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Proteomics of Carbon Fixation Energy Sources in *Halothiobacillus neapolitanus*

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Running title: Sulfur oxidation as energy for carbon fixation

Abstract

Through the use of proteomics, it was uncovered that the autotrophic, aerobic purple sulfur bacterium *Halothiobacillus neapolitanus* displays changes in cellular levels of portions of its carbon dioxide uptake and fixation mechanisms upon switch from bicarbonate to CO_2(g) as carbon source. This includes an increase in level of a heterodimeric bicarbonate transporter along with a potential switch between form I and form II of RubisCO. Additional changes are seen in several sulfur oxidation pathways, which may indicate a link between sulfur oxidation pathways as an energy source and carbon uptake/fixation mechanisms.

Introduction

Halothiobacillus neapolitanus is an obligate aerobic chemolithoautotroph capable of utilizing the complete oxidation of inorganic sulfur compounds as its sole source of metabolic energy (Garrity *et al.* 2005). While formally classified within the Purple Sulfur Bacteria (PSB) (Kelly and Wood 2000; Ghosh and Dam 2009), *H. neapolitanus* does not perform anoxygenic photosynthesis as it lacks the necessary photosynthetic reaction centers and associated antenna pigments (Lucas *et al.* 2009). It does, however, possess carboxysomes, which allow for aerobic autotrophic growth (Kerfeld *et al.* 2010; Bonacci *et al.* 2012).

H. neapolitanus genome sequence (Lucas *et al.* 2009) indicates that this species contains genes for a diverse set of sulfur oxidation (sox) activities including; a) a sulfur oxygenase/reductase homologous to that found in archaea species (Veith *et al.* 2012), b) several genes for homologs of sulfide:quinone reductases found in green sulfur bacteria (GSB) species (Gregerson *et al.* 2011), c) genes for a complete thiosulfate oxidizing multi-enzyme system (TOMES) pathway similar to that found in *Paracoccus pantotrophus* GB-17 (Friedrich *et al.* 2005; Bardichewsky *et al.* 2006; Reijerse *et al.* 2007; Zander *et al.* 2011), d) a flavocytochrome based sulfide dehydrogenase homologous to FccA/FccB (9) and e) a

unique tetrathionate forming thiosulfate dehydrogenase that appears to be hetero-oligomeric as opposed to the homo-dimeric enzyme from the PSB *Al. vinosum* reported by Denkmann *et al.* (Denkmann *et al.* 2012; Brito *et al.* 2014).

One distinguishing feature of the sox gene arrangement in *H. neapolitanus* is the unique arrangement of genes for the TOMES pathway. The majority of organisms studied to date display a general pattern of having a core set of enzymes (sox AXYZBCD) in either a single operon or at least closely spaced within the genome. *H. neapolitanus* shows no such arrangement with TOMES components widely dispersed through the genome and even on opposite strands (Figure 1).
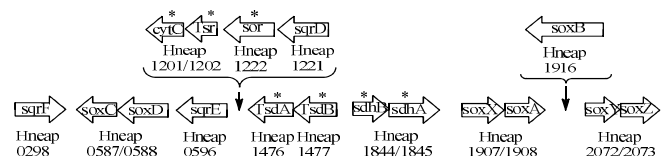
Figure 1. Relative arrangements of genes reported to be associated with oxidation of inorganic sulfur in *H. neapolitanus.* Note the lack of a single, contiguous sox operon.

Carbon fixation in *H. neapolitanus* appears to be primarily associated with a carbon concentration mechanism (ccm) and carboxysomes containing the carbon fixation mechanism. The ccm is composed of one heterodimeric bicarbonate transporter at Hneap_0211/0212 and a heterotrimeric transporter at Hneap_0907-0909 as shown in Figure 2. Both of these appear to be similar to those reported for *T. crunogena* by Scott and colleagues (Mangiapia *et al.* 2017). *H. neapolitanus* does not appear to possess genes for additional types of bicarbonate transporters (Scott *pers. comm.*).

