cells have evolved many different solutions to deal with reactive metabolic intermediates, including temporally segregating different metabolic processes such that the metabolites produced by one process do not interfere with other processes, channeling substrates to deliver intermediates directly to the next enzyme, and physically separating metabolic processes by compartmentalizing them into specific organelles or segregating them across the cytoplasmic membrane. While these solutions are well documented, there is less known about how chemical reactivity itself affects evolution. Here, we hypothesize that if the chemical reactivity of a metabolic intermediate has negative effects on the growth of a microbial cell, then this reactivity will increase the pace of molecular evolution. This could occur by two different mechanisms. First, the stress caused by chemical reactivity could increase the mutation rate. Second, the decrease in fitness due to chemical reactivity could lead to an increased availability of mutations with large beneficial effects, thus reducing the number of generations required to fix those mutations. We tested this hypothesis by experimentally evolving the denitrifying bacterium *Pseudomonas stutzeri*. During denitrification, the metabolic intermediate nitrite accumulates and has pH-dependent negative effects on growth. We exploited these pH-dependent negative effects to evolve *P. stutzeri* under different levels of chemical reactivity. We demonstrate that increased nitrite reactivity accelerates the pace of molecular evolution. We further demonstrate that this increase is due to an increase in the availability of beneficial mutations rather than an increase in the mutation rate. Together these results demonstrate that chemical reactivity does indeed affect the pace of evolution and is therefore likely to have had impacts on the life histories of microbial cells.
4.2 Introduction

Chemical reactivity is an important factor that shapes the spatial and temporal arrangement of metabolic processes within microbial cells (de Lorenzo et al., 2014). Reactive chemicals may form as intermediates or end products of metabolism, and there are many examples of how microbial cells arrange metabolic processes in space and time to prevent the accumulation of these reactive chemicals and reduce their negative effects on cell growth. Consider the denitrification process where some microbial cells sequentially reduce nitrate ($\text{NO}_3^-$) via nitrite ($\text{NO}_2^-$), nitric oxide (NO) and nitrous oxide ($\text{N}_2\text{O}$) into di-nitrogen gas ($\text{N}_2$) to yield energy (Fig. 4.1) (Ferguson, 1994). The intermediate nitric oxide is a free radical that has cytotoxic effects on cell division and forms metal-nitrosyl complexes with enzymes (Vollack and Zumft, 2001). Indeed, nitric oxide performs many of the antimicrobial functions of macrophages (Nathan and Hibbs, 1991). Thus, denitrifying microorganisms must maintain nitric oxide at low concentrations during denitrification. To achieve this, nitric oxide regulates the transcription of denitrification genes (Vollack and Zumft, 2001), suggesting regulatory solutions to prevent nitric oxide accumulation. In addition, denitrifying microorganisms typically produce and consume nitric oxide in the periplasmic space of the cell, thus preventing nitric oxide from reaching the cytoplasm and imposing its deleterious effects (Zumft, 1997).

While it is clear that chemical reactivity of metabolic intermediates have shaped the spatial and temporal arrangement of metabolic processes within microbial cells, it is less clear whether chemical reactivity itself affects the pace of molecular evolution. In this context, we use the term “pace of molecular evolution” to refer to the number of mutations fixed per generation and not to the number of mutations fixed per unit time. Chemical reactivity could affect the pace of molecular evolution by at least two distinct mechanisms. First, chemical reactivity (and stress in general) might increase the mutation rate, thus providing a larger set of mutations for selection or drift to act upon (Galhardo et al., 2007; MacLean et al., 2013). Second, chemical reactivity might decrease the fitness of the cell. That is, chemical reactivity might cause slower growth rates or lower yields due to the inhibitory effects of the metabolites. This could result in increased selection pressure and increased availability of mutations with large fitness benefits, thus reducing the number of generations required for beneficial mutations to become fixed within a population (Barrett, M’Gonigle, et al., 2006).

Our main objective was to experimentally test whether increased chemical reactivity of metabolic intermediates do indeed accelerate the pace of molecular evolution, and by which mechanisms such an effect might emerge (i.e., by increasing the mutation rate or by increasing the availability of mutations with large fitness benefits). To address this knowledge gap, we employed the experimental system described in Chapter 3 where we could manipulate the chemical reactivity of a single metabolite. We then experimentally evolved replicated populations of bacteria at two distinct levels of chemical reactivity, quantified the types and numbers of mutations that accumulated during experimental evolution, and tested whether chemical reactivity accelerated the pace of molecular evolution.
The experimental system that we used is based on the denitrifying bacterium *Pseudomonas stutzeri* A1501 (see Chapter 3), which is a facultative anaerobe with a fully sequenced genome (Yan *et al.*, 2010). In the absence of oxygen, *P. stutzeri* can use nitrogen oxides as terminal electron acceptors to support its growth (Laluca* et al.*, 2006). *P. stutzeri* sequentially reduces nitrate (NO$_3^-$) to nitrite (NO$_2^-$), nitric oxide (NO), nitrous oxide (N$_2$O), and finally to di-nitrogen gas (N$_2$) using different enzyme complexes for each reduction step (Fig. 4.1) (Zumft, 1997). An important feature of this experimental system is that nitrite accumulates in batch culture and has pH-dependent negative effects on growth (see Chapter 3). As the pH decreases, nitrite increasingly generates nitrous acid (HNO$_2$), which uncouples proton translocation (Sijbesma *et al.*, 1996; Zhou *et al.*, 2011). In addition, nitrite increasingly and spontaneously generates nitric oxide radicals that impose cytotoxic effects on cell division and form metal-nitrosyl complexes with enzymes (Zumft, 1993). The consequence is that, as the pH decreases, the increased chemical reactivity of nitrite slows growth (Almeida, Julio, *et al.*, 1995) (see Chapter 3). In general, nitrite has negligible negative effects at pH 7.5 and severe negative effects at pH 6.5, while pH itself has no observable negative effects under this pH range (see Chapter 3). The pH of the culture medium can therefore be used to manipulate the chemical reactivity of nitrite without creating confounding factors (see Chapter 3), thus allowing us to test the hypothesis that increased chemical reactivity accelerates the pace of molecular evolution.

![Figure 4.1 The denitrification pathway of *Pseudomonas stutzeri*.](image)

Nitrate is reduced to nitrite in the cytoplasm and then nitrite is actively transported to the periplasm where it is further reduced. The consequence is that nitrite accumulates in the periplasm. The nitrite reductase and the nitric oxide reductase are associated with the periplasmic membrane while the nitrite reductase and the nitrous oxide reductase are free in the periplasmic space.
4.3 Materials and methods

Strains and genetic manipulations
We obtained the wild-type bacterium *P. stutzeri* A1501 from the Biological Resource Center of Institut Pasteur (www.crbp.pasteur.fr) and used this strain to construct all of the *P. stutzeri* mutant strains described in this study (Table S4.3). We deleted the *comA* gene from all the strains to prevent the internalization of extracellular DNA as described elsewhere (see Chapter 3. We introduced DNA fragments that contain the isopropyl-β-D-thiogalactopyranosid (IPTG)-inducible *P*<sub>lac</sub> promoter located immediately upstream of the *egfp* or *echerry* (Minoia et al., 2008) gene into *P. stutzeri* A1601. These genes encode green or red fluorescent proteins, respectively. We introduced the DNA fragments using derivatives of the mini-Tn7T-LAC-Gm transposon and the pUC18T conditionally replicative delivery plasmid (Table S4.3) as described elsewhere (Lambertsen et al., 2004; Choi et al., 2005). Briefly, we constructed the derivative transposons by first purifying the pUC18T-mini-Tn7T-LAC-Gm plasmid from an overnight culture of *E. coli* DH5α/λpir (Table S4.3) (Miller and Mekalanos, 1988)(2836362). We next PCR amplified the *egfp* or *echerry* gene using GoTaq DNA polymerase (Promega, Madison, WI, USA) and the oligonucleotide primers listed in Supplementary Table S4.4. These primers contain the *BamHI* and *KpnI* restriction sites that we used to clone the PCR products into the pUC18T-mini-Tn7T-LAC-Gm plasmid (Table S4.3). We then digested the pUC18T-mini-Tn7T-LAC-Gm plasmid and the PCR products with *BamHI* and *KpnI* (Thermo Fisher Scientific, Waltham, MA, USA) and ligated the PCR products into the pUC18T-mini-Tn7T-LAC-Gm plasmid. We designated the assembled derivative plasmids as pUC18T-mini-Tn7T-LAC-Gm-*egfp* and pUC18T-mini-Tn7T-LAC-Gm-*echerry* respectively (Table S4.3). We replicated the assembled derivative plasmids in *E. coli* DH5α/λpir (Table S4.3) (Miller and Mekalanos, 1988).

We used conjugative four-parental mating to deliver the assembled pUC18T-mini-Tn7T-LAC-Gm derivative plasmids along with the helper pUX-BF13 plasmid that expresses the transposase gene into wild-type *P. stutzeri* A1501 (Table 1) as described elsewhere (Choi et al., 2005). We selected *P. stutzeri* exconjugants by plating on 3-(*N*-morpholino)propanesulfonic acid (MOPS) agar plates containing 0.2% of sodium citrate and 10 µg ml<sup>-1</sup> of gentamycin (Choi et al., 2005). We verified plasmid segregation by testing for ampicillin sensitivity on lysogeny broth (LB) agar plates containing 100 µg ml<sup>-1</sup> of ampicillin. We did not perform *FRT* excision of the gentamycin resistance marker (Choi et al., 2005) and all of the *P. stutzeri* mutant strains therefore retained gentamycin resistance (Table S4.3).

Culture conditions
We cultured all *P. stutzeri* strains under aerobic conditions in a defined asparagine-citrate synthetic medium (ACS medium) (Coyle et al., 1985) with 10 µg ml<sup>-1</sup> of gentamicin. We cultured all *P. stutzeri* strains under anaerobic conditions in di-nitrogen gas (N<sub>2</sub>)-sparged ACS medium amended with 10 mM of sodium nitrate (NaNO<sub>3</sub>) and 10 µg ml<sup>-1</sup> of gentamycin. We reported a complete description of the methods to prepare and inoculate di-nitrogen gas-sparged ACS
medium elsewhere (see Chapter 3). We incubated all *P. stutzeri* cultures at 30°C with shaking at 220 rpm.

**Experimental evolution**

We experimentally evolved four populations that carry the *egfp* gene and four populations that carry the *echerry* gene for each pH condition (pH 6.5 and 7.5) for a total of 16 populations. We did not add IPTG to the medium during experimental evolution to avoid the cost of expressing the fluorescent proteins, and to therefore minimize the probability of selecting for loss-of-function mutations in the *egfp* or *echerry* gene. We used the *egfp* and *echerry*-expressing traits to distinguish different strains during competition assays (see below) and to periodically assess for cross-contamination between populations. For the latter test, we plated a small aliquot of each population onto an individual LB agar plate containing 0.1 mM of IPTG. We never observed cross-contamination during the experiment (*i.e.*, we never observed populations that contained both *egfp* and *echerry*-expressing colonies).

For the evolution experiment, we serially transferred batch cultures containing di-nitrogen gas (N$_2$)-sparged ACS medium amended with 10 mM of sodium nitrate (NaNO$_3$) as the growth-limiting substrate. We transferred each population after entering stationary phase at a dilution of 1:200 vol:vol (although see the exceptions below) for a total of 700 generations. We transferred the populations evolved at pH 7.5 every day, with some exceptions during the beginning of the evolution experiment when growth was slow and unstable (*i.e.* some cultures grew slower or had long lag periods before growth was observed). We transferred the populations evolved at pH 6.5 every fourth day during the beginning of the evolution experiment and every second day at the end of the evolution experiment. In general, we performed each transfer at a 1:200 dilution (vol:vol) into fresh di-nitrogen gas-sparged ACS medium, with some exceptions at the beginning of the evolutionary experiments when growth was unstable and highly variable. At these times, we performed each transfer at a 1:50 or a 1:100 dilution (vol:vol) and we took these exceptions into account when estimating the total number of generations.

**Sequencing**

We streaked each evolved and ancestral population onto LB agar plates containing 10 μg ml$^{-1}$ of gentamicin and 0.1 mM of IPTG and picked a single colony from each population for whole genome sequencing. We grew the single clones in LB overnight and extracted the DNA with a Wizard Gemoic DNA purification kit (Promega, Madison, WI). We then sent the extracted DNA for sequencing at the ETH Quantitative Genomics Facility (Basel, Switzerland). There, the genomes were sequenced with whole genome Illumina sequencing (HiSeq 2000, paired-end 100bp), and primary data analysis, de-multiplexing and quality control analysis of the sequencing data using FastQC (illumina, San Diego, CA) were performed.

**Identification of mutations in the evolved clones.**

The genomic data was further analyzed at the ETH Genomic Diversity Center (Zürich, Switzerland). Quality filtering of the raw reads was done using PRINSEQ-lite v0.20.4 (Schmieder and Edwards, 2011) where duplicated reads were removed and ambiguous base pairs were trimmed. Bresq v.0.24rc5
(Deatherage and Barrick, 2014) was used to identify differences between the evolved genomes and the reference genome of the ancestral 1601 strains.

**Growth to stationary phase**
We estimated the time for populations to enter stationary phase by measuring the increase in optical density at 600 nm (OD<sub>600</sub>) over time. We plated each evolved population on LB agar plates containing 0.1 mM of IPTG, picked ten individual colonies, inoculated the colonies into separate one-mL cultures containing aerobic ACS medium, and let them grow overnight into stationary phase. I then mixed the individual one-mL cultures from each evolved population in equal volumes, inoculated 800 μL of the mixture into 20 mL of di-nitrogen gas (N<sub>2</sub>)-sparged ACS medium amended with 10 mM of sodium nitrate (NaNO<sub>3</sub>), and grew the mixture until reaching stationary phase. We used mixtures of ten individual clones from each population to maintain genetic variation of the population. We then inoculated 200 μL of this stationary phase culture into 20 mL of fresh di-nitrogen gas-sparged ACS medium amended with 10 mM of sodium nitrate and measured OD<sub>600</sub> over time in a Synergy plate reader (BioTek, Luzern, Switzerland) until reaching stationary phase.

