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Impact of Sulfur Starvation in Autotrophic and Heterotrophic Cultures of the Extremophilic Microalga *Galdieria phlegrea* (Cyanidiophyceae)

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In plants and algae, sulfate assimilation and cysteine synthesis are regulated by sulfur (S) accessibility from the environment. This study reports the effects of S deprivation in autotrophic and heterotrophic cultures of *Galdieria phlegrea* (Cyanidiophyceae), a unicellular red alga isolated in the Solfatara crater located in Campi Flegrei (Naples, Italy), where H_2S is the prevalent form of gaseous S in the fumarolic fluids and S is widespread in the soils near the fumaroles. This is the first report on the effects of S deprivation on a sulfurous microalga that is also able to grow heterotrophically in the dark. The removal of S from the culture medium of illuminated cells caused a decrease in the soluble protein content and a significant decrease in the intracellular levels of glutathione. Cells from heterotrophic cultures of *G. phlegrea* exhibited high levels of internal proteins and high glutathione content, which did not diminish during S starvation, but rather glutathione significantly increased. The activity of *O*-acetylserine(thiol)lyase (OASTL), the enzyme synthesizing cysteine, was enhanced under S deprivation in a time-dependent manner in autotrophic but not in heterotrophic cells. Analysis of the transcript abundance of the OASTL gene supports the OASTL activity increase in autotrophic cultures under S deprivation.

Keywords: O-Acetylserine(thiol)lyase • Cyanidiophyceae • Galdieria phlegrea • Glutathione • Heterotrophic cultures • Sulfur deficiency.

Abbreviations: GSH, reduced glutathioneOASTL, *O*-acetylserine(thiol)lyase; SAT, serine acetyltransferase.

Introduction

Acid hot spring systems are found throughout the world and share similar characteristics. The temperature is very high near the springs (approximately 100°C) and declines to 50—35°C in the soils surrounding the hot pools, where sulfate minerals such as alunite and free sulfuric acid are deposited in large amounts and the pH fluctuates between 0.5 and 1.5 (Ciniglia et al. 2005). The eukaryotes found in acid hot springs are represented by mixed populations of algae, with a prevalence of Cyanidiophyceae, one of the most ancient groups of algae, which diverged from the base of Rhodophyta approximately 1.3 billion years (Müller et al. 2001, Yoon et al. 2006). Three genera, *Cyanidium*, *Cyanidioschyzon* and *Galdieria*, are recognized in the class Cyanidiophyceae, and presently five *Galdieria* species, *G. sulphuraria*, *G. daedala*, *G. partita*, *G. maxima* and *G. phlegrea*, have been described based on morphological characteristics, such as cell shape, number and shape of plastids, structure of the cell wall, presence/absence of vacuoles, cell pattern, division and number of autospores in sporangia (Merola et al. 1981, Sentsova, 1991, Ott and Seckbach 1994, Albertano et al. 2000, Pinto et al. 2003). All *Galdieria* species are able to grow in the dark by using numerous carbon sources as organic substrates (Gross 1999). It has been hypothesized that the heterotrophic abilities of *Galdieria* are important for survival in cryptoendolithic habitats where light availability is severely compromised (Gross et al. 2001). Interestingly, *G. sulphuraria* and *G. phlegrea* can live in the same environments in separate populations, as observed in the hydrothermal system of Pisciarelli, which is situated on the eastern edge of the Solfatara crater in the central part of the Campi Flegrei Caldera, Naples, Italy (Valentino and Stanzione 2003). The *G. phlegrea* at this site is confined to the fissures of the rock walls (it is considered a strictly cryptoendolithic species), where light is almost absent, the temperature ranges from 35 to 55°C and the pH is between 0.5 and 1.5 (Pinto et al. 2007). Such a combination of facultative heterotrophy, thermophily, obligatory acidophily and a natural sulfur (S-)rich habitat is a unique feature of oxygenic photosynthesizing organisms and thus deserves attention.

The literature on S metabolism in algae is rather limited (Giordano et al. 2005, Norici et al. 2005, Carfagna et al. 2011a).The data available for algae, moreover, mostly concern green algae as a model for all algal systems (Yildiz et al.1994, Ravina et al. 2002, Zhang et al. 2004, Pootakam et al. 2010) or organisms of little ecological significance. The S metabolism in microalgae such as *Galdieria* and related genera is presently unknown.

In plant cells, H_2S, derived from the enzymatic reduction of SO_3^{2-}, is inserted into the backbone of *O*-acetylserine to form

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cysteine through the enzyme *O*-acetylserine(thiol)lyase (OASTL; EC 4.2.99.8). *O*-Acetylserine is provided by the enzyme serine acetyltransferase (SAT). In vascular plants *O*-acetylserine and cysteine are synthesized in the cytosol, the plastids and the mitochondria by compartment-specific SAT and OASTL isoforms all encoded by the nuclear genome (Giordano et al. 2008). In *Chlamydomonas reinhardtii*, all the messengers involved in cysteine synthesis appear to code for proteins with chloroplast transit peptides, suggesting that, in this alga, in contrast to vascular plants, cysteine synthesis takes place exclusively in the chloroplast (Ravina et al. 2002). In *Chlorella sorokininana*, protein gel blot analysis revealed the presence of at least two OASTL isoforms, one localized in the chloroplast and one localized in the cytosol in sulfate-starved cells (Carfagna et al. 2011a). While the S level within the cell is controlled by cysteine biosynthesis via OASTL, OASTL activity strongly depends on the S nutritional status of the algae (Carfagna et al. 2011a).

