Mobilization of sulfur by green sulfur bacteria – Physiological and molecular studies on *Chlorobaculum parvum* DSM 263

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Mobilization of sulfur by green sulfur bacteria – Physiological and molecular studies on *Chlorobaculum parvum* DSM 263

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TABLE OF CONTENTS

ABSTRACT

Green sulfur bacteria are photolithotrophs that use inorganic sulfur compounds as electron donors for photosynthesis. Elemental, solid sulfur is one of the electron donors used. Sulfur is produced by green sulfur bacteria during the oxidation of sulfide to sulfate, and during the oxidation of thiosulfate to sulfur and sulfate. Green sulfur bacteria have been known for long, and the genomes of 12 strains have been sequenced. Yet, it is not clear how green sulfur bacteria can access elemental sulfur, which is practically insoluble at the temperatures at which most of them grow.

The present work has been done using pure cultures of the mesophilic strain *Chlorobaculum parvum* DSM 263.

By studying the dynamics of inorganic sulfur compounds in growing cultures of *Cla. parvum* DSM 263, it was shown that the sulfur produced during thiosulfate oxidative disproportionation can be either oxidized immediately to sulfate, or released outside the cells. Extracellular sulfur oxidation needs a dedicated system, which is not synthesised in the presence of thiosulfate. Experiments conducted at low light intensities revealed that thiosulfate is not consumed exclusively *via* the Sox multienzymatic system, known thus far as the only thiosulfate oxidizing system present in green sulfur bacteria. It is not clear yet if this additional pathway for thiosulfate consumption allows strain DSM 263 to obtain electrons for thiosulfate, or if it is used by bacteria to consume the reducing power that cells might have in excess.

Physiological studies conducted on *Cla. parvum* DSM 263 fed with sulfur furnished evidence that the oxidation of elemental sulfur to sulfate is accompanied by the formation of yet unidentified sulfur compound(s). Similarly to what commented for thiosulfate consumption, these compounds could be intermediates in the oxidation of sulfur to sulfate, or could be side products of sulfur consumption.

Essential elements for the utilisation of elemental sulfur were shown to be present in the membranes of *Cla. parvum* DSM 263. Differential membrane proteomic studies were thus performed on cells of *Cla. parvum* DSM 263 grown on sulfur or on sulfide. Seven proteins were found to be overexpressed in sulfuric conditions, revealing that bacteria do possess different proteomic equipment for oxidizing the insoluble sulfur rather than the soluble sulfide. Identification of these overexpressed proteins and of their interaction partners will probably help revealing the cellular apparatus of extracellular solid sulfur mobilization.

ZUSAMMENFASSUNG

Grüne Schwefelbakterien bilden eine eigene Gruppe von photolithotrophen Bakterien, welche anorganische Schwefelverbindungen als Elektronenquelle für die Photosynthese nutzen. Elementarer Schwefel ist eine dieser Elektronenquellen. Er wird von Grünen Schwefelbakterien bei der Oxidation von Sulfid oder Thiosulfat zu Sulfat gebildet. Grüne Schwefelbakterien sind schon lange bekannt und vor kurzem wurde das Genom von 12 Stämmen sequenziert. Trotzdem ist es bisher nicht gelungen, zu verstehen, in welcher Form der elementare Schwefel zugänglich gemacht wird.

Für die vorliegende Arbeit wurde der mesophile Stamm *Chlorobaculum parvum* DSM 263 verwendet.

Die Untersuchung von anorganischen Schwefelverbindungen in wachsenden *Cla. parvum* DSM 263-Kulturen hat gezeigt, dass der durch Oxidation von Thiosulfat entstehende Elementarschwefel entweder sofort zu Sulfat umgewandelt oder aus der Zelle ausgeschieden wird. Um den elementaren Schwefel, welcher nicht sofort periplasmatisch zu Sulfat umgewandelt wird, oxidieren zu können, würde ein spezielles Schwefeloxidationssystem benötigt, dessen Synthese bisher nie in Gegenwart von Thiosulfat beobachtet wurde. Die Ergebnisse einiger bei niedrigen Lichtintensitäten durchgeführter Experimente lassen deshalb den Schluss zu, dass Thiosulfat nicht ausschließlich durch das Sox-Multienzymsystem verbraucht wird, welches bisher das einzige bekannte Thiosulfatoxidationssystem in Grünen Schwefelbakterien darstellt. Es ist bisher nicht nachgewiesen worden, dass dieser zweite Stoffwechselweg Stamm DSM 263 erlaubt, Elektronen von Thiosulfat zu gewinnen, oder ob er dazu dient Reduktionskraft abzubauen, welche ohnehin in der Zelle im Überfluss vorhanden ist.

Physiologische Studien an auf Schwefel gewachsenem *Chlorobaculum parvum* DSM 263 untermauerten die Vermutung, dass während der Oxidation von elementarem Schwefel zu Sulfat ein oder mehrere nicht identifizierte Zwischenprodukte entstehen. Ähnliches wird auch für die Oxidation von Thiosulfat vermutet. Die entstehenden Substanzen könnten Zwischenprodukte der Oxidation von Schwefel zu Sulfat sein, sie könnten aber auch Nebenprodukte des Schwefelverbrauchs sein.

Es konnte gezeigt werden, dass die essentiellen Elemente zur Verwendung von elementarem Schwefel auch in den Membranen von *Cla. parvum* DSM 263 vorhanden sind. Des Weiteren wurden differentielle Membran-proteomische Experimente an Zellen von *Cla. parvum* DSM 263 durchgeführt, welche auf Schwefel oder Sulfid gewachsen waren. Es konnte nachgewiesen werden, dass in Umgebung von elementarem Schwefel sieben Proteine überexprimiert wurden. Dieses Ergebnis lässt den Schluss zu, dass die Bakterien unterschiedliche proteomische Ausstattungen

besitzen, um eher den unlöslichen Schwefel als das lösliche Sulfid zu oxidieren. Die Identifizierung dieser überexprimierten Proteine und ihrer Interaktionspartner wird vermutlich dazu beitragen können, den zellulären Mechanismus aufzuklären, welcher für die Mobilisierung von extrazellulärem ungelöstem Schwefel verantwortlich ist.

– Chapter 1 –

GENERAL INTRODUCTION

Green sulfur bacteria (*Chlorobi*) are primary producers that use light energy to fix carbon dioxide into biomass. They also play a role in the sulfur cycle, oxidizing a variety of reduced sulfur compounds (sulfide, thisoulfate, sulfur) to sulfur or sulfate (Fig. 1).

Chlorobi are restricted to anoxic environments where light, carbon dioxide, and reduced sulfur compounds are present. They are present in microbial mats, anoxic sediments, and anoxic water basins, where they might even become numerically preponderant – it is the case of the chemoclines of Black Sea and Lake Cadagno (Manske et al., 2005; Halm et al., 2009).

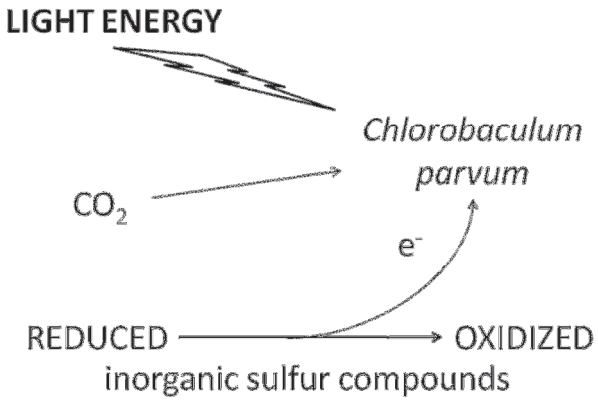

Fig. 1: Schematic representation of the metabolism of green sulfur bacteria, which reduce CO_2 photosynthetically using inorganic sulfur compounds as electron donors. *Chlorobaculum parvum* DSM 263/NCIB 8327 is the model strain used in the present study.

However, even if not among the most diffused bacteria, green sulfur bacteria possess unique features that renders their metabolism particularly interesting for basic research: they can perform photosynthesis at extremely low light intensities, they possess unique cellular structures – the chlorosomes, and they can use a solid compound (sulfur, S_8) as electron donor.

The clade of green sulfur bacteria has been known for long: already in the 30s, green sulfur bacteria served as models for Van Niel's formulation of the general equation of photosynthesis (Barker and Hungate, 1990). They have been studied extensively again in the 80s using biochemical methods (Fischer 1984; Steinmetz and Fischer, 1982 and 1985), and recently by genome analysis (Eisen et al, 2002; works by Bryant, Frigaard, and Hanson). However, part of their sulfur metabolism is still elusive (Fig. 2).

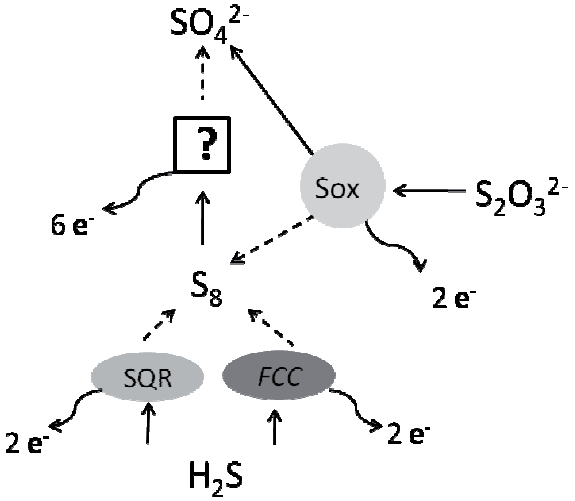

Fig. 2: Schematic representation of what is known and what is unknown about oxidation of inorganic sulfur compounds by GSBs. The oxidation of sulfide to sulfur is attributed to SQR and to FCC. The oxidation of thiosulfate to sulfur and sulfate is attributed to the Sox multienzymatic system. It is not known how sulfur is oxidized to sulfate. Dashed lines indicate that the first product of that enzymatic reaction is not known.

The present PhD thesis starts with a mini review on green sulfur bacteria (section 1.1), which aims to present the current knowledge available on the biology of this phylum. After an overview of environmental diffusion, methods of enrichment and cultivation, methods of molecular detection and characterization, the review treats with more detail the cell structures and the pathways responsible for the photosynthetic oxidation of sulfur compounds, following the ideal route of electrons from the activated photosystem to CO_2, and concluding with the mechanisms of oxidation of the electron donors – sulfide, thiosulfate, and sulfur. The review terminates illustrating what is currently unknown in the oxidation of sulfur compounds by green sulfur bacteria.

The general introduction gives then an overview of the sulfur cycle (section 1.2), and ends (section 1.3) elucidating the aims of the present PhD work and introducing the content of Chapters 2, 3, and 4, which describe and discuss the results obtained.

A final section (Chapter 5) summarizes the contributions of the present work to the knowledge of the group *Chlorobi*.

1.1 Green Sulfur Bacteria: photosynthesis and oxidation of sulfur compounds

Phylogenesis. Green sulphur bacteria are a monophyletic group of anoxygenic photolithotrophs. Imhoff (2008) lists 15 species of GSBs (green sulfur bacteria) for which there is at least an isolated strain, and groups them into 4 genera. The number of species might sum to 17 in a future, after the addition of *Chlorobaculum macestae* (Keppen et al, 2008) and *Prosthecochloris indica* (Anil Kumar et al, 2009). A review on the phylogenesis and taxonomy of GSBs based also on non-cultivated GSBs is presented by Imhoff and Thiel (2010).

GSBs are a monophyletic group placed into the phylum Chlorobi, order *Chlorobiales*, family *Chlorobiaceae* (Imhoff, 2008). Gruber et al (1998), basing on the phylogenetic analysis of the gene sequence of *recA* (a gene coding for a protein conserved among bacteria, which is involved in cell processes like homologous DNA recombination, SOS induction, and DNA damage-induced mutagenesis) in *Cla. tepidum*, placed GSBs as closest relative to the *Cytophaga-Flexibacter-Bacteroides* group. Whole genome analysis of representatives of the 5 taxa known to host photosynthesisers revealed that photosynthetic elements have been subjected to extensive horizontal gene transfer, and that *Chloroflexi*, *Cyanobacteria*, and *Heliobacteria* group more closely together than with the group formed by *Chlorobi* and *Proteobacteria* (Raymond et al, 2002). A different conclusion was reached by Frigaard et al (2003), who analyzed the genome of *Cla. tepidum* and found that several components of the energy metabolism (mentioned examples are some steps in the biosynthesis of carotenes and protoporphyrin, the structures of the complex I and of the rTCA-cycle enzyme ATP-citrate lyase, the production of chlorophyll *a*) place *Cla. tepidum* closer to cyanobacteria and plants than to other photosynthetic bacteria (purple bacteria, which belong to *Proteobacteria*, and *Heliobacteria*). The contrasting conclusions of Raymond et al (2002) and Frigaard et al (2003) can probably be explained by the extensive horizontal gene transfer reported by Raymond et al (2002) (Frigaard et al, 2003).

The characterization of cultivated species has revealed that GSBs have a limited physiological flexibility, being strictly photolithotrophs. Their peculiarities are an adaptation to extremely low light intensities (Overmann et al, 1992; Manske et al, 2005; Beatty et al, 2005), and the ability by some members to create highly evolved symbiosis with chemotrophs (consortia) (Overmann, 2006).

Except the iron oxidizer *Chlorobium ferrooxidans*, all known GSBs use inorganic sulfur compounds as electron donors for photosynthetic CO_2 fixation. GSBs live in anoxic environments where reduced sulfur compounds (reduced iron or H_2 for *Chl. ferrooxidans*) are present, in the water column, sediments, or microbial mats of freshwater, estuarine or marine systems (Frigaard and Dahl, 2009; Alexander and Imhoff, 2006).

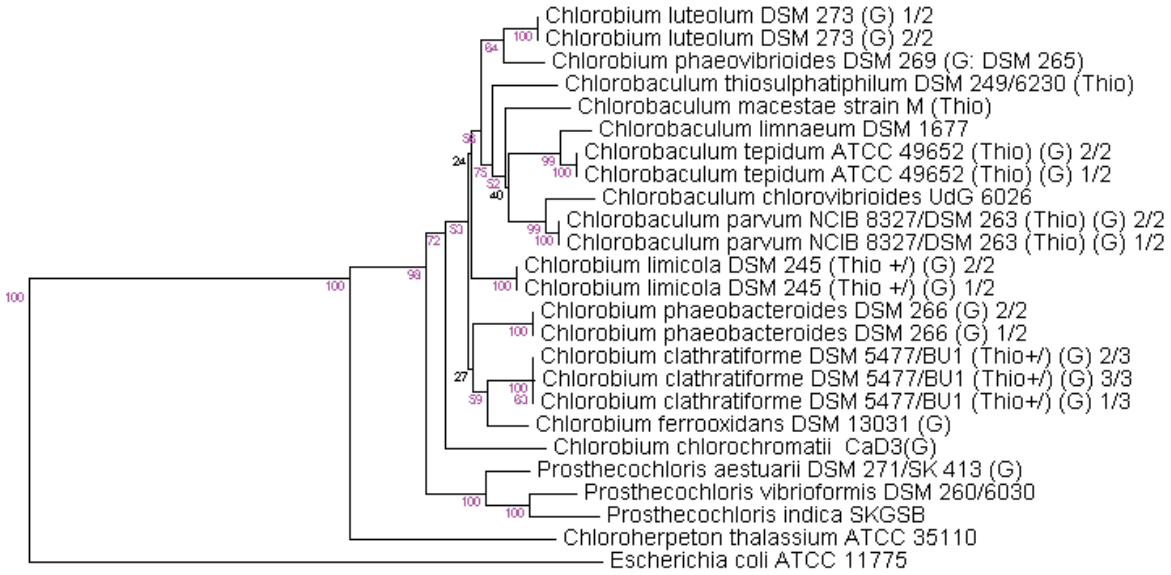

Fig. 3. Phylogenetic tree of the 16S rRNA genes from type-strain species of GSBs. The tree was calculated using the Weighbor weighted neighbour-joining tree-building algorithm available at RDB (Ribosomal Database Project) (Cole et al, 2007 and 2009). Numbers at the bifurcation of branches indicate bootstrap values obtained after 100 runs. Sequences of the species whose genome is available (indicated with G), were obtained from the Joint-Genome-Institute. The other sequences were obtained from NCBI (National Centre for Biotechnology Information). Fractions are used to distinguish the different 16S rRNA genes hosted by a single strain. (G) indicates that the genome of the relative species has been sequenced. (Thio) indicates that all the known strains of the relative species can use thiosulfate. (Thio+/) indicates that only some strains of the relative species can use thiosulfate. The 16S rRNA gene sequences of *Cla. limnaeum* DSM 1677 and *Cla. macestae* strain M are not complete, but comprise anyway more than 1200 and more than 1300 nucleotides respectively. *Escherichia coli* is the outgroup species.

Enrichment and cultivation. GSBs can be enriched in inorganic medium supplied with sulfide. Since the ecological niche of GSBs overlaps with that of purple sulfur bacteria (PSBs), which are also phototrophic sulfur oxidizers, some measures must be adopted in order to favour GSBs over PSBs. To outcompete PSBs, Overmann (2006) recommends to illuminate the enrichment with low light intensities (0.5-5 μE m^{-2} sec^{-1}) supplied by cool white fluorescent light, and to maintain the pH of the medium below 7. Even if GSBs generally tolerate higher sulfide concentrations than PSBs, the enrichment of GSBs should not be done with high concentration of sulfide if the target species is unknown, since gas-vacuolated freshwater species compete only when sulfide concentration is below 2 mM (Pfennig, 1975; Overmann, 2006). When low light intensities and pH values below 7 are not effective in separating GSBs from PSBs, a help can come from the addition of 4-aminobenzenesulfonate (sulfanilate), which inhibits the growth of PSBs (Anil Kumar et al, 2007). Alternatively, GSBs can be isolated directly without enrichment, by deep agar dilution series (Trüper, 1970). Deep agar growth was however unsuccessful when the target was a low-light adapted strain from the Black Sea chemocline (Manske et al, 2005). GSBs have also been grown on

agar plates inside anaerobic jars, using a reservoir of acidified thioacetamide as source of sulfide gas (Irgens, 1983).

Molecular biology. GSBs can be detected also using specific PCR (polymerase chain reaction) primers, FISH (fluorescence *in situ* hybridization) probe, and antibodies:

1) 16S rRNA gene primers specific for GSBs: F-99-GSB 5′-ACTTGGCGCAAGGGTGA-3′ (positions 99–117 according to *Escherichia coli* enumeration) and the general eubacterial R-1369 primer; the obtained PCR product is approximately 1240 bp, equal to more than 85% of the 16S rRNA gene length) (Alexander et al, 2002);
2) primers for the *fmo* gene, whose product – the FMO (Fenna-Matthew-Olson) protein – binds chlorophyll *a* in the region between the chlorosome and the cytoplasmic membrane (see Fig. 4). The *fmoA* primers are: F-Start-fmo 5′-ATGGCTCTTTTYGG-3′ and R-889-fmo 5′-CCGACCATNCCGTGRTG-3′ (positions according to *Cla. tepidum* ATCC 49652 enumeration); the obtained PCR product is 900 bp, equal to 82% of *fmo*A gene length (Alexander et al, 2002);
3) a specific FISH probe (GSB-532 (S-F-GSB-532-a-A-15): 5'-TGCCACCCCTGTATC-3') (Tuschak et al, 1999);
4) polyclonal antibodies against chlorosome polypeptides (Cahill and Stolz, 1995).

Phenotype. The phenotypic classification of GSBs is based on cell morphology, absorption properties, structure of photosynthetic pigments, and presence of gas vesicles. However, as it is often the case, the phenotype characters do not reflect the taxonomy. Basing on 16S rRNA gene and the *fmo* sequence analysis, and excluding the separated lineage hosting *Chloroherpeton*, 4 groups of GSBs have been recognized (Alexander et al, 2002), which are divided into 3 genera – *Chlorobium*, *Prosthecochloris*, and *Chlorobaculum* (Imhoff, 2003).

Genomics. Studies on cultivated members of the genus *Chlorobium* via PFGE (pulsed field gel electrophoresis) genome analysis revealed great genomic diversity both in chromosomic and in plasmidic material. PFGE analysis of the variability of restriction sites along the chromosome, ribotyping (which analyses the variability of restriction sites along the rRNA genes), and RAPD (random amplification of polymorphic DNA, a technique that analyses randomly distributed polymorphisms) are suggested to analyse the genetic variability among *Chlorobium* strains (Mendez-Alvarez et al, 2001). ERIC (enterobacterial repetitive intergenic consensus)-PCR revealed to be an effective fingerprinting technique to discriminate new isolates (Overmann and Tuschak, 1997).

N_2 fixation. GSBs are capable of nitrogen fixation but can also use ammonium and some organic compounds. Four strains, identified as *Chl. limicola* f. *thiosulphatophilum*, were shown to be able to use N_2, ammonia, urea, and glutamine as sole nitrogen sources. *Cla. parvum* DSM 263/NCIB 8327 could also use the aminoacids glutamate, aspartate, asparagine, proline, or valine. Its nitrogenase activity was switched off by 1 mM ammonia (Heda and Madigan, 1986).

Pigments. GSBs are green or brown, reflecting their pigment content. Cell cultures of green sulfur bacteria containing BChl (bacteriochlorophyll) *c* or *d* are dark green, whereas cultures of green sulfur bacteria containing BChl *e* appear brown. The brown colour of GSBs containing BChl *e* is attributed to the special optical properties of aggregated BChl *e*, which has a strong absorption maximum in the region 500 to 550 nm (Steensgaard et al, 2000), or to the 4-times higher content of carotenes (Overmann, 2006). Carotenoids of GSBs are studied as biomarkers for anoxic conditions (Brocks et al, 2005). Green strains contain chlorobactene, brown strains contain isorenieratene. The photosynthetic pigments of GSBs are BChl *a,* and BChl *c*, *d*, or *e*. Intact cells absorb at 745-755 nm (BChl *c*), 715-745 nm (BChl *d*), or 710-725 nm (BChl *e*) (Overmann, 2006). The presence of pigments allows an estimation of cell abundance in a suspension even in the presence of cell-size particles, like the sulfur globules that are produced by GSBs: instead of the absorbance at 675 nm, the parameter used to monitor growth is the difference between the maximum absorbance *in vivo* of bacteriochlorophyll *c*, *d*, or *e* and the absorbance at 830 nm (wavelength at which the bacteriochlorophyll peak is over) (Garcia-Gil and Abella, 1986).

Green pigmented GSBs are reported in thin layers below PSBs, in water depths up to 13 m, in dystrophic lakes, or illuminated by geothermal light (Beatty et al, 2005) where light of the blue or red range prevails. Brown pigmented GSBs are usually found deeper than green pigmented GSBs, up to 26 m below the sea surface and more (e.g. Black Sea chemocline), or below the phytoplankton, where the available light is restricted to the blue-green to green wavelengths (Overmann, 2006; Brocks et al, 2005). GSBs are favoured over the PSBs not only at low light intensities, but also in the presence of large amounts of dissolved humic and tannic materials, as found sometimes in lakes (Parkin and Brock, 1981).

Adaptation to low light intensities. GSBs can exploit very low light intensities. A brown GSB adapted to extremely low light intensities was isolated from the chemocline of the Black Sea, at 80-100 m depth. Such a strain (MN1) belonged to the species *Chl. phaeobacteroides*. In comparison to the reference strain 2430, strain MN1 grows and oxidizes sulfide faster at non-saturating light intensities (< 1 $\mu E\ m^{-2}\ sec^{-1}$), but is slower at saturating light intensities. Instead, the K_M values for sulfide are similar in the 2 strains. At light intensities < 1 $\mu E\ m^{-2}\ sec^{-1}$, strain MN1

contains the double amount of BChl *e* compared to strain 2430, while the efficiencies of energy transfer inside the chlorosomes are comparable, and in the range of other GSBs (40-60%). As third adaptation to constantly low illuminations, strain MN1 has lower maintenance energy (0 ± 0.001 h^{-1}) than strain 2430 (0.0031 ± 0.0016 h^{-1}) (Overmann et al, 1992). Light values at the chemocline of the Black Sea, as reported by Manske et al (2005), range between 2.2 and 0.75 nmol quanta m^{-2} sec^{-1}, and the calculated *in situ* doubling times of the GSB strain living there varied between 3.1-26 years. In laboratory, such a strain needed however 15 nmol quanta m^{-2} sec^{-1}. The specific pigment content, known to increase in GSBs with the decrease of illumination, was never higher than 220 µg BChl *e* (g protein)$^{-1}$, which thus represents the upper limit for pigment accumulation. Pigment aggregation and pigment-rods stability in the chlorosome are maximized respectively by a loss of [E,M]-BChl e_F in favour of [I,E]-BChl e_F, and by the presence of geranyl homologues of BChl *e* (Manske et al, 2005). Even if the contribution of this strain to sulfur and carbon cycle in the Black Sea was calculated to be marginal (Manske et al, 2005), the occupation of such a dark niche by an obligate phototroph represents quite well the efficiency that GSBs have in harvesting and using even low amounts of light.

Low light but of a different spectrum favoured instead the life of a green GSB in a deep-sea hydrothermal vent. The GSB isolated from such a depth (2391 m) was supposed to use geothermal infrared light, and absence of isorenieratene and presence of BChl *c* are in accordance with the geothermal light wavelengths measured at the site (Beatty et al, 2005).

Chlorosomes. Photosynthesis at extremely low light intensities is feasible in GSBs thanks to big antenna structures, the chlorosomes. Chlorosomes are the main cytological feature of GSBs, and have been extensively described by Frigaard and Bryant (2006). Chlorosomes are bags full of antenna pigments, carotenoids, quinones, and proteins, situated in the periplasm, attached to the cytoplasmic membrane. They are not exclusive to GSBs, but are present also in a subgroup of the family *Chloroflexaceae*, phylum *Chloroflexi* (Frigaard and Bryant, 2006).

Chlorosomes of GSBs contain BChl *c*, *d*, or *e*, small amounts of BChl *a*, carotenoids, 3 isoprenoid quinones (1'-oxomenaquinone-7, a derivative, and menaquinone-7), and 10 proteins (CsmA, B, C, D, E, F, H, I, J, X). The chlorosomes envelope is 2-3 nm wide, and consists of monogalactosyl diglyceride and polypeptides (Overmann, 2006). A chlorosome from *Chl. tepidum* is 170–260 nm long, 90–160 nm wide, and 30–40 nm high, and contains about 150,000–300,000 BChl *c* molecules, about 2,500 BChl *a* molecules, 20,000 carotenoid molecules, 15,000 chlorobiumquinone molecules, 3,000 menaquinone-7 molecules, 5,000 protein molecules (of which about half are CsmA), and 20,000 lipid molecules (glycolipids, phospholipids, and wax esters)

(Frigaard and Bryant, 2006). A detailed representation of a chlorosome as currently known is presented in Fig. 4.

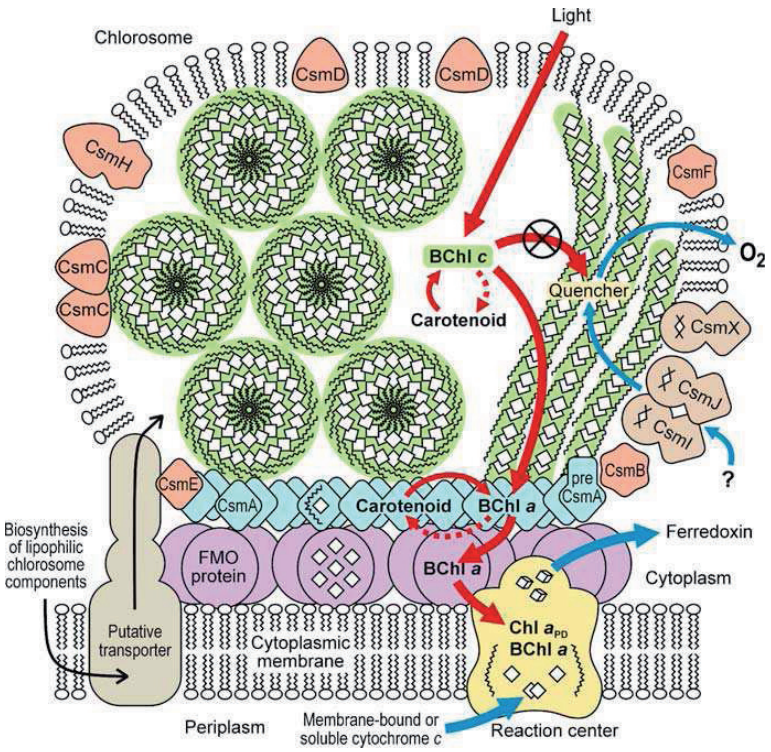

Fig. 4. From Frigaard and Bryant (2006). Model of chlorosome and photosynthetic membrane in *Chl. tepidum*. Csm are chlorosomes proteins, FMO protein is the Fenna-Matthew-Olson protein.There are currently 2 models describing BChl *c* aggregation: the rod-model is shown on the left side of the chlorosome interior, while the lamellar-model is shown on the right side. Continuous red lines indicate singlet excitation energy transfer, dotted red lines indicate the quenching of excited BChl triplets by carotenoids, and blue lines indicate electron transfer. In the presence of O_2, the quencher in the chlorosome is activated and prevents excitation transfer from BChl *c* to the reaction centre, thus preventing photosynthetic electron transfer. The quencher is activated by oxidation and it is inactivated by reduction probably by the chlorosome proteins CsmI and CsmJ. With kind permission from Springer Science+Business Media: *Complex Intracellular Structures in Prokaryotes*, Chlorosomes: antenna organelles in photosynthetic Green Bacteria, 2006, pp. 79-114, Frigaard NU & Bryant DA, Fig. 2.

Chlorosomes contain different homologs of bacteriochlorophylls *c*, *d*, or *e*, in dependence of light intensity and physiological state of the organism. Homologs have different substitutes at position C-8 (ethyl, propyl, isobutyl or neopentyl groups have been described) and C-12 (methyl or ethyl) of the porphyrin ring system (Glaeser et al, 2002). Saga et al (2005) showed that the homolog composition of BChl *c* in *Cla. parvum* DSM 263/8327(former *Chl. vibrioforme*) is influenced by sulfide. At sulfide concentrations 0.9-1.5 g/L, BChl *c* homologs with a methyl at position-12 accumulate. In contrast, the homolog composition of BChls in *Cla. tepidum* seems not to be influenced by sulfide.

