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ORIGINAL ARTICLE

Genomics of a phototrophic nitrite oxidizer: insights into the evolution of photosynthesis and nitrification

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Oxygenic photosynthesis evolved from anoxygenic ancestors before the rise of oxygen ~2.32 billion years ago; however, little is known about this transition. A high redox potential reaction center is a prerequisite for the evolution of the water-oxidizing complex of photosystem II. Therefore, it is likely that high-potential phototrophy originally evolved to oxidize alternative electron donors that utilized simpler redox chemistry, such as nitrite or Mn. To determine whether nitrite could have had a role in the transition to high-potential phototrophy, we sequenced and analyzed the genome of *Thiocapsa* KS1, a *Gammaproteobacteria* capable of anoxygenic phototrophic nitrite oxidation. The genome revealed a high metabolic flexibility, which likely allows *Thiocapsa* KS1 to colonize a great variety of habitats and to persist under fluctuating environmental conditions. We demonstrate that *Thiocapsa* KS1 does not utilize a high-potential reaction center for phototrophic nitrite oxidation, which suggests that this type of phototrophic nitrite oxidation did not drive the evolution of high-potential phototrophy. In addition, phylogenetic and biochemical analyses of the nitrite oxidoreductase (NXR) from *Thiocapsa* KS1 illuminate a complex evolutionary history of nitrite oxidation. Our results indicate that the NXR in *Thiocapsa* originates from a different nitrate reductase clade than the NXRs in chemolithotrophic nitrite oxidizers, suggesting that multiple evolutionary trajectories led to modern nitrite-oxidizing bacteria.

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Introduction

The evolution of oxygenic photosynthesis in the ancestors of *Cyanobacteria* was the most important metabolic innovation in Earth's history; however, the evolutionary trajectory from ancestral anoxygenic phototrophy to oxygenic photosynthesis remains uncertain. Comparative biology of extant phototrophic microbes places important constraints on this process. There are currently seven bacterial phyla that contain members capable of chlorophyll-based phototrophy: *Cyanobacteria*, *Alpha-*, *Beta-* and *Gammaproteobacteria*, *Chloroflexi*, *Chlorobi*, *Firmicutes*, *Acidobacteria* and *Gemmatimonadetes* (Bryant *et al.*, 2007; Overmann and Garcia-Pichel, 2013; Shih *et al.*, 2013; Zeng *et al.*, 2014). A fundamental division between phototrophs is based on the types of reaction centers that they use. In type I reaction centers (RCI and PSI) the final electron acceptor is a ferredoxin protein, whereas in type II reaction centers (RCII and PSII) the electrons are donated to quinones (Blankenship, 2014). Anoxygenic phototrophs only utilize a single reaction center, whereas oxygenic *Cyanobacteria* couple type I and type II reaction centers in series. Many substrates can be used as electron donors for phototrophy; however, there are important energetic limits (Supplementary Table S1). Extant anoxygenic reaction centers have redox potentials that range from +300 to +500 mV, and primarily oxidize lower potential (<+100 mV) electron donors (Supplementary Figure S1). In contrast, PSII used in oxygenic photosynthesis produces a very strong oxidant (~+1250 mV; Rappaport and Diner, 2008) that is capable of oxidizing water to molecular oxygen (+810 mV). The water-oxidizing complex in PSII could have only evolved after the origin of high-potential phototrophy (Fischer *et al.*, 2016), suggesting that some type of high-potential anoxygenic phototrophy bridged the evolutionary gap between low-potential anoxygenic and high-potential oxygenic phototrophy (Olson, 1970; Rutherford and Faller,

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2003). The highest redox potential substrate oxidized by known extant anoxygenic phototrophs is nitrite (+430 mV); therefore, phototrophic nitrite oxidizers might provide insight into the evolution of high-potential phototrophy (Griffin *et al.*, 2007; Schott *et al.*, 2010).

Phototrophic nitrite oxidizers are also important for understanding the evolution of chemolithotrophic nitrite-oxidizing bacteria (NOB). NOB catalyzes the second step of nitrification, which is a key process of the biogeochemical nitrogen cycle in oxic ecosystems. The highly structured intracytoplasmic membrane systems (ICMs) of the NOB *Nitrobacter* (*Alphaproteobacteria*) and *Nitrococcus* (*Gammaproteobacteria*) closely resemble the ICM of phototrophic purple bacteria. This ultrastructural similarity, and the relatively close phylogenetic affiliation of *Nitrobacter* and *Nitrococcus* with purple bacteria, led to the hypothesis that these chemolithotrophic NOB evolved from phototrophic ancestors (Teske *et al.*, 1994). This scenario seemed to gain support from the surprising discovery of anoxygenic phototrophic nitrite oxidizers in the genera *Rhodopseudomonas* and *Thiocapsa* within the *Alpha-* and *Gammaproteobacteria*, respectively (Griffin *et al.*, 2007; Schott *et al.*, 2010). Recent studies revealed a second evolutionary origin of chemolithotrophic nitrite oxidation, where this metabolism evolved independently in the non-proteobacterial NOB genera *Nitrospira* and *Nitrospina*, probably through horizontal gene transfer of the nitrite-oxidizing enzyme and other functionally important proteins with anaerobic ammonium oxidizers (Lücker *et al.*, 2010, 2013). Until now, it has remained unclear whether the extant anoxygenic phototrophic nitrite oxidizers developed along either of these two lines of NOB evolution or represent a third independent lineage.