In the present study, we evaluated the protein content, thiol levels and cysteine synthesis in *G. phlegrea* from a natural S-rich habitat under different regimes of S nutrition in either autotrophic or heterotrophic conditions; we compared the results with those previously reported for the green unicellular alga *C. sorokiniana* from mesophilic environments (Carfagna et al. 2011a, Salbitani et al. 2014). We also investigated the effects of S starvation on the OASTL activities and OASTL mRNA in *G. phlegrea* cells cultured in autotrophic or heterotrophic conditions.

Vascular plants have evolved organ-specific modes of nutrition: a phototrophic shoot closely interacts with a heterotrophic root using xylem and phloem as communication highways. The complex communication between autotrophic and heterotrophic metabolism could be studied in unicellular organisms and the results could be transferred to the multi-organ system of higher plants.

Results

Protein content of the cells

The protein content of the autotrophic and heterotrophic cells in the logarithmic phase of growth was 3.3 ± 0.3 and 4.6 ± 0.03 pg cell^{-1}, respectively. **Fig. 1** shows the 24 h variation in the protein content in S-starved cells under both autotrophic and heterotrophic conditions. The total soluble protein content in S-starved cells significantly decreased during the first 5 h under autotrophic conditions and then remained constant, while the total soluble protein content remained high and unchanged in S-starved heterotrophic cells.

Thiol contents of the cells

The total glutathione content was 3.3 ± 0.3 and 4.91 ± 0.009 pmol 10^{-5} cell^{-1} in cells cultured under autotrophic and heterotrophic conditions, respectively. In cells cultured under autotrophic conditions, the glutathione content was halved within 2 h from the start of S starvation, and it was strongly reduced after 24 h. In contrast, after 2 h of S starvation,

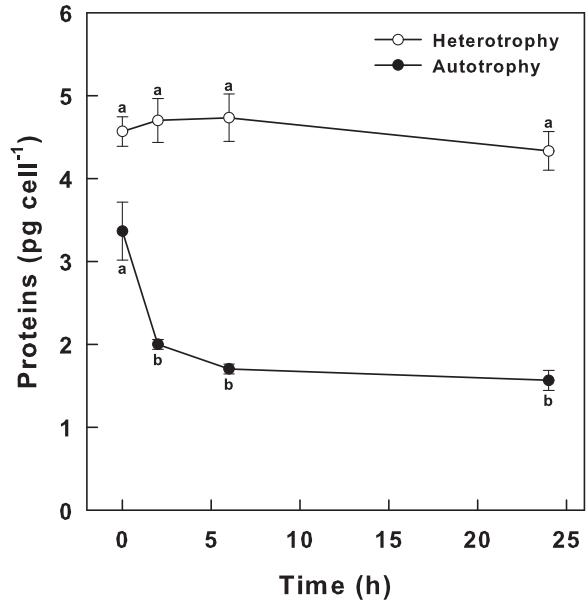

Fig. 1 The time course of total soluble protein concentration upon sulfur deprivation in the autotrophic or heterotrophic *G. phlegrea* cells. At the indicated times, cells were assayed for the total soluble protein content. The values reported are the means ± SE from five independent experiments (n = 5). Error bars smaller than the symbols are not shown. Different letters indicate statistically significant differences ($P < 0.001$, ANOVA, Tukey's multiple comparison). Further details are provided in the Materials and Methods.

the glutathione content significantly increased under heterotrophic conditions ($P < 0.001$), reaching 20.8 ± 0.4 pmol 10^{-5} cell^{-1}, and it remained high for the entire duration of the experiment (**Fig. 2**). The cellular glutathione content was always higher in the heterotrophic cells compared with the autotrophic cells in either the S-sufficient or the S-starved conditions.

The intracellular level of reduced glutathione (GSH) in autotrophic cells shifted from 30% in S-sufficient conditions to 50% under S starvation. On the other hand, in heterotrophic cells, the GSH level was maintained at around 30% of the total glutathione during the experiment.

Elemental cell contents

In cells cultured under autotrophic or heterotrophic conditions, the proportion of carbon (C) was similar and did not vary under S deprivation. Conversely, the cell quota of nitrogen (N) was different in the two types of cells but not affected by S starvation (**Table 1**). The amount of total N is halved in cells grown in heterotrophy compared with those grown in autotrophy. The total content of S and phophorus (P) was different in autotrophic and heterotrophic cells. However, total S decreases during S starvation in cells in autotrophy. In cells cultured in heterotrophy and S starved, total elemental S does not vary significantly.