In addition to the aforementioned carbon concentration mechanism, genes are present for a protein shell-enclosed carboxysome. These genes include shell proteins, a shell-based carbonic anhydrase

(CA) and cbbS, cbbM, cbbL subunits of RubisCO (Figure 3). The properties and role of carboxysomes in *H. neapolitanus* in carbon fixation have been well characterized by Heinhorst and colleagues (Kerfeld *et al.* 2010).
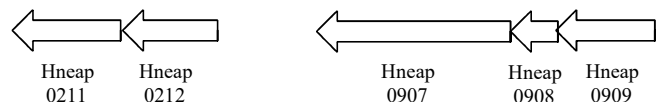

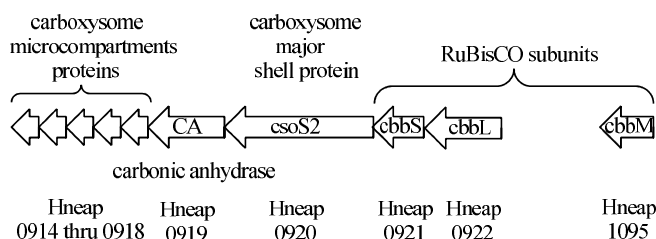Figure 2. Gene arrangement for the predicted bicarbonate transporters in *H. neapolitanus*.

Figure 3. Relative gene locations for genes comprising the ribulose-1, 5-bisphosphate carboxylase-oxygenase (RubisCO) of *H. neapolitanus*.

While the structure and role of carboxysomes have been well studied in a wide variety of autotrophic microbes, the unique gene arrangement of the sulfur oxidation (i.e. energy producing) pathways of *H. neapolitanus* gives rise to questions as to the relationship between sulfur oxidation as an energy source and carbon fixation in this species. This report uses proteomics technologies to explore the relationship between changes in dissolved inorganic carbon speciation and energy producing pathways in the obligate autotroph *H. neapolitanus*.

Materials and Methods

Halothiobacillus neapolitanus, DSM 15147, was obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) in Braunschweig, Germany. Cells were cultured at a constant pH of 7.0 +/- 0.2 in the media previously described by Heinhorst and coworkers, using thiosulfate as energy source (Kerfeld *et. al.* 2010). All cultures were performed at 30°C in a continuous flow chemostat using a dilution rate of 0.25. Constant aeration at 3L/min per liter of culture volume was used in order to maintain aerobic growth conditions.

The effect of different forms of dissolved inorganic carbon (DIC) was assessed by dividing replicate growths into two separate groups with carbon sources as follows: a) 5mM sodium bicarbonate supplemented into the growth media accompanied by aeration with CO_2 free (i.e. scrubbed) air and b) aeration with 5% (v/v) CO_2 in air.

Growths for each carbon source were performed in triplicate. Harvested cell mass from individual growths was flash frozen at -77°C and stored at -80°C until submitted to the University of Arkansas for Medical Sciences Proteomics Core facility for quantitative analysis.

Proteins were reduced, alkylated, and purified by chloroform/methanol extraction prior to digestion with sequencing grade modified porcine trypsin (Promega). Tryptic peptides were labeled using tandem mass tag isobaric labeling reagents (Thermo) following the manufacturer's instructions and combined into one multiplex sample group. The labeled peptide multiplex was separated into 36 fractions on a 100 x 1.0 mm Acquity BEH C18 column (Waters) using an UltiMate 3000 UHPLC system (Thermo) with a 40 min gradient from 99:1 to 60:40 buffer A:B ratio under basic pH conditions, and then consolidated into 12 super-fractions. Buffer A was composed of 0.1% formic acid and 0.5% acetonitrile in water. Buffer B was composed of 0.1% formic acid in 99.9% acetonitrile. Both buffers were adjusted to pH 10 with ammonium hydroxide. Each super-fraction was then further separated by reverse phase XSelect CSH C18 2.5 um resin (Waters) on an in-line 150 x 0.075 mm column using an UltiMate 3000 RSLCnano system (Thermo). Peptides were eluted using a 60 min gradient from 97:3 to 60:40 buffer A:B ratio. Eluted peptides were ionized by electrospray (2.15 kV) followed by mass spectrometric analysis on an Orbitrap Fusion Lumos mass spectrometer (Thermo) using multi-notch MS3 parameters. MS data were acquired using the FTMS analyzer in top-speed profile mode at a resolution of 120,000 over a range of 375 to 1500 m/z. Following CID activation with normalized collision energy of 35.0, MS/MS data were acquired using the ion trap analyzer in centroid mode and normal mass range. Using synchronous precursor selection, up to 10 MS/MS precursors were selected for HCD activation with normalized collision energy of 65.0, followed by acquisition of MS3 reporter ion data using the FTMS analyzer in profile mode at a resolution of 50,000 over a range of 100-500 m/z.

