**Title:** Super-resolution microscopy of the ß-carboxysome reveals a homogenous matrix

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**Running Head:**
SUPER-RESOLUTION OF THE ß-CARBOXYSOME

**Abbreviations:**
3D-Structured Illumination Microscopy (3D-SIM), ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), small subunit domain (SSU), CcmM35 (M35), CcmM58 (M58)

**Abstract:**

Carbon fixation in cyanobacteria makes a major contribution to the global carbon cycle. The cyanobacterial carboxysome is a proteinaceous microcompartment that protects and concentrates the carbon-fixing enzyme RuBisCO in a paracrystalline lattice, making it possible for these organisms to fix $CO_2$ from the atmosphere. The protein responsible for the organization of this lattice in beta-type carboxysomes of the freshwater cyanobacterium *Synechococcus elongatus*, CcmM, occurs in two isoforms thought to localize differentially within the carboxysome matrix. Here, we use widefield timelapse and 3D-structured illumination microscopy (3D-SIM) to study the recruitment and localization of these two isoforms. We demonstrate that this super-resolution technique is capable of successfully resolving the outer protein shell of the carboxysome from its internal cargo. We develop an automated analysis pipeline to analyze and quantify 3D-SIM images and generate a population level description of carboxysome shell protein, RuBisCO, and CcmM isoform localization. We find that both CcmM isoforms colocalize in space and time, prompting a revised model of the internal arrangement of the beta carboxysome.

**Introduction:**

RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase), the primary enzyme of carbon fixation, is ubiquitous in autotrophic organisms. However, it is notoriously inefficient and readily participates in energetically wasteful side-reactions with $O_2$ (Andersson *et al*., 2008). Many organisms that rely on RuBisCO to acquire inorganic carbon tune the enzyme's local microenvironment to achieve sufficient levels of carbon fixation. While plants can do this within membrane barriers, many single-celled organisms have evolved a strategy of proteinaceous compartmentalization.

The carboxysome is a bacterial microcompartment (BMC) found in cyanobacteria and prokaryotic chemoautotrophs. Composed of thousands of individual subunits, the carboxysome encapsulates RuBisCO and carbonic anhydrase (CA) within a selectively permeable icosahedral shell, making survival possible at atmospheric levels of $CO_2$ (Figure 1A) (Rae *et al*., 2013; Kerfeld *et al*., 2016).

While evolutionarily distinct carboxysomes share many similar structural features, the RuBisCO in the beta-type carboxysome (ß-carboxysome) of the freshwater cyanobacterium *Synechococcus elongatus* PCC7942 is organized into a paracrystalline lattice (Kaneko *et al*., 2006). The structural protein CcmM has been implicated in connecting individual RuBisCO enzymes into an ordered matrix, resulting in the observed dense crystalline packing (Long *et al*., 2007; Cot *et al*., 2008, Long *et al.,* 2010; Cameron *et al*., 2013). The full-length 58kDa CcmM protein of PCC7942 is characterized by an N-terminal carbonic anhydrase (CA) binding domain and three repeating domains toward the C-terminal end that have sequence homology to the small subunit of RuBisCO (Figure 1B) (Price *et al*., 1993). Due to an internal ribosomal entry site (IRES), CcmM is translated as two isoforms, a full-length protein (M58) and a 35kDa truncated version (M35) that consists of only the three small subunit like (SSU-like) domains (Long *et al*., 2007; Long *et al*., 2010).

The current model of ß-carboxysome internal structure suggests that the small subunit like domains (SSU-like) of CcmM displace some of the small subunits of the $L_8S_8$ RuBisCO holoenzyme and tether multiple enzymes together into a dense network (Figure 1C) (Long *et al*., 2007; Long *et al*., 2010; Rae *et al*., 2013; Kerfeld *et al.,* 2016). Co-purification and yeast 2-hybrid experiments have shown that RuBisCO readily complexes with both isoforms of CcmM in PCC7942 (Long *et al*., 2007; Cot *et al*., 2008). In addition, fluorescence tagging experiments have demonstrated that M35 alone is

capable of nucleating assembly of tagged RuBisCO if at least two of the SSU-like domains are present. However, M35 is not sufficient to cause encapsulation by recruiting shell components in the absence of M58 (Cameron *et al*., 2013). Densitometry analyses of western blot experiments have found imbalanced ratios of RbcS to RbcL (~5:8) in PCC7942 cell lysates, suggesting that the missing RbcS slots may be occupied by CcmM SSU domains (Long *et al*., 2011). These findings have supported a model of carboxysome architecture and step-wise assembly in which M35 nucleates a core of RuBisCO. In this model, M58 is limited to a layer beneath the shell where it can interact with CcmN, CA, and shell components. While this model predicts that the CcmM isoforms are differentially localized, these predictions have yet to be directly tested.

In order to test this model, we fluorescently tagged CcmM to visualize its assembly and the localization of CcmM isoforms *in vivo*. Previous work has shown that completely replacing CcmM with tagged fusions does not disrupt carboxysome function (Long *et al*., 2007; Cameron *et al*., 2015). Loss of either isoform of CcmM creates mutants that fail to produce carboxysomes and have a high $CO_2$ requirement (HCR) for growth (Woodger *et al.,* 2005; Long *et al*., 2010). However, complementation of such mutants with tagged copies of CcmM has been shown to rescue this phenotype (Long *et al*., 2007; Cameron *et al*., 2015).