OASTL activity

The two types of cells displayed similar levels of OASTL activity during the exponential phase of growth. The activity was found

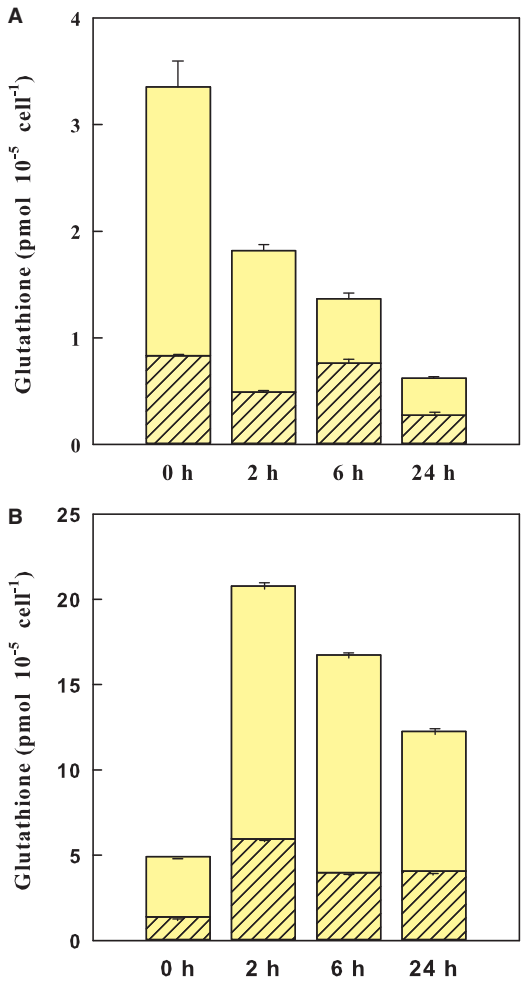

Fig. 2 Total glutathione content in cells of *G. phlegrea* under S starvation in autotrophic (A) or heterotrophic (B) conditions. The values reported are the means ± SE from five independent experiments (n = 5). The striped bar indicates reduced glutathione (GSH). Further details are provided in the Materials and Methods.

to be 1.3 ± 0.05 and 1.14 ± 0.01 U mg^{-1} under autotrophic and heterotrophic conditions, respectively. Moreover, the OASTL activity increased in a time-dependent manner in the autotrophic cells under S starvation (**Fig. 3**) but remained constant in the heterotrophic cells (**Fig. 3**).

OASTL transcript levels and expression analysis

By screening algal genomic databases, one candidate gene for cysteine synthase (CYS) was found in *G. sulphuraria*, with an open reading frame of 1,155 bp. The amino acid sequence showed significant homology with *C. merolae* cmOASTL1, with a percentage identity of 74%, as well as with red algal CYSs (*Pyropia yezoensis*, 73%; *Porphyra purpurea*, 73%; *Chondrus crispus*, 70%). These data suggest that GS_CYSA can be renamed GS_OASTL1 (**Fig. 4**). The phylogenetic analysis revealed that GS_CYSA clustered together with CYSs of red algae, and form a sister clade with green algae and plants,

Table 1 Effects of sulfur (S) deprivation on cellular contents of total carbon (C), nitrogen (N), sulfur (S)and phosphorus (P), expressed as mg g^{-1} dry biomass, in autotrophic or heterotrophic conditions

	N	C	S	P
Autotrophy				
Control	113.42 ± 2.32^{Aa}	486.05 ± 20.80^{Aa}	11.50 ± 0.30^{Aab}	4.59 ± 0.20^{Aa}
2 h	120.55 ± 2.82^{a}	529.01 ± 12.18^{a}	12.75 ± 0.22^{a}	4.98 ± 0.22^{a}
6 h	112.97 ± 0.88^{a}	496.54 ± 5.24^{a}	9.90 ± 0.47^{b}	3.43 ± 0.16 ^{b}
24h	115.01 ± 1.97^{a}	494.29 ± 8.39^{a}	10.80 ± 0.69^{b}	3.33^{b} ± 0.22
Heterotrophy				
Control	56.40 ± 1.18^{Ba}	470.21 ± 7.39^{Aa}	8.33 ± 0.23^{Ba}	3.18 ± 0.22^{Ba}
2 h	53.88 ± 1.18^{a}	470.18 ± 1.67^{a}	9.04 ± 0.50^{a}	4.06 ± 0.07^{b}
6 h	56.37 ± 0.66^{a}	477.78 ± 2.68^{a}	9.55 ± 0.31^{a}	3.47 ± 0.14^{ab}
24 h	50.93 ± 1.23^{a}	469.97 ± 2.57^{a}	9.28 ± 0.22^{a}	3.73 ± 0.17^{ab}

Data are presented as means ± SE (n = 4).
The superscript letters indicate the statistical significance: identical letters identify means that are not significantly different; different letters identify statistically different means ($P \leq 0.001$). Upper case letters indicate the comparison between autotrophic and heterotrophic conditions.

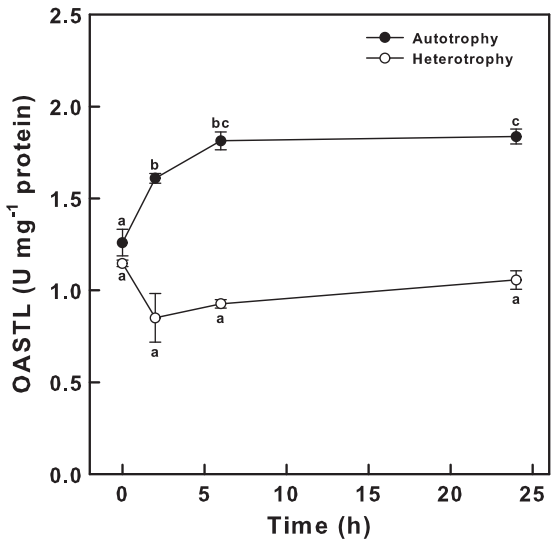

Fig. 3 Effects of sulfur deprivation on the OASTL activity in cells of *Galdieria phlegrea* cultured under autotrophic or heterotrophic conditions. The values reported are the means ± SE from five independent experiments (n = 5). Error bars smaller than the symbols are not shown. Different letters indicate statistically significant differences ($P < 0.001$, ANOVA, Tukey's multiple comparison).

thus confirming the eukaryotic origin of gene (Toda et al. 2001) (**Supplementary Fig. S2**).

