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REGULAR PAPER

Sulfur oxidation in mutants of the photosynthetic green sulfur bacterium *Chlorobium tepidum* devoid of cytochrome *c*-554 and SoxB

Chihiro Azai · Yusuke Tsukatani · Jiro Harada · Hirozo Oh-oka

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Abstract A mutant devoid of cytochrome *c*-554 (CT0075) in *Chlorobium tepidum* (syn. *Chlorobaculum tepidum*) exhibited a decreased growth rate but normal growth yield when compared to the wild type. From quantitative determinations of sulfur compounds in media, the mutant was found to oxidize thiosulfate more slowly than the wild type but completely to sulfate as the wild type. This indicates that cytochrome *c*-554 would increase the rate of thiosulfate oxidation by serving as an efficient electron carrier but is not indispensable for thiosulfate oxidation itself. On the other hand, mutants in which a portion of the *soxB* gene (*CT1021*) was replaced with the *aacC1* cassette did not grow at all in a medium containing only thiosulfate as an electron source. They exhibited partial growth yields in media containing only sulfide when compared to the wild type. This indicates that SoxB is not only essential for thiosulfate oxidation but also responsible for sulfide oxidation. An alternative electron carrier or electron transfer path would thus be operating between the Sox system and the reaction center in the mutant devoid of cytochrome *c*-554. Cytochrome *c*-554 might function in any other pathway(s) as well as the thiosulfate oxidation one, since even green sulfur bacteria that cannot oxidize thiosulfate contain a *cycA* gene encoding this electron carrier.

Keywords cyt *c*-554 · Electron transfer · Green sulfur bacteria · SoxB · Sulfur oxidation

Abbreviations

cyt	Cytochrome
MOPS	3-(*N*-morpholino)propanesulfonic acid
RC	Reaction center
Sox	Sulfur oxidation
SQR	Sulfide:quinone reductase

Introduction

Green sulfur bacteria (*Chlorobiaceae*) are strictly anaerobic phototrophic organisms that utilize reduced sulfur compounds (sulfide, thiosulfate, and/or elemental sulfur) as electron sources for carbon dioxide fixation (Trüper et al. 1988; Pfennig 1989; Imhoff 2003). All these sulfur compounds are oxidized to sulfate, and liberated electrons are finally supplied to the light-driven energy conversion system, where the type 1 reaction center (RC) generates a strong reductant, reduced ferredoxin, to produce NADPH through ferredoxin-$NADP^+$ oxidoreductase (Seo and Sakurai 2002; Oh-oka and Blankenship 2004). Reduced ferredoxin also serves as an electron donor for the reductive carboxylation reactions in the reverse tricarboxylic acid cycle (Yoon et al. 2001).

Two electron-donating paths to cytochrome (cyt) c_z of the RC complex are known to function in a green sulfur bacterium, *Chlorobium* (*Chl.*) *tepidum* (Oh-oka et al. 1998); one is through soluble cyt *c*-554, which contains a

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single *c*-type heme with an apparent molecular mass of about 10 kDa, and another is through membrane-bound menaquinol:cyt *c* oxidoreductase (CT0302-0303) (this soluble cyt *c* is named cyt *c*-555 after its α-absorption peak shift in the case of *Chlorobium limicola*; we use cyt *c*-554/555 when there is no need to distinguish them by their α-absorption peaks in the context). Recently, by the reconstitution experiments, cyt *c*-554 was shown not to shuttle between menaquinol:cyt *c* oxidoreductase and cyt c_z, contrary to the case of purple bacterial cyt c_2 (Tsukatani et al. 2008). Cyt *c*-554 was, therefore, considered to be involved only in the electron transfer from thiosulfate oxidation, as suggested by several in vitro biochemical experiments that were carried out in the early 1970s (Kusai and Yamanaka 1973a, b; Oh-oka and Blankenship 2004), although there has been no concrete in vivo evidence for this.

On the other hand, a subject concerning thiosulfate oxidation system, as well as its related electron transfer path(s), has not been resolved yet in green sulfur bacteria. A Sox multienzyme system, which is known to be involved in thiosulfate oxidation, has been elucidated to consist of at least four components (SoxAX, YZ, CD, and B) in the chemolithotrophic bacterium *Paracoccus pantotrophus* (Rother et al. 2001). This type of multienzyme system seems to be widely distributed in thiosulfate-utilizing bacteria (Friedrich et al. 2001). The genomic analysis of *Chl. tepidum* has also revealed the presence of the *sox* gene cluster, which is presumably involved in thiosulfate oxidation (Eisen et al. 2002; Frigaard and Bryant 2008). However, contrary to the case in *P. pantotrophus*, the *soxCD* genes, which are responsible for the oxidation of the outer sulfur atom covalently bound to a cysteine residue in SoxY, are missing in the *Chl. tepidum* genome (Frigaard and Bryant 2004). Other reaction mechanisms not involving SoxC/D have therefore been suggested to enable thiosulfate oxidation to sulfate (Frigaard and Bryant 2008). A recent study has demonstrated that SoxK (CT1020), which made a ternary complex along with SoxAX (CT1019, CT1016), was necessary to exhibit a maximal reduction rate of cyt *c*-554 when the thiosulfate oxidation activity was measured in an in vitro reconstitution system (Ogawa et al. 2008).