Here we show the successful complementation of a Δ*ccmM* mutant with a dually fluorescently tagged integration of *ccmM*. This dual tagging strategy does not significantly impair carboxysome formation, morphology, or function. In our timelapse fluorescence microscopy data we find no evidence of differential CcmM isoform assembly during carboxysome biogenesis. Using super-resolution 3D Structured Illumination Microscopy (3D-SIM), we examined the relative localization of carboxysome shell, CcmM, and RuBisCO proteins. Our 3D-SIM data does not support a model of carboxysome internal organization in which M58 is limited to a layer just beneath the external protein shell. These data support a revised model of carboxysome internal structure in which M35 and M58 isoforms are both integrated deep within the core of the carboxysome and are both involved with the early steps of carboxysome assembly.

## Results:

### Fluorescent fusions to CcmM complement a deletion of the wildtype

We integrated an N- and C-terminal fluorescently-tagged copy of *ccmM* (*mTagBFP2-ccmM-mNeonGreen*) driven by the IPTG inducible promoter P*trc* into the genome of an *S. elongatus* PCC7942 Δ*ccmM*::*HygR* mutant strain (Figure 2A-B) and verified expression with western blot analysis (Figure 2C). This strategy allowed us to visually separate full length CcmM from truncated M35 by blue fluorescence. We found that P*trc* was sufficiently leaky to produce protein without induction. Consequently we never induced the Δ*ccmM* + *mTagBFP2-CcmM-mNeonGreen* strain, including for western blot analysis, unless otherwise noted. We blotted lysates of WT, Δ*ccmM*, and Δ*ccmM* + *mTagBFP2-CcmM-mNeonGreen* strains for the presence of CcmM (Figure 2C). In WT 7942 cells, we detected two bands at ~60 kDa and ~40 kDa, corresponding to the two native CcmM isoforms M58 and M35. In the Δ*ccmM* strain, these bands are absent. In the Δ*ccmM* + *mTagBFP2-CcmM-mNeonGreen* gene complement, we detected two bands at ~140 kDa and ~70 kDa, corresponding to molecular weight shifts of 60kDa (both fluorescent tags) and 30kDa (mNeonGreen alone), respectively. The observed weight shift of the tagged CcmM demonstrates that the CcmM IRES is not impaired by the presence of N- and C-terminal tags, and that both long and short CcmM isoforms are translated. We also made a reciprocally tagged version of the CcmM complement

(mNeonGreen-CcmM-mTagBFP2), but did not use it for further analysis because western blotting showed the presence of protein degradation (Supplemental Figure S1).

Our tagged *ccmM* complement grew at a rate comparable to WT at atmospheric levels of $CO_2$ (Figure 2D). We grew WT 7942, the Δ*ccmM* mutant, and the Δ*ccmM* + *mTagBFP2-CcmM-mNeonGreen* complement in ambient air and 2% $CO_2$ conditions, measured optical density at 750nm ($OD_{750}$), and calculated doubling times between the 18- and 90-hour time points. Both WT and Δ*ccmM* cells cultured with 2% $CO_2$ grew well with similar doubling times of 17.6 ± 0.4 hours ($n$ = 5, Error = SD) and 15.8 ± 2.1 hours ($n$ = 6, Error = SD) respectively. As expected, Δ*ccmM* mutant growth was dependent on supplemented $CO_2$ (Long *et al*., 2010); these cells showed no growth in ambient air conditions. In contrast, the Δ*ccmM* + *mTagBFP2-CcmM-mNeonGreen* complement grew well in air with a doubling time of 23.3 ± 2.4 hours ($n$ = 6, Error = SD). This growth rate is similar to that of WT cells in air, which had a doubling time of 20.6 ± 1.8 hours ($n$ = 6, Error = SD), indicating that our engineered strain is capable of fixing carbon, and that carboxysome function is not severely impaired by the dual-fusion *ccmM* complement. Because both the M58 and M35 isoforms of CcmM are required for growth without $CO_2$ supplementation (Long *et al*., 2010), our data indicate that both isoforms produced by our dual fusion are functional.

Carboxysomes formed in the Δ*ccmM* + *mTagBFP2-CcmM-mNeonGreen* complement were structurally similar to those of WT cells. Using transmission electron microscopy, we imaged ultrathin sections of fixed cells from WT 7942, Δ*ccmM*, and Δ*ccmM* + *mTagBFP2-CcmM-mNeonGreen* strains (Figure 3). Carboxysomes in WT PCC7942 cells had an average maximum width of 179 ± 33 nm ($n$ = 26, Error = SD). Carboxysomes in Δ*ccmM* + *mTagBFP2-CcmM-mNeonGreen* cells generally had a normal appearance and shape with visibly angled facets and often clear hexagonal geometry, but were noticeably larger, with an average maximum diameter of 319 ± 92 nm ($n$ = 41, Error = SD). This is not unexpected; it has been previously reported that His-tagged *ccmM* complements increase carboxysome size (Long *et al.,* 2007). Occasionally, dense masses at the cell poles were observed in Δ*ccmM* + *mTagBFP2-CcmM-mNeonGreen* micrographs, most likely indicating protein aggregation. Because we are using this strain without induction and no effective repressors exist, we cannot know if reducing expression would prevent aggregate formation. However, IPTG-induced cells observed with 3D-SIM display a dramatic increase in polar protein aggregation, often filling a large portion of the cell, confirming that overexpression of this construct leads to aggregation (Supplemental Figure S2).