In both *G. sulphuraria* CYSA and *C. merolae* cmOASTL1, the consensus amino acid sequence (PXXSVKDR) for the putative PLP-binding domain is entirely conserved (**Fig. 4**).

Since no amplification fragments were obtained by using cmOASTL2 degenerate primers, qRT-PCR was performed using exclusively GS_CYS primer pairs. In our study, in agreement with 'minimum standard for the provision of information for qPCR experiments' (MIQE) (Bustin et al. 2009), we used two reference genes as internal controls in the normalization

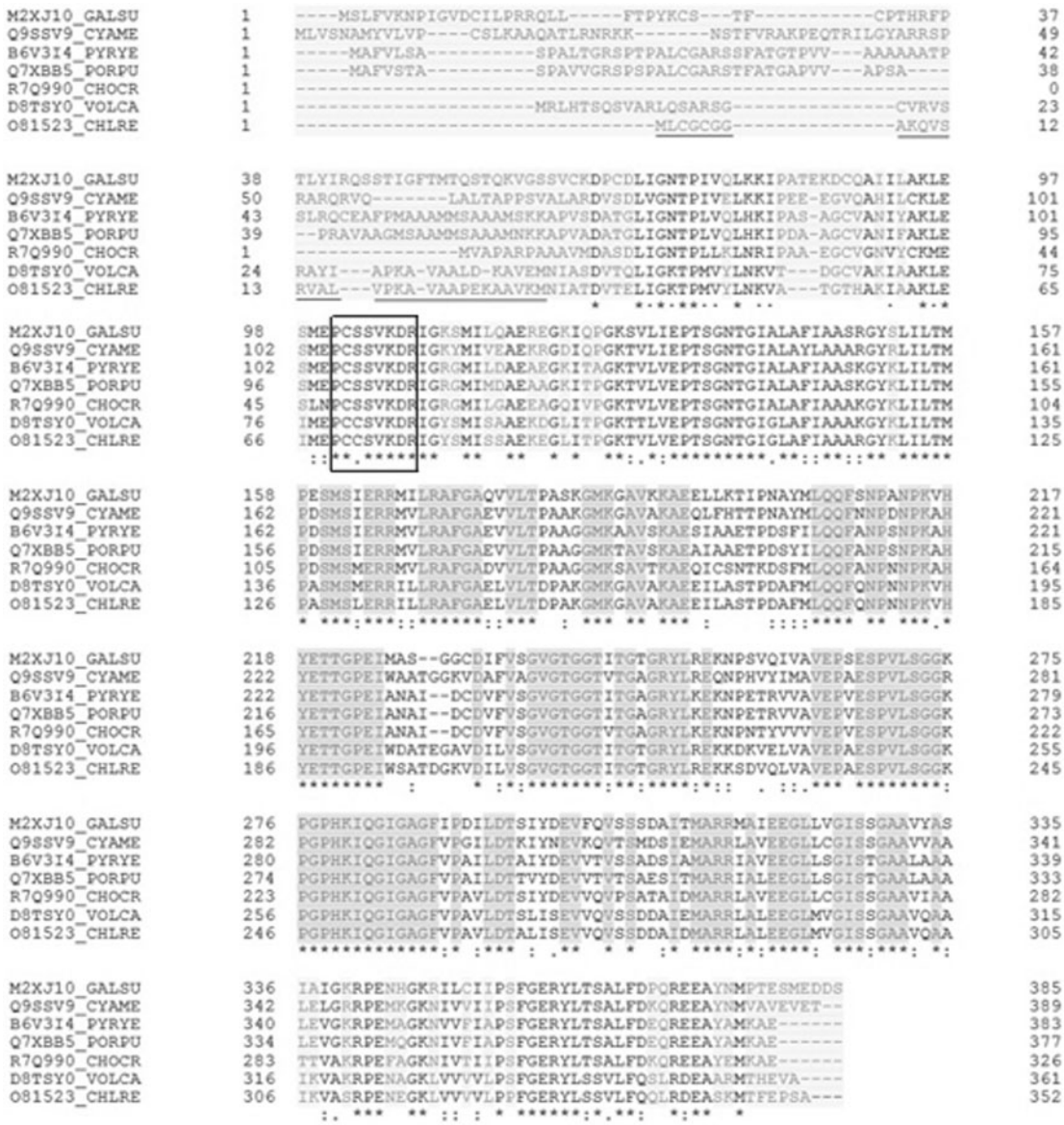

Fig. 4 Sequence alignment of the amino acid sequences of *G. sulphuraria* OASTL1 (M2XJ10) with the following peptide sequences: *C. merolae* cmOASTL1 (Q9SSV9), *P. yezoensis* OASTL (B6V3I4), *P. purpurea* CS (Q7XBB5), *C. crispus* CHC (R7Q990), *V. carteri* CYSK (D8TSY0) and *C. reinhardtii* Crcys-1A (O81523). The amino acid sequences were aligned using the multiple alignment program Clustal W (Bioedit). Gaps introduced to maximize similarity are shown with '—'. Sequence numbering is shown on the right. The conserved 5'-phosphate-binding site (PXXSVKDR) is indicated in the rectangle. The overall consensus sequence is indicated with an asterisk on the bottom line. Conserved motifs are shaded in gray.

strategy. Actin and elongation factor α showed no or only minimal changes in expression levels between the individual samples and experimental conditions. The right choice of reference genes is crucial to analyze the results of qRT-PCR accurately (Radonic et al. 2004) and to reduce the errors from variations among the samples, extraction and RNA quality and efficiency in cDNA synthesis, internal controls and the different experimental samples (Tichopad et al. 2003, Peters et al. 2004).

