# Title page

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**Changes in ATP sulfurylase activity in response to altered cyanobacteria growth conditions.**

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# Changes in ATP Sulfurylase activity in response to altered cyanobacteria growth conditions

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Here we investigated variations in cell growth and ATP sulfurylase activity when two cyanobacterial strains – *Synechocystis* sp. PCC6803 and *Synechococcus* sp. WH7803 – were grown comparatively between conventional media and media with low ammonium, low sulfate and a controlled high $CO_2$/low $O_2$ atmosphere, which might resemble some Precambrian environments. In both organisms, a transition and adaptation to the reconstructed environmental media resulted in a decrease in ATPS specific activity. This decrease in activity appears to be decoupled from growth rate, suggesting the enzyme is not rate-limiting in S assimilation and raising questions about the role of ATPS redox regulation in cell physiology and thorughout history.

**KEYWORDS**: ATPS, Sulfur, *Synechocystis*, *Synechococcus*, Proterozoic

Sulfur is a universal and integral component of metabolism and biomass. Able to vary oxidation states from +6 to -2, and to populate the 3d orbitals, sulfur forms bonds with both carbon and iron, creating a bridge between the inorganic and organic in the cell (Beinert, 2000). With this electronic and molecular versatility, sulfur is involved in diverse and unique metabolisms across the tree of life (Dahl and Friedrich, 2008). At the same time, it is found in conserved co-factors and biomass components such

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as S-adenosyl methionine (Bridwell-Rabb et al., 2018; Marsh et al., 2010), coenzyme A (Strauss, 2010), and proteogenic cysteine residues, with their attendant in Fe-S clusters (Beinert et al., 1997; Gao, 2020; Rouault, 2019). With these proprieties, sulfur involving reactions likely had a prominent role from the origin of life onward (De Duve, 1991; Goldford et al., 2017; Russell et al., 1994; Wächtershäuser, 1990). Much later in evolution, sulfur availability in the oxidized form of sulfate may have influenced oceanic phytoplankton primary productivity (Giordano and Prioretti, 2016; Norici et al., 2005), and the radiation and evolution of this group (Ratti et al., 2011). Going further, sulfate variability in the ocean may also have recorded a linkage between animal evolution and the geochemical record of sulfate deposits (Canfield and Farquhar, 2009).

A considerable literature exists describing sulfur in the metabolism, ecology, and evolution of phytoplankton (Giordano et al., 2005, 2008; Kopriva and Rennenberg, 2004; Prioretti and Giordano, 2016; Takahashi et al., 2011). Some prominent areas of discussion are the sulfonium compound metabolism of DMSP/DMS (Giordano and Prioretti, 2016; Giordano et al., 2005; Ratti and Giordano, 2008; Takahashi et al., 2011), and protein Fe-S cluster biosynthesis (Cassier-Chauvat and Chauvat, 2014; Gao, 2020; Rina et al., 2000). In unicellular algae and cyanobacteria (as in many other organisms), S acquisition from the environment into biomass begins from sulfate (Giordano and Prioretti, 2016). Sulfate is kinetically inert and requires activation, which is then followed by reduction to biomass appropriate oxidation states (**Fig. 1**). **ATP Sulfurylase** (**ATPS** - EC 2.7.7.4) has the key role of $SO_4^{2-}$ activation at the beginning of S assimilation pathway, hydrolyzing ATP and producing a sulfate-ester (Giordano and Prioretti, 2016; Prioretti et al., 2014; Schmidt, 1972, 1988; Takahashi et al., 2011; Ullrich et al., 2001).

In plants, S assimilation is regulated at different levels in response to growth conditions and in accordance with C and N metabolisms (Khan et al., 2010; Kopriva and Rennenberg, 2004; Koprivova and Kopriva, 2014; Martin et al., 2005; Takahashi et al., 2011; Vauclare et al., 2002). In both algae and cyanobacteria, diverse regulatory mechanisms exist (Schmidt, 1988; Takahashi et al., 2011) and have been investigated (Kopriva et al., 2002; MacRae et al., 2001; Patron et al., 2008; Prioretti et al., 2014). At the stage of sulfate activation, redox regulation of ATPS enzyme activity was postulated and subsequently

confirmed (Prioretti et al., 2014, 2016). Within cyanobacteria, the redox regulated isoform, **ATPS–B,** contains 5 conserved cysteine residues (Prioretti et al., 2014), whereas **ATPS–A**, with only 4 conserved cysteine residues (Prioretti et al., 2014), appears to lack the critical regulatory S-residue organization thought to allow disulfide bridge formation and the modulation of enzyme activity.