**Both CcmM isoforms share similar recruitment kinetics and localization**

Both CcmM isoforms are recruited to growing carboxysomes with similar timing and kinetics. Using widefield microscopy, we imaged the CcmM complement (mTagBFP2-CcmM-mNeonGreen) at 10 minute intervals (Figure 4). Particle tracking revealed that both the CcmM isoforms assembled slowly, with an average assembly time of approximately 10.50 ± 5.39 hours ($n$ = 8, Error = SD) for M58-mNeonGreen and M35-mNeonGreen, and 12.06 ± 7.27 hours ($n$ = 8, Error = SD) for mTagBFP2-M58 (Figure 4C). This is longer than the ~6-7 hour maturation time previously reported for RuBisCO (Chen *et al*., 2013), perhaps as a result of temperature or light levels during imaging. Assembly times were estimated based on the time it took a particle to go from the intensity required for automatic particle detection (methods) to the first value ≥ 0.8 on the normalized intensity plot.

Next, a four-parameter logistic equation was used to approximately model the particle tracks. These models highlight similar assembly kinetics for both CcmM isoforms within the same carboxysome. Based on these fit curves, half-max values of green and

blue particle assemblies were found to have an absolute average difference of 1.18 ± 0.68 hours ($n$ = 8, Error = SD). If one CcmM isoform precedes the other during carboxysome biogenesis, the half-max values of each isoform would be expected to be significantly different. However, a comparison of half-max values from eight measured assemblies found no significant difference (Wilcoxon matched-pairs signed rank test, number of pairs = 8, P = 0.945). In order to test if CcmM isoforms differ in rate of assembly, channel specific assembly times (average reported above) were analyzed and found not to be significantly different among paired fluorescent channels (Wilcoxon matched-pairs signed rank test, number of pairs = 8, P = 0.375). This indicates that CcmM isoforms M35 and M58 assembled at relatively similar rates.

**M58 is not confined to a sub-shell layer in the carboxysome**

Super-resolution microscopy of fluorescently tagged M58 and M35 show indistinguishable distributions in the carboxysome. We used 3D-Structured Illumination Microscopy (3D-SIM) to acquire super-resolution images of the Δ*ccmM* + *mTagBFP2-CcmM-mNeonGreen* strain, as well as fluorescently tagged RuBisCO (RbcL-sfGFP) and the shell protein CcmK4 (CcmK4-mTagBFP2 and CcmK4-sfGFP) in PCC7942. 3D-SIM images of strains with both GFP- and BFP-tagged CcmK4 resolved the external carboxysome shell as a ring structure in the xy-plane (Figure 5A-F), demonstrating that 3D-SIM provides sufficient resolution to identify peripherally localized proteins in the carboxysome. To confirm these rings were not an artifact of SIM reconstruction, we imaged a strain of PCC7942 integrated with a GFP labeled copy of the large subunit of RuBisCO (RbcL-sfGFP). This strain has been previously used successfully to study RuBisCO assembly and spacing (Savage *et al*., 2010; Chen *et al*., 2013). 3D-SIM of RbcL-sfGFP showed no ring-like structures, producing only solid regularly-spaced particles (Figure 5G-I). To test if M58 was in fact limited to a sub-shell layer, we imaged the Δ*ccmM* + *mTagBFP2-CcmM-mNeonGreen* gene complement with 3D-SIM. Both CcmM isoforms produced solid objects similar to those observed with the RbcL-sfGFP strain (Figure 5J-N). These qualitative results suggested that the tagged M58 isoform of CcmM was not excluded from integration into the carboxysome core as would be expected if the protein was only present as a thin layer just beneath the exterior shell. In order to check that variations in signal intensity between experiments and cyanobacterial strains did not lead to poor signal-to-noise ratios and consequent artifacts, the reconstruction quality of each SIM data set was verified by the reconstructed intensity histogram (RIH) score from the SIMcheck ImageJ plugin (Ball *et al*., 2015). RIH scores for all conditions and data sets were of similar quality (Supplemental Table S1).

We developed an automated image analysis pipeline and a metric to quantify the degree to which fluorescently-labeled proteins were localized at the perimeter of the carboxysome versus uniformly distributed throughout the matrix. The max-to-center intensity (MTC) ratio is calculated for each particle as the peak intensity of the average radial profile divided by the intensity at the particle centroid. A perfectly uniform protein distribution would result in the peak particle intensity overlapping the particle centroid, and yield an MTC ratio of 1.00. Higher MTC values result from particles with decreased intensity at their centers, indicating a more ring-link appearance.

