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

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In anaerobic enrichment cultures for phototrophic nitrite-oxidizing bacteria from different freshwater sites, two different cell types, i.e. non-motile cocci and motile, rod-shaped bacteria, always outnumbered all other bacteria. Most-probable-number (MPN) dilution series with samples from two freshwater sites yielded only low numbers ($\leqslant 3 \times 10^3$ 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 in both 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 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 its 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 and ferrous iron for auto-trophic cell carbon synthesis from carbon dioxide (Pfennig, 1967, 1977; Widdel *et al.*, 1993; Stackebrandt *et al.*, 1996). Photosynthetic bacteria can be found in almost every aquatic environment, including freshwater, marine, alkaline, acidic and hot or cold waters (Pfennig, 1976, 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 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 bacteria, such as *Rhodopseudomonas* spp. and *Rhodobacter* spp., 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 non-sulfur 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 was 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, strain KS1 and strain LQ17, enriched with nitrite as electron donor is studied in detail, and also with respect to its potential function in nitrite oxidation in nature.

METHODS

Chemicals. All chemicals were of analytical grade and were obtained from Fluka, Riedel-de Haën, Merck and Sigma. Gases were purchased from Messer-Griesheim and Sauerstoffwerke Friedrichshafen.

Sources of organisms. *Thiocapsa roseopersicina* strains DSM 221 and DSM 217 and *Rhodopseudomonas palustris* strain DSM 123 were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). 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-capped 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 ~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 illuminance 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. The 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 (per litre: 20 g NaCl, 3 g $MgCl_2 . 6H_2O$).

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 a wavelength of 578 or 660 nm (Spectronic 70 spectrophotometer, Bausch & Lomb, or Camspec M107 spectrophotometer, Camspec) for more than 2 months. Substrate utilization was tested in triplicate with more than 25 different substrates in the light, and the optical density was followed for 3 months. Measurements of optical density values were converted to biomass yields using an empirically determined

conversion factor (OD_{578} 1 equals 250 mg cell mass l^{-1}) that 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 with strain KS1 alone 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 HEPES, pH 7.2, and 1.5 mM fructose as electron and carbon source, in the presence and absence of 21 % oxygen under an N_2 atmosphere. A CO_2/bicarbonate buffer (5 mM, pH 7.2) was added as an additional carbon source. This buffer system was also used to test both the temperature range and the optimum for 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 % (w/v) HCl for 12 h and washed with double-distilled water. Three out of six independent dilution series each with three 1 : 10 dilution steps were provided with extra molybdenum (300 nM) and growth was followed by optical density 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 containing 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 (MPN) 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 DCMU. Tubes were inoculated with surface material from one of three different freshwater sediments: a small slightly dystrophic lake (Dingelsdorfer Ried), a large neutral oligotrophic pre-alpine hard-water lake (Lake Constance), and 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 the highest speed before transfer. Tubes were incubated for up to 3 months before final scores were taken. Growth was measured and scored positive if nitrite disappeared and detectable amounts of nitrate were formed. Nitrite was refed when the concentration dropped below 0.1 mM.

Toxicity tests. The toxicity of nitrite was tested with phototrophic enrichment cultures from the Konstanz sewage plant, with nitrite additions in the range 0.5–15 mM nitrite. The 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) equipped with a digital camera (Intas Science Imaging Instruments) using the software MagnaFire 2.0 (Optronics).

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

Chemical analyses. Nitrite and nitrate were quantified by HPLC using an anion exchange column (Sykam) and UV detection at 210 nm, and were determined semiquantitatively with Merckoquant test strips (nitrite test 2–80 mg NO_2 l^{-1}; nitrate test 10–500 mg NO_3 l^{-1}) (Merck). Sulfide was measured colorimetrically following Cline (1969).

DNA extraction and 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) 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′) and 533F (Weisburg *et al.*, 1991) (5′-GTG CCA GCA GCC GCG GTA A-3′). Sequences were aligned using DNASTAR (http://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.

A culture of *Thiocapsa* sp. strain KS1 has been deposited with the Japan Collection of Micro-organisms under accession 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 in order to include surface-attached bacteria also. 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 refed with 1 mM nitrite when the nitrite concentrations dropped below 0.1 mM. In the first 3 weeks, some enrichment cultures consumed the given nitrite without concomitant nitrate formation. After approximately 1 month and repeated refeedings (depending on the source of inoculum), the nitrite consumption rate decreased. After a few more weeks, nitrate accumulated to a 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 1 month. After repeated refeeding, 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 resampling 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 also oxidized nitrite to nitrate 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.