Quantitative data of OASTL gene expression in *G. phlegrea* in S-starved cells cultured in autotrophy and heterotrophy in comparison with S-sufficient control cells are shown in **Fig. 5**. Cells cultured in autotrophy significantly up-regulated the relative expression of OASTL after 2 h (15-fold) of the S starvation (**Fig. 5**), while the transcript levels in S-starved cells, under heterotrophic conditions, showed a slight increase after 24 h (3.3-fold) (**Fig. 5**).

Discussion

Sulfur represents an essential nutrient for vascular plants and microalgae, and sulfate uptake and assimilation have been widely described (Davidian and Kopriva 2010, Birke et al. 2012). In plants and green microalgae, such as *C. sorokiniana* and *C. reinhardtii*, the S assimilation pathway is repressed when sulfate is available and it is activated by sulfate starvation

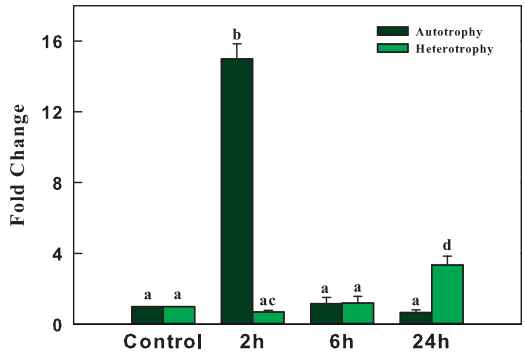

Fig. 5 OASTL gene expression in *Galdieria phlegrea* cells cultured in autotrophic (dark green bars) or heterotrophic (light green bars) conditions under S starvation. The mRNA levels were normalized with respect to the level of mRNA for the reference genes (actin and elongation factor α). Bars show the means ± SE from three independent experiments ($n = 3$). Different letters indicate statistically significant differences ($P < 0.001$, ANOVA, Tukey's multiple comparison).

(Ravina et al. 1999, Carfagna et al. 2011a, Carfagna et al. 2011b). Additionally, *C. reinhardtii* cells exposed to low sulfate levels exhibited elevated sulfate transport activity (Pootakam et al. 2010) and both the transcript levels and the activities of the enzymes, associated with S assimilation, increased (Ravina et al. 2002, Zhang et al. 2004), allowing for efficient scavenging and assimilation of the S available from the environment. Here we present the first report detailing the effects of S starvation under both autotrophic and heterotrophic culture conditions in a microalga inhabiting hot springs with high S and sulfate levels.

Galdieria phlegrea cells, in auto- and heterotrophy, contained a double content of soluble proteins compared with the green microalga *C. sorokiniana* (Salbitani et al. 2015). The protein content in plant cells represents an important indicator of both reversible and irreversible changes in metabolism, being influenced by a large variety of stressors (Carfagna et al. 2011b).

According to our data, the content of soluble protein is different between the two cell types, being higher in heterotrophic cells, whereas the amount of total N is halved in cells grown in heterotrophy compared with those in autotrophy. This apparent contradiction can be explained by the lower content in heterotrophic cells of insoluble light-harvesting pigment—protein complexes and photosynthetic electron transfer chain components, which are N rich, and form a large and variable fraction of algal cell biomass (Leonardos and Geider 2005). For *G. sulphuraria*, as well as *G. partita*, it has been reported that glucose down-regulates the number of thylakoid membranes and photosynthetic pigments (Oesterhelt at al. 2007). This would also explain the lower content of total S found in cells in heterotrophy: some important sulfolipids are present in the thylakoid membranes (Sugimoto et al., 2008). In the cells in autotrophy, many proteins are insoluble or involved in complex formation bound to the photosynthetic thylakoid membranes. Furthermore, possibly amino acids or inorganic N may contribute to N in cells from autotrophic culture.

Sinetova et al. (2006) assessed that *G. sulphuraria*, closely related to *G. phlegrea*, contained 50—55% of total proteins in the cell wall. In *G. phlegrea*, S starvation caused a decrease in the total soluble protein content in autotrophic cells, similarly to the case in *C. sorokiniana* (Carfagna et al. 2011a) and *C. reinhardtii* (Ravina et al. 1999). It could be assumed that the S-starved cells of *G. phlegrea* may utilize S from the internal protein pool to redistribute the amino acid resource in order to satisfy their nutritional requirements, as occurs in *C. sorokiniana* (Carfagna et al. 2011). The decrease in S compounds (Carfagna et al., 2011) could also cause a reduction of ex novo protein synthesis.

S starvation induces degradation of lipids of thylakoid membranes of *C. reinhardtii*, thus inhibiting photosynthetic activity (Sugimoto et al. 2008). This may explain the decrease in the intracellular protein concentration of the microalga in autotrophic cultures.