ANOVA testing confirmed that mean MTC values were significantly heterogeneous between all the labeled carboxysome proteins (Welch's ANOVA, $F_{4, 7378}$=160.48, $P$<0.001). A Games-Howell test with $q_{critical}$(k=5, df=∞, α=0.001)=5.484 was then used to make further pairwise comparisons of labeled carboxysome protein MTC values (Supplementary Table S2). This automated analysis of RbcL-sfGFP and CcmK4-sfGFP images confirmed our initial observations that carboxysomes appear fundamentally different between these strains (Games-Howell test, MD=0.02, $q$=22.26);

256 similar results were obtained from the comparison of RbcL-sfGFP and CcmK4-
257 mTagBFP2 (Games-Howell test, MD=0.03, *q*=26.38) (Figure 6A). RbcL-sfGFP particles
258 had an average diameter of 244 ± 45 nm and an average MTC value of 1.002 ± 0.005 (*n*
259 = 3505), with a maximum intensity ratio of 1.04. Based on this, we calculated an upper
260 ratio threshold of 1.05, below which objects are likely to be uniform, by adding two
261 standard deviations to the RbcL-sfGFP max ratio of 1.04. This threshold is denoted on
262 MTC ratio versus diameter plots as a horizontal dotted line (Figure 6).
263 In contrast to RbcL, we found that shell proteins displayed high MTC ratios.
264 CcmK4-sfGFP particles had an average diameter of 253 ± 34 nm (*n* = 3459), and in
265 comparison to RbcL-sfGFP particles, a significantly greater average MTC ratio of 1.023
266 ± 0.077 (Games-Howell test, MD=0.02, *q*=22.26) (Figure 6H). Approximately 10% of
267 CcmK4-sfGFP particles have MTC ratios ≥ 1.05, denoting a ring-like appearance (Figure
268 6B). Analysis of CcmK4-mTagBFP2 particles also revealed a significantly different mean
269 MTC ratio compared to labeled RbcL-sfGFP (Games-Howell test, MD=0.03, *q*=26.38)
270 (Figure 6H), with the CcmK4-mTagBFP2 particles having an average size of 244 ± 32
271 nm and average MTC ratio of 1.036 ± 0.093 (Figure 6C). Of note, the percent of ring-like
272 particles identified in CcmK4-mTagBFP2 cells (18.16% ≥ $MTC_{1.05}$) was significantly
273 greater than the percentage found in CcmK4-sfGFP cells (9.90% ≥ $MTC_{1.05}$) (Games-
274 Howell test, MD=0.01, *q*=8.51) (Figure 6H). This increase in the fraction of rings
275 identified with BFP labeled CcmK4 over those identified with GFP may be the result of
276 the improvement in resolution due to the shorter wavelength of blue versus green
277 fluorophores. Based on the smallest resolvable shell in the CcmK4-mTagBFP2 strain,
278 which had an apparent diameter of 197nm, and accounting for an expected shell depth
279 of 5-6nm, we do not expect to resolve by SIM even true ring-like particles smaller than
280 approximately 220nm in diameter (Kaneko *et al*., 2006). This lower resolvable limit is
281 denoted on plots of tagged M58 and of M35 particles by a grey region in Figure 6D-E.
282 Analysis of the Δ*ccmM* + *mTagBFP2-CcmM-mNeonGreen* gene complement
283 identified a small number of tagged M58 particles as ring-like (Figure 6D). Our analysis
284 found 0.58% (19 of 3303) of the mTagBFP2-M58 particles had MTC ratios of ≥ 1.05,
285 with an average size of 233 ± 46 nm and average MTC ratio of 1.003 ± 0.010 (Figure
286 6F). We found a significant difference in the MTC ratios of mTagBFP2-M58 particles vs
287 both shell-labeled CcmK4-mTagBFP2 (Games-Howell test, MD=0.03, *q*=25.27) and
288 CcmK4-sfGFP (Games-Howell test, MD=0.02, *q*=20.69) (Figure 6H). This supports
289 qualitative observations that particles of the labeled M58 strain did not appear as ring-
290 like as those in the strains with labeled carboxysome shells. mTagBFP2-M58 particles
291 also had significantly different MTC ratios compared to RbcL-sfGFP particles (Games-
292 Howell test, MD=0.001, *q*=10.05), but this difference was produced by only 19 ring-like
293 particles in the mTagBFP2-M58 strain (Figure 6H). Taken together, these test results
294 indicate that though mTagBFP2-M58 labeled carboxysomes have a small but statistically
295 significant number of MTC ratios ≥ 1.05, the mean ratio is not similar to that produced by
296 labeled carboxysome shell proteins, as would have been expected if the M58 isoform
297 followed a strict sub-shell localization.
298 The Δ*ccmM* + *mTagBFP2-CcmM-mNeonGreen* complement, which expresses
299 both full-length M58-mNeonGreen and the short isoform M35-mNeonGreen, produced 2
300 particles with MTC ratios ≥ 1.05 (Figure 6E). Only 0.06% of particles were found above
301 that threshold, with an average size of 244 ± 47 nm (*n* = 3437) and average MTC ratio of
302 1.002 ± 0.005 (Figure 6F). The mean MTC ratio of these particles was significantly
303 different than those of both labeled CcmK4-sfGFP (Games-Howell test, MD=0.02,
304 *q*=22.20) and CcmK4-mTagBFP2 (Games-Howell test, MD=0.03, *q*=26.34) (Figure 6H).
305 Pair-wise comparison of M58-mNeonGreen and M35-mNeonGreen particles to RbcL-
306 sfGFP found no significant difference in mean MTC ratios (Games-Howell test,

