Physiology, ecology and biochemistry of anaerobic, phototrophic oxidation of nitrite

Dissertation

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Joachim Schott

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1. Referent: Prof. Dr. Bernhard Schink

2. Referent: Prof. Dr. Alasdair Cook

Success consists of going from failure to failure
without loss of enthusiasm.

Winston Churchill (1874-1965)

Der einzige Mist, auf dem nichts wächst,
ist der Pessimist.

Theodor Heuss (1884-1963)

Die Naturwissenschaft braucht der Mensch zum Erkennen,
die Religion aber braucht er zum Handeln

Max Planck (1858-1947)

Danksagung

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Ben, der die Idee und die ersten Anreicherungskulturen Nitrit-oxidierender phototropher Bakterien hatte, und mir nach einer kurzen und guten Einführung das Thema übergab

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List of publications

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Anaerobic phototrophic nitrite oxidation by *Thiocapsa* sp. strain KS1 and *Rhodopseudomonas* sp. strain LQ17.
Microbiology 156, 2428-2437.

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Is the phototrophic nitrite oxidation of *Thiocapsa sp*. strain KS1 performed via a nitrate reductase?
Manuscript

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1 Summary

This thesis describes the novel process of anaerobic oxidation of nitrite to nitrate performed by phototrophic bacteria and its qualitative and, in parts, its quantitative distribution in the environment. Bicarbonate-buffered enrichment cultures which had 1 mM nitrite as sole electron donor, were obtaioned from many freshwater and some saltwater sites. In these cultures, nitrite was almost stoichiometrically oxidized to nitrate with concomitant increase in optical density in the light. Quantitative measurements of three sampling sites via the MPN-method revealed cell densities of 10^4 cells per ml in activated sewage sludge whereas sediments of Lake Constance and sediments of the slightly acidic lake Dingelsdorfer Ried contained substantially less cells per ml. Also in nitrite oxidation, enrichment cultures from activated sewage sludge were the most active ones, from which two morphological different bacterial strains could be isolated: strain KS1 and strain LQ17.

Both strains oxidized nitrite to nitrate anaerobically in the light with concomitant biomass formation. Without light, no growth or nitrite oxidation was detectable.

While strain LQ17 oxidized 1 mM nitrite incompletely to 0.6 mM nitrate within three months, strain KS1 oxidized nitrite stoichiometrically to nitrate within few days. If these strains were fed with nitrite at concentrations higher than 1.5 mM, the lag phase increased and growth was slowed down, and at concentrations above 4 mM no nitrite oxidation was observed and the OD of the cultured decreased permanently. Cultivation of strain KS1 in molybdenum-free medium with nitrite as sole electron donor revealed no nitrite oxidation or growth unless molybdenum (300 nM) was added. With organic electron donors in darkness, no anaerobic growth was observed with both strains, neither with nitrate nor with sulfate as alternative electron acceptor, whereas both strains were able to utilize organic substrates under air. When grown phototrophically, both strains utilized many organic and some inorganic substrates, and further physiological experiments such as, e.g., utilized nitrogen or sulfur sources or the *in-vivo* absorption spectra together with 16S rRNA gene analyses allowed to assign strain LQ17 to the genus *Rhodopseudomonas* and of strain KS1 to the genus *Thiocapsa*. Of already isolated strains, the two *Thiocapsa roseopersicina* strains DSM221 and DSM217 were also able to oxidize nitrite stoichiometrically to nitrate.

When grown with nitrite as sole electron donor, cell-free extracts of strain KS1 exhibited no nitrite oxidase but a specific nitrate reductase activity of more than 1 U per mg protein. Comparison with cell-free extracts of strain KS1 grown with fructose as e-donor and nitrate as N-source exhibited only few mU per mg protein. Subsequent SDS-PAGE analysis revealed two protein bands of 130-150 kDa and 55-60 kDa, which were strongly expressed specifically after growth with nitrite, and resembled the α- and β-subunit of the membrane-bound nitrate reductase.

2 Zusammenfassung

Die vorliegende Arbeit ist eine erste Beschreibung der anaeroben Oxidation von Nitrit zu Nitrat durch phototrophe Bakterien und deren qualitativem und teilweise quantitativem Vorkommen im Süß- und Salzwasser. Aus zahlreichen Süß- und einigen Salzwasserstandorten konnten in Hydrogencarbonat-gepuffertem Minimalmedium mit 1 mM Nitrit als einziger Elektronendonorquelle Nitrit-oxidierende Anreicherungskulturen kultiviert werden, die annähernd stöchiometrisch im Licht Nitrit zu Nitrat bei gleichzeitiger Zunahme der optischen Dichte oxidierten. In quantitativen Zellzahlmessungen durch die MPN-Methode drei Standorte konnten im Belebtschlamm der Konstanzer Kläranlage 10^4 Zellen pro ml bestimmt werden, wohingegen die beiden litoralen Seestandorte Bodenseesediment und Dingelsdorfer Ried deutlich geringere Zelldichten Nitrit-oxidierender phototropher Zellen aufwiesen. Aus der auch vom Nitritumsatz her aktivsten Anreicherungskultur der Konstanzer Kläranlage wurden zwei morphologisch verschiedene Bakterienstämme isoliert: Stamm KS1 und Stamm LQ17.

An beiden Stämmen konnte gezeigt werden, das Nitrit unter anoxischen Bedingungen bei gleichzeitiger Zunahme der OD nur im Licht zu Nitrat oxidiert wurde. Ohne Licht oder ohne Nitrit war kein Wachstum oder Nitritumsatz messbar. Während Stamm LQ17 1 mM Nitrit innerhalb von 3 Monaten unvollständig zu bis zu 0,6 mM Nitrat umwandelte, oxidierte Stamm KS1 1 mM Nitrit stöchiometrisch zu 1 mM Nitrat innerhalb von wenigen Tagen. Konzentrationen von mehr als 1,5 mM Nitrit erhöhten die Lag-Phase in Zellkulturen von KS1 und verlangsamten das Wachstum, und bei Konzentrationen von mehr als 4 mM Nitrit sank die OD der betroffenen Kultur dauerhaft, ohne weiteres Wachstum zu zeigen. In Molybdän-freiem Medium konnte kein Wachstum oder Umsatz von Nitrit durch Stamm KS1 beobachtet werden. Erst die Zugabe von Molybdän (300 nM) stellte das Wachstum wieder her. Anaerobes Wachstum in Dunkelheit konnte bei keinem Stamm festgestellt werden, auch nicht mit Nitrat oder Sulfat als Elektronenakzeptor. Unter oxischen Bedingungen von 21% O_2 konnten beide Stämme

organische Substrate veratmen. Bei phototropher Lebensweise wurde von beiden Stämmen eine Vielzahl von organischen und einige anorganische Verbindungen als Elektronendonor genutzt. Weitere physiologische Untersuchungen, wie nutzbare Stickstoff- oder Schwefelquellen oder das *in-vivo* Absorptionsspektren sowie die taxonomische Ähnlichkeiten des 16S rRNA Gens erlaubten eine Zuordnung von Stamm LQ17 zur Gattung *Rhodopseudomonas* und von Stamm KS1 zur Gattung *Thiocapsa*. Auch zwei weitere *Thiocapsa roseopersicina* Stämme, DSM221 und DSM217, konnten phototroph Nitrit stöchiometrisch zu Nitrat oxidieren.

Zellaufschlüsse von KS1 Kulturen, die mit Nitrit als einzigem Elektronendonor kultiviert wurden, zeigten in Enzymtests mit reduziertem Methyviologen als künstlichem Elekktronendonor und Nitrat oder Chlorat als Elektronenakzeptor vor allem in der Membranfraktion eine spezifische Nitratreduktaseaktivität von über 1 U pro mg Protein, die im Vergleich zu KS1 Kulturen, die mit Fructose als Elektronendonor und Nitrat als Stickstoffquelle inkubiert wurden, nur einige milliunits pro mg Protein erreichte. Nitrit oxidierende Enzymaktivitäten konnten nicht nachgewiesen werden. In anschließenden SDS-PAGE Analysen der Zellfraktionen konnten 2 starke Proteinbanden der Größen 55-60 kDa und 130-150 kDa ausgemacht werden, die parallel mit einer hohen Nitratreduktaseaktivität einhergingen und eine vergleichbare Größe zu α- und β-Untereinheiten membranständiger Nitratreduktasen hatten.

3 General introduction

The nitrogen cycle and its connections to microorganisms

In aquatic environments, nitrogen is the second most important element of living cells as it is an obligate constituent of many biological molecules, e. g., of proteins or nucleotide bases. Though nitrogen is present in most environments on the Earth's surface as dinitrogen gas or bound in sedimentary rocks, less than 2% is directly biologically available (Galloway, 1998; Mackenzie, 1998), and, often together with phosohorous, nitrogen is a limiting resource in many environments (Vitousek & Hamilton, 1991; Falkowski, 1997; Tyrell, 1999; Vitousek et al. 2002; Galloway et al. 2004; Mills et al, 2005; Elser et al., 2007; LeBauer & Treseder, 2008). As CO_2 concentrations increase continuously, some studies even predict higher CO_2 fixation rates only if higher bioavailable nitrogen levels are present in the environment, hence a sufficient nitrogen supply limits the effects of global warming (Reich et al., 2006). Therefore, all main reactions and organisms involved in the N cycle need to be investigated to estimate and regulate the N cycle as good as possible. In many environments, the nitrogen cycle is one of the major redox cycles, and nitrite appears as a key intermediate in several different metabolic pathways (fig.1).

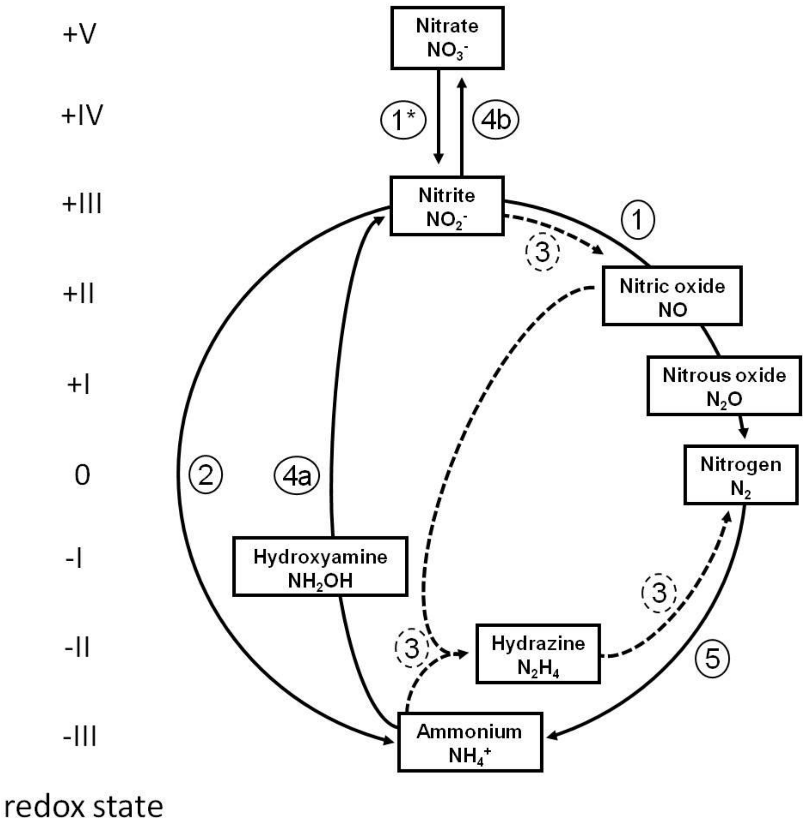

Fig. 1 The simplified nitrogen cycle. In ①, respiratory denitrification, nitrate is reduced via nitrite, nitric oxide and nitrous oxide to dinitrogen gas, whereas in ②, dissimilatory nitrate reduction to ammonium (DNRA), nitrate is reduced via nitrite and further to ammonium an six electron transfer reaction. The recently discovered ③, anammox reaction, converts nitrite and ammonium to dinitrogen and water, though the pathway is not clearly investigated yet and therefore drawn in dashed lines. During ④, aerobic nitrification, nitrite appears as an extracellular linker between two different groups of bacteria, (4a), nitrosobacteria, which convert ammonium to nitrite, and (4b), nitrobacteria, which convert nitrite to nitrate. Only in ⑤, nitrogen fixation, nitrite does not appear as an intermediate.
*Nitrate is also reduced to nitrite in DNRA and assimilatory reduction to ammonium.

Under anoxic condition, nitrate or nitrite may serve as electron acceptors in anaerobic respiration in two different reductive pathways with different end products (Bokranz et al., 1983, Strohm et al. 2007). In respiratory denitrification, nitrate is reduced via nitrite, nitric oxide, and nitrous oxide to dinitrogen (Zumpft, 1997, Stolz & Basu, 2002), whereas in dissimilatory nitrate reduction to ammonium (DNRA) nitrate is reduced via nitrite directly to ammonia. The responsible enzyme, nitrite reductase, reduces nitrite in a six-electron transfer to ammonia (Einsle et al., 2002). In the recently discover anammox reaction nitrite and ammonium are converted to dinitrogen gas, though the metabolic pathway is not terminatory resolved (Francis et al., 2007). The assimilatory reduction to ammonium is performed in a similar way, though different enzymes are used, with different regulation mechanisms; and this process is active both under oxic and anoxic conditions (Kerber et al., 1981; Stolz & Basu, 2002). Another assimilatory pathway is the reductive fixation of dinitrogen gas to ammonium, which needs anoxic conditions and 8 mol ATP equivalent per mol NH_4^+ (Seefeldt et al., 2009).

In many reactions of the nitrogen cycle, molybdenum is a necessary co-factor involved in enzymes such as in common nitrogenases or nitrate reductases (Kisker et al., 1997; Schwarz et al., 2009), though in some organisms different iso-enzymes are present which contain no molybdendum (Antipov et al., 1998; Afshar et al., 1998; Antipov et al., 2003).

As described above, the reduction of nitrate to nitrite takes place in three different metabolic pathways and is also performed by at least three different enzymes. The assimilatory nitrate reduction to ammonium is performed by a cytoplasmic nitrate reductase, encoded by nasA, and the DNRA and denitrification can be performed by either a periplasmic nitrate reductase encoded by napA, or a membrane-bound one, encoded by narG (Zumft 1997; Stolz & Basu, 2002).

Though there are many reactions and fluxes of different nitrogen compounds within the nitrogen cycle, naturally, only lightning (Galloway et al., 1995) and microbial nitrogen fixation increase the bio-available forms of nitrogen (Vitousek et al. 1997), whereas denitrification and the anammox reaction are the main sinks of bound nitrogen (Megonical et al., 2004).

Phototrophic bacteria using rhodopsin or chlorophyll derivates

Phototrophy is a process in which light energy is transformed into chemical energy, and is exploited by prokaryotes by two different systems (Bryant & Frigant, 2006).

Rhodopsin-based systems are found in Archaea and Eubacteria (Venter et al., 2004). Energy conservation is performed by photon-induced configuration changes of rhodopsins, and thereby building up a concentration gradient of ions (Oesterhelt and Stoeckenius, 1971). Detailed information is reviewed in Yizhar et al., (2011). As this kind of phototrophic metabolism does not involve electron transport processes or need electron donors, its activity does not directly participate in important nutrient cycles.

This is completely different with chlorophyll-based systems which perform photosynthesis, a process in which light energy is not only transferred to chemical energy, but also CO_2 is reduced to biomass (Bryant & Frigant, 2006). Photons are collected and shuttled via light-harvesting complexes to the reaction center where electrons are excited by photons. These electrons are passed onto electron transport systems, thereby producing a proton gradient.

Until now, chlorophyll-based systems were not found in Archaea but in five phyla of Eubacteria: *Cyanobacteria*, *Proteobacteria*, *Chlorobiaceae*, *Chloroflexaceae* and *Firmicutes*.

Only cyanobacteria have two different reaction centers and are able of a non-cyclic electron transport system, allowing them to photo-oxidize water and produce oxygen as a by-product. Therefore, this process is named oxygenic photosynthesis, though oxygenic photosynthesis is also capable of a cyclic electron transport (Munekage et al., 2006) and some cyanobacteria can oxidize other electron donors such as sulfide (Cohen et al., 1975; Garlick et al., 1977). In all other cases the electron transport is cyclic and contains only one type of reaction center (fig 2). There, other electron donors than water are oxidized and no oxygen is released, therefore named anoxygenic photosynthesis.

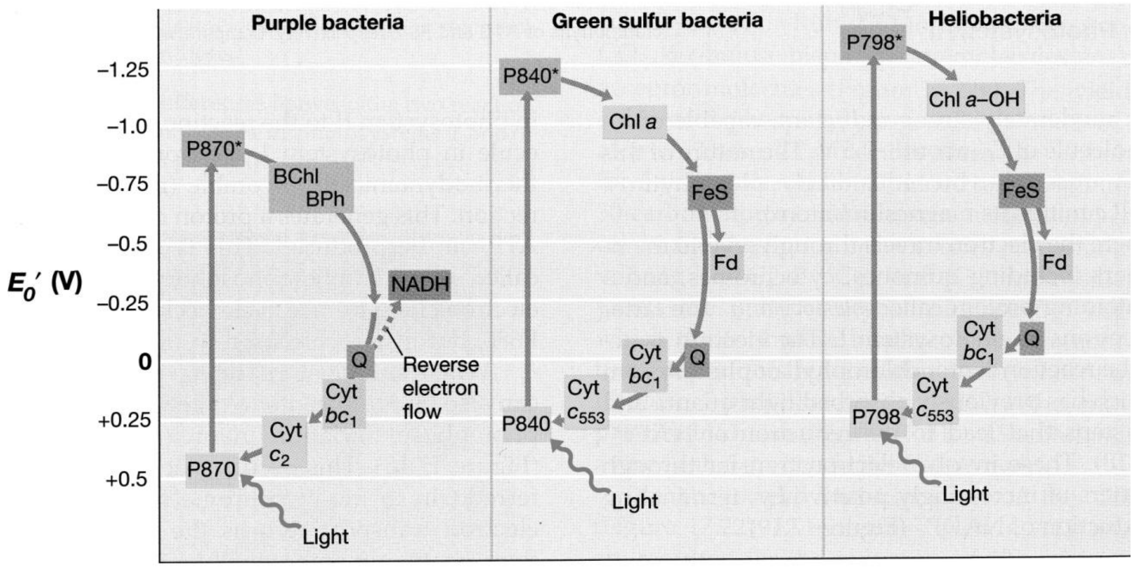

Fig. 2 Comparison of different types of electron transport chains after Madigan & Martinko (2006). In purple bacteria, the primary electron donor is the “special pair” bacteriochlorophyll of a type II reaction centre with an absorption maximum at 870 nm and a redox potential of about +490 mV (Cusanovich et al., 1968). When excited by a photon, the special pair bacteriochlorophyll releases an electron into the electron transport chain via bacteriopheophytin, ubiquinone, the cytochrome bc$_1$ complex and an additional cytochrome back to the special pair of the reaction centre, where the cycle starts again. Green sulfur bacteria and Heliobacteria contain a type I reaction centre at a redox potential of around +250 mV. Excited electrons are passed via an iron sulfur protein to quinones, the cytochrome bc$_1$ complex and additional cytochromes back to the reaction centre. Redox equivalents such as NADH, which are needed for CO_2 incorporation to biomass, derive in green sulfur and heliobacteria from reduced ferredoxin, whereas purple bacteria have to invest ATP into a reversed electron flow to transfer electrons to NADH.

Unlike Bryant & Frigant (2006), in this thesis phototrophy with organic electron donors also counts to photosynthesis. There, theoretically, the metabolites are oxidized to CO_2, and the harvested electrons are shuttled via the photosystems back to the reaction center and finally to NADH, which is needed to re-incorporate CO_2. The real metabolism may differ in detail, as electrons are needed only to fill the cyclic photosystems once, and photon-excited electron transfer cycles can run over and over again to create sufficient ATP. The main parts of organic compounds are photo-assimilated for anabolic reactions directly.