MPN counts

Estimations of cell densities of nitrite-oxidizing phototrophs by the MPN technique were performed with samples from 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 two subsamples,

one containing the upper 10 mm layer and one containing the sediment layer at 10–20 mm depth. In both samples from a littoral sediment of Lake Constance, phototrophic nitrite oxidizers were below 100 cells ml^{-1}. Sediments from the slightly acidic Dingeldorfer Ried contained 3×10^3 cells ml^{-1}, and the highest cell densities were found in activated sewage sludge at 1.5×10^4 cells ml^{-1}. In this sewage sludge, which also performed ammonia and nitrite oxidation, we found nitrite at a concentration of 10–50 µM; 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, and 2–3 µm in diameter (Fig. 1a). 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 optical density and nitrite oxidation rates as those of anaerobic cultures for several days, but were slightly pale in colour compared with anoxic cultures. Fructose was utilized aerobically in the dark as well as anaerobically in the light. No anaerobic growth with fructose was observed in the dark, with either nitrate or sulfate as electron acceptor.

Besides nitrite (1–2 mM), strain KS1 photo-assimilated the following substrates (concentrations in mM): fructose (1–4), 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. 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 optical density could be detected at sulfide concentrations of

5.5 mM or higher. Strain KS1 grew best in freshwater medium, but growth was possible at up to 18 g NaCl l^{-1}. Optimal growth was found at 28 °C, with limits of 4 and 37 °C. Growth was possible within a pH range of 6.5–8.8, with a broad optimum around pH 7.2. Vitamins were not required. Strain KS1 utilized sulfate, sulfite, thiosulfate and elemental sulfur as sulfur sources, and nitrate, nitrite, N$_2$ and ammonia as nitrogen sources. An *in vivo* absorption spectrum between 350 and 900 nm showed absorption maxima at wavelengths of 376, 486, 516, 552, 590, 799 and 854 nm (Fig. 2), indicating the presence of bacteriochlorophyll *a* and carotenoids of the spirilloxanthin series; no absorption peaks beyond 900 nm were evident.

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 colour. 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, in the presence of either 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. The *in vivo* absorption spectrum between 350 and 900 nm showed absorption maxima at wavelengths of 376, 459, 491, 525, 591, 805 and 863 nm (Fig. 2).
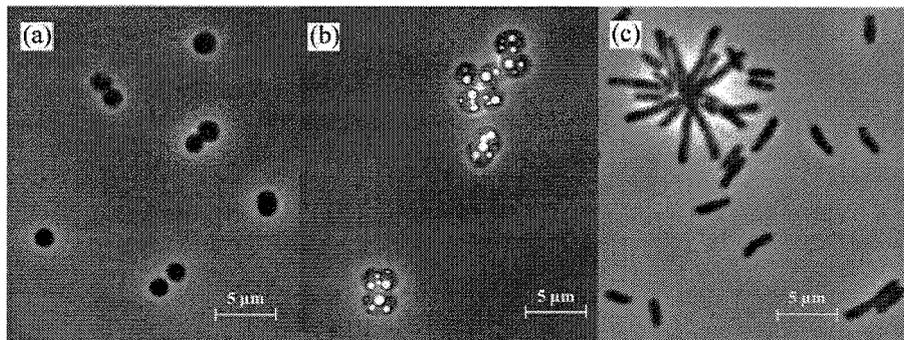
Fig. 1. Phase-contrast photomicrographs of nitrite-oxidizing phototrophs. (a) Strain KS1 grown with nitrite, (b) strain KS1 grown with thiosulfate, (c) strain LQ17 grown with nitrite as electron donor. Bars, 5 µm.

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

Substrate provided	Concentration (mM)	Measured ΔOD_{578}	Biomass produced (mg l^{-1})	Calculated biomass (mg l^{-1})	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	222.0	210	105.7

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 subcultures 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.

## 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 1 month, the nitrite concentration started to decrease with a simultaneous increase in optical density. Nitrate was produced non-stoichiometrically and reached up to 60 % of the consumed nitrite (Fig. 4).