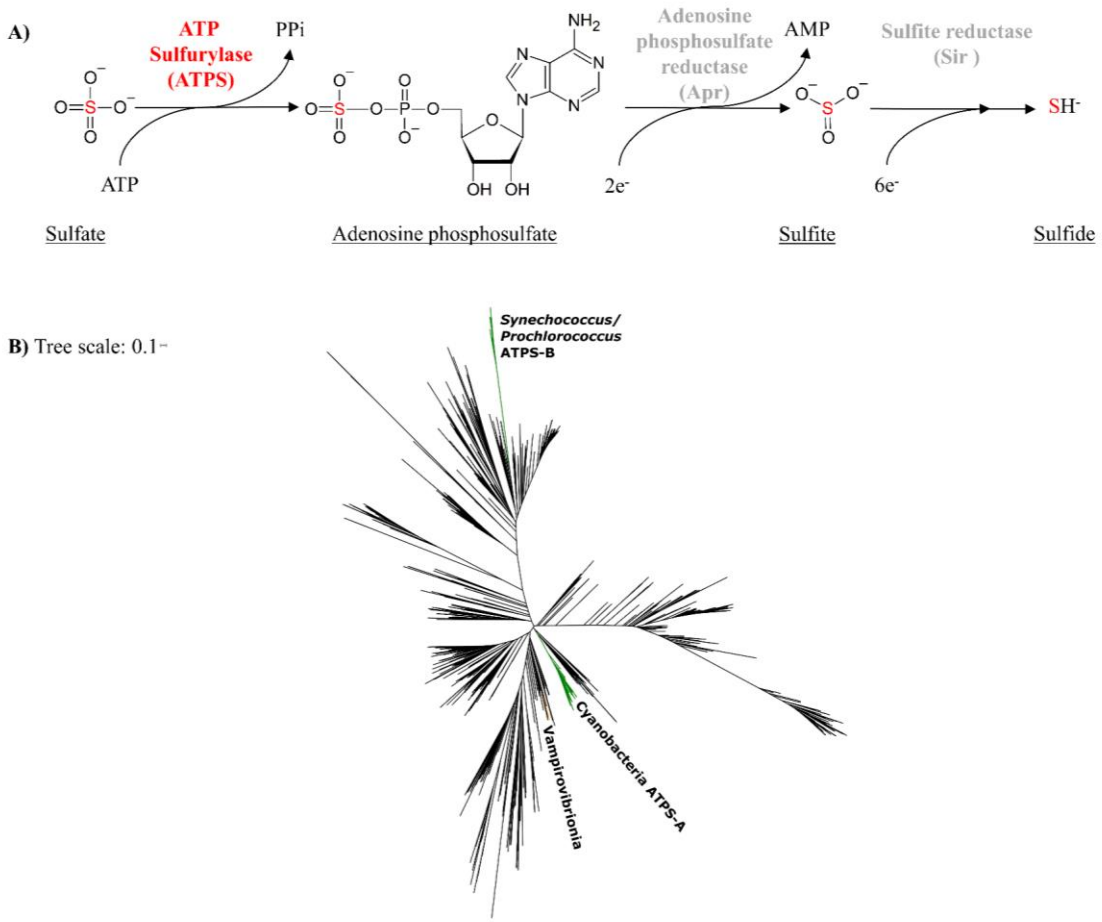


***Fig. 1 A)*** *The role of ATP Sulfurylase in sulfate activation and the downstream steps leading to sulfide. The two step PAPS path can also reduce adenosine phosphosulfate to sulfite but is not shown.* ***B)*** *Phylogeny of ATP sulfurylase in bacteria and archaea. Homologs from the Vampiriovibrionia (an ancestral non-photosynthetic cyanobacteria class) are in orange, while the oxygenic cyanobacteria are labelled in green as the Synechococcus/Prochlorococcus clade with the ATPS-B isoform, and those cyanobacteria with the ATPS-A isoform. Remaining archaea and bacteria are colored in black. A more detailed tree can be found in the* *Supplemental Materials*