Details of the genes for anoxygenic photosynthesis are available, e.g., in the well-examined α-proteobacterium *Rhodobacter sphaeroides*. There, the genes are clustered together in several operons. Located between the puh and the puf operons which encode genes for the light-harvesting complex I and the reaction center, are the genes for bacteriochlorophyll synthesis (bch) and carotenoid synthesis (crt), whereas the genes for light-harvesting complex II are localized further downstream on the puc operon (Ponnampalam et al., 1995).

Although the translation of the gene synthesis is enhanced by light and inhibited by oxygen via species-specific regulators, the anoxygenic photosystem does not depend on continuous anoxic conditions to be carried out (de Wit & van Gemerden, 1987), but only on anoxic periods for pigment synthesis (Schaub & van Gemerden, 1993). An exception are aerobic anoxygenic bacteria which depend also for pigment synthesis on oxic conditions (Okamura et al., 1985;Yurkov & Beatty, 1998).

The origin of chlorophyll-based systems is unclear, as *Cyanobacteria*, *Proteobacteria*, *Chlorobiaceae*, *Chloroflexaceae* and *Firmicutes* form no monophyletic group. The complexity of the chlorophyll-based system makes several distinct and independent geneses unlikely. Therefore Woese (1987) suggests a common phototrophic ancestor and secondary loss of photosynthetic genes, whereas lateral gene transfer is also discussed (Raymond et al., 2002; Blankenship, 1992). Nonetheless, the origin seems to be within the α-*Proteobacteria* (Xiong & Bauer, 2002).

Electron donors for anoxygenic phototrophic bacteria

Many organic compounds are known to serve as electron donors for anoxygenic photosynthesis, including sugars, fatty acids, amino acids, aromatic compounds, humic acids, dimethyl sulfide (Visscher & van Gemerden, 1991), sulfonates (Ryan et al., 2004) and many more. Beyond organic electron donors, there are also some inorganic compounds known to fuel anoxygenic photosynthesis, e.g., H_2S, S°, $S_2O_3^{2-}$, and polysulfides (Visscher et al., 1990; Sander & Dahl, 2008; Dahl, 2008), H_2 (Drews & Imhoff, 1991; Schwartz & Friedrich, 2006), Fe^{2+} (Widdel et al., 1993), and arsenite (Budinoff and Hollibaugh, 2008).

Especially many sulfur compounds are found to donate electrons to anoxygenic photosynthesis, ranking from the most reduced sulfide via polysulfides, sulfur, thiosulfate, tetrathionate, to more oxidized compounds such as DMS or sulfonates, though the utilization of the electrons released by the oxidation of the sulfonate functional group was not directly shown.

Neglecting the cross links and covalent bounds of many sulfur compounds within the metabolic pathways to each other, the sulfur cycle shares some similarities with the nitrogen cycle. All reductions of sulfate have sulfite as an intermediate, as the reductions of nitrate have nitrite as intermediate. The reduction of sulfite is performed in a 6-electron transferring reaction as nitrite is reduced to ammonium in a 6-electron transferring reaction, too. This reaction is performed in some cases even by the same enzyme (Einsle 2011). Despite these similarities, only assimilatory metabolic pathways of anoxygenic phototrophs were connected to the nitrogen cycle. Of amino acids which are already known as organic electron donors, only the carbon and sulfur parts are oxidized, whereas the amino-parts are excreted as ammonium.

Aim of this study

As this anaerobic phototrophic nitrite oxidation is a completely new alternative to the already known aerobic nitrite oxidation, the necessary biochemical equipment for this reaction should be identified and investigated. Therefore, pure strains should be isolated from already existing enrichment cultures. Besides physiological examinations of the ecological properties, abilities and limits of the strains, e.g. the range and optima for light intensity, temperature, or sulfide or oxygen tolerance, the appearance of nitrite-oxidizing phototrophs in different habitats and its frequency within single sampling sites should be investigated. As an alternative, already isolated strain should be tested for anaerobic phototrophic nitrite oxidation. A combination of these data may allow first conclusions of this new metabolic process onto possible influences onto the global nitrogen cycle.

4 Nitrite, an electron donor for anoxygenic photosynthesis

Benjamin Michael Griffin, Joachim Schott & Bernhard Schink

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Introduction, results and discussion

Although compounds of the sulfur cycle, and more recently the iron cycle, are well-studied electron donors for anoxygenic photosynthesis, no analogous oxidations in the nitrogen cycle are known. Here we report on a new process in which anoxygenic phototrophic bacteria use nitrite as an electron donor for photosynthesis, providing a microbial mechanism for the stoichiometric oxidation of nitrite to nitrate in the absence of oxygen. To examine nitrite as a possible electron donor for anoxygenic phototrophs, we established enrichment cultures derived from local sewage sludge and several freshwater sediments in anoxic, bicarbonate-buffered mineral medium (Supporting Online Material). Low amounts of nitrite (1-2 mM) were fed repeatedly to avoid toxicity, and the cultures were incubated continuously in the light.

After incubating in the light for several weeks, enrichment cultures from 10 out of 14 sampling sites oxidized nitrite to nitrate and developed pink coloration, as typical of anoxygenic phototrophs. Absorption spectra of intact cells revealed maxima at 799 nm and 854 nm, which are characteristic of bacteriochlorophyll *a* (Imhoff, 1995). No chlorophyll *a* or oxygen was observed in nitrite-oxidizing cultures, suggesting that nitrate did not form due to a combination of oxygenic photosynthesis and aerobic nitrification. No growth or nitrite oxidation occurred in cultures incubated in the dark or in uninoculated bottles; thereby ruling out that nitrate was produced by anaerobic ammonia oxidation (anammox) or abiotic, photochemical processes.

Light-dark shift experiments performed over several days with enrichment cultures transferred five times clearly showed that growth and nitrate production depended on both light and nitrite (Fig. 1). The rate of nitrite consumption increased upon multiple feedings and approached 2 mM per day after one week in the light. As expected for a photoautotrophic process, nitrite consumed, nitrate produced, and biomass formed were all tightly correlated; nitrate was formed from nitrite near stoichiometrically.

We isolated the numerically dominant coccus (2-3 µm in diameter) from the most active enrichment culture derived from Konstanz sewage sludge by dilution to extinction in liquid medium (Fig. 1C, Supporting Online Material). Analysis of the 16S ribosomal RNA gene sequence revealed that the strain, designated KS, is most closely related to *Thiocapsa roseopersicina* (98% identical). *Thiocapsa* species are widely distributed purple sulfur bacteria of the order Chromatiales and are metabolic generalists capable of photoautotrophic growth on a variety of common inorganic electron donors, in addition to aerobic chemlithoautotrophic growth (Imhoff, 2003).

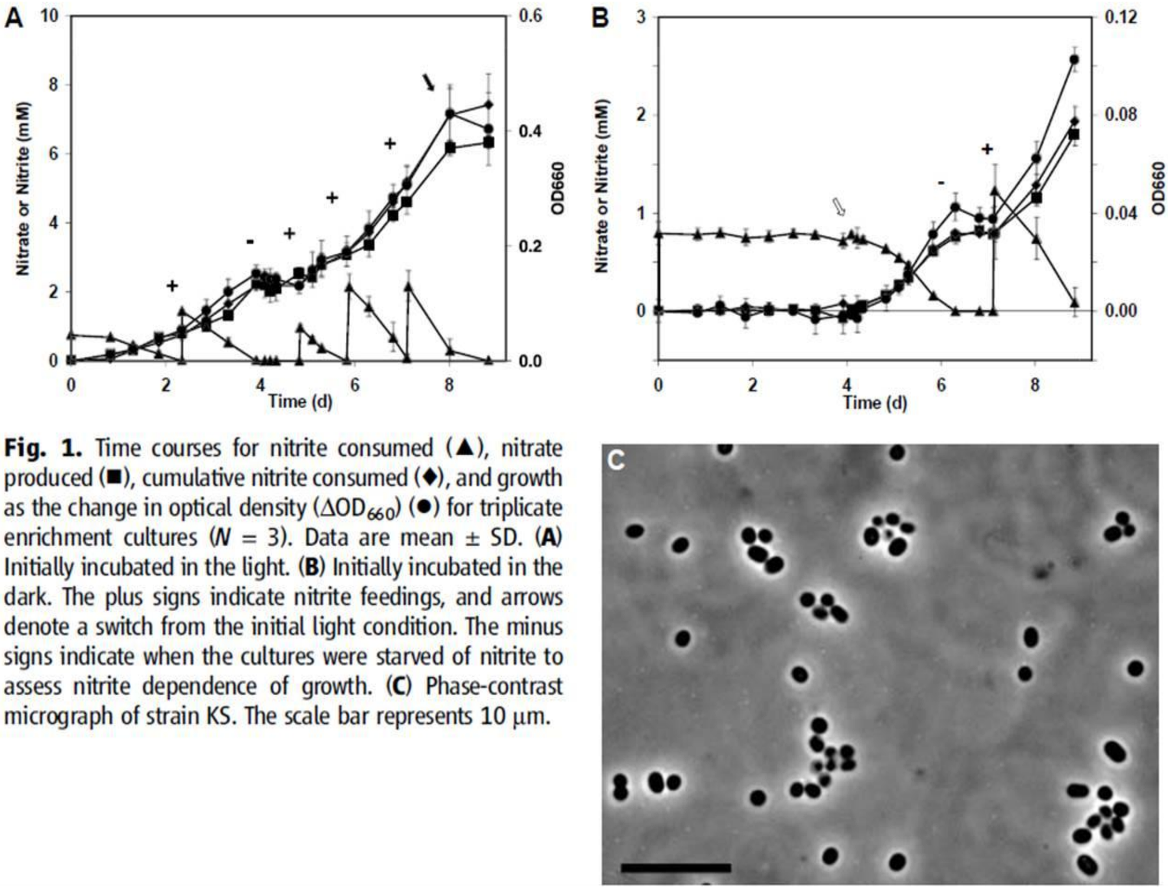

Fig. 1. Time courses for nitrite consumed (▲), nitrate produced (■), cumulative nitrite consumed (◆), and growth as the change in optical density (ΔOD_{660}) (●) for triplicate enrichment cultures (N = 3). Data are mean ± SD. **(A)** Initially incubated in the light. **(B)** Initially incubated in the dark. The plus signs indicate nitrite feedings, and arrows denote a switch from the initial light condition. The minus signs indicate when the cultures were starved of nitrite to assess nitrite dependence of growth. **(C)** Phase-contrast micrograph of strain KS. The scale bar represents 10 µm.

Although phototrophs were known to directly influence the nitrogen cycle through reductive processes such as nitrogen fixation, assimilation, and respiration (Megonigal et al., 2003), this is the only example of a photosynthetically driven oxidation in the nitrogen cycle. In principle, this photosynthetic process could compete for nitrite in the environment with other key nitrogen cycle processes such as denitrification, aerobic nitrification, or anammox.

In 1970, Olson proposed in detail how the water oxidizing activity of oxygenic photosynthesis may have evolved from anoxygenic photosynthesis through a series of inorganic nitrogen electron donors with increasing midpoint potentials (Olson, 1970). The nitrite/nitrate couple, with a standard redox potential of +0.43 V, could theoretically donate electrons to the quinone-type reaction center in purple sulfur bacteria, where the bacteriochlorophyll primary donor has a midpoint potential as high as +0.49 V (Cusanovich et al., 1965). This work demonstrates nitrite as the highest-potential electron donor for anoxygenic photosynthesis known so far and provides a modern example of an electron donor once proposed in the evolution of oxygenic photosynthesis.

Materials and Methods (Suporting online material)

Our approach to enriching nitrite oxidizing phototrophs was to limit the potential toxicity of nitrite by repeatedly feeding it in low amounts (1-2 mM). We also sought to avoid prolonged electron donor depletion during exposure of the phototrophs to light. This required frequent monitoring, which was accomplished using semi-quantitative nitrite/nitrate test strips (Merck, Germany). The enrichment medium was a defined bicarbonate-buffered mineral medium, pH 7.3 (Widdl & Bak, 1992). The medium was prepared anoxically, but was not further reduced since common reductants, such as sulfide or cysteine, could serve as potential electron donors for common anoxygenic phototrophs. Likewise, the sulfur source for this medium was sulfate (1 mM). Ammonium ions (1 mM) were frequently added to the medium as a supplementary nitrogen source. We found that ammonium ion addition was not necessary for growth or nitrite oxidation, but cultures grew better when it was added, presumably because the cells were not required to reduce nitrite or nitrate to biomass-N. Growth of oxygenic phototrophs was prevented in primary enrichment cultures with the photosystem II inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU, 10 µM). In later transfers DCMU was not necessary and not included. Nitrate and nitrite were quantified using an HPLC equipped with an anion-exchange column (Sykam, Germany) and UV detection at 210 nm.

Isolation of the organisms responsible for photosynthetic nitrite oxidation was hampered by their inability to grow on agar or agarose containing medium and by the necessary presence of nitrite, an excellent electron acceptor for undesired chemotrophs. Successful isolation, however, resulted from alternating nitrite and sulfide as electron donors in repeated dilution to extinction series in liquid medium. The resulting culture was microscopically pure and retained the ability to stoichiometrically oxidize nitrite to nitrate, however at a several fold reduced rate compared to the highly enriched mixed cultures.

5 Anaerobic phototrophic nitrite oxidation by *Thiocapsa sp.* strain KS1 and *Rhodopseudomonas sp.* strain LQ17

Joachim Schott, Benjamin Michael Griffin & Bernhard Schink

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Dedicated to the memory of Prof. Dr. Dr. h. c. Norbert Pfennig (1925-2008)

Abstract

In anaerobic enrichment cultures for phototrophic nitrite-oxidizing bacteria from different freshwater sites, two different cell types always outnumbered all other bacteria, i.e., non-motile cocci and motile, rod-shaped bacteria. Most-probable-number dilution series with samples from two freshwater sites yielded only low numbers ($\leq 3 \times 10^3$ cm^{-3}) of phototrophic nitrite oxidizers. Slightly higher numbers (about 10^4 cm^{-3}) were found in activated sewage sludge. Anaerobic phototrophic oxidation of nitrite was studied with two different isolates, the phototrophic sulfur bacterium strain KS1 and the purple nonsulfur bacterium strain LQ17, both of which were isolated from activated sludge collected from the municipal sewage treatment plant in Konstanz, Germany. Strain KS1 converted 1 mM nitrite stoichiometrically to nitrate with concomitant formation of cell matter within 2-3 days; whereas strain LQ17 oxidized only up to 60% of the given nitrite to nitrate within several months with the concomitant formation of cell biomass. Nitrite oxidation to nitrate was strictly light-dependent and required the presence of molybdenum in the medium. Nitrite was oxidized both in the presence and absence of oxygen. Nitrite inhibited growth at concentrations higher than 2 mM. Hydroxylamine and hydrazine were found to be toxic to the phototrophs in the range of 5-50 µM and did not stimulate phototrophic growth. Based on morphology, substrate utilization pattern, *in-vivo* absorption spectra, and 16S rRNA-gene sequence similarity, strain KS1 was assigned to the genus *Thiocapsa* and strain LQ17 to the genus *Rhodopseudomonas*. Also *Thiocapsa roseopersicina* strains DSM 217 and DSM 221 were found to oxidize nitrite to nitrate with concomitant growth. We conclude that the ability to use nitrite phototrophically as electron donor is widespread in nature, but low MPN counts indicate that their contribution to nitrite oxidation in the studied habitats is rather limited.

Introduction

Anoxygenic phototrophs are known to use organic substrates or reduced inorganic electron donors such as sulfur compounds, hydrogen, or ferrous iron for autotrophic cell carbon synthesis from carbon dioxide (Pfennig, 1967; Pfennig, 1977; Widdel et al., 1993; Stackebrandt et al., 1996). Photosynthetic bacteria can be found in almost every aquatic environment including freshwater, marine, alkaline, acidic, hot or cold waters (Pfennig, 1976; Pfennig, 1978; Stanier et al., 1981; Trüper & Pfennig, 1981; Van Trappen et al., 2004; Caumette et al., 2004; Herbert et al., 2005; Asao et al., 2007). In addition to these natural environments, purple anoxygenic phototrophs also inhabit engineered systems, such as wastewater treatment facilities, although their role there is not well understood. Until very recently, inorganic nitrogen compounds were not known as electron sources for photosynthesis. Nonetheless, nitrogen compounds are used in the assimilatory or dissimilatory metabolism. Although ammonia is usually their preferred nitrogen source, some phototrophic strains assimilate nitrate or nitrite if ammonia is absent (Malofeeva et al., 1974; Klemme, 1979; Pino et al., 2006; Olmo-Mira et al., 2006). Furthermore, fixation of molecular nitrogen is common among most anoxygenic phototrophs (Gogotov & Glinskii, 1973; Malofeeva & Laush, 1976; Madigan et al., 1984). In one counter-intuitive case, nitrogen is assimilated from nitrate via denitrification and subsequent nitrogen fixation (Dunstan et al., 1982).

In the dark, some purple non-sulfur purple bacteria such as *Rhodopseudomonas* sp. and *Rhodobacter* sp. can use nitrate as an electron acceptor for respiratory ATP generation (Castillo & Cárdenas, 1982; Satoh et al., 1976; Preuss & Klemme, 1983; Hougardy et al. 2000). Some of these denitrifying purple nonsulfur bacteria exhibit a taxis response to nitrate and nitrite if nitrite reductase is present (Lee et al., 2002). Denitrification by purple sulfur bacteria has not been reported so far.

Utilization of reduced nitrogen compounds as electron sources for anoxygenic photosynthesis has been predicted long ago (Olson, 1970; Broda, 1977) but was demonstrated only recently (Griffin et al., 2007). In the present study, the physiology of nitrite oxidation by two pure cultures enriched with nitrite as electron donor, strain KS1 and strain LQ17, is studied in detail, also with respect to its potential function in nitrite oxidation in nature.

Materials and methods

Chemicals

All chemicals were of analytical grade and were obtained from Fluka (Buchs, Switzerland), Riedel-de Haën (Seelze, Germany), Merck (Darmstadt, Germany), and Sigma (München, Germany). Gases were purchased from Messer-Griesheim (Darmstadt, Germany) and Sauerstoffwerke Friedrichshafen (Friedrichshafen, Germany).

Sources of organisms

Thiocapsa roseopersicina strains DSM 221 and DSM 217 and *Rhodopseudomonas palustris* strain DSM 123 were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). Strains KS1 and LQ17 were enriched and isolated from sewage sludge of the municipal sewage treatment plant at Konstanz, Germany.

Cultivation and isolation

Strains KS1 and LQ17 were enriched and isolated in a freshwater mineral medium, pH 7.2, buffered with 30 mM bicarbonate/CO_2 and supplemented with vitamin and trace element solutions (Widdel & Bak, 1992). The medium was prepared anoxically but was not further reduced since common reductants, such as sulfide or cysteine, could serve as potential electron donors for common anoxygenic phototrophs. The sulfur source was sulfate (1 mM). Enrichment cultures were established in 50 ml screw cap bottles containing 25 ml growth medium (Griffin et al., 2007). Nitrite served as sole electron donor and was added at 1 mM concentration to avoid toxicity. Inocula were added to ca. 1% (v/v) from different lake sediments or activated sewage sludge. Cultures were incubated at 20°C in the light of a tungsten bulb to provide a surface irradiance of 200 to 300 lx. Dichlorophenyldimethylurea (DCMU, 50 µM) was added in the first enrichment cultures to inhibit oxygenic photosynthesis.