To reveal the mechanism of phototrophic growth on nitrite, constrain the energetics of high-potential anoxygenic phototrophy, shed light on possible evolutionary links between photolithotrophic and chemolithotrophic nitrite oxidizers and elicudate the metabolic capabilities and ecophysiology of a phototrophic nitrite oxidizer, we sequenced and analyzed the genome of the nitrite-oxidizing phototroph *Thiocapsa sp.* strain KS1. In addition, we carried out biochemical experiments with fractionated cell-free extracts of a *Thiocapsa* KS1 pure culture to characterize the enzymatic nitrite-oxidizing and nitrate-reducing activities.

Materials and methods

Genome sequencing and analysis

Thiocapsa KS1 (JCM 15485) was grown as described in Supplementary Information. High-molecular-weight genomic DNA was isolated following the hexadecyltrimethylammonium bromide protocol as described elsewhere (Lücker *et al.*, 2013). Sequencing and assembly were performed at LGC Genomics (Berlin, Germany) using GS FLX Titanium-sequencing technology. The *Thiocapsa* KS1 draft genome was annotated using the MicroScope annotation platform (Vallenet *et al.*, 2013). Data from this sequencing project have been deposited at the European Nucleotide Archive under study ID PRJEB9229.

RCII and NXR sequences from genomes and metagenomes were retrieved from National Center for Biotechnology Information and DOE Joint Genome Institute. Sequence alignments were calculated using Muscle 3.8 (Edgar, 2004) or ARB (Ludwig, 2004). Phylogenetic analyses were performed on the CIPRES (Miller *et al.*, 2010) cluster or on a desktop PC using RAxML (Stamatakis, 2014) and MrBayes (Ronquist and Huelsenbeck, 2003).

Cell-free extracts and protein analyses

Cell-free extracts from *Thiocapsa* KS1 were prepared and fractionated as described in Supplementary Information. The enzymatic activities of NXR were measured continuously with spectrophotometric assays (Supplementary Information). SDS-PAGE was performed according to Muller *et al.* (2009). Protein bands were sent to TopLab (Martinsried, Germany) for tryptic digestion and peptide mass fingerprinting without destaining. The fingerprints were matched (Mascot search engine) against the NCBI protein database.

Results

Genome analysis

Thiocapsa KS1 is a gammaproteobacterium isolated from a sewage treatment facility in Konstanz, Germany, by selecting for photoautotrophic growth on nitrite (Griffin *et al.*, 2007; Schott *et al.*, 2010). This versatile phototroph can utilize a range of simple organic carbon molecules, sulfur compounds, H_2 and nitrite as electron donors. We briefly describe the genes associated with respiration, phototrophy, nitrogen metabolism and other functions that confer *Thiocapsa* KS1 with exceptionally high ecophysiological flexibility, which makes it the most metabolically versatile nitrite-oxidizing microorganism known (Figure 1 and Supplementary Table S2).

General respiration

The genome of *Thiocapsa* KS1 contained one copy of a 14-subunit NADH dehydrogenase (complex I) that allows organotrophic respiration. Under photolithoautotrophic growth conditions, complex I will operate in reverse to produce NADH required for carbon fixation (Elbehti *et al.*, 2000; Supplementary Figure S2). Biosynthetic pathways for both menaquinone and ubiquinone were present, consistent with the detection of both quinone types in the close relative *T. roseopersicina* (Imhoff, 1984). *Thiocapsa* KS1 had one cytochrome (cyt.) bc_1 complex (complex III) that conserves energy during phototrophic growth, aerobic respiration and denitrification. Three high-