307 MD=0.00005, *q*=0.57) (Figure 6G), indicating that the population of green fluorescent
308 particles produced in the Δ*ccmM* + mTagBFP2-CcmM-mNeonGreen strain were solid,
309 similar to those observed in the strain with labeled RuBisCO.
310
311 **Discussion:**
312 **The carboxysome can support the addition of large foreign proteins via direct**
313 **fusion to CcmM**
314 The function of CcmM and other components of the carboxysome are robust to
315 the addition of large protein fusions, further supporting these fusions as a means to
316 localize foreign cargo to the carboxysome for engineering applications. Previous work
317 has shown that tagged CcmM gene complements can rescue the HCR phenotype of
318 CcmM mutants without significant impairment of carboxysome function compared to WT
319 cells (Long *et al*., 2010; Cameron *et al*., 2015). We find that such complementation is
320 also possible when fluorescent tags are fused to both ends of CcmM, significantly
321 increasing the size of both the M58 and M35 isoforms. These results suggest that
322 heterologous proteins could be simultaneously fused to multiple loci in the matrix,
323 opening the door for localizing multipart enzymatic pathways inside the carboxysome.
324
325 **M35 and M58 assemble simultaneously during carboxysome biogenesis**
326 M35 has been hypothesized to coordinate the organized paracrystalline cargo
327 assembly of RuBisCO, with M58 limited to a peripheral sub-shell layer (Long *et al.,* 2007;
328 Cot *et al.,* 2008; Long *et al*., 2010; Long *et al*., 2011; Rae *et al*., 2013). Current models of
329 carboxysome biogenesis have proposed a primarily cargo-centric assembly pathway, in
330 which RuBisCO and internal structural components form prior to shell encapsulation
331 (Cameron *et al.,* 2013; Chen *et al.,* 2013). Based on FRAP imaging of carboxysomes
332 that suggests matrix components are relatively static (Chen *et al.,* 2013), we
333 hypothesized that M35 would be present in pre-carboxysome assemblies prior to M58,
334 with M58 appearing later during shell formation. However, we were unable to resolve
335 any significant temporal difference between the CcmM isoforms. This concurrent timing
336 indicates that M58 and M35 are present together even at the early stages of
337 carboxysome assembly, with both isoforms involved in organization of the pre-
338 carboxysome RuBisCO matrix. These findings are not necessarily in disagreement with
339 current models of carboxysome assembly. Rather, they highlight how both CcmM
340 isoforms are integrally involved in carboxysome biogenesis from the earliest stages.
341 Assuming that the carboxysome core grows by addition of material to the outside of a
342 stable paracrystalline RuBisCO assembly (Chen *et al*., 2013), these results also
343 suggest that M58 does not assemble as a separate layer around a pre-existing
344 M35/RuBisCO core.
345
346 **M58 is not exclusive to a subshell layer**
347 3D-SIM experiments and quantitative analysis do not support a model of
348 carboxysome internal structure in which M58 is limited to a sub-shell localization. The
349 preexisting model described above proposes that M58 is not present within the primary
350 M35-Rubisco matrix. This makes logical sense based on the fact that M35, which does
351 not contain the N-terminal domain, presumably can achieve denser packing of RuBisCO
352 enzymes.
353 We expected that if M58 was in fact limited to a sub-shell layer ~5-6nm beneath
354 the outer surface, it would appear as a ring similar to CcmK4 by 3D-SIM. While SIM
355 imaging of CcmK4 did reveal the expected ring-like distribution, carboxysomes with
356 fluorescently-labeled M58 appeared predominantly as solid objects, with only a very a
357 small population (0.58%) showing dips in fluorescence intensity at their centers.