Strain KS1 was purified from enrichment cultures in repeated dilution series to extinction with nitrite (1 mM) or sulfide (2 mM) as electron donor. Strain LQ17 was purified in deep-agar dilution series (Widdel & Bak, 1992) containing the same medium as described above with 1% washed agar. Purity of cultures was checked regularly by phase contrast microscopy after growth with nitrite alone or with nitrite plus 0.05% yeast extract. For enrichment cultures from saltwater sites, a modified freshwater medium with increased sodium chloride and magnesium chloride concentrations was used (20 g NaCl, 3 g $MgCl_2 * 6 H_2O$ per l).

Growth experiments

All growth experiments were performed at least in duplicate at 20°C in continuous light. If not specified otherwise, they were performed in sterile, O_2-free medium under a N_2/CO_2 atmosphere, and growth was measured via turbidity at 578 nm or 660 nm wavelength (Spectronics 70 spectrophotometer, Bausch & Lomb, Rochester, NY, USA or Camspec M107 spectrophotometer, Camspec, Camspec Ltd. Cambridge, UK) for more than two months. Substrate utilization was tested in triplicate with more than 25 different substrates in the light, and the OD was followed for 3 months. Measurements of OD values were converted to biomass yields using an empirically determined conversion factor (OD_{578} =1 equals 250 mg cell mass per l) which was applicable to both strains. Substrate conversion stoichiometries were calculated using the formula $<C_4H_7O_3>$ for cell material (Pfennig & Biebl, 1976). Vitamin dependency was tested only for strain KS1 in repeatedly transferred (1:10) liquid cultures using vitamin-free medium and fructose as substrate in the light. To examine the utilization of sulfur sources, cultures were starved for sulfur by repeated transfers (1:10) into sulfur-free medium supplemented with 4 mM fructose for strain KS1, and 4 mM acetate for strain LQ17 as electron donor. A sterile stock suspension of elemental sulfur was prepared as described by Pfennig & Biebl (1976). Utilization of nitrogen sources was tested in the light in nitrogen-free freshwater medium containing 3 mM fructose as electron donor for strain KS1 and 4 mM acetate for strain LQ17, with 1 mM sulfate as sulfur source. Tests for tolerance towards oxygen, sodium nitrite, sodium chloride, and sodium sulfide as well as tests for molybdenum requirement and the pH range were run only with strain KS1 with formate (2 mM) or fructose (1.5-4 mM) as substrate. Tolerance towards oxygen was measured with photoheterotrophically grown cultures of strain KS1 in mineral medium buffered with 30 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (pH 7.2) and 1.5 mM fructose as electron and carbon source, in the presence and absence of 21% oxygen under a N_2 atmosphere. A CO_2/bicarbonate buffer (5 mM, pH 7.2) was added as an additional carbon source. This buffer system was used also to test the temperature range and optimum of growth with 3 mM fructose as carbon and electron source and molecular oxygen as electron acceptor in the dark. The pH range was tested in the light with the described freshwater medium buffered with CO_2/bicarbonate and adjusted with HCl or Na_2CO_3, with 3 mM fructose as electron donor. In order to check for molybdenum requirement, cultures were starved for molybdenum in molybdenum-free medium for at least three transfers. For these tests, all glassware was soaked in 5% (m/v) HCl for 12 hours and washed with double-distilled water. Three out of six independent dilution series each with three one-tenth dilution steps were provided with extra molybdenum (300 nM) and growth was followed via OD measurement.

Short-term growth experiments with nitrite as sole electron donor are shown only for strain KS1. A nitrite-grown culture grown to OD_{660} = 1 was centrifuged and supplied with fresh medium with nitrite. After 5 mM nitrite was consumed, 10% of this culture was transferred into fresh medium containing 3 mM nitrite, and distributed in 50 ml aliquots into 120 ml serum bottles which were incubated under alternating light/dark conditions.

Most-probable-number counts (American Public Health association, 1965) were performed in dilution series in triplicate 1:10 dilution series in anoxic glass tubes sealed with black butyl rubber stoppers and filled with freshwater medium containing 50 µM dichlorophenyldimethylurea. Tubes were inoculated with surface material from either one of three different freshwater sediments; a small slightly dystrophic lake (Dingelsdorfer Ried), a large neutral oligotrophic pre-alpine hardwater lake (Lake Constance), and with activated sewage sludge from the municipal sewage treatment plant at Konstanz, Germany. To provide an almost homogeneous distribution, all tubes were mixed for 1 min with a Vortex mixer at highest speed before transfer. Tubes were incubated for up to three months before final scores were taken. Growth was measured and scored positive if nitrite disappeared and detectable amounts of nitrate were formed. Nitrite was re-fed when the concentration dropped below 0.1 mM.

Toxicity tests

Toxicity of nitrite was tested with phototrophic enrichment cultures from the Konstanz sewage plant with nitrite additions in the range of 0.5-15 mM nitrite. Toxicity of hydroxylamine and hydrazine was tested with the same enrichment culture with 1 mM nitrite plus 5-1000 µM hydroxylamine or hydrazine.

Microscopy and spectroscopy

Cultures were observed with an Axiophot phase-contrast microscope (Zeiss, Germany) equipped with a digital camera (Intas Science Imaging Instruments GmbH, Göttingen, Germany) using software MagnaFire 2.0 (Optronics, Goleta, CA, USA).

In-vivo absorption spectra were recorded with cells that were centrifuged and resuspended in saturated sucrose solution (Trüper & Pfennig, 1981) with an Uvikon 930 spectrophotometer (Kontron Instruments, Groß-Zimmern, Germany).

Chemical analyses

Nitrite and nitrate were quantified by HPLC using an anion exchange column (Sykam, Germany) and UV detection at 210 nm wavelength, and semi-quantitatively with Merckoquant test strips (Nitrite Test 2-80 mg/l NO_2^-, Nitrate Test 10-500 mg/l NO_3^-) (Merck, Darmstadt, Germany). Sulfide was measured colorimetrically after Cline (1969).

DNA extraction, amplification, and phylogenetic analysis

DNA was extracted from cell pellets of cultures centrifuged for 10 min at 10,000 *g*. The protocol described by Henckel et al. (1999) was used for DNA extraction, PCR amplification, and gel electrophoresis. PCR products were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced (GATC, Konstanz, Germany) with the following primers: 27F (Edwards et al., 1989) (5`- AGA GTT TGA TCC TGG CTC AG -3`), 1492R (Weisburg et al., 1991) (5`- TAC GGY TAC CTT GTT ACG ACT T -3`), 907R (Lane et al., 1985) (5`- CCC GTC AAT TCM TTT GAG TTT -3`) 533F (Weisburg et al., 1991) (5`- GTG CCA GCA GCC GCG GTA A -3`). Sequences were aligned using DNAStar (www.dnastar.com) and corrected manually. Phylogenetic analysis was done using the ARB software package (version 2.5b; http://www.arb-home.de) (Ludwig *et al.*, 2004). The new sequences were added to the ARB database and aligned against the consensus sequences of *Thiocapsa* or *Rhodopseudomonas*, respectively, using the FAST Aligner tool as implemented in ARB. Alignments were checked and manually corrected where necessary. Sequences of 1419 nucleotides were used for alignment. Only those positions which were identical in 50 % of all sequences were used to create a filter. Phylogenetic analysis was done using the maximum likelihood, neighbour-joining and maximum parsimony algorithms as implemented in ARB (Ludwig et al., 2004). Phylogenetic distances were also determined by using the similarity matrix in ARB without using any filter and also with *E. coli* as the filter. The 16S rRNA gene sequence of *Thiocapsa* strain KS1 was deposited with GenBank under the accession No. EF581005.

A culture of *Thiocapsa* strain KS1 was deposited with the Japan Collection of Microorganisms under the registration number JCM 15485.

Results

Enrichment and Isolation

Enrichment cultures for nitrite-oxidizing phototrophs were started with inocula from more than 30 different freshwater and 10 saltwater sites, including surface sediments from very small periodically flooded waterholes, small creeks and ditches, slow-flowing rivers, small ponds, lakes, and saltwater marshes. Water samples always included some surface sediment material to include also surface-attached bacteria. The pH of these water bodies was neutral and in one case (Dingelsdorfer Ried) slightly acidic (pH 5.5-6.0). Artificial environments such as sediments from fish tanks or activated sewage sludge from the municipal sewage plant in Konstanz were also sampled. All enrichment cultures were incubated either in continuous light or alternatively with a 16 h light/8 h dark cycle. Nitrite and nitrate concentrations of all samples were monitored over time, and cultures were re-fed with 1 mM nitrite when the nitrite concentrations dropped below 0.1 mM. In the first three weeks, some enrichment cultures consumed the given nitrite without concomitant nitrate formation. After approximately one month and repeated re-feedings (depending on the source of inoculum), the nitrite consumption rate decreased. After a few more weeks, nitrate accumulated to low concentration (0.1-0.3 mM) in many of the enrichment cultures incubated in continuous light. Cultures incubated under light/dark conditions also formed nitrate, but with a delay of one month. After repeated re-feeding, a purple-red cell pellet developed at the bottom of the culture tubes, and subcultures with nitrite as electron donor were established with 10% inocula. Although re-sampling of the same sampling sites did not always obtain nitrite-oxidizing phototrophs, there was almost no freshwater habitat from which such bacteria could not be enriched, except for profundal lake sediments and fish tank sediment samples.

In all nitrite-oxidizing phototrophic enrichment cultures, two cell types were dominant after several transfers, i. e., coccoid cells and short irregular rods. Since the cultures derived from the Konstanz sewage plant were the most active ones, we tried purification with these cultures. In deep-agar dilution series with 1% agar, only rod-shaped bacteria could be isolated. These bacteria grew well in deep-agar cultures but only poorly in subsequent liquid media especially if nitrite was the sole electron donor. The coccoid cells did not grow in agar medium and could not be separated from rod-shaped bacteria in repeated liquid dilutions with 1 mM nitrite or 1 mM thiosulfate as sole electron donor. Finally, the coccoid cells were isolated in repeated liquid dilution series with alternating 1 mM nitrite or 2 mM sulfide as electron donor. Early enrichment cultures on samples collected from saltwater oxidized nitrite to nitrate as well with concomitant formation of rose-red cell aggregates at the bottom of the flasks within several weeks. After two transfers, nitrite oxidation and growth rate in these cultures decreased severely. These cultures were not followed any further.

Toxicity tests with nitrite-oxidizing phototrophic enrichment cultures exhibited incomplete inhibition of growth by 100 µM hydroxylamine and complete inhibition by 200 µM hydroxylamine or 10 µM hydrazine. These compounds were not tested further for phototrophic utilization.

Most-probable-number counts

Estimations of cell densities of nitrite-oxidizing phototrophs by the most-probable-number technique were performed with three different freshwater sites. Inocula were taken from a freshwater ditch sediment from a layer about 5-15 mm deep, at 0.3-2 m water depth. A sediment core from Lake Constance was divided into 2 subsamples, one containing the upper 10 mm layer and one containing the sediment layer at 10 to 20 mm depth. In both samples from littoral sediment of Lake Constance, phototrophic nitrite oxidizers were below 100 cells per ml. Sediments from the slightly acidic Dingeldorfer Ried contained 3 x 10^3 cells per ml, and the highest cell densities were found in activated sewage sludge with 1.5 x 10^4 cells per ml. In this sewage sludge which also performs ammonia and nitrite oxidation, we found nitrite at 10 - 50 µM concentration; nitrite was not measurable (< 10 µM) at the pond and lake sites.

Strain characterization

Strain KS1

Cells of strain KS1 were coccoid and non-motile, 2-3 µm in diameter (Fig. 1a).

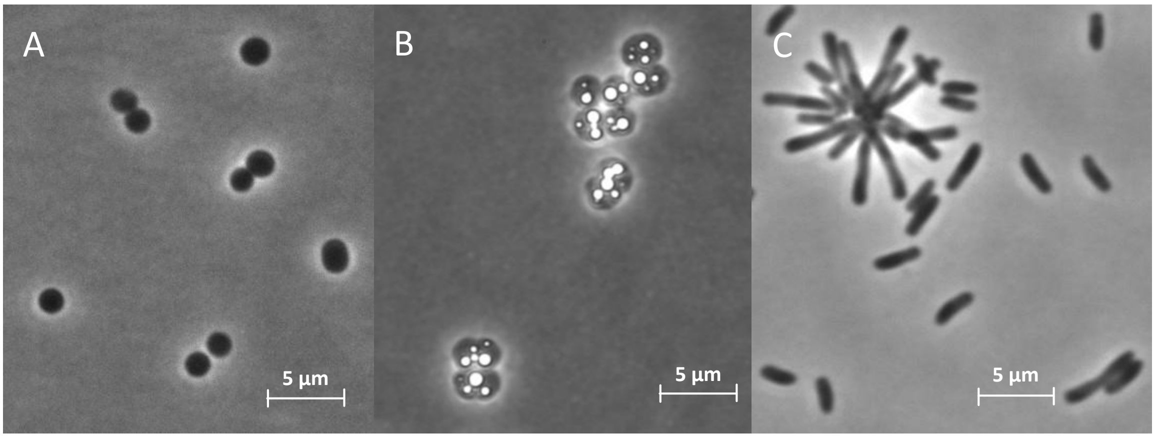

Fig. 1. Phase contrast photomicrographs of nitrite-oxidizing phototrophs. a) Strain KS1 grown with nitrite, b) Strain KS1 grown with thiosulfate, c) Strain LQ17 grown with nitrite as electron donor.

Nitrite was oxidized to nitrate only in the light. No growth or nitrite oxidation was observed under air in the dark. After exposure to air in continuous light, cultures of strain KS1 showed the same OD and nitrite oxidation rates as anaerobic cultures did for several days, but were slightly pale in color compared to the anoxic one. Fructose was utilized aerobically in the dark as well as anaerobically in the light. No anaerobic growth with fructose was observed in the dark, neither with nitrate nor with sulfate as electron acceptor. Besides nitrite (1-2 mM), strain KS1 photo-assimilated the following substrates (concentrations in mM): fructose (1-4 mM), formate (3), acetate (3), propionate (2.5), lactate (2.5), pyruvate (3), malate (3.5), succinate (2.5), glycerol (3), sulfur (2), thiosulfate (2.5), sulfide (1), and H_2 (20% in the headspace). Electron balances of substrate utilization and cell mass formation are shown in table 1.

Table 1: Stoichiometry of substrate oxidation and cell mass formation by strain KS1.

Substrate provided	Concentration (mM)	Measured ΔOD_{578}	Produced Biomass (mg per l)	Calculated Biomass (mg per l)	Electron recovery (%)
Nitrite	2	0.11	23.3	24	96.9
Thiosulfate	2.5	0.55	116.3	120	96.9
Sulfide	1	0.25	52.9	48	110.1
Acetate	3	0.57	120.5	144	83.7
Propionate	2.5	1.035	219.9	210	104.7
Fructose	2	1.5	317.1	288	110.1
Pyruvate	3	0.8	169.1	180	94.0
Lactate	2.5	0.85	179.7	180	99.8
Succinate	2.5	1.05	222	210	105.7

No growth was observed with glucose (2), galactose (2), butyrate (2), 2-oxoglutarate (2), glycolate (1), glyoxylate (1), alanine (2), citrate (2), tartrate (3.5), benzoate (0.25), methanol (2), or ethanol (3). After growth with sulfide or thiosulfate, sulfur globules were formed inside the cells (Fig. 1b). Good growth with sulfide was observed at concentrations up to 3 mM, with an optimum at 1.5 mM. No increase in OD could be detected at 5.5 mM or higher sulfide concentrations. Strain KS1 grew best in freshwater medium, but growth was possible up to 18 g NaCl per l. Optimal growth was found at 28°C, with limits below 4°C and at 37°C. Growth was possible within a pH range of 6.5 to 8.8, with a broad optimum around pH 7.2. Vitamins were not required. Strain KS1 utilized sulfate, sulfite, thiosulfate or elemental sulfur as sulfur sources, and nitrate, nitrite, N_2, or ammonia as nitrogen sources. An *in-vivo* absorption spectrum between 350 nm and 900 nm showed absorption maxima at 376, 486, 516, 552, 590, 799, and 854 nm wavelength (Fig. 2) indicating the presence of bacteriochlorophyll *a* and carotenoids of the spirilloxanthin series; no absorption peaks beyond 900 nm were evident.

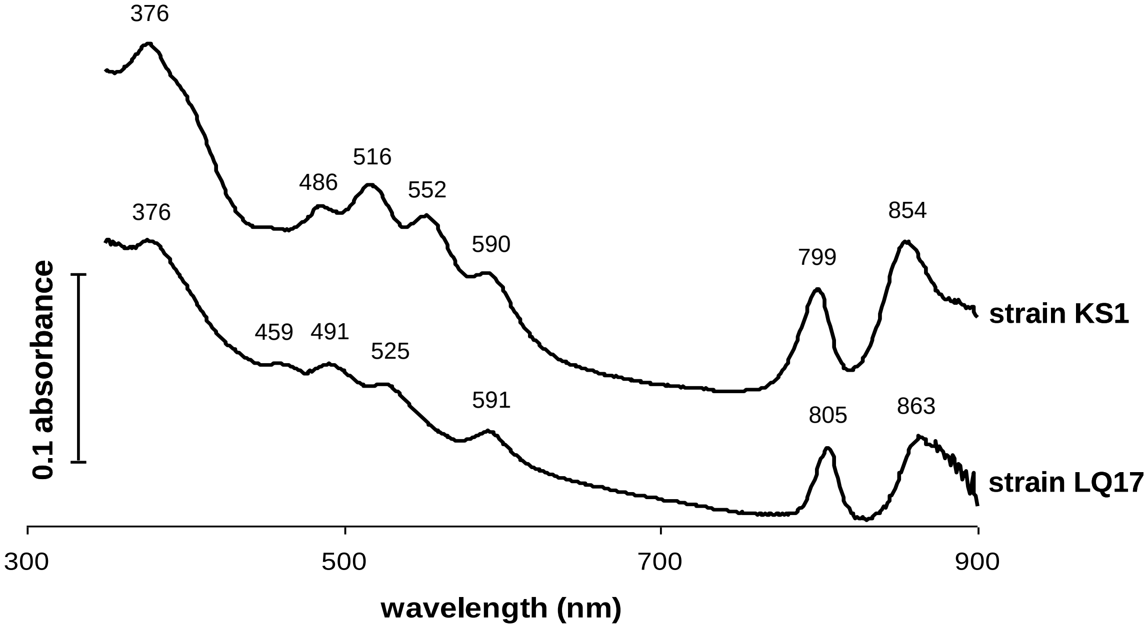

Fig. 2 Absorption spectra of dense cell suspensions of nitrite-oxidizing phototrophs after growth with nitrite. Cells were suspended in saturated sucrose solution.

Strain LQ17

Cells of strain LQ17 were motile, irregular rods, 0.6-1 µm in diameter (Fig 1c), and did not form gas vesicles. Phototrophically grown cultures were pink to purple-red in color. Cells stained Gram-negative and were catalase-positive after aerobic growth. Malate could be utilized aerobically in the dark, as well as anaerobically in the light. No anaerobic growth with malate was observed in the dark, not either in the presence of nitrate or sulfate.

Besides nitrite (1 mM), strain LQ17 photo-assimilated the following substrates (concentrations in mM): formate (2), acetate (1), propionate (0.5), butyrate (0.5), caprylate (1), caproate (1), lactate (1), pyruvate (1), malate (1), succinate (1.5), tartrate (0.5), alanine (0.5), glutamine (1), 2-oxoglutarate (0.5), malate (0.5), fumarate (1.5), glycerol (0.5), benzoate (0.5), thiosulfate (0.5) and hydrogen (20% in the headspace). No growth was observed with fructose (1), galactose (0.5), glucose (0.5), mannitol (0.5), sorbitol (1), glycolate (1), glyoxylate (1), citrate (0.5), methanol (1.5), ethanol (1.5), sulfide (0.5) or sulfur (2). After growth with thiosulfate, no sulfur globules were observed in or around the cells. Strain LQ17 utilized sulfate, sulfite, sulfide, sulfur and thiosulfate as sulfur sources and nitrate, nitrite, dinitrogen and ammonia as nitrogen sources. *In-vivo* absorption spectrum between 350 nm and 900 nm showed absorption maxima at 376, 459, 491, 525, 591, 805, and 863 nm wavelength (Fig. 2).