It has been hypothesized that freshwater and marine cyanobacteria species are characterized by different evolutionary histories (Sánchez-Baracaldo, 2015; Sánchez-Baracaldo et al., 2017). Consequently, the different environments in which they evolved (perhaps including less or more oxidizing) could have influenced the presence/absence of redox regulation in specific proteins. The phylogenetic distribution pattern of ATPS homologs (**Fig. 1B** – see Supplemental Material for the explanation of how the tree was constructed) is suggestive this. The tree shows how the marine photosynthetic *Synnecococcus*/*Prochloroccoccus* (Syn/Pro) clade are well separated from other photosynthetic cyanobacteria (including the freshwater group). Notably, the Vampiriovibrionia class, since it represents an ancient and non-photosynthetic cyanobacteria taxon (Soo et al., 2019), was expected to be on a different branch and indeed it is possible to recognize as such (**Fig. 1B**). Within freshwater cyanobacteria species (and those marine species not enclosed in the Syn/Pro clade) the ATPS–A isoform without redox regulation is found, while, in the more derived Syn/Pro clade (which constitutes the picocyanobacteria plankton) the ATPS–B isoform with redox-regulation is found (Giordano and Prioretti, 2016; Prioretti et al., 2014, 2016). This pattern of distribution also corresponds to ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), and carboxysomes (Badger and Price, 2003; Rae et al., 2013): cyanobacteria with ATPS–A match the freshwater and brackish β-cyanobacteria which possess RubisCO-1B and β-carboxysomes; while those with ATPS–B coincide with the marine α-cyanobacteria that have RubisCO-1A and α-carboxysomes (Prioretti et al., 2016).

Protein phylogenies can be linked with gene transfer events during evolution, and several studies indeed confirmed the importance of horizontal gene transfer (HGT) during the evolution of the oxygenic photosynthesis in the cyanobacteria taxon (Fischer et al., 2016 and references in it). It has been observed for example that the Syn/Pro clade seems to have received a large number of genes via HGT from Proteobacteria: several genes involved in the formation of the α–carboxysome have been transfer from this group to Cyanobacteria along with bacteriochlorophylls synthesis genes (Bryant et al., 2012; Ward and Shih, 2020). The ATPS phylogeny (where Proteobacteria are more distributed across the tree; Supplemental Materials) is consistent with this protein having been part of this exchange (**Fig. 1B**), with

the ATPS–B sequences nested within a clade primarily made up of proteobacterial sequences and very distant from the ATPS–A clade made up of other cyanobacteria. Moreover, a small number of Vampiriovibrionia species having a different version of ATPS protein is consistent with them acquiring it via HGT after their divergence from oxygenic Cyanobacteria, similar to how they've acquired those proteins involved in the aerobic respiration (Soo et al., 2017, 2019).

To further our understandings of the ATPS protein and its regulation in cyanobacteria, we grew two cyanobacteria species: the freshwater *Synechocystis* sp. PCC6803 (referred to simply as *Synechocystis* from now on) with non-redox regulated ATPS–A and, and the marine *Synechococcus* sp. WH7803 (referred to as *Synechococcus* from now on) with redox-regulated ATPS–B in multiple growth conditions and measured the resulting enzyme activity with a crude cell extract assay. The experiments allowed a comparison of enzyme activity levels between the two isoform types when expressed in cells exposed (and adapted) to the modern environment and a possible Precambrian condition. In particular, we considered the Proterozoic Eon, which lasted from 2.5Gyr to 0.6/0.5Gyr ago (refer to the following for more description: Fischer, 2008; Knoll and Nowak, 2017; Lyons et al., 2014; Rasmussen et al., 2008) and saw the oxygenation of Earth's atmosphere (Lyons et al., 2014). During this period is where most evolutionary theories place the differentiation and radiation of the cyanobacteria taxon (Sánchez-Baracaldo, 2015; Schirrmeister et al., 2016; Shih et al., 2017 - despite most of the extant diversity of this taxon having been accumulated during the Phanerozoic Eon - Louca et al., 2018).

For each specie, 3 experimental conditions (with 3 biological replicates each) were set up in a growth chamber (12h light/12h dark cycle, temperature 20°C and light with a white LED lamp at 50μmol photon/$m^2$s of irradiance). The cells were grown as semi-continuous cultures with the dilution volume based on growth rate data (Gastoldi et al in prep.) The three conditions analysed were: **Standard Condition** (**ST – Table S1**), the **Possible Proterozoic Condition** (**PPr – Table S2**) and the **Transitional Condition** (**TR – Table S3**). AMCONA medium (Fanesi et al., 2014) was used for the marine *Synechococcus* specie while the BG11 medium (Stanier et al., 1971) was used for the freshwater *Synechocystis*. All the liquid cultures were bubbled continuously with the atmosphere corresponding to

the specific condition and experiments were performed after an adaptation period (2/3 months). The PPr condition had a higher $CO_2/O_2$ ratio than today (10'000ppm of $CO_2$ ensured by a controlled gas system) while the TR and PPr growth media had a decrease in sulfate, a switch from nitrate to ammonium, and, in the case of the *Synechococcus*, lowered Fe concentrations compared to the ST. TR and PPr were identical except for the gas composition, with TR using air and PPr bubbled with a $CO_2$ mix (details available in Supplemental Materials). These alterations might capture some historical variability during the Proterozoic Eon, but this can and should be debated.