**Competition assay**
We competed a randomly picked clone from each evolved population against the ancestral population that expresses a different fluorescent protein-encoding gene (i.e., we competed the egfp-expressing evolved populations against the echerry-expressing ancestral population and vice versa). This allowed us to distinguish and quantify the frequencies of different strains when competed against each other. To perform the competition assays, we first plated each evolved population on LB agar plates containing 0.1 mM of IPTG, picked a random clone from a single colony, inoculated the colonies into one-mL cultures of aerobic ACS medium, and let them grow overnight into stationary phase. We then inoculated 800 μL of the mixture into 20 mL of di-nitrogen gas-sparged ACS medium amended with 10 mM of sodium nitrate (NaNO<sub>3</sub>) and grew the mixture until reaching stationary phase. We finally mixed an aliquot of the culture of each evolved clone with an aliquot from the ancestral culture (50:50 or 5:95 vol:vol, depending on the experiment). We then inoculated 100 μL of this mixture into 20 mL fresh di-nitrogen gas-sparged ACS medium amended with 10 mM of sodium nitrate and grew the cultures until reaching stationary phase. We plated this mixture onto LB agar plates (two per competition) containing 0.1 mM IPTG prior to incubation and after reaching stationary phase to determine the initial and final frequencies of evolved and ancestral cells. We calculated the fitness of the evolved populations relative to the ancestral populations using the following formula (Lenski et al., 1991; Lee et al., 2009), where W is the relative fitness of the evolved population, R<sub>0</sub> is the frequency of evolved cells before competition, R<sub>1</sub> is the frequency of evolved cells after competition, and F is the fold increase of cell during the competition as determined by the dilution.

\[
W = \frac{log \left( \frac{R_1 \times F}{R_0} \right)}{log \left( \frac{(1-R_1) \times F}{1-R_0} \right)}
\]
**Biolog assay**

We plated each population on LB agar plates containing 0.1 mM IPTG, picked ten individual colonies, inoculated the colonies into separate one-mL cultures of aerobic ACS medium, and let them grow overnight until reaching stationary phase. We then mixed the individual one-ml cultures from each population in equal volumes, diluted the mixture into aerobic ACS medium lacking a carbon source (no citrate or asparagine), and pipetted the mixture into each well of a 96-well PM1 Biolog plate (Biolog, Hayward, CA, USA), where each well contains a different carbon source. We evaluated one PM1 Biolog plate per evolved population and three PM1 Biolog plates for each ancestral population (*i.e.*, three PM1 Biolog plates for the *egfp*-containing strain and three PM1 Biolog plates for the *echerry*-containing strain). We incubated the plates at 30°C and shaking at 220 rpm for 24 hours and measured the OD$_{600}$ with an Eon plate reader (BioTek, Luzern, Switzerland) every second hour. Note that we did not use a respiration indicator as is typical for Biolog analyses, and we therefore measured cell density rather than respiration activity. To estimate the performance on each carbon source, we calculated the area under the curve (growth over time) to account for both growth rate and yield (Guckert *et al.*, 1996). We relativized the data by calculating the average for the evolved clone measurements and dividing that value by the average of the ancestor measurements for each carbon source.

**Statistical analyses**

We used the non-parametric Wilcoxon rank sum test to test for differences between different treatments. This test is more robust to outliers than its parametric equivalent, which is particularly relevant to this study. We found that one population followed a very different evolutionary trajectory than the other populations (see results section), thus reducing the power of parametric tests and justifying the use of non-parametric tests. For multiple comparison tests we used analysis of variance (ANOVA) with a subsequent Tukey’s Honest Significant Difference (HSD) post hoc-test.
4.4 Results

**Increased nitrite reactivity accelerates molecular evolution**

Our first objective was to experimentally test whether increased reactivity of the metabolic intermediate nitrite (NO$_2^-$) accelerates molecular evolution. To achieve this objective, we experimentally evolved eight populations of *P. stutzeri* at pH 6.5 (high reactivity of nitrite) and eight populations at pH 7.5 (low reactivity of nitrite) for approximately 700 generations (see Chapter 3 for experimental testing of reactivity at different pH conditions). We then randomly selected a single clone from each evolved population, sequenced its genome, and quantified the number of mutations within that clone. We found more mutations in clones evolved at pH 6.5 than at pH 7.5, thus supporting our main hypothesis (Wilcoxon rank sum test; $P<0.005$, $n_1=n_2=8$) (Fig. 4.2). We further found that one clone evolved at pH 6.5 had five-times more mutations than the other clones evolved at pH 6.5. This clone contained a loss of function mutation in the *uvrA* gene, which encodes for excinuclease ABC subunit A, a protein involved in DNA repair by nucleotide excision in other microorganisms (Goosen and Moolenaar, 2001; Jaciuk *et al.*, 2011). Thus, this mutation likely caused an increased mutation rate. We therefore further tested whether there were more mutations in clones evolved at pH 6.5 than in clones evolved at pH 7.5 when this clone was removed from the statistical analysis, and this was indeed the case (Wilcoxon rank sum test; $P<0.005$, $n_1=8$, $n_2=7$) (Fig. 4.2). This was also true when we only took into account non-synonymous mutations in coding regions (Fig. S4.1). Thus, our data supports the hypothesis that the increased reactivity of the metabolic intermediate nitrite accelerates the pace of molecular evolution.

**Figure 4.2** The number of mutations in the sequenced evolved clones for each pH condition used for experimental evolution.

Horizontal bars and P-values indicate the outcomes of two-sample Wilcoxon rank-sum tests. Stars indicate a P value less than 0.05. The red arrow indicates the clone with a mutation in *uvrA*. Data are presented as Tukey box-plots.
**Increased mutation rates cannot account for the accelerated pace of molecular evolution**

The increased numbers of mutations in clones evolved at pH 6.5 (high reactivity of nitrite \([\text{NO}_2^-]\)) could be due to either increased mutation rates or increased availability of mutations with large fitness benefits. We investigated this by categorizing all mutations by mutation type: point mutations (non-synonymous, synonymous or intergenic), indels (genic and intergenic), large deletions (>250 bp), mutations conferred by insertion sequences, and mutations in ribosomal RNAs (Fig. 4.3). We reasoned that if the accelerated pace of molecular evolution was predominantly caused by increased mutation rates, then we would expect increased numbers of synonymous substitutions to be fixed within those clones. We found that this was not the case. There was no significant difference in the relative abundance of synonymous substitutions (Wilcoxon rank-sum test, \(P>0.5\), \(n_1=n_2=8\)) or the absolute abundance of synonymous substitutions (Wilcoxon rank-sum test, \(P>0.6\), \(n_1=n_2=8\)) for clones evolved at pH 6.5 compared to clones evolved at pH 7.5 (Fig 4.3). This was true regardless of whether we included or removed the clone with the mutation in the \(uvrA\) gene from the analysis. Indeed, for clones evolved at pH 6.5, we did not detect any synonymous mutations except in the clone previously described as having a mutation in the \(uvrA\) gene. Taken together, we have no evidence that differences in the mutation rates generally explain the increased numbers of mutations in clones evolved at pH 6.5 (high reactivity of nitrite) relative to pH 7.5 (low reactivity of nitrite). Instead, we found that clones evolved at pH 6.5 had significantly more non-synonymous substitutions than other types of mutations (analysis of variance [ANOVA] followed by Tukey’s HSD post hoc analysis; \(P<0.01\), (Fig S4.3). In contrast, clones evolved at pH 7.5 had significantly more indels in coding regions than other types of mutations (analysis of variance followed by Tukey’s HSD post hoc analysis; \(P<0.01\), (Fig.S4.2). Taken together, we have no evidence that differences in the mutation rates generally explain the increased numbers of mutations fixed in clones evolved at pH 6.5 (high reactivity of nitrite) relative to pH 7.5 (low reactivity of nitrite).

The difference in the type of mutation occurring at the two conditions may be partly due to the type of genes they occur in. However, consider one of the genes that have mutations at both conditions; \(oprQ\). This gene has mutations in two clones evolved at low nitrite reactivity, and in six clones evolved at high nitrite reactivity. In the two clones evolved at low reactivity the mutations are deletions but in five of the six clones evolved at high reactivity the mutation is a single base substitution. Thus, the differences in the types of mutations are likely a consequence of the different environments.
Each mutation was categorized by type and the relative frequency of each mutation type was calculated for each clone (number of mutation type $n$ / total number of mutations in clone). The mutations were further sorted based on the pH of the culture medium. Data are presented as Tukey box-plots.

**Increased nitrite reactivity leads to larger increases in fitness**

If the increased numbers of mutations in clones evolved at pH 6.5 (strong reactivity of nitrite) was not due to increased mutation rates but instead due to increased availability of mutations with large fitness benefits, then we would expect the populations evolved at pH 6.5 to have larger increases in fitness than the populations evolved at pH 7.5. We tested this expectation using growth assays and measuring the time for populations to reach stationary phase, which is equivalent to the time required for complete substrate consumption. We calculated the relative time for complete substrate consumption as the time for the evolved populations to enter stationary phase ($t_{\text{evol}}$) divided by the time for the ancestral populations to enter stationary phase ($t_{\text{anc}}$). We report the values as $t_{\text{evol}}/t_{\text{anc}}$ in Figure 4.4. We found that the populations evolved at pH 6.5 have significantly smaller values of $t_{\text{evol}}/t_{\text{anc}}$ than those evolved at pH 7.5 (Wilcoxon rank-sum test, $P<0.001$, $n_1=n_2=8$) (Fig. 4.4). Overall, the populations evolved at pH 6.5 require about 50% of the ancestral time to reach stationary phase while the populations evolved at pH 7.5 require about 80% of the ancestral time (Fig. 4.4). From these growth assays, we conclude that the increased numbers of mutations fixed in populations evolved at pH 6.5 (high reactivity of nitrite) is likely to due to increased availability of mutations with large fitness benefits.
Figure 4.4 The time to reach stationary phase for evolved populations divided by the time to stationary phase for ancestral populations.

Smaller values indicate increasingly shorter times for the evolved populations to consume all of the available substrates. The horizontal bar and P-value indicate the outcome of a two-sample Wilcoxon rank-sum test. Stars indicate a P-value less than 0.05. Data are presented as Tukey box-plots.

**Increased nitrite reactivity leads to larger increases in competitive fitness**

Because evolution at pH 6.5 (high reactivity of nitrite [NO₂⁻]) led to both increased numbers of mutations and larger increases in fitness, we further investigated whether evolution at pH 6.5 also results in increased competitive fitness. We define competitive fitness as the relative fitness of an evolved clone when competed directly with its ancestor in co-culture. To address this, we performed four different competition assays where we competed evolved clones against ancestral clones. We initiated the competition assays with 5% of evolved cells and 95% of ancestral cells, as our previous competition assays using an initial ratio of 50% of evolved cells and 50% of ancestral cells did not yield any significant differences (Fig. S4.2).

We first tested whether the clones evolved at pH 6.5 improved in competitive fitness more than the clones evolved at pH 7.5, which would be expected given that the clones evolved at pH 6.5 fixed more mutations and improved more in the time for complete substrate consumption. To accomplish this, we compared the competitive fitness of the evolved clones when they were competed against the ancestor at the same conditions as they were evolved. We found that the clones evolved at pH 6.5 increased in competitive fitness significantly more than the clones evolved at pH 7.5 (ANOVA with a Tukey’s HSD post hoc analysis, P<0.01)
(Fig. 4.5; first boxplot compared to the last boxplot), thus supporting our expectation.

We next assessed competitive fitness in the non-selected environment. We compared the competitive fitness of clones evolved at pH 7.5 when competed against the ancestor at pH 6.5 or pH 7.5. We found that the clones evolved at pH 7.5 increased in competitive fitness significantly more when competed at pH 6.5 than at pH 7.5 (ANOVA with a Tukey’s HSD post hoc analysis, \( P<0.01 \)) (Fig. 4.5; third boxplot compared to the last boxplot). This indicates that the mutations fixed in clones evolved at pH 7.5 have larger benefits at pH 6.5 than at pH 7.5. We further compared the competitive fitness of clones evolved at pH 6.5 or pH 7.5 against the ancestor at pH 6.5. We found that the clones evolved at pH 6.5 increased in competitive fitness significantly more when competed at pH 6.5 than at pH 7.5 (ANOVA with a Tukey’s HSD post hoc analysis, \( P<0.01 \)) (Fig. 4.5; first boxplot compared to the third boxplot). This indicates that, even though the mutations fixed in clones evolved at pH 7.5 had larger benefits at pH 6.5, this is not sufficient to account for all of the increased fitness of clones at pH 6.5. Finally, we compared the competitive fitness of the clones evolved at pH 6.5 or pH 7.5 when competed against the ancestor at pH 7.5. We found that there is no significant difference in the competitive fitness between the two evolutionary conditions when assayed at pH 7.5 (ANOVA with a Tukey’s HSD post hoc analysis, \( P>0.01 \)) (Fig. 4.5; second boxplot compared to the last boxplot). These results indicate that there are indeed mutations that have a beneficial effect on fitness at pH 6.5 that have no effect at pH 7.5. Taken together with the outcomes from the other competition assays, our data supports the idea that the larger increase in competitive fitness found after evolution at pH 6.5 is both due to an increased benefit of the same type of mutations that are found at pH 7.5 as well as additional types of mutations that have a benefit only at pH 6.5.
Figure 4.5 Competitive fitness of evolved clones relative to the ancestor.

Evolved cells were initially present in the culture at a frequency of 5%. Differences were compared using an ANOVA test followed by a post hoc Tukey’s HSD test. Significant differences correspond to $P<0.01$. Alphabetic assignments indicate groups that are statistically different from each other.