Physiology of phototrophic nitrite oxidation by strain KS1

Strain KS1 was grown in bicarbonate-buffered mineral medium in the light with nitrite as sole electron donor. A dense culture was distributed into sub-cultures and incubated under three different light regimes (Fig. 3). In the dark, the optical density was stable or decreased only slightly while nitrite and nitrate concentrations did not change (Fig. 3A, C). In the light and without nitrite, no nitrate was produced and the optical density decreased slightly. Growth was restored when both nitrite and light were supplied again (Fig. 3B). Filter-sterilized culture supernatants placed in the light showed no nitrite oxidation. Under oxic conditions in the dark with or without bacteria, no nitrite oxidation or increase of optical density was observed. Nitrite at concentrations higher than 1.5 mM increased lag phases and inhibited growth; no growth was observed with 4 mM nitrite. In the absence of molybdenum, strain KS1 grew with fructose but not with nitrite, and no nitrite oxidation was observed. After addition of molybdenum to molybdenum-starved cells, nitrite oxidation and nitrite-dependent growth resumed immediately.

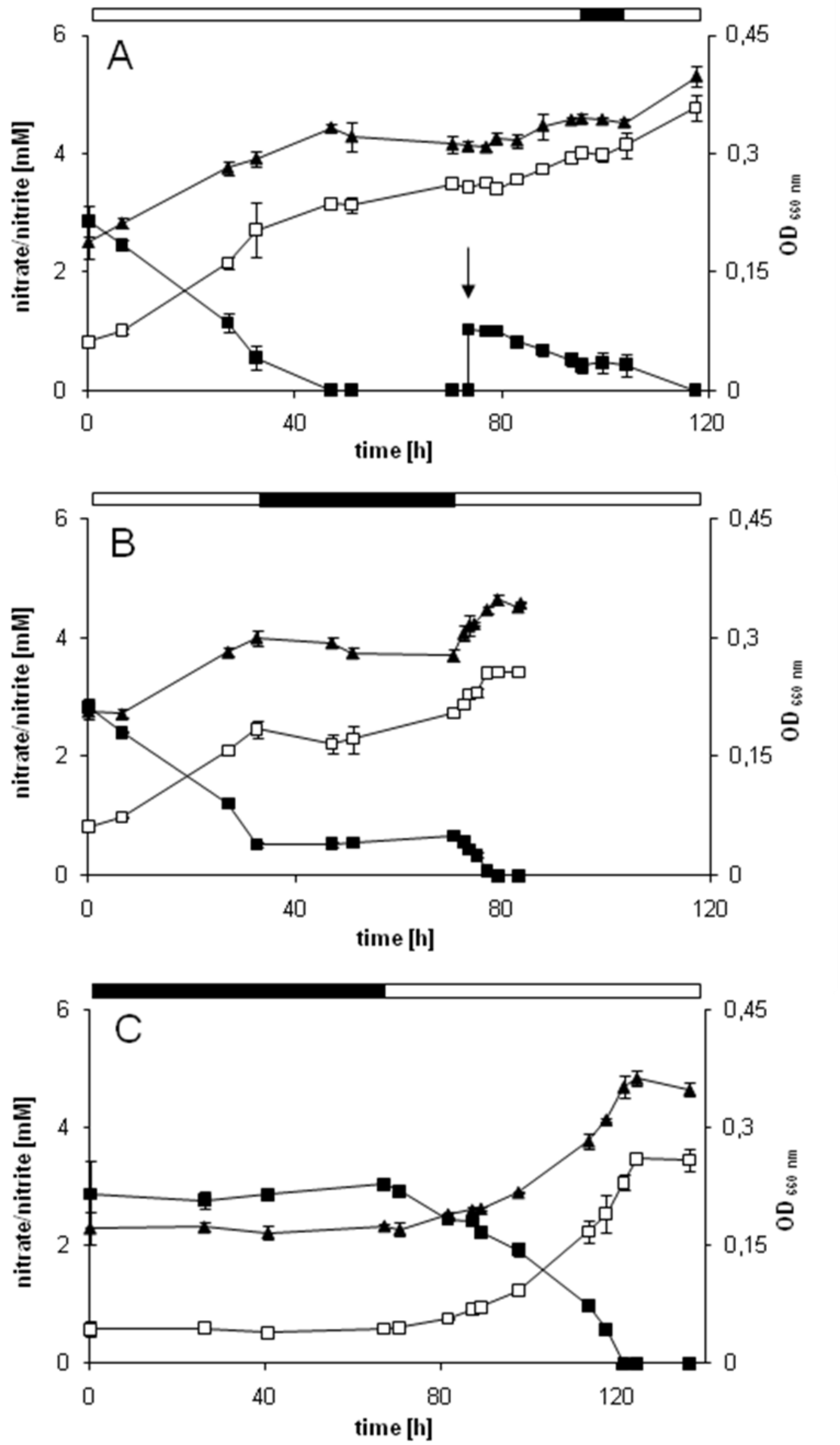

Fig. 3 Phototrophic growth and nitrite oxidation by strain KS1. One culture was distributed and incubated under three different light regimes. The bar above the diagrams displays the illumination patterns (black = darkness; white = illumination at 300 lx by a 100 W tungsten bulb. Arrows indicate re-feedings with nitrite. The standard deviation is often smaller than symbol size. ▲optical density, ■ nitrite concentration, □ nitrate concentration. A) N = 4, B) N =3, C) N = 4.

Physiology of phototrophic nitrite oxidation by strain LQ17

Strain LQ17 was grown in bicarbonate-buffered mineral medium in the light with nitrite as sole electron donor and sulfate as sulfur source. After one month, the nitrite concentration started to decrease with simultaneous increase in OD. Nitrate was produced non-stoichiometrically and reached up to 60% of the consumed nitrite (Fig. 4).

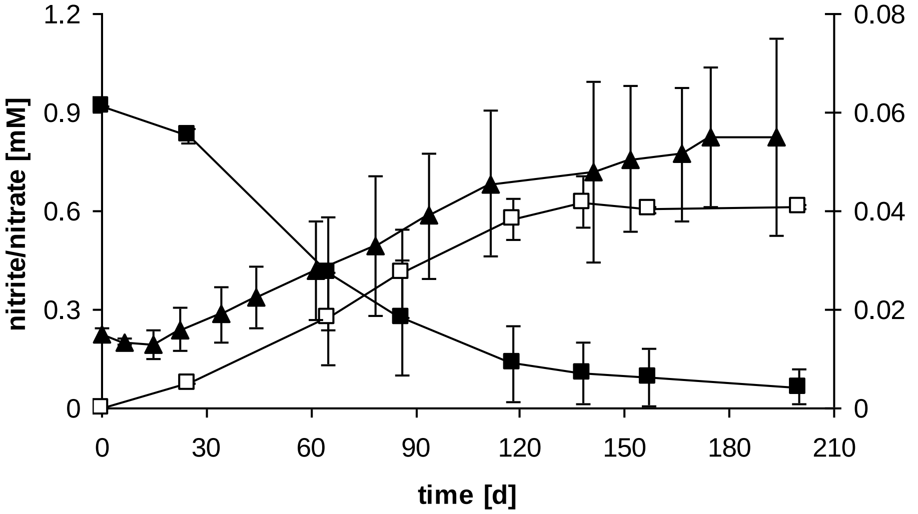

Fig. 4 Growth of strain LQ17 in the light with nitrite as sole electron donor. ▲optical density, ■ nitrite concentration, □ nitrate concentration. (N = 2)

Taxonomic assignment

Analyses of the 16S rRNA sequence of strains KS1 and LQ17 revealed high similarities to *Thiocapsa* strains (Gammaproteobacteria) or to *Rhodopseudomonas* strains (Alphaproteobacteria), respectively (Fig. 5).

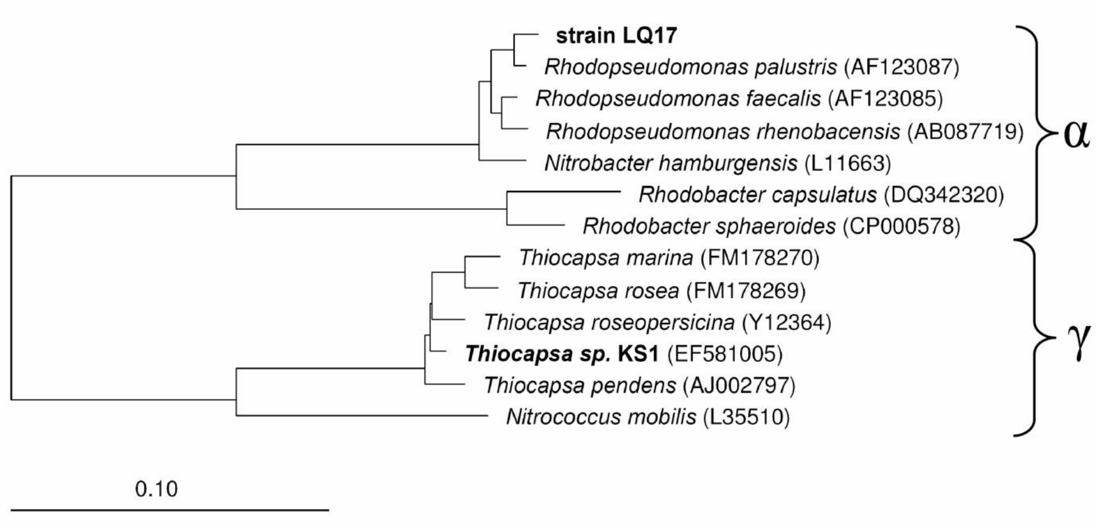

Fig. 5 Phylogenetic tree based on 16S rDNA sequences of representatives of phototrophic and related chemotrophic bacteria. The sequences of strains KS1 and LQ17 were aligned and integrated into the existing phylogenetic tree of the database Silva using integrated features of the program arb ubuntu. α and γ stand for α and γ Proteobacteria. Bar indicates 10% difference in nucleotide sequence.

Two strains of *Thiocapsa roseopersicina,* strains DSM 217 and DSM 221, were tested for possible phototrophic nitrite oxidation. After three transfers, a small purple pellet developed at the bottom of the flasks with simultaneous disappearance of nitrite and formation of nitrate in both cultures. Growth and nitrite oxidation to nitrate by *T. roseopersicina* strain DSM 221 is shown in Fig. 6.

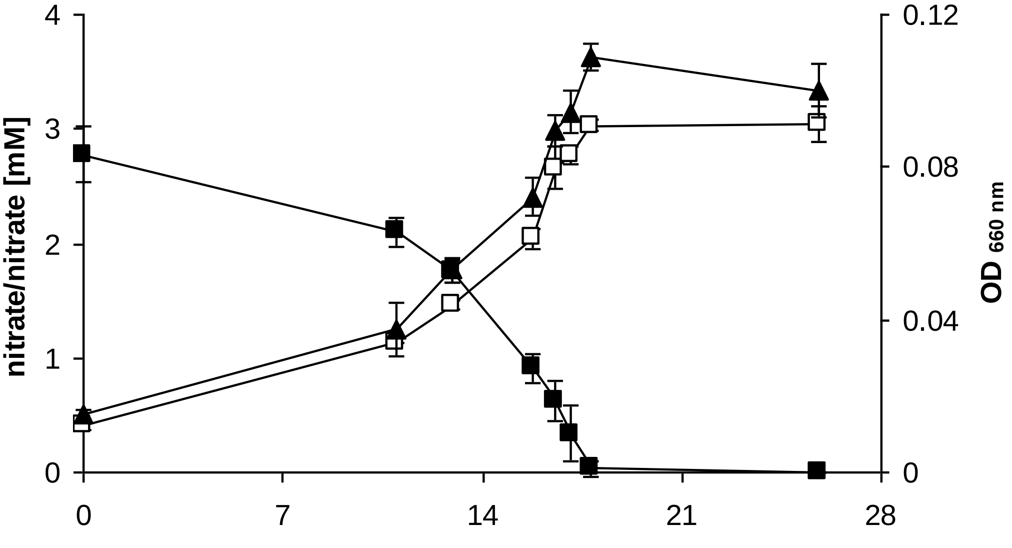

Fig. 6. Growth of *Thiocapsa roseopersicina* strain DSM 221 in the light with nitrite as sole electron donor. ▲optical density, ■ nitrite concentration, □ nitrate concentration. The standard deviation is often smaller than symbol size (N = 3)

Table 2: Substrate utilization patterns of strains KS1 and LQ17 and their closest phylogenetic neighbours.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Acetate	+	+	+	+	+	+	+	+
Alanine	-	n.d.	n.d.	n.d.	n.d	+	n.d.	n.d
2-Oxoglutarate	-	-	n.d.	n.d.	-	+	n.d.	n.d
Benzoate	-	-	-	-	-	+	+	-
Butyrate	-	-	+	n.d.	n.d	+	+	+
Caproate	n.d.	n.d.	n.d.	n.d.	n.d	+	+	n.d
Caprylate	n.d.	n.d.	-	n.d.	-	+	+	n.d
Citrate	-	n.d.	-	-	n.d	-	±	-
Ethanol	-	-	-	-	n.d	-	±	+
Formate	+	n.d.	-	-	+	+	+	+
Fructose	+	+	+	+	+	-	±	-
Fumarate	+	+	+	+	n.d	+	+	n.d
Galactose	-	n.d.	-	-	n.d	-	n.d.	n.d
Glucose	-	n.d.	+	-	n.d	-	±	-
Glutamine	n.d.	n.d.	n.d.	n.d.	n.d	+	n.d.	n.d
Glycerol	+	+	-	n.d.	+	+	+	n.d
Glycolate	-	n.d.	n.d.	n.d.	n.d	-	+	n.d
Glyoxylate	-	n.d.	n.d.	n.d.	n.d	-	n.d.	n.d
Hydrogen	+	+	n.d.	n.d.	+	+	+	n.d
Iron (+2)	-	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d
Lactate	+	-	+	n.d.	+	+	+	+
Malate	+	+	+	+	n.d	+	+	+
Mannitol	n.d.	n.d.	-	n.d.	n.d	-	±	n.d
Methanol	-	-	-	-	n.d	-	±	-
Nitrite	+	+	n.d.	n.d.	n.d	+	n.d.	n.d
Propionate	+	-	+	+	n.d	+	+	-
Pyruvate	+	+	+	+	n.d	+	+	+
Sorbitol	n.d.	n.d.	-	n.d.	n.d	-	+	n.d
Succinate	+	+	+	-	+	+	+	+
Sulfide	+	+	+	+	+	-	+	n.d
Sulfur	+	+	+	+	+	-	n.d.	n.d
Tartrate	-	-	-	-	-	+	-	+
Thiosulfate	+	+	+	+	+	+	+	n.d
Yeast extract	+	n.d.	+	n.d.	+	+	+	+

1 = strain KS1, 2 = *T. roseopersicina,* 3 = *T. litoralis,* 4 = *T. rosea,* 5 = *T. marina,* 6 = strain LQ17, 7 = *R. palustris*, 8 = *R. rhenobacensis*, n.d. = not determined

Discussion

In this paper, we document anaerobic oxidation of nitrite by two new isolates, *Thiocapsa sp.* strain KS1 and *Rhodopseudomonas sp.* strain LQ17. We also show that the known *Thiocapsa roseopersicina* strains DSM217 and DSM221 can grow with nitrite as electron donor. A short communication described this novel process with strain KS1 (Griffin et al., 2007). In phototrophic nitrite oxidation, nitrite serves as electron donor for anoxygenic photosynthesis, and the electrons are used for autotrophic CO_2 fixation. To date, nitrite was subject to three other types of anaerobic energy metabolism: dissimilatory nitrate reduction to ammonia, denitrification to N_2, and the “Anammox” reaction. In these anaerobic processes, nitrite acts as electron acceptor, either with organic or inorganic electron donors. The only known reaction in which nitrite is oxidized was so far aerobic nitrification (Bock et al. 1991) in which electrons from nitrite are transferred to oxygen, and CO_2 acts only as the carbon source.

Anoxygenic phototrophic nitrite oxidation by the purple sulfur and nonsulfur bacteria described here showed many similarities but also differences. Nitrite was oxidized only in the presence of bacteria, and required light. No growth and no nitrite oxidation was found in the dark, and in the absence of nitrite, no increase of optical density was observed. Sterile controls in the light showed no nitrite oxidation, hence ruling out a chemical oxidation of nitrite in the light. If cultures of strain KS1 were placed aerobically in the dark, no growth and no nitrite oxidation was observed either, indicating that strain KS1 cannot switch to aerobic nitrite oxidation. The tight stoichiometric coupling between nitrite disappearance and nitrate formation and corresponding cell matter formation from CO_2 by *Thiocapsa sp.* strain KS1 and *Thiocapsa roseopersicina* DSM217 and DSM221 proves that nitrite was oxidized to nitrate only by anoxygenic photosynthesis.

With the purple nonsulfur bacterium *Rhodopseudomonas sp.* strain LQ17, the stoichiometry of nitrite consumption and nitrate formation was incomplete. In our experiments only a maximum of 60% of the added nitrite was recovered as nitrate although the nitrite electrons appeared nearly quantitatively in the produced biomass. Theoretically, during the long incubation time, some nitrite could have been dismutated to nitrate and dinitrogen gas, a reaction that is exergonic but has never been observed so far as a biological activity (Strohm et al., 2007), or some nitrate simply was lost. The reason why strain LQ17 grew much slower in pure culture than in the enrichment cultures in which it competed well with strain KS1 is unknown. In co-cultures with strain KS1, growth of strain LQ17 could not be enhanced (results not shown). Thus, its growth in the enrichments may be supported by cross-feeding from other bacteria.

Phototrophic nitrite oxidation by strain KS1 was not influenced by short-term exposure to air in the culture headspace, in accordance with earlier observations which showed that under semi-oxic conditions in the presence of light photosynthetic metabolism is preferred over respiratory metabolism if the electron donor is limiting (de Wit & van Gemerden, 1986). Nevertheless, phototrophic nitrite oxidation is probably feasible only if anoxic periods are provided from time to time to allow pigment synthesis (Schaub & van Gemerden, 1993). Phototrophic growth under continuous exposure to oxygen has been shown only with one anoxygenic purple nonsulfur bacterium (Yildiz et al., 1991) and has not been reported for a purple sulfur bacterium so far.

Growth experiments with strain KS1 and nitrite as electron donor in the presence or absence of molybdenum were carried out to elucidate whether this transition metal has a function in this new process. In nitrite oxidation by aerobic nitrite oxidizing bacteria, molybdenum is an essential constituent of the molybdopterin cofactor of the nitrite-oxidizing enzyme (Fukuoka et al., 1987; Krüger et al., 1987; Meincke et al., 1992; Kroneck & Abt, 2002). Furthermore, almost all nitrate reductases which catalyze the reverse reaction from nitrate to nitrite contain the molybdenum cofactor (Stolz & Basu, 2002). Our results show that strain KS1 exhibited growth and nitrite oxidation only if provided with molybdenum, indicating that also the nitrite-oxidizing enzyme of phototrophic bacteria contains the molybdopterin cofactor.