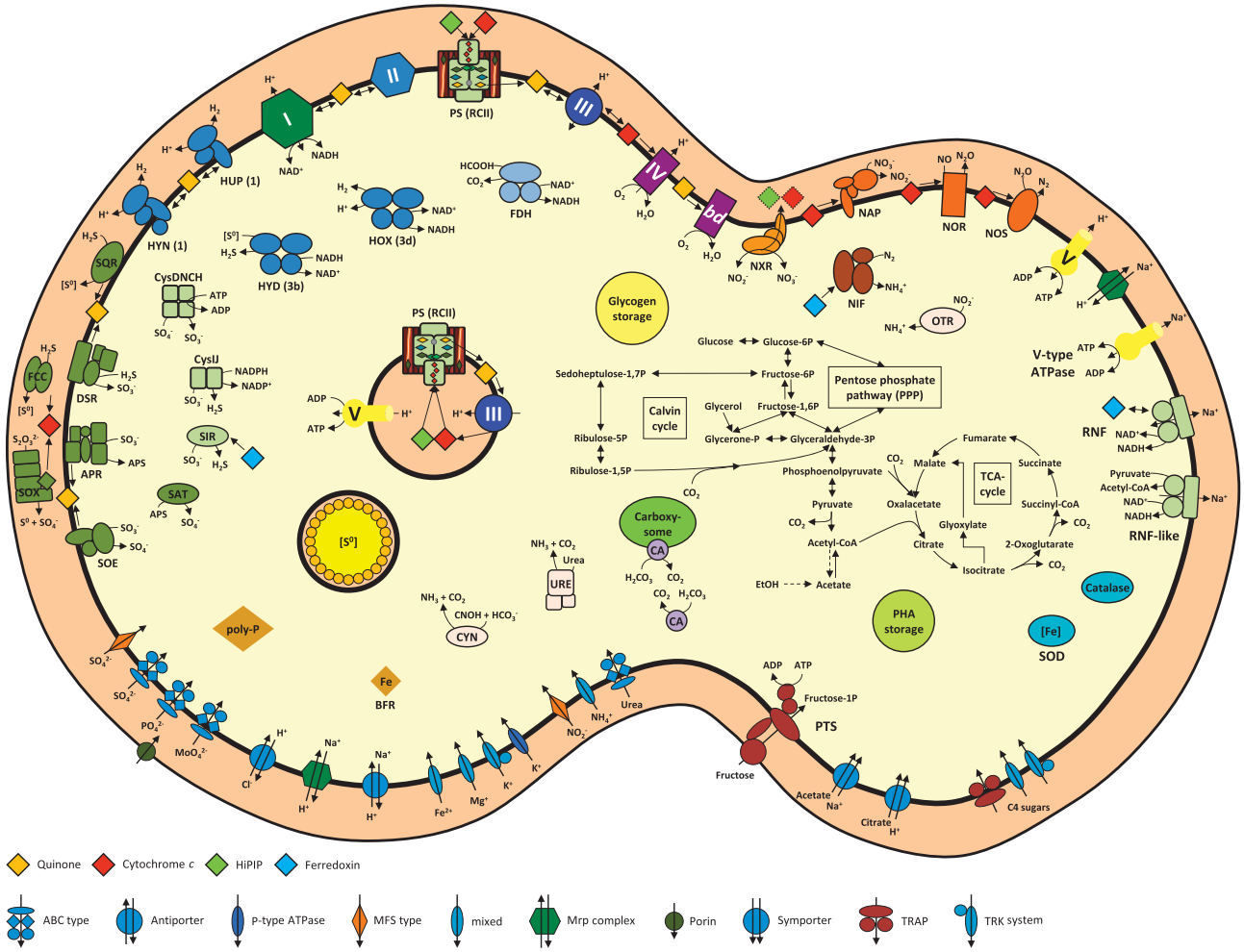

Figure 1 Metabolic diversity of *Thiocapsa* KS1. For details see main and supplemental text. APR, adenylylsulphate reductase complex; *bd*, cytochrome *bd* quinol oxidase; BFR, bacterioferritin; CA, carbonic anhydrase; CYN, cyanate hydratase; Cys, assimilatory sulfate reduction complexes; DSR, reverse dissimilatory sulfite reductase; FCC, sulfide dehydrogenase; FDH, formate dehydrogenase; HOX, HUP, HYD, HYN, hydrogenases (with enzyme classification indicated in brackets); NAP, periplasmic nitrate reductase; NIF, nitrogenase; NOR, nitric oxide reductase; NOS, nitrous oxide reductase; NXR, nitrite oxidoreductase; OTR, octaheme tetrathionate reductase; PHA, polyhydroxyalkanoate; PS, photosystem (type II reaction center); PTS, phosphotransferase system; RNF, H^+/Na^+-translocating NAD-ferredoxin reductase; SAT, sulfate adenylyltransferase; SIR, sulfite reductase; SOD, superoxide dismutase; SOE, sulfite-oxidizing enzyme; SOX, sulfur/thiosulfate oxidation protein complex; SQR, sulfide-quinone reductase; TCA cycle, tricarboxylic acid cycle; URE, urease. Enzyme complexes of the electron transport chain are labeled by Roman numerals: I, NADH dehydrogenase; II, succinate dehydrogenase/fumarate reductase; III, cytochrome bc_1 complex; IV, cbb_3-type cytochrome *c* oxidase; Orange, red, green and blue diamonds represent quinones, cytochrome *c* proteins, HiPIPs and ferredoxins, respectively.

affinity oxygen reductases were present; two C-family heme–copper oxidoreductases (cyt. cbb_3 oxidase, complex IV) and a quinol-oxidizing cyt. *bd* oxidase. Low-affinity A-family oxygen reductases were absent. Details on ATP production and reverse electron transport are provided in Supplementary Information.

Phototrophy

Thiocapsa KS1 encoded one set of RCII genes: *PufL*, *PufM*, *PufC* and *PuhA*. These had very high sequence identities (98% for PufL and PufM) to the closest sequenced strain, *T. marina* DSM 5653, which was confirmed using PCR. *Thiocapsa* KS1 and *T. marina* contained a second copy of the RCII cyt. *c* subunit PufC, which appeared to be fused to an outer membrane protein. Multiple copies of genes for the light-harvesting complexes LH1 and LH2 were also present. *Thiocapsa* KS1 utilizes bacteriochlorophyll *a* for phototrophic growth, with genes responsible for its biosynthesis distributed throughout the genome. The pathway required for the biosynthesis of spirilloxanthin, the major pigment in *T. roseopersicina* (Kovács *et al.*, 2003), was complete. Several cyt. c_4-like di-heme proteins, along with two copies of high-potential iron–sulfur proteins (HiPIPs), were available to act as diffusible periplasmic electron carriers.