Two substrains of *Cla. parvum* DSM 263/8327 (former *Chl. vibrioforme*) are known to differ only for the kind of main antenna pigment: one substrain produces BChl *c*, the other produces BChl *d*. The difference between the 2 bacteriochlorophylls is a methyl group at the 20-position of the tetrapyrrole ring, present in BChl *c* or substituted by a hydrogen atom in BChl *d*. Production of BChl *d* instead of BChl *c* in the 2 substrains of *Cla. parvum* DSM 263/8327 is due to a single-basepair frameshift mutation in the gene *bchU*, which encodes a methyltransferase. The mutation of *bchU* is reversible, and after repeated cultivation, BChl *d*-strains produce BChl *c* (Saga and Tamiaki, 2004). Having BChl *c* or *d* results in differences at chlorosomal level (Saga and Tamiaki, 2004; Harada et al, 2005):

1) chlorosomes containing BChl *c* absorb at 459 and 751, while chlorosomes containing BChl *d* absorb at 448 and 734 nm;
2) chlorosomes containing BChl *c* transfer energy more efficiently (Bchl *a* in the baseplate absorbs at 795 nm)
3) the substrain that produces BChl *c* grows faster at low light intensities;
4) chlorosomes containing BChl *d* are structurally more stable (aggregates of BChl *d* resist more than aggregates of BChl *c* to disruption by a solvent);
5) cells containing BChl *c* are more resistant to oxygen than cells containing BChl *d*.

The transfer of singlet energy from carotenoids to BChls is inefficient, and the role of carotenoids seems rather to be protection of bacteriochlorophylls from photobleaching. Carotenoids mainly interact with BChl *a* in the baseplate, increasing the stability of the chlorosomes baseplate (Glaeser et al, 2002). Under oxic conditions, the BChl *c* of *Cla. tepidum* does not transfer energy to chlorosomal BChl *a*, but to a quencher, yet unidentified, avoiding in this way the formation of (low potential) reductants (e.g reduced ferredoxin), which would in turn react with oxygen, forming radicals. The quencher might be chlorobiumquinone (1'-oxomenaquinone-7) (Frigaard and Matsuura, 1999). The presence of a quencher has been demonstrated also in *Cla. parvum* DSM 263/8327 (former *Chl. vibrioforme* f. *thiosulphatophilum*) and in strain known as *Chl. limicola* f. *thiosulphatophilum* ATCC 17092 (Wang et al, 1990).

The GSBs biosynthetic pathways of BChl *a*, BChl *c*, and carotenoids have been revealed by Frigaard and Bryant (2004).

The role of chlorosome proteins is not clear, but it seems that only pigment-pigment interactions are relevant for the aggregation of BChl in chlorosomes, while the interaction between pigments and proteins seems to have a marginal role (Frigaard et al, 2004). A mutant of *Cla. tepidum* unable to synthesize BChl *c*, produces chlorosomes filled with carotenoids and devoid of all proteins but CsmA and CsmD. The chlorosome baseplate is a paracrystalline structure contaning

BChl *a* and stabilized by chlorosome-specific glycolipids, and serves to attach the chlorosomes to the Fenna-Matthew-Olson protein and to the cytoplasmic membrane. The chlorosome baseplate functions independently from BChl *c* and chlorosomes proteins other than CsmA, which binds all BChl *a*, in ratio 1:1 (Frigaard et al, 2005), and which is probably the only protein binding BChl *a*. In addition to the already mentioned CsmA and CsmD, the chlorosomes of *Cla. tepidum* contain other 8 proteins (CsmB, C, E, F, H, I, J, X), present in non-fixed ratio with the major antenna pigment present in the chlorosomes. Apart from mutation of *csmA*, which is lethal, mutation of each of the other 9 genes still leads to functional chlorosomes. Only 4 mutants have recognizable phenotype and allow attributing a function to the relative proteins: CsmB might play a role in organizing chlorosomes; CsmH might play a role, together with CsmC, in determining the lengths of BChl *c* aggregates; CsmJ might have a role in the turnover of photosynthetically generated reductants (Frigaard et al, 2004).

A connection has been found between vitamin B_{12} and chlorosome formation. Some strains of GSBs need in fact vitamin B_{12} – e.g. *Chl. limicola* strain 1230 does, *Cla. parvum* DSM263/NCIB 8327 does not (Overmann, 2006). Fuhrmann et al (1993) showed that in the absence of vitamin B_{12}, *Chl. limicola* strain 1230 does not form any chlorosomes, and forms 80% less BChl *c* and 60% less BChl *a*.

ADAPTATIONS TO LOW LIGHT INTENSITY (from Overmann, 2006)

- ☼ Decreased cell size.
- ☼ Bigger and/or more numerous chlorosomes.
- ☼ Chromatic adaptation: increased ratio BChl *a*/BChl *c* (or BChl *d*) and higher degree of alkylation of the bacteriochlorophyll tetrapyrrole (low light intensities: isobutyl or neopentyl side chains as main side chains; high light intensities: methyl, etyl, n-propyl side chains).
- ☼ Very high metabolic efficiency: only 4 moles of photons are needed to fix a mole of CO_2.
- ☼ Gas vacuoles (in some strains): increased buoyancy and proximity with light.

A hypothesis on chlorosome formation has been formulated by Hohmann-Marriot and Blankenship (2007). Chlorosomes might originate as a sort of lipid body, formed by accumulation and association of BChls, carotenoids, and quinones between the 2 layers of the cytoplasmic membrane. Glycosyl diacylglycerols (a component of the chlorosomes membrane) would accumulate in the vicinity of the so-formed liposome, associating with chlorosomes proteins. The chlorosomes might then separate from the cytoplasmic membrane, or stay connected to it in some points. No special assembly machinery is required to for a chlorosomes. The presence of chlorosomes in both GSBs and some FAPs seems to be the consequence of horizontal gene transfer

involving probably proteins of the baseplate, chlorosomes proteins, and proteins for BChls. The organism which received those genes had a membrane system that could interact with proteins and pigments acquired via horizontal gene transfer, and a photosynthetic reaction centre and antenna system capable of interfacing with the chlorosome. That would be enough for "chlorosome-gene transfer".

The Fenna-Matthews-Olson (FMO) protein. The interface between the antenna pigments and the reaction centre is the Fenna-Matthews-Olson protein, which contains BChl *a*, and is found exclusively in GSBs. The Fenna-Matthews-Olson (FMO) protein is soluble and is constituted by 3 subunits, each containing 7 BChl *a* molecules. It is embedded into the cytoplasmic membrane, between the chlorosomes and the reaction centres. The FMO protein is related to the reaction centre protein of GSBs (PscA), and derives probably from a primitive reaction centre (Olson, 2004). The FMO protein is bound to the inner surface of the plasma membrane probably by an aminoglycosphingolipid, which Jensen et al (1991) found in the plasma membrane but not in the chlorosomes of *Cla. thiosulphatiphilum* DSM 249 (former *Chl. limicola* f. *thiosulphatophilum* 6230). The aminoglycosphingolipid was proposed to have the additional or alternative role of phospholipids substitute during phosphate limitation.

The reaction centre. The photosynthetic reaction centre of GSBs is an iron-sulfur-type (RC I). The genes of photosynthetic reaction centre of GSBs are related to the photosystem I of chloroplasts and cyanobacteria (Büttner et al, 1992). The reaction centre (RC) of GSBs is composed of 5 subunits: the homodimeric core reaction RC P840 (2 subunits of 65 kDa each), the FMO protein (41 kDa), a Fe-S protein that includes the electron acceptors F_A and F_B (31 kDa), a cytochrome c_z (22 kDa), and a 18kDa-protein. Additional soluble cytochromes are involved in the electron transfer from inorganic sulfur compounds to the RC (Oh-oka and Blankenship, 2004). The electron transfer in the homodimeric RC of GSBs might occur in both branches of the RC, differently to what is known for the heterodimeric RC of PSBs, in which only 1 branch is active. While the antenna chromophores are photochemically inactive, serving only to capture photons, the pigments in the reaction centre are photochemically active, and undergo charge separation upon photon absorption. Charge separation in the core RC results in the production of an oxidized bacteriochlorophyll and of a reduced chlorine pigment. From the reduced chlorine, the electrons migrate to a quinone, then to the iron-sulfur centres, and finally to ferredoxin, as shown in Fig. 5 (Hillier and Babcock, 2001).

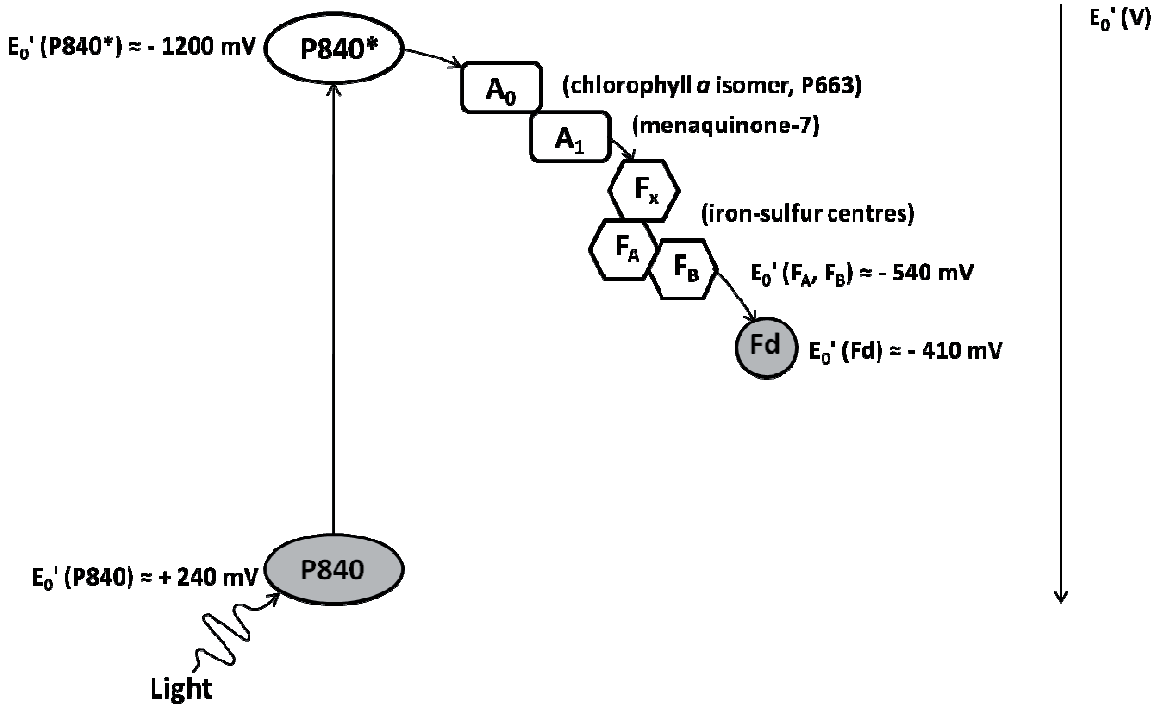

Fig. 5: Schematic representation of the electron flux in GSBs, which brings to the production of reduced ferredoxin (Fd). Electrons to reduce the oxidized reaction centre (P840) derive from the oxidation of sulfide to sulfur, of sulfur to sulfate, and, in some strains, from the oxidation of thiosulfate to sulfate or to sulfur and sulfate. The reaction centre (P840), once excited by light, can reduce the chlorophyll *a* P663. Electrons are then transferred to menaquinone-7, to iron-sulfur centres, and finally to ferredoxin. Information for the figure was taken from Overmann (2006).

GSBs are strict photolithotrophs that fix CO_2 via the reverse TCA cycle. Electron flow from the RC result in the production of reduced ferredoxin, which is the electron donor for the rTCA cycle and can reduce NAD^+ as well. Cyclic photophosphorylation in GSBs is the same as in PSBs (Overmann, 2006). GSBs are very efficient in fixing CO_2: Larsen (1954) calculated light requirements of 9-10 quanta per fixed CO_2 in a strain that was called *Chl. limicola* f. *thiosulphatophilum*, while Brune (1989) reports for GSBs even lower values (3.3-4.5 quanta per fixed CO_2). Small organic molecules such as acetate can be photoassimilated by GSBs, but cannot support growth if CO_2 is absent. Sadler and Stanier (1960) proposed that GSBs cannot grow on the sole acetate because they lack the enzyme to oxidize it, thus being unable to get reducing power or CO_2 from it. Tang and Blankenship (2010) showed that *Cla. tepidum* has a complete rTCA cycle and an incomplete TCA cycle, and that *Cla. tepidum* needs both pathways for assimilation of acetate and pyruvate.

Cla. parvum NCIB 8346 (former *Chl. thiosulfatophilum* strain 8346) was shown to store polyglucose in form of 30 nm-diameter granules. In the absence of an electron donor, polyglucose is degraded, both in light and in dark conditions, and accompanied by excretion mainly of acetate, but also of propionate, caproate, and succinic acid (Sirevåg and Ormerod, 1977).

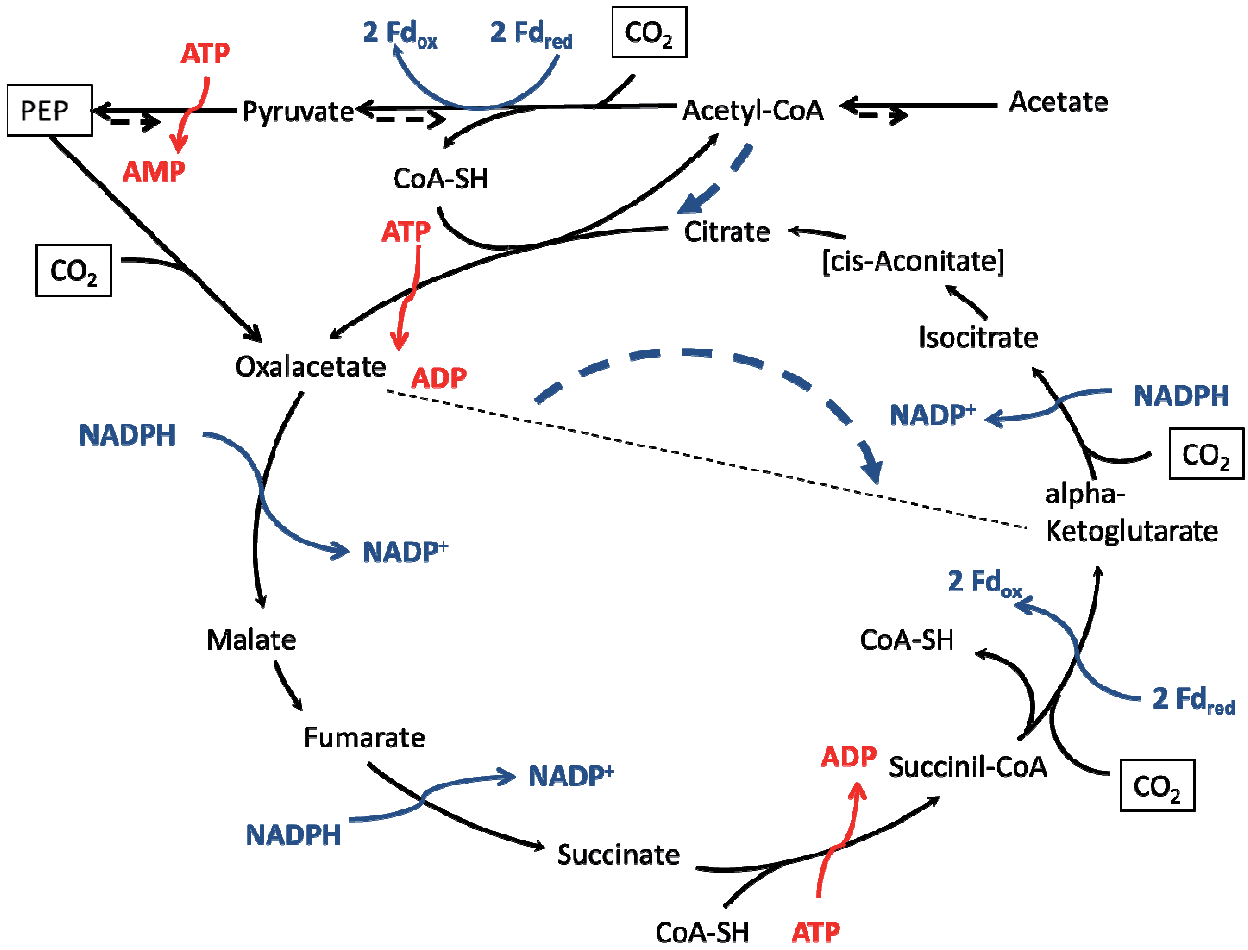

Fig. 6: Schematic representation of the reverse TCA cycle (black arrows). Reversible reactions are indicated by the co-presence of a dashed black arrow. The oxidative cycle, indicated by dashed blue arrows, is incomplete, since the alpha-ketoglutarate dehydrogenase is missing in GSBs. Only the tract between oxalacetate and alpha-ketoglutarayte is possible. The function of the oxidative branch has been speculated to be the formation of alpha-ketoglutarate, which is needed for ammonia assimilation (Tang and Blankenship, 2010).

Oxidation of inorganic sulfur compounds. The electron donors for the reduction of the oxidized RC (P840*) derive from inorganic sulfur compounds. All sulfur-oxidizing GSBs can use sulfide and sulfur, while only some strains can use thiosulfate. Among all the 38 sulfur-oxidizing strains described so far, 10 (26%) can use thiosulfate as electron donor, 7 were not tested for thiosulfate, the remaining 21 (55%) can get electrons from sulfide and sulfur (Imhoff, 2008; Keppen et al, 2008; Anil Kumar et al, 2009). *Cla. parvum* (former *Chl. vibrioforme* f. *thiosulphtophilum*) and a strain named by Larsen (1952) *Chl.limicola* have been reported to be able to grow on tetrathionate (Khanna and Nicholas, 1982; Larsen, 1952).

The sulfur metabolism of GSBs has recently been reviewed by Sakurai et al (2010), Hanson et al (2010), Frigaard and Dahl (2009), and Frigaard and Bryant (2008a and 2008b), at the light of comparative genomics among the 12 sequenced GSBs, and of mutational studies conducted mainly

on *Cla. tepidum*. Fig. 7 is a schematic representation of what is known or hypothesized about the oxidation of inorganic sulfur compounds in GSBs.

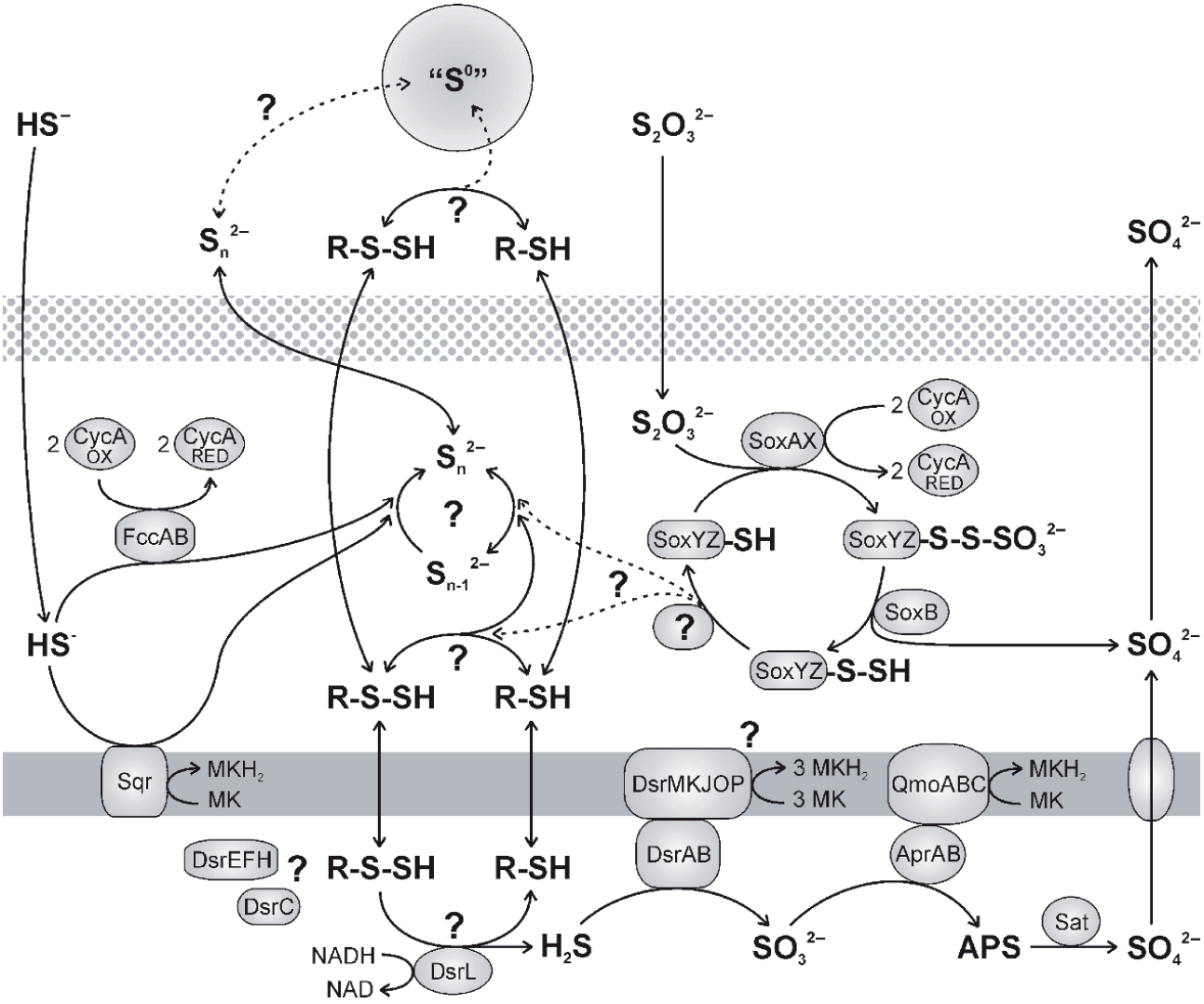

Fig. 7: From Frigaard and Bryant (2008b). Overview of known or hypothesized pathways in the oxidation of inorganic sulfur compounds in GSBs. With kind permission from Springer Science+Business Media: *Sulfur metabolism in phototrophic organisms*, chapter 17, Genomic insights into the sulfur metabolism of phototrophic green sulfur bacteria, 2008, pp. 337-355, Frigaard NU & Bryant DA, Fig. 2.
If not otherwise stated, the distribution and function – known or hypothesized – of the complexes present in the figure has been taken from Frigaard and Dahl (2009):

- ***sqr*** (sulfide:quinone oxidoreductase): it is present in all sequenced GSBs. *Cla. tepidum* 12025, *Chl. ferrooxidans* 13031, *Chl. phaeobacteroides* BS-1, *Chl. luteoulum* DSM 273 (Frigaard and Bryant, 2008b), and *Cla. parvum* DSM 263 possess an additional SQR-homolog, which was shown by Chan et al (2009) to be involved as well in sulfide oxidation. *Cla. tepidum* 12025 and *Cla. parvum* DSM 263 have a 3rd SQR-homolog, whose function is not known (Chan et al, 2009; Frigaard and Dahl, 2009).
- ***fcc*** (flavocytochrome *c*:sulfide dehydrogenase): present in all sequenced GSBs but *Chl. ferrooxidans* DSM 13031 and *Chl. luteolum* 273.
- ***soxFXYZAKBW***: it is present in all 5 sequenced genomes of the thiosulfate-oxidizing GSBs (*Cla. parvum* DSM 263, *Cla. tepidum* DSM 12025, *Chl. chlorochromatii* CaD3, *Chl. chlathratiforme* DSM 5477/BU1, *Chl. phaeovibrioides* DSM 265). SoxF, which has been called also SoxJ, is a membrane-bound FCC (Verté et al, 2002; Sakurai et al, 2010; Ogawa et al, 2010).

- ***dsrNCABLUEFHTMKJOPVW***: it is present as a single cluster in all sequenced GSBs but *Chl. ferrooxidans* DSM 13031 and *Chloroherpeton thalassium* ATCC 11775. *DsrEFH*, however, is not present in *Cla. parvum* DSM 263/NCIB 8327 (Holkenbrink et al, 2011). The gene units are *dsrAB*, whose product clusters with proteins from other sulfur oxidizers, and *dsrTMKJOP*, which seems instead acquired from a sulfate-reducer. *Dsr* gene products have been extensively studied in PSBs, and they are speculated to have the same function as in GSBs. DsrAB is a cytoplasmic sulfite reductase. DsrN and DsrR are cytoplasmic proteins, probably involved in the biogenesis of DsrAB. DsrEFH, DsrC, DsrL, and DsrS are also soluble cytoplasmic proteins. DsrL is an iron-sulfur flavoprotein with NADH:acceptor oxidoreductase activity. DsrKMJOP is probably a transmembrane electron-transporting system: DsrP is an integral membrane protein, DsrM is a membrane-bound cytochrome *b*, DsrJ a cytochrome *c*, DsrO and DsrK are iron-sulfur proteins.
- ***aprBA***: it is present in the genomes of *Cla. tepidum* DSM 12025, *Chl. chlorochromatii* CaD3, *Chl. chlathratiforme* DSM 5477/BU1, *Ptc.* BS1. *AprBA* codifies a potential dissimilatory APS (adenosine-5'-posphosulfate, also called adenylylsulfate) reductase, which catalyses the oxidative phosphorylation of sulfite, with generation of APS and reducing equivalents from sulfite and AMP (adenosine monophosphate).
- ***sat****:* it has the same distribution of *aprBA*. *Sat* codifies ATP (adenosine triphosphate) sulfurylase, which catalyses the formation of ATP and sulfate from APS and pyrophosphate (PP_i).
- ***qmo***: it has the same distribution of *aprBA*. *Qmo* (quinone-interacting membrane-bound oxidoreductase) codifies a membrane-bound redox complex, as deduced from comparison with the action of a Qmo complex in a sulfate-reducer (*Desulfovibrio desulfuricans*).
  Sat-aprBA-qmoABC in *Cla. tepidum* are downstream of *dsr*.
- ***PSRLC3***: it is present in *Chl. chlorochromatii* CaD3 (which possesses also the Sat-AprBA-QmoABC system), *Chl limicola* DSM 245, *Chl. luteolum* DSM 273, *Chl. phaeobacteroides* DS266, *Chl. phaeovibrioides* DSM 265, *Chloroherpeton thalassium* ATCC 35110, *Ptc. aestuarii* DSM 271. PSRLC3 is a cytoplasmic homolog of the periplasmic polysulfide-reductase system of *Wolinella succinogenes*, where it catalyzes polysulfides respiration with H_2 as electron donor. Frigaard and Dahl (2009) hypothesize that PSRLC3 could oxidize to sulfate the sulfite produced from Dsr, in those GSBs that do not possess the system Sat-AprBA-QmoABC.
  PSRLC1 *and* ***PSRLC2***: periplasmic homologs of the polysulfide reductase system of *Wol. succinogenes*. Every sequenced GSB but *Chl. ferrooxidans* DSM 13031 has one or both these complexes, whose function is not known.

Sulfide oxidation to sulfur. GSBs oxidize sulfide to sulfur, which is generally detected as extracellular sulfur. However, it has also been reported that GSBs fed with low amounts of sulfide do not form sulfur as intermediate in the oxidation of sulfide to sulfate (Pfennig, 1975). The observed direct oxidation of sulfide to sulfate is probably due to the simultaneous oxidation of sulfide and sulfur, which was shown to happen at low sulfide concentrations (Cork et al, 1985). GSBs have higher affinities for sulfide than PSBs (Van Gemerden, 1984). On the other hand, sulfur oxidation by GSBs is inhibited by sulfide, while PSBs can oxidize sulfur in the presence of sulfide (Brune, 1989).

At low sulfide concentrations, some GSBs might be advantaged by the capacity of adsorbing metals (Mn^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+}, but not Ni^{2+}) demonstrated for strains *Chl. limicola* DSM 249 and *Chl. phaeobacteroides* UdG 6030. FeS and MnS were oxidized by both *Chl. limicola* DSM 249 and *Chl. phaeobacteroides* UdG 6030 (Borrego and Garcia-Gil, 1995), but *Chl. phaeobacteroides* UdG 6030 bound $Mn^{2+}$and Fe^{2+} more efficiently than *Chl. limicola* DSM 249. Strain UdG 6030 was isolated from a meromictic lake where maxima of BChl *e* often coincided

with maxima of Fe^{2+} (Garcia-Gil and Borrego, 1997), which suggests that the presence of Fe^{2+} favours the growth of strain UdG 6030. *Chlorobium* might take advantage of $Mn^{2+}$and Fe^{2+} attached to membrane to trap S^{2-}, which would be relevant when sulfide concentration is low (Borrego and Garcia-Gil, 1995).

The oxidation of sulfide to sulfur has been attributed to the sulfide:quinone oxidoreductase (SQR) and to the flavocytochrome-*c*:sulfide dehydrogenase (FCC) (Frigaard and Dahl, 2009, and references therein). In GSBs, the product of SQR activity on sulfide might be polysulfides, as shown by Griesbeck et al (2002) in activity assays on SQR from the purple non-sulfur bacterium *Rhodobacter capsulatus*.

Differently from SQR, FCC is not present in all GSBs (e.g. *Chl. luteolum* DSM 273 lacks it). In the PSB *Allochromatium vinosum*, FCC does not seem to contribute to sulfide oxidation in an obvious way: a mutant of *Alc. vinosum* devoid of FCC was able to oxidize sulfide at the same rate of the wild-type (Frigaard and Dahl, 2009). Brune (1995) hypothesized that FCC might be advantageous for cells in environments with low concentrations of sulfide.

A sulfide-oxidizing activity by SoxF too was detected *in vitro* (Ogawa et al, 2010). SoxF is a component of the Sox system and was shown to have sulfide-dehydrogenase activity also when isolated from *P. pantotrophus*, where it was speculated to have the *in vivo* function of activating SoxYZ (Quentemeier et al, 2008).