S starvation did not affect the high levels of soluble proteins found in heterotrophic cells.

In algae and plants, glutathione represents an essential S-containing compound formed from cysteine, the first amino acid regarded as the terminal metabolite of S assimilation. Our results show that in *G. phlegrea* both in autotrophic cells but especially in cells cultured in heterotrophy, the glutathione content is much higher than that found in *C. sorokiniana* (Salbitani et al. 2015). Edwards et al. (2013) also speculated on the existence of a large organic S pool as glutathione in the red alga *Cyanidioschyzon merolae*.

During 24 h of S starvation, the glutathione level of *G. phlegrea* cells from autotrophic cultures strongly decreased, particularly during the first 6 h. A decrease in both the cysteine and glutathione intracellular levels was observed in *C. sorokiniana* cells grown under S deficiency within the first 4 h from the start of the S deprivation. In this regard, it is noteworthy that under S deficiency, in autotrophic cells the glutathione pool decreased and the GSH/total glutathione ratio increased, as already previously observed in *C. sorokiniana* cells (Salbitani et al. 2015).

Interestingly, in *G. phlegrea* cultured under heterotrophic conditions, the protein and glutathione concentrations were higher than those observed in the autotrophic cells. Also, S starvation increased glutathione intracellular levels, while the total soluble protein content remained high during the 24 h of S deprivation. Thus, it can be hypothesized that heterotrophic cells contain abundant reserves of organic S. Under S starvation, in heterotrophic cells, most probably the excess of C added to the medium was also stored in proteins and glutathione (Perez-Garcia et al. 2011). In cells cultured in heterotrophy and S starved, total elemental S did not vary significantly.

The elemental analysis of dry biomass from autotrophic and heterotrophic cultures under S starvation has revealed that the total C content was similar in the two types of cells, while the cell quota of N was strongly affected. However, it is very interesting to note, and in agreement with our data, that the total S decreased during the S starvation only in cells in autotrophy. In cells cultured in heterotrophy and S starved, total elemental S did not vary significantly.

On the other hand, the GSH/total glutathione ratio appears particularly low in heterotrophic cells under S deficiency, indicating the occurrence of a putative intracellular oxidative perturbation.

It has been proposed that glutathione can also act as signal to control the sulfate uptake rate in higher plants (Davidian and Kopriva 2010); however, sulfate transport, although well described in plants (Takahashi et al. 1997) and green algae (Yildiz et al. 1994), still requires investigations in Cyanidiophyceae.

It has been demonstrated that H_2S represents an S source for plants, being absorbed through stomata (Riemenschneider et al. 2005, Birke et al. 2015). It is reasonable to argue that similar metabolic routes also occur in other photosynthetic organisms and particularly in microalgae inhabiting S springs with high H_2S emissions. Birke et al. (2015) have recently demonstrated that during H_2S exposure in Arabidopsis, a large amount of excess sulfide was fixed and stored in the form of cysteine and glutathione, but also as thiosulfate. Unfortunately, nothing is known about the synthesis of thiosulfate in microalgae and whether it could be an S storage compound.

Among enzymes of the assimilatory sulfate reduction pathway, OASTL was of special interest because of the presence of multiple isoforms and because it was strongly affected by the nutritional status of plants (Carfagna et al. 2011b, Wirtz et al. 2012) and algae (Ravina et al. 1999, Carfagna et al. 2011a). S starvation causes a conspicuous time-dependent increase in the specific activity of OASTL in many organisms (Ravina et al. 1999, Davidian and Kopriva 2010, Carfagna et al. 2011a, Carfagna et al. 2011b, Wirtz et al. 2012). *Galdieria phlegrea* autotrophic cells seem to respond quickly to conditions of S deprivation, as indicated by the prompt induction of OASTL activity and mRNA transcription and by the concomitant reduction of their protein and thiol content. S starvation induced OASTL activity and decreased the levels of glutathione in autotrophic cells of *G. phlegrea* as well as in *C. sorokiniana* (Carfagna et al. 2011a). The increased OASTL activity in the autotrophic cells of *G. phlegrea* was able to compensate for the S deficiency in the culture medium by exerting significant control of the thiol metabolite concentrations and cysteine homeostasis. Expression analysis revealed an increase in mRNA encoding OASTL in cells cultured in autotrophy, more pronounced after 2 h (15-fold) of S starvation.

Neither OASTL activity nor mRNA encoding OASTL was altered in heterotrophic cells under S deprivation at least in the first 6 h. However, after 24 h, S-starved cells of *G. phlegrea* in heterotrophic conditions showed an increase in OASTL mRNA.

A possible explanation of this trend is ascribable to the high protein and glutathione content of heterotrophic cells and organic S which would not require OASTL activation. Furthermore, it is possible that in *G. phlegrea*, under heterotrophic conditions, *sac* genes are repressed (Ravina et al. 2002). However, the late increase of OASTL transcript levels (3.3-fold) could be considered as a response of cells to prolonged heterotrophic stress.