Data Analysis

Proteins were identified and reporter ions quantified by searching the UniprotKB *Halothiobacillus*

neapolitanus database (2,353 entries) using MaxQuant (Max Planck Institute) with a parent ion tolerance of 3 ppm, a fragment ion tolerance of 0.5 Da, a reporter ion tolerance of 0.001 Da, fixed modifications including carbamidomethyl on C, TMT-10 plex on K and the peptide N-terminus, and variable modifications including oxidation on M, and acetylation of the protein N-terminus. Scaffold Q+S (Proteome Software) was used to verify MS/MS based peptide and protein identifications (protein identifications were accepted if they could be established with less than 1.0% false discovery and contained at least 2 identified peptides; protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii *et al.* 2003) and to perform reporter ion-based statistical analysis. Quantitative comparisons between samples grown using $CO_{2(g)}$ versus the bicarbonate control were done using the Mann-Whitney test with the Benjamini-Hochberg correction in order to compare triplicate samples grown using either $CO_{2(g)}$ or bicarbonate as DIC source.

Results

A change in DIC species from 5mM bicarbonate ion to dissolved $CO_{2(aq)}$ appears to induce changes in cellular levels of a number of proteins including bicarbonate transporters, several enzymes in sulfur oxidation pathways and specific terminal oxidases. Only slight changes of less than 0.5 log2fold are seen in cellular levels of traditional stress response proteins in both cytoplasmic and extracytoplasmic spaces (Figure 4). Since 0.5 log2fold change is the generally accepted minimum to be considered a significant change, these low levels of change indicate that the observed changes in cellular levels of other proteins are probably not linked to a general stress response type of mechanism. It is interesting to note that those chaperones traditionally associated with the cytoplasm display a general trend of increases with log2fold increases of +0.37, +0.31 and +0.38 for groL, groS and dnaK respectively, while those associated with extracytoplasmic activities display decreases of -0.12 and -0.36 for surA and ompH.

Changes that are more significant are seen in the cellular levels of proteins associated with carbon uptake and fixation. Figure 5 shows that the dimeric bicarbonate transporter located at gene loci Hneap_0211/0212 displays a log2fold increase of +1.16 and +1.26 for the two respective subunits. The two detected subunits of the trimeric transporter show no significant change in level with log2fold changes between -0.05 and +0.17. These results indicate that

only one of the two bicarbonate transporters appears to be sensitive to CO_2 as carbon source. No information is available on the relative sensitivity of either transporter to bicarbonate concentrations.
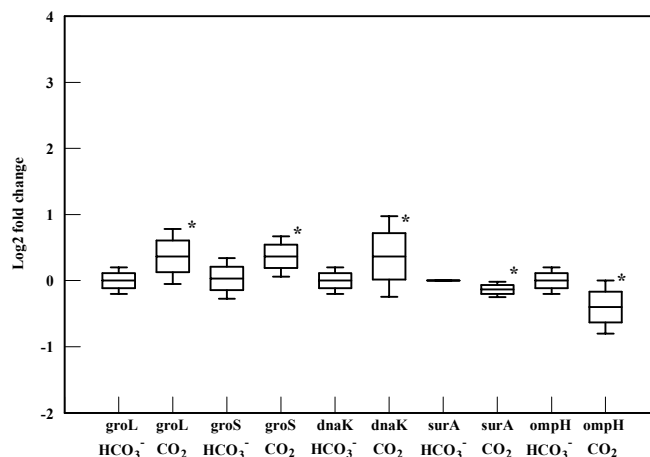