358 Consequently, we find no evidence by super-resolution microscopy to support a model
359 of carboxysome structure in which M58 is excluded from the carboxysome core,
360 suggesting that the internal carboxysome structure contains a mixed population of both
361 M35 and M58 coordinating with RuBisCO. The finding that M58 is present throughout
362 the carboxysome core raises questions about the compartment's internal structure. For
363 example, CcmM has been reported to interact with other carboxysome components
364 (such as CcaA, CcmN, and CcmK) in yeast two hybrid and pulldown experiments (Long
365 *et al.,* 2007; Cot *et al*., 2008; Kinney *et al.,* 2012), although the strength of these
366 interactions is unknown. CcmN is essential for shell recruitment and assembly (Kinney *et*
367 *al.,* 2012), and thus is thought to localize at the carboxysome periphery (Rae *et al.,*
368 2013). In addition, crystal structures of CcmM from *Thermosynechococcus elongatus*
369 has revealed a homotrimer complex (Peña *et al.,* 2010). Thus, CcmM may be involved in
370 multiple structural and functional roles throughout the carboxysome (Figure 7).
371 Our findings also raise new questions about the structure and biogenesis of
372 carboxysomes. For example, M58 is thought to play a role in recruitment of shell
373 components (Cot *et al*., 2008; Long *et al.,* 2011; Kinney *et al*., 2012; Cameron *et al*.,
374 2013). However, shell proteins are recruited to carboxysomes only late in their
375 maturation process (Chen *et al.,* 2013). Therefore, if M58 is present even at the early
376 stages of carboxysome assembly, other factors must be responsible for delaying shell
377 recruitment. Furthermore, as our data indicate that CcmM58 is present deep within the
378 carboxysome, it is possible that this protein is interacting with the carbonic anhydrase
379 CcaA throughout the internal matrix of RuBisCO (Figure 7D). The nature of these factors
380 is a promising avenue for future work.
381
382 **Materials and Methods:**
383 **Western blot analysis**
384 For SDS-PAGE, log-phase ($OD_{750}$ = 1.0) cultures of cyanobacterial strains were
385 pelleted and frozen at -20°C. Frozen pellets were thawed in B-PER Bacterial Protein
386 Extraction Reagent (ThermoScientific), without DNAse or Lysozyme, and normalized by
387 wet pellet mass. Resuspended cells were mixed directly with SDS loading buffer and
388 boiled. Boiled samples were run on a 4-20% Tris-Glycine NuPAGE gel (Invitrogen) at
389 120V. SDS-PAGE separated proteins were transferred to a nitrocellulose membrane
390 with an iBlot2 transfer device (ThermoScientific).
391 The membrane was blocked with 5% skim milk in TBST (50mM Tris, 150mM
392 NaCl, 0.1% Tween 20), then probed with 1:10,000 of anti-CcmM primary antibody (a gift
393 of Ben Long), followed by a horseradish peroxidase-conjugated goat anti-rabbit
394 secondary antibody (Agrisera AS09 602). The membrane was incubated with
395 SuperSignal West Dura Extended Duration chemiluminescent substrate
396 (Thermoscientific) and imaged with the Bio-Rad ChemiDoc system.
397
398 **Transmission Electron Microscopy**
399 For TEM experiments, 1mL of mid-log cultures were pelleted (6k rpm, 5 min) and
400 concentrated 2x in sterile $dH_2O$. The cells were then mixed 1:1 with fixative (1.25%
401 formaldehyde, 2.5% glutaraldehyde, 0.03% picric acid, 0.1M sodium cacodylate buffer,
402 pH 7.4), incubated at RT for 30 min, then pelleted and stored in fixative at $4^oC$ overnight.
403 Fixed cells were embedded in epon resin and cut into ultra-thin sections (~60-80nm).
404 Sections were stained with uranyl acetate and lead citrate, then imaged on a Tecnai G2
405 Spirit Bio TWIN TEM.
406
407 **Cell culture**

Wild-type, mutant, and modified strains of *Synechococcus elongatus* PCC7942 were grown in BG-11 media, with 1 g/L HEPES adjusted to pH 8.5. Cultures were grown at 30-35°C under constant light with an approximate intensity of 1000 lm/m$^2$. Cultures were grown in 125ml or 250ml flat bottom flasks, shaking at 150 rpm. Wild type 7942 and knockout complements were grown with ambient air conditions. Mutant Δ*ccmM*::*HygR* cultures were supplemented with 2% $CO_2$ and 25 µg/mL hygromycin B. Strains with fluorescently labeled carboxysome components, carrying kanamycin or spectinomycin resistance genes, were grown with 25 µg/mL kanamycin or 25µg/mL spectinomycin respectively. For the CcmM complement strain, Δ*ccmM* + *mTagBFP2-CcmM-mNeonGreen*, cultures were grown with 5 µg/mL kanamycin and 25 µg/mL hygromycin B. For growth curve experiments, biological replicates were made by isolating colonies grown on BG11 agar with 1 g/L HEPES, 50mM $HCO_3^-$, and 1mM $Na_2S_2O_3$. Isolates were screened by PCR to confirm the presence or absence of the WT *ccmM* gene. Growth curve cultures were prepared by washing starter cultures in fresh BG11 pH 8.5 media, then diluting to OD750 of ~0.1 in 40 mL BG11 in 250mL flat bottom flasks.

**Image acquisition**

For both conventional fluorescence and 3D-SIM experiments, 5µL of either mid-log cells ($OD_{750}$ = 1-2) for timelapse experiments or early-log cells ($OD_{750}$ = 0.5 - 1.2) for 3D-SIM were spotted on 2% agarose BG11 pads. Agarose pads were placed sample-side down in MatTek 35mm glass bottom microwell dishes with No. 1.5 coverslips. Sterile water was spotted within the dish to mitigate dehydration of the agarose pad, and the dish was sealed with parafilm. Though strains expressing labeled CcmM were under the control of the IPTG inducible promoter P*trc*, no induction was used with these cells due to adequate basal expression. Cells expressing RbcL-sfGFP were imaged on BG11 agarose pads with the addition of 50µM IPTG following previously reported protocols (Savage *et al*., 2010). SIM of uninduced RbcL-sfGFP cells also produced solid particles, but were not as bright as induced cells and produced several false-positives in our analysis pipeline (Supplemental figure S3). For timelapse experiments, samples were recovered for >1h at 34-35°C, under ~1000 lm/m$^2$, and in ambient air, before being imaged using a Nikon TE2000 and a 100x oil objective. Cells were kept at 30°C under ~800 lm/m$^2$ light intensity during timelapse, and imaged every 10min.