Sulfur metabolism

Thiocapsa KS1 encoded a wide diversity of enzymes involved in oxidative sulfur metabolism (Gregersen *et al.*, 2011; Figure 1, Supplementary Information). Systems for the oxidation of sulfur, thiosulfate and

sulfide were identified, which allow for the utilization of these sulfur compounds as electron donors for anoxygenic photosynthesis. During phototrophic growth with sulfide or thiosulfate, elemental sulfur (S^0) is stored in extracytoplasmic sulfur globules within chromatophores (Pattaragulwanit *et al*., 1998), a property shared with other purple sulfur bacteria. These stores can later be used as electron donors when environmental sulfide and thiosulfate concentrations decrease. The complete assimilatory pathway for sulfate reduction via adenosinephosphosulfate and 3'-phosphoadenosinephosphosulfate was also present in the genome.

Hydrogenases

Thiocapsa KS1 encoded at least five Ni–Fe hydrogenases that can oxidize H_2 as an electron source for photosynthesis, recycle H_2 formed during diazotrophic growth or produce H_2 during phototrophic growth on reduced sulfur or carbon compounds (Maróti *et al*., 2010; Supplementary Information).

Carbon metabolism

Thiocapsa KS1 assimilates CO_2 via the Benson–Bassham cycle, which was complete in the genome. Genes for the ribulose-bisphosphate carboxylase (type I RuBisCO) large and small subunits were duplicated and a type IV RuBisCO, which is not involved in carbon fixation (Tabita *et al*., 2007), was also present. Carboxysome shell proteins and carbonic anhydrases indicated the presence of carboxysomes for concentrating CO_2 (Yeates *et al*., 2008). Phosphoglycolate formed by the oxygenase activity of RuBisCO may be fed into the tricarboxylic acid cycle via the glycolate salvage pathway and glyoxylate shunt. The complete C_4-dicarboxylic acid cycle allows additional CO_2 fixation by phosphoenolpyruvate carboxylation. *Thiocapsa* KS1 also possessed a full gene inventory for photo- or chemoorganoheterotrophic growth (Supplementary Information).

Nitrogen metabolism

As a diazotroph, *Thiocapsa* KS1 encoded a complete set of *nif* genes for nitrogen fixation including molybdenum-iron nitrogenase (NifDK) and nitrogenase reductase (NifH). *Thiocapsa* KS1 can assimilate ammonium and also utilize nitrite and nitrate as nitrogen sources in culture (Schott *et al*., 2010), although the organism did not possess genes for canonical assimilatory nitrate or nitrite reductases. Interestingly, the genome encoded several alternative mechanisms (Supplementary Information). In addition, the presence of genes encoding nickel-dependent urease, cyanate hydratase, thiocyanate hydrolase and ethanolamine ammonia-lyase indicated a broad spectrum of reduced nitrogen sources for *Thiocapsa* KS1.

Nitrite oxidation in *Thiocapsa* KS1 was mediated by a Mo-*bis*-MGD-binding nitrite oxidoreductase (NXR), an enzyme that can catalyze nitrite oxidation and nitrate reduction ($NO_2^- + H_2O \leftrightarrow NO_3^- + 2e^- + 2H^+$; Tanaka *et al*., 1983; Sundermeyer-Klinger *et al*., 1984). The NXR of *Thiocapsa* KS1 was similar to the NXR forms found in the chemolithotrophic NOB *Nitrobacter*, *Nitrococcus* and *Nitrolancea* (Starkenburg *et al*., 2008; Sorokin *et al*., 2012) and to the dissimilatory membrane-bound nitrate reductase (NAR) system found in many nitrate-reducing organisms. The NXR complex consisted of the α subunit (NxrA), which contains the catalytic site, the electron-channeling β subunit (NxrB) with four cysteine-rich binding motifs for [Fe-S] clusters and the γ subunit (NxrC), a membrane protein that putatively binds two heme *b* groups. Electrons derived from nitrite flow from NxrA through NxrB to NxrC, which anchors NXR in the membrane and transfers the electrons to the downstream electron carriers. Like in *Nitrobacter* (Kirstein and Bock, 1993; Spieck *et al*., 1996) the NxrA and NxrB subunits were oriented toward the cytoplasm and resembled the NarGH subunits of bacterial NARs. A TorD-like chaperone, which probably inserts the Mo-*bis*-MGD cofactor into NxrA (Blasco *et al*., 1998; Ilbert *et al*., 2003), was encoded between the *nxrB* and *nxrC* genes.

Although *Thiocapsa* KS1 has candidate genes for a complete denitrification pathway (Figure 1) and can use a range of organic and inorganic low-potential electron donors for energy conservation, no growth was observed under anoxic conditions in the presence of these electron donors and nitrate as electron acceptor (Griffin *et al*., 2007; Schott *et al*., 2010). Nitrate reduction to nitrite could be performed by a periplasmic NAR (NapDAGHB). This complex is missing NapC, similar to the periplasmic NARs found in many *Epsilonproteobacteria* (Simon *et al*., 2003), suggesting that an alternative electron transfer pathway to NapA is utilized. In addition, NXR could function as a membrane-bound NAR (see below). Iron- (NirS) or copper- (NirK) dependent nitrite reductases are missing. Instead, the *Thiocapsa* KS1 genome contained two copies of hydroxylamine dehydrogenase-related proteins, which have been implied in nitrite reduction to NO in *Methylococcus capsulatus* strain Bath (Campbell *et al*., 2011). In addition, strain KS1 had both a nitric oxide reductase and a nitrous oxide reductase that would enable the sequential reduction of NO to N_2.