Increased nitrite reactivity selects for mutations in genes with a broader variety of functional annotations

We next tested whether evolution at pH 6.5 (high reactivity of nitrite $[\text{NO}_2^-]$) not only promotes more mutations to become fixed, but also affects the functional composition of those mutations. To address this, we functionally categorized each gene with a mutation according to existing annotations (Winsor et al., 2011; Yan et al., 2008). In this analysis we only included non-synonymous substitutions in coding regions and point mutations in intergenic regions likely to be involved in transcription (Fig. 4.6). Clones evolved at pH 7.5 only have mutations in three functional categories: cell motility, signal transduction and unknown functions. Mutations in these types of functions are also commonly found in clones evolved at pH 6.5. The number of mutations in the clones does not differ between the two conditions (low and high nitrite reactivity) in these functional categories. Clones evolved at pH 6.5 had mutations in all of the same categories discussed above, but also have mutations in additional categories: carbohydrate transport and metabolism, lipid transport and metabolism, energy production and conversion, inorganic ion transport and metabolism and transcription. However, the only functional category where this is statistically significant from the clones evolved at pH 7.5 is carbohydrate transport and metabolism (Wilcoxon rank sum test, $n_1=n_2=8$, $P<0.001$). Indeed, each of the
clones evolved at pH 6.5 have a mutation in a gene encoding for an enzyme involved in carbohydrate metabolism, while none of the clones evolved at pH 7.5 have a mutation in gene involved in carbohydrate metabolism. The additional functional categories are those with mutations only found in the clone with a mutation in uvrA (Fig. 4.6). A few clones evolved at pH 6.5 also have mutations in genes involved in denitrification that were not observed in clones evolved at pH 7.5 (denitrification-related genes are distributed among a variety of functional categories). In conclusion, evolution at pH 6.5 (strong reactivity of nitrite) leads not only to more mutations but also to mutations with a larger variety of functional annotations, including mutations affecting carbohydrate metabolism.

![Figure 4.6](image)

**Figure 4.6 Functional categorization of genes that contain mutations in evolved clones.**

Each mutation was categorized by type, and the number of mutations of a functional type was determined for each clone. The mutations were further sorted based on the pH of the culture medium. P values are the outcomes of two-sample Wilcoxon rank-sum tests. Stars indicate a P-value less than 0.05. Data are presented as Tukey box-plots.
Populations evolved at increased nitrite reactivity have decreased efficiency in the use of alternative carbon substrates. Evolutionary adaptations to one environment can lead to trade-offs in other environments. As we identified many clones with mutations in cell motility functions, we tested if this lead to a decrease in cell motility. All evolved clones had severely diminished motility compared to the ancestor (Fig S4.3). However, because clones evolved at pH 6.5 (high reactivity of nitrite) accumulated more mutations in a wider variety of genes than clones evolved at pH 7.5 (low reactivity of nitrite), we tested whether the increased numbers of mutations correlates with increased pleiotropic effects under non-selected environments. To accomplish this, we measured the performance of the ancestral and all of the evolved populations when growing on a variety of alternative carbon sources using Biolog PM1 plates. These plates contain 95 individual carbon sources and can be used to measure growth over time. We measured performance as the area under the growth curve (OD600 vs time) for each carbon substrate as described elsewhere (Guckert et al., 1996). We only took into account those carbon sources for which there was visible growth.

First, we compared the populations evolved at pH 6.5 with the ancestor grown at pH 6.5 and the populations evolved at pH 7.5 with the ancestor grown at pH 7.5 (Fig. 7A and B). We found that populations evolved at pH 7.5 had no significant overall change in their ability to use alternative carbon substrates when compared to the ancestor; the average of evolved/ancestral (area = area under curve) does not deviate from one (Fig. 7A; one sample Wilcoxon rank-sum test; P>0.2). In contrast, populations evolved at pH 6.5 had an overall decrease in carbon source utilization; the average of evolved/ancestral is significantly lower than one (Fig. 7B; one-sample Wilcoxon rank-sum test; P<0.05). We also calculated the differences between the two pH conditions for both the ancestors and the evolved populations. That is, we calculated the average at pH 6.5 and divided it by the average at pH 7.5 for each carbon source for the ancestors and for the evolved populations (Fig. 7C and D). The average of pH6.5/7.5 does not deviate from one for the ancestors, indicating that pH itself has no effect (Fig. 7C; one-sample Wilcoxon signed-rank test, P>0.3). In contrast, the average of pH6.5/7.5 is significantly lower than one for the evolved populations, indicating that evolution at pH 6.5 does indeed have negative consequences on the utilization of non-selected substrates (Fig. 7D; one-sample Wilcoxon signed-rank test, P<0.00001). Taken together, our data supports the conclusion that the increased accumulation of mutations at pH 6.5 led to increased antagonistic pleiotropic effects in non-selected environments. This is probably due to the increased accumulation of mutations in carbohydrate utilization and transport genes at pH 6.5 which are likely to have negative effects on the ability of those evolved populations to utilize different carbon substrates.
Figure 4.7 Performance of evolved and ancestral clones to utilize various carbon substrates.

Performance is quantified as the area under the growth curve (OD<sub>600</sub> vs time), which takes into account both growth rate and yield. Histograms are the mean area under the curve for evolved clones divided by the mean area under the curve for the ancestral cells for each carbon substrate at (A) pH 7.5 or (B) pH 6.5. Additionally, histograms are provided for the mean area under the curve for (C) ancestral cells at pH 6.5 divided by ancestral cells at pH 7.5 or (D) evolved cells at pH 6.5 divided by evolved cells at pH 7.5.
4.5 Discussion

Our results provide experimental evidence that increased chemical reactivity of a single metabolic intermediate accelerates the pace of molecular evolution, and this leads to larger increases in both absolute and relative fitness. We conclude that the increased rate of evolution is most likely due to the increased availability of mutations with large beneficial effects and not due to an increase in the mutation rate in response to stressful conditions, which is also commonly observed (MacLean et al., 2013; Galhardo et al., 2007; Ram and Hadany, 2012; Bjedov et al., 2003). This is based on evidence that there is no increase in synonymous mutations at high nitrite reactivity (Fig. 4.3) and further supported by the fact that the additional mutations that are fixed during evolution at high nitrite reactivity do not confer a significant additional increase in fitness at low nitrite reactivity (Fig. 4.5). Furthermore, we identified a single clone with five-fold more mutations than the other clones (Fig. 4.2), but this clone did not show any difference in the increase in fitness when compared to the other clones (Fig. 4.4).

The larger increases in fitness observed after evolution at high nitrite reactivity are due to two mechanisms. The first mechanism is that the fitness benefits of mutations in genes encoding for certain functions have larger beneficial effects as nitrite reactivity increases. We base this conclusion on the fact that clones evolved at low nitrite reactivity share mutations in genes with similar functions with clones evolved at high nitrite reactivity, including mutations in genes involved in cell motility (e.g. flgD, fleQ), signal transduction (phoP), and in genes encoding for membrane proteins (oprE3, PST_2380 [a porin]) (Tables S4.1 and S4.2). This suggests that there are adaptations common to both conditions. Yet, the clones evolved at low nitrite reactivity have a larger increase in competitive fitness when grown at high nitrite reactivity than at low nitrite reactivity (Fig. 4.5) suggesting that the adaptations common to both conditions have a larger benefit at higher nitrite reactivity. This idea that the benefit of mutations can be larger (and thus requiring fewer generations to fix within the population) in a less fit background is consistent with several previous studies. For example, a recent study with yeast demonstrated that beneficial mutations have increasing benefits in more stressful backgrounds, where the initial fitness was determined by the genotype rather the environment (Kryazhimskiy et al., 2014). As another example, mutations conferring antibiotic resistance have increasing benefits at increasing antibiotic treatments (MacLean and Buckling, 2009). Thus, in the case of nitrite reactivity, mutations in certain functions have beneficial effects at both low and high reactivity, but those benefits increase when reactivity increases.

The second mechanism is that mutations emerged that are only beneficial at increased nitrite reactivity. That is, bacteria evolved at high nitrite reactivity have a larger increase in competitive fitness than bacteria evolved at low nitrite reactivity when competed at high nitrite reactivity (Fig 4.5). Additionally, bacteria evolved at high nitrite reactivity have no significant difference in the increase of competitive fitness compared to bacteria evolved at low reactivity at low reactivity conditions (Fig. 4.5). This idea is further supported by the fact that mutations in genes encoding for proteins involved in carbon metabolism (PykA, Fbp, Gap-2) and in denitrification were only identified in clones evolved at high
nitrite reactivity (Table S4.1 and Table S4.2). These results indicate that mutations in functions such as carbon metabolism and denitrification only have a beneficial effect at high nitrite reactivity.

Our data additionally demonstrates that increased chemical reactivity not only accelerates molecular evolution, but also reduces the niche breadth of the cells. We found that the increase in the number of mutations for high nitrite reactivity lead to increased negative pleiotropy in non-selected environments, as demonstrated by the decrease in efficiency on different carbon sources (Fig. 4.7). While statistically significant, we caution that these results may not be biologically significant, as the effects are typically small for many of the substrates. However, the fact that all lineages evolved at high nitrite reactivity have mutations in important enzymes involved in glycolysis and gluconeogenesis supports the idea that carbon use would be affected in these lineages. This suggests that evolution at increased reactivity of a metabolic intermediate leads to increased metabolic specialization and decreased niche breadth. This decrease in niche breadth may not be unexpected, as there is some evidence supporting the idea that adaptation to more stressful environments leads to increased trade-offs in non-selected environments. For example, when *Escherichia coli*, a bacteria that normally grows best at neutral pH was experimentally evolved at different environmental pH, evolution at lower pH lead to reduced fitness at higher pH to a much larger extent than the opposite (Hughes *et al.*, 2007). Given the large numbers of substrates and intermediates that are present in the natural environment, chemical reactivity may be an important factor that limits niche breadth and, in turn, promotes biodiversity and the coexistence of different metabolically specialized genotypes.
4.6 Supplementary material

Supplementary methods

Motility Assay

We picked a single clone from each ancestral strain and each evolved lineage as a single colony from an agar plate and grew them over night in aerobic ACS medium at 30°C. We spotted 2μL from each over night culture onto LB agar plates containing 0.4% agar. We measured the diameter of the spreading bacteria after 72 hours of growth at 30°C we measured the diameter of the spreading bacteria.

Supplementary figures

Figure S 4.1 Number of non synonymous mutations in coding regions in the evolved clones. Horizontal bars and P-values indicate the outcomes of two-sample Wilcoxon rank-sum tests. Stars indicate a P value less than 0.05. Data are presented as Tukey box-plots
Figure S 4.2 Ratio of type of mutation in clones evolved at pH 7.5

Differences were compared using an ANOVA test followed by a post hoc Tukey's HSD test. Significant differences correspond to $P<0.01$. Alphabetic assignments indicate groups that are statistically different from each other.

Figure S 4.3 Ratio of type of mutation in clones evolved at pH 6.5

Differences were compared using an ANOVA test followed by a post hoc Tukey's HSD test. Significant differences correspond to $P<0.01$. Alphabetic assignments indicate groups that are statistically different from each other.
Figure S 4.4 The relative fitness of the evolved clones calculated from competition with the ancestor at the conditions of the evolution when the evolved cultures are 50% at the start of the competition.

There is no significant difference in the increase in fitness between pH 7.5 and pH 6.5 (wilcoxon rank sum test, p>0.4, n₁=n₂=8)

Figure S 4.5 Diameter on a plate after 72 hours.

Dark gray is the ancestor, medium gray are clones evolved at pH 6.5, light gray are clones evolved at pH 7.5. All evolved lineages spread less than the ancestor (Welch two sample t-test, p<0.001, n₁=n₂=3, for each evolved clone), demonstrating decreased cell motility
### Table S 4.1 Novel mutations in clones evolved at pH 7.5.

<table>
<thead>
<tr>
<th>clone</th>
<th>position</th>
<th>mutation</th>
<th>Annotation*</th>
<th>gene</th>
<th>description</th>
</tr>
</thead>
<tbody>
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<td>outer membrane protein OprE3</td>
</tr>
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<td>C2</td>
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<td>A -&gt; C</td>
<td>R401R (CGA -&gt; CGC)</td>
<td>for</td>
<td>fosmidomycin resistance protein</td>
</tr>
<tr>
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<td>coding (972-983/1293 nt)</td>
<td>Pst_2380</td>
<td>porin</td>
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<td>+C</td>
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<td>flrR</td>
<td>flagellar biosynthesis protein FlrR</td>
</tr>
<tr>
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<td>noncoding (1207/2901 nt)</td>
<td>Pst_2318</td>
<td>23S ribosomal RNA</td>
</tr>
<tr>
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<td>9bp x 2</td>
<td>duplication</td>
<td>flIP</td>
<td>flagellar MS-ring protein</td>
</tr>
<tr>
<td>C3</td>
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<td>10bp x 2</td>
<td>duplication</td>
<td>Pst_3150</td>
<td>hypothetical protein</td>
</tr>
<tr>
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<td>D51Y (GAC -&gt; TAC)</td>
<td>phoP</td>
<td>two-component response regulator PhoP</td>
</tr>
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<td>flagellar hook protein FliE</td>
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<td>porin</td>
</tr>
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<td>hypothetical protein / general stress protein</td>
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<td>Intergenic (-21/-75)</td>
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<td>two-component response regulator PhoP</td>
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<td>D3</td>
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<td>Δ1 : RP10 (-) +3bp : +C</td>
<td>coding (2-4/681 nt)</td>
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<td>R702R (CGA -&gt; CGG)</td>
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<td>+TGA</td>
<td>coding (944/1005 nt)</td>
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<td>basal-body rod modification protein FliD</td>
</tr>
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<td>+TGA</td>
<td>coding (944/1005 nt)</td>
<td>Pst_3150</td>
<td>hypothetical protein</td>
</tr>
</tbody>
</table>

Definitions: C clones, clones evolved from the ancestral strain 1601ech. D clones, clones evolved from the ancestral strain 1601gp.

*The type of change that the mutation have caused. D51Y (GAC -> TAC) means that a base pair substitution from an G to a T has caused amino acid 51 in this protein to change from aspartic acid (D) to tyrosine (Y). * signifies a stop codon. Intergenic (+186/-26) means that this mutation is located 186 base pairs downstream of one gene and 26 base pairs upstream of another. Coding (972-983/1293 nt) means the mutation is in (between) base pairs 972-983 out of 1293 nucleotides in the coding region of a protein.
Table S 4.2 Novel mutations in clones evolved at pH 6.5.