We previously reported that cyt *c*-554 is not essential for the phototrophic growth of *Chl. tepidum* (Tsukatani et al. 2006). As a full growth of the insertion mutant of the *cycA* gene (*CT0075*) (*cycA*::*aadA* strain) was observed in a medium containing both sulfide and thiosulfate, we simply considered that a tight coupling reaction between menaquinol:cyt *c* oxidoreductase and the RC, which enabled electron supply from sulfide oxidation by a membrane-bound sulfide:quinone reductase (SQR/CT0117, CT0876, and CT1087), would sustain its photosynthetic growth. In this study, a mutant devoid of SoxB (CT1021) (*soxB*::*aacC1* strain) and a double mutant of cyt *c*-554 and SoxB (*cycA*::*aadA*/*soxB*::*aacC1* strain) were constructed in order to reinvestigate the intrinsic role of cyt *c*-554 in vivo with modern molecular biological techniques in combination with traditional assays of sulfur compound concentrations in media. We examined the capabilities of three mutant strains so far obtained to oxidize both sulfide and thiosulfate in media. A complete oxidation of thiosulfate was unexpectedly observed in the *cycA*::*aadA* strain.

Materials and methods

Bacterial strains and growth conditions

The strain WT2321 of *Chl. tepidum* (Wahlund and Madigan 1995) was used as the wild type and host for transformation. The *cycA*::*aadA* strain was obtained in previous study (Tsukatani et al. 2006). Pf-7 medium (Wahlund et al. 1991), which contained 4 mM thiosulfate and 2.5 mM sulfide as electron sources, was used for the growth analyses shown in Fig. 2. However, in order to measure sulfur compounds more precisely as well as to get full growth of culture, an additional buffering reagent, 3-(*N*-morpholino)propanesulfonic acid (MOPS), was added to the Pf-7 medium at a final concentration of 10 mM (Frigaard and Bryant 2001), and the concentration of thiosulfate contained was increased to 9 mM, which was then designated as Pf-7-MOPS. As the upper limit of sulfide tolerance for *Chl. tepidum* was reported to be about 4 mM (Wahlund et al. 1991), the concentration of sulfide in Pf-7-MOPS was adjusted at 2.5 mM. Pf-7-MOPS containing only thiosulfate or sulfide as a sole electron source was prepared basically according to the method described by Frigaard and Bryant (2001) as follows. One liter of Pf-7-MOPS medium without $Na_2S_2O_3 \cdot 5H_2O$, $Na_2S \cdot 9H_2O$, and $NaHCO_3$ was autoclaved at 121°C for 20 min and cooled in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) overnight in order to completely remove any residual oxygen from the medium. In an anaerobic chamber, 2.3 g of $Na_2S_2O_3 \cdot 5H_2O$ or 0.6 g of $Na_2S \cdot 9H_2O$ was dissolved into a freshly prepared solution of 2.0 g of $NaHCO_3$ in 50 ml. After filter sterilization, each solution was added to the medium to make a 9 mM thiosulfate- or 2.5 mM sulfide-containing medium. Growth on agar CP plates was carried out as described previously (Tsukatani et al. 2004).

Plasmid construction for natural transformation

A 2.7-kb DNA fragment containing the *soxB* gene was amplified by polymerase chain reaction (PCR) using an

SOB-F primer (5′-*ACATGCCATGG*TCATCTTCGCCGCT GATC) and an SOB-R primer (5'-*GCTCTAGA*AGGTA AAGCCCTGCTTGG), both of which were designed on the basis of the complete genomic sequence of *Chl. tepidum* (Eisen et al. 2002). In these primer sequences, an *Nco*I site for SOB-F and an *Xba*I site for SOB-R, heterologous bases are italicized and recognition sites are underlined, respectively. Plasmid pSB was produced by digesting this DNA fragment with *Nco*I and *Xba*I and cloning the product thus obtained into the same recognition sites of pKF3 (Takara Bio Inc.). Plasmid pSB was then cut at two *Hin*dIII sites located at both ends of the *soxB* gene and ligated with the gentamycin resistance (Gm^r) cassette, which was produced by *Hin*dIII digestion of pUCGM (Schweizer 1993), as shown in Fig. 1a. The resultant plasmid, pSB-Gm^r, was prepared in a large amount with a MIDI-prep kit (Invitrogen). About 1 μg of pSB-Gm^r was linearized by cutting at the *Nco*I site and applied to natural transformation in strain WT2321 of the *Chl. tepidum* and the *cycA*::*aadA* strain as described previously (Frigaard and Bryant 2001). The transformants grown on selective (Gm^r) CP plates were restreaked three times onto Gm^r CP plates. Single colonies on the third plate were inoculated into the liquid media, and the grown cells were inoculated again into selective liquid media containing appropriate antibiotics (gentamycin for the *soxB*::*aacC1* strain and streptomycin/gentamycin for the *cycA*::*aadA*/*soxB*::*aacC1* strain). These growth cultures were used for further investigations.

Genomic DNAs of all the strains were prepared according to the method described previously (Tsukatani et al. 2004). Plasmid constructions and other routine molecular biological procedures were carried out using chemically competent *Escherichia coli* DH5α as a host.

Growth rate measurements

The wild type and mutant strains of *Chl. tepidum* were routinely grown at 40°C in a home-built growth chamber illuminated from the front with a light intensity of 30 μmol of photons m^{-2} s^{-1} using incandescent lamps, although the