Thiosulfate oxidation. Thiosulfate-oxidizing GSBs oxidize thiosulfate to sulfur and sulfate (oxidative disproportionation), or directly to sulfate, by the Sox system and probably the Dsr system. The oxidation of thiosulfate to sulfur and sulfate in GSBs is attributed to the Sox multienzymatic system (also known as TOMES) (Frigaard and Bryant, 2008 and 2008b; Frigaard and Dahl, 2009; Sakurai et al 2010). GSBs and PSBs share 7 genes with *P. pantotrophus* (see Fig. 8). Among them, there are the genes for the core set of the Sox system: SoxAX, SoxYZ, and SoxB, which are reported to have the same function as in *P. pantotrophus* (Frigaard and Dahl, 2009, Sakurai et al, 2010): SoxAX catalyses the oxidative binding of thiosulfate to SoxYZ and transfers electrons to cytochrome *c*-554/555, SoxYZ binds thiosulfate, and SoxB catalyses the hydrolysis of the internal thiosulfate-sulfur as sulfate. However, Ogawa et al (2010) showed that SoxYZ and SoxAX extracted from *Cla. tepidum* are not sufficient to catalyze the oxidative binding of thiosulfate *in vitro*, but SoxB is needed as well, even if it is not clear why.

THE SOX SYSTEM IN *PARACOCCUS PANTOTROPHUS*.

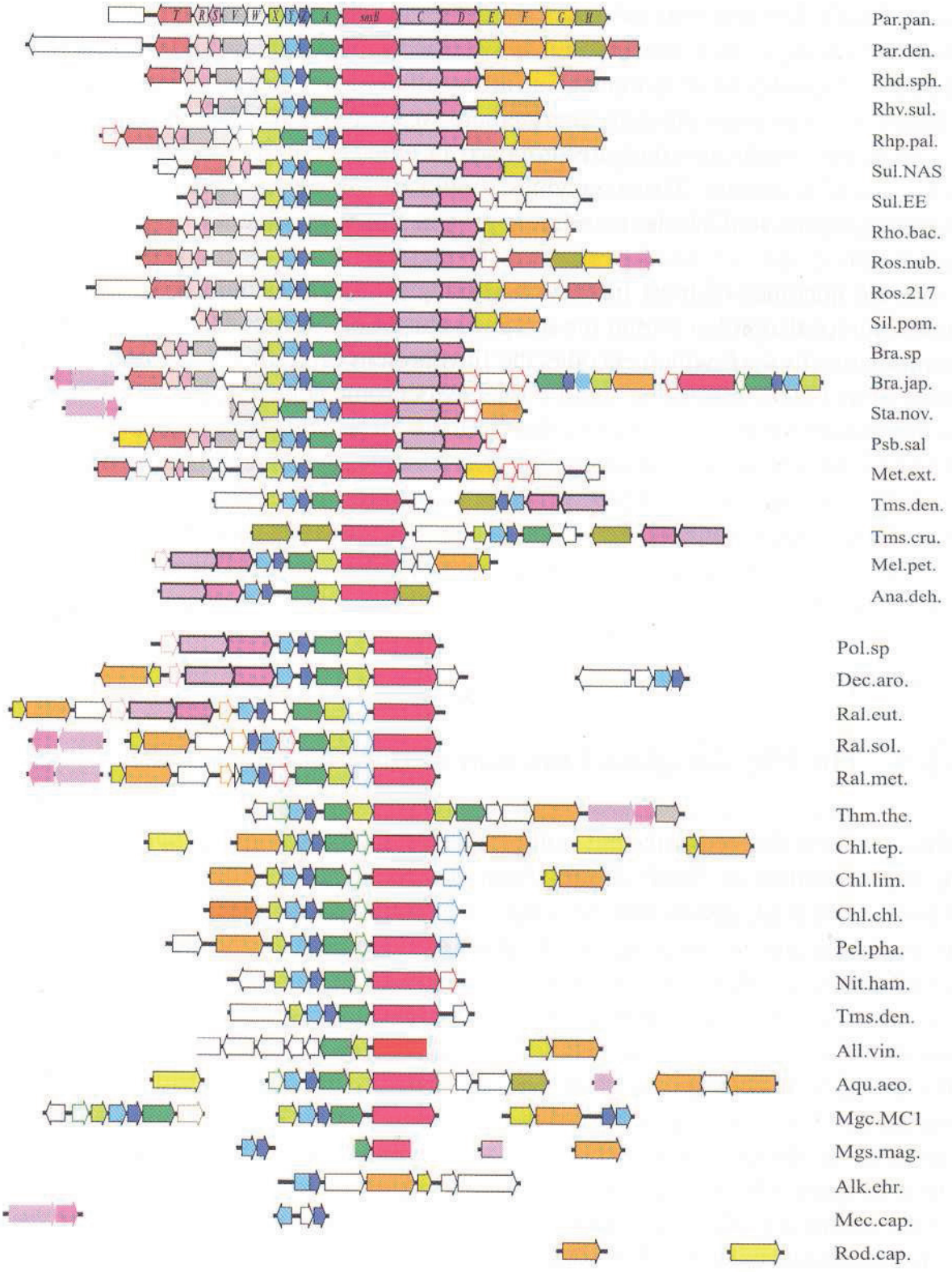

Fig. 8: Map of the *sox* gene cluster of *P. pantotrophus* and of *sox* gene homologs of other 38 bacteria. Open reading frames (ORFs) predicting homologous proteins are indicated by the same colour. Pink/violet arrows without frame indicate genes encoding sulfide dehydrogenases and their cytochromes. Bright yellow arrows, as for Rod.cap, indicate sulfide-quinone oxidoreductase genes. White arrows indicate ORFs not encoding Sox homologous. Par.pan, *Paracoccus pantotrophus* GB17; Par.den, *Paracoccus denitrificans* 1222;

Rhd.sph., *Rhodobacter sphaeroides*; Rhv.sul., *Rhodovulum sulfidophilum*; Rhp.pal., *Rhodopseudomonas palustris*; Sul.NAS, *Sulfitobacter* sp. NAS-14.1; Sul.EE, *Sulfitobacter* sp. EE-36, Rho.bac., rhodobacterale bacterium; Ros.nub. *Roseovarius nubinhiensis*; Ros.217, *Roseovarius* sp. 217; Sil.pom., *Silicibacter pomeroyi*; Bra.sp., *Bradyrhizobium* sp.; Bra.jap., *Bradyrhizobium japonicum*; Sta.nov., *Starkeya novella*; Psb.sal., *Pseudaminobacter salycilatoxidans* KCT001; Met.ext., *Methylobacterium extorquens*; Tms.den., *Thiomicrospira denitrificans*; Tms.cru. *Thiomicrospira crunogena*; Mel.pet. *Methylobium petroleophilum*; Ane.deh., *Anaeromyxobacter dehalogenans*; Pol.sp., *Polaromonas* sp.; Dec.aro., *Dechloromonas aromatica*; Ral.eut., *Ralstonia eutropha*; Ral.sol., *Ralstonia solanacearum*; Ral.met., *Ralstonia metallidurans*; Thm.the., *Thermus thermophilus*; Chl.tep., *Chlorobaculum tepidum*; Chl.lim., *Chlorobium limicola*; Chl.chl., *Chlorobium chlorochromatii* CaD3; Pel.pha., *Pelodictyon phaeoclathratiforme*; Nit.ham., *Nitrobacter hamburgensis*; Tms.den. *Thiomicrospira denitrificans*; All.vin., *Allochromatium vinosum*; Aqu.aeo., *Aquifex aeolicus*; Mgc.MC1, *Magnetococcus* MC-1; Mgs.mag., *Magnetospirillum magnetotacticum*; Alk.ehr., *Alkalilimnicola ehrlichii*; Mec.cap., *Methylococcus capsulatus*; Rod.cap., *Rhodobacter capsulatus*. With kind permission from Springer Science+Business Media: *Microbial Sulfur Metabolism*, chapter 12, Redox control of the chemotrophic sulfur oxidation of *Paracoccus pantotrophus*, 2008, pp. 139-150, Friedrich CG, Quentmeier A, Bardischewsky F, Rother D, Orawski G, Hellwig P, & Fischer J, Fig. 12.2.

In *Paracoccus pantotrophus* 15 open-reading frames are identified in one *sox* gene cluster, organized in three transcriptional units: *soxRS*, *soxvW*, *soxXYZABCDEFGH*. 7 polypeptide-coding genes originate 4 periplasmic proteins (SoxYZ, SoxAX, SoxB, and SoxCD) which, *in vitro*, can oxidize sulfide, sulfur, thiosulfate, and sulfite with cytochrome *c* as electron acceptor (reviewed by Friedrich et al, 2008).

SoxY forms a covalently bound complex with SoxZ. **SoxYZ** is the protein scaffold to which thiosulfate is oxidatively bound, and it interacts with SoxAX, SoxB, SoxCD, SoxS, and SoxF. The enzyme **SoxAX**, composed by the *c*-type cytochromes SoxA and SoxX, catalyzes the oxidative binding of thiosulfate to a cysteine residue of SoxY. **SoxCD** is a cytochrome complex containing molybdenum, and catalyzes the oxidation of the outer (sulfane) sulfur bound to SoxY to the sulfone state, transferring 6 electrons to an electron acceptor. **SoxB** catalyses the hydrolysis of the sulfane sulfur atoms formed by the action of SoxAX and SoxCD, releasing a sulfate molecule each time (reviewed by Friedrich et al, 2008). Quentmeier et al (2003) reported further that *in vitro* SoxB can convert the covalently bound subunits of SoxYZ (active form), in associate subunits, which aggregate into $Sox(YZ)_2$ tetramers (inactive form). SoxB has been used as a taxonomic gene marker for sulfur oxidizers (Petri et al, 2001; Meyer et al, 2007). *In vitro*, SoxY is linked to **SoxS** via a specific cysteine (Rother et al, 2008). **SoxS**, together with **SoxR**, mediates the regulation of thiosulfate oxidation. SoxR, which binds the intergenic regions *soxS-soxV* and *soxW-soxX*, is a repressor protein for the expression of *sox* genes (Rother et al, 2005). SoxS is a thiol-disulfide oxidoreductase that activates SoxY by specific reduction of the disulfide bonds between 2 SoxY subunits (Carius et al, 2009). SoxYZ can be isolated in different forms, 2 of which are inactive. One inactive for is the tetramer SoxY-$Y(Z)_2$, which is likely to be reduced and activated *in vivo* by SoxS, even if *in vitro* it can be activated by sulfide. The second inactive form is obtained by reaction with TCEP (tris(2-carboxyethyl)phosphine) and cannot be reactivated by sulfide *in vivo*, but it is probably activated *in vivo* by **SoxF** (Quentmeier et al 2008). **SoxF** is a flavocytochrome that *in vitro*

has cytochrome *c* – dependent sulfide dehydrogenase activity. SoxF does not act on thiosulfate, and its disruption does not affect thiosulfate oxidation, but its formation is induced by thiosulfate. SoxF is inhibited by sulfur, which is the product of sulfide oxidation, by sulfite, and by cyanide. **SoxE** is a small *c*-type cytochrome thought to be a partner of SoxF, even if *in vitro* this could not be shown (Quentmeier et al, 2004). **SoxW** and **SoxV** were described by Bardischewski et al (2006). According to Bardischewski et al (2006), SoxW and SoxV are present only in strains harbouring *SoxCD*, but Verté et al (2002) call SoxW a thiol-disulfide interchange protein homologue. SoxV is a transmembrane protein that maintains the periplasmic SoxW in a reduced state. Elimination of SoxV affects thiosulfate oxidation only *in vivo*, not *in vitro*. SoxW is not essential to thiosulfate oxidation, an indication that probably its role is to accept electrons from SoxV, which can anyway transfer electrons to some other partner. The hypothesis of Bardischewski et al (2006) is that SoxVW is involved in a catalytic cycle, for example they recurrently reduce cysteine residues of some Sox protein. Friedrich et al (2008) report SoxS as the *in vivo* electron acceptor of SoxV. The route of the electron from the cytoplasm for the activation of the Sox system would thus be SoxV-SoxS-SoxYZ.

GSBs and a number of other bacteria, among which the PSB *A. vinosum*, possess SoxK, also called SAXB (SoxAX binding protein) because *in vitro* it enhances the binding of SoxA with SoxX (Sakurai et al, 2010). SoxF, already mentioned in the previous section because of its sulfide dehydrogenase activity, was found to stimulate thiosulfate oxidation *in vitro* (Ogawa et al, 2010).
The Sox system has been reported also to oxidize sulfite *in vitro*, when cytochrome *c*-554 is added as electron acceptor (Sakurai et al, 2010).

In *P. pantotrophus* the sulfane intermediate is oxidized to valence VI by the complex SoxCD, which transfers 6 electrons to a yet unidentified acceptor (Friedrich et al, 2008, and references therein). SoxCD is absent in GSBs and in *A. vinosum*, which in fact are reported to produce sulfur as intermediate in the oxidation of thiosulfate to sulfate. A polysulfide chain might accumulate on SoxYZ (Sauvé et al, 2007), and at a certain point might detach spontaneously or might be transferred to an organic residue (Chan et al, 2008; Frigaard and Dahl, 2009; Sakurai et al, 2010). However, sulfur is not always a product of thiosulfate oxidation, as shown for *Cla. thiosulphatiphilum* and *Cla. tepidum* (Fischer, 1984; Chan et al., 2008; Frigaard and Dahl, 2009). Holkenbrink et al (2011) demonstrated that in *Cla. tepidum* the knock-out of the Dsr system causes sulfur accumulation from thiosulfate oxidation, and concluded that the Dsr system is responsible for the oxidation to sulfate of the sulfur produced during thiosulfate oxidation.

Sulfur produced during sulfide or thiosulfate oxidation, or added externally, is oxidized by GSBs to sulfate. It is not known how the sulfur formed from sulfide or thiosulfate oxidation is transferred outside the cells, where it is detected as "sulfur-globules". Transfer of sulfur from the periplasm to the extracellular environment might be performed by a homolog of the *E. coli* DsbD thiol:disulfide interchange protein, which is named *CT1075* in *Cla. tepidum*, and is present in all 12 sequenced GSBs at a minimum of 57% aminoacid sequence identity. An alternative hypothesis is that DsbD transports sulfur into the cytoplasm, where there is the Dsr system (Sakurai et al, 2010). Mutational studies on *Cla. tepidum* oxidizing thiosulfate showed that the Dsr system is needed for the oxidation of sulfur to sulfate (Holkenbrink et al, 2011), similarly to what proposed for PSBs (Grimm et al, 2008, and references therein). In PSBs Dsr produces sulfite from the oxidation of sulfur. If sulfite is a product of Dsr in GSBs as well, a system must exist for sulfite oxidation in GSBs. The oxidation of sulfite to sulfate in GSBs might be performed by QmoABC and AprBA (Frigaard and Dahl, 2009; Sakurai et al, 2010). In support to this idea there is the work of Rodriguez et al (2011), who showed that *Cla. tepidum* mutants defective of *qmoB* or of *qmoC* accumulate intracellular sulfite. However, not all GSBs possess QmoABC-AprBA (Frigaard and Dahl, 2009). Thiosulfate-oxidizing GSBs encode also SoxW (Sakurai et al, 2010), which is a thiol-disulfide interchange protein homologue (Verté et al, 2002) of unknown function.

Holkenbrink et al (2011) explained the variable or absent production of sulfur during thiosulfate oxidation by *Cla. tepidum* with the contemporary oxidation of S^0 to sulfate, catalyzed by the Dsr system. The Dsr system action might be also invoked to explain the absence of sulfur during oxidation of low amounts of sulfide, which was reported by Pfennig (1975) and Cork et al (1985).
It is not known how GSBs mobilize extracellular sulfur. Two proteins have been proposed to be involved in extracellular sulfur utilization: an excreted protein identified as CT0893 in *Cla. tepidum*, which is retained in the periplasm in a mutant that cannot grow on sulfur (Hanson and Tabita, 2003) and a protein identified as CT2230 in *Cla. tepidum*, which is related to the sulfur-induced protein identified in *Atb. ferrooxidans* by Ramírez et al (2004) and is present in the genome of all GSBs able to oxidize externally added sulfur (Frigaard and Dahl, 2009). However, the real function of these two proteins has not been further investigated.

Electrons from inorganic sulfur compounds are transferred to the reaction centre via soluble or membrane-bound cytochromes. Cytochromes in GSBs have been reviewed by Oh-oka and Blankenship (2004). The electron pathway from sulfide through the membrane-bound SQR seems to be all located inside the cytoplasmic membrane or in elements bound to it: sulfide → SQR

→ membrane-bound quinol oxidoreductase (a complex between a cytochrome *b* and a Rieske-type iron-sulfur protein) → cytochrome *c*-556, bound to the cyt-*b*/Rieske ISP → cytochrome c_z (also known as PscC or bound *c*-551), bound to RC → P840. However, a mutant of *Cla. tepidum* that has no cytochrome *c*-556 is still able to grow on sulfide, even if at a slower growth rate (Tsukatani et al, 2006).

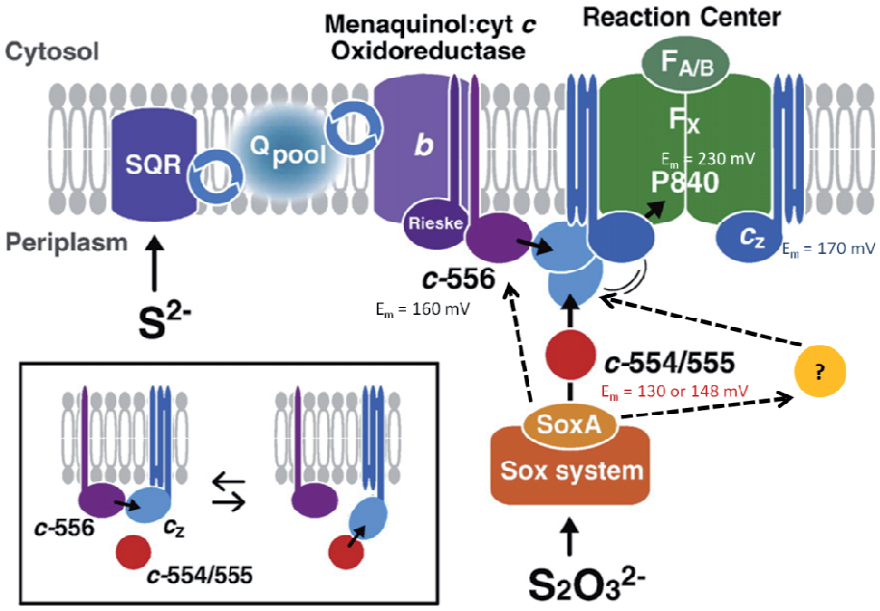

Fig. 9: From Tsukatani et al, 2008. Modified after Azai et al, 2009. Dashed lines indicate hypothesized pathways for the electrons deriving by thiosulfate oxidative binding, alternative to the involvements of c-555/554.

Electrons deriving from the oxidation of thiosulfate to sulfur and sulfate by SoxAX (SoxX is a cytochrome bound to the monoheme protein SoxA) would follow a "soluble" pathway: SoxX (soluble cytochrome *c*-551) → cytochrome *c*-554/555 (the maximum absorbance varies slightly according to the species that hosts it) → cytochrome c_z (also known as PscC or bound *c*-551), bound to RC → P840. Azai et al (2009) showed however that a mutant of *Cla. tepidum* devoid of cytochrome *c*-554/555 was able anyway to grow on thiosulfate, even if more slowly, which suggests that a cytochrome of the sulfide-way (cytochrome *c*-556), a yet unidentified cytochrome, or both are part of the electron transport chain from thiosulfate to P840.

It is not known how electrons deriving from the oxidation of sulfur – produced as intermediate of sulfide or thiosulfate oxidation, or added as substrate – are transferred to the reaction centre.

1.2 The sulfur cycle

Sulfur compounds exist in a variety of oxidation states, and can undergo many reactions, both chemical and biological. GSBs live in anoxic environments, at circumneutral pH, and have a response to temperature ranging from mesophilic to moderate termophilic. Under these conditions, the action of GSBs on inorganic sulfur compounds flanks transformations by other microorganisms, which might be present and active in the same environment, and chemical reactions. Luther et al (2011) compared the rates of sulfide oxidation to sulfur by the GSB *Cla. tepidum*, with the rates of chemical oxidation of sulfide to sulfur by oxygen. They concluded that biological oxidation of sulfide to sulfur is faster than chemical oxidation by oxygen in the absence of trace metals (Luther et al, 2011) Information reported below on the transformations of sulfur compounds in marine sediments is mainly taken from Zopfi et al (2004) and Jørgensen and Nelson (2004), who reviewed chemical reactions and transformations by chemotrophs.

Oxidation of sulfide to sulfate, whether it is biologically or chemically driven, results in the formation of intermediates, such as thiosulfate, sulfur, and sulfite, which are produced by a reaction of metal oxides with sulfide, or by incomplete bacterial sulfide oxidation. Of all sulfide produced by sulfate reduction, only 5-20% is buried into the sediments as pyrite (FeS_2) or iron sulfide (FeS), while the remaining 80-95% is eventually reoxidized to sulfate.

Pyrite (FeS_2) comprises the main sulfur pool in marine sediments, and undergoes slow transport and oxidation. In sediments, the immediate products of pyrite oxidation are thiosulfate and polythionates. Pyrite oxidation is chemical, and has been proposed to be performed by Fe(II)/Fe(III) electrons shuttling to Mn(IV). FeS can be oxidized by Mn(IV), with polysulfides and sulfur as oxidation products (Jørgensen and Nelson, 2004).

Oxidation of sulfide with oxygen leads to the formation of sulfite, which in turn reacts with oxygen to form sulfate or thiosulfate. Thiosulfate and sulfate are chemically stable at environmental temperatures and neutral conditions, and can thus accumulate. In the presence of trace metals, sulfur can also be formed by the reaction of sulfide with oxygen. Elemental sulfur can react with sulfite to form thiosulfate, and with sulfide to form polysulfides. Under oxic conditions, polysulfides decompose in sulfur and thiosulfate (Zopfi et al, 2004).

In marine sediments, oxygen is however present only in the top millimetres or centimetres. Below it, in the anoxic zone, oxidation of organic matter is performed directly by heterotrophic iron or manganese reducing bacteria, or indirectly, which is by a reaction of organic matter with the sulfide produced by sulfate-reducing bacteria (Jørgensen and Nelson, 2004). Under anoxic conditions, sulfide is chemically oxidized by Mn(IV) and Fe(III). Sulfide oxidation by Mn(IV) results in the formation mainly of sulfur, but also of thiosulfate and sulfate. Sulfide oxidation by

Fe(III) has sulfur as dominant product, while polysulfides, sulfite, and thiosulfate are formed in minor amounts. These intermediates of sulfide oxidation to sulfate can be transformed by microorganisms, which can oxidize or reduce them (Zopfi et al, 2004). There are also anaerobic sulfide oxidizers that use nitrate as electron acceptor. Cells that can store nitrate can couple sulfide oxidation to nitrate reduction even if nitrate and sulfide are spatially separated (Jørgensen and Nelson, 2004).

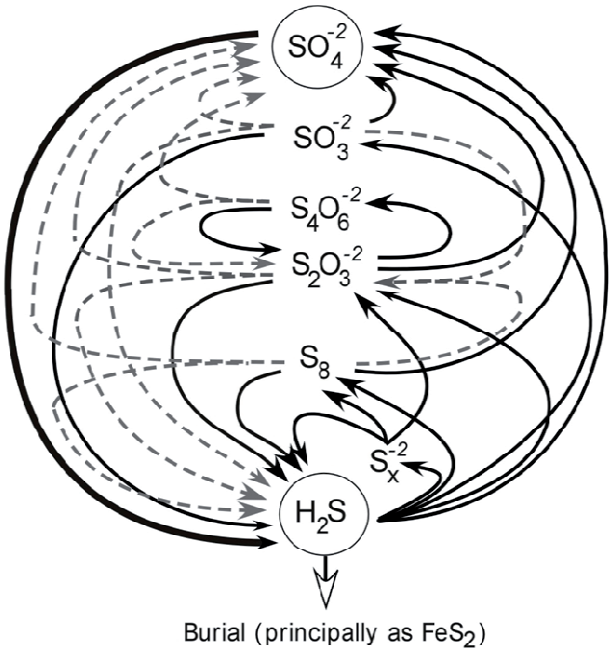

Fig. 10: From Zopfi et al (2004). Scheme of main inorganic sulfur compounds transformations in sediments. Reductive pathways are shown on the left, oxidative pathways on the right side of the cycle. Dashed lines on the left indicate disproportionations.

Sulfur is the product of the chemical oxidation of FeS by Mn(IV), of the chemical reaction between tetrathionate and sulfide, of polysulfides decomposition, and is also an intermediate in bacterial oxidation of sulfide or in bacterial oxidative disproportionation of thiosulfate.While thiosulfate can be a product of both oxidative and reductive processes (e.g during sulfate reduction under organic substrate limitation), elemental sulfur is produced only during oxidative pathways in the sulfur cycle (Zopfi et al, 2004). In the absence of H_2S, sulfur is chemically inert in natural environments, but is subjected to biological oxidation, disproportionation, or reduction (Schippers et al, 2004). In marine hydrothermal systems, chemolithoautotrophic S^0-oxidizers are responsible for much of the biomass synthesis. Among hyperthermophiles, S^0 reduction is much more common than sulfate-reduction (Amend et al, 2004).

Thiosulfate is chemically stable at pH neutral, so that when low thiosulfate concentrations are observed, this is due to biological action (Zopfi et al, 2004).

Sulfite has a high chemical reactivity, thus it is usually observed in the environment at low concentrations. Sulfite may be formed by enzymatic reduction of thiosulfate. Bacteria that cannot use sulfite release it into the environment, where sulfite might react with sulfur, to form thiosulfate. Sulfite is also a product of thiosulfate disproportionation, but such a process is cytoplasmatic, and normally sulfite is not released into the environment (Zopfi et al, 2004).

The three products of sulfide oxidation – sulfur, thiosulfate, and sulfite – can undergo disproportionation:

$S_2O_3^{2-} + H_2O \rightarrow H_2S + SO_4^{2-}$

$4\ SO_3^{2-} + 2\ H^+ \rightarrow H_2S + 3\ SO_4^{2-}$

$4\ S^0 + 4\ H_2O \rightarrow 3\ H_2S + SO_4^{2-} + 2\ H^+$

For disproportionations, bacteria do not need any external reductant or oxidant. Thiosulfate disproportionation can itself support energy requirements of autotrophs or heterotrophs. Sulfur disproportionation at normal environmental temperatures is purely a biological process, but requires continuous sulfide removal to be exergonic. Elemental sulfur may also react with sulfide to form polysulfides, which in turn combine with FeS to give pyrite (Jørgensen and Nelson, 2004).

Tetrathionate forms 1) during chemical oxidation of sulfide, FeS, and pyrite; 2) as a product of microbial aerobic oxidation of sulfide, sulfur or thiosulfate; 3) as a product of anaerobic chemical oxidation of thiosulfate with (MnIV); 4) from microbial oxidation of thiosulfate with nitrate. Tetrathionate reacts with sulfide to form sulfur and thiosulfate. Tetrathionate can also be reduced to thiosulfate by microorganisms, and thiosulfate can then be oxidized to tetrathionate by Mn(IV). Reduction of tetrathionate was found not to be directly coupled to the oxidation of organic matter, and to be repressed by electron acceptors as oxygen and nitrate. Tetrathionate reduction might be a way that fermenting microorganisms use to consume the excess reducing power, and to recycle NAD^+ or NADP. In any way tetrathionate might be formed, under anoxic conditions it will be primarily reduced to thiosulfate. Thiosulfate is consumed more slowly than tetrathionate is produced (Zopfi et al, 2004).

1.3 Focus of the PhD work

The general aim of the present work is contributing to explain how GSBs can use elemental sulfur as electron donor.

Only recently was it demonstrated that the moderate thermophile *Cla. tepidum* needs the cytoplasmic dissimilatory-sulfur-reductase (Dsr) system to oxidize the sulfur produced from thiosulfate oxidation (Holkenbrink et al, 2011). However, it has not been clarified yet how extracellular sulfur is transported into the cytoplasm, or if the cytoplasmic Dsr system is the only system for sulfur oxidation. The situation in *Cla. tepidum* might anyway be different from the situation in mesophilic strains of GSBs: at 45-55°C, which is the temperature of the habitat from which *Cla. tepidum* was isolated (Wahlund et al, 1991), sulfur is more soluble than at 28°C (Kamyshny, 2009), the temperature at which mesophiles are generally cultivated. *Cla. tepidum* might thus have access to solid sulfur without the need of any mobilization system.

GSBs are phylogenetically closely related, and have quite a simple metabolism – they are strict photolithotrophs, yet the genomic comparison of the 12 sequenced strains has revealed an unexpected variety in their genetic set (Frigaard and Dahl, 2009). Their genomes have been subjected to extensive lateral gene transfer (Raymond et al, 2002; Frigaard and Dahl, 2009). It might thus be particularly interesting to understand how the variations in the gene sets influence the metabolism of GSBs, or, on the other hand, how GSBs perform more or less the same tasks despite having different genes. Since GSBs have been subjected to lateral gene transfer, the discovery of a new pathway in GSBs or the attribution of a function to a non-characterized protein might be relevant also for other groups of microorganisms. On the other hand, if each strain has its own peculiarities, it might be risky to extend results obtained from one strain to the whole phylum of GSBs.

Studies on cytochromes, like those of Azai et al (2009) or Tsukatani et al (2006), have shown that it is often difficult to shut completely down a part of the sulfur metabolism in a GSB, partly because of the complex redox chemistry of sulfur compounds, which can be subjected to several chemical reactions (Zopfi et al, 2004), partly because the enzymes involved in electron transfer from sulfur compounds to cytochromes might accept more than one electron donor.

At the light of these difficulties, it was considered particularly valuable to keep simple the system studied. The model strain and the growing conditions reflected such a search of simplicity:

1) The strain chosen as a model (*Cla. parvum* DSM 263/NCIB 8327) has been known for long. Several works – among them Steinmetz and Fischer (1982 and 1985), Fuhrmann et al (1993), Borkenstein (2006) – documented different aspects of the strains's physiology and biochemisty

(e.g growth requirements; intermediates in sulfide, sulfur, and thiosulfate oxidation; cytochromes; production of surfactants).