<table>
<thead>
<tr>
<th>clone</th>
<th>position</th>
<th>mutation</th>
<th>annotation*</th>
<th>gene</th>
<th>description</th>
</tr>
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<tbody>
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<td>LI78F (TTC → TCC)</td>
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<td>two-component sensor NarX</td>
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<td>pyruvate kinase</td>
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<td>flagellum-specific ATP synthase</td>
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<td>outer membrane OprQ</td>
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<td>A95P (GCC → CCC)</td>
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<td>C→T</td>
<td>A317T (GGG → AGG)</td>
<td>pykA</td>
<td>pyruvate kinase</td>
</tr>
<tr>
<td>H2</td>
<td>1,510,239</td>
<td>RP6(+)6bp</td>
<td>coding (726-731/1773nt)</td>
<td>PST_1412</td>
<td>EAL/GGDEF domain-containing protein</td>
</tr>
<tr>
<td>H2</td>
<td>1,575,460</td>
<td>G→A</td>
<td>K89K (AAG → AAA)</td>
<td>PST_1469</td>
<td>type I restriction-modification system, M subunit</td>
</tr>
<tr>
<td>H2</td>
<td>1,623,732</td>
<td>G→A</td>
<td>L383L (CTG → CTA)</td>
<td>PST_1694</td>
<td>acyl-CoA dehydrogenase</td>
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<tr>
<td>H2</td>
<td>1,948,354</td>
<td>Δ1bp</td>
<td>coding (600/1440nt)</td>
<td>cydA2</td>
<td>cytochrome d ubiquinol oxidase, subunit I</td>
</tr>
<tr>
<td>H2</td>
<td>2,332,524</td>
<td>G→A</td>
<td>R259Q (GGG → GAG)</td>
<td>PST_2148</td>
<td>hypothetical protein</td>
</tr>
<tr>
<td>H2</td>
<td>2,404,122</td>
<td>C→T</td>
<td>C3C (TGC → TGT)</td>
<td>PST_2202</td>
<td>hypothetical protein</td>
</tr>
<tr>
<td>H2</td>
<td>2,532,546</td>
<td>C→T</td>
<td>noncoding (1207/2901nt)</td>
<td>PST_2318</td>
<td>23S ribosomal RNA</td>
</tr>
<tr>
<td>H2</td>
<td>2,812,452</td>
<td>T→C</td>
<td>E299G (GAG → GGG)</td>
<td>fiF</td>
<td>flagellar MS-ring protein</td>
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<tr>
<td>H2</td>
<td>3,039,855</td>
<td>Δ3bp</td>
<td>coding (194-196/1296nt)</td>
<td>oprQ</td>
<td>outer membrane protein OmpE3</td>
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<tr>
<td>H2</td>
<td>3,086,463</td>
<td>G→A</td>
<td>R89F (TTC → TTT)</td>
<td>PST_2869</td>
<td>gamma-glutamyltranspeptidase</td>
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<tr>
<td>H2</td>
<td>3,495,820</td>
<td>G→A</td>
<td>R423C (GGC → GAC)</td>
<td>PST_3235</td>
<td>alpha-alpha-trehalose-phosphate synthase</td>
</tr>
<tr>
<td>H2</td>
<td>3,590,596</td>
<td>C→T</td>
<td>G131D (GGT → GAT)</td>
<td>folP</td>
<td>dihydropteroate synthase</td>
</tr>
<tr>
<td>H2</td>
<td>3,937,070</td>
<td>T→C</td>
<td>D262G (GAC → GGC)</td>
<td>PST_3647</td>
<td>hypothetical protein</td>
</tr>
<tr>
<td>H2</td>
<td>3,942,203</td>
<td>C→T</td>
<td>R291R (GGA → GGG)</td>
<td>PST_3650</td>
<td>hypothetical protein</td>
</tr>
<tr>
<td>H2</td>
<td>4,391,436</td>
<td>C→T</td>
<td>S34F (TCT → TTC)</td>
<td>tctD</td>
<td>transcriptional regulatory protein TctD</td>
</tr>
<tr>
<td>H3</td>
<td>379,227</td>
<td>A→G</td>
<td>H39R (CAC → CGG)</td>
<td>fbp</td>
<td>fructose-1,6-bisphosphatase</td>
</tr>
<tr>
<td>H3</td>
<td>442,534</td>
<td>G→C</td>
<td>N188K (AAC → AAG)</td>
<td>phoP</td>
<td>two-component response regulator PhoP</td>
</tr>
<tr>
<td>H3</td>
<td>2,772,535</td>
<td>Δ1bp</td>
<td>coding (71/151nt)</td>
<td>fleQ</td>
<td>transcriptional regulator FleQ</td>
</tr>
<tr>
<td>H3</td>
<td>3,040,113</td>
<td>T→C</td>
<td>L151P (CTG → CGG)</td>
<td>oprQ</td>
<td>outer membrane protein OmpE3</td>
</tr>
<tr>
<td>H4</td>
<td>379,374</td>
<td>C→T</td>
<td>A80V (GCT → GTT)</td>
<td>fbp</td>
<td>fructose-1,6-bisphosphatase</td>
</tr>
<tr>
<td>H4</td>
<td>2,771,479</td>
<td>T→C</td>
<td>N376S (AAC → AGC)</td>
<td>fleQ</td>
<td>transcriptional regulator FleQ</td>
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<tr>
<td>H4</td>
<td>3,040,113</td>
<td>T→C</td>
<td>L151P (CTG → CGG)</td>
<td>oprQ</td>
<td>outer membrane protein OmpE3</td>
</tr>
<tr>
<td>H4</td>
<td>3,993,470</td>
<td>G→A</td>
<td>P66S (CCG → TGG)</td>
<td>PST_3693</td>
<td>tetrahydrofolate reductase subunit A</td>
</tr>
<tr>
<td>H4</td>
<td>4,231,226</td>
<td>T→C</td>
<td>H18V (ATT → GTT)</td>
<td>PST_3901</td>
<td>hypothetical protein</td>
</tr>
</tbody>
</table>

Definitions: G clones, clones evolved from the ancestral strain 1601ech. H clones, clones evolved from the ancestral strain 1601gfp.
The type of change that the mutation have caused. L17BF (CTC -> TTC) means that a base pair substitution from an C to a T has caused amino acid 178 in this protein to change from leucine (L) to phenylalanine (F). * signifies a stop codon. Intergenic (+45/-407) means that this mutation is located 45 base pairs downstream of one gene and 407 base pairs upstream of another. Coding coding (941-952/1293 nt) means the mutation is in (between) base pairs 941-952 out of 1293 nucleotides in the coding region of a protein.

**Table S 4.3 Strains and plasmids.**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. stutzeri</strong> strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1601<strong>gfp</strong></td>
<td>A1501 with ΔcomA and mini-Tn7-LAC-Gm-egfp; Gm, egfp*</td>
<td>This study</td>
</tr>
<tr>
<td>A1601<strong>ech</strong></td>
<td>A1501 with ΔcomA and mini-Tn7-LAC-Gm-echerry; Gm6, echerry*</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli</strong> strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α/λpir</td>
<td>Used for replication of pUC18T and pAW19 derivatives; λpir80dlacZΔM15 Δ(lacZYA-argG)U169 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA</td>
<td>(Miller and Mekalanos, 1988)</td>
</tr>
<tr>
<td>SM10/λpir</td>
<td>Used for replication of pUX-BF13; thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu, Km8, λpir</td>
<td>(Miller and Mekalanos, 1988)</td>
</tr>
<tr>
<td>BW20767</td>
<td>Used for conjugal transfer of pAW19 derivatives; RP4-2-Tc::Mu-1 Kan::Tn7 integrant leu-63::IS10 recA1 zbf-5 creB510 hsdR17 endA1 thi uildA (ΔM15)::pir*</td>
<td>(Metcalf et al., 1995)</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC18T-mini-Tn7T-LAC-Gm</td>
<td>pUC18-based conditionally replicative delivery plasmid for mini-Tn7-LAC-Gm; Ap6, Gm6, mob*</td>
<td>(Choi et al., 2005)</td>
</tr>
<tr>
<td>pUC18T-mini-Tn7T-LAC-Gm-egfp</td>
<td>pUC18T-mini-Tn7T-LAC-Gm containing egfp immediately downstream of Ploc; Ap6, Gm6, mob<em>egfp</em></td>
<td>This study</td>
</tr>
<tr>
<td>pUC18T-mini-Tn7T-LAC-Gm-echerry</td>
<td>pUC18T-mini-Tn7T-LAC-Gm containing echerry immediately downstream of Ploc; Ap6, Gm6, mob<em>echerry</em></td>
<td>This study</td>
</tr>
<tr>
<td>pUX-BF13</td>
<td>R6K replicon-based helper plasmid that provides the Tn7T transposition function in trans; Ap6, mob*</td>
<td>(Bao et al., 1991)</td>
</tr>
</tbody>
</table>

**Table S 4.4 Oligonucleotide PCR primers used for cloning the egfp, echerry, or ecfp gene into the pUC18T-mini-Tn7T-LAC-Gm plasmid.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>*Primer sequence (5'-3')</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>egfp</td>
<td>Forward</td>
<td>CGGGGATCCCTGATTAACCTTTATAAGGAGGAAAAAC ATATGAGTAAAGGAGAAGAACTTTTACACT</td>
<td>BamHI</td>
</tr>
<tr>
<td>Reverse</td>
<td>CGGGGATCCCTTCTGATAGTATCCTACTCCATGTTGAGG</td>
<td>KpnI</td>
<td></td>
</tr>
<tr>
<td>echerry</td>
<td>Forward</td>
<td>CGGGGATCCCTGATTAACCTTTATAAGGAGGAAAAAC ATATGAGTAAAGGAGAAGAACTTTTACACT</td>
<td>BamHI</td>
</tr>
<tr>
<td>Reverse</td>
<td>CGGGGATCCCTTCTGATAGTATCCTACTCCATGTTGAGG</td>
<td>KpnI</td>
<td></td>
</tr>
</tbody>
</table>

5 Substrate cross-feeding affects the trajectory but not the pace of evolutionary adaptation.

5.1 Abstract

Substrate cross-feeding is common in microbial populations, but the evolutionary consequences of such interactions have thus far been less investigated. Here, we use experimental evolution of isogenic nitrite cross-feeding cell-types and completely denitrifying cell-types to investigate how substrate cross-feeding interactions affect the pace and trajectory of molecular evolution. To achieve this, we performed an evolution experiment at both low and high nitrite reactivity (pH 7.5 and pH 6.5). We imposed a commensal substrate cross-feeding interaction at pH 7.5, where the nitrite-consuming strain depends on the nitrite-producing strain for its growth substrate. We imposed a mutualistic substrate cross-feeding interaction at pH 6.5, where the nitrite-consuming strain depends on the nitrite-producing strain for its growth substrate while the nitrite-producing strain depends on the nitrite-consuming strain to reduce the inhibitory effects of nitrite. We found that substrate cross-feeding did not accelerate molecular evolution through co-evolutionary interactions: substrate cross-feeding clones did not fix more mutations than completely-denitrifying clones. However, substrate cross-feeding did result in the fixation of more mutations at the population level, likely because it prevented clonal interference. Moreover, substrate cross-feeding lead to different adaptations (i.e. mutations in genes encoding for different functions) than those observed for complete denitrification. The difference between substrate cross-feeding and complete denitrification were pronounced at high nitrite reactivity where it is clear that completely denitrifying cell-types and nitrite cross-feeding cell-types use distinctly different mechanisms to deal with nitrite reactivity. Taken together, our data demonstrates that substrate cross-feeding affects the trajectory but not the pace of molecular evolution.
5.2 Introduction

Substrate cross-feeding interactions, where different cell-types consume metabolites produced by other cell-types, is frequently observed within microbial communities (De Vuyst and Leroy, 2011; Seth and Taga, 2014; Martienssen and Schöps, 1999; Schink, 1997; McInerney et al., 2009; Pelz et al., 1999). Thus, substrate cross-feeding interactions have likely shaped and influenced the evolution of microorganisms in the environment. However, there are few experimental tests on how substrate cross-feeding itself influences evolution (Hillesland and Stahl, 2010; Hillesland et al., 2014). Substrate cross-feeding interactions could accelerate evolution because cells must coexist with and adapt to other cell-types as well as to the abiotic characteristics of the environment. For example, substrate cross-feeding interactions could accelerate the loss of functions over time as the cross-feeding cell-types develop further dependencies on each other (Morris et al., 2012). Moreover, if one cell-type changes its output with regards to the cross-fed substrate, than the other cell-type might have to adapt to that change. Finally, even if the substrate cross-feeding interaction is obligate, there may also be competition for other common resources, which could further accelerate evolution. If cell-types are interacting more strongly (for example mutualism (Hillesland and Stahl, 2010) compared to commensalism (Christensen et al., 2002)) then this could have even stronger effects on the adaptive rate of the cell-types to each other. Indeed, in the case of strong antagonistic interactions, coevolution of antagonistically interacting bacteria and phage has been shown to accelerate the pace of evolution (Paterson et al., 2010).

Here, our main objective was to compare the evolutionary pace and trajectory of substrate cross-feeding cell-types to that of an isogenic completely-consuming cell type. In this context, we use the term “pace of molecular evolution” to refer to the number of mutations fixed per generation and not the number of mutations fixed per unit time. We hypothesized that the substrate cross-feeding interaction will accelerate molecular evolution. In addition, we investigated how the strength of the substrate cross-feeding interaction affects the pace of molecular evolution, where increasing the strength of the interaction should further accelerate molecular evolution. To achieve this objective we used the experimental system described below (but also established in Chapters 3 and 4) that allowed us to manipulate the strength of the substrate cross-feeding interaction by manipulating the toxicity of the cross-fed intermediate.

Our experimental system is based on the bacterium Pseudomonas stutzeri A1501, which is a facultative anaerobic bacterium with a fully sequenced genome (Yan et al., 2008). Wild-type P. stutzeri A1501 uses N-oxides as terminal electron acceptors in the absence of oxygen and expresses all of the enzymes required to completely reduce nitrate (NO$_3^-$) via nitrite (NO$_2^-$), nitric oxide (NO) and nitrous oxide (N$_2$O) into di-nitrogen gas (N$_2$) (Zumft, 1997; Lalucat et al., 2006; Yan et al., 2008). The denitrification enzymes are encoded by separate operons (Yan, 2005) (nar encodes the nitrate reduction, nir encodes the nitrite reduction, nor encodes the nitric oxide reduction and nos encodes the nitrous oxide reduction) making it possible to delete different steps of the denitrification pathway from
different strains. We can then use the deletion mutants to create nitrite-producing (strain A1603) and nitrite-consuming (strain A1602) strains that can be assembled together into nitrite cross-feeding co-cultures (Chapter 3).