Enzymatic activities of NXR

Cell-free extracts were prepared from *Thiocapsa* KS1 cultures to test the nitrite-oxidizing and nitrate-reducing enzyme activities. Although cultures grown photolithoautotrophically with nitrite as the sole electron donor were analyzed, no nitrite-oxidizing enzyme activity was detected by the two assays applied (Meincke *et al*., 1992). In contrast, a pronounced nitrate-reducing activity was measurable (Supplementary Table S3). The highest specific NAR activity (up to 1700 mU·(mg protein)$^{-1}$) was found in the membrane fraction, whereas the cytosolic

fraction showed less than half of this activity (Supplementary Table S3). The protein contents of the membrane and cytosolic fractions were roughly equal. No activity was detected with NAD(P)H as an alternative electron donor and dithiothreitol as reducing agent. Cells grown with the alternative electron donor H_2 had no NAR activity when grown with ammonium as nitrogen source (Supplementary Table S3), and grew only poorly with nitrate as nitrogen source so that no cell extracts could be prepared from these cultures. Extracts from cells grown on fructose and nitrate had a very low NAR activity that was found exclusively in the cytosolic fraction (Supplementary Table S3), indicating the involvement of a distinct enzyme system for assimilatory nitrate reduction.

Cell fractions were also analyzed using one-dimensional SDS-PAGE (Supplementary Figure S3). The protein patterns from nitrite-grown cells contained two strong bands that were absent from H_2-grown cells and thus assumed to be involved in nitrite oxidation (Supplementary Figure S3). These bands had estimated sizes of 130–150 and 55–60 kDa, respectively, which resemble the sizes of NxrA and NxrB from *Nitrobacter* (Meincke *et al.*, 1992). Consistently, mass spectrometric fingerprint analysis and *in silico* comparison of the obtained peaks to publicly available protein sequences confirmed that the large bands (130–150 kDa) in the cytosolic and membrane fractions represented the NXR α subunit, whereas the smaller bands (55–60 kDa) were identified as the NXR β subunit (Supplementary Figure S3).

Reaction center analyses

A key step in the transition from anoxygenic phototrophy to oxygenic photosynthesis was the evolution of a high-potential reaction center. RCIIs using menaquinones as electron acceptors (*Chloroflexi* (Collins *et al.*, 2011) and the gammaproteobacterium *Halorhodospira halophila* (Schoepp-Cothenet *et al.*, 2009)) have special pair P/P^+ redox potentials ranging from +270 to +390 mV, whereas those utilizing ubiquinone have redox potentials in the range of +400 to +505 mV.

The redox potential of the P/P^+ transition in RCII is determined by intrinsic chemical properties of bacteriochlorophyll, the electronic coupling of the special pair and their interactions with the surrounding protein (Allen and Williams, 2014). All known RCIIs use bacteriochlorophylls coupled in a special pair, with the redox potential predominately tuned by the protein environment surrounding the special pair. Experimental studies have shown that it is possible to increase the P/P^+ redox potential of RCII by (1) forming hydrogen bonds with the 2-acetyl and 9-keto groups of the special pair of bacteriochlorophylls, (2) converting one of the special pair bacteriochlorophylls into a bacteriopheophytin by altering its Mg^{2+} ligand or (3) adding positive charges that electrostatically interact with the special pair. In *Rhodobacter sphaeroides* the effects of adding hydrogen bonds to the special pair were shown to be additive, with three additional hydrogen bonds increasing the redox potential from +505 to +765 mV (Lin *et al.*, 1994; Allen and Williams, 2011; Figure 2). In *Blastochloris viridis* the replacement of a special pair bacteriochlorophyll histidine ligand with leucine resulted in the loss of Mg^{2+} (converting it to a bacteriopheophytin), shifting the redox potential from +517 to +772 mV (Ponomarenko *et al.*, 2009). Furthermore, extensive mutations in *R. sphaeroides* also showed that the addition of a positive charge