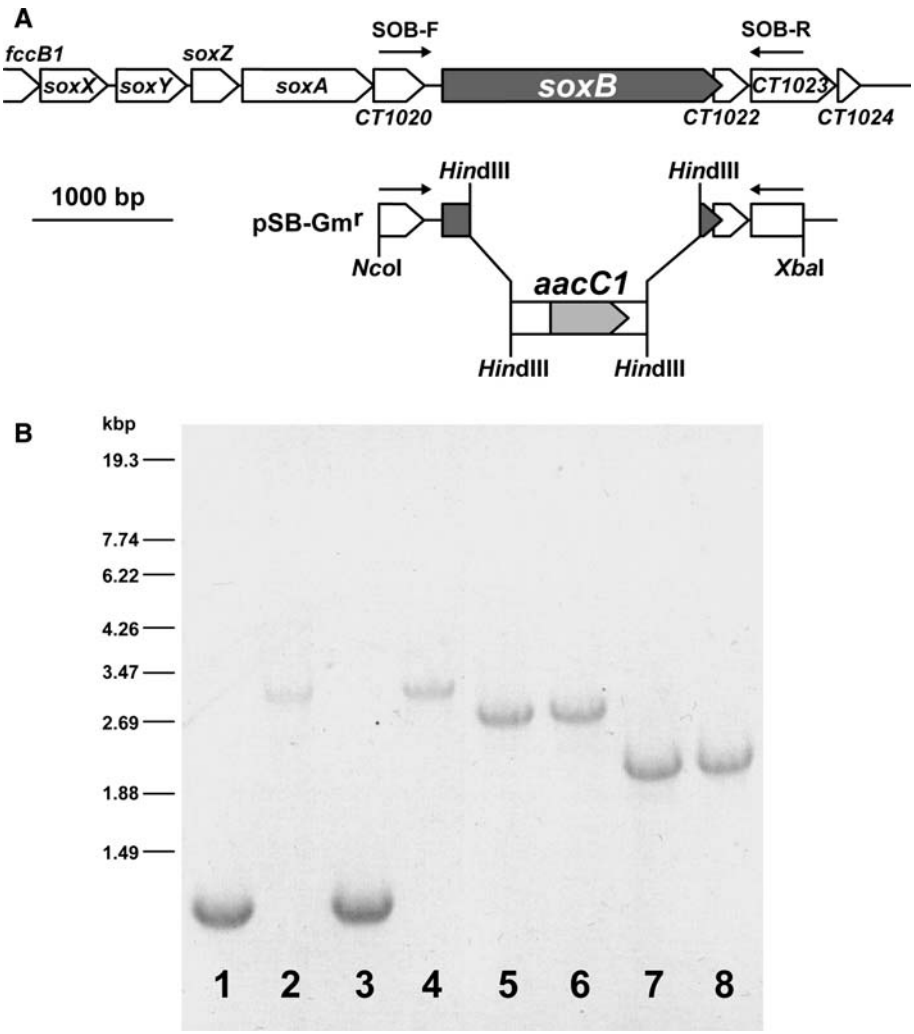

Fig. 1 **a** Schematic map for the construction of mutants. Genes are indicated by rectangles. The arrows represent the oligonucleotide primers used for the cloning of a *soxB* gene. Plasmid pSB-Gm^r was digested at the AhdI site for natural transformation and then introduced into *Chl. tepidum*. **b** PCR analysis of the genomes from the wild type (lanes 1 and 5), *cycA*::*aadA* strain (lanes 2 and 6), *soxB*::*aacC1* strain (lanes 3 and 7), and *cycA*::*aadA*/*soxB*::*aacC1* strain (lanes 4 and 8). The loci of *cycA* and *soxB* were amplified by PCR with the primer sets of C554F and C554R (lanes 1–4) and of SOB-F and SOB-R (lanes 5–8), respectively. The numbers indicate the lengths of the DNA fragments in kilobases

optimum temperature for growth is 47°C (Wahlund et al. 1991). In the case of the growth measurements of mutants, no antibiotic was added into liquid media in order to avoid any influence to their growth behavior. It has recently been demonstrated that temperature for growth affects cellular physiology, that is, compositions of bacteriochlorophyll *c* homologs and physical properties of chlorosomes (Morgan-Kiss et al. 2009). Although the phenotypes observed in this study were expected to be more severe at the optimum temperature, the same conclusions concerning sulfur oxidation would be drawn from experiments at different temperatures.

For a quick estimation of the growth rate, the optical density at 660 nm was routinely monitored using a photometer (mini photo 518R, TAITEC). For a more reliable estimation, the protein content in each culture was determined basically according to the method described by Mukhopadhyay et al. (1999). Inoculation was done by transferring an aliquot amount (1 μg protein) of early stationary-phase cells, which were grown preliminarily under the same condition, into a freshly prepared medium in a 30-ml screw-capped tube without a headspace. Each culture was kept in the dark for 1–2 h before measurements were begun. The average value of five independent measurements was plotted against the time elapsed. The optical density was not monitored consecutively in each tube, but it was discarded after measurement in order to avoid any interference of growth.

Quantitative determinations of sulfur compounds

After centrifugation of each culture, the resultant supernatant was kept at −80°C until use except for sulfide determination. The content of the sulfur compounds in the supernatant was determined as follows: the sulfide content was estimated by the formation of methylene blue (Trüper and Schlegel 1964), the thiosulfate content by cyanolysis in the presence of Cu^{2+} ion followed by Fe-SCN complex formation (Westley 1987), and the sulfate content by the formation of $BaSO_4$ precipitates after the reaction with $BaCl_2$ under acidic conditions (Sörbo 1987). As Pf-7-MOPS contained 0.8 mM $MgSO_4$, it was impossible to clarify physiological meanings of values <0.8 mM obtained in the present measurement (see Table 1).

Table 1 Cell yields and concentrations of sulfur compounds in stationary-phase cultures (after 72 h of growth) of the wild type and three mutants