OASTL activity was enhanced in *G. phlegrea* under conditions of S deprivation, but only if the cells were cultured under autotrophic conditions, indicating that metabolic energy from photosynthesis is most probably essential. Moreover, under heterotrophic conditions, i.e. in continuous darkness and on glucose, the Calvin cycle was inactivated in *G. sulphuraria* (Oesterhelt et al. 2007). Numerous pieces of experimental evidence led to the hypothesis of a possible involvement of light in the assimilation of sulfate in plants, although it is still not clear which stages of S assimilation could be more affected. The ATP-sulfurylase activity increases with light irradiation in barley, corn andoats, and decreases with the addition of inhibitors of electron transport in photosynthesis (Astolfi et al. 2001). In *Arabidopsis thaliana*, mRNA levels of APS kinase, sulfite reductase, OASTL and SAT are higher in green leaves than in etiolated tissues (Kopriva et al. 1999).

Our results seem to indicate a strict link between the lack of photosynthesis and the regulation of S assimilation in *G. phlegrea* heterotrophic cells under S deficiency.

Cysteine biosynthesis in autotrophic S-starved *G. phlegrea* cells is regulated by the combination of transcriptional and post-transcriptional mechanisms, since there is a clear correlation between OASTL mRNA abundance and enzyme activities, and GSH intracellular levels, whereas in cells cultured in heterotrophy, S starvation does not affect the already high levels of proteins and glutathione, and the enzyme OASTL is not upregulated to produce more cysteine.

The results obtained here show for the first time that *G. phlegrea* accumulates glutathione under heterotrophic conditions; even under S starvation, the intracellular levels of glutathione in heterotrophic cells are higher than those found in cells grown in autotrophy. If the decline in glutathione levels under S starvation is due to the limitation of the amino acid cysteine, and then to the slowing down of S assimilation, we can speculate that the cysteine, required to maintain high levels of glutathione, derives from other reserves, such as proteins. The high content of total protein in heterotrophic cells of *Galdieria* would reinforce this hypothesis. Proteins in plant cells represent an important sink for reduced S in the form of cysteine also in Arabidopsis (Birke et al. 2015). On the other hand, the C backbone of glutathione derives from the utilization of glucose added to the culture and not from the products newly synthesized by photosynthesis. This would lead us to assume that in *G. phlegrea* heterotrophic cells an unusual co-regulation between C and S metabolism occurs.

In conclusion, *G. phlegrea* cells, cultured under either autotrophic or heterotrophic conditions, exhibit a distinct suite of responses when exposed to S deprivation. In autotrophic cells, the removal of S from the culture medium caused a decrease in the protein content and in the intracellular levels of glutathione, while cells from heterotrophic cultures exhibited high levels of internal proteins and high glutathione content, which did not diminish during S starvation.

In this study, we have shown that *G. phlegrea* cells are rich in proteins and glutathione, an S-containing molecule known as a powerful antioxidant. This finding opens up a promising avenue of research for the large-scale production of this molecule from *Galdieria* cultures.

Materials and Methods

Algal strains and cultivation

Experiments were performed with pure cultures of the red algae *Galdieria phlegrea* (strain 002/329) from the ACUF collection of the Department of Biology of the University of Federico II, Naples, Italy (http://www.biologiavegetale.unina.it/acuf.html).

Galdieria was grown in Allen's autotrophic medium (Allen 1959) containing 40 mg l^{-1} $(NH_4)_3PO_4$ as an N source. The initial algal concentration was set at 5×10^4 cells ml^{-1}. The flasks were placed on a Plexiglas shaking apparatus under continuous irradiance (150 µE m^{-2} s^{-1}) provided by daylight fluorescent Philips lamps (TLD 30 W/55). Carbon dioxide was supplemented by sparging filtered air into the medium. In heterotrophic cultures, each flask was wrapped with aluminum foil, and organic carbon was supplemented with 2% (w/v) glucose.

The pH was set at 1.5 and controlled daily, whereas the temperature was maintained at 38 ± 1°C.

The cultures were sampled daily, and growth was followed by measuring the optical density (OD) of the cultures at 550 nm with a spectrophotometer (Thermo, Helios Biomate5). The number of cells was determined by direct counting of the cells in the growth medium using a Bürker chamber.

Under autotrophic conditions, *G. phlegrea* grew exponentially for 39 d after a lag phase of 8 d; the stationary phase began on day 50. The cells cultured in autotrophic conditions had a growth rate of 0.26 d^{-1} (**Supplementary Fig. S1**). The microalgae grew faster heterotrophically in darkness with 2% glucose and, after a lag phase of 12 d, their growth rate was 0.28 d^{-1} (**Supplementary Fig. S1**).

In the experiments on S starvation, control cells were harvested during the logarithmic phase of growth (culture OD between 0.8 and 1.0) by low speed centrifugation at 4,000×*g* for 10 min and then washed twice with S-free Allen's medium where $(NH_4)_3PO_4$, $MgCl_2$ and $FeCl_2$ were substituted for their respective sulfuric salts and without changing the molarity of the other individual ions. Furthermore, the culture medium was adjusted to pH 1.5 with hydrochloric acid to replace sulfuric acid and to obtain S-starved cells. Then, the supernatant was removed, and the algal pellets were re-suspended in S-free Allen's medium and cultured for up to 24 h. In heterotrophic cultivation, the flasks were covered with an aluminum sheet to ensure cultivation in the dark.