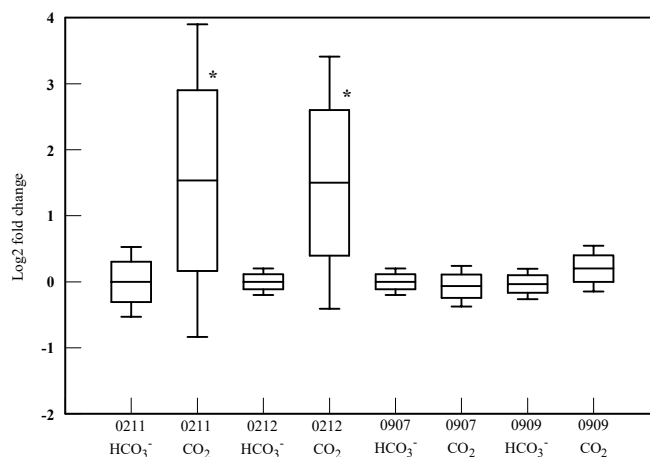Figure 4. Changes in cellular levels of chaperones associated with changes in DIC species. * p-values <0.05.

Figure 5. Changes in cellular level of the dimeric bicarbonate transporter (Hneap_0211/0212) and the trimeric bicarbonate transporter (Hneap_0907/0908/ 0909). * p-values < 0.05

In addition to the carbon concentration mechanism, minor changes in the relative levels of individual peptides associated with the RubisCO and the carboxysome were detected. A general trend of increased levels of peptide associated with form I was detected with log2fold changes of +0.49 and +0.56 for carboxysome shell protein 1 (csS1) and the RubisCO small subunit (cbbS) respectively. Other proteins displayed increases less than 0.25. Changes in level of subunit cbbM, form II, are not as clear. While log2fold change is calculated at 0.02, the p-values of < 0.05

coupled with the wide error of means in the HCO_3^- reference sample makes interpretation difficult (Fig. 6).
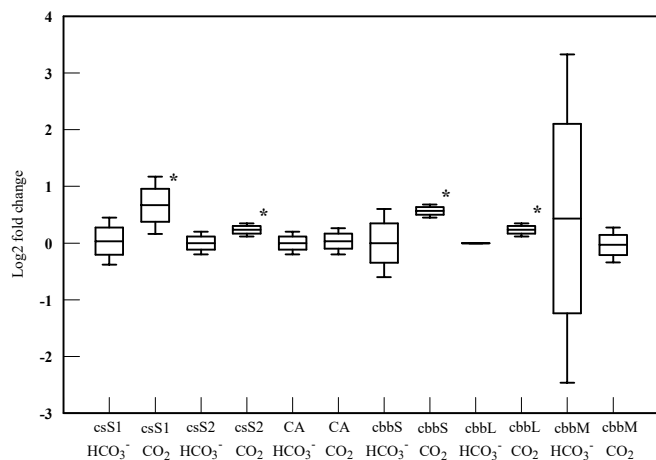

Figure 6. Changes in cellular levels of proteins associated with the carboxysome and RubisCO. * p-values < 0.05.

Changes in levels of proteins involved in cellular energy production include both those involved with substrate level oxidations and terminal oxidases. Figure 7 shows changes in the levels of proteins involved in sulfide oxidation. The sulfide:quinone reductase homologs show several significant changes with sqrF increasing 0.92 log2fold and the sqrD and sqrE decreasing by 0.70 and 0.37 log2fold respectively. Subunit B of the cytochrome linked sulfide dehydrogenase only increased by 0.34 log2fold. It is interesting to note the general trend for these sulfide oxidation proteins. Those that show slight increases are
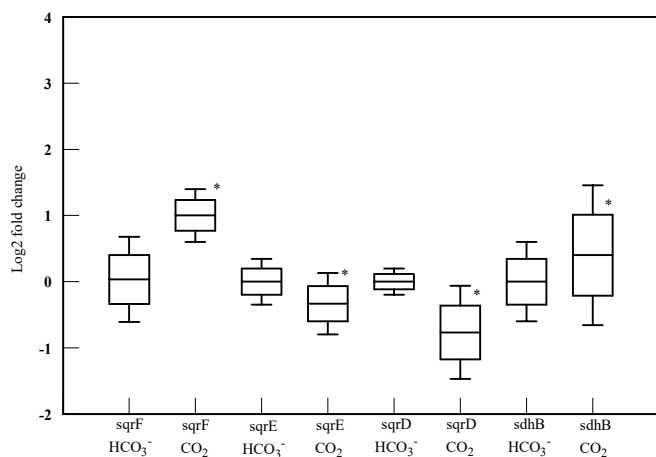

Figure 7. Sulfide:quinone oxidoreductases and sulfide dehydrogenase levels show a general trend of increases for those protein predicted to face the extracytoplasmic space. * p-values < 0.05.

homologs of proteins in the green sulfur bacteria (GSB), which are predicted to face the extracytoplasmic space increase while those that display decrease would be predicted to face the cytoplasm in GSB (9).