Strains	Electron sources in Pf-7-MOPS	Cell yields (μg protein/ml)	Sulfide concentrations (mM)	Thiosulfate concentrations (mM)	Elemental sulfur concentrations (mM)	Sulfate concentrations (mM)
Wild type	Sulfide/ thiosulfate	184.3 (±28.8)	ND[a]	ND[a]	0.1 (±0.0)	21.6 (±1.2)
	Thiosulfate	211.0 (±10.9)	ND[a]	ND[a]	0.9 (±0.1)	17.4 (±1.9)
	Sulfide	31.9 (±0.9)	ND[a]	ND[a]	0.1 (±0.0)	3.0 (±0.1)
cycA::*aadA*	Sulfide/ thiosulfate	170.5 (±22.6)	ND[a]	ND[a]	0.1 (±0.0)	22.1 (±1.0)
	Thiosulfate	211.6 (±11.3)	ND[a]	ND[a]	0.4 (±0.0)	18.9 (±1.1)
	Sulfide	33.0 (±2.0)	ND[a]	ND[a]	0.1 (±0.0)	2.8 (±0.1)
soxB::*aacC1*	Sulfide/ thiosulfate	11.8 (±0.5)[b]	ND[a]	9.5 (±0.1)[b]	ND[a]	0.4 (±0.0)[b]
	Thiosulfate	ND[a]	ND[a]	9.2 (±0.1)[b]	ND[a]	0.1 (±0.0)[b]
	Sulfide	14.7 (±0.9)[b]	ND[a]	0.3 (±0.0)	ND[a]	0.9 (±0.1)[b]
cycA::*aadA*/ *soxB*::*aacC1*	Sulfide/ thiosulfate	21.1 (±3.3)[b,c]	ND[a]	9.1 (±0.6)[b]	ND[a]	1.2 (±0.1)[b,c]
	Thiosulfate	ND[a]	ND[a]	9.2 (±0.0)[b]	ND[a]	0.1 (±0.0)[b]
	Sulfide	19.3 (±2.6)[b,c]	ND[a]	0.2 (±0.0)	ND[a]	1.7 (±0.1)[b,c]
None[d]	Sulfide/ thiosulfate	–	2.1 (±0.1)	9.2 (±0.1)	ND[a]	0.1 (±0.0)
	Thiosulfate	–	ND[a]	9.0 (±0.1)	ND[a]	0.1 (±0.0)
	Sulfide	–	2.4 (±0.1)	0.1 (±0.0)	ND[a]	0.7 (±0.0)

[a] ND, Not detectable

[b] $P < 0.001$, for comparison to the wild type within the same condition

[c] $P < 0.05$, for comparison to the *soxB*::*aacC1* strain within the same condition

[d] Concentrations of sulfur compounds were measured in freshly prepared media as described in the text. Note that Pf-7-MOPS contains 0.8 mM $MgSO_4$

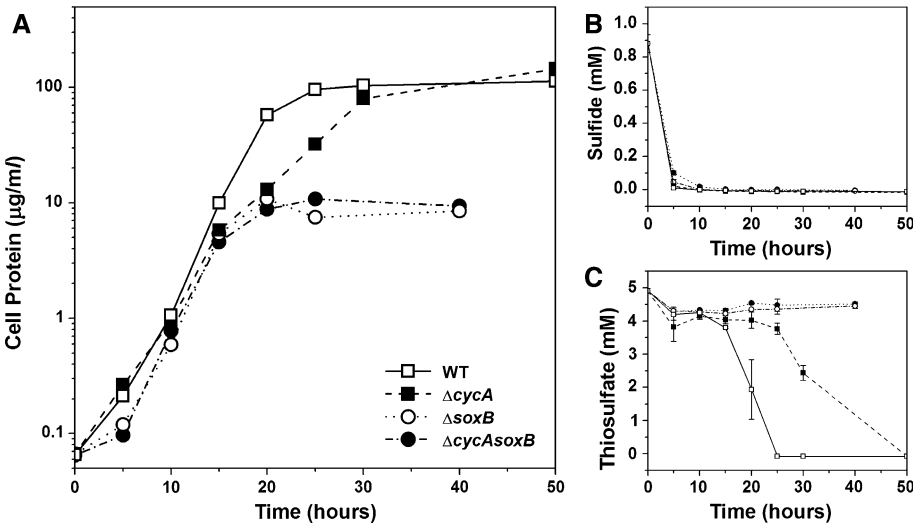

Fig. 2 **a** Growth profiles for the wild type (*open squares*), *cycA*::*aadA* strain (*closed squares*), *soxB*::*aacC1* strain (*open circles*), and *cycA*::*aadA*/*soxB*::*aacC1* strain (*closed circles*) cultivated in Pf-7 media at 40°C. **b** and **c** Sulfide and thiosulfate consumptions during the cultivation of wild type and three mutants. The symbols are the same as in (**a**). The average of five independent measurements of the cell density (μg protein/ml) and the sulfide and thiosulfate concentrations (Morgan-Kiss et al. 2009) in media were plotted against time. Standard deviations are also indicated by bars in (**b**) and (**c**), but too small to be noted other than three points indicated in (**c**). Measurement values were missing at 30 h in the *soxB*::*aacC1* and *cycA*::*aadA*/*soxB*::*aacC1* strains in (**c**) because they were plotted at 40 h instead

The elemental sulfur was also quantified after hexane extraction of the centrifugation pellet, followed by further extraction with methanol and subsequent measurement of the absorption of the finally obtained extract at 260 nm (Stal et al. 1984). The hot cyanolysis method was also applied to estimate it and almost the same result was obtained (Schedel and Trüper 1980) (data not shown).

Although a freshly prepared liquid medium (both Pf-7 and Pf-7-MOPS) contained 2.5 mM sulfide, the actual measurement value of sulfide immediately after inoculation was found to be only 1 mM due to its volatilization and/or oxidation during a centrifugation step to collect the supernatant (see Fig. 2b). The initial sulfide content was therefore confirmed to be about 2.4 mM by a direct addition of reagents into the medium in a culture tube after the removal of its screw cap and a subsequent color development in it (see Table 1). The cyanolysis method tends to react slightly with elemental sulfur and/or polysulfide derived from sulfide oxidation by air (Westley 1987), which would, thus, overestimate the thiosulfate content to be 0.1–0.3 mM in sulfide-containing media (also see Table 1).