Compared to other electron donors used in anoxygenic photosynthesis such as organic compounds, HS^-, H_2 or Fe^{2+}, nitrite is the electron donor with the highest redox potential used so far ($E°'$ nitrate/nitrite = +430 mV; Thauer et al., 1977). Such electrons enter the photosynthetic apparatus via a cytochrome or other electron carrier, close to the redox potential of the reaction center primary donor, i. e., up to +490 mV (Cusanovich et al.,1968). It is not surprising, therefore, that our enrichments with nitrite so far yielded only purple bacteria. The reaction centers of Chlorobiaceae have substantially lower acceptance potentials, i. e., at +250 mV (Madigan & Martinko, 2006), and nitrite oxidation by these bacteria would require reversed electron transport to allow electron flow to the reaction center. Both obtained isolates, strain KS1 and strain LQ17, resembled known types of purple bacteria. The *in vivo* absorption spectra of both strains indicated the presence of bacteriochlorophyll a (Fig. 2) (Kondratieva et al., 1976; Puchkova et al., 2000; Herbert et al., 2005). Both strains were catalase-positive, utilized many organic compounds phototrophically and grew as well aerobically in the dark with malate (strain LQ17) or fructose (strain KS1) as electron and carbon source. A broad pattern of substrate utilization by strain LQ17 and the formation of intracellular sulfur globules in cells of strain KS1 when grown on sulfide or thiosulfate support the results of 16S rRNA gene analysis, which indicated close relationships of strain LQ17 to *Rhodopseudomonas* strains and of strain KS1 to *Thiocapsa* strains (Table 2). Most noteworthy, strain LQ17 is closely related to the aerobic nitrite oxidizer *Nitrobacter hamburgensis* within the α Proteobacteria, and *Thiocapsa* strain KS 1 is related to *Nitrococcus mobilis* (Fig. 5). Thus, the capacity for nitrite oxidation appears to be shared between phototrophic and chemotrophic bacteria within both taxonomic groups.

With its independence of vitamins, its salt tolerance and high tolerance towards oxygen, strain KS1 is a typical representative of the genus *Thiocapsa* (Puchkova et al., 2000; Caumette et al., 2004). Also two known strains of *Thiocapsa roseopersicina*, strain DSM 221 and DSM 217, were found to oxidize nitrite phototrophically. Representatives of both genera, *Rhodopseudomonas* and *Thiocapsa*, are known as generalists among the purple bacteria which are found frequently in various aquatic habitats (Herbert, 1985; Visscher et al., 1990), which is consistent with the high frequency of positive enrichments of nitrite-oxidizing phototrophs from nearly all sampling sites. Nonetheless, MPN counts revealed only low numbers of nitrite-oxidizing phototrophs at two lake sites, and slightly higher numbers in activated sewage sludge. MPN counts use to underestimate real cell numbers in natural samples, e. g., because cultivation media and conditions may not be well adapted or because cell aggregates cannot be efficiently disassembled (Bussmann et al. 2001). Moreover, especially nitrite-oxidizing bacteria tend to form microcolonies which might be overlooked in the scoring. For this reason, we did not only look for growth but also for nitrate formation in the dilution series. Since our cultivation conditions are certainly sub-optimal and also our isolates tend to aggregate and obviously have difficulties to resume growth from single cells, all these factors may have contributed to an underestimation of the cell densities of nitrite-oxidizing phototrophs. Nonetheless, the highest numbers were found in activated sewage sludge where aerobic nitrifiers are active and nitrite was measurable in 10-100 micromolar concentrations. Fortunately, the latter is not true for pristine lake sites, and phototrophic nitrite oxidation may contribute only to a small extent to nitrogen cycling in such habitats. From an early study on phototrophic bacteria in sewage sludge, it was concluded that phototrophs can compete with other bacteria only under anaerobic conditions in the light although their exposure to light in such devices is rather limited, due to the high density of biomass (Siefert et al., 1978). To which extent phototrophic nitrite oxidation contributes to the overall nitrate oxidation activity in activated sludge may be worth a separate study.

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6 Is phototrophic nitrite oxidation by *Thiocapsa sp*. strain KS1 performed through a nitrate reductase?

Joachim Schott & Bernhard Schink

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Abstract

In cell-free extracts of phototrophically grown cells of *Thiocapsa sp.* strain KS1 with nitrite as sole electron donor, no direct oxidation of nitrite could be detected as it was shown earlier for *Nitrobacter sp.* by other authors. Instead, high nitrate reductase activity was measurable almost exclusively in cells grown phototrophically with nitrite as electron donor and ammonia as nitrogen source. Cells grown with hydrogen or fructose as electron donor and ammonium as nitrogen source had no nitrate reductase activity, and cells grown with fructose and nitrate showed only low activities. Cell-free extracts exhibiting nitrate reductase activities revealed two bands of 130-150 kDa and 55-60 kDa in SDS-PAGE, which were specifically strong expressed after growth with nitrite and resembled the α- and β-subunit of the membrane bound nitrate reductase.

Introduction

Anaerobic phototrophic oxidation of nitrite nitrite to nitrate was described first for enrichment cultures (Griffin et al., 2007). More qualitative and quantitative data were obtained later with two pure cultures of nitrite –oxidizing phototrophs, and also data on the abundances of phototrophic nitrite oxidizers were provided (Schott et al., 2010). Also two strains of *Thiocapsa roseopersicina* DSM 221 and DSM 217, were found to be able to photooxidize nitrite anaerobically. These strains were described long ago, e. g. in an enrichment culture by Winogradsky in 1888, and later in pure culture (Schmidt et al., 1965; Pfennig & Trüper, 1971). Strain DSM 217 was originally isolated as *Thiocapsa floridana* strain 1711 (Dr. B.J. Tindall, personal correspondence).

This novel type of metabolism was predicted long before (Olsen 1970, Broda 1977). With the cultivation of phototrophic nitrite oxidizers, now also the biochemistry of anaerobic nitrite oxidation can be studied at the enzymatic level. The only nitrite oxidation process known so far is the aerobic nitrification performed by, e. g., *Nitrobacter spp.*. In this process, the oxygen introduced into nitrite to form nitrate does not derive from oxygen but from water, through the action of a molybdopterin-containing enzyme (Aleem & Sewell, 1981; Sundermeyer-Klinger et al., 1984). These nitrite-oxidizing enzymes exhibited also nitrate reductase activities. Basically, photonitrification could be performed by a similar nitrite:acceptor oxidoreductase, and the released electrons could be shuttled to the membrane-bound photosystem of purple sulfur bacteria which has been studied in detail in the past (Blankenship, 1994; Allen & Williams, 1998; Kramer et al., 2008; Swierczek et al., 2010,).

In these bacteria, the primary electron donor is bacteriochlorophyll P-870, and electrons are shuttled via accessory bacteriochlorophylls, bacteriopheophytin *a*, quinones and various cytochromes or HiPIP back to bacteriochlorophyll *a* (Fig. 1) (Jenney et al., 1996; Meyer & Cusanovich, 2003; Madigan & Martinko, 2006), establishing a proton gradient across the cytoplasmic membrane at the cytochrome bc_1 complex and Q-cycle to generate ATP (Crofts, 2004; Kramer et al., 2008). Electrons released by nitrite oxidation have a midpoint potential of +430 mV and could be shuttled via unknown electron carriers to bacteriochlorophyll P870 at +450 mV. Part of the electrons is transferred via reversed electron flow to NADH, which is needed as electron donor for autotrophic CO_2 fixation (McEvan, 1994).

In the present paper we tried to identify the nitrite-oxidizing enzyme of *Thiocapsa sp.* strain KS1, and to compare it with known nitrite-oxidizing enzymes.

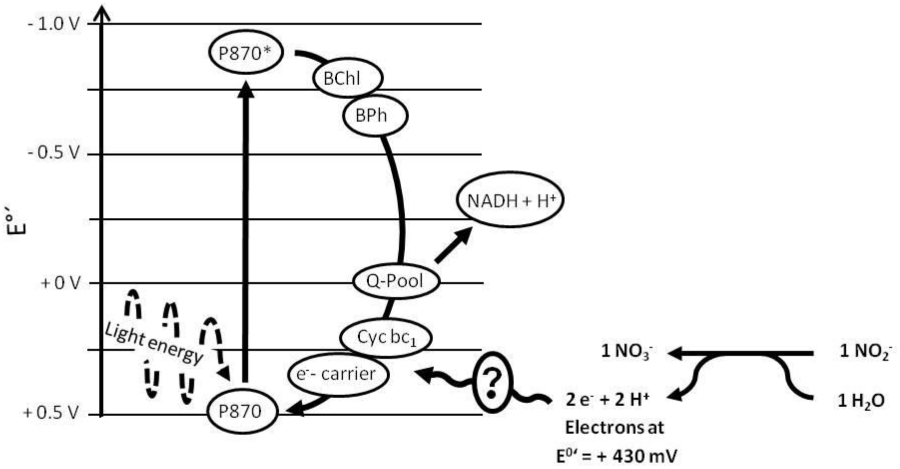

Fig. 1. Edited scheme of the electron transport chain of photosystem II in purple bacteria (Ermler et al., 1994; Blankenship, 1994; Madigan & Martinko, 2006; Kramer et al., 2008). The hypothetical entrance into the phototrophic electron transport chain of the electrons released by nitrite oxidation at +430 mV is marked by the arrow with the question mark. Possible electron carriers are listed below and within the discussion. The redox potential of each compound may differ between different organisms and isolation methods.
Abbreviations: cyt = cytochrome; P870 = special bacteriochlorphyll a pair with a absorption maximum at 870 nm; * = bacteriochlorophyll, excited by a photon; BChl = accessory bacteriochlorophyll; BPh = bacteriopheophytin; Q-Pool = pool of ubiquinon within the cytoplasmic membrane; Cyt bc$_1$ = Complex of cyt b and Rieske-Protein associated in γ- proteobacteria with cyt c$_1$; e$^-$ -carrier = electron carrier, e.g., cyt c$_2$, cyt c$_y$, cyt c´, cyt c$_{8,}$ cyt c$_{4,}$ high potential iron–sulfur protein (HiPIP) and others (Meyer & Cusanovich, 2002).

Materials and Methods

Source of organism and growth conditions

*Thiocapsa sp. s*train KS1 was isolated from sewage sludge of the municipal sewage treatment plant at Konstanz, Germany. Cultures were grown as described before (Schott et al., 2010) except that cultures were incubated at 28°C and re-fed with nitrite at 2 mM increments. Growth was measured via turbidity at 660 nm wavelength with a Camspec M107 spectrophotometer (Camspec, Camspec Ltd.11, High Street, Sawston, Cambridge, UK).

Cultivation and preparation of cell-free extract

Cells were grown in 1-l cultures to an OD of 0.4 to 0.6 and harvested at a remaining nitrite concentration of 0.3 to 0.6 mM by centrifugation (Du Pont de Nemours, Bad Homburg, Germany) for 20 min at 6,000 × g. Fructose (2 mM) or H_2 (in the headspace of the bottle) was provided as alternative electron donor. Harvested cells were washed twice with oxygen-free 50 mM phosphate-buffer, pH 8.0, with half volumes and equal centrifugation steps as described above. The cell pellet was resuspended in 3-5 ml modified cell-cracking buffer (Meincke et al., 1990) containing 50 mM phosphate buffer, pH 8.0, 750 mM sucrose and 3 mM EDTA. Cells were broken by repeated treatments in a cooled French pressure cell (Aminco, Silver Spring, USA) at 137 MPa under N_2 atmosphere on ice. Alternatively, cultures were treated before the French press treatment additionally with protease inhibitor cocktail 50µl · ml^{-1}), lysozyme (4 mg · ml^{-1}), EDTA (0-10 mM), or shock-freezing in liquid nitrogen alone or in various combinations. Alternatively, cells were lysed with mutanolysin (2.5 U · ml^{-1}), lysozyme (4 mg · ml^{-1}), or the mild detergent hexadecyltrimethylammonium bromide (CTAB, 0.1 – 30 mM) (Brune and Schink, 1990). Remaining intact cells and fragments were removed by centrifugation at 6,000 × *g* for 20 min. The membrane fraction was separated from the cytoplasmic and the periplasmic fraction by ultracentrifugation (Optima TL-ultracentrifuge, TLA-100.4-rotor; Beckman, München, Germany) at 120,000 × g for 60 min. Protein concentrations were determined by the microprotein assay (Bradford, 1976) with bovine serum albumin as standard.

Enzyme assays

If not described otherwise, enzyme activities were assayed continuously by following absorption changes with a spectrophotometer 100-40 (Hitachi, Tokyo, Japan) connected to an analogous recorder (SE 120 Metrawatt, BBC Goerz, Vienna, Austria). Assays were performed anoxically in 1 ml volume in rubber-stoppered cuvettes at 30°C. Additions were made with microlitre syringes from anoxic stock solutions. One unit of specific enzyme activity was defined as 1 µmol of substrate consumed or product formed per minute at 30°C, and normalized to milligram of protein.

Nitrate reductase or Nitrite:acceptor oxidoreductase (EC 1.7.99.4)

Nitrate reductase activity assay contained 50 mM Tris-HCl, pH 7.5-8.0, or 50 mM phosphate buffer, pH 7.6-8.0, 1 mM methyl viologen (ε_{578}=9.78 cm^{-1} mM^{-1}) or, alternatively, benzyl viologen (ε_{578}= 8.65 cm^{-1} mM^{-1}) pre-reduced with 1-3 mM sodium dithionite, 10 mM sodium nitrate and 10-20 µl cell-free extract. Viologen oxidation was measured photometrically at 578 nm. The reaction was started by addition of nitrate or cell-free extract. Cell-free extract boiled for 5 min served as control. The formation of nitrite at the end of the reaction was determined by HPLC analysis.

Nitrite oxidase or Nitrite:acceptor oxidoreductase (EC 1.7.99.4)

The nitrite:acceptor oxidoreductase activity assay contained 50 mM Tris-HCl, pH 8.0, or 50 mM phosphate buffer, pH 8.0, 10 mM potassium ferricyanide ($K_3[Fe(CN)_6]$, ε_{420} = 1.02 mM^{-1} cm^{-1}) (Schellenberg and Hellerman,1958; Estabrook, 1961; Nisimoto et al., 2010), 2 mM sodium nitrite, 0 to 20 mM $MgCl_2$, and 10-200 µl cell-free extract, and was measured photometrically at 420 nm as described (Sundermeyer-Klinger et al., 1984). As an alternative artificial electron acceptor, ferricenium hexafluorophosphate (ε_{300} = 4.3 mM^{-1} cm^{-1}, Lehman and Thorpe, 1990) was used.

Alternatively, nitrite oxidation activity was measured by a discontinuous test with nitrite as electron donor and chlorate as electron acceptor as described by Meincke (1990), using HPLC analysis to determine nitrate/nitrite concentrations over time.

Furthermore, different buffer concentrations between 10 and 200 mM of potassium/sodium phosphate or Tris buffer were used; the tested pH range was between 5.0 and 8.0. The reaction mixture was equilibrated at temperatures between 20 and 40°C before starting the reaction with cell-free extract or substrate. As reducing agents, up to 2 mM DTT, DTE, sulfide or dithionite was used.

SDS-PAGE and peptide mass fingerprinting

SDS-PAGE was performed as described in Müller et al. (2009) as minigels (Protean II; Bio-Rad) with 10% polyacrylamide in the resolving gel and 4% polyacrylamide in the stacking gel. Gels were run at 20 mA until the marker front reached the anodic end of the gel, and gels were stained with colloidal Coomassie blue (Neuhoff et al., 1988). Protein bands of interest were blotted to a PVDF membrane (Simeonova et al. 2008) except that the blotting buffer contained additionally 0.4% SDS. Protein bands were sent to TopLab GmbH (Martinsried, Germany) for tryptic digestion and peptide mass fingerprinting without destaining. Cystein side chains were reduced with DTT and alkylated with iodoacetamide. The fingerprints were matched (Mascot search engine) against the amino acid sequences in the database of NCBI.

Chemical analyses

Nitrite and nitrate were quantified by HPLC using an anion exchange column (Sykam, Germany) and UV detection at 210 nm wavelength.

Microscopy and spectroscopy

In order to test for contaminations and for proper cell disruption, cultures and lysates were observed with an Axiophot phase-contrast microscope (Zeiss, Germany).

Chemicals

All chemicals were of analytical grade and were obtained from Fluka (Buchs, Switzerland), Riedel-de Haën (Seelze, Germany), Merck (Darmstadt, Germany), and Sigma (München, Germany). Gases were purchased from Messer-Griesheim (Darmstadt, Germany) and Sauerstoffwerke Friedrichshafen (Friedrichshafen, Germany).

Results

To measure nitrite oxidation of nitrite-grown cells at the enzymatic level, the two enzyme assay systems described by Sundermeyer-Klinger et al. (1984) and Meinke et al. (1992) were applied with various modifications. The only observed difference in absorption decrease appeared between untreated and boiled crude extract when ferricenium was used as artificial electron acceptor. This absorption decrease was not coupled to formation of nitrate and appeared even if the only electron donor nitrite was omitted.

Table 1: Acitivities of the nitrate-reducing enzyme in different cell fractions of *Thiocapsa sp.* strain KS1 after growth with different substrate combinations

Electron donor	**Nitrogen source**	**Cell fraction**	**Specific activity (mU· (mg protein)$^{-1}$))**
NO_2^-	5 mM NH_4^+	Cell-free extract	200-1400
NO_2^-	5 mM NH_4^+	Cytosolic fraction	300-700
NO_2^-	5 mM NH_4^+	Membrane fraction	700-1700
H_2	5 mM NH_4^+	Cell-free extract	0*
H_2	5 mM NH_4^+	Cytosolic fraction	0*
H_2	5 mM NH_4^+	Membrane fraction	0*
Fructose	2 mM NO_3^-	Cell-free extract	0* - 300
Fructose	2 mM NO_3^-	Cytosolic fraction	0* - 200
Fructose	2 mM NO_3^-	Membrane fraction	0* - 50

*** = the enzyme test system had a high background reaction of up to 300 mU, therefore enzyme activities below 100 mU were counted as samples with no enzyme activity**

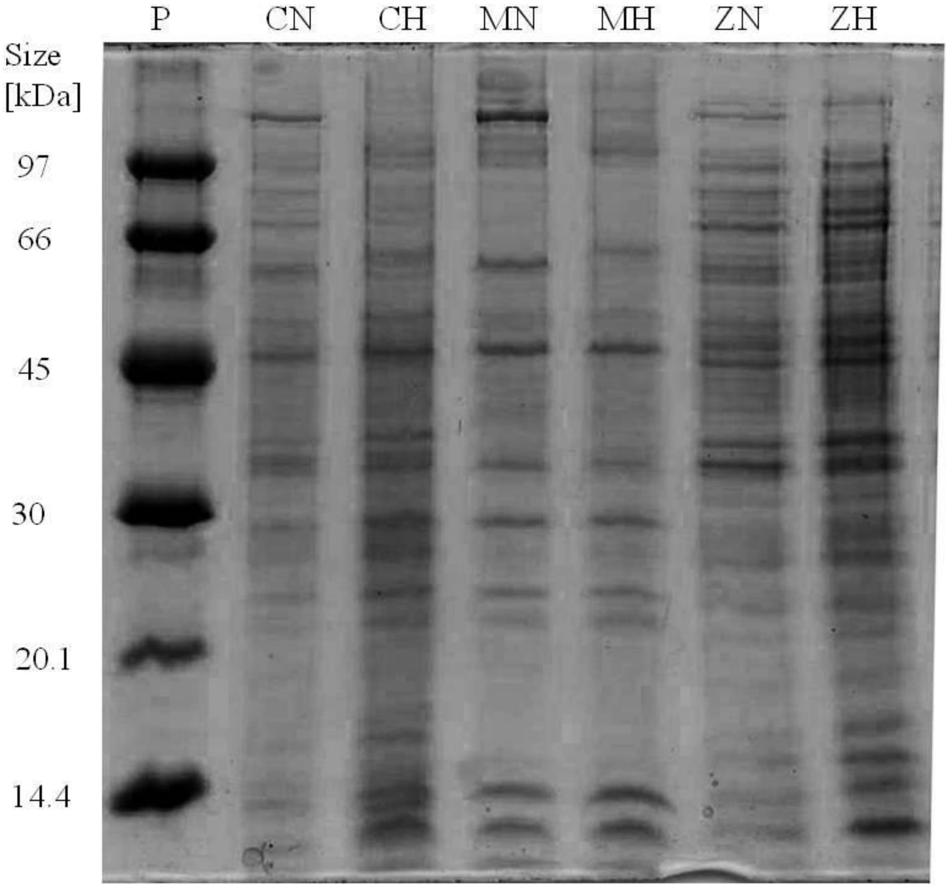

Fig.2 Comparison of 10% acrylamide 1D-SDS-protein-gel-pattern of *Thiocapsa sp.* KS-1 grown phototrophically with nitrite or hydrogen as sole electron donor. 2 mM ammonia served as nitrogen source. Each line was charged with ca. 5.5 µg of protein fraction. P = peptide marker, C = cell-free extract, M = membrane fraction, Z = cytosolic fraction, N = nitrogen grown cells, H = hydrogen grown cells.