The key feature of our experimental system is that nitrite becomes increasingly reactive at lower pH (Sijbesma et al., 1996; Zhou et al., 2011; Zumft, 1993) and can cause severe growth inhibition for a variety of different bacteria (Almeida, Julio, et al., 1995), including P. stutzeri A1501 (Chapters 3, and 4). In general, nitrite has no growth-inhibiting effects at pH 7.5 but severe growth-inhibiting effects at pH 6.5. We could therefore impose a weak cross-feeding interaction by growing co-cultures at pH 7.5 (the nitrite-consuming strain depends on the nitrite-producing strain to provide its substrate) or a strong cross-feeding interaction by growing co-cultures at pH 6.5 (the nitrite-consuming strain depends on the nitrite-producing strain to provide its substrate while the nitrite-producing strain depends on the nitrite-consuming strain to detoxify its local environment). We then used experimental evolution by serial batch transfer (also described in Chapter 4) to compare the evolution of substrate cross-feeding cell-types to completely-consuming cell-types at both low (pH 7.5) and high (pH 6.5) nitrite reactivity.

Figure 5.1 Strains used for experimental evolution.

Arrows indicate the metabolic processes that are performed by each strain. The colors of the arrows indicate whether the strain expresses the echerry or egfp fluorescent protein. Definitions: Nar, nitrate reductase encoded by the nar gene cluster; Nir, nitrite reductase encoded by the nir gene cluster; Nor, nitric oxide reductase encoded by the nor gene cluster; Nos, nitrous oxide reductase encoded by the nos gene cluster.
5.3 Materials and methods

Bacterial strains, genetic manipulations, and growth conditions
We obtained wild-type *P. stutzeri* A1501 (Yan et al., 2008) from the Biological Resource Center of Institut Pasteur (www.crbip.pasteur.fr) and used this strain to construct all of the isogenic mutant strains used in this study. The genetic modifications of all mutant strains are summarized in Table S5.3. In brief, we deleted the *narG* gene to prevent nitrate (NO$_3^-$) reduction (designated as the nitrite-consuming strain [strain A1602]) and the *nirS* gene to prevent nitrite (NO$_2^-$) reduction (designated as the nitrite-producing strain [A1603]) as described elsewhere (see Chapter 3). We additionally deleted the *comA* gene from all the strains to prevent the internalization of extracellular DNA as described elsewhere (see Chapter 3). The main purpose for deleting *comA* was to reduce the probability that the nitrite-consuming (strain A1602) and –producing (strain A1603) strains would recombine with each other via natural transformation when grown together. Finally, we introduced DNA fragments that contain the isopropyl-β-D-thiogalactopyranosid (IPTG)-inducible P$_{lac}$ promoter located immediately upstream of the *egfp* or *echerry* (Minoia et al., 2008) gene into the nitrite-consuming strain (strain A1602) and the nitrite-producing strain (strain A1603). The *egfp* and *echerry* genes encode green or red fluorescent proteins, respectively. We introduced the DNA fragments using derivatives of the mini-Tn7T-LAC-Gm transposon and the pUC18T conditionally replicative delivery plasmid as described elsewhere (see Chapter 4).

We cultivated all *P. stutzeri* strains under aerobic conditions with a completely defined asparagine-citrate synthetic medium (ACS medium) (Coyle et al., 1985) in 1-ml mixed batch reactors. We cultivated all *P. stutzeri* strains under anaerobic conditions with di-nitrogen gas (N$_2$)-sparged ACS medium in 25-ml serum bottles fitted with gas-tight stoppers. We provide a detailed description of methods to prepare and inoculate anaerobic ACS medium elsewhere (see Chapter 3). We incubated all *P. stutzeri* cultures at 30°C with shaking at 220 rpm.

Experimental evolution
We experimentally evolved a total of eight nitrite (NO$_2^-$) cross-feeding populations at each pH condition (pH 6.5 and 7.5) for a total of 16 populations. Half of the populations for each pH condition consisted of the nitrite-consuming strain (strain A1602) carrying the *egfp* gene and the nitrite-producing strain (strain A1603) carrying the *echerry* gene (Table S5.3). The other half of the populations for each pH condition consisted of the nitrite-consuming strain (strain A1602) carrying the *echerry* gene and the nitrite-producing strain (strain A1603) carrying the *egfp* gene (Table S5.3). We did not add IPTG to the medium during experimental evolution to avoid the cost of expressing the fluorescent proteins, and to therefore minimize the probability of selecting for loss-of-function mutations in the *egfp* or *echerry* gene. We used both combinations of *egfp* and *echerry*-expressing traits to periodically assess for cross-contamination between populations and to control for potential
differential effects of the different fluorescent proteins on the evolutionary outcome.

For the evolution experiment, we streaked the nitrite (NO\(_2\))-consuming strain (strain A1602), and the nitrite-producing strain (strain A1603) onto lysogeny broth (LB) agar plates, inoculated one colony of each strain into a different test-tube containing 1 ml of aerobic ACS medium set to pH 7.5 or 6.5, and incubated the test-tubes for 24 hours at 30°C with continuous shaking (220 rpm). We then mixed the nitrite-producing (strain A1603) and –consuming (strain A1602) strains together at a 50:50 vol:vol mixture and diluted the mixture at a dilution of 1:25 (vol:vol) into serum bottles containing anaerobic ACS medium amended with 10 mM sodium nitrate to achieve a final volume of 20 ml. We next serially transferred batch cultures containing di-nitrogen gas (N\(_2\))-sparged ACS medium amended with 10 mM of sodium nitrate (NaNO\(_3\)) as the growth-limiting substrate. We transferred each population after entering stationary phase at a dilution of 1:200 vol:vol (except for a few exceptions in the beginning of the evolutionary experiment when growth was very slow and highly variable) for a total of 700 generations.

**Sequencing**

We streaked each evolved and ancestral population of nitrite cross-feeding strains onto LB agar plates containing 10 \(\mu\)g ml\(^{-1}\) of gentamicin and 0.1 mM of IPTG and picked a single colony of the nitrite-producing and nitrite-consuming strains (each colony expressed a different fluorescent protein) from each population for whole genome sequencing. We grew the single clones in LB medium overnight and extracted the DNA with a Wizard Genomic DNA purification kit (Promega, Madison, WI). We then sent the extracted DNA for sequencing at the ETH Quantitative Genomics Facility (Basel, Switzerland). There, the genomes were sequenced with Illumina sequencing (HiSeq 2000, paired-end 100bp), and primary data analysis, de-multiplexing and quality control analysis of the sequencing data using FastQC (Illumina, San Diego, CA) were performed.

**Identification of novel mutations in the evolved clones**

We analyzed the genomic data further in collaboration with the ETH Genomic Diversity Center (Zürich, Switzerland). Quality filtering of the raw reads was done using PRINSEQ-lite v0.20.4 (Schmieder and Edwards, 2011), and duplicate reads were removed and ambiguous base pairs were trimmed. Breseq v.0.24rc5 (Deatherage and Barrick, 2014) was used to identify differences between the evolved genomes and the reference genomes of strains A1602 or A1603.
5.4 Results

Substrate cross-feeding does not accelerate evolution
We first tested whether nitrite cross-feeding interactions lead to more mutations fixed per generation than complete denitrification. To achieve this, we plated the evolved populations on LB agar plates, randomly picked one nitrite-producing colony and one nitrite-consuming colony from each population, and quantified the number of mutations within these clones. We then compared the number of mutations that were acquired in these clones with the number of mutations that were acquired by randomly selected clones from each evolved completely-denitrifying population (see Chapter 4). We first made this comparison for clones evolved at pH 7.5. We found that there was no difference in the number of mutations in the nitrite cross-feeding clones as compared to the completely denitrifying clones (Wilcoxon rank-sum test, \( P=1, n_1=n_2=8 \)) (Fig. 5.2).

![Figure 5.2](image)

Figure 5.2 The number of mutations in single randomly picked clones after 700 generations of evolution at pH 7.5.

The horizontal bar and \( P \)-value indicate the outcome of a two-sample Wilcoxon rank-sum test. Data are presented as Tukey box-plots.

We next performed the same comparison for the clones evolved at pH 6.5. As discussed in Chapter 4, one of the completely-consuming clones fixed significantly more mutations than the other clones, most likely due to a mutation in the \( uvrA \) gene. This gene is involved in DNA repair in other bacteria (Goosen and Moolenaar, 2001; Jaciuk et al., 2011). We therefore made the comparison both including and excluding this clone. When the completely-denitrifying clone containing the mutation in the \( uvrA \) gene was excluded from the test, the completely-denitrifying clones had significantly more mutations than the nitrite-
producing clones (Wilcoxon rank-sum test, \( P<0.005, n_1=7, n_2=8 \)) but not more than the nitrite-consuming clones (Wilcoxon rank-sum test, \( P>0.6, n_1=7, n_2=8 \)) (Fig. 5.3A). When we included the completely-denitrifying clone with a mutation in the \( uvrA \) gene into the test, the completely-denitrifying clones had significantly more mutations than both the nitrite-producing and –consuming clones (Wilcoxon rank-sum test, \( P<0.005, n_1=n_2=8 \)) (Fig. 5.3B). Taken together, we have no empirical evidence to support our hypothesis that substrate cross-feeding should accelerate the pace of molecular evolution, and instead our data points towards the opposite outcome when nitrite imposes strong growth-inhibiting effects (i.e., pH 6.5).

**Figure 5.3** The number of mutations in single randomly picked clones after 700 generations of evolution at pH 6.5.

The horizontal bar and \( P \)-value indicate the outcome of a two-sample Wilcoxon rank-sum test. The star indicates a \( P \)-value less than 0.05. Data are presented as Tukey box-plots.

**Substrate cross-feeding leads to more mutations within populations than complete consumption**

We next compared the sums of mutations in the nitrite-producing and –consuming clones obtained from the same populations to the numbers of mutations in the completely-consuming clones from the same evolutionary conditions (i.e., the same pH). At pH 7.5, we found that the sums of mutations among the nitrite-producing and –consuming clones from the same populations were significantly greater than the numbers of mutations in the completely consuming populations (Wilcoxon rank-sum test, \( P<0.01, n_1=n_2=8 \)), (Fig. 5.4). This is true at pH 6.5 if the clone with the mutation in the \( uvrA \) gene is removed from the analysis (Wilcoxon rank-sum test, \( P<0.05, n_1=7, n_2=8 \)) (Fig. 5.5A) but is not true if that clone is retained in the analysis (Wilcoxon rank-sum test, \( P>0.1, n_1=n_2=8 \)) (Figure 5.5B). Our results therefore provide weak evidence that substrate cross-feeding accelerates the pace of molecular evolution at the population level but not at the individual level. This conclusion assumes that two randomly selected clones from the completely-consuming populations are likely to be genetically identical (i.e., that the completely-consuming populations are dominated by a single genotype).
**Figure 5.4** The sum of mutations in the nitrite-consuming and –producing clones from the same population compared to the number of mutations in the completely consuming clones after 700 generations of evolution at pH 7.5.

The horizontal bar and \( P \)-value indicate the outcome of a two-sample Wilcoxon rank-sum test. The star indicates a \( P \)-value less than 0.05. Data are presented as Tukey box-plots.

**Figure 5.5** The sum of mutations in the nitrite-consuming and –producing clones from the same population compared to the number of mutations in the completely consuming clones after 700 generations of evolution at pH 6.5.

The horizontal bar and \( P \)-value indicate the outcome of a two-sample Wilcoxon rank-sum test. The star indicates a \( P \)-value less than 0.05. Data are presented as Tukey box-plots.
Increased nitrite reactivity leads to more mutations in nitrite consumers but not in nitrite producers

We next compared the numbers of mutations in the nitrite cross-feeding clones to determine if increased nitrite reactivity leads to more mutations in nitrite cross-feeding clones as well as in completely-consuming clones (see Chapter 4). First, we compared the sums of mutations in the nitrite –consumer and –producer clones evolved at pH 6.5 with the sums of mutations in the nitrite –producer and –consumer clones evolved at pH 7.5 and found that this difference was not significant (Wilcoxon rank-sum test, $P>0.05$, $n_1=n_2=8$), (Fig. 5.6). Second, we compared the numbers of mutations in nitrite-producing clones evolved at pH 7.5 to the numbers of mutations in nitrite-producing clones evolved at pH 6.5 and found no difference (Wilcoxon rank-sum test, $P<0.5$, $n_1=n_2=8$), (Fig. 5.7A). Third, we compared the numbers of mutations in consuming clones evolved at pH 7.5 to the numbers of mutations in nitrite consuming clones evolved at pH 6.5 and found that there were significantly more mutations in the consuming clones evolved at pH 6.5 than at pH 7.5 (Wilcoxon rank-sum test, $P<0.05$, $n_1=n_2=8$), (Fig. 5.7B). Thus, it seems that increased nitrite reactivity leads to more mutations only in the nitrite-consuming cells when nitrite is cross-fed.

*Figure 5.6 The sum of mutations in the nitrite-consuming and –producing clones from the same population after 700 generations of evolution at pH 7.5 compared to the sum of mutations in the nitrite-consuming and –producing clones from the same population compared number of mutations in the completely consuming clones after 700 generations of evolution at pH 6.5.*

The horizontal bar and $P$-value indicate the outcome of a two-sample Wilcoxon rank-sum test. The star indicates a $P$-value less than 0.05. Data are presented as Tukey box-plots.
Figure 5.7 Comparison of the number of mutations in cross-feeding clones after 700 generations at pH 7.5 and pH 6.5.

The number of mutations in nitrite producers evolved at pH 7.5 compared to the number of mutations in nitrite producers evolved at pH 6.5 (A). The number of mutations in nitrite consumer evolved at pH 7.5 compared to the number of mutations in nitrite consumers evolved at pH 6.5 (B). The horizontal bar and P-value indicate the outcome of a two-sample Wilcoxon rank-sum test. The star indicates a P-value less than 0.05. Data are presented as Tukey box-plots.