Results

Construction of *Chl. tepidum* mutants

The *soxB* gene was disrupted in both the *Chl. tepidum* wild type and the *cycA*::*aadA* strain by replacing the central portion of a *soxB* gene with the *aacC1* gentamycin cassette as depicted in Fig. 1a. The insertion of the *aacC1* gene into the correct locus in the genomic DNA was verified by PCR analyses using a primer set of SOB-F/SOB-R. Although 2.7-kb fragments containing a full size of the *soxB* gene were amplified from the wild type and *cycA*::*aadA* strain, products with a size of 2.0 kb were obtained from the *soxB*::*aacC1* and *cycA*::*aadA*/*soxB*::*aacC1* strains (Fig. 1b). This was interpreted to be due to the deletion of a 1.6-kb fragment within the coding region of the *soxB* gene when an *aacC1* cassette with the size of 0.9 kb was inserted (see Fig. 1a). On the other hand, a primer set of C554F/C554R, which was applied to cause the insertional disruption of the *cycA* gene in our previous study (Tsukatani et al. 2006), confirmed that both mutants were never affected by the present transformation procedures. Furthermore, the same results were also obtained in mutants after several generations of cultivation in non-selective Pf-7 and/or Pf-7-MOPS media (data not shown), indicating that the *aacC1* cassette was stably incorporated into the genome.

Growth rates and oxidations of sulfide and thiosulfate in complete media

Figure 2a shows the growth profiles of the wild type and three mutants (*cycA*::*aadA*, *soxB*::*aacC1*, and *cycA*::*aadA*/*soxB*::*aacC1* strains) cultivated in Pf-7 medium (Wahlund et al. 1991), which contains both sulfide and thiosulfate (Fig. 2a). All of them grew almost at the same rate during the initial 15 h, and their doubling times were estimated to

be 2 h, nearly the same as that reported originally in *Chl. tepidum* under optimal conditions (Wahlund et al. 1991). During this period, sulfide was consumed completely in all cultures, while the thiosulfate contents remained almost unchanged (Fig. 2b, c). Although sulfide appeared to be exhausted more slowly in the cultures of the disruption mutants of the *soxB* gene (*soxB*::*aacC1* and *cycA*::*aadA*/*soxB*::*aacC1* strains) than in those of the wild type and the *cycA*::*aadA* strain, their growth rates did not clearly reflect these small distinctions.

After 15 h, the wild type and the *cycA*::*aadA* strain still continued to grow to attain their full growth, although the *cycA*::*aadA* strain was slightly retarded compared to the wild type ($P < 0.001$) (Fig. 2a). The doubling time of the wild type was thus almost the same as before, while that of the mutant strain was estimated to be about 4.5 h. In agreement with their respective growth profiles, the wild type consumed thiosulfate more rapidly than the *cycA*::*aadA* strain (Fig. 2c), and no significant difference was observed between their final cell yields.

On the other hand, the *soxB*::*aacC1* and *cycA*::*aadA*/*soxB*::*aacC1* strains ceased to grow after 15 h, when sulfide in their cultures was completely exhausted, and their final cell yields were estimated to be less than one tenth of that of the wild type (Fig. 2a, b; also see Table 1). Since the SoxB is a component in a multienzyme system involved in thiosulfate oxidation, it was conceivable that both mutants could not utilize thiosulfate as an electron source (Friedrich et al. 2001). In fact, the thiosulfate contents in their cultures were constant during the measurements of their growths, as shown in Fig. 2c.

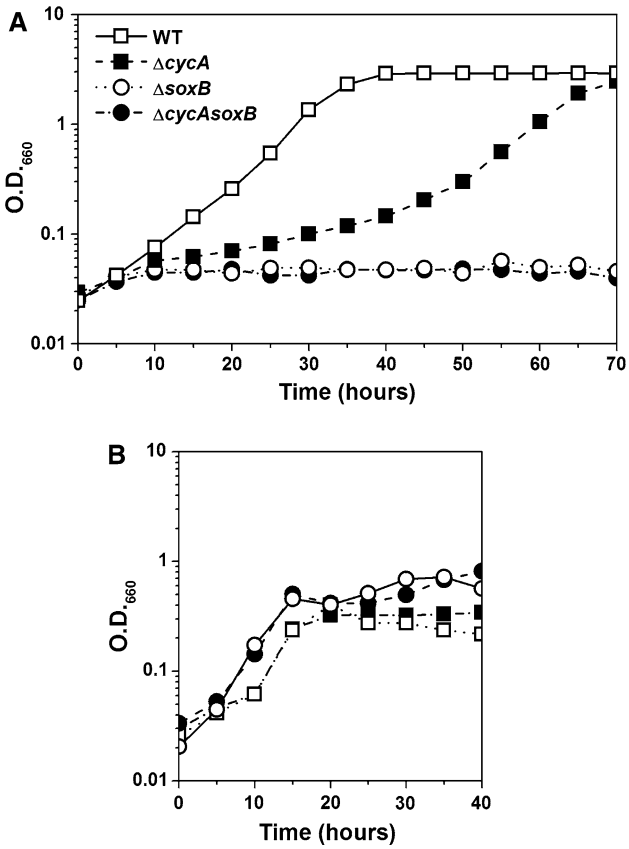

Fig. 3 Growth profiles for the wild type (*open squares*), *cycA*::*aadA* strain (*closed squares*), *soxB*::*aacC1* strain (*open circles*), and *cycA*::*aadA*/*soxB*::*aacC1* strain (*closed circles*) cultivated in Pf-7-MOPS media with **a** thiosulfate or **b** sulfide at 40°C. The average of five independent measurements of the cell density at 660 nm was plotted against time

Utilization of sulfide and thiosulfate as electron sources

It still remained unknown to what degree the mutants could efficiently utilize sulfide and thiosulfate for their photosynthetic growths. The growth profiles, therefore, were observed after transferring into freshly prepared media containing only sulfide or thiosulfate as the sole electron source (Fig. 3). Cell numbers were roughly estimated by measuring optical densities at 660 nm for the sake of convenience because there was no need to discriminate subtle differences in growth rates. The final cell yields were nevertheless measured by determining the protein contents in order to avoid overestimates due to light scattering (Table 1). The sulfur content in each culture after the stationary phase was also measured to estimate the degree of oxidation of the two electron sources.