Taxonomic assignment

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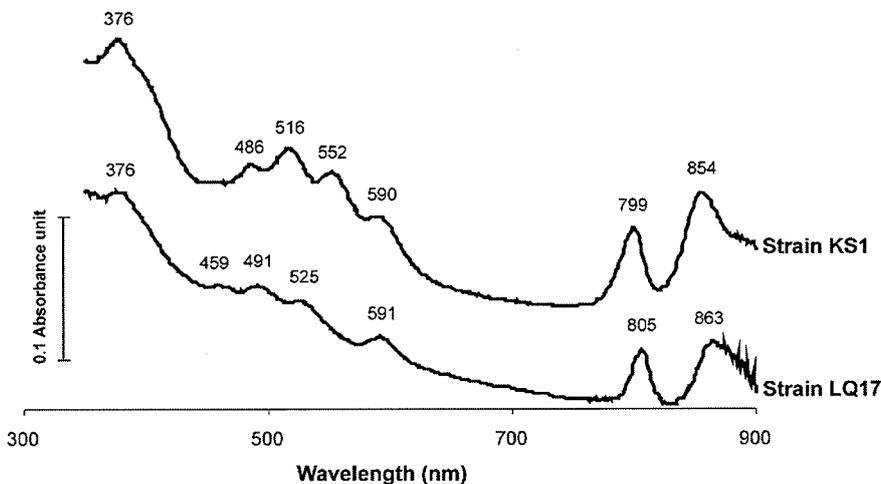

Fig. 2. Absorption spectra of dense cell suspensions of nitrite-oxidizing phototrophs after growth with nitrite. Cells were suspended in saturated sucrose solution.
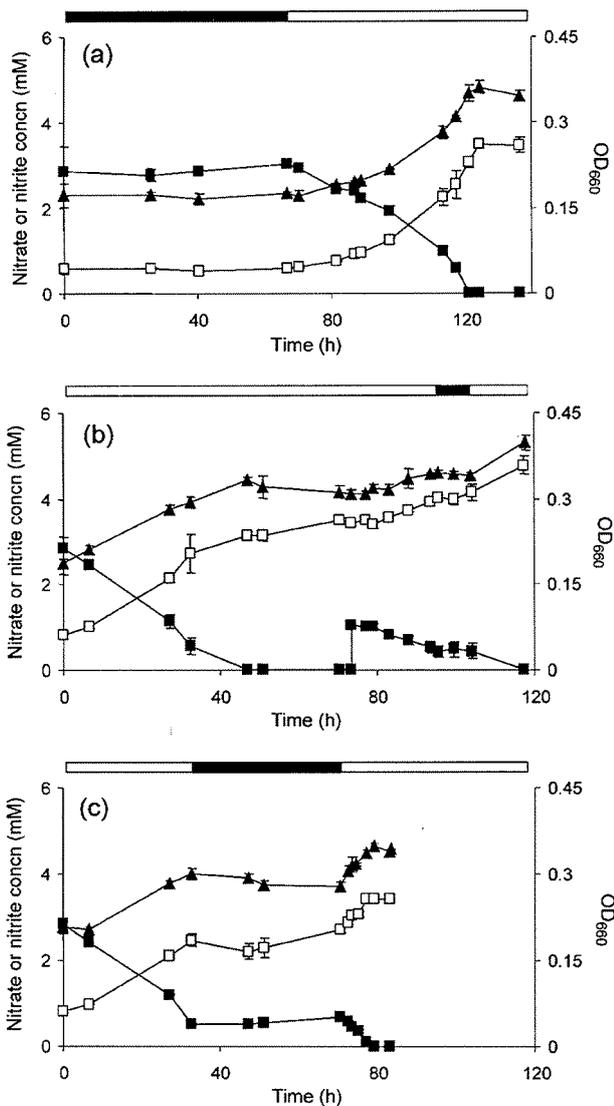
Fig. 3. Phototrophic growth and nitrite oxidation by strain KS1. One culture was distributed and incubated under three different light regimes. The bar above the diagrams displays the illumination patterns (black=darkness; white=illumination at 300 lx) by a 100 W tungsten bulb. Arrows indicate refeedings with nitrite. The error bars (SD) are often smaller than the symbol size. Symbols: $\blacktriangle$, OD_{660}; $\blacksquare$, nitrite concentration; $\square$, nitrate concentration. (a) $n=4$, (b) $n=3$, (c) $n=4$.