Increased nitrite reactivity leads to different adaptations in cross-feeding clones than in completely-consuming clones

We next asked whether the mutations identified within nitrite cross-feeding populations have different functional annotations than those identified in completely-denitrifying populations. Stated alternatively, is there evidence for evolution in direct response to the cross-feeding interaction? To test this, we categorized and compared genes with mutations by their functional annotations (Winsor et al., 2011; Yan et al., 2008). At pH 7.5, we found that both completely-consuming and nitrite cross-feeding clones had mutations in genes involved with cell motility, signal transduction and unknown functions (Fig. 5.8). Nitrite cross-feeding clones also sometimes acquired mutations in genes with other functional annotations (for example secondary metabolite biosynthesis, transport and catabolism and cell wall/membrane/envelope biosynthesis). However, mutations in each of these additional functional categories were rare and none were detected in more than three clones (usually just in one), making it difficult to draw any general conclusions. It seems that the most important adaptations occurring at pH 7.5 (low nitrite reactivity) do not differ significantly whether nitrite is completely consumed within one cell-type or if nitrite is cross-fed between different cell-types. However, mutations in a few specific genes were reoccurring in the nitrite cross-feeding clones and were never detected in the completely-consuming clones (PST_3282 and lpxC, see Table S5.1).
Next, we compared the mutations that were identified in clones after 700 generations of evolution at pH 6.5 (high nitrite reactivity) (Fig 5.9). Here, mutations in genes encoding for proteins involved in cell motility, signal transduction and unknown functions were also frequently acquired in both completely-consuming clones and nitrite cross-feeding clones. There were also mutations in genes encoding for proteins involved in additional functions in both completely-consuming clones and nitrite cross-feeding clones. Most of these functional categories only include genes that were acquired by very few clones, with two notable exceptions. First, all of the completely-consuming clones acquired non-synonymous point mutations in genes encoding for proteins involved in carbon metabolism (*pykA*, *fbp* or *gap-2*) (Table S4.2). Second, all of the nitrite-consuming clones acquired a point mutation in the intergenic region downstream of *nirS* (cytochrome cd1 nitrite reductase) and the upstream region of *nirT* (tetraheme cytochrome nirT) (Table S5.2). These genes therefore encode for proteins directly involved in nitrite reduction. In Fig. 5.9, these mutations are annotated to energy production and conversion. In addition, mutations in *narX* or *narL*, which encode for a two-component response sensor and regulator.
respectively (Härtig et al., 1999), were found in 7 out of 8 of the nitrite-consuming clones (Table S5.2) but only in one completely-consuming clone (Table S4.2). This means that at high nitrite reactivity where nitrite has strong inhibitory effects on growth, there are important adaptations that differ significantly between completely-consuming and nitrite cross-feeding cell-types.

**Figure 5.9** Number of mutations in clones evolved for 700 generations at pH 6.5 sorted by functional annotation.

The horizontal bar and *P*-value indicate the outcome of a two-sample Wilcoxon rank-sum test. Stars indicate a *P*-value less than 0.05. Data are presented as Tukey box-plots.
5.5 Discussion

Our results do not support the hypothesis that substrate cross-feeding accelerates the pace of molecular evolution. Instead, the consequence of substrate cross-feeding on evolution is that it changes the targets of beneficial mutations, but only significantly if the intermediate that is cross-fed has inhibitory effects and changes the relative efficiency of cross-feeding compared to complete consumption.

While we did not observe more mutations in individuals that cross-feed, we did observe more mutations in populations that cross-feed (i.e., the sum of mutations among the nitrite-producing and –consuming populations is greater than the number of mutations in completely-consuming populations). The underlying cause of this is likely because nitrite-cross-feeding prevents clonal interference between the nitrite-producing and –consuming cells. Clonal interference occurs when beneficial mutations occur in different clones. In the absence of recombination (which would allow the mutations to eventually end up in the same clone) this leads to competition between beneficial mutations. This will thus allow only the most beneficial mutation to persist in the population (Maddamsetti et al., 2015; Desai et al., 2007). Thus, as the nitrite-producing and consuming clones do not share the same niche space, they cannot outcompete each other in this experimental system, thus preventing clonal interference between the two cell-types and allowing more mutations to persist in the population. That this effect seems weak when nitrite reactivity is high might be due to the fact that the completely-consuming cells started at a much lower fitness than the nitrite cross-feeding cells at the beginning of experimental evolution (Chapter 3), which may lead to increased availability of beneficial mutations (see Chapter 4), or simply because the mutations in response to increased nitrite reactivity may only be possible in the nitrite-consuming strain and not in the nitrite-producing strain. Considering the fact that the mutation in the nir operon was acquired in every sequenced nitrite-consuming clone evolved at high nitrite reactivity, but no such pattern is evident for the nitrite producing clones, this is a strong possibility. In addition, high nitrite reactivity also lead to more mutations in the nitrite-consuming strains but not in the nitrite-producing strains, which further supports the idea that mutations in response to increased nitrite reactivity are only likely to occur in cells which consume nitrite.

At increased nitrite reactivity there are different mechanisms for adaptation to nitrite reactivity depending on whether nitrite is cross-fed or not. Specifically, mutations in carbon metabolism always occurred in completely-consuming clones and mutations in denitrification genes always occurred in the nitrite-consuming clones. If the mutations that we identified in only the completely-consuming clones alleviates competition between the nitrite and nitrate reductases (see Chapter 3) and thus leads to lower accumulation of nitrite during denitrifying growth, then these types of mutations would have little effect in nitrite cross-feeding clones when intra-enzyme competition is already eliminated (see Chapter 3). Furthermore, if the mutation in the nir operon causes the nitrite reductase enzyme to simply have a higher catalytic rate then this mutation may be less beneficial in completely-consuming cells if the competition
between the enzymes is the main factor that limits nitrite reduction during most of the growth cycle. However, one of the evolved completely-consuming clones does have a mutation in the nir operon in the same intergenic region as the nitrite-consuming clone (Table S4.2), although in this case this mutation is a one-basepair deletion instead of a substitution. However, as the mutation is intergenic its phenotypic effect may be similar. To directly test the fitness benefit of these specific mutations (nir operon, carbon metabolism) the mutations could be introduced in the ancestral completely-consuming and nitrite-consuming strains and their fitness effects directly measured. This would then elucidate if the reason for these different mechanisms for adaptation to increased nitrite reactivity is due to the fact that the benefit of the mutations is dependent on whether nitrite is cross-fed or not. All mutations that occur frequently and only occur in the nitrite cross-feeding strains evolved at high reactivity occur in the nitrite-consuming strain, which reinforces the idea that the new opportunities for adaptations in the cross-feeding populations may be due to the fact the nitrite reduction is no longer inhibited by nitrate reduction as in the complete consumer (Chapter 3).

At low nitrite reactivity, the potential difference in the function of mutations of completely-consuming and nitrite cross-feeding clones is not as clear. This suggests that when nitrite does not have negative effects on growth (at pH 7.5) and when nitrite cross-feeding does not change the fitness of the population (see Chapter 3) there are also less significant effects on the adaptive trajectories. However, as some genes had mutations in several clones from only the nitrite cross-feeding populations, this suggests that there are still adaptive mechanisms that might be specific to nitrite cross-feeding even at low nitrite reactivity. This idea is reinforced by the example of mutations in the gene PST_3282, which has a mutation in two cross-feeding clones evolved at low reactivity and in two cross-feeding clones evolved at high reactivity, but no mutation in that gene was identified in any of the completely-consuming clones, indicating that mutations in this gene are specific to nitrite cross-feeding.

In conclusion, substrate cross-feeding does change the trajectory of evolutionary adaptation, and the extent to which this occurs is affected by the growth-inhibiting effects of the cross-fed intermediate. However, biotic interactions between different cell-types did not affect the pace of evolution. Instead, the reactivity of the intermediate seems to cause strong selection pressure regardless of whether the intermeidate is cross-fed or not.
## 5.6 Supplementary materials

### Supplementary tables

**Table S 5.1** Novel mutations in cross-feeding clones evolved at pH 7.5

<table>
<thead>
<tr>
<th>clone</th>
<th>position</th>
<th>mutation</th>
<th>annotation*</th>
<th>gene</th>
<th>description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1 Δnar</td>
<td>2,796,851</td>
<td>C -&gt; T</td>
<td>G176D (GGC -&gt; GAC)</td>
<td>fabA</td>
<td>flagellar biosynthesis protein FabA</td>
</tr>
<tr>
<td>A1 Δnar</td>
<td>2,771,093</td>
<td>A -&gt; G</td>
<td>*505Q (TAG -&gt; GAG)</td>
<td>fecQ</td>
<td>transcriptional regulator FleQ</td>
</tr>
<tr>
<td>A1 Δnar</td>
<td>3,552,142</td>
<td>C -&gt; A</td>
<td>G176V (GGG -&gt; GTA)</td>
<td>PST_3282</td>
<td>fatty acid alpha hydroxylase</td>
</tr>
<tr>
<td>A2 Δnar</td>
<td>733,863</td>
<td>C -&gt; A</td>
<td>intergenic (+902/-360)</td>
<td>PST_0639 PST_0640</td>
<td>type I restriction-modification system / phage Hau3 resistance protein</td>
</tr>
<tr>
<td>A2 Δnar</td>
<td>1,486,000</td>
<td>RP3(+)+1bp</td>
<td>coding (431-433/1317nt)</td>
<td>flgE</td>
<td>flagellar hook protein FlgE</td>
</tr>
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<td>PST_1694</td>
<td>hypothetical protein</td>
</tr>
<tr>
<td>A2 Δnar</td>
<td>3,552,584</td>
<td>G -&gt; T</td>
<td>Q29K (CAG -&gt; AAG)</td>
<td>PST_3282</td>
<td>fatty acid alpha hydroxylase</td>
</tr>
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<td>A2 Δnar</td>
<td>2,063,012</td>
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<td>flagellar MS-ring protein</td>
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<td>G -&gt; A</td>
<td>L175L (CTG -&gt; TTG)</td>
<td>algI</td>
<td>alginate O-acylation protein AlgI</td>
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<td>A2 Δnar</td>
<td>3,392,365</td>
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<td>+TGA</td>
<td>coding (944/1005nt)</td>
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<td>hypothetical protein</td>
</tr>
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<td>flagellar hook protein FlgE</td>
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<td>+CGA</td>
<td>coding (833/1005)</td>
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<tr>
<td>A4 Δnar</td>
<td>443,079</td>
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<td>duplication</td>
<td>phoP</td>
<td>two-component response regulator PhoP</td>
</tr>
<tr>
<td>A4 Δnar</td>
<td>2,808,475</td>
<td>G -&gt; A</td>
<td>Q448* (CAA -&gt; TAA)</td>
<td>fis</td>
<td>flagellum-specific ATP synthase</td>
</tr>
<tr>
<td>A4 Δnar</td>
<td>3,036,324</td>
<td>G -&gt; A</td>
<td>H199Y (CAC -&gt; TAG)</td>
<td>aspS</td>
<td>aspartyl-tRNA synthetase</td>
</tr>
<tr>
<td>A4 Δnar</td>
<td>442,808</td>
<td>T -&gt; A</td>
<td>L97V (GAC -&gt; GTC)</td>
<td>phoP</td>
<td>two-component response regulator PhoP</td>
</tr>
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<td>A4 Δnar</td>
<td>520,300</td>
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<td>R73P (CGC -&gt; GCC)</td>
<td>crc</td>
<td>catabolite repression control protein</td>
</tr>
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<td>A4 Δnar</td>
<td>2,804,423</td>
<td>G -&gt; A</td>
<td>Q254* (TAG -&gt; TAC)</td>
<td>fisK</td>
<td>flagellar hook-length control protein FisK</td>
</tr>
<tr>
<td>B1 Δnar</td>
<td>336,930</td>
<td>A -&gt; G</td>
<td>E102G (GAG -&gt; GGG)</td>
<td>ompR</td>
<td>osmolality response regulator</td>
</tr>
<tr>
<td>B1 Δnar</td>
<td>2,532,546</td>
<td>C -&gt; T</td>
<td>noncoding (1207/2901nt)</td>
<td>PST_2318</td>
<td>23S ribosomal RNA</td>
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<td>B1 Δnar</td>
<td>2,771,721</td>
<td>Δ39bp</td>
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<td>transcriptional regulator FleQ</td>
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<td>L304R (CTG -&gt; AG)</td>
<td>PST_3634</td>
<td>thymidine phosphohydrolase</td>
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<tr>
<td>B1 Δnar</td>
<td>2,804,555</td>
<td>C -&gt; A</td>
<td>E210* (TAA -&gt; TAG)</td>
<td>fisK</td>
<td>flagellar hook-length control protein FisK</td>
</tr>
<tr>
<td>B1 Δnar</td>
<td>3,392,358</td>
<td>A -&gt; C</td>
<td>I543S (ATC -&gt; AGC)</td>
<td>PST_3150</td>
<td>hypothetical protein</td>
</tr>
</tbody>
</table>
| B2 Δnar | 1,199,102 | C -> T | A265V (GGG -> GTG) | lpxC | UDP-3-O-β-D-glucosyl-4-N-
| Δnir | 2,500,449 | A -> G | G395G (GGT -> GCC) | PST,2291 recombination factor protein RarA |
| Δnir | 2,808,847 | C -> T | A324T (GGG -> AGG) | fil flagellum-specific ATP synthase |
| Δnar | 2,809,371 | G -> A | F114L (CGG -> CTG) | fil flagellum-specific ATP synthase |
| Δnir | 3,392,466 | C -> A | G278V (GGC -> GTG) | PST,3150 hypothetical protein |
| Δnar | 4,005,451 | G -> A | K219R (AAG -> AAA) | PST,3703 hypothetical protein |
| Δnir | 2,799,641 | 7bp x 2 | duplication | flIR flagellar biosynthesis protein FlIR |
| Δnar | 3,332,325 | 35bp x 2 | duplication | PST,3097 / PST,3098 hypothetical protein / flagellar biosynthesis protein FlIR |
| Δnar | 3,392,466 | C -> A | G278V (GGC -> GTG) | PST,3150 hypothetical protein |
| Δnar | 1,198,532 | C -> T | T751I (ACT -> ATT) | ipxC UDP-3-O-[3-hydroxymyristoyl]N-acetylglucosamine deacylase |
| Δnar | 2,792,897 | T -> C | Y69C (TAC -> TGC) | flaA flagellar biosynthesis sigma factor |
| Δnar | 4,057,685 | Δ332bp | intergenic (+1212/+764) | PST,3751 / PST,3752 / CRISPR-associated Cas2 family protein / hypothetical protein |
| Δnir | 1,199,102 | C -> T | A265V (GCG -> GTG) | ipxC UDP-3-O-[3-hydroxymyristoyl]N-acetylglucosamine deacylase |
| Δnar | 1,486,023 | RP3(-Δ1)bp | coding (454-456/1317nt) | flaE flagellar protein FlgE hook |
| Δnar | 1,486,453 | Δ88bp | coding (588-893/1317nt) | flaE flagellar protein FlgE hook |
| Δnar | 3,107,691 | C -> A | P359Q (CCG -> GAG) | PST,2886 sensory box kinase/response regulator |
| Δinar | 3,392,466 | C -> A | G278V (GGC -> GTG) | PST,3150 hypothetical protein |

Definitions: A Δnir clones, clones evolved from the ancestral strain 1602gfp; A Δnar clones, clones evolved from the ancestral strain 1603ech. B Δnir clones; clones evolved from the ancestral strain 1602ech. B Δnar clones; clones evolved from the ancestral strain 1603gfp. A1 Δnir and A1 Δnar are nitrite-producing and -consuming clones, respectively, from the same evolved population, A2 Δnir and A2 Δnar are from the same population, A3 Δnir and A3 Δnar are from the same population, etc.