When 9 mM thiosulfate was provided as the sole electron source, the *soxB*::*aacC1* and *cycA*::*aadA*/*soxB*::*aacC1* strains did not grow completely (Fig. 3a), and no consumption of thiosulfate was observed in either culture (Table 1). The *cycA*::*aadA* strain grew more slowly than the wild type, as expected from the result obtained in the Pf-7 medium, and required much more time to attain full growth (Figs. 2a and 3a). This could be interpreted as a result reflecting the relatively slower oxidation rate of thiosulfate in the mutant compared to that in the wild type (Fig. 2c). The cell yield of the *cycA*::*aadA* strain was, however, almost the same as that of the wild type in Pf-7-MOPS medium with or without thiosulfate (Table 1). The entire consumption of thiosulfate by both the wild type and the *cycA*::*aadA* strain indicated that they could utilize thiosulfate for their photosynthetic growth and completely oxidize it to sulfate. In fact, the amounts of sulfate accumulated after their full growths were nearly equivalent to those of thiosulfate added into freshly prepared media in terms of the sulfur contents (Table 1).

On the other hand, when 2.5 mM sulfide was added to the media as a sole electron source, all four strains showed a similar growth profile (Fig. 3b). It is well known that polysulfide and/or elemental sulfur globules, which are the products of sulfide oxidation, are excreted outside of the

cells during phototrophic growth and then oxidized further to sulfate (Brune 1989). The small bulges in the growth curves of the wild type and the *cycA*::*aadA* strain, which could be observed at around 15 h in Fig. 3b, were thus ascribable to their transient appearance detected as a scattering artifact. From the amounts of sulfate accumulated in the cultures after full growth, most of the sulfide added into freshly prepared media seemed to be completely oxidized to sulfate by the wild type and the *cycA*::*aadA* strain (Table 1). Although the *soxB*::*aacC1* and *cycA*::*aadA*/*soxB*::*aacC1* strains could consume sulfide judging from Fig. 2b, they were found to oxidize it only partially (Table 1). Almost the same amount of sulfate was detected in the *soxB*::*aacC1* strain as compared to that in the control medium, and a half amount of sulfate expected from a complete sulfide oxidation seemed to be accumulated in the *cycA*::*aadA*/*soxB*::*aacC1* strain. In fact, the final cell yields of the wild type and the *cycA*::*aadA* strain were about two times larger than those of the *soxB*::*aacC1* and *cycA*::*aadA*/*soxB*::*aacC1* strains (Table 1), in agreement with the incomplete oxidation of sulfide to sulfate by the latter two mutants. In the present experiment, the maximal sulfide concentration in media was limited to 2.5 mM because of its toxic effect on cells at higher concentrations.

Discussion

The *cycA*::*aadA* strain could grow even in the medium containing only thiosulfate as an electron source but exhibited a rather retarded growth rate compared to that of the wild type (Fig. 3a), which was attributable to a relatively slower oxidation rate of thiosulfate by the mutant than the wild type (Fig. 2c). However, it is noteworthy that the mutant still retained a capability to completely oxidize thiosulfate to sulfate. This was also supported by the fact that the final cell yield of the *cycA*::*aadA* strain was almost the same as that of the wild type, implying that the mutant can utilize all the reducing power derived from thiosulfate oxidation for its own growth. Therefore, the present results have clearly demonstrated that cyt *c*-554 functions as an efficient electron carrier between the Sox system and cyt c_z as expected but is not indispensable for thiosulfate oxidation.

It has been demonstrated in vitro that the Sox multienzyme system, which oxidizes sulfite, thiosulfate, sulfur, and/or sulfide, consists of four protein components, namely, SoxAX, SoxYZ, SoxB, and SoxCD, in *P. pantotrophus* (Rother et al. 2001). On the other hand, the genome sequence analysis of *Chl. tepidum* has revealed that no orthologs of the *soxC* and *D* genes, which encode sulfur dehydrogenase (SoxCD), are found in the *sox* gene cluster or at other locations (Eisen et al. 2002). Our results clearly demonstrate that the *soxB* gene is indispensable for the oxidation of thiosulfate (see Fig. 3a and Table 1), suggesting that some other component(s) than the SoxCD or a different oxidation mechanism must be operating in *Chl. tepidum*. Although an alternative carrier would also accept electrons from this Sox system, it is not at all clear at present what could play the role of cyt *c*-554 in the *cycA*::*aadA* strain.

Furthermore, an additional interesting phenotype was recognized in mutants devoid of SoxB, i.e., neither the *soxB*::*aacC1* strain nor the *cycA*::*aadA*/*soxB*::*aacC1* one oxidized sulfide completely to sulfate (Table 1). They did not also excrete any polysulfide/sulfur globules outside of the cells (Table 1), although the cultures of the *soxB* mutants appeared to become somewhat turbid and show slight aggregations. One possible interpretation might be that the Sox system in *Chl. tepidum* is somehow involved in the oxidation of intermediate(s) produced during sulfide oxidation. This should be resolved by a more intensive analysis of all possible intermediate(s) and/or in vitro reconstitution experiments in the future.