T. roseopersicina, 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 are shown in Fig. 6.
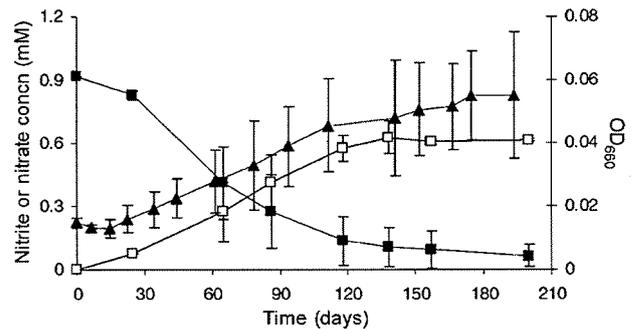

Fig. 4. Growth of strain LQ17 in the light with nitrite as sole electron donor. Symbols: $\blacktriangle$, OD_{660}; $\blacksquare$, nitrite concentration; $\square$, nitrate concentration ($n=2$).

DISCUSSION

In this paper, we document anaerobic oxidation of nitrite by two novel isolates, *Thiocapsa* sp. strain KS1 and *Rhodopseudomonas* sp. strain LQ17. We also show that the known *T. roseopersicina* strains DSM 217 and DSM 221 can grow with nitrite as electron donor. A short communication has 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 has been 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, with either organic or inorganic electron donors. The only reaction known so far in which nitrite is oxidized is aerobic nitrification (Bock *et al.* 1991), in which electrons from nitrite are transferred to oxygen, and CO_2 acts only as the carbon source.
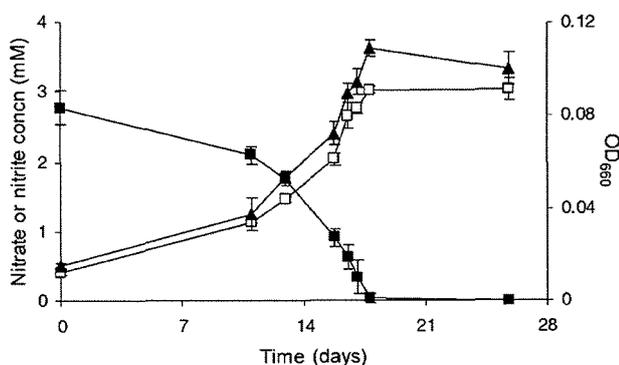
Anoxygenic phototrophic nitrite oxidation by the purple sulfur and nonsulfur bacteria described here showed many similarities but also differences. Nitrite was oxidized only in the presence of bacteria, and required light. No growth or nitrite oxidation was found in the dark, and in the absence of nitrite, no increase of optical density was observed. Sterile controls in the light showed no nitrite oxidation, hence ruling out chemical oxidation of nitrite in the light. If cultures of strain KS1 were placed aerobically in the dark, no growth or 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 *T. roseopersicina* DSM 217 and DSM 221 prove that nitrite is oxidized to nitrate by anoxygenic photosynthesis alone.

With the purple nonsulfur bacterium *Rhodopseudomonas* sp. strain LQ17, the stoichiometry of nitrite consumption
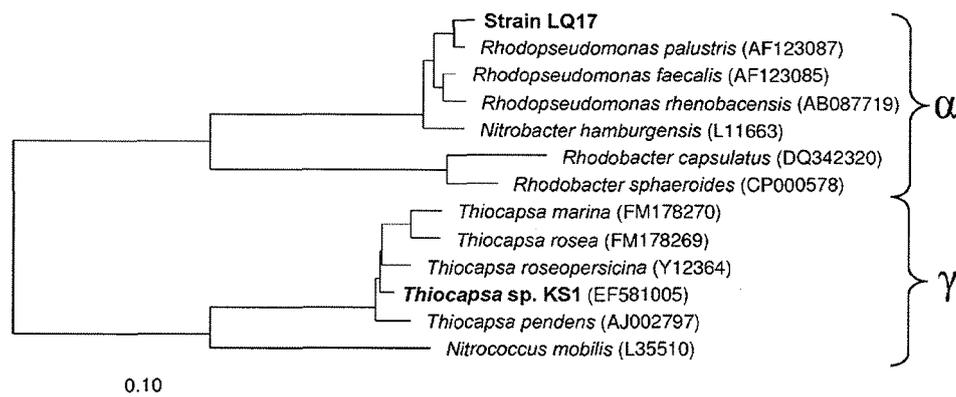
Fig. 5. Phylogenetic tree based on 16S rDNA sequences of representatives of phototrophic and related chemotrophic bacteria. The sequences of strains KS1 and LQ17 were aligned and integrated into the existing phylogenetic tree of the database Silva (http://www.arb-silva.de/) using integrated features of the program arb ubuntu (http://packages.ubuntu.com/jaunty/science/arb). α and γ, Alpha- and Gammaproteobacteria, respectively. Bar, 10 % difference in nucleotide sequence.