2) *Cla. pravum* DSM 263/NCIB 8327 is a mesophile, which is believed to reveal more insights about sulfur mobilization than a termophile – sulfur solubility increases with temperature (Kamyshny, 2009).
3) The chosen growing strain can oxidize thiosulfate, and produces sulfur both from sulfide oxidation and from thiosulfate oxidative disproportionation.
4) The genome of *Cla. pravum* DSM 263/NCIB 8327 was sequenced and published by the Joint Genome Institute (Lucas et al, 2008). The availability of the genome sequence allowed comparisons with the gene content of other GSBs, and facilitated the proteomic study – the genome sequencing permits to choose an easier approach for peptide spots identification after differential 2-dimensional gel electrophoresis.
5) Cultivation of *Cla. pravum* DSM 263/NCIB 8327 was carried on under continuous light, offering CO_2 as the sole carbon source, despite GSBs being able to assimilate small organic molecules.

Cla. parvum DSM 263 was studied using physiological (Chapters 2 and 4) and molecular methods (Chapter 3).

Chapters 2 deals with thiosulfate oxidation by *Cla. parvum* DSM 263. Thiosulfate oxidation has been attributed to the Sox multienzymatic system. However, such model is incomplete, since it does not explain how sulfur is released from the Sox enzyme. Research presented in Chapter 2 tries to clarify if the Sox-model proposed for phototrophic sulfur oxidizers holds *in vivo*, despite the incompleteness of the model itself. A physiological approach was used, and light intensity was chosen as the parameter to be changed while measuring how the dynamics of inorganic sulfur compounds and biomass varied.

A molecular approach was instead chosen to try to identify the proteins involved in the oxidation of sulfur. Results are presented and discussed in **Chapter 3**. Since GSBs do not oxidize sulfur in the presence of high concentrations of sulfide, it was possible to perform differential proteomics on sulfide- vs. sulfur-grown populations. Sulfide is a soluble compound, while sulfur is solid, but both are used by the bacterium as source of electrons. It was thus expected to observe few differences in the proteomes of cells grown on sulfide respect to cells grown on sulfur. *Cla. parvum* DSM 263/NCIB 8327 was shown to need contact with sulfur in order to oxidize it, thus the membrane proteome of bacteria was studied.

Chapter 4 contains preliminary results on sulfur oxidation by *Cla. parvum* DSM 263/NCIB 8327, investigated by physiological methods.

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– Chapter 2 –

THIOSULFATE OXIDATION BY THE GREEN SULFUR BACTERIUM *CHLOROBACULUM PARVUM* DSM 263 – The Sox system studied *in vivo*

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Abstract

Thiosulfate can be used by some strains of green sulfur bacteria as electron donor for photosynthesis. Thiosulfate oxidation has been proposed to be performed by the Sox multienzymatic system. To test whether the Sox model for thiosulfate oxidation holds *in vivo*, we analyzed the dynamics of sulfide, thiosulfate, sulfur, sulfate, and biomass under physiological conditions in cultures of *Chlorobaculum parvum* DSM 263 illuminated by different light intensities and fed with different combinations of sulfide and thiosulfate. Sulfide – the first sulfur compound to be consumed – was oxidized in 2 phases. Two-phases sulfide oxidation could be an evidence of the different roles of the SQRs encoded in the genomes of green sulfur bacteria. Sulfur was always the last electron donor to be consumed, which suggests that the expression of the system for extracellular-sulfur oxidation starts only after sulfide and thiosulfate depletion. Conversely 1) more sulfate than sulfur was produced from thiosulfate oxidation as the illumination of the cultures increased, and 2) the growth rate during thiosulfate consumption was positively correlated with the sulfate yield on thiosulfate. These two apparently contradictory results, i.e., lack of expression of the sulfur-oxidizing system in the presence of thiosulfate and simultaneous oxidation of sulfur and thiosulfate, indicate that the site of sulfur oxidation is periplasmic or extracellular. Periplasmic sulfur oxidation would occur during thiosulfate oxidation, while extracellular sulfur oxidation would need the synthesis of an inducible system. Finally, the sulfate yields on thiosulfate and the formation of unidentified sulfur that we observed in some experiments suggest the existence of a pathway for thiosulfate consumption alternative to Sox.

Introduction

Green sulfur bacteria (GSBs) are photolithoautotrophic microorganisms that use inorganic sulfur compounds as electron donors for CO_2 fixation. The only exception known thus far is the GSB *Chlorobium ferrooxidans* that uses iron. The genomes of a dozen species of GSBs have been recently sequenced and analyzed. Reviews on the state of the art of genomics and physiology are presented by Frigaard and Bryant (2008a, 2008b), Frigaard and Dahl (2008), Chan et al. (2008a), and by Hanson et al. (2010). With the exception of the aforementioned *Chl. ferrooxidans,* all GSBs can oxidize sulfide (HS^-) and sulfur (S^0), while only few strains can oxidize thiosulfate (*Chlorobaculum limnaeum* 1549; *Cla. parvum*; *Cla. tepidum*; *Cla. thiosulphatiphilum*; *Chl. clathratiforme* DSM 5477; *Chl. limicola* 1630, 9330, and DSM 257; *Chl. phaeovibrioides* DSM 265) (Frigaard & Dahl, 2008).

Thiosulfate ($S_2O_3^{2-}$), whose sulfur moieties have valences of -1 and +5 (Vairavamurthy et al., 1993), has been recognized as a key compound in the sulfur cycle of marine and freshwater sediments, and is generally detected in the sediment porewater at low concentrations (nM-µM; Zopfi et al., 2004). $S_2O_3^{2-}$ can serve as electron donor or sink, or can be disproportionated, linking the oxidative and reductive parts of the sulfur cycle (Fossing & Jørgensen, 1989; Jørgensen, 1990a, b).

The commonly accepted mechanism for $S_2O_3^{2-}$ oxidation by GSBs is an oxidative cleavage, resulting in the production of S^0 and sulfate (SO_4^{2-}) (Frigaard & Dahl, 2008). Already in 1965, Trudinger had formulated the hypothesis that GSBs start $S_2O_3^{2-}$ oxidation with an oxidative binding of $S_2O_3^{2-}$ to a thiol. However, only recently was it possible to attribute $S_2O_3^{2-}$ oxidation in GSBs to the Sox complex, confirming Trudinger's idea. The Sox complex was first discovered and studied in *Paracoccus pantotrophus* (Kelly, 1971; Kelly et al., 1997; Friedrich, 1998; Friedrich et al., 2001; Quentmeier & Friedrich, 2001; Quentmeier et al., 2003, 2004; Quentmeier et al., 2007; Quentmeier et al., 2008). Later, a core of 3 protein components (SoxYZ, SoxAX, SoxB) was shown to be present and functional in the purple sulfur bacterium (PSB) *Allochromatium vinosum* (Hensen et al., 2006; Welte et al., 2009), and was retrieved in the genomic sequence of the GSB *Cla. tepidum* (former *Chl. tepidum*) and of the other $S_2O_3^{2-}$ - oxidizing GSBs (Eisen et al., 2002; Frigaard & Dahl, 2008). Research on the action of the Sox complex in GSBs, conducted so far *in vitro* and by mutagenesis (Verté et al., 2002; Ogawa et al., 2008a, b; Chan et al., 2008b), has confirmed that the Sox complex of GSBs acts similarly to the Sox complex of the PSB *Alc. Vinosum*: SoxYZ is the scaffold to which $S_2O_3^{2-}$ is bound, SoxAX catalyses the oxidative binding of $S_2O_3^{2-}$ to SoxYZ, and SoxB catalyses the hydrolysis of the $S_2O_3^{2-}$ sulfonate-sulfur as SO_4^{2-}.

However, the capability of GSBs to oxidize $S_2O_3^{2-}$ has also been shown to involve genetic elements additional to those encoded in the *sox* cluster. Chan et al. (2008b) performed genomic,

transcriptional, and mutational analysis, and discovered that a genomic region external to the *sox* cluster (*CT0868-0876*) is required for $S_2O_3^{2-}$ oxidation in the GSB *Cla. tepidum.* Méndez-Alvarez et al. (1994) showed that the GSB *Chl. limicola* DSM 245 becomes able to oxidize $S_2O_3^{2-}$ if transformed by a plasmid possessed by the GSB *Cla. thiosulphatiphilum* (formerly *Chl. limicola* f. *thiosulphatophilum*) DSM 249, and hypothesised that such a plasmid contains one or more genes for a $S_2O_3^{2-}$-oxidizing enzyme or for an expression-regulator of a gene for $S_2O_3^{2-}$-oxidation. The coexistence of 2 distinct pathways for $S_2O_3^{2-}$ oxidation has been already demonstrated in bacteria like the PSB *Alc. vinosum* (Hensen et al., 2006) and the facultative heterotroph *Starkeya novella* (Kappler et al., 2001), which oxidize $S_2O_3^{2-}$ to sulfate via tetrathionate ($S_4O_6^{2-}$) or - using the Sox system – via S^0.

The aim of our study was to clarify if the proposed model for $S_2O_3^{2-}$ oxidation by the Sox system is sufficient to explain the *in vivo* turnover of $S_2O_3^{2-}$, S^0, and SO_4^{2-}, as well as the biomass formation, in a GSB. Our investigation, conducted on cultures of *Cla. parvum* (former *Chl. vibrioforme* f. *thiosulphatophilum*) DSM 263, examined the preferential oxidation of HS^- or $S_2O_3^{2-}$ over S^0, and the stoichiometry of $S_2O_3^{2-}$ oxidation in dependence on different light intensities.

Materials and Methods

Medium and cultivation methods. Cultivation was carried out in the medium suggested by the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) for green sulfur bacteria, which was prepared dissolving in 1 litre of distilled water: 0.25 g $CaCl_2 \cdot 2\ H_2O$, 0.34 KH_2PO_4, 0.34 g NH_4Cl, 0.34 g KCl, 0.50 g $MgSO_4 \cdot 7\ H_2O$, and 10.00 g NaCl. After autoclaving and cooling, 1 mL L^{-1} of vitamin B_{12} (0.002 %), 1 mL L^{-1} of trace element solution SL10B (7.7 ml L^{-1} HCl 25%, 1.5 g L^{-1} $FeSO_4 \cdot 7\ H_2O$, 70 mg L^{-1} $ZnCl_2$, 100 mg L^{-1} $MnCl_2 \cdot 4\ H_2O$, 300 mg L^{-1} H_3BO_3, 190 mg L^{-1} $CoCl_2 \cdot 6\ H_2O$, 2 mg L^{-1} $CuCl_2 \cdot 2\ H_2O$, 24 mg L^{-1} $NiCl_2 \cdot 6\ H_2O$, 36 mg L^{-1} $Na_2MoO_4 \cdot 2\ H_2O$), and 20 ml L^{-1} $NaHCO_3$ (0.89 M) were added. The medium was first saturated with CO_2 and then gassed with N_2 until a pH of 6.8. Cultivation was carried out in 5 L or 50 mL bottles. In the latter case, medium was aliquoted in the final cultivation bottles under N_2 flush. Ascorbic acid (4 mM) and MOPS (3-(N-morpholino)propanesulfonic acid, 50 mM, pH 6.8) were subsequently added to scavenge residual oxygen and to ensure pH stability during the course of the experiments. Cultures were incubated at 28°C, illuminated by photon flux densities ranging from less than 1 to 80 µE m^{-2} sec^{-1} and continuously stirred at 240 rpm. Illumination was provided by neon light tubes (Biolux L18W/72 Osram, Munich, Germany). HS^- was added to a final concentration of 2 mM, $S_2O_3^{2-}$ to final concentrations of 5-11 or 45-85 mM.

Source of the organism. *Chlorobaculum parvum* DSM 263 (Imhoff, 2003) was purchased as a dried culture from the DSMZ and revitalized in the inorganic medium described above, containing HS^- (2 mM) and $S_2O_3^{2-}$ (10 mM) as electron donors. A single colony was isolated by the agar-shake method, in revitalizing medium supplemented with 0.1% $CaCl_2$ and 2.4% agar, according to the procedure described by Trüper (1970). The isolated colony was then grown in the same liquid medium. For long term storage, aliquots were kept in 20 % glycerol at -80°C. PCR-amplification and sequencing of the 16S rRNA gene was performed according to standard methods (Sambrook, 2001).

For each experiment, a glycerol stock (1.5 mL) was grown in 50 mL-inorganic medium supplied with ascorbic acid (4 mM), sulfide (2 mM), and $S_2O_3^{2-}$ (10 mM), incubated at a light intensity of 25 µE m^{-2} sec^{-1} and a temperature of 28ºC. This pre-culture was used as inoculum (1 or 2% volume) for the experiments.

Analytical procedures. Consumption and production of inorganic sulfur compounds and growth were monitored in dependence of time. Samples were withdrawn from the cultures using N_2-flushed syringes and injecting an equal volume of N_2 to maintain a slight overpressure and to avoid O_2 penetration into the culture bottles.

Withdrawn samples were fixed in 0.25 volumes of 2% zinc acetate solution and used for the quantification of bacteriochlorophyll *c*, proteins, HS^-, S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$, and SO_4^{2-}. Samples used for sulfite (SO_3^{2-}) quantification were fixed in a solution of monobromobimane (mBrB), essentially according to Rethmeyer et al (1997). The quantity of mBrB employed was however increased, in consideration of the high amounts of $S_2O_3^{2-}$ (30 mM) present in the analyzed samples: 50 µl samples were fixed with 250 µl mBrB (48 mM) and the reaction was stopped after 30 min by the addition of 2 mL methanesulfonic acid (65 mM). Non-fixed samples were used for the quantification of S-atoms belonging to polysulfides.

Bacteriochlorophyll was extracted by mixing one part of a fixed sample with four parts of 100% methanol. After incubation for 4-5 hours at 4ºC in the dark, samples were centrifuged at 13,000 rpm for 5 min in a benchtop microcentrifuge (Biofuge pico Heraeus, Hanau, Germany). Absorbance of the supernatant was measured at 670 nm against a blank of 80% methanol. Bacteriochlorophyll *c* concentration was determined using the absorbance coefficient of 86.0 cm L g^{-1} according to Stal et al (1984). Protein content was determined using the Bradford microassay, as described by Mukhopadhyay et al (1999).

HS^- concentration was determined according to Cline (1969). S-atoms belonging to organic polysulfides were quantified according to Yücel et al (2010). Briefly, 1 mL culture was extracted with 10 mL methanol:toluene (3:1), subsequently acidified with 10 mL 1N HCl, flushed for 10 min,

re-extracted with 10 mL methanol:toluene (3:1). Organic polysulfides were measured as S_8 from the second methanol-toluene extract.

S^0, $S_2O_3^{2-}$, SO_3^{2-}, $S_4O_6^{2-}$, and SO_4^{2-} were quantified by HPLC using a Merck-Hitachi intelligent pump (L-6220), an autosampler (AS-2000A), an oven (L-7350), UV/VIS- (L-4250), fluorescence- (F-1050), and conductivity-detector (L-3730). $S_2O_3^{2-}$, $S_4O_6^{2-}$, and SO_4^{2-} were quantified from fixed samples after filtration (0.2 µm pore size), based on the method described by Miura and Kawaoi (2000) and modified as follows: separation was achieved by a LiChrospher 60 RP-select B column (125 x 4 mm, 5 µm; Merck), eluting with 10 mM tetrapropylammonium bromide (Fluka) in 10% HPLC-grade acetonitrile pH 5, pumped at a flow rate of 0.8 mL min^{-1}. Temperature was kept constant at 30ºC. $S_2O_3^{2-}$ and $S_4O_6^{2-}$ were detected at 230 nm, SO_4^{2-} by conductivity. For S^0 quantification, fixed samples were diluted in HPLC-grade methanol, incubated overnight at 4ºC and subsequently filtered (0.2 µm pore size). S^0 was analyzed according to Zopfi et al. (2001), using a LiChrospher 100 RP-18 column (125 x 4 mm, 5 µm; Merck, Darmstadt, Germany).

SO_3^{2-} was quantified essentially according to Rethmeier et al. (1997), but using a slightly modified gradient: 0-13 min 10% B, 19 min 30% B; 23 min 50% B, 30-33 min 100% B, 34-39 min 10% B.

Results

1. Thiosulfate oxidation in dependence on light intensity

In batch cultures of strain *Cla. parvum* DSM 263, HS^-, $S_2O_3^{2-}$, and S^0 were depleted one after the other, as shown in Fig. 1.A. HS^- was always the first compound to disappear. Simultaneous to HS^- decrease was S^0 increase. HS^- was consumed in two phases, very fast at the beginning (Fig. 1.A: 58 ± 4 µmol in 15 h, time 0-15 h) and more slowly afterwards (Fig. 1.A: 26 ± 3 µmol in 75.5 h, time 15-90.5 h). Once HS^- had been depleted, $S_2O_3^{2-}$ amount started decreasing as well, while the amounts of SO_4^{2-} and S^0 increased. S^0 was always the last compound be depleted. While S^0 amount decreased, the amount of SO_4^{2-} increased. A lag-phase existed between the depletion of $S_2O_3^{2-}$ and the consumption of S^0: the increase of SO_4^{2-} amount slowed down and stopped for about 15 h (Fig. 1.A., time 146.8-161.5 h) after $S_2O_3^{2-}$ amount had been reduced to zero, and proceeded then again when the amount of S^0 started to decrease (Fig. 1.A). Protein production stopped as well during the same 15 h (Fig. 1.B, time 146.8-161.5 h).

Strain DSM 263 favoured HS^- over $S_2O_3^{2-}$ and S^0, and $S_2O_3^{2-}$ over S^0 independent of light intensity (from less than 1 to 80 µE m^{-2} sec^{-1}) or temperature (28 or 38ºC) (data not shown). In contrast, the observed stoichiometry of $S_2O_3^{2-}$ oxidation (Tab. 1) was influenced by the light intensity at which strain DSM 263 was cultivated. With increasing light intensities, the amount of SO_4^{2-} produced per mole of oxidized $S_2O_3^{2-}$ increased, while the amount of S^0 decreased. At all

tested light intensities, more SO_4^{2-} than S^0 was produced from $S_2O_3^{2-}$ oxidation. At light intensities ≤ 2 µE m^{-2} sec^{-1}, S^0 and SO_4^{2-} were not the only products of $S_2O_3^{2-}$ oxidation: 0.3-0.5 mol of S-atoms, which did not belong to sulfide, S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$, or SO_4^{2-}, were produced per mole of consumed $S_2O_3^{2-}$. The concentration of S-atoms belonging to unidentified sulfur species was greatest (6.4 ± 0.9 mM) in cultures illuminated with less than 1 µE m^{-2} sec^{-1}.

Growth parameters of strain DSM 263 were also influenced by the light intensity at which strain DSM 263 was cultivated (Tab. 1). The growth rate of strain DSM 263 had a maximum at 15 µE m^{-2} sec^{-1} and slightly declined at higher illuminations (Fig. S1). Protein and bacteriochlorophyll *c* yields on $S_2O_3^{2-}$ generally increased with light intensity. Growth rates and SO_4^{2-} yields on $S_2O_3^{2-}$ were positively correlated (Fig. 3).

2. Cultivation on excess of sulfide

As shown in Fig. 2, the oxidation of both S^0 and $S_2O_3^{2-}$ by strain DSM 263 was inhibited by HS^- maintained at concentrations of 2-4 mM by successive additions over a period of days. SO_4^{2-} concentration did not increase during the course of the experiment. $S_2O_3^{2-}$ also remained present at low concentrations during this experiment.

3. Cultivation on excess of thiosulfate

$S_2O_3^{2-}$ preferential oxidation over S^0 was further investigated by feeding batch cultures of strain DSM 263 with an excess of $S_2O_3^{2-}$. A summary of $S_2O_3^{2-}$ oxidation products as well as bacteriochlorophyll *c* and protein contents are presented in Tab. 2. In cultures illuminated with 2 µE m^{-2} sec^{-1} (case A) an equimolar amount of SO_4^{2-} was produced from $S_2O_3^{2-}$ oxidation, while in cultures illuminated with 25 µE m^{-2} sec^{-1} (case B) at the end of the experiment (5 days for cultures illuminated by 25 µE m^{-2} sec^{-1}, versus 16 days for cultures or 2 µE m^{-2} sec^{-1}) there was 17% less SO_4^{2-} than what is expected from an oxidative cleavage of $S_2O_3^{2-}$ (Fig. 4). At both light intensities, less S^0 than expected was found. Such a deficit was higher in cultures illuminated with 25 µE m^{-2} sec^{-1}.

As presented in Tab. 2, at the end of the experiment 4 ± 2 (case A) or 38 ± 2 (case B) mM of S-atoms could not be assigned to any compound. In case B, SO_3^{2-} and S-atoms belonging to polysulfides were measured too. The concentration of SO_3^{2-} was 1 µM. The concentration of S-atoms belonging to polysulfides was 1 mM, which only accounted for 3% of the total unidentified S-atoms.

Discussion

1. Oxidation of sulfide

The dynamics of HS^- oxidation in strain DSM 263 suggests the presence of two distinct systems for sulfide oxidation, one acting at high sulfide concentrations (1.6 mM), the other, which seems to need induction, working at lower sulfide concentrations (0.5 mM at the beginning of the second phase, Fig. 1.A; S2). A 2-phased oxidation of HS^- was observed also in cultures illuminated with less than 1 $\mu E\ m^{-2}\ sec^{-1}$ (Fig. S2). Based on genomics, transcriptomics, and mutational studies, Chan et al. (2009) hypothesised that 2 of the 3 sulfide:quinone reductases (SQRs) encoded in the genome of the GSB *Cla. tepidum* (genes *CT1087* and *CT0117*) are needed to oxidize high concentrations of HS^- ($\geq$ 4 mM), while the third SQR (gene *CT0876*) would function at low HS^- concentrations. The genome of strain DSM 263 encodes 3 SQRs whose aminoacid sequences are more than 90% identical to the SQRs encoded by the GSB *Cla. tepidum* (genes *Cpar_0875*, *Cpar_1010*, and *Cpar_0061*, homologues of *CT0876*, *CT1087*, and *CT0117* respectively). The two distinct phases of HS^- oxidation by strain DSM 263 might thus be attributed to the action of the gene products of: 1) *Cpar_1010* and *Cpar_0061* at the beginning of the batch incubation, when HS^- concentration is higher than 0.5 mM; 2) *Cpar_0875* when HS^- concentration drops to 0.5 mM. Mutational studies on strain DSM 263 and analysis of the transcripts of the 3 SQRs during the 2 phases of HS^- oxidation are however needed to draw definitive conclusions.

We show that the GSB *Cla. parvum* DSM 263 prefers to oxidize HS^- over $S_2O_3^{2-}$ and S^0. This pattern is consistent with the general characteristics of GSBs as described by Brune (1989). However, this preference for sulfide over thiosulfate and elemental sulfur differs to what results recently ascribed to the GSB *Cla. tepidum* (Holkenbrink et al., 2011).

Expression of the $S_2O_3^{2-}$ oxidizing system does not appear to increase after HS^- depletion. More likely it was already complete when HS^- was still present. This is supported by the observation that during $S_2O_3^{2-}$ consumption following HS^- depletion, the ratio between consumed $S_2O_3^{2-}$ and produced proteins increased at a constant rate (Fig. S3). We conclude that HS^- does not inhibit the expression of the $S_2O_3^{2-}$ oxidizing system in strain DSM 263. The observed preference for HS^- over $S_2O_3^{2-}$ may be due to the faster kinetics of HS^- oxidation, competition of HS^- with $S_2O_3^{2-}$ for the $S_2O_3^{2-}$ oxidizing system, or inhibition by HS^- of the activity of the $S_2O_3^{2-}$ oxidizing system.

The preferential oxidation of HS^- over S^0 might be consequence of expression inhibition of the S^0 oxidizing system by HS^-, $S_2O_3^{2-}$, or both. Elements needed for S^0 oxidation have to be synthesised after $S_2O_3^{2-}$ depletion, as shown by the 15-h lag-phase between $S_2O_3^{2-}$ depletion and S^0 oxidation. Thus, at least part of the S^0-oxidizing system is not constitutively expressed in cells of strain DSM 263. Activation of the S^0 oxidizing system by S^0 itself, although theoretically possible,

seems to be unlikely, because S^0 is present during the course of the whole experiment, and – due to its hydrophobic nature – tends to stick to cell membranes, thus being continuously available to bacteria. In conclusion, the preferential oxidation of HS^- over $S_2O_3^{2-}$ and S^0, which we observed in cultures of strain DSM 263 even in a day-long period, seems to be consequence of 1) fast HS^- oxidation, competitive interaction between $S_2O_3^{2-}$ and HS^- for the $S_2O_3^{2-}$-oxidizing system, or inhibition by HS^- of the activity of the $S_2O_3^{2-}$-oxidizing system; 2) inhibition of the expression of the S^0-oxidizing system by HS^-, $S_2O_3^{2-}$, or both.

2. Oxidation of thiosulfate

The currently accepted system for $S_2O_3^{2-}$ oxidation in GSBs is the Sox complex in the phototrophic variation (Frigaard & Dahl, 2008; Ghosh & Dam, 2009) (Fig. 4). This model predicts that phototrophic sulfur bacteria (GSBs and PSBs) oxidize $S_2O_3^{2-}$ to S^0 and SO_4^{2-}. Both S^0 and SO_4^{2-} should be produced in a ratio 1:1 with consumed $S_2O_3^{2-}$, according to the equation

$$2\ S_2O_3^{2-} + CO_2 + H_2O \rightarrow 2\ SO_4^{2-} + 2\ S^0 + [CH_2O] \qquad (A)$$

S^0 can be considered an intermediate in the complete oxidation of $S_2O_3^{2-}$ to SO_4^{2-}, since GSBs can oxidize S^0 to SO_4^{2-}:

$$2\ S^0 + 3\ CO_2 + 5\ H_2O \rightarrow 2\ SO_4^{2-} + 3\ [CH_2O] + 4\ H^+ \qquad (B)$$

Our aim was to understand if the Sox system is the only enzymatic complex responsible for $S_2O_3^{2-}$ oxidation in the GSB strain DSM 263, or if $S_2O_3^{2-}$ could be oxidized to SO_4^{2-} via an intermediate other than S^0:

$$S_2O_3^{2-} + 5\ H_2O \rightarrow [SX] \rightarrow 2\ SO_4^{2-} + 10\ H^+ + 8\ e^- \qquad (C)$$

Our strategy consisted in comparing the predictions of the Sox model (Fig. 4; reaction A) with the ratios between S^0, SO_4^{2-}, and $S_2O_3^{2-}$ detected during the oxidation of $S_2O_3^{2-}$ by strain DSM 263 grown at different light intensities.

The ratios between the amounts of S^0, SO_4^{2-}, and $S_2O_3^{2-}$ detected in a culture can be faithful representation of the stoichiometry of $S_2O_3^{2-}$ oxidation to S^0 and SO_4^{2-} by the Sox system (reaction A) only if S^0 is not oxidized to SO_4^{2-} at the same time (reaction B). An analysis of literature indicates that no general rule exists among GSBs about the preference for $S_2O_3^{2-}$ over S^0. The GSBs *Cla. thiosulphatiphilum* and *Cla. tepidum* do not necessarily release S^0 during $S_2O_3^{2-}$. They were interpreted to oxidize S^0 together with $S_2O_3^{2-}$ (Fischer, 1984; Chan et al., 2008b). In contrast, the GSB *Cla. parvum* has been reported not to consume S^0 until $S_2O_3^{2-}$ is available, releasing S^0-globules during $S_2O_3^{2-}$ oxidation (Steinmetz & Fischer, 1982). We detected a time-lag of about 15 hours between the depletion of $S_2O_3^{2-}$ and the consumption of S^0 by strain DSM 263, which suggests that S^0 oxidation needs novel transcription and translation of genes. Mobilization of

extracellular S^0 by a non-constitutive system would explain why S^0 always accumulated exponentially during $S_2O_3^{2-}$ oxidation and was the last inorganic sulfur compound to be depleted, as shown in this study and as reported by Steinmetz and Fischer (1982). As discussed in the previous section, the expression of the S^0 oxidizing system seems to be inhibited by HS^-, $S_2O_3^{2-}$, or both.

However, we found also that strain DSM 263 produced less S^0 and generally more SO_4^{2-} than what expected from $S_2O_3^{2-}$ oxidation by Sox (reaction A and Fig. 4, right panel). SO_4^{2-} was likely derived from photosynthetic oxidation of $S_2O_3^{2-}$, as indicated by the positive correlation between the rate of SO_4^{2-} produced per unit of biomass and the growth rate during $S_2O_3^{2-}$ oxidation. What can have happened is that part of S^0 produced by the Sox system was oxidized in the periplasm immediately after having being produced (reactions A and B). Candidates for S^0 oxidation in the periplasm are the heterodisulfide reductase complex and the Dsr system (Frigaard & Dahl, 2008). Holkenbrink et al. (2011), analysing several mutants of the GSB *Cla. tepidum*, showed that the Dsr system is indeed responsible for S^0 oxidation in the periplasm. The GSB *Cla. parvum*, in contrast to *Cla. tepidum*, lacks *dsrEFH* (Holkenbrink et al., 2011). This might explain the release of S^0 during $S_2O_3^{2-}$ oxidation to SO_4^{2-} by strain DSM 263, but not by *Cla. tepidum*. Strain DSM 263 might lack the capacity of oxidizing $S_2O_3^{2-}$ to SO_4^{2-} in the periplasm. In summary, we propose that S^0 produced by the action of the Sox system has two possible fates: 1) S^0 can be excreted, needing then an inducible system to be oxidized to SO_4^{2-}; 2) S^0 can be immediately oxidized SO_4^{2-} in the periplasm, by a periplasmic S^0 oxidation system acting in-line with the Sox complex and thus varying the observed stoichiometry of $S_2O_3^{2-}$ oxidation.