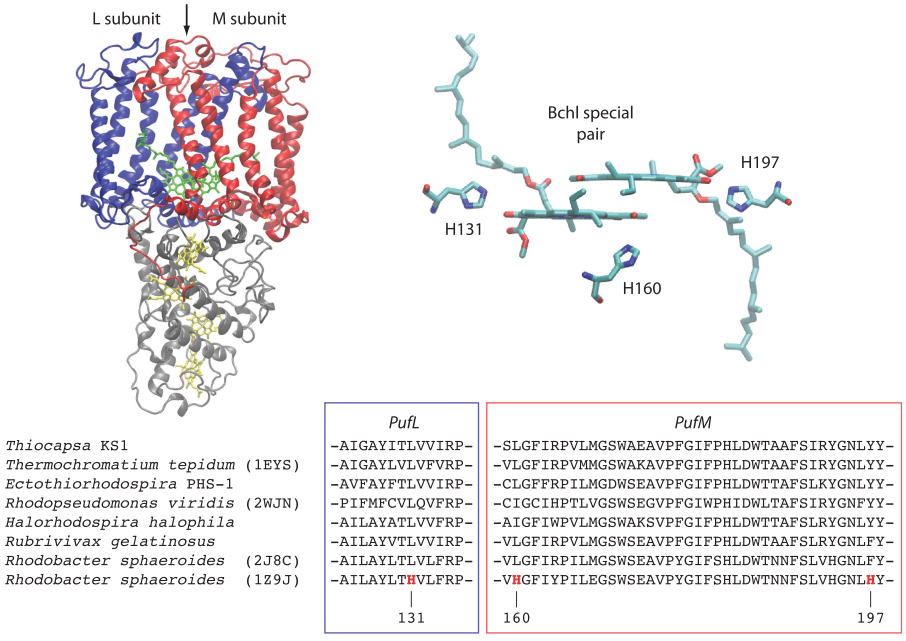

Figure 2 High-potential RCIIs? Shown are known mutations that produce high-potential RCIIs. Sequence analysis detected no natural variants capable of forming hydrogen bonds at these positions (Supplementary Table S4). PufL is blue, PufM is red and PufC is gray.

within 10 Å of the special pair increased the redox potential by 50 mV. Protein modifications such as these can be easily identified in multiple sequence alignments.

The RCII from *Thiocapsa* KS1 has none of the modifications described above that could raise its redox potential, and has the exact same residues interacting with the special pair as *Thermochromatium tepidum* (Ivancich *et al.*, 1996), implying a redox potential of ~+500 mV (Figure 2, Supplementary Table S4).

To determine whether RCIIs found in nature exhibited any modifications that would enable high-potential phototrophy, we analyzed the RCII genes from all sequenced genomes and publically available metagenomes. Sequence alignments of >3000 RCII proteins (Supplementary Table S5) identified no variants in positions that could form additional hydrogen bonds with either the 2-acetyl and 9-keto groups of the special pair (Supplementary Table S4). In addition, no sequences were found that had modified bacteriochlorophyll ligands, and no variants were identified that would modify electrostatic interactions with the special pair (Krammer *et al.*, 2009). Together, this implies that extant RCIIs are unable to achieve redox potentials greater than ~+500 mV.

Discussion

Ecophysiology of Thiocapsa KS1

Although chemolithoautotrophic NOB have been studied for decades, we are just beginning to understand the physiology of phototrophic nitrite oxidizers. Traditionally, the chemolithoautotrophic NOBs were assumed to be highly specialized and metabolically restricted organisms. This picture changed with discoveries such as the chemoorganoheterotrophic *Nitrobacter* (Bock, 1976) and the aerobically H_2-oxidizing, or anaerobically formate-consuming and nitrate-reducing, *Nitrospira* (Koch *et al.*, 2014, 2015). However, the *Thiocapsa* KS1 genome has revealed an exceptionally high degree of metabolic versatility not found in other NOB. This flexibility likely allows *Thiocapsa* KS1 to colonize a wide variety of ecological niches and to persist under changing environmental conditions, using photolithotrophic nitrite oxidation, as only one of several alternative lifestyles. Further studies on the ecophysiology of phototrophic NOB will be crucial for assessing the relative contribution of photolithotrophic nitrite oxidation to overall nitrification in the environment. The whole-genome analysis of *Thiocapsa* KS1 presented here provides a number of hypotheses on the ecophysiology of phototrophic NOB that can be tested in targeted experiments in either pure cultures of *Thiocapsa* KS1 or in microbial communities.

Evolution of nitrite oxidation in Thiocapsa KS1 and chemolithotrophic nitrite oxidizers

Thiocapsa KS1 is the first identified (Griffin *et al.*, 2007) and genomically characterized (this study) nitrite-oxidizing phototroph. As several genomes of chemolithotrophic NOB had been sequenced earlier, we were able to compare the nitrite-oxidizing systems of these organisms to elucidate whether and how the evolutionary pathways of photo- and chemolithotrophic nitrite oxidation are related.

To enable photolithotrophic growth on nitrite, *Thiocapsa* KS1 utilizes NXR. Notably, in the known aerobic chemolithotrophic NOB two different forms of NXR have been identified. They differ in the localization of their α and β subunits on either the cytoplasmic or periplasmic side of the membrane, and thus in the bioenergetics of nitrite oxidation. The NXR of *Nitrospira* (Lücker *et al.*, 2010) and *Nitrospina* (Lücker *et al.*, 2013) is located on the periplasmic side of the membrane, indicating that nitrite oxidation occurs in the periplasm of these organisms. This is energetically ideal, given that protons generated by the reaction directly contribute to the *pmf*. In contrast, NOB from the *Proteobacteria*, such as *Nitrobacter* (Starkenburg *et al.*, 2006), and from the *Chloroflexi* (Sorokin *et al.*, 2012), oxidizes nitrite on the cytoplasmic side of the membrane. In this case protons from nitrite oxidation are released into the cytoplasm, with no contribution to *pmf*, and electrons are transferred across the membrane to a soluble cyt. c_{550} on the periplasmic side (Figure 1 and Supplementary Figure S4). The redox potential of this cyt. c_{550} appears to be low (~+280 mV in *Nitrobacter*; Ketchum *et al.*, 1969), and this step of the electron transport chain might require energy from the electrochemical membrane potential (Cobley, 1976; Ferguson, 1982). Assuming a membrane potential of ~150 mV, the electrons from the nitrite/nitrate couple may reach a potential of ~+280 mV when transferred along the electrochemical gradient to the positive side of the membrane.