In the medium containing both sulfide and thiosulfate, the wild type as well as the *cycA*::*aadA* strain utilized sulfide exclusively during the early stage of growth and then oxidized thiosulfate after sulfide exhaustion (see Fig. 2) (Chan et al. 2008). This suggests that an activity of thiosulfate oxidation might be somehow regulated, presumably by the redox state of the quinone pool, because the sulfide oxidation by SQR could control its redox balance. It is noteworthy that the *cycA*::*aadA* strain commenced thiosulfate consumption about 10 h later after the wild type did (Fig. 2c). In accordance with this, the mutant strain also resumed its growth after a time lag in media containing only thiosulfate as an electron donor as well (Fig. 3a). It may suggest that an electron-carrying component other than cyt *c*-554 or an alternative electron transfer path was induced to oxidize thiosulfate, although there is no genetic information at present from the relevant genomic analysis. Recently, the RT-PCR analysis of three SQR homologs has indicated that the transcription level of *CT1087* increased when sulfide was supplemented to cultures (Chan et al. 2009). It will therefore be interesting issues to study regulation mechanisms of sulfur oxidation pathway in detail.

The mechanisms of sulfur oxidation as well as its metabolic pathways remain enigmatic in green sulfur bacteria, although several in vitro reconstitution experiments had intensively been conducted in the early 1970s (Kusai and Yamanaka 1973a, b). Recent study has demonstrated that SoxK, gene of which is located between the *soxA* and *soxB* genes in the genome of *Chl. tepidum* (Frigaard and Bryant 2008), drastically enhanced the thiosulfate-dependent reduction of cyt *c*-554 by forming a ternary complex with SoxAX, when added into a reaction mixture containing SoxYZ (CT1017-1018) and SoxB (Ogawa et al. 2008).

However, it still remains an unknown and critical issue how thiosulfate is completely oxidized to sulfate in vivo. Now that genomic databases are available in some sulfur-oxidizing bacteria, comparative studies as well as molecular genetic analyses using *Chl. tepidum* will clarify the components involved in sulfide and/or thiosulfate oxidation and enable us to envisage the entire picture of the sulfur metabolism in green sulfur bacteria in the near future.

A *cycA* gene encoding a low-molecular weight and soluble monoheme-type cyt *c*-554/555 is distributed in all species of green sulfur bacteria of which genome sequences have so far been determined. Their amino acid sequence identity is approximately 57–84% to each other and 74–84% among species containing the sox gene cluster in particular. *Chlorobium ferroxidans*, which does not use reduced sulfur compounds (sulfide, thiosulfate, and elemental sulfur) as electron sources but oxidizes ferrous ion to ferric ion, contains neither the sox gene cluster nor other relevant genes. The amino acid sequence of *Chl. ferroxidans* cyt *c*-554/555, nevertheless, shows about 73% identity compared to that of *Chl. tepidum*. This could imply two possibilities concerning the function of cyt *c*-554/555. One is that cyt *c*-554/555 has the same function as an electron carrier among all species of green sulfur bacteria; *Chl. tepidum* cyt *c*-554 not only accepts electrons from the Sox system but would also operate in (an)other unknown but common pathway(s). The other is that cyt *c*-554/555 has different functions depending on the species; *Chl. tepidum* cyt *c*-554 accepts electrons from the Sox system, while *Chl. ferroxidans* cyt *c*-554/555 would do so from the oxidation system of ferrous ion. At present, there is no biochemical and/or molecular biological data concerning cyt *c*-554/555 from *Chl. ferroxidans* or any other species which cannot oxidize thiosulfate at all.

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References

Brune DC (1989) Sulfur oxidation by phototrophic bacteria. Biochim Biophys Acta 975:189–221. doi:10.1016/S0005-2728(89)80251-8

Chan LK, Weber TS, Morgan-Kiss RM, Hanson TE (2008) A genomic region required for phototrophic thiosulfate oxidation in the green sulfur bacterium *Chlorobium tepidum* (syn. *Chlorobaculum tepidum*). Microbiology 154:818–829. doi:10.1099/mic.0.2007/012583-0

Chan LK, Morgan-Kiss RM, Hanson TE (2009) Functional analysis of three sulfide:quinone oxidoreductase homologs in *Chlorobaculum tepidum*. J Bacteriol 191:1026–1034. doi:10.1128/JB.01154-08

Eisen JA et al (2002) The complete genome sequence of *Chlorobium tepidum* TLS, a photosynthetic, anaerobic, green-sulfur bacterium. Proc Natl Acad Sci USA 99:9509–9514. doi:10.1073/pnas.132181499

Friedrich CG, Rother D, Bardischewsky F, Quentmeier A, Fischer J (2001) Oxidation of reduced inorganic sulfur compounds by bacteria: emergence of a common mechanism? Appl Environ Microbiol 67:2873–2882. doi:10.1128/AEM.67.7.2873-2882.2001

Frigaard N-U, Bryant DA (2001) Chromosomal gene inactivation in the green sulfur bacterium *Chlorobium tepidum* by natural transformation. Appl Environ Microbiol 67:2538–2544. doi:10.1128/AEM.67.6.2538-2544.2001

Frigaard N-U, Bryant DA (2004) Seeing green bacteria in a new light: genomics-enabled studies of the photosynthetic apparatus in green sulfur bacteria and filamentous anoxygenic phototrophic bacteria. Arch Microbiol 182:265–276. doi:10.1007/s00203-004-0718-9

Frigaard N-U, Bryant DA (2008) Genomic insights into the sulfur metabolism of phototrophic green sulfur bacteria. In: Hell R, Dahl C, Knaff D, Leustek T (eds) Sulfur metabolism in phototrophic organisms. Springer, Dordrecht, The Netherlands, pp 337–355

Imhoff JF (2003) Phylogenetic taxonomy of the family *Chlorobiaceae* on the basis of 16S rRNA and *fmo* (Fenna–Matthews–Olson protein) gene sequences. Int J Syst Evol Microbiol 53:941–951. doi:10.1099/ijs.0.02403-0

Kusai A, Yamanaka T (1973a) Cytochrome *c* (553, *Chlorobium thiosulfatophilum*) is a sulphide-cytochrome *c* reductase. FEBS Lett 34:235–237. doi:10.1016/0014-5793(73)80801-4