If S^0 is oxidized during $S_2O_3^{2-}$ oxidation, (reactions A and B at the same time), the observed stoichiometry will not reflect what is predicted by the Sox model (Fig. 4), regardless of whether alternative non-Sox enzymes for $S_2O_3^{2-}$ oxidation exist or not. As electrons deriving from the oxidation of sulfur compounds are used for photosynthetic light-dependent carbon fixation in GSBs, minimizing the photon flux should help to decipher which processes are involved in $S_2O_3^{2-}$ oxidation and SO_4^{2-} formation. We expect that in cultures of strain DSM 263 illuminated with low light intensities 1) S^0 produced from $S_2O_3^{2-}$ is only minimally consumed (reaction B); 2) possible intermediates in S^0 or $S_2O_3^{2-}$ oxidation to SO_4^{2-} accumulate (reaction B modified as $2\ S^0 + 3\ CO_2 + 5\ H_2O \rightarrow [SY] \rightarrow 2\ SO_4^{2-} + 3\ [CH_2O] + 4\ H^+$; reaction C). We observed that in cultures of strain DSM 263 illuminated with a light intensity lower than 1 $\mu E\ m^{-2}\ sec^{-1}$, for every mole of $S_2O_3^{2-}$ consumed, only 0.86 ± 0.09 moles of SO_4^{2-} were produced (Tab. 1). Such a deficit of SO_4^{2-} detected during $S_2O_3^{2-}$ oxidation to S^0 and SO_4^{2-}, suggests that $S_2O_3^{2-}$ is consumed not only by Sox. In fact, if no intermediate in the oxidation of $S_2O_3^{2-}$ to SO_4^{2-} by a hypothetical system alternative to Sox (reaction C) accumulated, the yield of SO_4^{2-} on $S_2O_3^{2-}$ would be equal to 1 – or higher, if S^0 were oxidized to SO_4^{2-} during $S_2O_3^{2-}$ consumption (reaction B). A S^0 yield on $S_2O_3^{2-}$ lower than 1 cannot

be interpreted univocally, since it might be the result of incomplete S^0 oxidation to SO_4^{2-} (reaction B modified as 2 S^0 + 3 CO_2 + 5 H_2O → [SY] → 2 SO_4^{2-} + 3 [CH_2O] + 4 H^+), of the action of a hypothetical $S_2O_3^{2-}$ oxidizing system different from Sox (reaction C), or of both. The observed deficits of S^0 and SO_4^{2-} during $S_2O_3^{2-}$ consumption cannot be completely justified by sulfur assimilation into biomass. We know that in cells of strain DSM 263 grown at 2 µE m^{-2} sec^{-1}, S-atoms are the 0.6% (wt/wt) of the dry biomass and the 1.9% (wt/wt) of the protein mass (experiment not described). Since at the lowest tested light intensity, 2.3 ± 0.4 g of proteins are formed for every mole of $S_2O_3^{2-}$ oxidized (Tab. 1), it can be estimated that less than 2 mmol of S-atoms were assimilated into biomass for every mole of consumed $S_2O_3^{2-}$. In conclusion, a light intensity lower than 1 µE m^{-2} sec^{-1} allowed the accumulation of unidentified product(s) of $S_2O_3^{2-}$ consumption, which might be attributed to the existence of a pathway for $S_2O_3^{2-}$ oxidation to SO_4^{2-} alternative to the Sox system (reaction C), or for the consumption of $S_2O_3^{2-}$ without SO_4^{2-} production (reaction C stopped at SX).

As an alternative to low light intensity, another way to facilitate the accumulation of intermediates in the oxidation of $S_2O_3^{2-}$ to SO_4^{2-} (reaction C) might consist in giving cells a large excess of substrate ($S_2O_3^{2-}$). When strain DSM 263 was fed with 4 mmol $S_2O_3^{2-}$ and cultivated at 25 µE m^{-2} sec^{-1}, for every mole of $S_2O_3^{2-}$ which was consumed, only 0.84 ± 0.03 mol of SO_4^{2-} were formed (Tab. 2). As for cultures grown at low light intensities, results obtained feeding strain DSM 263 with an excess of $S_2O_3^{2-}$ suggest the existence of a pathway for $S_2O_3^{2-}$ consumption alternative to Sox.

In cultures of strain DSM 263 fed with an excess of thiosulfate and not buffered by MOPS (Tab. 2, case A), yields of S^0 and SO_4^{2-} on $S_2O_3^{2-}$ were very close to the value predicted by the Sox model (Fig. 4; reaction A), even if cultivation was carried on at a lower light intensity (2 compared to 25 µE m^{-2} sec^{-1} of case B, Tab. 2). Since MOPS is not used by strain DSM 263 (data not shown), it might be that a pH constantly equal to 6.8 favours the accumulation of intermediates or products in the consumption of $S_2O_3^{2-}$ by a system different from Sox, which might mean that at pH 6.8 the system for $S_2O_3^{2-}$ consumption alternative to Sox works better than Sox.

In literature, additional pathways are reported for oxidation of $S_2O_3^{2-}$ by GSBs, but they do not seem to be active under the conditions described in this paper. The tetrathionate-pathway for $S_2O_3^{2-}$ oxidation (2 $S_4O_6^{2-}$ + 7 CO_2 + 13 H_2O → 8 SO_4^{2-} + 7 [CH_2O] + 12 H^+) was found to be active at saturating light intensities in *Cla. thiosulphatiphilum* (Larsen et al., 1952; Khanna & Nicholas, 1982). However, in experiments presented here, $S_4O_6^{2-}$ was not detected in amounts high enough to account for the unidentified compound. In fact, in the light-intensity experiments no $S_4O_6^{2-}$ was detected at the end of $S_2O_3^{2-}$ oxidation (detection limit: 1 µmol), while in cultures fed with excess of $S_2O_3^{2-}$ the amount of $S_4O_6^{2-}$ detected at the stationary phase was lower than 25 µmol.

In addition, strain DSM 263 was unable to grow on $S_4O_6^{2-}$ at a saturating light intensity (25 μE m^{-2} sec^{-1}, data not shown). An enzyme for long thought to be involved in $S_2O_3^{2-}$ consumption by GSBs is rhodanese, which would transfer 1 S-atom of $S_2O_3^{2-}$ to a nucleophilic compound. Steinmetz and Fischer (1985) reported the presence of two distinct enzymes with rhodanese activity in cell extracts of strain DSM 263. The action of rhodanese on $S_2O_3^{2-}$ would result in the production of SO_3^{2-}. However, strain DSM 263 was unable to grow on SO_3^{2-} (data not shown). The inability of strain DSM 263 to grow on SO_3^{2-} is in agreement with the current knowledge on the oxidizing capabilities of GSBs (Brune, 1989) and with the gene content of strain DSM 263, which is the only sequenced GSB to lack any putative system for SO_3^{2-} oxidation (*psr* or *sat-aprBA-qmoABC* genes) (Frigaard & Bryant, 2008a; Frigaard & Dahl, 2008).

The gene content of strain DSM 263 is somehow exceptional among the other GSBs whose genome is known, since it lacks *dsrEFH* (Holkenbrink et al., 2011) and a putative system for SO_3^{2-} oxidation (Frigaard & Bryant, 2008a; Frigaard & Dahl, 2008). Differences in the genomes might justify the different behaviours of strain DSM 263 and *Cla. tepidum* during $S_2O_3^{2-}$ oxidation (Holkenbrink et al., 2011). Analysis of the stoichiometry of $S_2O_3^{2-}$ oxidation in other $S_2O_3^{2-}$-oxidizing GSBs that have a complete Dsr system and a putative system for SO_3^{2-} oxidation might help to understand if *dsrEFH* and SO_3^{2-} are involved in the oxidation of $S_2O_3^{2-}$ via a system alternative to Sox (reaction C), or in the oxidation to SO_4^{2-} of the S^0 produced from $S_2O_3^{2-}$ by Sox (reaction B). A comparison with the behaviour of other $S_2O_3^{2-}$ oxidizing GSBs would clarify if strain DSM 263 oxidizes $S_2O_3^{2-}$ in an exceptional way or if it has a more widespread but yet not studied mechanism for $S_2O_3^{2-}$ consumption. Additional physiological experiments might help to understand whether the unidentified S-atoms are side products of $S_2O_3^{2-}$ consumption or intermediates of $S_2O_3^{2-}$ oxidation to SO_4^{2-}. Results presented here could not clarify if all the unidentified S-atoms present in cultures of strain DSM were produced only from $S_2O_3^{2-}$ oxidation or also from S^0 oxidation. Experiments conducted with the GSB *Cla. tepidum* suggest in fact the involvement of a not-yet characterized thiol (Hanson et al., 2010). Studies on S^0 oxidation seem thus to be necessary to fully understand the oxidation of $S_2O_3^{2-}$ by strain DSM 263.

Figures and Tables

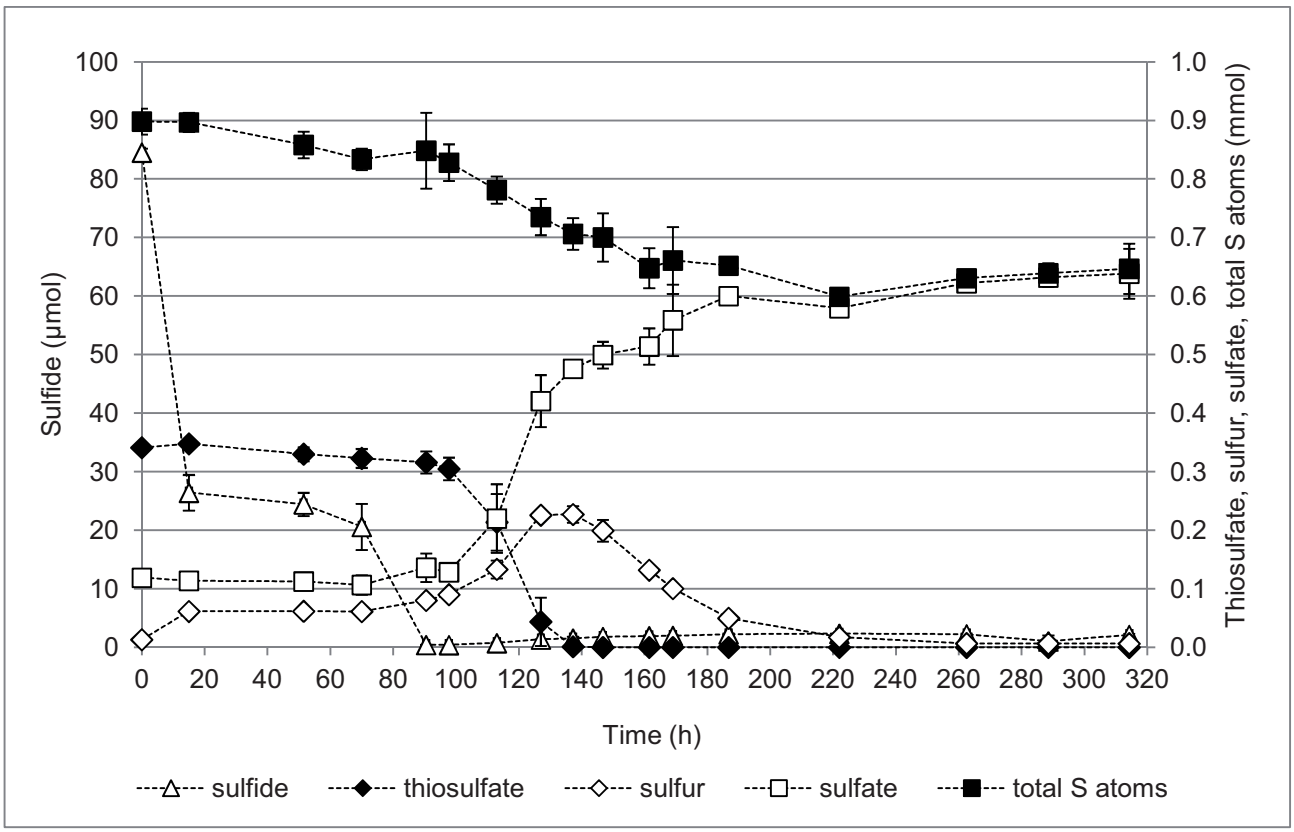

Fig. 1.A. Inorganic sulfur compounds dynamics during growth of *Cla. parvum* DSM 263 with sulfide and thiosulfate at pH 6.8, 28ºC, and 2 µE m^{-2} sec^{-1} in 50-mL vessels. Error bars represent the range of values between two biological replicates inoculated at the same time. Total S atoms were calculated summing the S-atoms present in sulfide, sulfur, thiosulfate, and sulfate.

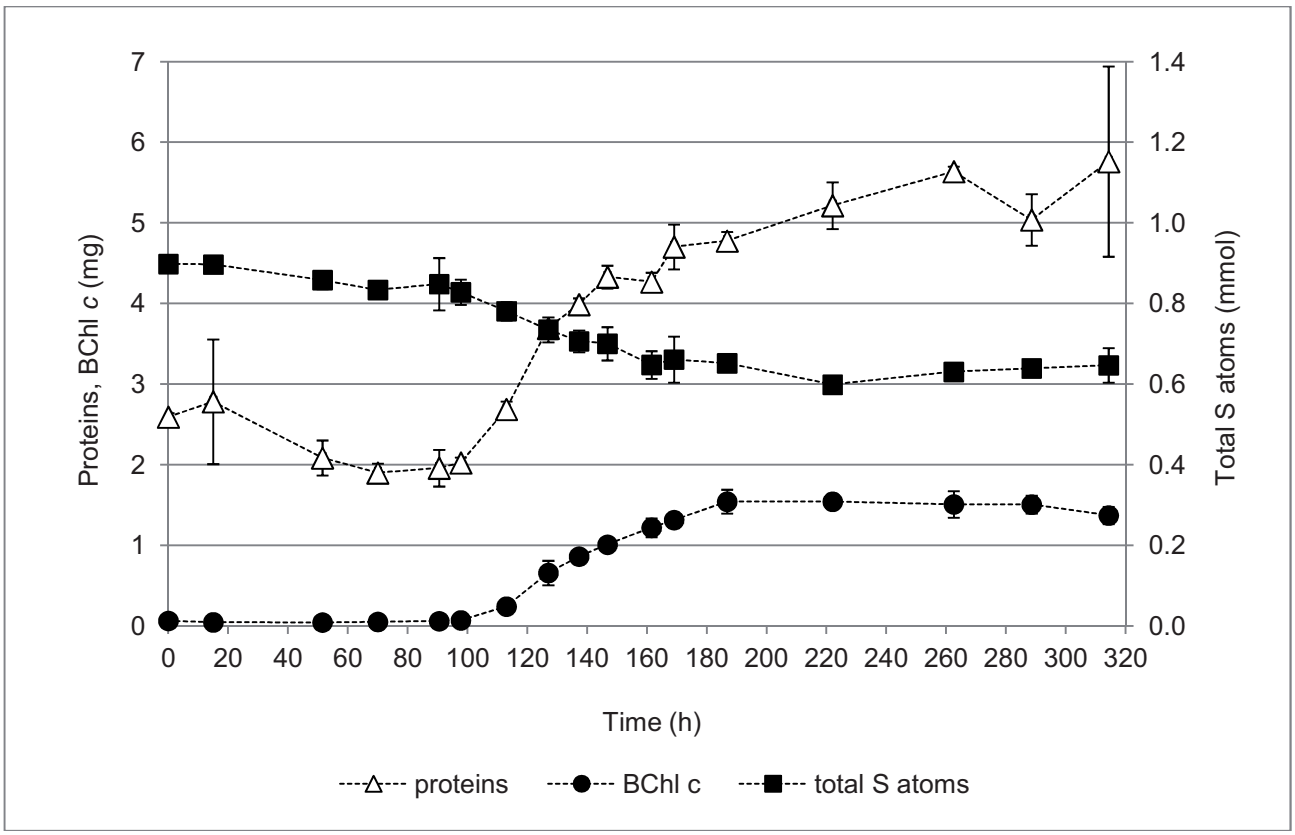

Fig. 1.B. Protein-, bacteriochlorophyll *c*-content, and total S-atoms during growth of *Cla. parvum* DSM 263 at pH 6.8, 28ºC, and 2 µE m^{-2} sec^{-1} in 50-mL vessels. Error bars represent the range of values between two biological replicates inoculated at the same time. Total S- atoms were calculated summing the S atoms present in sulfide, sulfur, thiosulfate, and sulfate.

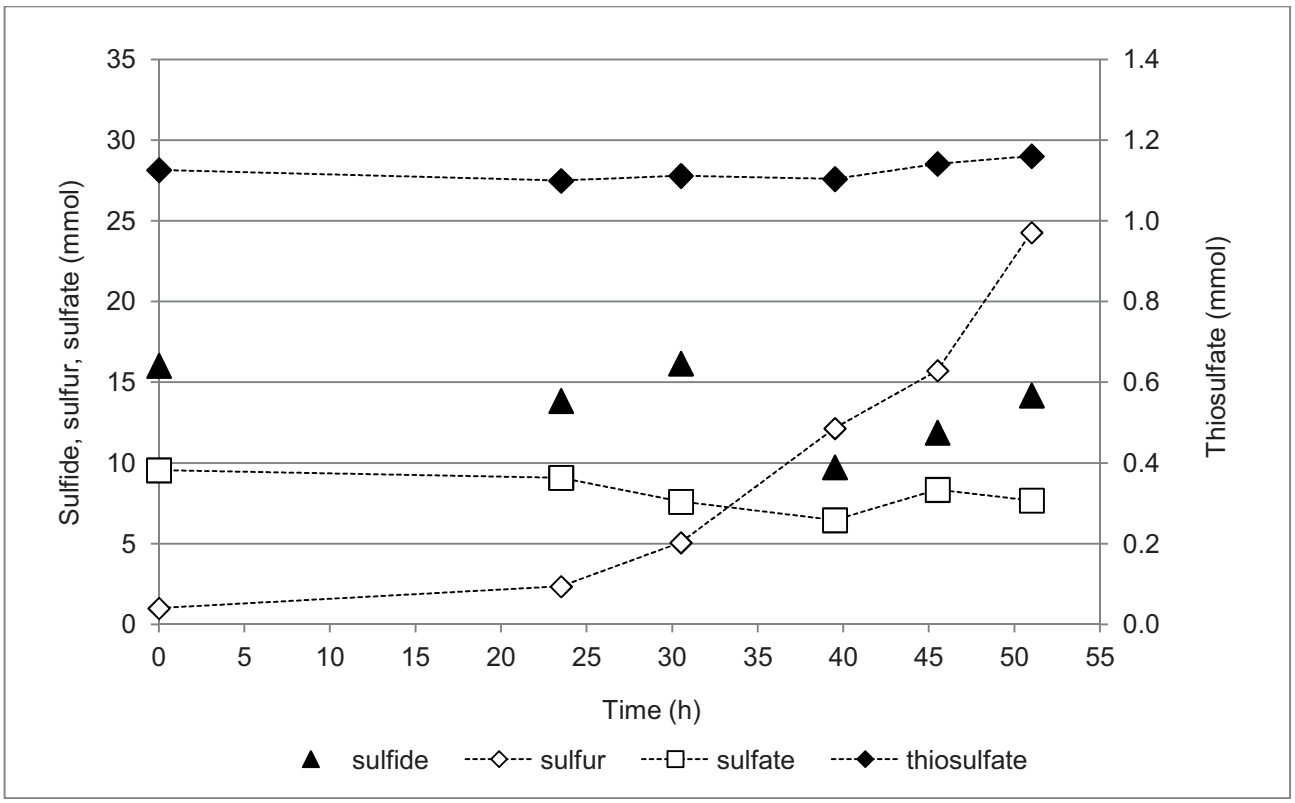

Fig. 2. Dynamics of inorganic sulfur compounds in a 5 L culture of *Cla. parvum* DSM 263 growing on sulfide at pH 6.8, 28ºC, and 2 µE m^{-2} sec^{-1}. Sulfide was added at certain times, in order to maintain sulfide concentration in the culture between 2 and 4 mM. A total of 30 mmol sulfide were added during the course of the experiment.

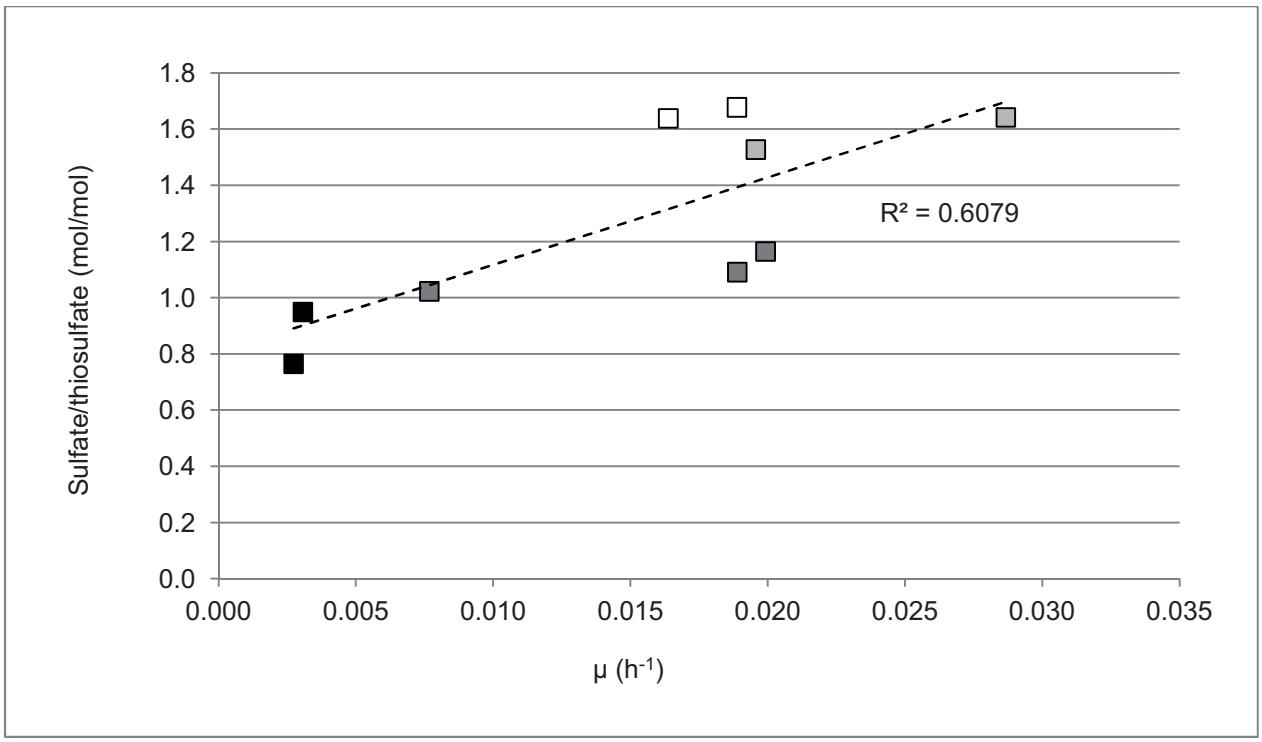

Fig. 3. Correlation between SO_4^{2-} yields on $S_2O_3^{2-}$ and growth rate (μ) during thiosulfate consumption, in cultures illuminated by different light intensities. Data are derived from 50-mL batch cultures of *Cla. parvum* DSM 263 grown at pH 6.8, 28ºC, and illuminated with light intensities from < 1 to 80 μE m^{-2} sec^{-1}. The colour of the squares indicates the light intensity at which the cultures were illuminated: black < 1 μE m^{-2} sec^{-1}; dark grey: 2 μE m^{-2} sec^{-1}; grey: 15 μE m^{-2} sec^{-1}; light grey: 25 μE m^{-2} sec^{-1}; white: 80 μE m^{-2} sec^{-1}.

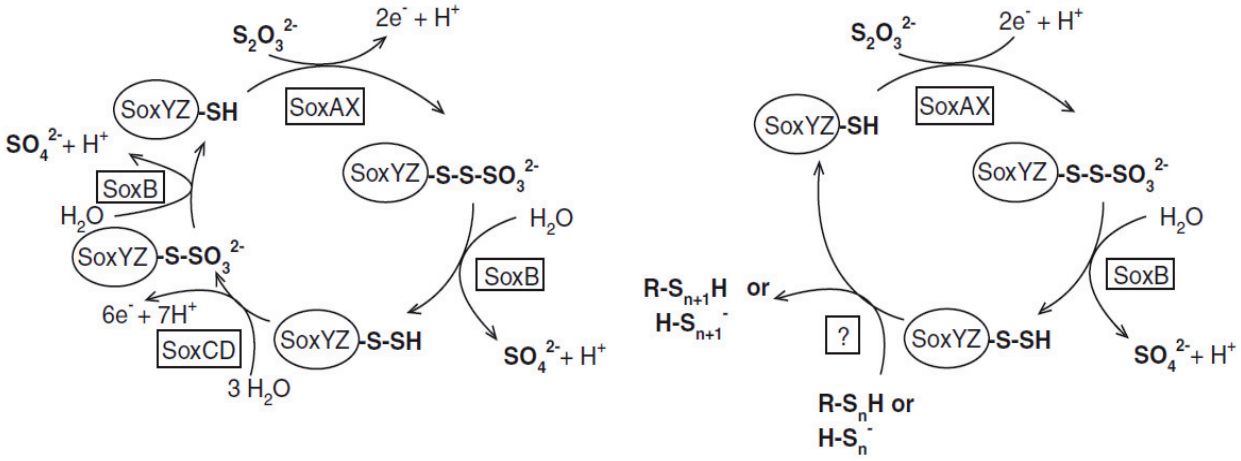

Fig. 4. Reproduced from Frigaard and Dahl (2008). Models of $S_2O_3^{2-}$ oxidation by the Sox system in organisms that possess SoxCD, like *P. pantotrophus* (left panel), and in organisms that lack SoxCD, like PSBs and GSBs (right panel). According to the proposed model, SoxYZ is the scaffold to which $S_2O_3^{2-}$ is bound. SoxAX catalyses the oxidative binding of a $S_2O_3^{2-}$ molecule to SoxYZ by releasing 2 electrons. The $S_2O_3^{2-}$ sulfone-sulfur is then hydrolysed by SoxB as SO_4^{2-}. In *P. pantotrophus*, SoxCD oxidizes the resulting S^0, recycling thus SoxYZ and transferring the last 6 electrons of the $S_2O_3^{2-}$ molecule. In PSBs and GSBs, which lack SoxCD, S^0 is instead an intermediate of $S_2O_3^{2-}$. It is not know how SoxYZ is recycled in PSBs and GSBs, or if S^0 released from SoxYZ forms inorganic polysulfides or is bound to an organic thiol.

Tab.1. Cultural parameters measured during thiosulfate oxidation by cultures of *Cla. parvum* DSM 263 grown at pH 6.8, 28° C, illuminated at different light intensities. Values indicated are moles of sulfur and sulfate, g of proteins and bacteriochlorophyll *c* produced per mole of oxidized thiosulfate. The last column shows bacterial growth rate (μ), based on protein content, during thiosulfate oxidation.

Light intensity ($\mu E\ m^{-2}\ sec^{-1}$)	$S^0 / S_2O_3^{2-}$	$SO_4^{2-} / S_2O_3^{2-}$	$(S^0+SO_4^{2-}) / S_2O_3^{2-}$	proteins / $S_2O_3^{2-}$	BChl c / $S_2O_3^{2-}$	μ ($10^{-3}\ h^{-1}$)
<1	0.67[a] ± 0.08	0.86[a] ± 0.09	1.53[a] ± 0.02	2.3[a] ± 0.4	1.8[a] ± 0.1	2.9[a] ± 0.2
2	0.57[b] ± 0.05	1.09[b] ± 0.07	1.66[b] ± 0.02	5.6[b] ± 1.5	2.4[b] ± 0.6	15[b] ± 7
15	0.42[a] ± 0.15	1.58[a] ± 0.06	2.00[a] ± 0.10	6.1[a] ± 1.6	3.3[a] ± 0.5	24[a] ± 5
25	0.46[c]	1.64[c]	2.10[c]	7.9[c]	3.5[c]	16[c]
80	0.36[c]	1.68[c]	2.03[c]	5.6[c]	2.0[c]	19[c]

[a] average of 2 values
[b] average of 3 values
[c] results of a single experiment

Tab. 2. Products of thiosulfate oxidation, detected in the stationary phase of 50-mL cultures of *Cla. parvum* DSM 263 grown at 28ºC, illuminated with light intensities of 2 or 25 μE m^{-2} sec^{-1}. The cultures were fed with an excess of thiosulfate (45-50 mM for cultures illuminated with 2 μE m^{-2} sec^{-1}, 70-85 mM for those illuminated with 25 μE m^{-2} sec^{-1}). Cultures illuminated with 2 μE m^{-2} sec^{-1} were not buffered with MOPS. Values indicate the average and range of variation between two biological replicates.

	without MOPS	with MOPS
Light intensity (μE m^{-2} sec^{-1})	2 (case A)	25 (case B)
Initial thiosulfate amount (mmol)	2.5 ± 0.1	4.0 ± 0.2
$S_2O_3^{2-}$ consumed (mmol)	1.98 ± 0.02	2.43 ± 0.03
SO_4^{2-} produced (mmol)	1.97 ± 0.07	2.04 ± 0.09
S^0 produced (mmol)	1.76 ± 0.01	0.92 ± 0.09
Protein content (mg)	5.5 ± 0.3	13.62 ± 0.09
BChl *c* (mg)	3.4 ± 0.3	2.5 ± 0.4
$SO_4^{2-}/S_2O_3^{2-}$	0.99 ± 0.05	0.84 ± 0.03
$S^0/S_2O_3^{2-}$	0.89 ± 0.01	0.38 ± 0.03

Supplementary material

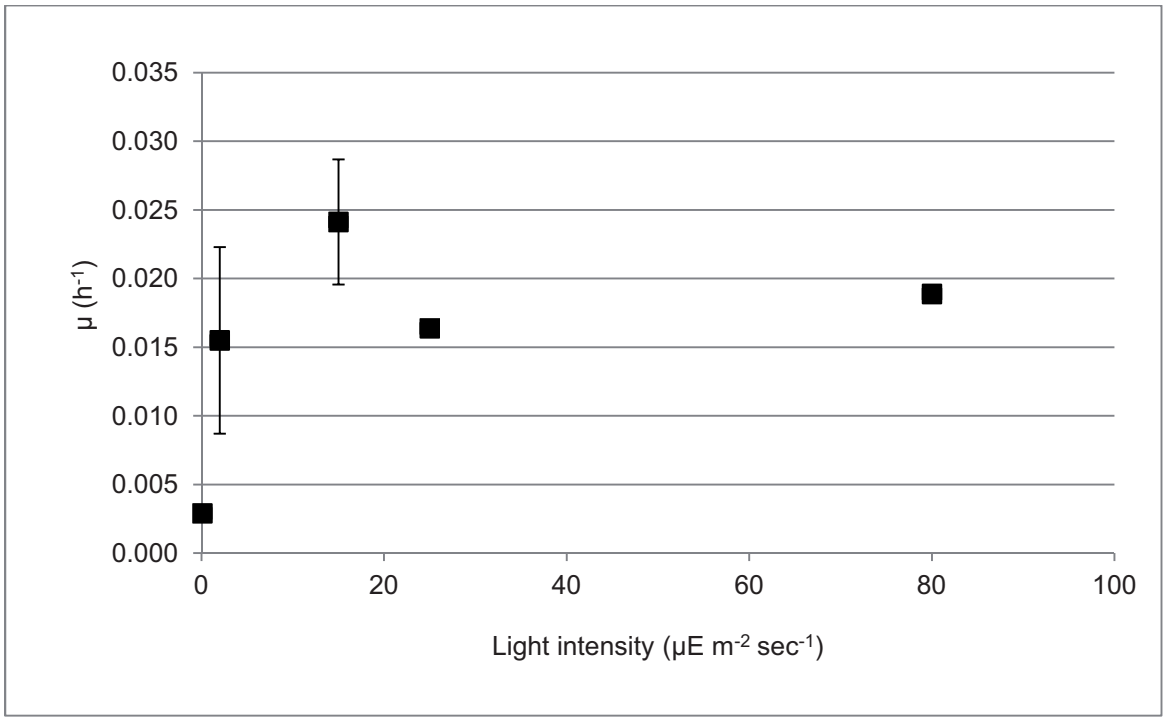

Fig. S1. Growth rate (μ) of 50-mL batch cultures of *Clb. parvum* DSM 263 incubated at pH 6.8, 28ºC, and illuminated with light intensities ranging from less than 1 to 80 μE m^{-2} sec^{-1}, in dependence on light intensity. Error bars represent the range of values between two (light intensity lower than 1 μE m^{-2} sec^{-1}) or the standard deviation of three (2 μE m^{-2} sec^{-1}) biological replicates inoculated at the same time.