Growth curves were determined as explained in other works (Gastoldi et al.; in preparation). ATPS activity was measured using crude cell extracts (refer to Supplemental Materials for a detailed procedure - which folllowed Giordano et al., 2000): the activity was observed spectrophotometrically at 25°C for 15 minutes and the linear phase of the assay was considered for the data analyses, as in previous works (Burnell, 1984; Giordano et al., 2000; Prioretti et al., 2016). The specific activity of ATPS (expressed in $\mu mol/min \cdot ml^{-1}$) in the crude extract was then normalized to the concentration of the protein (expressed in *mg/ml*) of the extract itself which was determined through the Lowry/Peterson technique (Lowry et al., 1951; Peterson, 1977).

For both organisms, the specific activity of the ATPS enzyme in the cells was very different between conditions. In *Synechocystis*, the mean activity value was 1459nmol/min $mg^{-1}$ in the ST condition, higher than in the TR condition (164nmol/min $mg^{-1}$ – ANOVA, *p-value* = 0.000753, Tukey as post doc, n=9, **Fig. 2**), where S, and Fe were lowered in concentration compared to the standard media and N changed from nitrate to ammonium. The TR condition showed a lower value than also PPr condition, where the value was 634nmol/min $mg^{-1}$, though the difference between TR and PPr was less significant judged by a Tukey test (used as post hoc) which gave a higher *p-value* (*p-value* = 0.071).

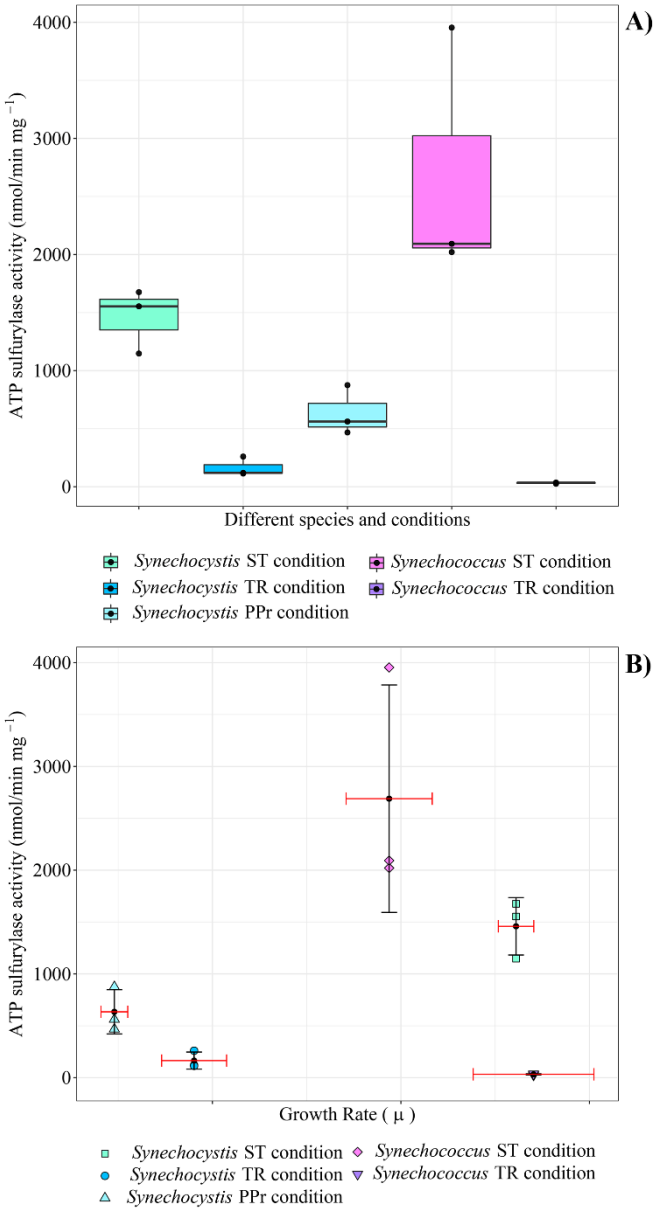


***Fig. 2*** *ATP sulfurylase activity.* ***A)*** *Each box represents the interquartile range of a specific condition and the black dots are the biological replicates for each one: the black line is the median and represents the second quartile Q2. Above the line is the upper quartile Q3 while below the line is the lower quartile Q1. The lines that come out from each box represent the minimum (lower) and the maximum (higher) value in the data.* ***B)*** *Each point represents a biological replicate; each condition is characterized with different shape and color. The black dot in each set of values is the mean of both the ATPS activity and the Growth Rate for the specific dataset. For each mean a Standard Deviation was also added, black SD*