The NXR of *Thiocapsa* KS1 faces similar constraints as the *Nitrobacter* system because its α subunit with the active site is also located on the cytoplasmic side of the membrane. In phototrophic bacteria, for example, *R. capsulatus*, there are at least two electron transport pathways to the reaction center that involve *c*-type cytochromes, one via the soluble cyt. c_2 and one via the membrane-bound cyt. c_y (Jenney and Daldal, 1993; Jenney *et al.*, 1994). With a range of +345 to +395 mV (Pettigrew *et al.*, 1978), the redox potential of various cyt. c_2 from different phototrophic purple bacteria would be in the right range to allow electron transfer from nitrite to the reaction center. Alternatively, a HiPIP protein could be employed as soluble electron carrier instead of cyt. *c*. For example, the purple sulfur bacterium *Allochromatium vinosum* preferentially uses HiPIP instead of *c*-type cytochromes during photo-organotrophic growth (Van Driessche *et al.*, 2003). HiPIP potentials range from +50 to +500 mV (Heering *et al.*, 1995), and the HiPIP protein of *T. roseopersicina* has a redox potential of +342 mV (Przysiecki *et al.*, 1985). Hence, electron transfer from nitrite via NXR to a HiPIP or cyt. *c* should be

possible and might be facilitated by the membrane potential as outlined above for the chemolithotrophic NOB and cyt. c_{550}.

Although the NXRs from *Thiocapsa* KS1 and *Nitrobacter* are both membrane-associated and oriented toward the cytoplasm, we identified the key differences in these enzymes. In *Thiocapsa* KS1, the transfer of electrons from NXR across the cell membrane and onto the soluble electron carrier most likely involves a unique di-heme cyt. *c* that is fused onto the γ subunit of NXR (NxrC; Supplementary Figure S4). This fusion is unique among the known nitrite oxidizers, and its functional analog in all known chemolithotrophic NOBs with a cytoplasmic NXR, such as *Nitrobacter*, is a di-heme cyt. *c* encoded by a separate gene upstream of the *nxrA* gene (Sorokin *et al*., 2012). Protein sequence analyses indicate different evolutionary origins for these cyt. *c* moieties. The separate cyt. *c* occurs also in several dissimilatory membrane-bound NARs from heterotrophic denitrifiers such as *Thermus thermophilus*, where it belongs to a unique electron transport chain from a special NADH oxidase via NAR to nitrite-, NO- and N_2O reductases (Cava *et al*., 2008).

Phylogenetic analyses of NxrA and NxrB protein sequences showed that the periplasmic NXR forms of *Nitrospira* and *Nitrospina* are phylogenetically distinct from the cytoplasmic forms of *Nitrobacter*, *Nitrococcus* and *Nitrolancea* (Lücker *et al*., 2010, 2013; Sorokin *et al*., 2012; Figure 3, Supplementary Figure S5). Interestingly, the NxrA and NxrB sequences from *Thiocapsa* KS1 are not closely related to either of these two NXR groups in phylogenetic trees, but instead form a separate deep-branching lineage, which is only distantly related to the cytoplasmic NXRs from chemolithotrophic NOB (Supplementary Figure S5). Both cytoplasmic NXR lineages are related to NARs from phylogenetically diverse bacteria and archaea (Figure 3, Supplementary Figure S5). Consistent with this phylogenetic placement, the NXRs of *Nitrobacter* (Sundermeyer-Klinger *et al*., 1984) and of *Thiocapsa* KS1 (this study) have strong nitrate-reducing activity. The lack of detectable nitrite-oxidizing activity in the whole-cell extracts from strain KS1 might reflect the experimental design, for example, the absence of a membrane potential in the cell-free extracts that could facilitate the electron transfer from nitrite to the soluble acceptor. Thus, the topologies of the phylogenetic trees (Figure 3, Supplementary Figure S5) and the nitrate-reducing activities strongly suggest that the cytoplasmic NXR of *Thiocapsa* KS1 originated from a different NAR ancestor than the NXRs found in chemolithotrophic NOB. Similar conversions of reductases into oxidases have been described previously in other nitrogen cycle enzymes (Klotz and Stein, 2008).

Two *T. roseopersicina* strains are also capable of phototrophic nitrite oxidation (Schott *et al*., 2010); however, NXR is not found in *T. marina* DSM 5653 or any other sequenced member of the *Chromatiaceae*. This suggests that the common ancestor of *Thiocapsa* KS1 and *T. roseopersicina* acquired NXR via lateral gene transfer after divergence from *T. marina*. The donor of the *nxr* genes in this lateral gene transfer event remains unknown, but cannot be a member of the known chemolithotrophic NOB clades because the NXR of *Thiocapsa* is not closely affiliated with the NXR forms of any of these organisms (Figure 3; Supplementary Figure S5).