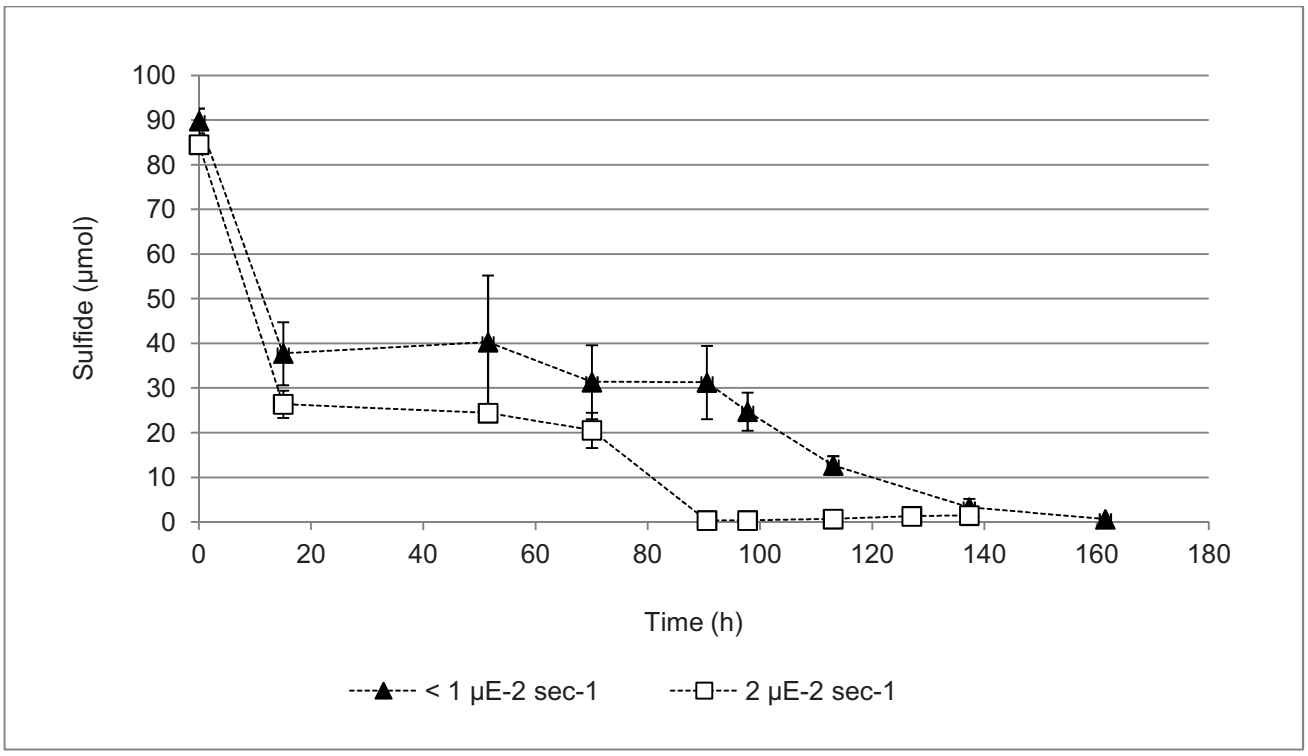

Fig. S2. Sulfide oxidation in 50-mL batch cultures of *Clb. parvum* DSM 263 incubated at pH 6.8, 28ºC, and illuminated with light intensities ≤ 2 µE m^{-2} sec^{-1}. Error bars represent the range of values between two biological replicates inoculated at the same time. The protein content during the represented period of time was constant and equal to 1.0 ± 0.1 mg for cultures illuminated with less than 1 µE m^{-2} sec^{-1}, 2.3 ± 0.4 mg for cultures illuminated with 2 µE m^{-2} sec^{-1}.

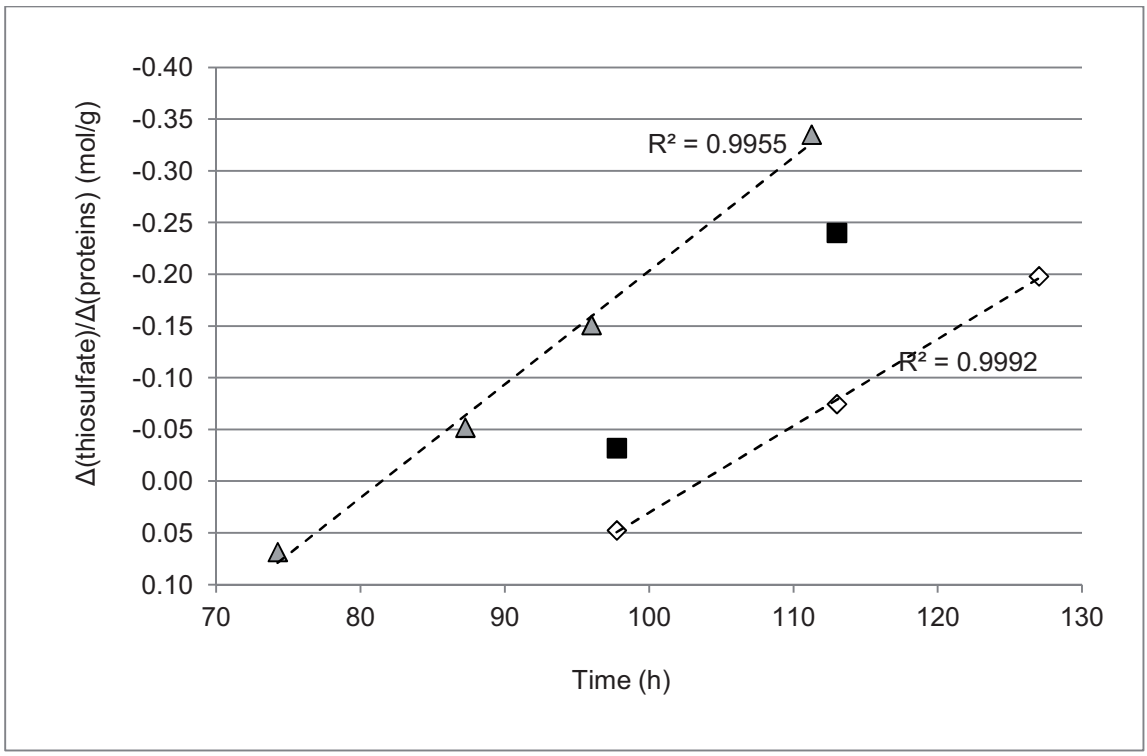

Fig. S3. Thiosulfate consumption (Δ(thiosulfate)) relative to protein production (Δ(proteins)) calculated at time intervals during $S_2O_3^{2-}$ oxidation by 50-mL batch cultures of *Cla. parvum* strain DSM 263 incubated at pH 6.8, 28°C, and illuminated with 2 µE m^{-2} sec^{-1}. Different symbols represent different biological replicates.

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– Chapter 3 –

ELEMENTAL SULFUR MOBILIZATION BY THE GREEN SULFUR BACTERIUM *CHLOROBACULUM PARVUM* DSM 263

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Abstract

Green sulfur bacteria are anaerobic anoxygenic photolithoautotrophs that couple the fixation of CO_2 to the oxidation of inorganic sulfur compounds or, in the case of *Chlorobium ferrooxidans*, ferrous iron. When sulfide or thiosulfate is the electron donor, elemental sulfur (S^0) is an intermediate product, which can be further oxidized to sulfate. S^0 is thus a key substrate, whose insolubility in water constitutes a special task for the living cells. In the present study, cells of the green sulfur bacterium *Chlorobaculum parvum* DSM 263 were shown to need a contact with sulfur in order to mobilize it. Cultures of *Cla. parvum* DSM 263 were then subjected to differential membrane proteomic analysis. Two proteins, a ferredoxin and a putative signal transduction protein with NTPase activity, were found only under sulfur-oxidizing conditions. Other 5 proteins were overexpressed under sulfuric conditions. Even if they could not be identified or better characterized in the present study, they indicate that sulfur oxidation is accompanied by the production of specific proteins, which are not expressed when bacteria oxidize sulfide. The contact between cells and sulfur might trigger the production of excreted proteins or membrane proteins, which in turn would allow the cells to use electrons from sulfur.

Introduction

Green sulfur bacteria (GSBs) are photolithotrophs that use inorganic sulfur compounds as electron donors for photosynthetic CO_2 fixation. Sulfur globules are the intermediate product of the oxidation of sulfide to sulfate. The chemical nature of sulfur produced by photosynthetic bacteria has not been completely clarified yet. Sulfur globules produced by phototrophs were claimed to be polymeric (Prange et al., 2002) or to consist of cyclooctasulfur (Pickering et al., 2001; George et al., 2008), depending on the interpretation of spectra obtained with XANES (X-ray absorption near edge structure). In any case, at the temperature at which mesophilic GSBs grow (28°C), sulfur is practically insoluble (the solubility of cyclooctasulfur is in the order of 20 nM. Kamyshny, 2009). Sulfur mobilization represents thus a special task for the bacteria.

Sulfur could be chemically attacked and solubilised by sulfide (A) or sulfite (B) (Brune, 1995; Hinsley and Berks, 2002):

$$n/8\ S_8 + HS^- \leftrightarrow S_n^{2-} + H^+ \qquad (A)$$

$$n/8\ S_8 + SO_3^{2-} \rightarrow S_2O_3^{2-} \qquad (B)$$

Sulfite and sulfide could be already present in the natural environment or in the medium, or they could be formed by GSBs. Recently, thiols were detected during the oxidation of sulfur by *Chlorobaculum tepidum*, and were proposed to act as electron-shuttles between insoluble sulfur and cells (Hanson et al., 2010). Instead, thiols detected in cultures of the purple sulfur bacterium *Allochromatium vinosum* could not be specifically associated to sulfur oxidation (Franz et al, 2009). Whether sulfur is mobilized by organic or inorganic sulfur molecules, cells would not access directly to the electrons released from the oxidation of sulfur to sulfate. A yet unexplored possibility in GSBs is the direct transfer of electrons between sulfur and cells, which would likely occur via membrane proteins.

GSBs offer the possibility to study the proteins differentially expressed under sulfide- or sulfur-oxidizing conditions. Cork et al. (1985) showed that a strain known as *Chlorobium limicola* f. *thiosulfatophilum* ATCC 17092 oxidizes sulfur even in the presence of low concentrations of sulfide (0.5 mM), but at higher sulfide concentrations sulfur is not consumed (Brune, 1989). It is thus possible to differentiate populations that oxidize sulfide from those oxidizing sulfur, provided that the sulfide concentration is maintained at least above 0.5 mM.

In the present work, we investigated the direct involvement of bacterial cells in the mobilization of sulfur, and subsequently the membrane proteins involved in this process in the mesophilic GSB *Chlorobaculum parvum* DSM 263, whose genome is available (Lucas et al, 2008).

Materials and methods

Cultural conditions

Chlorobaculum parvum strain DSM 263 (Imhoff 2003) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany).

Cultivation was carried out in anoxic Pfennig's medium (10 g/L NaCl, 0.50 g/L $MgSO_4$, 0.25 g/L $CaCl_2 \cdot 2\ H_2O$, 0.34 g/L KH_2PO_4, 0.34 g/L NH_4Cl, 0.34 g/L KCl, 1.5 g/L $NaHCO_3$, 1 mL/L vitamin B_{12} solution (0.002% in H_2O), 1 mL/L trace element solution SL-10B) supplemented with ascorbic acid (4 mM) as oxygen scavenger. Trace element solution SL-10B contained: 7.7 mL/L HCl 25%, 1.5 g/L $FeSO_4 \cdot 7\ H_2O$, 70 mg/L $ZnCl_2$, 100 mg/L $MnCl_2 \cdot 4\ H_2O$, 300 mg/L H_3BO_3, 190 mg/L $CoCl_2 \cdot 6\ H_2O$, 2 mg/L $CuCl_2 \cdot 2\ H_2O$, 24 mg/L $NiCl_2 \cdot 6\ H_2O$, 36 mg/L $Na_2MoO_4 \cdot 2\ H_2O$). Cultures were incubated at 28°C, illuminated by neon light (Biolux L18W/72 Osram, Munich, Germany), and continuously stirred at 240 rpm. Light intensity on the surface of the 50 mL or 5 L bottles used for cultivation was 25 $\mu E\ m^{-2}\ sec^{-1}$.

The dried culture obtained from DSMZ was revitalized in anoxic medium supplemented with thiosulfate (10 mM) and sulfide (2 mM). In order to ensure that the culture used in the experiments was pure, a single colony was isolated by agar-shaking in anoxic inorganic medium supplemented with thiosulfate (10 mM) and sodium sulfide (2 mM) as electron donors and with 0.1% $CaCl_2$ and 2.4% agar, according to the procedure described by Trüper (1970). The isolated colony was then grown in the same medium devoid of agar. Aliquots of the amplified isolate were used to prepare glycerol stocks (20% glycerol final concentration), which were stored at – 80°C until used. Sequencing of the PCR-amplified 16S rRNA gene confirmed that the isolate was *Clb. parvum* DSM 263.

Preparation for the investigation on the contact between cells and sulfur

For studies on cell-sulfur contact, biogenic sulfur (prepared as described in the section below) was embedded into 1.5% agar and brought in contact with a living culture of *Cla. parvum* DSM 263. Tubes (15 mL) containing cells and agar-embedded sulfur were incubated for several days at 30° C, continuously illuminated by 25 $\mu E\ m^{-2}\ sec^{-1}$. The appearance of cleared zones at the border between cell culture and agar was observed.

Chemically-made sulfur was prepared by polysulfides oxidation, according to Moser and Nealson (1996): a polysulfide solution made by boiling together commercial sulfur and sulfide, was shaken overnight in air to allow sulfide dispersal and sulfur precipitation. The obtained chemically-made sulfur was then washed twice in sterile water.

Growing conditions for proteomics

Cells of strain DSM 263 to be used for proteomics were grown in 5 L batch cultures. Precultures (50 mL) were cultivated with thiosulfate (10 mM) and sulfide (2 mM) as electron donors. One 50-mL preculture was used to inoculate two 5 L-vessels, which constituted a biological replicate for the comparison between growth on sulfide and on zero-valent sulfur. Three biological replicates were analysed for the experiment.

To maintain the pH constantly at 6.8 during the cultivation, the medium was supplemented with 50 mM filter-sterilized MOPS (3-(N-morpholino)propanesulfonic acid, Sigma Aldrich, St. Louis, MO, USA). In one of the biological replicates grown on biogenic sulfur, the medium was instead supplemented with 50 mM filter-sterilized Bis-Tris (2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol, Sigma-Aldrich), pH 6.8.

Sulfide was prepared from washed crystals of $Na_2S \cdot 9\ H_2O$. Filter-sterilized sulfide (0.1-1 mM final concentration) was added to the culture at the beginning and during growth to keep the concentration of sulfide between 3 and 5 mM.

Biogenic sulfur was prepared from a culture of strain DSM 263 grown on sulfide (2 mM) and excess of thiosulfate (40 – 50 mM). Cells and sulfur were harvested by centrifugation at 17,000 *g* for 10 min at 4°C (rotor JA-10, Beckman Coulter, Brea, CA, USA). Cells were then separated from sulfur via centrifugation in 2.5 M sucrose at 2,000 *g* for 20 min at 10°C (rotor JA-20, Beckman Coulter). After this treatment, cells remained mainly in the upper phase, while sulfur was all dispersed in the sucrose phase or could be found as pellet at the bottom of the centrifugation tube. The upper phase was discarded and the sucrose phase was mixed with an equal amount of milliQ water. Subsequent centrifugation at 2,000 *g* for 20 min at 10°C allowed the pelleting of sulfur, while cells could be found on the surface of the pellet as well as in the supernatant. The whole procedure was repeated 2-3 times. Biogenic sulfur was finally washed three times with milliQ water, pelleted by centrifugation at 10,000 *g* for 5 min (rotor F2402H, Beckman Coulter), pasteurized at 80°C for 45 min, and stored at -80°C until use.

The prepared sulfur was added to the 5 L vessel at a concentration of 0.4 g/L.

Analysis of cultural parameters

Consumption or production of inorganic sulfur compounds and growth were monitored during cultivation. Samples (0.8 mL) were withdrawn from the culture at different intervals of time, using N_2-flushed syringes and injecting an equal volume of N_2 to maintain a slight overpressure and avoid O_2 penetration into the culture vessel.

The difference between the optical density at 750 nm and at 830 nm was used to monitor growth (Garcia-Gil & Abella 1986). Additional samples were fixed in 4 volumes ZnAc 2% and used for the

analysis of sulfide, zero-valent sulfur (S^0), thiosulfate, sulfate, bacteriochlorophyll *c*, and protein content. Sulfide concentration was determined immediately, according to Cline (1969). The remaining part of the fixed samples was stored at -80ºC until further processing.

Zero-valent sulfur (S^0), thiosulfate, and sulfate were quantified by HPLC using a Merck-Hitachi device equipped with an intelligent pump (L-6220), an autosampler (AS-2000A), an oven (L-7350), a UV/VIS- (L-4250), and a conductivity-detector (L-3730).

For S^0 quantification, fixed samples were diluted in HPLC-grade methanol, incubated overnight at 4ºC and subsequently filtered (0.2 µm). S^0 was analysed using a LiChrospher 100 RP-18 column (125 x 4 mm, 5 µm; Merck, Darmstadt, Germany). Running conditions were set according to Zopfi et al. (2001): 1 mL/min of 100% HPLC-grade methanol, detection at 265 nm. Temperature was kept constant at 30ºC.

Thiosulfate and sulfate were quantified from fixed samples after filtration (0.2 µm), using a modification of the method described by Miura & Kawaoi (2000): separation was achieved by a LiChrospher 60 RP-select B column (125 x 4 mm, 5 µm; Merck), eluting with 10 mM tetrapropylammonium bromide (Fluka) in 10% HPLC-grade acetonitrile pH 5, and a flow rate of 0.8 ml/min. Temperature was kept constant at 30ºC. Thiosulfate was detected at 230 nm, sulfate by conductivity.

Bacteriochlorophyll was extracted by mixing one part of fixed sample with four parts of 100% methanol. After incubation for 4-5 hours at 4ºC in the dark, samples were centrifuged at full speed for 5 min in a benchtop microcentrifuge (Biofuge pico Heraeus, Hanau, Germany). Absorbance of the supernatant was measured at 670 nm against a blank of 80% methanol. Bacteriochlorophyll *c* concentration was determined using the absorbance coefficient of 86.0 cm L g^{-1}, according to Stal et al. (1984).

Protein extraction

When the culture was in the early log-phase ($OD_{750\text{-}830}$ of 0.7-0.8), the 5 L bottle remained standing unstirred for 5-10 minutes, allowing sulfur to precipitate, thus separating from bacteria. Then, cells were harvested by centrifugation at 17,000 *g* for 15 min at 4ºC (rotor JA-10, Beckman Coulter), washed three times in anoxic ice-cold PBS (NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4 2.9 g/L, KH_2PO_4 0.2 g/L, pH 7.0) supplemented with 5 mM PMSF (polymethylsulfonil fluoride), and stored at -80ºC in aliquots of 0.5 g until further utilization. Lysis was conducted essentially according to Frigaard et al. (2005): 0.5 g of cells were resuspended in 4 mL of lysis buffer (50 mM Tris HCl, 10 mM ascorbic acid, 1 mM DTT (dithiothreitol), 2 mM PMSF, pH 8.0) and passed through a cooled French press three times at 18,000 psi. Unbroken cells, cell debris, and sulfur were separated by centrifugation at 10,000 *g* for 5 min at 4ºC (benchtop centrifuge 5415R Eppendorf, Hamburg,

Germany). The supernatant was then ultracentrifuged at 70,000 rpm for 30 min at 4ºC (rotor TLA-110, Beckman Coulter) to separate the soluble fraction from membranes. In order to separate the membrane-associated protein fraction, membranes were washed with lysis buffer supplemented with 0.5 M NaCl, pelleted again by centrifugation at 40,000 rpm for 15 min at 4ºC (rotor TLA-110, Beckman Coulter), and resuspended in lysis buffer supplemented with 4% SDS. Proteins were precipitated overnight at -20ºC in five volumes of 0.1 M ammonium acetate in methanol, harvested by centrifugation at full speed for 15 min at 4º C (benchtop centrifuge 5810R, Eppendorf), washed three times in the precipitation solution, resuspended in protein buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% ampholytes 3-10 (BioRad, Hercules, CA, USA)), and stored at -80ºC until further processing. Urea and thiourea used in the sample buffer had been treated previously with the mixed-bed ion exchanger Amberlite IRN-150 (Fluka), as indicated by Westermeier et al. (2008). Protein concentration was determined using the RC DC protein assay kit from BioRad.

2-dimensional-gel-electrophoresis

The first dimension was isoelectric focusing (IEF), carried out essentially as described by Oetjen et al. (2009) and Hurek et al. (1995) with 20 mM NaOH as cathode solution and 10 mM phosphoric acid as anode solution. Gel rods were polymerized in 230 x 2.5 (inner diameter) mm glass tubes, with an actual length of the gel of 170 mm. Gel solution contained 3.8% acrylamide/bisacrylamide (30:1), 9 M urea, 2 % CHAPS (3-(3-(Cholamidopropyl)dimethylammonio)-1-proanesulfonate), 3.0% ampholytes 3-10, 1.5% ampholytes 4-6, 1.5% ampholytes 5-8 (Serva, Heidelberg, Germany), 0.05 % ammonium persulfate, and 0.1% TEMED. Once polymerized, gel rods were overlaid with sample buffer and overlay buffer (6 M urea, 0.4% ampholytes 3-10 (BioRad), 100 mM DTT, 2% CHAPS) and prefocused 10 min at 100 V, 30 min at 200 V, 1 h at 300 V, and 2 h at 400 V. Before application on top of the gel, samples were sonicated 5 times for 10 sec with 30 sec intervals on ice and afterwards centrifuged at full speed for 2 min at 4ºC (rotor F45-30-11 5417R, Eppendorf) to eliminate any particle. 500 µg protein extracts were applied to each gel and overlaid with overlay buffer. Focusing conditions were 20 min at 100 V, 20 min at 200 V, 4 h at 300 V, and 14 h at 400 V. Gel rods were then stored at -20ºC for a maximum of 1 week until the second dimension was run.

The second dimension was a vertical SDS-PAGE in 12.5% acrylamide according to Laemmli (1970) but without stacking gel (Westermeier et al. 2008). Before SDS-PAGE, extruded gel rods were equilibrated for 30 min in a buffer containing 6 M urea, 20% glycerol, 60 mM Tris HCl pH 8.8, 1% SDS, 50 mM DTT, and bromophenol blue. Electrophoresis was conducted for 1 h at 40 V, followed by 12-14 h at 70 V.

Gels were stained in Colloidal Coomassie Brilliant Blue G250, as indicated in Westermeier et al. (2008).

Gel images were acquired using a scanner Epson Perfection V750Pro (Epson, Nagano, Japan) and the imaging software SilverFast Ai (LaserSoft Imaging AG, Kiel, Germany). Spot patterns were analysed with ImageMaster 2D 4.01 (GE Healthcare, Muenchen, Germany).

Peptide Mass Fingerprint analysis

Trypsin (sequencing grade; Roche, Mannheim, Germany) digests for peptide-mass-fingerprinting were obtained from selected spots following the procedure indicated by Shevchenko et al (2006). Trypsin digests were obtained from 3 different gels for each spot and each growth condition in which the spot was detected.

Dried peptides were resuspended in ACN / TFA 0.1% (1:1) and mixed with α-Cyano-4-hydroxycinnamic acid (CHCA) (1:10 of a saturated solution in 75% ACN/TFA 0.1%) onto the spectrometer target. Dried spots were subsequently washed twice with $NH_4H_2PO_4$ (10 mM) / TFA (0.1%) and recristallized with ethanol:acetone: 0.1% TFA (6:3:1). Spectra were obtained using a MALDI/TOF AutoflexII (Bruker Daltonics, Bremen, Germany), summing at least 5,000 shots per spectrum, and were analysed using Mascot Distiller 2.3.2 (Matrix Science, London, UK). Masses obtained were compared with the annotated genome of *Chlorobaculum parvum* DSM 263 (database UniProtKB/TrEMBL) using the software Aldente (ExPASy Proteomics Server, Swiss Institute of Bioinformatics, Geneve, Switzerland).

The software that was used to assign a protein identity to a mass spectrum (Aldente) associates a score to each assignment. Such a score allows distinguishing between unspecific and reliable assignments. In the present study, a protein was considered identified if the peptide mass fingerprinting (PMF) analysis of all replicates of its spots scored higher than the limit fixed by Aldente, and if the detected peptide masses could cover more than the 30% of the protein.

Cell localization of proteins was calculated using the software PSORTb v.3.0 (Brinkman Laboratory, Simon Fraser University, British Columbia, Canada).

Results and Discussion

Contact between cells and sulfur.

For the investigation of the contact cell-sulfur, biogenic sulfur produced from *Cla. parvum* DSM 263 was used. Even if it cannot be excluded that biogenic sulfur was altered during purification, such sulfur formed a homogeneous suspension in the medium and was anyway considered to be closer to the sulfur produced by GSBs than the colloidal sulfur prepared from

polysulfides (Moser and Nealson, 1996) or by acidification of thiosulfate (Roy and Trudinger, 1970), or than the commercial crystalline "sulfur flower". As can be seen from Fig. 1A, living cells were unable to solubilise agar-embedded biogenic sulfur, since no clear zone appeared at the interface between cells and agar.

For a comparison, in Fig. 1B it is visible what happened when the sulfur was prepared from polysulfides, as described by Moser and Nealson (1996). It is likely that the sulfur used for the agar-sulfur preparation visible in Fig. 1B contained sulfide, which attacked chemically sulfur, solubilising it. Moser and Nealson (1996) report images of the facultative anaerobic sulfur-reducer *Shewanella putrefaciens* grown inside agar plates. As indicated by the clear zone visible around the colonies (Fig. 2 by Moser and Nealson, 1996), sulfur was solubilised even if not in contact with cells of *S. putrefaciens*.

Results shown in Fig. 1A indicate instead that *Cla. parvum* DSM 263 needs to be in contact with sulfur to utilise it.A similar conclusion was reached by using a dialysing membrane to separate sulfur and cells of the same *Cla. parvum* DSM 263 (Borkenstein, 2006), or of the purple sulfur bacterium *Alc. vinosum* (Franz et al., 2007).

A contact between cells and sulfur might be necessary because electrons are transferred directly from sulfur to a cellular acceptor *via* structures such as spinae (scenario A), as observed with the electron microscope in several GSBs (Brooke et al., 1992 and 1995; Pibernat and Abella, 1996), or *via* one or more outer-membrane proteins. Alternatively, cell contact with sulfur would be necessary to activate the release of a soluble electron shuttle (scenario B). The electron shuttle could be an organic thiol, such as the thiol detected by Hanson and coworkers (2010) in cultures of *Cla. tepidum*. If a direct electron transfer takes place between sulfur and bacteria (scenario A), the contact between cells and sulfur should probably be quite stable, while if the contact with sulfur was needed only to activate the release of an electron shuttle (scenario B), a less stable mechanism of attachment would probably be sufficient. In Chapter 2 (pp. 55-56) and in the following paragraph of the present chapter it is concluded that *Cla. parvum* overexpresses some proteins (membrane proteins) when growing on sulfur. It could be reasonably speculated that in scenario A (direct electron transfer) to be overexpressed during growth on sulfur are the outer membrane proteins involved in sulfur oxidation. Conversely, it might be speculated that if sulfur was an activator (scenario B), the compound acting as sulfur-sensor would not necessarily need to be overexpressed after cell contact with sulfur; the elements to be overexpressed in the membrane would rather be the components involved in the increased production of the electron shuttle and, if the electron shuttle cannot pass the membrane, the "docking component" or the transporter of the electron shuttle.