Cell fractions were analyzed also by 1-D SDS-PAGE (Fig. 2). Comparison of protein patterns between hydrogen- and nitrite-grown cells revealed two strong bands that were assumed to be involved in nitrite oxidation (Fig. 2). The protein bands had an estimated size of approximately 130-150 kDa and 55-60 kDa, respectively.

The 130-150 kDa band was blotted to a PVDF membrane and sent to TopLap for trypsin digestion and mass spectrometric protein fingerprint analysis (Fig. 3). The resulting peaks were compared with the mascot program to proteins in the NCBI database and resulted with a score of 66 and a sequence coverage of 42% in a similarity to the α-subunit of a putative sarcosine oxidase of the α-proteobacterium *Sinorhizobium meliloti* strain 1021.

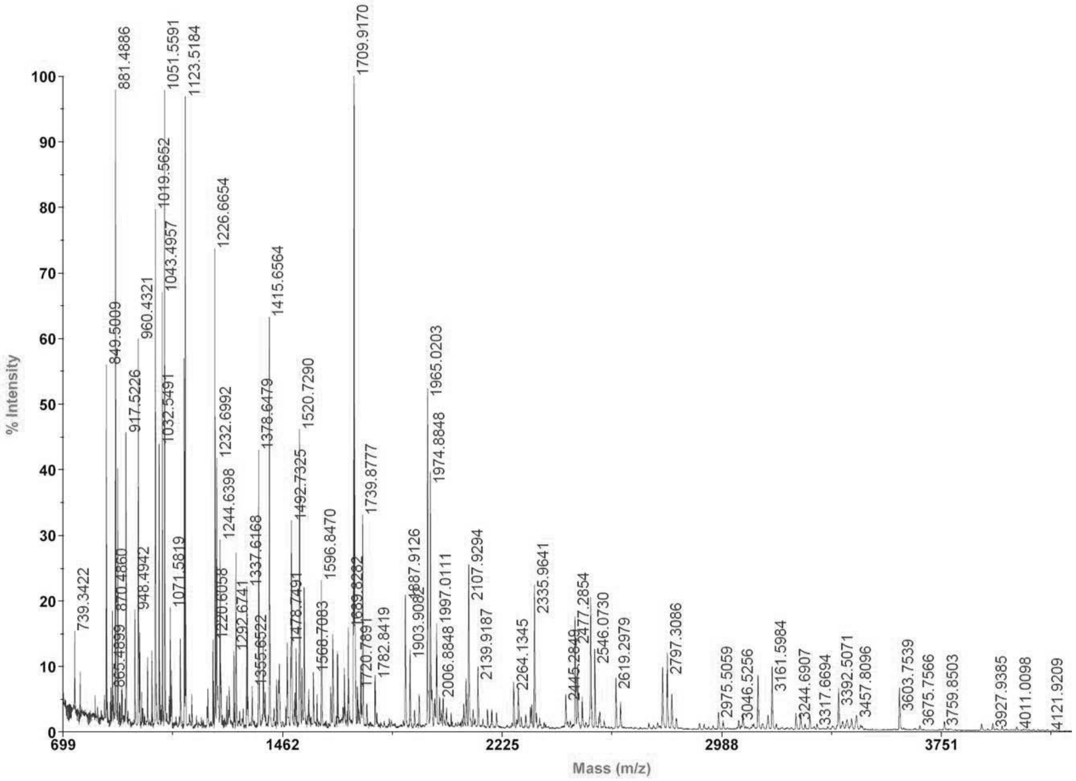

Fig. 3 Peakdiagramm of the peptide mass fingerprint of the 130-150 kDa band. The sample was digested with trypsin, reduced with DTT and alkylated with iodoacetamide.

Cells grown with fructose as electron donor and nitrate as nitrogen source exhibited low nitrate reductase activity (0-300 mU · (mg protein)$^{-1}$) and showed a faint band at 120 kDa in SDS-PAGE (not shown).

Discussion

Cells of *Thiocapsa* sp. strain KS1 grown phototrophically with nitrite as sole electron donor exhibited no nitrite-oxidizing reaction in the assay for nitrite:acceptor oxidoreductase described by Sundermeyer-Klinger et al. (1984) or by Meinke at al. (1992). Alternative discontinuous assays with cytochromes or ferricenium as electron acceptors in combination with light exposure could not detect any activity either (data not shown).

Hence enzyme assays in the reverse direction using reduced methyl or benzyl viologen as artificial electron donor and nitrate as electron acceptor were applied. These assays revealed nitrate reductase activities up to several units per mg protein, and were present at high activity only in cells grown phototrophically with nitrite as electron donor. Cells grown with hydrogen showed no nitrate reductase activity even if grown with nitrate as nitrogen source, but growth with hydrogen plus nitrate as nitrogen source was very poor. In contrast, growth with fructose as electron donor and nitrate as nitrogen source exceeded an OD of 1 and exhibited moderate nitrate reductase activities (up to 300 mU · (mg protein)$^{-1}$).

In comparative SDS-PAGE analysis of various protein fractions, two strong protein bands of a size of approximately 130-150 kDa and 55-60 kDa were prominent specifically in cells grown phototrophically with nitrite as electron donor. These protein bands resembled those of nitrite oxidoreductase of *Nitrobacter hamburgensis* (Meinke et al., 1992; Stolz and Basu 2002): the 130-150 kDa band could represent the catalytically active α subunit, encoded by narG, whereas the 55-60 kDa band could be the 4 Fe 4S-containing β subunit encoded by narH. Interestingly, although strain KS1 can grow aerobically with fructose in the dark and a nitrate reductase activity could be measured in various disrupted cell fractions, no growth was obtained with whole cells and nitrate as sole electron acceptor and fructose as electron donor (Schott et al, 2010). To this first indications of a different kind of nitrate reductase adds the first peptide mass fingerprint analysis of the 130-150 kDa band that revealed a similarity to the α subunit of a putative sarcosine oxdidase of the α-proteobacterium *Sinorhizobium meliloti* strain 1021, though these results have to be confirmed further.

The often found γ subunit of nitrate reductase (20-30 kDa, encoded by narI (Zumft, 1997), which is typically associated with a cytochrome *b*, e.g., in *Escherichia coli* (Rothery et al., 2001), could not be detected in our SDS-PAGE protein analysis. The same was true for nitrite oxidoreductase of *N. hamburgensis* for which no γ subunit could be detected either (Meinke et al., 1992).

Supported by inhibition experiments, Aleem & Sewell (1981) speculated that in *Nitrobacter agilis* one nitrite oxidase is present with cytochrome a_1 (E= +352 mV, Sewell et al., 1972) as natural electron acceptor, and one additional dissimilatory nitrate reductase where cytochrome *b* and cytochrome *c* are involved in the electron transfer.

In phototrophic bacteria, e.g. *Rhodobacter capsulatus*, there are at least two pathways from the cytochrome bc_1 complex to the reaction center, one via the soluble cytochrome c_2 and one via the membrane bound cytochrome c_y (Daldal et al., 2002; Hochkoeppler et al., 1995). With a range of +345 to +395 mV (Pettigrew et al., 1978), the redox potential of various cytochrome c_2 from different phototrophic purple bacteria is high enough to allow an electron transfer from nitrite to the phototrophic reaction center. Whether this transport is directly mediated by different c-type cytochromes (Meyer & Cusanovic, 2003; Axelrod et al., 2002), or if other cytochromes, e.g. cyt c_4 (Branca et al., 2007; Akazaki et al., 2008) or other electron carrier like the membrane-bound high potential iron-sulfur protein (HiPIP, E= +330 mV) (Meyer & Cusanovich, 2003, Menin et al., 1998; Kennel et al., 1972) are involved, has to be investigated further. The remaining gap between the redox couple of nitrate/nitrite and the yet unknown primary electron-accepting carrier may be even shortened at slightly alkaline pH (Aleem & Sewell, 1981).

Still missing is a direct linkage between nitrate oxidation and the 130-150 kDa band, as protein purification attempts as well as in-gel enzyme assays in native gels showed no enzyme activities at all.

Nonetheless, the SDS-PAGE pattern itself, its appearance at specific growth conditions as well as a molybdenum requirement for growth (Schott et al., 2010), and the measured enzyme activities with chlorate as additional electron acceptor have a lot in common with a molybdenum-containing membrane-bound nitrate reductase, in contrast to the molybdenum-containing periplasmic nitrate reductase which is restricted to nitrate as electron acceptor and consists of two subunits (catalytic α subunit of around 90 kDa, endcoded by napA, and the dihaem cytochrome containing β subunit of around 18 kDa, encoded by napB, respectively) and an associated cytochrome, e.g. the tetrahaeme cytochrome *c* encoded by napC (Zumft, 1997, Steenhoudt et al., 2001; Stolz & Basu, 2002; Jepson et al., 2007, Coelho et al., 2011).

Further insights might by uncovered when the genome of our *Thiocapsa* strain is sequenced (in preparation), which would allow a better identification and an access for methods at the genome level of the two bands and possibly some parts of the transduction pathway.

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7. General Discussion

Phototrophic oxidation of nitrite is a new metabolic pathway within the N cycle which was not regarded in any description, calculation or estimation of the nitrogen cycle so far (Falkowski, 1997; Galloway, 1998; Galloway et al., 2004; LeBauer & Treseder, 2008). Hence, as it was done for the anammox reaction, efforts need to be undertaken to study the diversity and environmental distribution of phototrophic bacteria oxidizing nitrite as well as the detailed reaction mechanism of phototrophic nitrite oxidation and its influence on the N cycle.

This thesis described the enrichment and isolation of nitrite oxidizing phototrophs and aspects of nitrite oxidation itself as well as of specific strain characteristics (chapter 4 and chapter 5), whereas chapter 6 concentrates on the necessary enzymatic components. Detailed discussions are given within these chapters. This general discussion has a more critical view on the overall description process including unpublished data and further alternative enrichment and detection methods.

Anaerobic phototrophic nitrite oxidation of strain KS1

Strain KS1 oxidized 1 mM nitrite stoichiometrically to nitrate with concomitant biomass production within 1 day. It exhibited nitrate reductase activity which was found at high activities only in cells grown phototrophically with nitrite as electron donor (chapter 6). SDS-PAGE analysis revealed two bands of 130-150 kDa and 55-65 kDa, respectively, which resemble the α- and β-subunit of a membrane-bound nitrate reductase. In accordance, strain KS1 depended on molybdenum for growth with nitrite, and in enzyme assays the electron acceptor nitrate could be substituted with chlorate, two features which are also found in membrane-bound nitrate reductases (Zumft, 1997). Nonetheless a first peptide mass fingerprint of the 130-150 kDa band obtained from SDS-PAGE did not show similarities to known nitrate reductases, and, interestingly, strain KS1 was able to grow aerobically in the dark with fructose as electron source but not anaerobically with nitrate as alternative electron acceptor (chapter 5). Furthermore, a direct connection between the appearance of these two peptide bands and the measurable nitrate reductase activity could not be established yet, as purification attempts and in-gel activity measurements need to be adapted.

Though nitrate reductase was present at high activities specifically in cells grown phototrophically with nitrite as electron donor, and already described enzyme assays for the forward reaction (Sundermeyer-Klinger et al., 1984; Meinke et al., 1992) could not detect a nitrite-dependent activity, as neither assays with different cell fractions incubated in the light did, it still remains questionable if the observed nitrate reductase activity can really be related to the phototrophic nitrite oxidation reaction *in vivo*.

Either way, if strain KS1 uses a nitrate reductase to oxidize nitrite to nitrate, or if it possesses a new, unknown type of nitrite oxidase, the already existing enzyme assay for a nitrite oxidation reaction have to be adapted further, e.g., with different concentrations of substrates, co-factors, co-substrates or helping enzymes, to pull the electron transfer more towards nitrite oxidation. The other remaining question, how the electrons are passed to the reaction center, could not be investigated yet but might be easier to solve when the genome of strain KS1 is sequenced (in preparation).

Anaerobic, phototrophic nitrite oxidation of strain LQ17

Different nitrite oxidases might be found in other bacteria, e.g., *Rhodopseudomonas sp.* strain LQ17. Tests for nitrite oxidation or nitrate reduction reactions were omitted as growth with 1 mM of nitrite as sole electron donor was incomplete and took over 3 month which is far too long to produce substantial amounts of biomass for biochemical tests. Reasons for this long incubation times were not obvious. On other substrates like pyruvate or benzoate, growth and consumption of the electron donor took only several days which was comparable to the nitrite oxidation rates in enrichment cultures, excluding general growth inhibitions due to the mineral medium composition. Even in repeated liquied dilution series to extinction on 1 mM nitrite, growth in the first dilution steps took only few days, and in the last growing dilution steps, the rod-shaped bacteria could not be separated from the coccus-shaped ones, indicating a kind of interaction between these two bacteria. Subsequent co-cultivation of the isolated strain LQ17 with strain KS1 could not enhance growth of strain LQ17, thus its growth in the enrichment cultures may need some kind of adaptation of one or both bacteria or may be supported by cross-feeding from other bacteria.

Enrichment strategy and used mineral media composition

Difficulties with growth on nitrite were also observed with saltwater enrichment cultures. In 10 out of 20 enrichment from 10 different sites from saltwater marshes around Woods Hole, Ma., USA, a thin reddish layer became visible at the bottom of the flasks. Microscopic pictures showed mainly rod- and coccus-shaped bacteria similar to the ones enriched in freshwater samples, though some differently shaped bacteria were also visible. These cultures oxidized nitrite to nitrate not fully stoichiometrically but only to 50 to 70%. First direct isolation attempts in deep-agar dilution series revealed no nitrite-oxidizing pure culture, and even from the most active four enrichment cultures which could be transferred in saltwater liquid dilution series five to six times, no pure culture was obtained. After the first transfer, the lag and growth phase of all enrichment cultures increased by every further transfer dilution series. After six transfers, growth or nitrite oxidation was not visible for more than 3 months. Probably the provided saltwater medium was not the ideal medium, e.g. missing essential micronutrients. Maybe the addition of small amounts of more reduced sulfur compounds, e.g., sulfide or thiosulfate, or reduced nitrogen compounds such as ammonium, may have avoided growth problems, as some bacteria need a reduced sulfur source (Arunasri et al., 2005). Our saltwater medium did not contain sulfide but sulfate as sole sulfur source to detect even slow phototrophic growth with nitrite by turbidity. If missing nutrients within the saltwater medium are not easy to be identified, growth inhibition by missing nutrients should be ruled out using additionally filter-sterilized saltwater from the sampling site for medium preparation.

Similar enrichment approaches with freshwater minimal medium amended with reduced sulfur compounds may also result in new nitrite oxidizing bacteria, as some bacteria depend on reduced sulfur sources for growth, too. Interestingly, in some of our first freshwater enrichments, there were long filamentous bacteria present which disappeared in further transfers (data not shown). This might be due to missing components in the medium, but also to the transfer procedure, which was carried out with syringes and small needles of 0.4 mm in diameter. Different transfer methods, different cultivation media, or reduced substrate concentrations might enrich for different nitrite-oxidizing phototrophs, as it was shown for aerobic ammonium-oxidizing bacteria at low substrate concentrations (Bollmann & Laanbroek, 2001) and predicted by Costa et al. (2006).

Anaerobic phototrophic nitrite oxidation by already isolated strains

In experiments with the already isolated strains sulfide was added as additional sulfur source. Among other purple sulfur bacteria, e. g. *Thiocapsa bogorowi* (forma *Thiocapsa roseopersicina* strain BBS), *Thiocapsa pendens* strain DSM236, *Allochromatium vinosum* strain DSM180, *Thiobaca trueperii* strain DSM13587, only *Thiocapsa roseopersicina* strains DSM217 and DSM221, the closest known relatives of strain KS1 which were isolated more than 50 years ago under different conditions, were found to oxidize nitrite phototrophically, indicating that this metabolism is not an uncommon capacity of a specialized strain, but may be part of main metabolic processes in several *Thiocapsa roseopersicina* strains. Whether also other bacterial strains are able to oxidize nitrite phototrophically remains to be investigated in further studies.

A first hint might be the utilization of nitrate as nitrogen source, though information in the literature are sometimes irritating. Some strains of *Thiocapsa roseopersicina* are known to utilize nitrate as nitrogen sources (Malofeeva & Laush, 1976, Castillo & Cárdenas, 1982), but not all (Bast, 1977), whereas Imhoff describes the Chromatiaceae to be unable of nitrate utilization (Imhoff, 2006). Perhaps also due to the late discovery of phototrophic nitrite oxidation, some earlier descriptions of new purple sulfur bacteria lack information for the metabolization of N sources, e.g. *Thiocapsa halophila* (Caumette et al., 1991), *Thiorhodococcus minus* (Guyoneaud et al., 1997), T*hiorhodospira sibirica* (Bryantseva et al., 1999), *Thioflavicoccus mobilis* (Imhoff & Pfennig, 2001), *Thiocapsa* *marina* (Caumette et al., 2004) whereas others lack only information of nitrite or nitrate as N sources, e.g. *Marichromatium indicum* (Arunasri et al.,2005), and *Thioalkalicoccus limnaeus* (Bryantseva et al., 2000).

Environmental distribution of anaerobic nitrite-oxidizing phototrophic bacteria

Nitrite oxidizing phototrophs were found in almost all natural sampled litoral sediments ranking from very small periodically flooded waterholes, small creeks and ditches, slow-flowing rivers, small ponds, lakes to saltwater marshes which supported a basic role in the N cycle in these environments. Even in artificial environments such as sewage plant nitrite oxidizing phototrophs could be enriched, though never in fish tank sediments which might be due to its isolated habbitat and cleaning procedures of these sediments.

But estimations on the abundance of nitrite-oxidizing bacteria in natural environments by MPN-counts in 3 different environments revealed only low cell numbers. Sediments of Lake Konstanz contained cell densities below 100 cells per ml and sediments from the slightly acidic Dingeldorfer Ried 3 x 10^3 cells per ml. The highest densities of phototrophic nitrite oxidizers were found in activated sewage sludge with 1.5 x 10^4 cells per ml. As already discussed before, real cell numbers are probably higher due to not idela cultivation and media conditions. In many cases, the inoculum for culture transfer need to be higher than 0.1 %, otherwise no growth occurred in some samples. Although sediment fractions were mixed thoroughly, phototrophic nitrite-oxidizing cells tended to form aggregates with each other and with sediment particles which further contributes to an underestimation.