Content of thiols

The reduced (GSH) and total glutathione were determined, by adapting the method of Anderson (1985) for algal cells. The cell pellet from 200 ml of algal culture was re-suspended in 3 ml of 5% (w/v) sulfosalicylic acid. Cells were lysed by a passage at 1,000 p.s.i. through a French pressure cell (Aminco) and centrifuged at 16,000 r.p.m. for 20 min at 4°C; the clear supernatant was used as crude extract. The concentration of total glutathione and GSH was determined as previously described (Salbitani et al. 2015). Thiol levels were expressed as pmol $cell^{-1}$.

Elemental cell content determination

Dry algal samples were powdered by a Fritsch Pulverisette (type 00.502) equipped with an agate mortar and ball mill. Elemental contents were determined by combustion in an Elemental Analyzer NA 1500 (Carlo Erba Strumentazione).

OASTL extraction and assay

Algal cells harvested by low-speed centrifugation (4,000×*g* for 10 min) were re-suspended in cold extraction buffer [50 mM potassium phosphate buffer (pH 7.5), 1 mM dithiothreitol, 10 µM pyridoxal 5′-phosphate] and were lysed by passage through a French pressure cell (Aminco) (1,000 p.s.i.). The homogenate was centrifuged at 16,000×*g* for 20 min at 4°C (Sorvall RC5C plus with a Sorvall SS34 rotor), and the clear supernatant was used as the crude extract. Enzymatic OASTL activity was determined colorimetrically by measuring the amount of cysteine formed in a reaction mixture, as described previously (Carfagna et al. 2011a). OASTL activity was expressed in units that correspond to the formation of 1 µmol of cysteine min^{-1}. The OASTL activity was correlated with the soluble protein content of the samples.

In cell extracts (from 100 ml of culture) the concentration of protein was determined by the Bio-Rad protein assay based on the Bradford method (1976), using bovine serum albumin as the standard. The number of cells (reported to 1 ml of culture) was determined by direct counting of the cells by a Bürker chamber. Then the protein concentration was reported as pg $cell^{-1}$.

cDNA preparation, real-time PCR primer design and sequence alignment

Specific sequences for the OASTL1 gene were searched in the *G. sulphuraria* genome available in GenBank (Schönknecht et al. 2013). The OASTL gene was labeled as cysteine synthase A (GASU_24750; Gene ID: 17088846). The deduced amino acid sequences were aligned with the CLUSTAL W program (Thompson et al. 1994). Multiple alignments with OASTL amino acid sequences belonging to red algae, green algae, bacteria, cyanobacteria and plants were performed, in order to ascertain the appropriate primers. Although no nucleotide sequence ascribable to OASTL2 was identified in the *G. sulphuraria* genome, a degenerate PCR strategy was used to confirm the absence of this gene also in the *G. phlegrea* genome. Then, primer pairs for OASTL1 were designed on the *G. sulphuraria* genome while degenerate primer pairs for OASTL2 were designed on the *C. merolae* genome and are reported in **Supplementary Table S1**.

PCR and real-time quantitative PCR analysis

Galdieria phlegrea cells were collected in logarithmic phase under autotrophy or heterotrophy and S starved for 2, 6 and 24 h. Total DNA was isolated from *G. phlegrea* by using the DNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. Total RNA of *G. phlegrea* was isolated by using the RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. The extracted total RNA was treated with DNase (Invitrogen Life Technologies) and the concentration of RNA determined by UV/visible spectroscopy, while its structural integrity was checked on a non-denaturing agarose gel, followed by ethidium bromide staining. First-strand cDNA was synthesized from total RNA using oligo$(dT)_{20}$ primers and the ThermoScript RT-PCR System (Invitrogen Life Technologies), according to the manufacturer's recommendations. PCR and quantitative real-time PCR were performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad) to analyze the specific expression patterns of the OASTL gene. cDNA was amplified in 96-well plates using the SsoAdvanced™ SYBR® Green Supermix (Bio-Rad), 25 ng of cDNA and 300 nM specific sense and antisense primers in a final volume of 20 µl for each well. Each sample was analyzed in triplicate. A sample without template was used as negative control, and a sample with non-retrotranscribed mRNA instead of template cDNA was used as control for genomic DNA contamination. Cycling parameters were denaturation at 95°C for 10 s and annealing/extension at 54°C for 30 s (repeated 40 times). For OASTL2, PCRs were performed for 40 cycles of 94°C (1 min), 54°C (1 min) and 72°C (1 min).

In order to verify the specificity of the amplification, a melt-curve analysis was performed immediately after the amplification protocol. The reference genes (actin and elongation factor α) were measured with three replicates in each PCR run, and the average Ct value was used for relative expression analysis. The amplification efficiency (E) and correlation coefficient (R^2) of each reference/target gene were determined using a pool representing all cDNA samples by a five-point standard curve based on a 10-fold dilution series. Relative fold changes in gene expression were calculated using the comparative $2^{-\Delta\Delta^{Ct}}$ method using the geometric mean of all the reference genes for normalization (Vandesompele et al. 2002, Schmittgen and Livak 2008). Three biological and three technical repetitions were performed for each treatment and time point.

Statistical analysis

Experimental data analyses were carried out using Sigmaplot 12 software. Data of the mean ± SE of 3—6 independent experiments were presented.

The statistical analysis was performed by one-way or two-way analysis of variance (ANOVA) with a Tukey post-hoc test to determine differences between autotrophic and

heterotrophic algae, S-starved cells or non-S-starved cells; $P < 0.001$ as significant. If necessary, the data were log + 1 (x) transformed before the analysis.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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