Kusai K, Yamanaka T (1973b) The oxidation mechanisms of thiosulphate and sulphide in *Chlorobium thiosulfatophilum*: roles of cytochrome *c*-551 and cytochrome *c*-553. Biochim Biophys Acta 325:304–314. doi:10.1016/0005-2728(73)90106-0

Morgan-Kiss RM et al (2009) *Chlorobaculum tepidum* regulates chlorosome structure and function in response to temperature and electron donor availability. Photosynth Res 99:11–21. doi:10.1007/s11120-008-9361-7

Mukhopadhyay B, Johnson EF, Ascano M Jr (1999) Conditions for vigorous growth on sulfide and reactor-scale cultivation protocols for the thermophilic green sulfur bacterium *Chlorobium tepidum*. Appl Environ Microbiol 65:301–306

Ogawa T et al (2008) SoxAX binding protein, a novel component of the thiosulfate-oxidizing multienzyme system in the green sulfur bacterium *Chlorobium tepidum*. J Bacteriol 190:6097–6110. doi:10.1128/JB.00634-08

Oh-oka H, Blankenship RE (2004) Green bacteria: secondary electron donor (cytochromes). In: Lennarz WJ, Lane MD (eds) Encyclopedia of biological chemistry. Academic Press, Oxford, pp 521–524

Oh-oka H, Iwaki M, Itoh S (1998) Membrane-bound cytochrome c_z couples quinol oxidoreductase to the P840 reaction center complex in isolated membranes of the green sulfur bacterium *Chlorobium tepidum*. Biochemistry 37:12293–12300. doi:10.1021/bi9800799

Pfennig N (1989) Green bacteria. In: Staley JT, Bryant MP, Pfennig N, Holt JC (eds) Bergey's manual of systematic bacteriology. Williams and Wilkins, Baltimore, MD, pp 1682–1707

Rother D, Henrich HJ, Quentmeier A, Bardischewsky F, Friedrich CG (2001) Novel genes of the sox gene cluster, mutagenesis of the flavoprotein SoxF, and evidence for a general sulfur-oxidizing system in *Paracoccus pantotrophus* GB17. J Bacteriol 183:4499–4508. doi:10.1128/JB.183.15.4499-4508.2001

Schedel M, Trüper HG (1980) Anaerobic oxidation of thiosulfate and elemental sulfur in *Thiobacillus denitrificans*. Arch Microbiol 124:205–210. doi:10.1007/BF00427728

Schweizer HD (1993) Small broad-host-range gentamycin resistance gene cassettes for site-specific insertion and deletion mutagenesis. Biotechniques 15:831–834

Seo D, Sakurai H (2002) Purification and characterization of ferredoxin-NAD(P)$^{(+)}$ reductase from the green sulfur bacterium *Chlorobium tepidum*. Biochim Biophys Acta 1597:123–132

Sörbo B (1987) Sulfate: turbidimetric and nephelometric methods. Methods Enzymol 143:3–6. doi:10.1016/0076-6879(87)43003-6

Stal LJ, van Gemerden H, Krumbein WE (1984) The simultaneous assay of chlorophyll and bacteriochlorophyll in natural microbial communities. J Microbiol Methods 2:295–306. doi:10.1016/0167-7012(84)90048-4

Trüper HG, Schlegel HG (1964) Sulfur metabolism in *Thiorhodaceae*. I. Quantitative measurements in growing cells of *Chromatium okenii*. Antonie Van Leeuwenhoek 30:225–238. doi:10.1007/BF02046728

Trüper HG, Lorenz C, Schedel M, Steinmetz M (1988) Metabolism of thiosulfate in *Chlorobium*. In: Olson JM, Ormerod JG, Amesz J, Stackebrandt E, Trüper HG (eds) Grenn photosynthetic bacteria. Plenum Press, New York, pp 189–200

Tsukatani Y, Miyamoto R, Itoh S, Oh-Oka H (2004) Function of a PscD subunit in a homodimeric reaction center complex of the photosynthetic green sulfur bacterium *Chlorobium tepidum* studied by insertional gene inactivation. Regulation of energy transfer and ferredoxin-mediated $NADP^+$ reduction on the cytoplasmic side. J Biol Chem 279:51122–51130. doi:10.1074/jbc.M410432200

Tsukatani Y, Miyamoto R, Itoh S, Oh-oka H (2006) Soluble cytochrome *c*-554, CycA, is not essential for photosynthetic electron transfer in *Chlorobium tepidum*. FEBS Lett 580:2191–2194. doi:10.1016/j.febslet.2006.03.016

Tsukatani Y, Azai C, Kondo T, Itoh S, Oh-Oka H (2008) Parallel electron donation pathways to cytochrome c_z in the type I homodimeric photosynthetic reaction center complex of *Chlorobium tepidum*. Biochim Biophys Acta 1777:1211–1217. doi:10.1016/j.bbabio.2008.05.002

Wahlund TM, Madigan MT (1995) Genetic transfer by conjugation in the thermophilic green sulfur bacterium *Chlorobium tepidum*. J Bacteriol 177:2583–2588

Wahlund TM, Woese CR, Castenholz RW, Madigan MT (1991) A thermophilic green sulfur bacterium from New Zealand hot springs, *Chlorobium tepidum* sp. nov. Arch Microbiol 156:81–90. doi:10.1007/BF00290978

Westley J (1987) Thiocyanate and thiosulfate. Methods Enzymol 143:22–25. doi:10.1016/0076-6879(87)43008-5

Yoon KS, Bobst C, Hemann CF, Hille R, Tabita FR (2001) Spectroscopic and functional properties of novel 2[4Fe-4S] cluster-containing ferredoxins from the green sulfur bacterium *Chlorobium tepidum*. J Biol Chem 276:44027–44036. doi:10.1074/jbc.M107852200