Differentiation of populations growing on sulfur or on sulfide

Bacteria oxidizing sulfide to sulfur did not consume sulfur, as can be seen in Fig. 2. Small amounts of thiosulfate were present, but not used, as indicated by the constant concentration of sulfate (Fig.2), a product of thiosulfate oxidation. Steinmetz and Fischer (1982) showed that in cultures of *Cla. parvum* DSM 263 (former *Chlorobium vibrioforme* f. *thiosulphatophilum*) thiosulfate was produced during sulfide oxidation. However, in the case reported in Fig. 2 thiosulfate did not increased, even if sulfide oxidation continued during the whole experiment. As shown in Fig. 2, thiosulfate was present since the beginning of the experiment, and remained more or less constant during time, similarly to what Steinmetz and Fischer (1981) documented for a culture of *Chl. limicola* 6330. Presence of thiosulfate in cultures of *Chl. limicola* 6330 was attributed to chemical reaction between sulfite and sulfur (Steinmetz and Fischer, 1981). The presence of thiosulfate can be explained by chemical reactions even in the case described here. Small amounts of oxygen might have been present at the beginning of the experiment in the cultivation medium. Reaction between sulfide and oxygen produces sulfite, which in turn can react with oxygen or with sulfur to form thiosulfate (Zopfi et al., 2004).

Since cells growing on high sulfide concentrations do not use sulfur, it is possible to cultivate *Cla. parvum* DSM 263 only on sulfide. Thus, it is possible to perform differential proteomics between sulfide- and sulfur-oxidizing populations, despite the presence of sulfur in both types of culture.

Differential proteomics

Gels of membrane proteins from populations grown on sulfide or sulfur are shown in Fig. 4. As visible, more protein material was loaded onto the gels that displayed extracts from populations grown on sulfide (from now on called sulfide-gels); nevertheless, some spots present in the gels loaded with extracts from populations grown on sulfur (from now on called sulfur-gels) are weaker or not detectable in the sulfide-gels. Tab. 1 summarizes the average normalized volumes (ANVs) and the standard deviations of spots detected for each condition in the majority of gels. Spot 299 was used as internal standard to calibrate finely the amount of proteins loaded onto each gel, and is thus presented with a normalized volume of 100.

Ten spots were detected only in the sulfur-gels included in the analysis. However, 8 of these 10 spots had a standard deviation higher than the 60% of their ANV. The analysis of additional 4 sulfide-gels, which were prepared to optimise the protocol used for the present analysis but were not included in the calculation of ANVs, revealed that those 8 spots were not exclusive of sulfur grown-populations. Spot 164 had an ANV equal to 25% of the reference spot and standard deviations 44% of its ANV. It was detected only in sulfur-gels. An additional spot, number 136, had an ANV equal

to 19% of the reference spot, and standard deviation 26% of its ANV. However, its presence cannot be excluded in 1 of the sulfide-gels (Fig. 4, framed panels). In general, the inability of detecting a spot might mean that the expression of the relative protein is below the detection limit, rather than being totally absent. This is probably the case of spot 136, which was detected as weak signal in a sulfide-gel that had been loaded with higher amounts of proteins than the sulfur-gel. Thus, spot 136 was also considered differentially expressed, and was further subjected to peptide-mass-fingerprinting, as was spot 164. The protein identities associated to spots 136, 164, and 207 are presented in Tab. 2.

A number of 75 differently located spots were detected in both sulfide- and sulfur-gels. The ANV of each spot of sulfur-gels was divided by the ANV of the corresponding spot present in sulfide-gels. The obtained ratio represents the spot X-folds-expression under sulfuric respect to sulfidic conditions. The reciprocal of the so-calculated ratios gave the spot X-folds-expression under sulfidic respect to sulfuric conditions. X-folds-expressions higher than 1.0 were called overexpressions and are shown in Fig. 3, in which each plotted data-point is a protein spot detected in both sulfur- and sulfide-gels. The expression of the majority of the protein spots did not vary between sulfuric- and sulfidic-growing conditions, as indicated by the clouds of data points mainly located below the limit of 2-folds overexpression (Fig. 3). Only 5 protein spots were more than 2-folds overexpressed: the ANVs of spots 155, 207, 214, 225, and 269 were more than 2-fold higher in sulfur-gels than in sulfide-gels. These 5 spots were further subjected to peptide-mass-fingerprinting. The obtained results are summarized in Tab. 2.

The high volume variability of some spots might reflect variability in the expression by cells: i.e. some other, non-identified factors than the electron donors used might control the expression or modification of some protein. Alternatively, high spot volume variability might be due to non controllable processes happening during protein extraction, precipitation, solubilisation, electrophoresis, or during gel staining. The higher variability in the volume of two spots might be also produced by the presence of 2 proteins in the same spot, one or both of which are differentially expressed.

The seven overexpressed protein-spots all derived from populations of *Cla. parvum* DSM 263 grown on sulfur. Overexpression or exclusive expression of proteins under sulfur-oxidizing conditions might indicate that sulfur oxidation needs synthesis of novel proteins respect to those present in cells oxidizing sulfide. This is in agreement with results presented in Chapter 2 of the present thesis (p. 54), where it is reported that S^0 is always the last compund to be consumed, and that a 15 h-lag phase exists between the depletion of thiosulfate and the consumpion of S^0. In Chapter 2 it is then concluded that S^0 oxidation needs translation and transcription of novel genes (pp. 55-56).

It is also possible that proteins found to be overexpressed under sulfuric conditions were instead present in the biogenic sulfur used as substrate for the sulfur-oxidizing cultures. However, sulfur was discarded together with cell debris during membrane proteins extraction, and it is probably more likely that some proteins were lost because hydrophobically associated with sulfur, rather than been added to the protein extract. A 2-dimensional gel electrophoresis of extracts from the sulfur used to feed bacteria would help clarifying such a doubt.

Only 3 out of 7 proteins could be considered identified by peptide mass fingerprinting (PMF) analysis, according to the definition given in the section Material and Methods. While one of them could not be assigned to any cell compartment, the other 2 are classified as cytoplasmic. These 2 proteins might effectively be cytoplasmic proteins that ended in the membrane fraction because they formed complexes with membrane proteins or lipids, or because they aggregated and were pelletted during ultracentrifugation. The 2 proteins identified as cytoplasmic might also be membrane proteins assigned to the wrong compartment by the localization software used (PSORTb v.3.0). While no function could be assigned to the protein corresponding to spot 207, it might be speculated that the other 2 proteins, which were detected only in sulfur-oxidizing populations, could be involved in the transfer of electrons from sulfur (protein corresponding to spot 164) and in the transfer of regulatory signals (protein corresponding to spot 136).

The relative failure of the spots identification might be due to insufficient quality of the obtained mass spectra, which might be caused by low amount and purity of material obtained from the gel. A second reason that would prevent spots identification could be the incompleteness of the database against which the peptide-mass search was performed (in this case, UniProtKB/TrEMBL). Additionally, the target protein could be absent from the protein database derived from the genome annotation. Improvement of the quality of spectra by cleaning the peptide extracts, search performed against an additional database (e.g. NCBI), and in particular search performed against the whole translated genome rather then against the genome segments identified as “protein coding” might be successful strategies for identifying the remaining peptides.

Conclusions

Results here presented allow to conclude that cells of *Cla. parvum* DSM 263 need to be in contact with sulfur (S_8) in order to mobilize S^0. The present work could thus attribute the mobilization of sulfur to the action of cells of *Cla. parvum* DSM 263, rather than to a mere chemical attack by a compound present in the medium (e.g. sulfide, or sulfite). Cells membranes could be identified as the key compartment for S_8 mobilization.

Seven protein spots visualized by 2-dimensional gel electrophoresis were found to be overexpressed or exclusively expressed when *Cla. parvum* DSM oxidized sulfur. Such an evidence is in agreement with the conclusion reached in Chapter 2 (p. 55), which states that *Cla. parvum* DSM 263 growing on thiosulfate or sulfide does not express all the components needed for sulfur oxidation. The identification of these proteins or even of fragments of them will open the way to the identification abd description of the genes and the pathways responsible for sulfur mobilization.

We finally propose two alternative models for sulfur mobilization:

a) The electron transfer between cells and sulfur takes place at the membrane/sulfur interface. The proteins overexpressed are those responsible for sulfur adhesion and oxidation.

b) The contact between cells and sulfur triggers the release of "electron shuttles" that mobilize sulfur. The proteins to be overexpressed are the proteins responsible for the production maybe the docking/transport of the electron shuttle.

Further characterization of the overexpressed proteins detected in this study will help elucidating which is the mechanism used by *Cla. parvum* DSM 263.

Figures and Tables

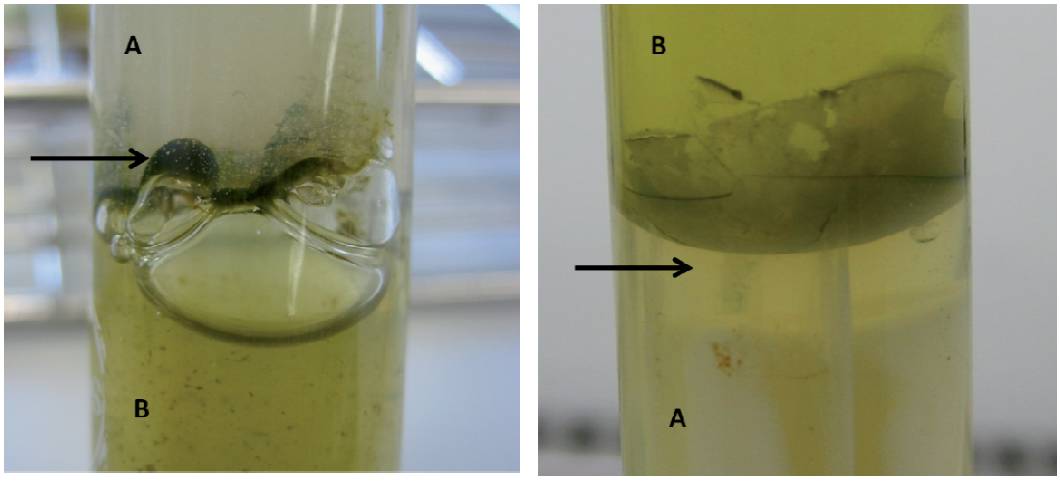


Fig. 1: Cultures of *Cla. parvum* DSM 263 (B) in contact with agarose containing biogenic sulfur (A, left panel) or sulfur produced chemically by polysulfides oxidation (A, right panel). Photos were taken after 2 weeks of incubation of cultures and agar-sulfur. The culture tube shown in the left panel was put upside-down only to take the photo, and the bubble that is visible below the agar (below A, left panel) is formed by headspace gas (N_2 and CO_2). As indicated by the arrows, bacteria are attached to agar containing biogenic sulfur, without any visible clear zone in the agar (arrow, left panel). A clear zone would look like that one indicated by the arrow of the right panel, which shows agar that contained sulfur formed by polysulfide oxidation.

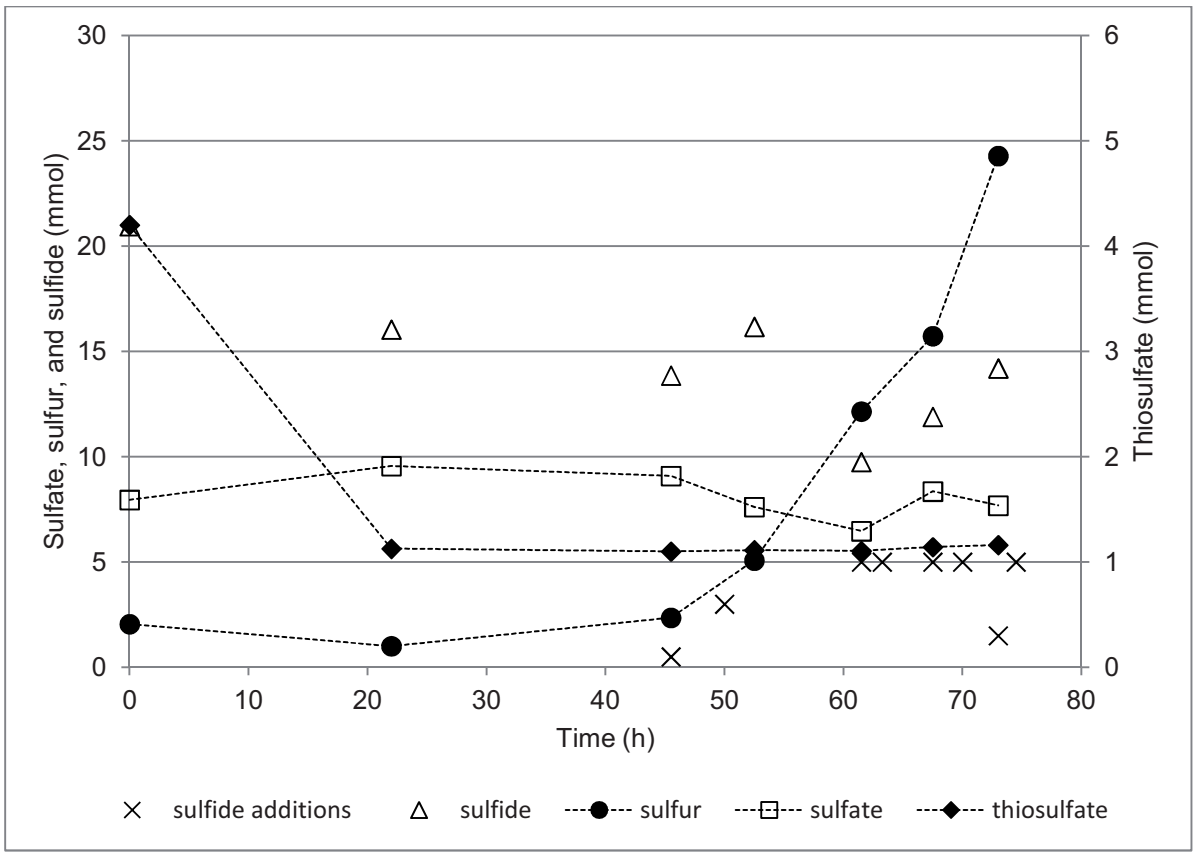


Fig. 2: Dynamics of inorganic sulfur compounds measured in a 5 L culture of *Clb. parvum* DSM 263 growing on sulfur at pH 6.8, 28ºC, and 25 µE m^{-2} sec^{-1} irradiance. Sulfide (0.5-5 mmol) was added at certain times (crosses) in order to maintain sulfide concentrations in the culture between 2 and 4 mM (corresponding to 10-20 mmol sulfide amount in the 5 L bottle used for cultivation). A total of 30 mmol sulfide was added during the course of the experiment.

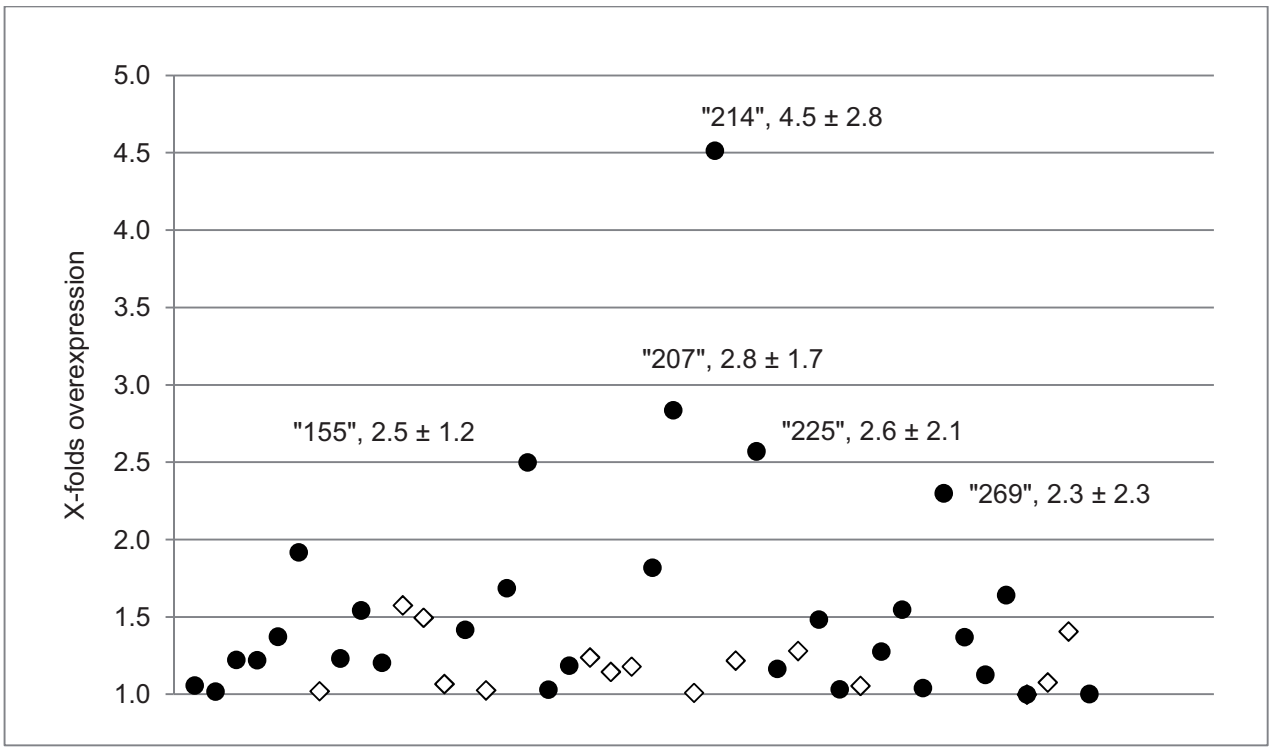
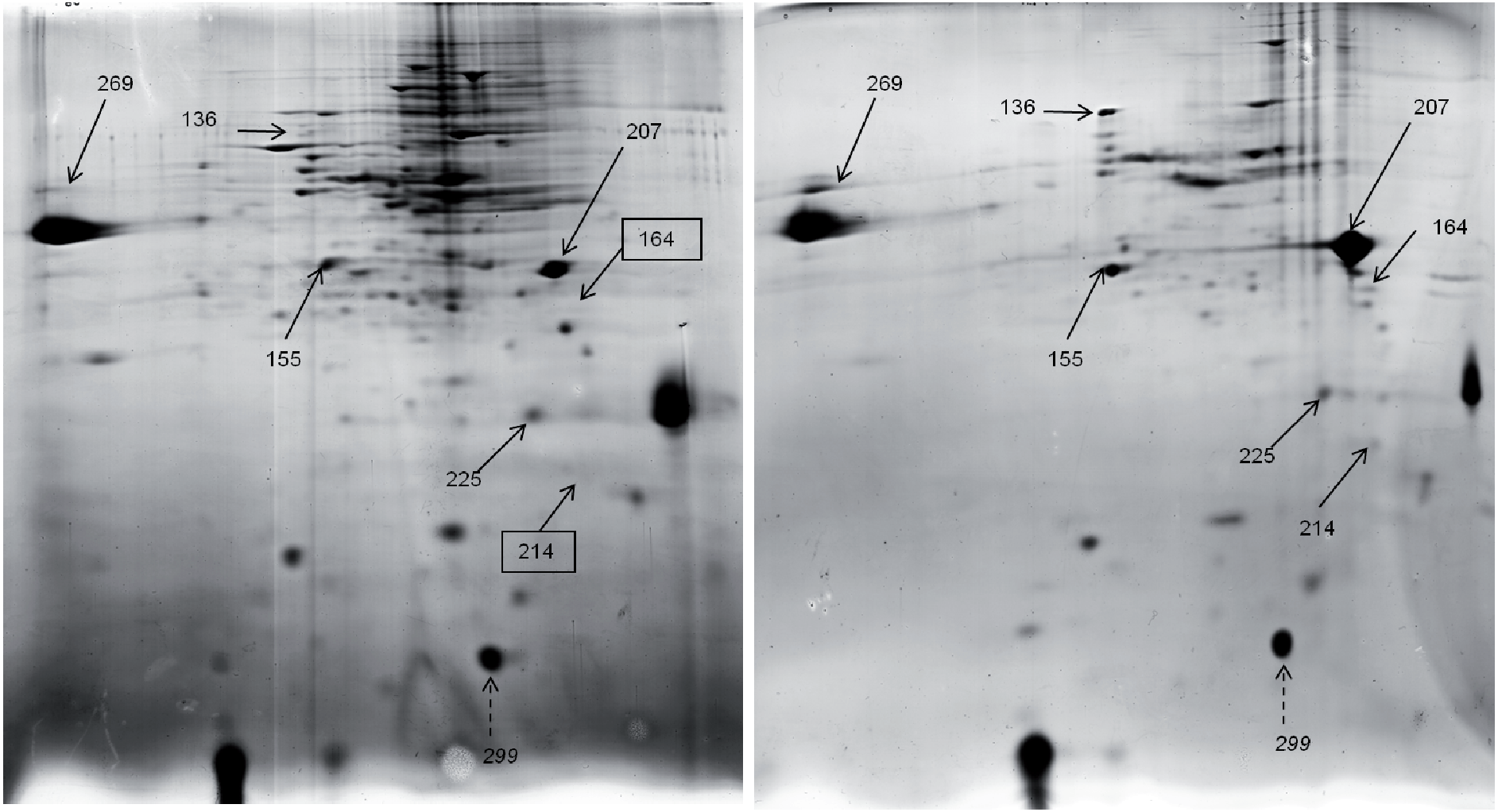


Fig. 3: Protein-spots detected in both sulfur- and sulfide-oxidizing cultures of *Cla. parvum* DSM 263 are represented here in terms of average increased expression under sulfur- (black dots) or sulfide-oxidizing conditions (white diamonds). Spots whose expressions were more than doubled are labelled with their identification number and with the value of their average overexpression plus/minus their standard deviation.


269
136
207
164
155
225
214
299
269
136
207
164
155
225
214
299

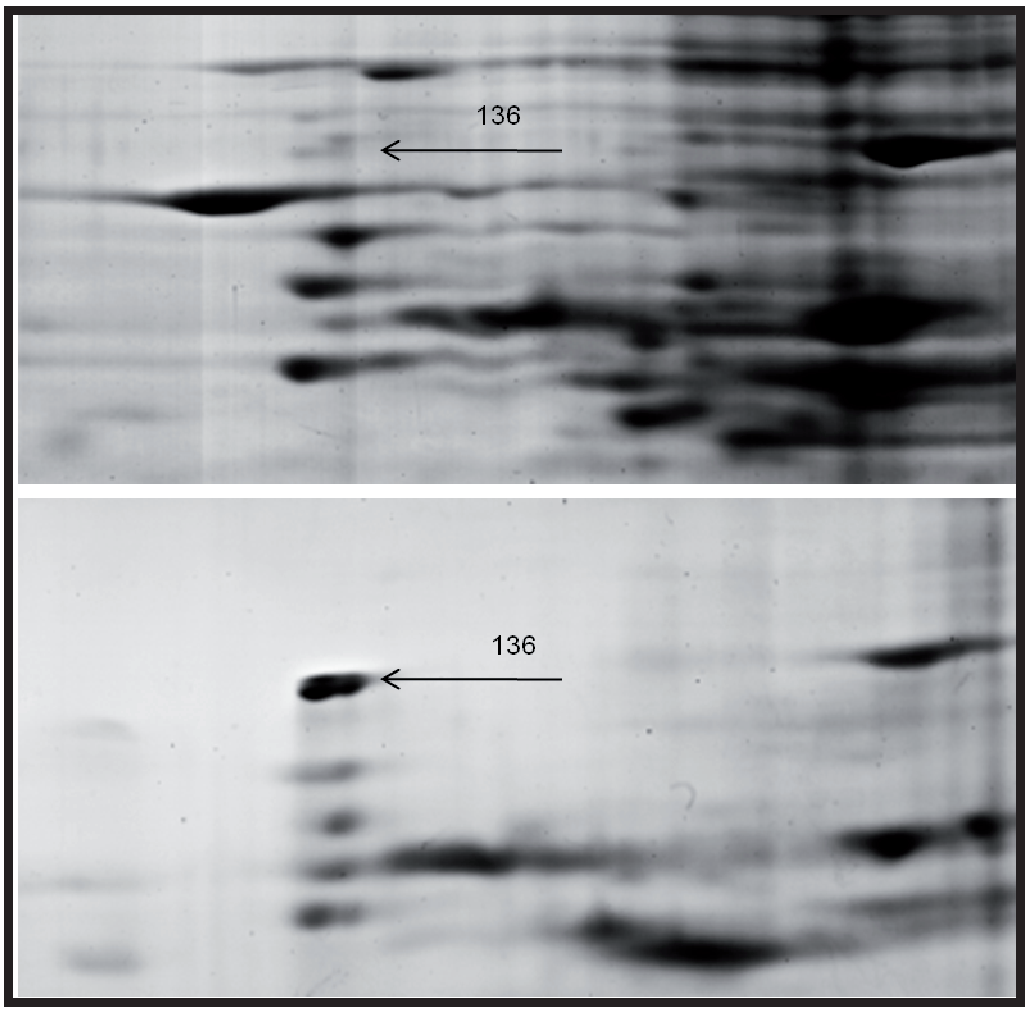


Fig. 4: Comparison between 2-dimensional gels patterns of membrane proteins from a population of *Cla. parvum* DSM 263 grown on sulfide (whole gel, left panel) or sulfur (whole gel, right panel). The dashed arrow (299) indicates the reference protein-spot used for internal calibration. The other arrows and the relative numbers indicate protein-spots that were more than 2-folds overexpressed. Framed numbers indicate absence of the protein-spot. Spot 214, absent in this sulfide-gel (left panel), was instead present in the other 2 replicates of sulfide-gels. Spot 136, whose presence cannot be excluded in this sulfide-gel (left panel), was instead absent from the other replicates of sulfide-gels. In the lower, bordered frame it is visible a detail of the 2 gels (sulfide-gel on the top, sulfur-gel on the bottom), which shows the position of spot 136.

Tab. 1: Summary of the average normalized intensities and standard deviations of spots detected in the majority of gels loaded with extracts from sulfur-oxidizing and sulfide-oxidizing populations of *Cla. parvum* DSM 263.

Spot ID	Sulfur	Sulfide
6	28 ± 9	27 ± 8
8	3 ± 5	12 ± 4
11	2 ± 2	*absent*
15	26 ± 6	25 ± 2
22	40 ± 10	33 ± 21
35	2 ± 4	*absent*
36	5 ± 7	8 ± 17
37	6 ± 6	5 ± 2
41	32 ± 10	26 ± 11
46	11 ± 9	17 ± 11
92	59 ± 56	94 ± 25
97	57 ± 34	41 ± 16
100	17 ± 3	9 ± 8
101	15 ± 10	15 ± 3
102	17 ± 3	2 ± 5
103	9 ± 5	2 ± 4
104	9 ± 11	8 ± 5
105	14 ± 5	12 ± 5
107	5 ± 5	*absent*
109	11 ± 7	7 ± 3
114	16 ± 7	13 ± 8
120	11 ± 3	17 ± 5
125	25 ± 10	37 ± 22
128	20 ± 12	21 ± 9
131	7 ± 7	4 ± 4
136	25 ± 11	*absent*
138	13 ± 4	11 ± 11
140	13 ± 5	9 ± 6
143	18 ± 5	19 ± 8
147	36 ± 6	22 ± 3
155	42 ± 12	17 ± 6
156	62 ± 6	60 ± 27
157	41 ± 11	35 ± 11
161	25 ± 18	31 ± 11
163	19 ± 5	21 ± 7
164	19 ± 5	*absent*
173	12 ± 10	8 ± 6
174	12 ± 20	35 ± 51
184	24 ± 15	29 ± 10
185	25 ± 19	14 ± 4
186	7 ± 7	5 ± 3
187	12 ± 13	3 ± 3
192	30 ± 12	3 ± 7

Spot ID	Sulfur	Sulfide
201	14 ± 14	4 ± 3
203	11 ± 16	7 ± 4
207	277 ± 68	97 ± 54
210	14 ± 4	14 ± 5
214	45 ± 16	10 ± 5
220	22 ± 15	*absent*
221	34 ± 27	22 ± 18
222	16 ± 8	20 ± 6
225	47 ± 16	18 ± 13
226	24 ± 22	14 ± 30
233	38 ± 37	50 ± 54
236	19 ± 11	16 ± 9
237	27 ± 16	*absent*
241	22 ± 3	33 ± 34
242	18 ± 3	24 ± 4
243	30 ± 9	20 ± 3
245	26 ± 8	25 ± 7
258	15 ± 11	10 ± 7
259	180 ± 13	190 ± 26
263	28 ± 7	22 ± 9
264	29 ± 11	18 ± 8
268	317 ± 40	305 ± 162
269	66 ± 53	29 ± 10
272	13 ± 12	*absent*
275	34 ± 21	57 ± 39
277	21 ± 14	17 ± 14
278	17 ± 24	3 ± 7
281	3 ± 5	4 ± 8
282	4 ± 5	6 ± 9
287	11 ± 10	12 ± 5
288	34 ± 14	25 ± 2
289	27 ± 3	24 ± 9
295	10 ± 10	*absent*
296	8 ± 8	2 ± 5
297	7 ± 11	6 ± 4
298	15 ± 6	9 ± 1
299	**100**	**100**
302	24 ± 8	25 ± 20
303	44 ± 18	61 ± 15
304	18 ± 11	9 ± 10
305	70 ± 66	*absent*
306	327 ± 95	326 ± 92

Tab. 2: Peptide-mass fingerprinting analysis of the selected protein-spots obtained from cells of *Cla. parvum* DSM 263 grown under sulfuric or sulfidic conditions.

Spot ID	Protein name	Reference	MW (Kda)	pI	Cell localization
155	n.d.				
207	conserved hypothetical protein	YP_001999240.1	42	4.6	unknown
214	n.d.				
225	n.d.				
269	n.d.				
136	putative signal transduction protein with NACHT domain	YP_001999268.1	120	6.0	cytoplasmic
164	4Fe-4S ferredoxin iron-sulfur binding domain protein	YP_001998113.1	31	5.6	cytoplasmic

n.d. : not determined.