Hence, our analyses of *Thiocapsa* KS1 do not support the earlier hypothesis that nitrite oxidation in *Nitrobacter* and other chemolithotrophic NOB evolved from a phototrophic ancestor. Instead, the evolutionary history of nitrite oxidation seems to be more complex and comprises at least three

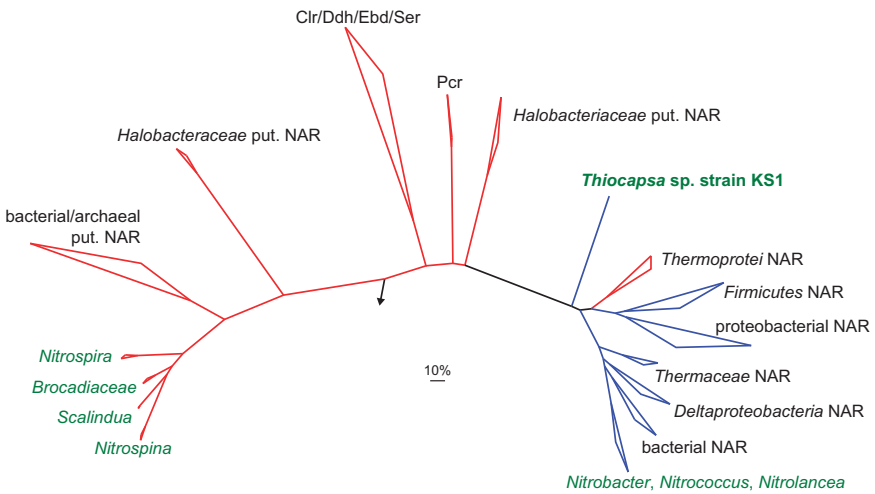

Figure 3 Phylogenetic analyses of *Thiocapsa* KS1 NXR and related enzymes of the dimethyl sulfoxide reductase type II family. Bayesian inference tree of the large (NxrA) subunit. Names of validated enzymes are indicated (Clr, chlorate reductase; Ddh, dimethylsulfide dehydrogenase; Ebd, ethylbenzene dehydrogenase; NAR, nitrate reductase; Pcr, perchlorate reductase; Ser, selenatereductase). The arrow indicates the outgroup; the scale bar represents 10% estimated sequence divergence. Enzyme complexes with the active center located on the cytoplasmic side of the membrane are indicated in blue, complexes oriented toward the periplasm in red. Names of organisms containing a NXR are shown in green. Note the three independent origins of NXR.

independent origins: two for the cytoplasmic NXRs and one for the periplasmic forms (Figure 3, Supplementary Figure S5).

The limits of high-potential anoxygenic phototrophy

The high redox potential of nitrite (+430 mV) poses a challenge for anoxygenic phototrophy. Currently, studied RCIIs from *Proteobacteria* and *Chloroflexi* have redox potentials in the range of +270 to +505 mV (Supplementary Table S1). Expectedly, RCIIs that reduce menaquinol, such as those found in *Chloroflexi* (Collins *et al.*, 2011) and *Halorhodospira halophila* SL1 (Schoepp-Cothenet *et al.*, 2009), have redox potentials at the lower end of this range. The RCIIs from *Chloroflexi*, *Gemmatimonadetes* and the majority of *Proteobacteria* contain a tetra-heme cyt. *c* protein (PufC) that is bound on the periplasmic side of the RCII complex and serves as a wire connecting the soluble electron donor with the special pair. The redox potentials of these hemes vary, with the direct donor to the special pair (heme c_{559} in Figure 2) having a potential of ~+380 mV (Nogi *et al.*, 2005). The majority of electron donors for anoxygenic phototrophy have redox potentials ~0 mV or lower, providing a significant thermodynamic driving force for the overall electron transfer reaction (Supplementary Figure S1). Nitrite has a much higher redox potential—higher than PufC and very close to the redox potential of the RCII special pair in *Thiocapsa* (+490 mV in *T. pfennigii;* Prince, 1978). This raises a key question: how do anoxygenic phototrophs use nitrite as an electron donor for photoautotrophic growth?

To drive nitrite oxidation, anoxygenic phototrophs have two options: either transfer electrons from nitrite to the electron carrier pool utilized by other electron donors, possibly by expending energy, or use a high-potential cyt. *c* (similar to those found in acidophilic iron oxidizers) and a modified reaction center that is able to generate a higher redox potential that can produce the overpotential needed to drive the reaction (Supplementary Figure S2). *Thiocapsa* KS1 employs the first scenario. This shows that a high-potential reaction center is not required for nitrite oxidation. However, it remains possible that other anoxygenic phototrophs might utilize a high-potential reaction center (Supplementary Figure S2) to oxidize nitrite or other high-potential substrates. In the analyses of ~3000 RCII sequences from genomic and environmental metagenomic data sets, we observed no mutations that would confer a higher potential on any RCII found to date (Supplementary Table S4). This implies that RCII can oxidize only substrates with redox potentials lower than ~+500 mV, and that high-potential anoxygenic phototrophy using RCII is either uncommon or absent in modern environments. Thus, extant RCIIs offer a limited understanding of the evolution of the high-potential phototrophy required for the evolution of oxygenic photosynthesis.

Conflict of Interest

The authors declare no conflict of interest.

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