No significant changes in cellular levels of either the tetrathionate forming (tsdA and tsdB) or TOMES (soxY and soxB) thiosulfate oxidizing pathway proteins appears to occur with log2fold changes ranging from -0.20 to 0.01. Although the remainder of the sox pathway proteins (Z, AX, CD) are not shown for clarity, they follow the same trend with log2fold changes ranging from -0.24 to 0.08. The sulfur oxygenase-reductase however, shows a 0.75 log2fold decrease as shown in Figure 8.

In addition to sulfur oxidation proteins, changes in levels of caa3, bd and cbb3 terminal oxidases were also detected (Figures 9 and 10). Figure 9 shows a clear trend in decrease in levels of the majority of subunits of the caa3 type terminal oxidase with decrease ranging from 2.26 to 0.83 log2fold. Other terminal oxidases display a lower sensitivity to the DIC source (Figure 10). While the bd-quinol oxidase at Hneap_1294/1295 displays a decrease of 0.57 log2fold in the detected subunit, the cbb3 type terminal oxidase displays log2fold changes ranging from -0.06 to +0.20 for the Hneap_1876 through 1880 subunits respectively, indicating a slight sensitivity to DIC species for the bd- quinol oxidase and little or no sensitivity for the cbb3 type terminal oxidase.
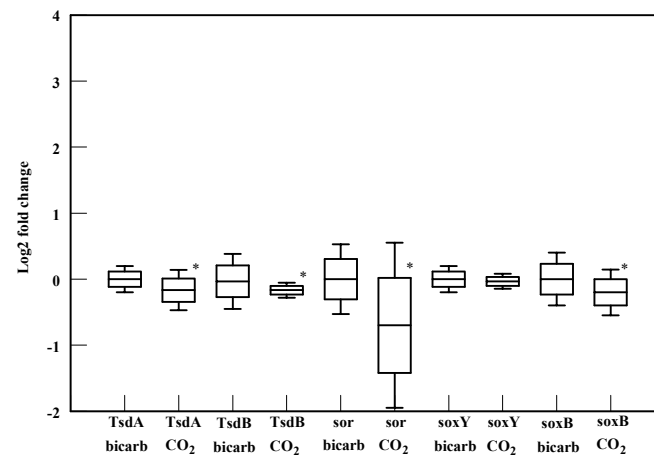

Figure 8. Thiosulfate oxidation by thiosulfate dehydrogenase (tsdAB) and TOMES (soxY and soxB). Sulfur oxidation (sor) is included for comparison. * p-values < 0.05.

Discussion

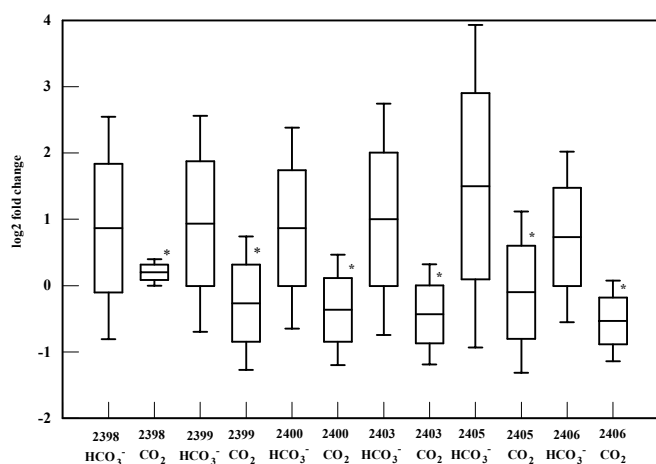The low level of change in cellular levels of stress response related proteins indicates that the shift in DIC