3D-SIM data was collected on a DeltaVision OMX V4 Blaze system (GE Healthcare) equipped with a 60x / 1.42 N.A. Plan Apo oil immersion objective lens (Olympus), and three Edge 5.5 sCMOS cameras (PCO). mTagBFP2 fluorescence was excited with a 405nm laser and collected with a 477/35 emission filter, mNeonGreen with a 514nm laser and 541/22 emission filter, and sfGFP with a 488nm laser and a 528/48 emission filter. Z-stacks of ~2 microns were acquired with a z-step of 125 nm and with 15 raw images per plane (five phases, three angles). Spherical aberration was minimized using immersion oil matching (Hiraoka *et al*., 1990). Super-resolution images were computationally reconstructed from the raw datasets with a channel-specific measured optical transfer function (OTF) and a Wiener filter constant of 0.001 using CUDA-accelerated 3D-SIM reconstruction code based on Gustafsson *et al.* (2008). Axial chromatic aberration was measured using TetraSpeck beads (Thermo Fisher) a nano-grid control slide and multi-channel datasets were registered using the image registration function in softWoRx.

**Image analysis**

SIM datasets were imported into MATLAB (Mathworks) using the bioformats reader, followed by thresholding and background subtraction. The threshold value was

459 empirically determined and assessed by visualizing the resultant centroid position and
460 mask in MATLAB and Imaris (Bitplane AG) respectively. Segmented objects were further
461 analyzed if they met a cutoff for volume, surface area, sphericity, major-minor axis ratio,
462 and mean radius.
463 We calculated the MTC (max to center) ratio for each object. From the centroid,
464 the radially averaged intensity for each sub-sampled (0.5 pixels) radius was measured.
465 This was done for each Z-plane and then averaged over the Z-axis. A polynomial curve
466 was fit to the averaged radial profile of each segmented object in MATLAB. The most
467 suitable polynomial fit (up to 6th order) was selected based on meeting the Bayesian
468 Information Criterion. Then, the 1st and 2nd derivative of each fitted curve were
469 calculated to find the root with the global maximum. The intensity at this root was
470 calculated and divided by the intensity at the centroid. This ratio was calculated for each
471 object and plotted versus its mean radius in MATLAB.
472 Notably, the resolvable carboxysome ring limit of 220nm that we estimate in our
473 analysis is well above the ~110nm theoretical resolution limit of 3D-SIM (at 520nm
474 wavelength; Gustafsson *et al.,* 2000; Gustafsson *et al*., 2008). The difference is likely
475 due to a combination of practical limitations along with the probable overestimation of
476 carboxysome diameter in our analysis pipeline due to convolution of the true object size
477 with the point spread function in the fluorescence images.
478 Timelapse data of Δ*ccmM* + *mTagBFP2-CcmM-mNeonGreen* were processed
479 with ImageJ (FIJI) (Schindelin *et al*., 2012). Timelapse data from two sequential
480 acquisitions of the same field of view were imported into FIJI with the Bio-Formats Import
481 plugin with autoscaling, concatenated, and separated by channel. Channels were
482 registered using rigid registration in the StackReg FIJI plugin, then background
483 subtracted with a 50-pixel radius rolling ball. Particle tracking was performed in FIJI with
484 the TrackMate plugin using the LoG detector (Laplacian of Gaussian filter) with 0.5μm
485 estimated blob diameter, 0.5 threshold, and sub-pixel localization. Automatically
486 generated tracks were manually extended backwards in time 20-30 frames by selecting
487 0.5μm diameter regions at or near site where the particle was first detected in order to
488 capture a background fluorescence level. Particle tracks were corrected for
489 photobleaching by dividing intensities by an average of 7 reference particles, normalized
490 to the first frame of the time lapse, in each channel. Reference particles were objects
491 observed to be relatively stable compared to particles that lost or gained fluorescence
492 due to carboxysome biogenesis. The same reference particles were used for correction
493 in both channels. To generate the timelapse montage, brightness and contrast values
494 were manually adjusted separately in each channel to enhance visibility in the figure.
495
496 **Cloning and strain construction**
497 Plasmids used for *S. elongatus* strain construction were made by Gibson
498 assembly. Tagged CcmM plasmids were cloned by inserting the *ccmM* gene, along with
499 mNeonGreen and mTagBFP2 coding sequences with additional flexible linker
500 sequences, into the previously described pDFS21 neutral site II (NS2) integration
501 plasmid downstream of the IPTG inducible P*trc* promoter (Savage *et al*., 2010). The
502 *ccmM* gene was obtained directly from the *S. elongatus* PCC7942 genome via PCR with
503 Phusion polymerase (NEB). The *ccmK4* gene for cloning shell fusion plasmids was
504 obtained as a synthesized gBlock (IDT) and cloned into the pDFS21 integration plasmid.
505 *S. elongatus* strains were transformed with integration plasmids by incubating ~5
506 fold concentrated $OD_{750}$ 1-2 culture with 10-100ng plasmid at 30$^{o}$C in the dark overnight,
507 followed by plating transformants on selective BG11 agar (Clerico *et al*., 2007). The
508 mutant Δ*ccmM* strain was made by integrating a hygromycin B resistance gene into the
509 native *ccmM* ORF, followed by selective plating and screening (Sachdeva G., 2014).