Outlook and further experiments

A hint for phototrophic nitrite oxidation in natural environments would be the oxidation of nitrite in the presence of alternative electron donors, though these experiments were not yet executed. Furthermore, in many environments a competition for nitrite may take place between bacteria which use respiratory denitrification, DNRA, anammox reaction, aerobic nitrification, or phototrophic nitrite oxidation. Under stable unchanging conditions, most probably one metabolic group would outcompete all others, but such conditions are rare in nature and occur most often only in scientific laboratories. Natural environments for anoxygenic, nitrite-oxidizing phototrophs such as salt- or freshwater sediments are dominated by changes, e.g., different light regimes, changes in temperature or oxygen tensions, which is also reflected by the metabolic versatility of the found nitrite-oxidizing phototrophs, increasing the difficulties of estimating the influence of nitrite-oxidizing phototrophs on the N cycle. Nonetheless, competition experiments for nitrite might offer clues for estimations of nitrite fluxes and metabolic pathway in nature. One interesting short-term experiment would be a distribution of a sample from the site of interest into subsamples which are then placed under different conditions, e.g., anaerobic in darkness, anaerobic and illuminated, aerobic in darkness and aerobic and illuminated. As nitrification depends on oxygen and nitrite-oxidizing phototrophic depend on light, samples of anoxic condition without light resemble the metabolic activity of denitrifying and DNRA bacteria. Samples placed in anoxic conditions in the light add nitrite-oxidation of phototrophic bacteria, whereas aerobic incubation in darkness adds the metabolic activity of nitrifying bacteria. Under oxic conditions in the light, all nitrite-consuming metabolic types are active. Besides nitrite consumption, the production of nitrate, ammonium, or dinitrogen gas could be measured which are the end-products of three different nitrite-consuming metabolic pathways nitrification, DNRA and denitrification. In addition, the use of specific inhibitors for one metabolic group could inhibit its metabolic activity, e.g., acetylen inhibits denitrification (Yoshinari & Knowles, 1976, Smith et al., 1978). Though using inhibitors always raises questions if they do not affect something else, e.g. acetylene also inhibits hydrogenases in nitrogen fixation or monooxygenases in methane oxidation.

Hypothetical impact of anaerobic nitrite oxidation in history

Phototrophic nitrite oxidation does not contribute as an alternative to the discussions of the origin of the banded iron formations. Although Fe(+II) can be oxidized anaerobically with nitrate as electron acceptor (Straub et al., 1996), and the estimated euphotic zone of precambrian oceans might be sufficient to allow phototrophic activity (Kappler et al., 2005), nitrite is already a highly oxidized N compound which probably was formed to detectable amounts only after oxygenic photosynthesis developed. Only if an anaerobic way of ammonium oxidation to nitrite would have existed, the phototrophic oxidation of nitrite could have been an alternative to phototrophic Fe(II) oxidation. In speculations about the origin of oxygenic photosynthesis, phototrophic ammonium oxidation as well as phototrophic nitrite oxidation were discussed as intermediate steps (Olsen, 1970). But up to date, phototrophic ammonium oxidation could not be proven, and also own enrichments for anaerobic, phototrophic ammonium-oxidizers failed (data not shown). Perhaps long-term enrichments under low substrate concentrations such as suggested for bacteria which could oxidize ammonium completely to nitrate (Costa et al., 2006) are more efficient.

Concluding remarks

Nontheless, the recent discoveries of new electron donors for anoxygenic photosynthesis, e.g. arsenite (Kulp et al., 2008) and nitrite (Griffin et al., 2007) encourages for further investigations, leaving nitrite with a redox potential of +430 mV as the highest known electron donor for anoxygenic photosynthesis so far.

The influence of this new metabolic reaction on the N cycle in the environment could not be exactly determined. Nitrite-oxidizing phototrophs could be enriched from many freshwater and some saltwater sites as well as in artificial environments such as sewage treatment plants. In most sampling sites, nitrite concentrations were below the detection limit of 1 µM, but even if nitrite concentrations are low, the turnover rates of nitrate and nitrite might be high as described for other metabolites (Suttle et al., 1990?), which leaves space for metabolic interaction. For instance, nitrite-oxidizing phototrophs could re-oxidize nitrite excreted by denitrifying bacteria, or could in parts substitute nitro-bacteria in nitrifying bacterial aggregates. But due to the versatility of utilized electron donors of strains KS1 and LQ17, the phototrophic nitrite oxidation in natural environment remains questionable until further investigations.

8. References

Afshar, S., Kim, C., Monbouquette, H.G. & Schröder, I. (1998). Effect of tungstate on nitrate reductase by the hypertermophilic archeon *Pyrobaculum aerophilum*.
Appl Environ Microbiol **64**, 3004-3008.

Akazaki, H., Futami, Y., Shibayama, N., Shirasaki, I., Nakade, H., Chida, H., Hakamata, W., Park, S.-Y., Nishio, T. & Oku, T. (2008). Physicochemical properties of diheme cytochrome c_4 of unknown function from *Vibrio parahaemolyticus* strain RIMD2210633.
Biosci Biotechnol Biochem **72**, 2791-2794.

Aleem, M.I.H. & Sewell, D.L. (1981). Mechanism of nitrite oxidation and oxidoreductase systems in *Nitrobacter agilis*.
Curr Microbiol **5**, 267-272.

Allen, J.P. & Williams, J.C. (1998). Photosynthetic reaction centers.
FEMS Microbiol Lett **438**, 5-9.

American Public Health Association (1965). Standard methods for the examination of water and wastewater including bottom sediments and sludge,
American Public Health Association Inc. New York, USA, pp. 604-609.

Antipov, A.N., Lyalikova, N.N., Khijniak, T.V. & L'vov, N.P. (1998). Molybdenum-free nitrate reductases from vanadate-reducing bacteria.
FEBS Lett **441**, 257-260.

Antipov, A.N., Sorokin, D.Y., L'vov, N.P. & Kuenen, J.G. (2003). New enzyme belonging to the family of molybdenum-free nitrate reductases.
Biochem J **369**, 185-189.

Arth, I., Frenzel, P. & Conrad, R. (1998). Denitrification coupled to nitrification in the rhizophere of rice.
Soil Biol Biochem **30**, 509-515.

Arunasri, K., Sasilkala, C., Ramana, C.V., Süling, J. & Imhoff, J.F. (2005). *Marichromatium indicum* sp. nov., a novel purple sulfur gammaproteobacterium from mangrove soil of Goa, India.
Int J Syst Evol Microbiol **55**, 673-679.

Asao, M., Takaichi, S. & Madigan, M.T. (2007). *Thiocapsa imhoffii*, sp. nov., an alkaliphilic purple sulfur bacterium of the family *Chromatiaceae* from Soap Lake, Washington (USA).
Arch Microbiol **188**, 665–675.

Axelrod, H.L., Abresch, E.C., Okamura, M.Y., Yeh, A.P., Rees, D.C. & Feher, G. (2002). X-Ray structural determination of the cytochrome c_2: reaction center electron transfer complex from *Rhodobacter sphaeroides*.
J Mol Biol **319**, 501–515.

Bast, E. (1977). Utilization of nitrogen compounds and ammonium assimilation by *chromatiaceae*.
Arch Microbiol **113**, 91-94.

Blankenship, R.E. (1994). Protein structure, electron transfer and evolution of prokaryotic photosynthetic reaction centers.
Antonie van Leeuwenhoek **65**, 322-329.

Bock, E., Koops, H.-P., Ahlers, B. & Harms, H. (1991) Oxidation of inorganic nitrogen compounds as energy source.
In *The Prokaryotes,* edited by Balows, A., Trüper, H. G., Dworkin, M., Harder, W., Schleifer, K.-H. 2nd ed, Springer Verlag, New York, USA, pp. 414 - 430.

Bokranz, M., Katz, J., Schröder, I., Roberton, A.M. & Kröger, A. (1983). Energy metabolism and biosynthesis of *Vibrio succinogenes* growing with nitrate or nitrite as terminal electron acceptor.
Arch Microbiol **135,** 36–41.

Bollmann, A. & Laanbroek, H.J. (2001). Continuous culture enrichments of ammonia-oxidizing bacteria at low ammonium concentrations.
FEMS Microbiol Ecol **37**, 211-221.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.
Anal Biochem **72**, 248-254.

Branca, R.M.M., Bodó, G., Babyinka, C. & Prokai, L. (2007). *De novo* sequencing of a 21-kDa cytochrome c_4 from *Thiocapsa roseopersicina* by nanoelectrospray ionization ion-trap and Fourier-transform ion-cyclotron resonance mass spectrometry.
J Mass Spectrom **42**, 1569-1582.

Broda, E. (1977). Two kinds of lithotrophs missing in nature.
Z Allg Mikrobiol **17**, 491-493.

Brune, A. & Schink, B. (1990). Pyrogallol-to-phloroglucinol conversion and other hydroxyl-transfer reactions cataylized by cell extracts of *Pelobacter acidigallici*.
J Bacteriol **172**, 1070-1076.

Bryant, D.A. & Frigaard, N.U. (2006). Prokaryotic photosynthesis and phototrophy illuminated.
Trends Microbiol **14**, 488–496.

Bryantseva, I.A., Gorlenko, V.M., Kompantseva, E.I., Imhoff, J.F., Süling, J. & Mityushina, L. (1999). *Thiorhodospira sibirica* gen. nov., sp. nov., a new alkaliphilic purple sulfur bacterium from a Siberian soda lake.
Int J Syst Bacteriol **49**, 697-703.

Bryantseva, I.A., Gorlenko, V.M., Kompantseva, E.I. & Imhoff, J.F. (2000). *Thioalkalicoccus limnaeus* gen. nov., sp. nov., a new alkaliphilic purple sulfur bacterium with bacteriochlorophyll b.
Int J Syst Bacteriol **50**, 2157-2163.

Bussmann, I., Philipp, B. & Schink, B. (2001). Factors influencing the cultivability of lake water bacteria.
J Microbiol Methods **47**, 41.

Castillo, F. & Cárdenas, J. (1982). Nitrate reduction by photosynthetic purple bacteria.
Photosynth Res **3**, 3-18.

Caumette, P., Baulaigue, R. & Matheron, R. (1991). *Thiocapsa halophila* sp. nov., a new halophilic phototrophic purple sulfur bacterium.
Arch Microbiol **155**, 170-176.

Caumette, P., Guyoneaud, R., Imhoff, J. F., Süling, J. & Gorlenko, V. (2004). *Thiocapsa marina* sp. nov., a novel, okenone-containing, purple sulfur bacterium isolated from brackish coastal and marine environments.
Int J Syst Evol Microbiol **54**, 1031–1036.

Cline, J.D. (1969). Spectrophotometric determination of hydrogen sulfide in natural waters. *Limnol Oceanogr* **14**, 454-458.

Coelho, C., González, P.J., Moura, J.J.G., Moura, I., Trincão, J. & Romão, M.J. (2011). The crystal structure of *Cupriavidus necator* nitrate reductase in oxidized and partially reduced states.
J Mol Biol **408**, 932-948.

Cohen, Y., Padan, E., & Shilo, M. 1975. Facultative anoxygenic photosynthesis in the cyanobacterium *Oscillatoria limnetica*.
J Bacteriol **123**, 855-861.

Cohen, Y., Jörgensen, B.B., Revsbech, N.P. and Paplawski, R. (1986). Adaptation to hydrogen sulfide of oxygenic and anoxygenic photosynthesis among cyanobacteria.
Appl Environ Microbiol **51**, 398-407.

Costa, E., Pérez, J. & Kreft, J.-U. (2006). Why is metabolic labour divided in nitrification? *Trends Microbiol* **14**, 213–219.

Croft, A.R. (2004). The Q-cycle – a personal perspective.
Photosynth Res **80**, 223-243.

Cusanovich, M.A, Bartsch, R.G. & Kamen, M.D. (1968). Light-induced electron transport in *Chromatium* strain D. II. Light-induced absorbance changes in *Chromatium* chromatophores.
Biochim Biophys Acta **153**, 397-417.

Dahl, C. (2008). Inorganic sulfur compounds as electron donors in purple sulfur bacteria. In *Sulfur in Phototrophic Organisms* edited by Hell, R., Dahl, C., Knaff, D. B. & Leustek, T.

In *Advances in Photosynthesis and Respiration* **27**, edited by Gvindjee, Amesz, Barber, Blankenship, Murata, Orgen & Ort. Springer Verlag, New York, USA, pp. 289–317

Daldal, F., Deshmukh, M. & Prince, R.C. (2003). Membrane-anchored cytochrome *c* as an electron carrier in photosynthesis and respiration: past, present and future of an unexpected discovery.
Photosynth Res **76**, 127-134.

de Wit, R. & van Gemerden, H. (1986). Chemolithotrophic growth of the phototrophic sulfur bacterium *Thiocapsa roseopersicina.*
FEMS Microbiol Ecol **45**, 117-126.

Dunstan, R.H., Kelley, B.C. & Nicholas, D.J. (1982). Fixation of dinitrogen derived from denitrification of nitrate in a photosynthetic bacterium, *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*.
J Bacteriol **150**, 100–104.

Edwards, U., Rogall, T., Blöcker, H., Emde, M. & Böttger, E. C. (1989). Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA.
Nucleic Acids Res **17**, 7843–7853.

Einsle, O. (2011). Structure and function of formate-dependent cytochrome *c* nitrite reductase, NrfA.
Meth Enzymol **496**, 399-422.

Einsle, O., Messerschmidt, A., Huber, R., Kroneck, P.M.H. & Neese, F. (2002). Mechanism of the six-electron reduction of nitrite to ammonia by cytochrome *c* nitrite reductase.
J Am Chem Soc **124**, 11737-11745

Elser, J.J., Bracken, M.E., Cleland, E.E., Gruner, D.S., Harpole, W.D., Hillebrand, H., Ngai, J.G., Seabloom, E.W., Shurin, J.B. & Smith, J.E. (2007). Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems.
Ecol Lett **10**, 1-8.

Ermler, U., Fritzsch, G., Buchanan, S.K. & Michel, H. (1994). Structure of the photosynthetic reaction centre from Rhodobacter sphaeroides at 2.65 Å resolution: cofactors and protein-cofactor interactions.
Structure **2**, 925–936.

Estabrook, R.W. (1991). Studies of oxidative phosphorylation with potassium ferricyanide as electron acceptor.
J Biol Chem **236**, 3051-3057.

Falkowski, P.G. (1997). Evolution of the nitrogen cycle and its influence on the biological sequestration of CO_2 in the ocean.
Nature **387**, 272-275.

Francis, C.A., Beman, J.M. & Kuypers, M.M.M. (2007). New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation.
ISME J **1**, 19-27

Fukuoka, M., Fukumori, Y. & Yamanaka, T. (1987). *Nitrobacter winogradskyi* cytochrome a_1c_1 is an iron-sulfur molybdoenzyme having hemes a and c.
J Biochem **102**, 525-530.

Galloway, J.N. (1998). The global nitrogen cycle: changes and consequences.
Environ Pollut **102**, 15-24.

Galloway, J.N., Schlesinger, W.H., Levy Il, H., Michaels, A. & Schnoor, J.L. (1995). Nitrogen fixation: anthropogenic enhancement – environmental response.
Global Biochem Cy **9**, 235-252.

Galloway, J.N., Dentener, F.J., Carpone, D.G., Boyer, E.W., Howarth, R.W., Seitzinger, S.P., Asner, G.P., Cleveland, C.C., Green, P.A., Holland, E.A., Karl, D.M., Michaels, A.F., Porter, J.H., Townsend, A.R. & Vörösmarty, C.J. (2004). Nitrogen cycles: past, present, and future.
Biogeochemistry **70**, 153–226.

Garlick, S., Oren, A. & Padan, E. (1977). Occurrence of facultative anoxygenic photosynthesis among filamnentous and unicellular cyanobacteria.
J Bacteriol **129**, 623-629.

Gogotov, I.N. & Glinskii, V. P. (1973). Comparative study of nitrogen fixation in the purple bacteria.
Microbiology (Engl. Transl. Mikrobiologiya) **42**, 877-880.

Griffin, B.M, Schott, J. & Schink, B. (2007). Nitrite, an electron donor for anoxygenic photosynthesis.
Science **316**, 1870.

Guyoneaud, R., Matheron, R., Liesack, W., Imhoff, J.F. & Caumette, P. (1997). *Thiorhodococcus minus*, gen. nov., sp. nov., a new purple sulfur bacterium from coastal lagoon sediments.
Arch Mircobiol **168**, 16-23.

Henckel, T., Friedrich, M. & Conrad, R. (1999). Molecular analyses of the methane - oxidizing microbial community in rice field soil by targeting the genes of the 16S rRNA, particulate methane monooxygenase, and methanol dehydrogenase.
Appl Environ Microbiol **65**, 1980–1990.

Herbert, R. A. (1985). Development of mass blooms of photosynthetic bacteria on sheltered beaches in Scapa Flow, Orkney Islands.
Proc R Soc Edinburgh **87 B**, 15-25.

Herbert, R. A., Ranchou-Peyruse, A., Duran, R., Guyoneaud, R. & Schwabe, S. (2005). Characterization of purple sulfur bacteria from the South Andros Black

Hole cave system: highlights taxonomic problems for ecological studies among the genera *Allochromatium* and *Thiocapsa*.
Environ Microbiol **7**, 1260–1268.

Hochkoeppler, A., Jenney, F.E.Jr., Lang, S.E., Zannoni, D. & Daldal, F. (1995). Membrane-associated cytochrome c_y of *Rhodobacter capsulatus* is an electron carrier from the cytochrome bc_1 complex to the cytochrome *c* oxidase during respiration.
J Bacteriol **177**, 608-613.

Hougardy, A., Tindall, B. J. & Klemme, J.-H. (2000). *Rhodopseudomonas rhenobacensis* sp. nov., a new nitrate -reducing purple non-sulfur bacterium.
Int J Syst Bacteriol **50**, 985–992.

Imhoff, J.F. (1995). Taxonomy and physiology of phototrophic purple bacteria and green sulfur bacteria in *anoxygenic photosynthetic bacteria*.
In *Anoxygenic Photosynthetic Bacteria* edited by Blankenship, R.E., Madigan, M.T. & Bauer, C.E., Vol. 2, Springer Verlag, Springer, The Netherlands, pp. 1-15.

Imhoff, J.F. (2006). The *Chromatiaceae*.
In *The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community*, edited by Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E. 3rd ed, Vol. 6, Springer-Verlag, New York, USA, pp. 846-873.

Imhoff, J.F. & Pfennig, N. (2001). *Thioflavicoccus mobilis* gen. nov., sp. nov., a novel purple sulfur bacterium with bacteriochlorophyll b.
Int J Syst Evol Microbiol **51**, 105-110.

Jenkins, M.C. & Kemp, W.M. (1984). The coupling of nitrification and denitrification in two estuarine sediments.
Limnol Oceanogr **29**, 609-619.

Jenney, F.E.Jr., Prince, R.C. & Daldal, F. (1994). Roles of the soluble cytochrome c_2 and membrane-associated cytochrome c_y of *Rhodobacter capsulatus* in photosynthetic electron transfer.
Biochemistry **33**, 2496-2502.

Jepson, B.J.N., Mohan, S., Clarke, T.A., Gates, A.J., Cole, J.A., Butler, C.S., Butt, J.N., Hemmings, A.M. & Richardson, D.J. (2007). Spectropotentiometric and structural analysis of the periplasmic nitrate reductase from *Escherichia coli*.
J Biol Chem **282**, 6426-6437.

Kappler, A., Pasquero, C., Konhauser, K.O. & Newman, D.K. (2005). Deposition of banded iron formation by anoxygenic phototrophic Fe(II)-oxidizing bacteria.
Geology **33**, 865-868.

Kennel, S.J., Bartsch, R.G. & Kamen, M.D. (1972). Observations on light-induced oxidation reactions in the electron transfer system of *Chromatium*.
Biophys J **12**, 882–896.

Kerber, N.L., Caballero, F.J. & Cárdenas, J. (1981). Assimilatory nitrite-reductase from *Rhodopseudomonas capsulatus* E_1F_1.
FEMS Microbiol Lett **11**, 249-252.