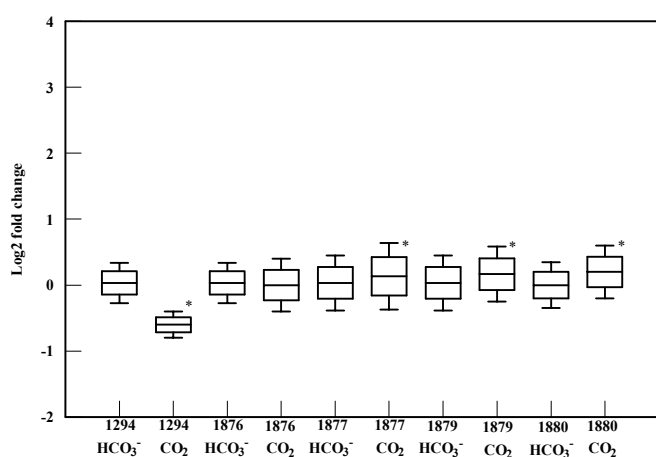Figure 9. Changes in level of the caa3 type terminal oxidase upon DIC species change. * p-values < 0.05

Figure 10. Changes in the cellular levels of the bd-quinol and cbb3 type terminal oxidases. * p-values < 0.05

species from bicarbonate to carbon dioxide does not trigger a strong stress response. This supports the observed changes in levels of other protein systems as being a result of selective sensitivity to dissolved inorganic carbon species and not a general stress response. The changes in levels of carbon concentration mechanisms and carbon fixation mechanisms are remarkably similar to those reported for *Thiomicrospira crunogena* cultured under low DIC conditions (Mangiapia *et al.* 2017). The *T. crunogena* heterodimeric bicarbonate transporter, Tcr_0853/0854 is increased, while the trimeric version Tcr_ 1081/1082/1083 shows no significant changes. In addition to changes in proteins involved in the carbon concentration mechanism, T. *crunogena* is reported to display a higher abundance of cbbL peptides when cultured under DIC limitations (Mangiapia *et al.* 2017). *H. neapolitanus* displays a

slight increase in cbbL (log2fold = +0.25) upon switching from 5mM bicarbonate to $CO_2(g)$ as carbon source indicating that the $CO_2(g)$ may represent a carbon limitation situation for *H. neapolitanus* when compared to 5mM bicarbonate. In addition, the changes in levels of cbbM may be indicative of a change from form II to form I RubisCO in response to carbon limitation.

Changes in levels of proteins involved in energy producing pathways are more difficult to interpret since there has been no report from other sulfur oxidizing species of such changes based on changes in carbon species. The complexity of interpretation is especially true in the case of sulfide:quinone reductases such as sqr D, E and F and the sulfide dehydrogenase sdhAB since the sulfur substrate in the growth media being thiosulfate and not sulfide. Even if the thiosulfate in the media were to undergo decomposition during autoclaving, it is expected that only trace amounts of sulfide would be produced. That being the case, the increase in level of sqrF and sdhB proteins upon change from bicarbonate to $CO_2(g)$ would be involved in processing only a fraction of the available substrate. Other than the decrease in sulfur oxygenase-reductase (sor), no significant change is seen in the levels of other sulfur oxidizing systems.

Changes in levels of terminal electron acceptors seem to fit a general pattern amongst other bacteria. The cbb3 terminal oxidase is the primary oxidase under aerobic conditions. This is consistent with there being no significant changes observed. The bd-quinol oxidase is a minor contributor. Several reports (Zhou *et al.* 2013; Osamura *et al.* 2017) indicate that caa3 type oxidases serves as a survival mechanism under substrate starvation conditions. The decrease in caa3 related proteins seen in Figure 9 may therefore represent an overall 'favor' of $CO_2(g)$ as dissolved carbon species.

Conclusions

Changes in DIC species does appear to elicit changes in levels of proteins with a total of 99 out of 1990 detected proteins showing log2fold change >0.5. The tandem-mass-tagged MS (TMT-MS) technique proved sufficient to identify and quantitate peptides associated with both carbon uptake/fixation and energy production. Since the changes witnessed in bicarbonate uptake and carbon fixation mechanisms are similar to those previously reported for *T. crunogena*, this supports the validity of the results seen within both the carbon concentration mechanism and the energy producing pathways.

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