*for the ATPS activity value and red SD for the Grow Rate value. Single values used for this figure can be found in* ***Table S4*** *in the* *Supplemental material*

In *Synechococcus*, the average value was 2689nmol/min $mg^{-1}$ in the ST condition while in the TR condition was only 32nmol/min $mg^{-1}$ (t-Test *p-value* = 0.0137, n=6). The experiment was not possible for the seawater PPr condition since the *Synechococcus* specie was not able to survive in that condition despite several attempts for its adaptation.

In both *Synechocystis* and *Synechococcus*, the difference between the ATP sulfurylase activity in the ST and the TR conditions points out that media components other than supplied gas can have strong effects on the enzyme activity and growth, since the atmosphere was the same in both conditions. It may be that, in these organisms, a limitation in more than one nutrient (in our work we lowered sulfate and switched from nitrate to ammonium between ST and TR conditions), could decrease ATPS expression. This result is contrary to previous results where sulfate alone was varied and the activity increased in *Synechococcus* sp. WH7803 (Prioretti and Giordano, 2016). Further work is needed to address the factors that regulate ATPS, but we can now see that both nutrients supplied as dissolved salts can strongly affect the enzyme specific activity.

Plotting enzyme activity vs. growth rate for both the organisms, a trend between growth rate and ATPS activity was not observed, suggesting that the enzyme activity itself is not limiting growth in these conditions (**Fig. 2B**). In the marine *Synechococcus*, the growth rate in the TR condition was higher than the ST condition, but the ATPS enzyme activity was much lower, despite being in a medium with lower sulfate. In the case of *Synechocystis*, which could grow in the PPr condition and does not have a redox regulated ATPS (Prioretti et al., 2016), a higher value of ATPS activity was found in the PPr condition than in the TR condition, despite a lower growth rate value in the PPr condition (**Fig. 2B**). One hypothesis stemming from this observation is that the lower $O_2$ available, which occurred in the PPr environment, promotes the activity of the enzyme or its expression. Although still preliminary, more data of this type – together with sulfur quota data which can be related with growth rates for some algae (Prioretti and

Giordano, 2016) – will aid in assessing the relationships between ATPS enzyme activity, growth rate, and sulfur cell content. It is curious that in *Synechococcus*, while the enzyme activity decreased the S content of the cells increased (Gastoldi et al.; in prep). This could again imply that the enzyme is not limiting in the uptake of S. Together, our results pose questions as to why the enzyme is redox regulated in some organisms, and also why the activity varies so greatly between conditions.

We analyzed the phylogenetic distribution, and regulation of enzyme activity at the first step in ATP and electron requiring sulfate assimilation. By studying two organisms – one with redox regulation at the ATPS step and one without, our motivation was to gain insight into intra-cell redox responses in relationship to environmental redox. Our results highlight that cyanobacterial lineages display unique phenotypes in these conditions. This variability adds richness – and some complication – to theories about cyanobacteria evolution and adaptation to the oxygenation of the planet, as other works started recently to point out (Herrmann et al., 2020; Uchiyama et al., 2020). A long term goal is to investigate the possible linkages between metabolic regulation and Earth history.

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## LIST OF FIGURES

**Fig. 1 A)** The role of ATP Sulfurylase in sulfate activation and the downstream steps. **B)** Phylogeny of ATP sulfurylase in bacteria and archaea. Homologs from the Vampiriovibrionia (an ancestral non-photosynthetic cyanobacteria class) are in orange, while the oxygenic cyanobacteria are labelled in green as the Syne/Pro clade with the ATPS-B isoform, and those cyanobacteria with the ATPS-A isoform. Remaining archaea and bacteria are colored in black. A more detailed tree can be found in the Supplemental Materials 4

**Fig. 2** ATP sulfurylase activity. **A)** Each box represents the interquartile range of a specific condition and the black dots are the biological replicates for each one: the black line is the median and represents the second quartile Q2. Above the line is the upper quartile Q3 while below the line is the lower quartile Q1. The lines that come out from each box represent the minimum (lower) and the maximum (higher) value in the data. **B)** Each point represents a biological replicate; each condition is characterized with different shape and color. The black dot in each set of values is the mean of both the ATPS activity and the Growth Rate for the specific dataset. For each mean a Standard Deviation was also added, black SD for the ATPS activity value and red SD for the Grow Rate value 8