Kisker, C., Schinkelin, H. & Rees, D.C. (1997). Molybdenum-cofactor-containing enzymes: structure and mechanism.
Annu Rev Biochem **66**, 233-267.

Klemme, J.H. (1979). Occurrence of assimilatory nitrate reduction in phototrophic bacteria of the genera *Rhodospirillum and Rhodopseudomonas.*
Microbiologiya **2**, 415- 420.

Kondratieva, E.N., Zhukov, V.G., Ivanovsky, R.N., Petrushkova, Y.P. & Monosov, E.Z. (1976). The capacity of phototrophic sulfur bacterium *Thiocapsa roseopersicina* for chemosynthesis.
Arch Microbiol **108**, 287-292.

Kramer, D.M., Nitschke, W., Cooley, J.W. (2008). The cytochrome bc_1 and related *bc* complexes: the Rieske/cytochrome *b* complex as the functional core of a central electron/proton transfer complex.
In *The purple phototrophic bactria* eds. Hunter, C.N., Daldal, F., Thurnauer, M.C. & Beatty, J.T. Springer Verlag, The Netherlands, pp. 451-473.

Kroneck, P.M.H. & Abt, D.J. (2002). Molybdenum in nitrate reductase and nitrite oxidoreductase.
In *Molybdenum and Tungsten. Their Roles in Biological Processes*, edited by A. Sigel, H. Sigel. New York: M. Dekker Inc., USA, pp. 369-403.

Krüger, B., Meyer, O., Nagel, M., Andreesen, J.R., Meincke, M., Bock, E., Blümle, S., & Zumft, W.G. (1987). Evidence fort he presence of bactopterin in the eubacterial molybdoenzymes nicotinic acid dehydrogenase, nitrite oxidoreductase, and respiratory nitrate reductase.
FEMS Microbiol Lett **48**, 225-227.

Kulp, T.R., Hoeft, S.E., Asao, M., Madigan, M.T., Hollibaugh, J.T., Fischer, J.C., Stolz, J.F., Culbertson, C.W., Miller, L.G. & Oremland, R.S. (2008). Arsenic (III) fuels anoxygenic photosynthesis in hot spring biofilms from Mono Lake, California.
Science **321**, 967-970.

Lane, D.J., Pace, B., Olsen, G.J., Stahl, D.A., Sogin, M.L. & Pace N.R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses.
P Natl Acad Sci USA **82**, 6955-6959.

LeBauer, D.S. & Treseder, K.K. (2008). Nitrogen limitation of net primary productivity in terrestrial ecosystems is globassy distributed.
Ecology **89**, 371-379.

Lee, D.Y., Ramos, A., Macomber, L. & Shapleigh, J.P. (2002). Taxis response of various denitrifying bacteria to nitrate and nitrite.
Appl Environ Microbiol **68**, 2140-2147.

Lehman, T.C. & Thorpe, C. (1990). Alternative electron acceptors for medium-chain acyl-CoA dehydrogenase: use of ferricenium salts.
Biochemistry **29**, 10594-10602.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., König, A., Liss, T., Lüssmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., Schleifer, K.H.. (2004). ARB: a software environment for sequence data.
Nucl Acids Res **32**, 1363-13.

Mackenzie, F.T. (1998). Our changing planet: An introduction to earth system science and global environmental change, 2nd ed. Prentice-Hall, Upper Saddle River, New Jersey, USA.

Madigan, M. T. & Martinko, J. M. (2006). *Brock Biology of Microorganisms*. 11th ed. Pearson Prentice Hall, New Jersey, USA, p. 992.

Madigan, M., Cox, S.S. & Stegeman, R.A. (1984). Nitrogen fixation and nitrogenise activities in members of the family *Rhodospirillaceae*.
J Bacteriol **157**, 73-78.

Malofeeva, I.V. & Laush, D. (1976). Utilization of nitrogen compounds by phototrophic bacteria.
Mikrobiologiya **45**, 512-514.

Malofeeva, I.V., Bogorov, L.V. & Gogotov, I.N. (1974). Utilization of nitrates by purple bacteria.
Mikrobiologiya **43**, 967-972.

McEwan, A.G. (1994). Photosynthetic electron transport and anaerobic metabolism in purple non-sulfur phototrophic bacteria.
Antonie van Leeuwenhoek **66**, 151-164.

Meincke, M., Bock, E., Kastrau, D. & Kroneck, P.M.H. (1992). Nitrite oxidoreductase from *Nitrobacter hamburgensis:* redox centers and their catalytic role.
Arch Microbiol **158**, 127-131.

Meinke, M. (1990). Untersuchungen zur Struktur und Funktion von Redoxzentren im Enzym Nitrit-Oxidoreductase, Dissertation, Universität Hamburg, Germany.

Megonigal, J.P., Hines, M.E. & Visscher, P.T. (2003) Anaerobic metabolism: linkages to trace gases and aerobic processes.
In *Treatise on Geochmistry*, edited by Schlesinger, W.H., Vol. 8, Elsevier, Amsterdam, The Netherlands, pp. 317-424.

Menin, L., Gaillard, J., Parot, P., Schoepp, B., Nitschke, W. & Verméglio, A. (1998). Role of HiPIP as electron donor to the RC-bound cytochrome in photosynthetic purple bacteria.

Photosynth Res **55**, 343–348.

Meyer, T.E. & Cusanovich M.A. (2003). Discovery and characterization of electron transfer proteins in the photosynthetic bacteria.
Photosynth Res **76**, 111-126.

Mills, M.M., Ridame, C., Davey, M., La Roche, J. & Geider, R.J. (2004). Iron and phosphorus co-limit nitrogen fixation in the eastern tropical North Atlantic.
Nature **429**, 292-294.

Müller, N., Schleheck, D. & Schink, B. (2009). Involvement of NADH:acceptor oxidoreductase and butyryl coenzyme A dehydrogenase in reveserd electron transport during syntrophic butyrate oxidation by *Syntrophomonas wolfei*.
J Bacteriol **191**, 6167-6177.

Nisimoto, Y., Jackson, H.M., Ogawa, H., Kawahara, T., & Lambeth, J.D. (2010). Constitutive NADPH-dependent electron transferase activity of the Nox4 dehydrogenase domain.
Biochemistry **49**, 2433-2442.

Olmo-Mira, M.F., Cabello, P., Pino, C., Martínez-Luque, M., Richardson, D.J., Castillo, F., Roldán, M.D. & Moreno-Vivián, C. (2006). Expression and characterization of the assimilatory NADH-nitrite reductase from the phototrophic bacterium *Rhodobacter capsulatus* E1F1.
Arch Microbiol **186**, 339-344.

Olson, J. M. (1970). The evolution of photosynthesis.
Science **168,** 438-446.

Oren, A., Padan, E., & Avron, M. (1977). Quantum yields for oxygenic and anoxygenic photosynthesis in the cyanobacterium *Oscillatoria limnetica*.
P Natl Acad Sci USA **74**, 2152-2156.

Pettigrew, G.W., Bartsch, R.G., Meyer, T.E. & Kamen, M.D. (1978). Redox potentials of the photosynthetic bacterial cytochromes c_2 and the structural bases for variability. *Biochim Biophys Acta* **503**, 509-523.

Pfennig, N. & Biebl, H. (1976). *Desulfuromonas acetoxidans* gen. nov. and sp. nov., a new anaerobic, sulfur reducing, acetate -oxidizing bacterium.
Arch Microbiol **110**, 3-12.

Pfennig, N. & Trüper, H.G. (1971). Type and neotype strains oft he species of phototrophic bacteria maintained in pure culture.
Int J Syst Bacteriol **21**, 19-24.

Pfennig, N. (1967). Photosynthetic Bacteria.
Annu Rev Microbiol **21**, 285-324.

Pfennig, N. (1976). Phototrophic green and purple bacteria: Adaption to the aquatic environment and role in the sulfur cycle.
Proc Soc Gen Microbiol **4**, 19-20.

Pfennig, N. (1977). Phototrophic green and purple bacteria: a comparative, systematic survey. *Annu Rev Microbiol* **31**, 275-290.

Pfennig, N. (1978). General physiology and ecology of photosynthetic bacteria. In *The Photosynthetic Bacteria,* edited by Sistrom, W.R. & Clayton, R.K. Plenum Press, New York, USA, pp. 3-18.

Pino, C., Olmo-Mira, F., Cabello, P., Martínez-Luque, M., Castillo, F., Roldán, M.D. & Moreno-Vivián, C. (2006). The assimilatory nitrate reduction system of the phototrophic bacterium *Rhodobacter capsulatus* E1F1. *Biochem Soc Trans* **34**, 127-129.

Preuss, M. & Klemme, J.-H. (1983). Purification and characterization of a dissimilatory nitrite reductase from the phototrophic bacterium *Rhodopseudomonas palustris*. *Z Naturforsch Sect C* **38**, 933–938.

Puchkova, N.N., Imhoff, J.F. & Gorlenko, V.M. (2000). *Thiocapsa litoralis* sp. nov., a new purple sulphur bacterium from microbial mats from the White Sea. *Int J Syst Evol Microbiol* **50**, 1441–1447.

Reich, P.B., Hungate, B.A. & Luo, Y. (2006). Carbon-nitrogen interactions in terrestrial ecosystems in response to rising atmospheric carbon dioxide. *Annu Rev Evol Syst* **37**, 611-636.

Rothery, R.A., Blasco, F., Magalon, A. & Weiner, J.H. (2001). The diheme cytochrome *b* subunit (NarI) of *Escherichia coli* nitrate reductase A (NarGHI): structure, function, and interaction with quinols. *J Mol Microbiol Biotechnol* **3**, 273-283.

Satoh, T., Hoshino, Y. & Kitamura, H. (1976). *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*, a denitrifying strain as a subspecies of *Rhodopseudomonas sphaeroides*. *Arch Microbiol* **108**, 265–269.

Schaub, B.E.M. & van Gemerden, H. (1994). Simultaneous phototrophic and chemotrophic growth in the purple sulfur bacterium *Thiocapsa roseopersicina* M1. *FEMS Microbiol Ecol* **13**, 185-196.

Schellenberg, K.A. & Hellerman, L. (1958). Oxidation of reduced diphosphopyridine nucleotide. *J Biol Chem* **231**, 547–556.

Schmidt, K., Pfennig, N. & Liaaen Jensen, S. (1965). Carotenoids of thiorhodaceae. *Arch Microbiol* **52,** 132-146.

Schott, J., Griffin, B.M. & Schink, B. (2010). Anaerobic phototrophic nitrite oxidation by *Thiocapsa* sp. strain KS1 and *Rhodopseudomonas* sp. strain LQ17. *Microbiology* **156**, 2428-2437.

Schwartz, G., Mendel, R.R. & Ribbe, M.W. (2009). Molybdenum cofactors, enzymes and pathways.
Nature **460**, 839-847.

Sewell, D.L., Aleem, M.I.H. & Wilson, D.F. (1972). The oxidation-reduction potentials and rates of oxidation of the cytochromes of *Nitrobacter agilis*.
Arch Biochem Biophys **153**, 312-319.

Siefert, E., Irgens R.L. & Pfennig, N. (1978). Phototrophic purple and green bacteria in a sewage treatment plant.
Appl Environ Microbiol **35**, 38-44.

Simeonova, D.D., Susnea, I., Moise, A., Schink, B. & Przybylski, M. (2009). “Unknown genome” proteomics: a new NAD(P)-dependent epimerase/dehydratase revealed by N-terminal sequencing, inverted PCR, and high resolution mass spectrometry.
Mol Cell Proteomics **8,** 122–131.

Stackebrandt, E., Rainey, F.A. & Ward-Rainey, N. (1996). Anoxygenic phototrophy across the phylogenetic spectrum: current understanding and future perspectives.
Arch Microbiol **166**, 211-223.

Stanier, R. Y., Pfennig, N. & Trüper, H. G. (1981). Introduction to the phototrophic prokaryotes.
In *The Prokaryotes*, edited by Starr, M.P., Stolp, H., Trüper, H.G., Balows, A., Schlegel H.G. 8th ed, Springer Verlag, Berlin, Germany, pp. 197-211.

Straub, K.L., Benz, M., Schink, B. & Widdl, F. (1996). Anaerobic, nitrate-dependent microbial oxidation of ferrous iron.
Appl Environ Microbiol **62**, 1458-1460.

Seefeldt, L.C., Hoffman, B.M. & Dean, D.R. (2009) Mechanism of Mo-dependent nitrogenase. *Annu Rev Biochem* **78**, 701-722.

Steenhoudt, O., Keijers, V., Okon, Y. & Vanderleyden, J. (2001). Identification and characterization of a periplasmic nitrate reductase in *Azospirillum brasilense* Sp245.
Arch Mircobiol **175**, 344-352.

Stolz, J.F. & Basu, P. (2002). Evolution of nitrate reductase: molecular and structural variations on a common function.
Chem Biochem **1**,198-206.

Strohm, T.O., Griffin, B., Zumft, W. & Schink, B. (2007). Growth yields in bacterial denitrification and nitrate ammonification.
Appl Environ Microbiol **73**, 1420-1424.

Sundermeyer-Klinger, S., Meyer, W., Warninghoff, B. & Bock, E. (1984). Membrane-bound nitrite oxidoreductase of *Nitrobacter*: evidence for a nitrate reductase system.
Arch Microbial **140**, 153-158.

Swierczek, M., Cieluch, E., Sarewicz, M., Borek, A., Moser, C.C., Dutton, P.L. & Osyczka, A. (2010). An electronic bus bar lies in the core of cytochrome *bc*$_1$.
Science **329**, 451-454.

Thauer, R.K., Jungermann, K. & Decker, K. (1977). Energy conservation in chemotrophic anaerobic bacteria.
Bacteriol Rev **41**, 100-180.

Trüper, H.G. & Pfennig, N. (1981). Characterization and identification of the anoxygenic phototrophic bacteria.
In *The Prokaryotes*, edited by Starr, M.P., Stolp, H., Trüper, H.G., Balows, A., Schlegel H.G. 8th ed, Springer Verlag, Berlin, Germany, pp. 299-312.

Tyrell, T. (1999). The relative influences of nitrogen and phosphorus on oceanic primary production.
Nature **400**, 525-531

Van Trappen, S., Mergaert, J. & Swings, J. (2004). *Loktanella salsilacus* gen. nov., sp. nov., *Loktanella fryxellensis* sp. nov. and *Loktanella vestfoldensis* sp. nov., new members of the Rhodobacter group, isolated from microbial mats in Antarctic lakes.
Int J Syst Evol Microbiol **54**, 1263–1269.

Venter, J.C., Remington, K., Heidelberg, J.F., Halpern, A.L., Rusch, D., Eisen, J.A., Wu, D., Paulsen, I., Nelson, K.E., Nelson, W., Fouts, D.E., Levy, S., Knap, A.H., Lomas, M.W., Nealson, K., White, O., Peterson, J., Hoffman, J., Parsons, R., Baden-Tillson, H., Pfannkoch, C., Rogers, Y.-H. & Smith, H.O. (2004). Environmental Genome Shotgun Sequencing of the Sargasso Sea.
Science 304, 66-74

Vitousek, P.M. & Howarth, R.W. (1991). Nitrogen limitation on land and in the sea: How can it occur?
Biogeochemistry **13**, 87-115.

Vitousek P.M., Hattenschwiler, S., Olander, L. & Allison, S. (2002). Nitrogen and nature.
Ambio **31**, 97–101.

Visscher, P.T., Nijburg, J.W. & van Gemerden, H. (1990). Polysulfide utilization by *Thiocapsa roseopersicina.*
Arch Microbiol **155**, 75–81.

Weisburg, W.G., Barns, S.M., Pelletier, D.A. & Lane, D.J. (1991). 16S ribosomal DNA amplification for phylogenetic study.
J Bacteriol **173**, 697–703.

Widdel, F. & Bak, F. (1992). Gram-negative mesophilic sulfate-reducing bacteria.
In *The Prokaryotes*, edited by Balows, A., Trüper, H.G., Dworkin, M., Harder, W. & Schleifer, K.-H. 2nd ed., Springer-Verlag, New York, USA, pp. 3352- 3378.

Widdel, F., Schnell, S., Heising, S., Ehrenreich, A., Assmus, B. & Schink, B. (1993). Ferrous iron oxidation by anoxygenic phototrophic bacteria. *Nature* **362**, 834–836.

Yildiz, F.H., Gest, H. & Bauer, C.E. (1991). Attenuated effect of oxygen on photopigment synthesis in *Rhodospirillum centenum*. *J Bacteriol* **173,** 5502–5506.

Yurkov, V.V., & Beatty, J.T. (1998). Aerobic anoxygenic phototrophic bacteria. *Microbiol Mol Biol Rev* **62**, 695–724.

Zumft, W.G. (1997). Cell biology and molecular basis of denitrification. *Microbiol Mol Biol R* **61**, 533-616.

9 Appendix

Abbreviations

°C	degree Celsius
µ	micro- (10^{-6})
ARB	*Arbor* (software package comprising various tools for sequence and phylogenetic analysis)
ATP	adenosine triphosphate
bch	genes encoding for bacteriochlorophyll biosynthesis
BChl	bacteriochlorophyll
BLAST	basic local alignment search tool
bp	base pairs
c	centi- (10^{2})
crt	genes encoding for carotinoid biosynthesis
CTAB	cetyl trimethyl ammonium bromide
cyt	cytochrom
d	day
Da	dalton
DCMU	Diuron (3-(3,4-Dichlorophenyl)-1,1-dimethylurea)
DMS	dimethylsulfide
DNA	desoxyribonucleic acid
DNRA	Dissimilatory Nitrate Reduction to Ammonium
DSM	Deutsche Sammlung für Mikroorganismen
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen
DTE	dithioerythritol
DTT	dithiothreitol
EDTA	ethylendiaminotetraacetic acid
e.g.	*exempli gratia* (for example)
et al.	*et alii* (and others)
f	forward
fig.	figure
g	gram or gravitational constant
h	hour
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HiPIP	high potential iron-sulfur protein
HPLC	high performance liquid chromatography
i.e.	*id est* (that is)
k	kilo (10^{3})
l	liter
lx	lux
M	mega (10^{9}) or mol $\cdot l^{-1}$
Ma	Massachusetts
m	milli- (10^{-3}) or meter
min	minute
MPN	most probable number
N	nitrogen
n	nano- (10^{-9})
n.d.	not determined
NADH	nicotinamimde adenine dinucleotide, reduced form

NCBI	National Center for Biotechnology Information
NY	New York
OD	optical dencity
Pa	pascal
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMF	peptide mass fingerprinting
puc	genes encoding for light harvesting complex II
puf	genes encoding for reaction center type II
puh	genes encoding for light harvesting complex I
PVDF	polyvinylidenfluorid
r	reverse
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecylsulfate
T.	*Thiocapsa*
U	enzyme unit
UK	United Kingdom
USA	United States of America
UV	ultraviolett
V	volt
v/v	volume / volume
W	watt
w/v	weight / volume

Declaration of author contribution

If not mentioned otherwise, results described in chapter 4, 5, and 6 were exclusively performed by myself or under my direct supervision. The subsequent analyses of these results and the composition oft the manuscripts were discussed with my supervisor Prof. Schink.

Following exceptions were made in:

- **the introduction, where the idea of drawing the nitrogen cycle with nitrite as central intermediate first arose from Benjamin M. Griffin.**

- **chapter 4, where Benjamin M. Griffin planned and performed most oft the experiments and the composition of the manuscript, whereas my contributions concentrated on the cultivation of enrichment cultures and isolation attempts. The micrographical picture was taken by myself as well.**

- **chapter 5, where the saltwater samples were collected by Benjamin M. Griffin. Also the idea of isolating the coccus shaped bacterium by altering the electron donors between 1 mM nitrite and 2 mM sulfide in liquid dilutions came from Benjamin M. Griffin.**

- **the discussion, where the idea for a competitive nitrite consumption experiment came from Prof. Schink**