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 may simply have been lost. The reason why strain LQ17 grew much more slowly in pure culture than in the enrichment cultures, in which it competed well with strain KS1, is unknown. In co-cultures with strain KS1, the 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.

Fig. 6. Growth of *T. roseopersicina* strain DSM 221 in the light with nitrite as sole electron donor. Symbols: ▲, OD_{660}; ■, nitrite concentration; □, nitrate concentration. The error bars (SD) are often smaller than symbol size ($n=3$).

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 show 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, 1987). Nevertheless, phototrophic nitrite oxidation is probably feasible only if anoxic periods are provided from time to time to allow pigment synthesis (Schaub & van Gemerden, 1994). 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 novel 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 catalyse 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 the nitrite-oxidizing enzyme of phototrophic bacteria also contains the molybdopterin cofactor.

Compared with other electron donors used in anoxygenic photosynthesis, such as organic compounds, HS^-, H_2 and Fe^{2+}, nitrite is the electron donor with the highest redox potential that has been used so far ($E^{0\prime}$ 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 centre primary donor, i.e. up to +490 mV (Cusanovich et al., 1968). It is not surprising, therefore, that our enrichments with nitrite have so far yielded only purple bacteria. The reaction centres of Chlorobiaceae have substantially lower acceptance potentials, i.e. +250 mV (Madigan & Martinko, 2006), and nitrite oxidation by these bacteria would require reversed electron transport to allow electron flow to the reaction centre. Both obtained isolates, strain KS1 and strain LQ17, resemble known types of purple bacteria. The in vivo absorption spectra of both strains indicate the presence of bacteriochlorophyll a (Fig. 2) (Kondratieva et al., 1976; Puchkova et al., 2000; Herbert et al., 2005). Both strains are catalase-positive, utilize many organic compounds phototrophically, and also grow aerobically in the dark with malate (strain LQ17)

or fructose (strain KS1) as electron and carbon source. The 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 indicate close relationships of strain LQ17 to Rhodopseudomonas strains and of strain KS1 to Thiocapsa strains (Table 2). Most noteworthily, strain LQ17 is closely related to the aerobic nitrite oxidizer Nitrobacter hamburgensis within the Alphaproteobacteria, and strain KS1 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

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

Strains: 1, strain KS1; 2, T. roseopersicina; 3, Thiocapsa litoralis; 4, Thiocapsa rosea; 5, Thiocapsa marina; 6, strain LQ17; 7, R. palustris; 8, Rhodopseudomonas rhenobacensis. −, No utilization, ±, slight utilization, +, utilization; ND, not determined.

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

representative of the genus *Thiocapsa* (Puchkova *et al.*, 2000; Caumette *et al.*, 2004). Also, two known strains of *T. roseopersicina*, 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 and are frequently found 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 tend to underestimate true cell numbers in natural samples, e.g. because the cultivation media and conditions may not be well adapted or because cell aggregates cannot be efficiently dispersed (Bussmann *et al.*, 2001). Moreover, nitrite-oxidizing bacteria in particular tend to form microcolonies, which might be overlooked in the scoring. For this reason, we looked not only for growth but also for nitrate formation in the dilution series. Our cultivation conditions were certainly suboptimal and our isolates also tended to aggregate and had obvious difficulties in resuming 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 at concentrations of 10–100 μM. Fortunately, the latter is not true for pristine lake sites, and phototrophic nitrite oxidation may contribute to only a small extent to nitrogen cycling in such habitats. In an early study of phototrophic bacteria in sewage sludge (Siefert *et al.*, 1978), it was concluded that phototrophs can compete with other bacteria only under anaerobic conditions in the light, although their exposure to light in the devices employed was rather limited, due to the high density of biomass. To what extent phototrophic nitrite oxidation contributes to the overall nitrate oxidation activity in activated sludge may be worth a separate study.

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