The type of change that the mutation have caused. G176D (GGC -> GAC) means that a base pair substitution from a G to a A has caused amino acid 176 in this protein to change from glycine (G) to aspartic acid (D). * signifies a stop codon. Intergenic (+902/-360) means that this mutation is located 904 base pairs downstream of one gene and 360 base pairs upstream of the next. Coding (431-433/1317nt) means the mutation is in (between) base pairs 431-433 out 1317 nucleotides in the coding region of a protein.
Table S 5.2 Novel mutations in cross-feeding clones evolved at pH 6.5.

<table>
<thead>
<tr>
<th>clone</th>
<th>position</th>
<th>mutation</th>
<th>annotation</th>
<th>gene</th>
<th>description</th>
</tr>
</thead>
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| E1 Δnir | 2,771,801 | T → C | M269V (ATG → GTG) | fleQ | transcriptional regulator }
<p>|         |          |          |            |      | FleQ       |
| E1 Δnir | 4,391,420 | C → T | R29W (CGG → TGG) | tctD | transcriptional regulatory protein TctD |
| E1 Δnir | 4,506,466 | G → A | intergenic (+298/+10) | pgm / adhA | phosphoglucomutase / alcohol dehydrogenase |
| E1 Δnir | 2,075,529 | A → G | I90T (ATC → ACC) | acdA | acyl-CoA dehydrogenase |
| E1 Δnir | 2,808,450 | +G | coding (1367/1380nt) | fbl | flagellum-specific ATP synthase |
| E1 Δnir | 3,818,976 | G → A | intergenic (-54/+16) | nirT / nirS | tetrahydrofuran nitrite reductase |
| E1 Δnir | 4,391,438 | G → T | A35S (GCC → TCC) | tctD | transcriptional regulatory protein TctD |
| E2 Δnir | 5,201,417 | C → G | intergenic (-8j-78) | crc / pyrE | catabolite repression control protein / orotate phosphoribosyltransferase |
| E2 Δnir | 2,795,867 | A → G | L50P (CTG → CGG) | fiaA | flagellar biosynthesis protein FiaA |
| E2 Δnir | 3,379,533 | C → G | P173A (CCG → GG) | envZ | osmolarity sensor protein EnvZ |
| E2 Δnir | 993,754 | T → G | T299P (ACC → GCC) | narX | two-component sensor NarX |
| E2 Δnir | 1,485,633 | Δ1:RP10(–) +3bp::+C | coding (66–68/1317nt) | fgaE | flagellar hook protein FgaE |
| E2 Δnir | 2,599,477 | Δ6bp | coding (997–1002/1293nt) | PST_2380 | porin |
| E2 Δnir | 3,818,976 | G → A | intergenic (-54/+16) | nirT / nirS | tetrahydrofuran nitrite reductase |
| E3 Δnir | 1,108,945 | Δ1bp | coding (386/1446nt) | PST_0997 | sulfite reductase (NADPH) flavoprotein α-component |
| E3 Δnir | 2,772,133 | RP4(+) +8bp::Δ1bp | coding (466–473/1515nt) | fleQ | transcriptional regulator FleQ |
| E3 Δnir | 3,355,746 | G → A | A20T (GCC → ACC) | PST_3117 | hydroxypropionate reductase |
| E3 Δnir | 4,370,532 | Δ15bp | coding (270–284/513nt) | PST_4028 | long-chain acyl-CoA thioester hydrolyase family protein |
| E3 Δnir | 994,349 | A → G | N10N (AAT → ACC) | NarX | two-component sensor NarX |
| E3 Δnir | 1,485,571 | Δ1:RP10(–) +3bp::+C | coding (4–6/1317nt) | fgaE | flagellar hook protein FgaE |
| E3 Δnir | 3,818,977 | C → T | intergenic (-55/+15) | nirT / nirS | tetrahydrofuran nitrite reductase |
| E4 Δnir | 248,848 | A → G | intergenic (-120/129) | PST_0212 / PST0213 | hypothetical protein / hypothetical protein |
| E4 Δnir | 1,108,945 | Δ1bp | coding (386/1446nt) | PST_0997 | sulfite reductase (NADPH) flavoprotein α-component |
| E4 Δnir | 3,552,536 | +G:RP10(+) +2bp | coding (132/133/1251nt) | PST_3282 | fatty acid α-hydroxylase |
| E4 Δnir | 4,392,032 | G → A | A233T (GCC → ACC) | tctD | transcriptional regulatory protein TctD |
| E4 Δnir | 992,029 | Δ1bp | coding (396/609nt) | narL | two-component response regulator NarL |
| E4 Δnir | 2,808,450 | +G | coding (1367/1380nt) | fbl | flagellum-specific ATP synthase |
| E4 Δnir | 3,818,976 | G → A | intergenic (-54/+16) | nirT / nirS | tetrahydrofuran nitrite reductase |
| E4 Δnir | 2,431,226 | T → C | I18V (ATT → GTT) | PST_3901 | hypothetical protein |
| F1 Δnir | 2,097,947 | C → T | A484T (GGG → ACC) | PST_1939 | molybdopterin oxidoreductase |
| F1 Δnir | 2,772,023 | T → C | K195E (AAG → AAG) | PST_2380 | transcriptional regulator |</p>
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<thead>
<tr>
<th>Type</th>
<th>Start</th>
<th>Change</th>
<th>Description</th>
<th>Location</th>
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<td>G → A</td>
<td>A233T (GCC → ACC)</td>
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<td>transcriptional regulators protein TctD</td>
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<td>Δ1bp</td>
<td>coding (390/609nt)</td>
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<td>T → C</td>
<td>R182G (GAA → GGA)</td>
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<td>6bp x2</td>
<td>duplication</td>
<td>FST_2110</td>
<td>methyl-accepting chemotaxis protein</td>
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<tr>
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<td>G → A</td>
<td>R148* (CGA → TGA)</td>
<td>flbN</td>
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<tr>
<td>F1 Δnar</td>
<td>3,818,977</td>
<td>C → T</td>
<td>intergenic (-55/+15)</td>
<td>nirT/nirS</td>
<td>tetrahemeheme protein nirT / cytochrome cd1 nitrite reductase</td>
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<tr>
<td>F1 Δnar</td>
<td>4,085,150</td>
<td>Δ266bp</td>
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<td>tetrahemeheme protein nirT / cytochrome cd1 nitrite reductase</td>
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</table>

Definitions: E Δnir clones, clones evolved from the ancestral strain 1602gfp; E Δnar clones, clones evolved from the ancestral strain 1603e. F Δnir clones, clones evolved from the ancestral strain 1602e. F Δnar clones; clones evolved from the ancestral strain 1603gfp. E1 Δnir and E1 Δnar are nitrite-producing and -consuming clones, respectively, from the same evolved population, E2 Δnir and E2 Δnar are from the same population, E3 Δnir and E3 Δnar are from the same population, etc.

*The type of change that the mutation have caused. M269V (ATG → GTG) means that a base pair substitution from an A to a G has caused amino acid 269 in this protein to change from methionine (M) to Valine (V). * signifies a stop codon intergenic (-54/+16) means that this mutation is located 16 base pairs downstream of one gene and 54 base pair upstream of another. coding (1367/1380nt) means the mutation is in base pair 1367 out of 1380 nucleotides in the coding region of a protein.
<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<td>A1601 with ΔnarG and mini-Tn7T-LAC-Gm-egfp; Gm&lt;sup&gt;8&lt;/sup&gt;, egfp&lt;sup&gt;*&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>A1602gfp</td>
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<td>This study</td>
</tr>
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<td>pUC18T-mini-Tn7T-LAC-Gm</td>
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<td>(Choi et al., 2005)</td>
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<tr>
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<td>pUC18T-mini-Tn7T-LAC-Gm containing egfp immediately downstream of P&lt;sub&gt;lac&lt;/sub&gt;; Ap&lt;sup&gt;8&lt;/sup&gt;, Gm&lt;sup&gt;8&lt;/sup&gt;, mob&lt;sup&gt;+&lt;/sup&gt;egfp&lt;sup&gt;*&lt;/sup&gt;</td>
<td>Chapter 4</td>
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<td>Chapter 4</td>
</tr>
</tbody>
</table>
6 General discussion and future perspectives

Here, I have used the pH-dependent reactivity of nitrite to show that chemical reactivity can be very important in determining both the ecological and evolutionary consequences of dividing metabolic labor among different cell-types.

6.1 The potential benefit of substrate cross-feeding for bioengineering

In Chapter 3 of this dissertation I show that the relative benefit of cross-feeding nitrite between two different cell-types as compared to complete consumption of nitrate into di-nitrogen gas by a single cell-type depends on the inhibitory effects of nitrite. This benefit is due to the accumulation of nitrite as a consequence of intracellular competition between the nitrate and the nitrite reductases when the same cell performs both steps of the metabolic pathway. Because of intracellular competition, the activity of the nitrate reductase has a negative effect on the activity of the nitrite reductase, thus resulting in the accumulation of the intermediate nitrite and the manifestation of its growth-inhibiting effects. This outcome should hold true for any metabolic pathway where the following two conditions are met: the activity of one enzyme inhibits the activity of the following enzyme and the intermediate that then accumulates has growth-inhibiting effects on the cell. Thus the reactivity of metabolic intermediates should be an important consideration when one wants to design efficient microbial metabolic processes for the production or degradation of a particular substrate, potentially representing an important engineering design principle. Dividing a single metabolic pathway among different cell-types thus has analogies to industrial assembly lines. The argument that I pose is that dividing metabolic labor reduces intra-enzyme competition and the accumulation of intermediates. Similarly, industrial assembly lines also seek to reduce competition between different industrial tasks and prevent the accumulation of manufacturing intermediates. The fact that division of labor can prevent the accumulation of intermediates (and thus minimize the negative effects of their accumulation) might explain why more diverse microbial communities tend to be more productive than less diverse communities (here I assume that different cell-types specialize at different metabolic processes) (Bell et al., 2005; Hooper et al., 2005; Fiegna et al., 2014). Therefore, gaining a better understanding on which intermediates in metabolic processes have inhibitory effects could help in designing more productive communities by choosing the appropriate cross-feeding strains to optimize certain processes.

6.2 When would substrate cross-feeding emerge from complete consumption?

Although nitrite cross-feeding is more efficient than complete consumption when nitrite is reactive, it is not clear when nitrite cross-feeding cell types would emerge from completely-consuming cell-types through natural selection. One way to gain a better understanding of this is to test if there are conditions in
which substrate cross-feeding cell-types (or, more generally, partially-consuming cell-types) can invade into populations of completely-consuming cell-types. From the results in Chapter 3, it is expected that a nitrite-consuming cell-type that has lost nitrate reductase activity should be able to invade into a population of completely-consuming cell types (at least at high nitrite reactivity conditions), as the nitrite-consuming cell-type would be able to start using the nitrite as soon as it is produced, thus making possible the frequency dependent co-existence of completely-consuming and nitrite-consuming cell-types. Based on my preliminary experiments, however, a method is needed that can detect and count cells at very low frequencies. This excludes plate count assays, but flow cytometry or microscopy could be workable options. However, I did not identify any clone from the evolved populations (either at low or high nitrite activity) which had lost nitrate (or nitrite) reductase function (Chapter 4, Tables S4.1 and S4.2). This indicates that at these experimental conditions it is unlikely for cross-feeding of nitrite to emerge within the limited number of generations that I investigated, even though it would increase the population fitness. Considering the fact that a nitrite consuming cell-type would detoxify the environment also for the complete-consuming cell-types, it is likely that a clone losing the nitrate reductase function could never rise to high enough frequencies to survive the bottlenecks of the experimental evolution. Perhaps a more constant environment (such as a chemostat) where nitrate is continuously supplied would allow for a more appropriate system to investigate if increasing nitrite reactivity has the possibility of promoting the evolutionary emergence of substrate cross-feeding from complete consumptions (Doebeli, 2002). Nitrite would be diluted out from a chemostat, however, and thus high concentrations of nitrate might then be necessary to promote cross-feeding at those conditions. In addition, as previously discussed in Chapter 3, spatial structure may promote the emergence of cross-feeding by preventing the rapid dispersal of nitrite away from the nitrite-producing cells and facilitate close cell-to-cell interactions. Thus performing invasion assays in spatially-structured environments or in chemostats might be a good starting point to identify the conditions that would promote the emergence of substrate cross-feeding from complete consumption. If then conditions where substrate cross-feeding strains can invade completely-consuming populations are identified, the next step would be to experimentally evolve a completely consuming cell-type at those conditions.

6.3  Nitrite cross-feeding in the natural environment

While nitrite cross-feeding improves denitrification at low pH in the laboratory, it is unclear whether this is also true in the natural environment. One way to test this would be to extend the research into the natural environment (i.e., field studies) and isolate denitrifiers from environments with different pH and determine if the acidity of the environment correlates to the abundance of cross-feeding cell-types as compared to completely-consuming cell-types. Ideally, this would be done from a specific site with a clear pH gradient (Rousk et al., 2010) where there are few alternative factors that change between the different microbial samples. If partially-consuming cell-types are common in acid soils but not in alkaline soils, this would reinforce the idea that nitrite reactivity is
important in determining the prevalence of cross-feeding in denitrification also outside of the laboratory. The fact that increasing the reactivity of nitrite results in cross-feeding of nitrite being more efficient than complete consumption does suggest that this is one possible explanation as to why some bacterial species only perform part of the denitrification pathway and appear to assemble together into nitrite cross-feeding consortia (Martienssen and Schöps, 1999; Van de Pas-Schoonen et al., 2005; Sorokin et al., 2007).