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– Chapter 4 –

SULFUR OXIDATION BY THE GREEN SULFUR BACTERIUM *CHLOROBACULUM PARVUM* DSM 263

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Introduction

Sulfur-oxidizing green sulfur bacteria (GSBs) are photolithotrophs that produce and consume elemental sulfur during the oxidation of sulfide and thiosulfate to sulfate (Frigaard and Dahl, 2009).

Sulfur produced by bacteria has been recognized to be particularly hydrophilic respect to cyclooctasulfur (S_8) (Dahl and Prange, 2006 and references therein). Biogenic sulfur has been investigated by Pickering et al (1998 and 2001), Prange et al (2002), and George et al (2008). These research groups used XANES (X-ray absorption near edge spectroscopy) to analyse the sulfur produced by the purple sulfur bacterium *Allochromatium vinosum*, and obtained substantially comparable spectra, but interpreted them differently. The variations observed in the spectra of biogenic sulfur produced by different bacteria, respect to spectra obtained from S_8, were attributed by Prange et al (2002) to the presence of organic residues on sulfur. Instead, George et al (2008) attributed those variations to artefacts caused by the dimensions of the sulfur particles analyzed. The green sulfur bacterium strain analyzed with XANES by Prange et al (2002), which is the same strain used in the present study, was concluded to produce mainly polymeric sulfur, with organic residues bound or complexed to it. Whatever the nature of sulfur globules produced by GSBs is, the difference between polymeric sulfur and S_8 might be crucial, as it is for *Chlorobaculum tepidum*, which can grow on biogenic sulfur, but cannot use S_8 (TE Hanson, personal communication).

Sulfur mobilization by GSBs has been already investigated by Borkenstein (2006), who analyzed in particular the production of sulfide, spinae, and biosurfactants in batch cultures of *Chlorobaculum parvum* DSM 263 (former *Prosthecochloris vibrioforme*) fed with commercial sulfur (S_8). He did not detect surfactants or spinae (Brooke et al, 1992 and 1995), but sulfide, whose presence was interpreted as an evidence of sulfur mobilization *via* reduction to sulfide (Borkenstein, 2006, and references therein). In the present study, we used the sulfide-sink FeOOH (iron oxyhydroxide) to test whether sulfide which might be present during sulfur oxidation to sulfate is the compound actually used by bacteria. Additionally, we investigated the consumption of biogenic sulfur by *Cla. parvum* DSM 263.

Material and Methods

Chlorobaculum parvum DSM 263 (Imhoff, 2003) was obtained as dried culture from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), and treated as described in Chapters 2 and 3.

Cultivation was carried out in inorganic medium, prepared as described in Chapters 2 and 3 but without MOPS (3-(N-morpholino)propanesulfonic acid). 50-mL batch cultures were incubated at 28°C, continuously stirred, and illuminated with 2 or 25 μE m^{-2} sec^{-1} furnished by neon light tubes (Biolux L18W/72 Osram, Munich, Germany).

Biogenic sulfur was prepared as described in Chapter 3, and added to the cultivation medium in a concentration of 0.9 g/L. Before being used in the iron-hydroxide experiment, commercial sulfur flower (Riedl-de-Haën), was pasteurised at 80°C overnight.

Amorphic Fe(III) oxyhydroxide was prepared according to Lovely and Phyllips (1986), bringing a solution of 0.4 M $FeCl_3$ to pH 7 by addition of sterile NaOH. The amorphic Fe(III) oxide was allowed to settle down, and washed 3 times with sterile water. The obtained iron oxide was considered sterile for our purposes.

The preculture used in the experiment with biogenic sulfur was grown on sulfide (2 mM) and thiosulfate (10 mM). The preculture used in the experiment with commercial sulfur was also grown on commercial sulfur. The inoculum for the cultures used in the experiments was in any case 2% of the final volume.

Analysis of sulfide, sulfur, thiosulfate, sulfate, and bacteriochlorophyll were performed on zinc acetate-fixed samples, as described in Chapters 2 and 3. Three samples which had been already fixed with zinc acetate were derivatized with monobromobimane as described by Rethmeier et al (1997), and analysed by HPLC (high performance liquid chromatography), as described in Chapter 2.

Results and Discussion

Biogenic sulfur oxidation.

In the experiments reported here, sulfur was the only electron donor furnished to the culture. As shown in Fig. 1A and 1B, sulfur was consumed during time, while BChl *c*, which indicated bacterial growth, and sulfate increased. Sulfate and BChl *c* started to increase after a lag-phase of 15 h for sulfate, 40 h for BChl *c*. Another culture inoculated at the same time but fed with thiosulfate did not present any lag in the growth or in the production of sulfate (data not shown). As already discussed in Chapter 3, sulfur oxidation probably needs proteins that are not expressed under thiosulfate oxidising conditions. *Cla. parvum* uses the lag-phase to synthesise them.

Sulfate, the final product of sulfur oxidation, and BChl *c* increased linearly, and no exponential phase was observed. Since light and CO_2 – the only other substrates needed by GSBs – were not limiting, it might be concluded that sulfur, or sulfur availability, is the limiting factor of bacterial growth. Growth and production of sulfate were linear also in a culture fed with sulfur and illuminated with 2 μE m^{-2} sec^{-1} (data not shown) and during sulfur oxidation following sulfide and thiosulfate oxidation (see Chapter 2).

Both in the culture illuminated with 2 and with 25 μE m^{-2} sec^{-1}, sulfate and BChl *c* were linearly correlated with sulfur. In the culture illuminated with 2 μE m^{-2} sec^{-1}, which grew and oxidized sulfur more slowly than the culture illuminated with 25 μE m^{-2} sec^{-1}, it was possible to identify three phases in the consumption of sulfur:

A. Initially (S^0 amounts between 1.5 and 1.2 mmol), S^0 is consumed (- 0.3 mmol), without any increase of SO_4^{2-} or biomass.
B. S^0 is consumed (S^0 amounts between 1.2 and 0.4 mmol), while sulfate and BChl *c* are produced: 0.4 mol of SO_4^{2-} and 1.1 mg of BChl c are formed for each mole of sulfur which is consumed.
C. Sulfur is stable, but biomass and SO_4^{2-} increase: 0.1 mmol of sulfate and 0.1 mg of BChl *c* are produced.

Results shown in Fig. 2 could be explained by a bad sampling/extraction of S^0. However, this seems to be excluded by the good linearity of the relation between sulfur and BChl *c*, described in phase B. The production of an intermediate in the oxidation of sulfur to sulfate might be as well the explanation for results shown in Fig. 2: an intermediate would be produced at the beginning, and partially consumed at the end of sulfur oxidation. Thiosulfate and tetrathionate were present below detection limit (10 μmol). Analyses of the bimane-fixed samples showed however that thiosulfate was present (data not shown). Sulfite was not detected, but could have been oxidized during fixation with zinc acetate, thus its presence cannot be excluded. Additional biological replicates are however needed to assess the reproducibility of the observed phenomenon.

Presence of sulfide.

Sulfide was present as well during sulfur oxidation, even if in low amounts (0.8-2.5 μmol, corresponding to concentrations 16-50 μM). It initially decreased, to increase again when bacteria started growing (fig. 1B). This pattern has been observed also when the substrate furnished was thiosulfate instead of sulfur. In the case of thiosulfate oxidising bacteria, filtering the sample before fixation with zinc-acetate revealed that most of the initial sulfide is in the medium, while sulfide detected afterwards is filtered away with sulfur and cells, being probably intracellular. Cells are lysed by the high HCl concentrations used in sulfide (data not shown). Thus, even if it cannot be excluded that sulfide is indeed the sulfur mobilizing agent used by *Chlorobaculum parvum* DSM 263 (Borkenstein, 2006), it might as well be an intracellular component, yet involved in sulfur compounds oxidation, but not released to attack sulfur. In favour of the latter interpretation, Borkenstein (2006) documents sulfide appearance only after sulfate and bacteriochlorophyll have increased.

Fig. 3 shows that bacteria could grow despite the presence of iron oxyhydroxyde, a sulfide sink, and that they grew associated with sulfur. However, it cannot be excluded that sulfide was produced from sulfur at the membrane level, where FeOOH was ineffective in removing it (J Overmann, personal communication).

Figures

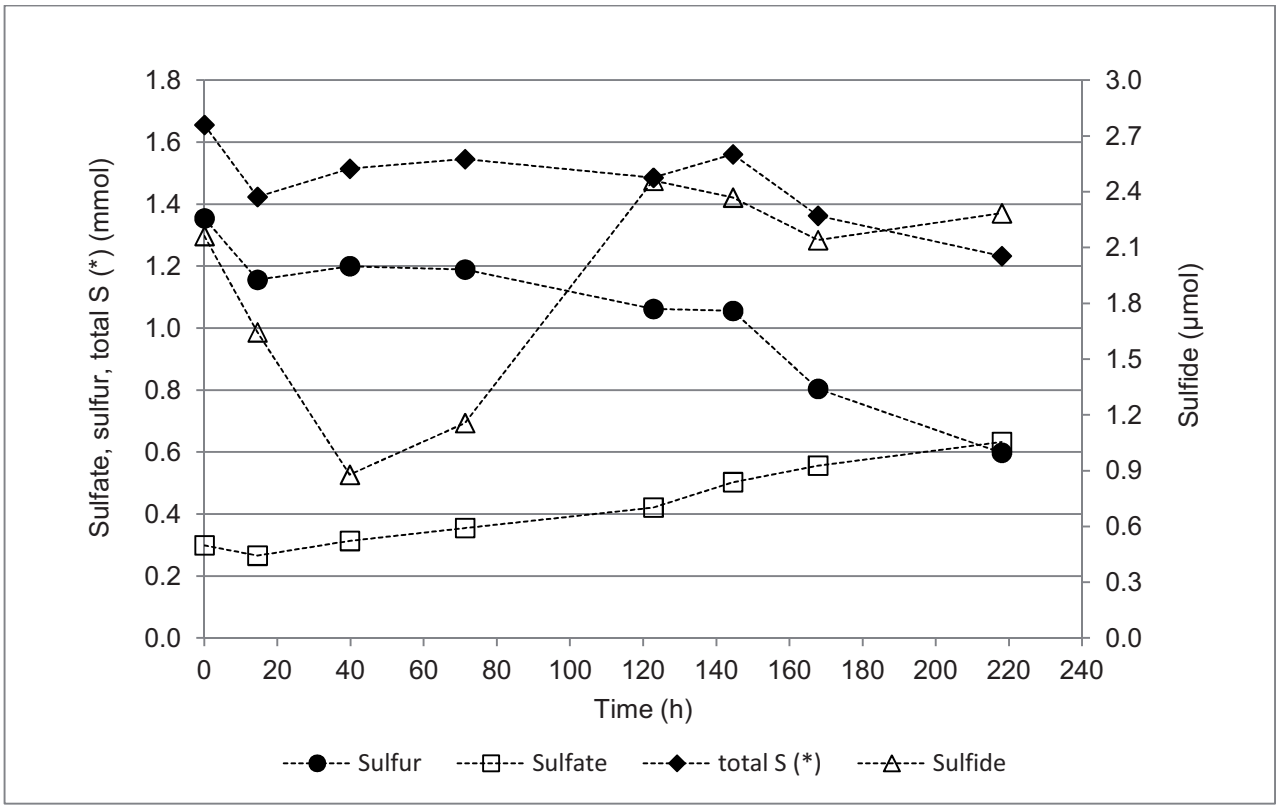

Fig. 1A: Sulfide, sulfur, sulfate, and total sulfur (tot S) variations in dependence on time, in a 50-ml batch culture of *Clb. parvum* DSM 263 that was growing on biogenic sulfur at pH 6.8, 28ºC, and 25 µE m^{-2} sec^{-1} irradiation. (*) Total sulfur was calculated summing the measured amounts of sulfide, sulfur, thiosulfate, and sulfate.

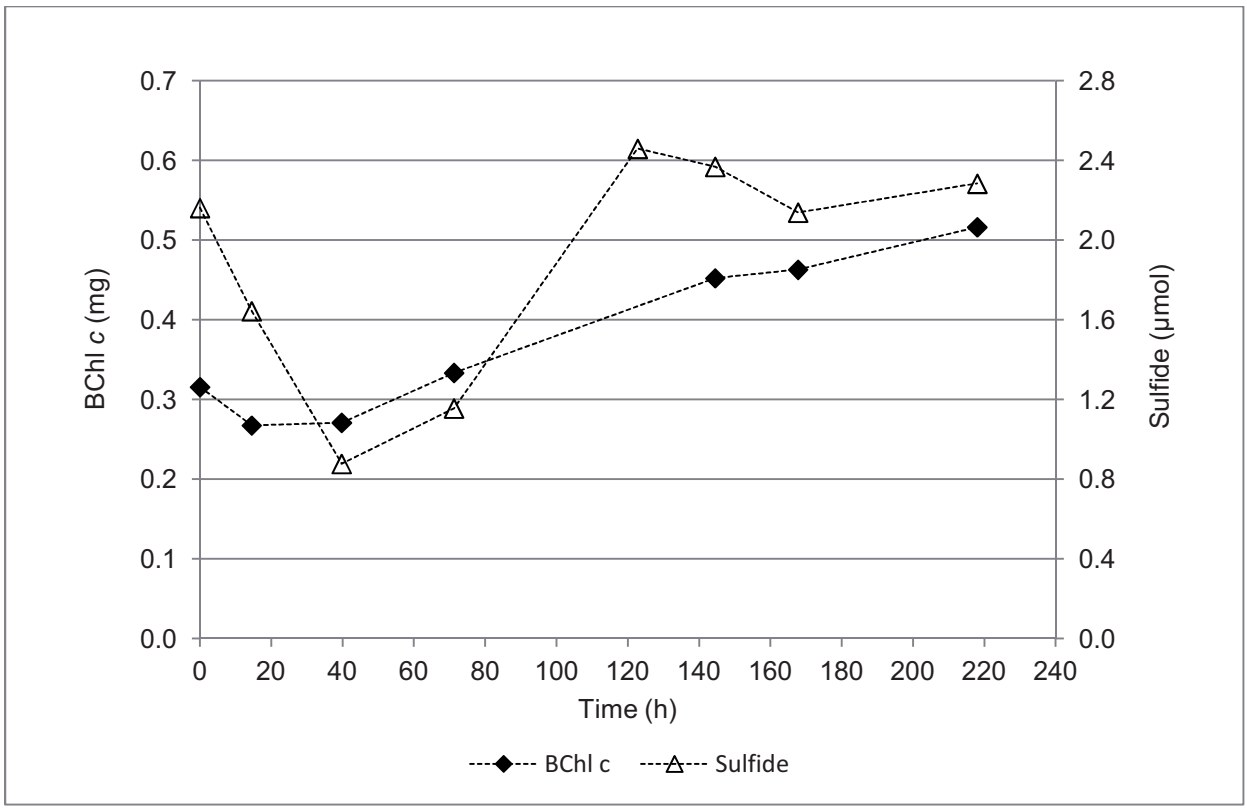

Fig. 1B: Sulfide and BChl *c* variations in dependence on time, in a 50-ml batch culture of *Clb. parvum* DSM 263 that was growing on biogenic sulfur at pH 6.8, 28ºC, and 25 µE m^{-2} sec^{-1} irradiation.

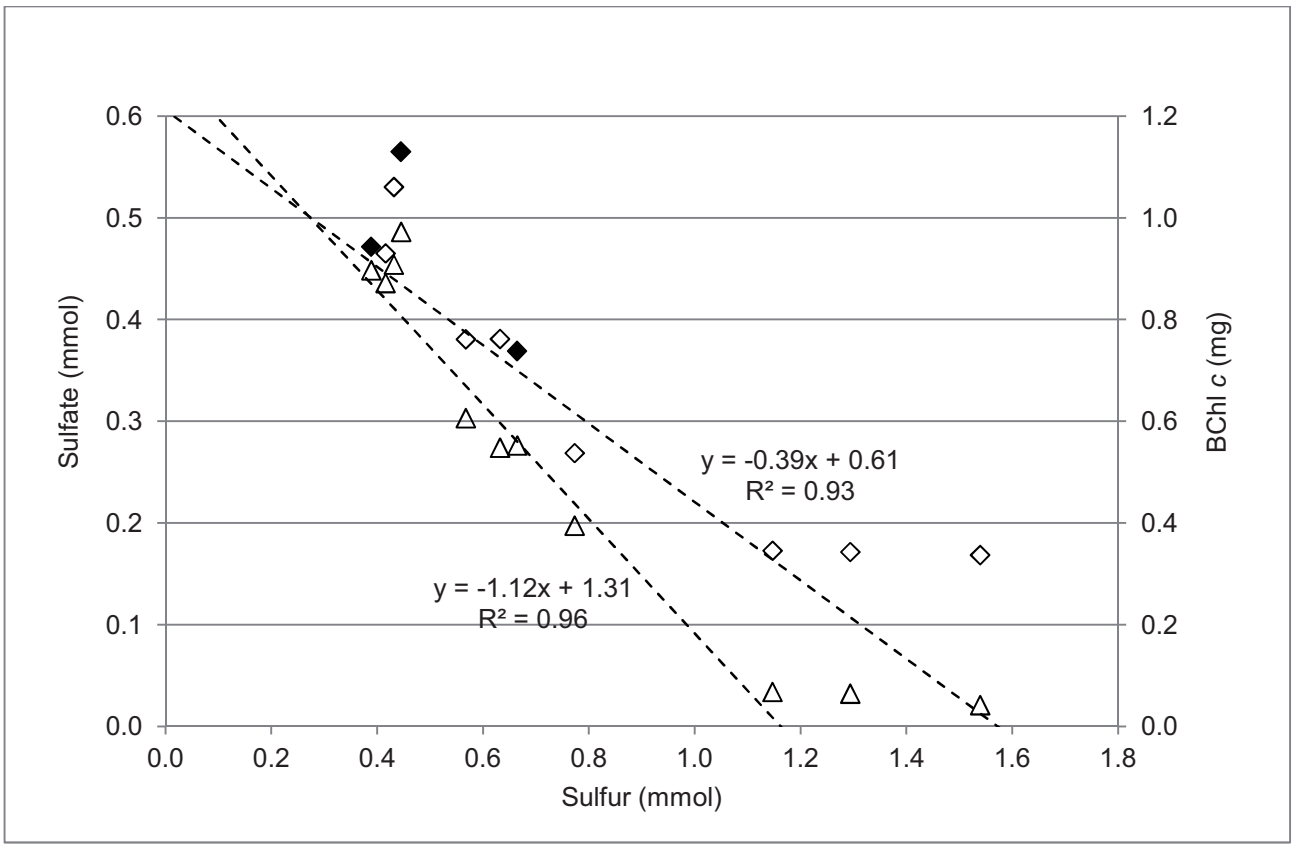

Fig. 2 Relation between sulfate (diamonds) or BChl *c* (triangles) and oxidized biogenic sulfur. Samples were withdrawn from a 50-ml batch culture of *Clb. parvum* DSM 263 that was growing on biogenic sulfur at pH 6.8, 28ºC, and 2 µE m^{-2} sec^{-1} irradiation. The linear correlations between sulfur and sulfate or sulfur and BChl *c* – visible in the range 0.4-1.2 mmol sulfur – are indicated by dotted lines.

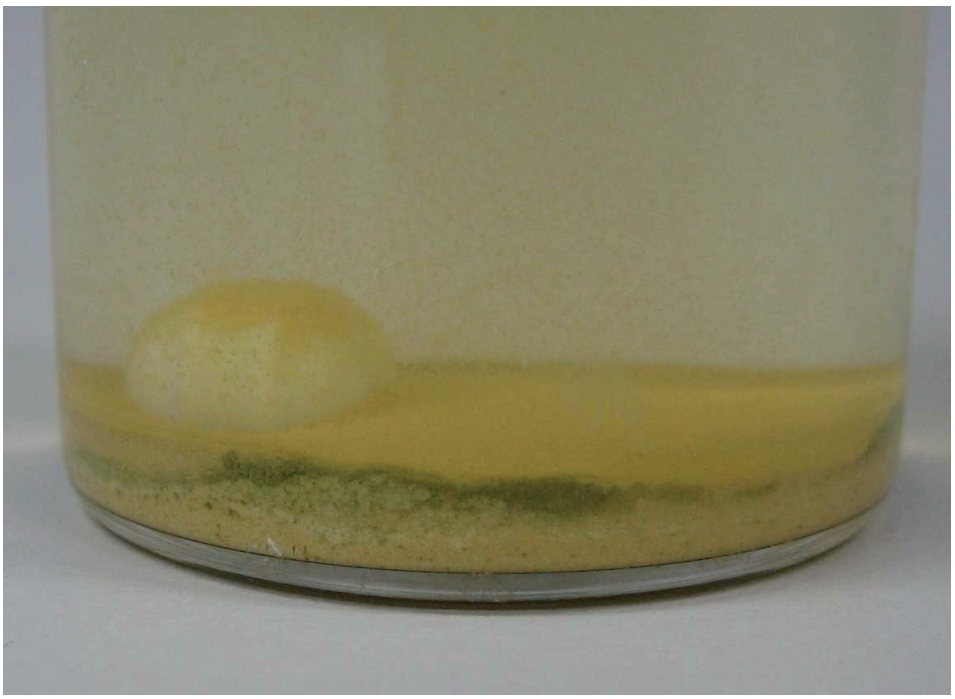

Fig. 3: Detail of the bottom of a 50-mL bottle containing inorganic medium, sulfur, FeOOH and living cells of *Clb. parvum* DSM 263. On the left side, it is visible the magnetic stirrer used during the experiment to keep the cultural conditions homogeneous throughout the whole bottle (to take the photo, sulfur, cells, and FeOOH were let settle down).

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– Chapter 5 –

CONCLUDING REMARKS

The present dissertation focuses on part of the energetic metabolism of green sulfur bacteria. To contribute understanding the oxidation of sulfur to sulfate, it was considered essential to document also the oxidation of sulfide and thiosulfate, from which sulfur is produced. Data on inorganic sulfur compounds dynamics collected under different growth conditions allowed a comparison between genomic predictions and observed physiology in the strain chosen as model, and revealed some unexplored ways of consuming inorganic sulfur compounds by GSBs.

Thiosulfate oxidation by the GSB strain *Cla. parvum* DSM 263 is discussed in Chapter 2. Results there presented describe a complex situation in which it is difficult to recognize the Sox-model in the observed patterns of production and consumption of inorganic sulfur compounds. It is clear, however, that 1) sulfur is always less than expected, 2) extracellular sulfur is not oxidized while thiosulfate is still present, and 3) a system alternative to Sox can consume thiosulfate releasing a yet unidentified sulfur compound. Sulfate yields on thiosulfate followed a saturation curve dependent on the light intensity used to illuminate cultures; the growth rates, however, did not follow the same curve, and growth rates declined at light intensities higher than 15 $\mu E\ m^{-2}\ sec^{-1}$. In other words, at light intensities higher than 15 $\mu E\ m^{-2}\ sec^{-1}$, thiosulfate was oxidised more completely than at lower light intensities, i.e., more sulfate than sulfur was produced from thiosulfate oxidation; however, at least part of the electrons deriving from oxidation of thiosulfate to sulfate were not used to synthesise biomass. Where did electrons go? One possible explanation is that electrons are indeed used to fix CO_2, but organic material is then excreted by cells. Excretion of organic acids by a GSB has been already observed by Sirevåg and Ormerod (1977). Another possibility is that a photolabile sulfur compound – organic or inorganic – is formed from thiosulfate, and then photochemically oxidized to sulfate. This latter possibility would justify the existence of the unidentified sulfur compound detected in bacterial cultures during thiosulfate oxidation.

Excretion of organic material or production of reduced sulfur compounds are ways that *Cla. parvum* might use to avoid photosynthetic saturation at high light intensities. The need of avoiding photosaturation seems particularly realistic for GSBs, which are adapted to low light intensities. Waste of reducing power by cells has been suggested also by Zopfi et al (2004) to justify the uncoupling between tetrathionate reduction and organic matter oxidation that they observed in marine sediments.

Growth rates and sulfate yield on thiosulfate were anyway positively correlated, independently from light intensity. The correlation between growth rates and sulfate yields indicates that part of the thiosulfate that was oxidised directly to sulfate in the periplasm did serve to form biomass. According to a conservative interpretation, which would consider the Sox system as the only thiosulfate-oxidising system in GSBs (Frigaard and Dahl, 2009 and references therein), part of the zero-valent sulfur produced from thiosulfate by Sox would be oxidised to sulfate in the periplasm. Alternatively, another enzymatic system would exist for thiosulfate oxidation, which could be as well the aforementioned system for thiosulfate "consumption" *via* unidentified intermediate (reaction A), most probably organic sulfur ([SX]):

$$S_2O_3^{2-} + 5\ H_2O \rightarrow \mathbf{[SX]} \rightarrow 2\ SO_4^{2-} + 10\ H^+ + 8\ e^- \qquad \text{(reaction A)}$$

In summary, three pools of zero-valent sulfur compounds might be produced from thiosulfate oxidation: A) extracellular sulfur globules; B) periplasmic zero-valent sulfur, oxidized to sulfate in the periplasm; C) organic sulfur compounds ([RS]). A schematic representation of the proposed model for thiosulfate and sulfur oxidation is presented in Fig. 1.

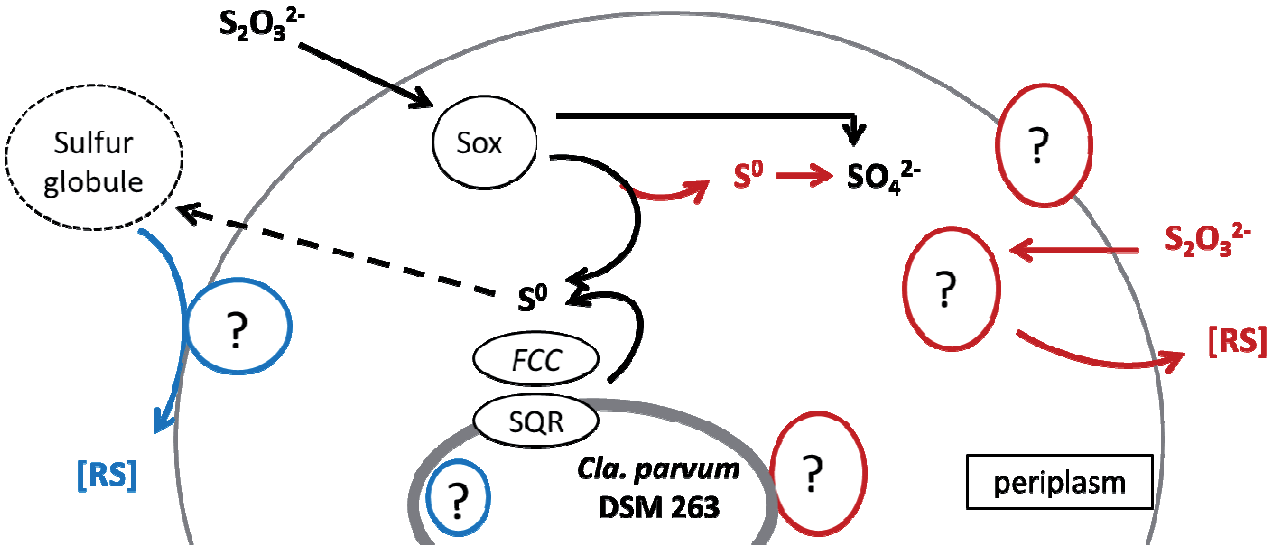

Fig. 1 Proposed model for the oxidation of sulfur compounds by the green sulfur bacterium *Cla. parvum* DSM 263. The proportion between cell and periplasm is not realistic. Black arrows indicated what was known on green sulfur bacteria before the present PhD work. The arrow between periplasmic S^0 and sulfur globule is dotted because it is not know in which form and by which modalities S^0 exits the periplasm. Coloured lines indicate the contributions of the present PhD work: in red the part about thiosulfate oxidation, in blue the part about sulfur oxidation. The proposed systems for thiosulfate and sulfur oxidation, which are responsible for the production of organic sulfur ([RS]) are indicated by question marks cirled in red and blue respectively. Since the cell localization of these putative systems is not known, the circles were located in different positions of the cell. [RS]: organic sulfur; Sox: Sox complex; FCC: flavocytochrome *c*; SQR: sulfide:quinone oxidoreductase. More details in the text.

The dynamics of the sulfur oxidation presented in Chapter 4 evidence that sulfur oxidation proceeds also *via* a yet unidentified intermediate (reaction B):

$$2\ S^0 + 3\ CO_2 + 5\ H_2O \rightarrow \mathbf{[SY]} \rightarrow 2\ SO_4^{2-} + 3\ [CH_2O] + 4\ H^+ \qquad \text{(reaction B)}$$

It cannot be excluded that [SX] and [SY] are the same compound, or at least that they belong to the same class of compounds. In Fig. 1 they are collectively indicated as [RS] (organic sulfur).

The characterization of the unidentified sulfur compound formed during thiosulfate oxidation (reaction A) seems necessary to understand further this yet unexplored pathway for thiosulfate consumption. Its identity might in fact furnish information on which enzymes were involved in its production. Knowing the enzymes and the genes codifying for them would allow checking the universality or less of this pathway in the other GSBs or in other bacterial lineages.

The same holds true for the unidentified compound formed during sulfur oxidation (reaction B). In this latter case proteomics has already revealed successful in detecting the proteins that are overexpressed under sulfur oxidizing condition (Chapter 3). The characterization of those proteins, as well as the identification of their cellular partners, are the next steps to be taken to characterize the cellular apparatus that GSBs use to oxidize sulfur, which might in turn help understanding the role of [SY].

The identification of the membrane players of sulfur mobilization will help understanding also the mechanism of sulfur mobilization.

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

Hiermit versichere ich, dass ich die vorliegende Dissertation mit dem Titel:

"Mobilization of sulfur by green sulfur bacteria – Physiological and molecular studies on *Chlorobaculum parvum* DSM 263"

1) ohne unerlaubte Hilfe angefertigt habe,

2) keine anderen, als die von mir angegebenen Quellen und Hilfsmittel benutzt habe und

3) die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Bremen, den 19. Mai 2011