6.4 Stress and the pace of evolution

Increasing the reactivity of nitrite, and thus changing the initial fitness of the population, lead to more mutations being fixed per generation. Nitrite reactivity may be considered as a stress to the cells, and thus the question is whether this is a general phenomenon that would occur to in response to any type of stress. As stress in the case of nitrite is caused by the reactivity of a metabolic intermediate, the answer to this is not clear. Although increasing the strength of antibiotics lead to larger benefits of resistance mutations (MacLean and Buckling, 2009), it is less clear if increasing the strength of selection would actually lead to more mutations. That is, when a stress might be mitigated by developing resistance towards that stress, this might lead to very different adaptive solutions than when the stress is caused by a molecule that the cell also has to use a source of energy. Although several evolutionary studies using experimental evolution have investigated the mutational response to stresses such as organic solvents (Minty et al., 2011) and temperature (Blaby et al., 2012), these studies were not concerned about different levels of stress. Thus it would be of interest to use experimental evolution at different magnitudes of stress, where the strength of selection was varied (i.e. low vs high stress) to see if increased stress generally selects for more mutations, or if it would more often lead to a similar number of mutations but with different effects depending on the level of stress. Perhaps the most straightforward way to investigate if stress caused by metabolic intermediates poses different challenges to cells than stress from external toxicants would be to evolve P. stutzeri at aerobic conditions in the same media used in Chapter 4 at different levels of nitrite reactivity. Under these conditions nitrite is not a source of energy and cannot be consumed by the cells, suggesting that beneficial mutations would be in different genes under aerobic conditions.

6.5 Limits to adaptation?

In the context of evolution, 700 generations is a relatively short period of time, and the long-term evolutionary trajectory is therefore unknown. Populations that were evolved at increased reactivity of nitrite increased significantly more in both absolute and competitive fitness, but they still grow significantly slower/have a lower fitness at high reactivity than the ancestor does at low reactivity. This poses the question if wild-type Pseudomonas stutzeri even has the potential to overcome the inhibitory effects of nitrite completely or if there are limits to the amount of evolutionary adaptation possible for the wild-type (complete consumer) genotype at the conditions of the experimental evolution.
The experimental evolution was limited to 700 generations and it is thus possible that increasing the number of generations would lead to further significant fitness increases. Indeed, in the long term evolution experiment of *E. coli* fitness is still increasing after 40,000 generations (Wiser *et al.*, 2013), although at a much slower rate than the initial improvement. However, it has also been suggested that strong selection might lead to evolution of the population to a local fitness peak (i.e. a quick fix) that makes better solutions less available or lost (Barrick and Lenski, 2013). One way to use the already performed experimental evolution to gain insight of the possibilities for further adaptation would be to use the frozen record and measure how fitness increased over evolutionary time for the populations evolved at both low and high reactivity. From other studies of experimental evolution, it is clear that fitness tends to increase very rapidly initially (due to mutations with beneficial fitness effects large enough to fix quickly) and the fitness increase then slows as novel mutations have less of an effect on fitness (Wiser *et al.*, 2013). If the increase in fitness over evolutionary time has slowed down less in the lineages evolved at high reactivity as compared to those evolved at low reactivity, this would suggest that there are still more potential for large increases in fitness at high nitrite reactivity if the experimental evolution were further progressed. Another experimental solution is then to restart the evolution from the final frozen stock and test whether continuing the experimental evolution still yields larger fitness increases at low pH (and high nitrite reactivity). None of the clones that were evolved at low nitrite reactivity had mutations in denitrification genes (Table S4.1), suggesting that the denitrification system under low nitrite reactivity was already close to optimal. At high nitrite reactivity, mutations in denitrification genes were found in a few cases (Table S4.2), but not in most of the sequenced clones. This might suggest that continuing the evolution experiment would lead to further adaptations in the denitrification pathway at high reactivity. It also cannot be excluded that there are possible mutations in denitrification genes that would have (a small) benefit at low reactivity and would fix in the population if the evolution experiment were progressed for a sufficient period of time.

### 6.6 Evolutionary trade-offs

Adaptive loss-of-function mutations are prevalent in evolutionary adaptation (Behe, 2010; Schneider *et al.*, 2000; Cooper *et al.*, 2001). All lineages from the experimental evolution at both low and high nitrite reactivity had loss-of-function mutations causing loss or severe decrease of cell motility. Our experimental evolution was propagated under well-mixed conditions and loss of motility has been shown before to readily occur when it is not needed (Bailey *et al.*, 2014). In addition, it has been shown that motility can be costly at certain conditions as deletion of the flagellar machinery can have various beneficial effects (Martínez-García *et al.*, 2014). Loss-of function adaptation is a clear example where a mutation can easily cause trade-offs. That is, a mutation that is beneficial in one environment has negative effects in other environments. When it comes to loss-of-function mutations it is evident that it will cause negative effects in an environment where the lost function is beneficial or even necessary for growth or survival.
Trade-offs are a well-established concept within evolutionary theory and have been used to explain a variety of adaptive outcomes. Trade-offs occur because of limitations by organisms to optimize all traits simultaneously, promoting biodiversity (Meyer et al., 2015). However, with regards to experimental investigations of trade-offs, the results are usually not straightforward. One example is the experimental adaptation of E.coli to temperature, where replicates were first adapted to different temperatures for 2000 generations and then all of the replicates were further adapted to 20°C for another 2000 generations (Bennett and Lenski, 2007). Here, investigators found that the degree of improvement at 20°C had no relation to the ancestral temperature, and that trade-offs at 40°C were not found in a third of the populations, with some instead also showing improvement at 40°C. In addition, the degree of improvement at 20°C was not correlated to the degree of trade-offs at 40°C. Another study (Rodriguez-Verdugo et al., 2014) where replicate populations of E.coli were adapted to high temperature (42.2°C) for 2000 generations did not find trade-offs in all populations at low temperature. Rather, although many populations had shifted their thermal niche to higher temperatures, others had instead expanded their thermal niche. Furthermore, the potential trade-offs for adaptation on either single-carbon (methanol) or multi-carbon (succinate) of Methylobacterium extorquens has been investigated (Lee et al., 2009) and here trade-offs were also prevalent but not always occurring. Adaptation on succinate lead to either trade-offs or improvements on other carbon sources while adaptation on methanol did not lead to any trade-offs on the carbon sources investigated.

Evolution at high nitrite reactivity lead to more mutations and mutations in genes with a larger variety of functional annotations. The clones evolved at high nitrite reactivity (but none of those evolved at low nitrite reactivity) have mutations in genes encoding for functions in carbohydrate transport and metabolism, lipid transport and metabolism, energy production and conversion, replication, recombination and repair and transcription. Significantly, every clone sequenced from evolution at high reactivity also has a mutation in a gene encoding for a protein involved in carbohydrate transport and metabolism. Actually, each clone has a mutation in either fbp (fructose-1,6-biphosphatase), pykA (pyruvate kinase), or gap-2 (glyceraldehyde-3-phosphate dehydrogenase) (See table S4.2) with the most common being a mutation in fbp and with all mutations leading to a single amino acid change, thus likely to be mutations that causes a modification of function of the protein. It is clear by the high level of parallelism that this is an important adaptation and also probably an important adaptation specifically to deal with the increased reactivity of nitrite, as no mutations in genes encoding for functions in carbon metabolism were identified in the clones evolved at low reactivity. This demonstrates that the increased reactivity of an intermediate in one metabolic pathway can lead to changes of other parts of metabolism, highlighting how intertwined and dependent microbial metabolic processes are to each other.

Obtaining mutations in enzymes involved in central carbon metabolism have the potential to lead to trade-offs when grown on carbon sources in non-selected environments. The evolution experiment that I performed was with a synthetic
minimal medium containing citrate and L-asparagine as the carbon sources. Thus I assayed growth on a number of different carbon sources to determine whether this had lead to an over all trade-off on other carbon sources. I found that this was indeed the case for the populations evolved at high nitrite reactivity (Fig 4.7) (i.e. the ones with mutations in genes encoding for functions involved in carbohydrate metabolism).

In this case the trade-off on other carbon sources is probably not a direct result from growing constantly at a specific carbon source but rather an adaptation to the inhibitory effects of accumulated nitrite, as the lineages evolved at low nitrite reactivity but on the same carbon sources did not exhibit this trade-off (Fig 4.7) or have mutations in genes involved in carbon metabolism (Table S4.1) Thus, when we investigate trade-offs with experimental evolution, it may be beneficial to investigate trade-offs that seem, at first glance, less straight forward. Adaptive mutations can also lead to unexpected beneficial effects in non-selected environments. One example of perhaps unexpected trade-offs is from 45,000 generations long term experimental evolution of E.coli, where the evolved strains became increasingly susceptible to phage T6* but developed resistance to phage λ in the complete absence of phage (Meyer et al., 2010). With easily available whole genome sequencing, less expected trade-offs after experimental evolution of microorganisms may be potentially predicted based on the genetic changes and thusly assayed in the laboratory.

6.7 Competition between cross-feeding cell types?
Evolution of nitrite cross-feeding strains at high nitrite reactivity lead to distinctly different adaptations than at low nitrite reactivity (Chapter 5). However, the phenotypic consequences of these adaptations have not yet been investigated. At high nitrite reactivity there are mutations that are unique to the nitrite-consuming strains (i.e. nir operon mutations and narXL mutations) (Table S5.2), but at low reactivity the mutations that occur in several of the clones tend to be mutations occurring in both nitrite-producing and –consuming strains (Table S5.1). Thus, at low reactivity nitrite-producing and –consuming strains have similar adaptations. This suggests that nitrite-producing and –consuming strains may compete with each other, even though they continue to co-exist due to their use of nitrate/nitrite. However, at high reactivity competition may be less pronounced. To test if there are differences in competition for common resources between the cross-feeders evolved at high or low nitrite reactivity, a consumer and producer from each evolved population could be competed against each other on for example nitrous oxide (which they can both use) or in aerobic ACS media to see if they differ in adaptations not specific to anaerobic growth but to for example the carbon sources.

6.8 Prevalence of cross-feeding of reactive intermediates?
Thus far, I have investigated the effect of cross-feeding within one single pathway. However, all cells have many different metabolic pathways. Is there a way to predict which metabolic pathways are likely to be divided into more than one cell type and thus be likely to promote biodiversity? My results suggest that if we know that metabolic intermediates have growth inhibiting effects this pathway will be more likely to be divided into different cell types. Another way
to get a better idea of which pathways are often divided into cross-feeding is to screen sequenced genomes for a large number of different metabolic pathways and determine for each metabolic pathway if genomes tend to have the genes encoding for an entire metabolic pathway or only for a part of the pathway. If such a screen yields a large number of potential partial metabolic pathways, these pathways could then be furthered investigated to elucidate if they produce reactive metabolic intermediates. This kind of pattern would then support the idea that chemical reactivity of metabolic intermediates is an important factor in driving metabolic diversity.
7 Acknowledgements

This research was supported by the Swiss National Foundation.

I would like to thank my advisor David Johnson for many helpful discussions and suggestions, for providing the mathematical modeling to inform my research, and for supporting me in pursuing my own ideas. Martin Ackermann for useful discussions and for his support, and Martin and Paul Rainey for being on my committee and providing the final feedback on my doctoral work. I also thank the Microbial Ecology group at ETHZ for useful discussions, as well as the Theoretical Biology, the Evolutionary Biology, the Experimental Ecology and the Microbial Populations groups at ETHZ for their input and discussion during our joint meetings. I specifically thank Jan Dolinsek, Felix Goldschmidt, Frank Schreiber, Simon van Vliet and Sebastian Bonhoeffer for their input on chapter 3. I thank Susan Schlegel for providing the German summary of this dissertation.

I thank Jan Roelof van der Meer and William Metcalf for generously providing plasmids and strains used for this study, Anja Bernet and Selina Derksen-Müller for assistance with the genetic manipulations, Thomas Fleischmann for assistance with the chemical analyses, Jean-Claude Walser from the Genomic Diversity center at ETHZ for sequence analysis and identifying novel mutations, Christian Beisel and Ina Nissen from the ETH Quantitative Genomics Facility for the sequencing and Benedict Borer and Claudia Keller for their work as undergraduate students.

I would also like to thank everyone who made my experiences in Zürich and at Eawag and ETH more pleasant, including everyone in the Environmental Science department at Eawag. I thank Nela Nikolic and Markus Arnoldini for being friendly and helpful when I started out, Konstanze Schiessl, Paolo Ocampo, Daan Kiviet and Daniel Angst for being great friends to travel, go out, or have dinner with, Colette Bigosch for being a great running companion as well as for all her help in the lab, Caitlin Proctor and Alejandra Rodriguez for being up for having fun on short notice and Olaya Rendueles Garcia, Kimberly Chen, Deborah Patsch, Flor Inés Arias Sánchez, Louis du Plessis, Dominique Cadosh, Ana Karina Pitol, Jenna Gallie, Felix Goldschmidt, Simon van Vliet, Roland Mathis, Alejandra Manjarrez and Alma Dal Co for being great company both when it is about work and when it is not.

I would also like to thank my parents for always supporting me in whatever I am doing, and my sister for always being my friend as well as my sister, and for listening to me even when I talk about my research.
8 **References**


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9 Curriculum vitae

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EDUCATION

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PRESENTATIONS

1st ASM Conference on Experimental Microbial Evolution 2014 (19th-22nd June), Washington, DC. *Hostile environmental conditions accelerate the pace of molecular evolution and niche specialization in bacterial populations.* oral presentation.

New Approaches and Concepts in Microbiology 2013 (14th-16th October), EMBL, Heidelberg, Germany. *Do mutualistic interactions promote an increase in molecular evolution?* poster presentation

ISME 14, Copenhagen, Denmark. 2012 (19th-24th August). *Adaptive evolution of Pseudomonas stutzeri under denitrifying conditions.* poster presentation

TEACHING EXPERIENCE

2011-2015: Teaching assistant each year for 2 days in the lab course *Introduction to microbiology* at ETH Zürich.

2011, May – October: Supervision of Bachelor project.

2013, February- July: Supervision of Bachelor project.