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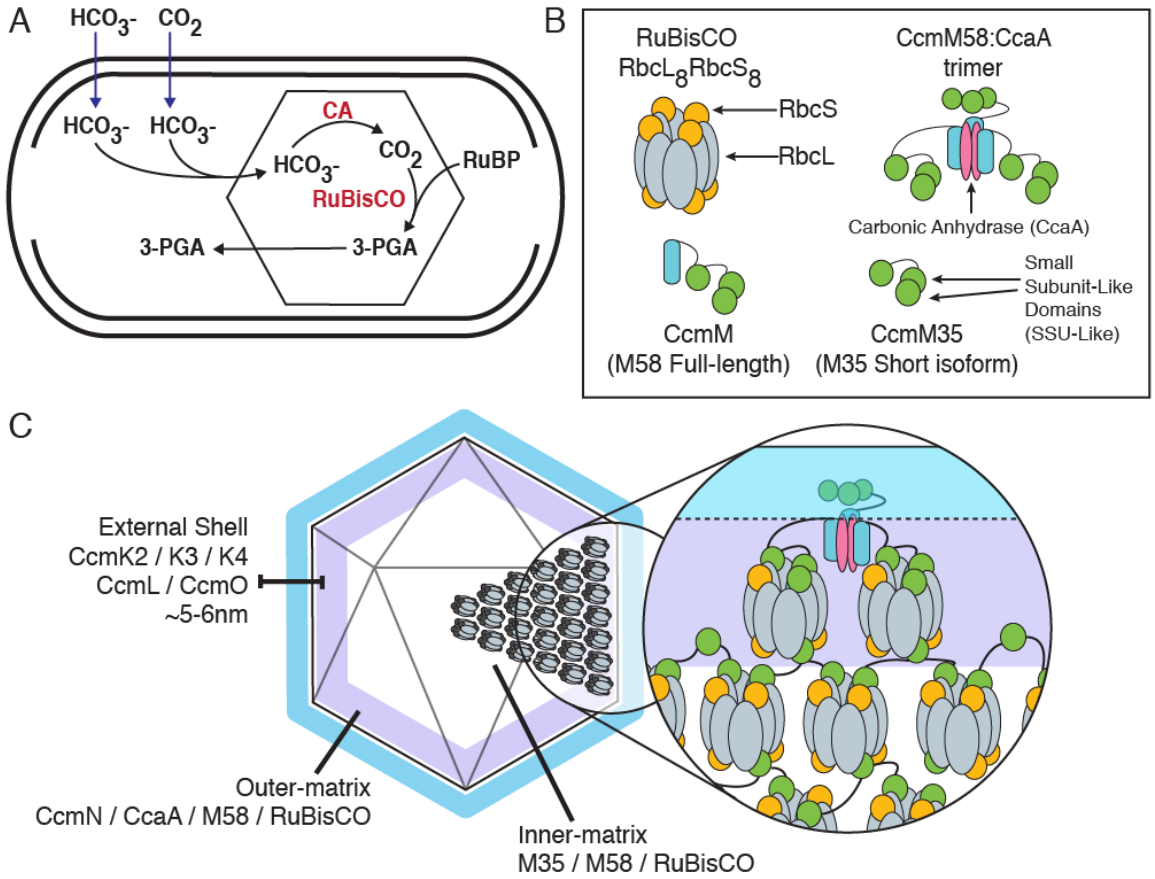



**Figure 1.** Current model of the cyanobacterial carboxysome internal structure. (A) *S. elongatus* utilizes the carboxysome to concentrate and protect its RuBisCO enzymes. Through a multi-step process, bicarbonate passes into the carboxysome, is converted to $CO_2$ by carbonic anhydrase, and is then fixed by RuBisCO to form 3-phosphoglyceric acid (3-PGA). (B) Legend of proteins and protein complexes of the carboxysomes interior. *CcmM* is an essential carboxysome gene that is translated as two different isoforms, the full-length 58kDa M58 and a truncated 35kDa form, M35. CcmM has an N-terminal domain that complexes with CcaA carbonic anhydrase, and three repetitive C-terminal domains that have homology with the small subunit of RuBisCO. (C) These small subunit-like domains (SSU-like), which make up the majority of the M35 protein, are believed to displace a portion of the RuBisCO small subunits and physically tether the enzymes together within the core of the carboxysome, to the exclusion of the long M58 isoform which has been hypothesized to remain just beneath the carboxysome shell.

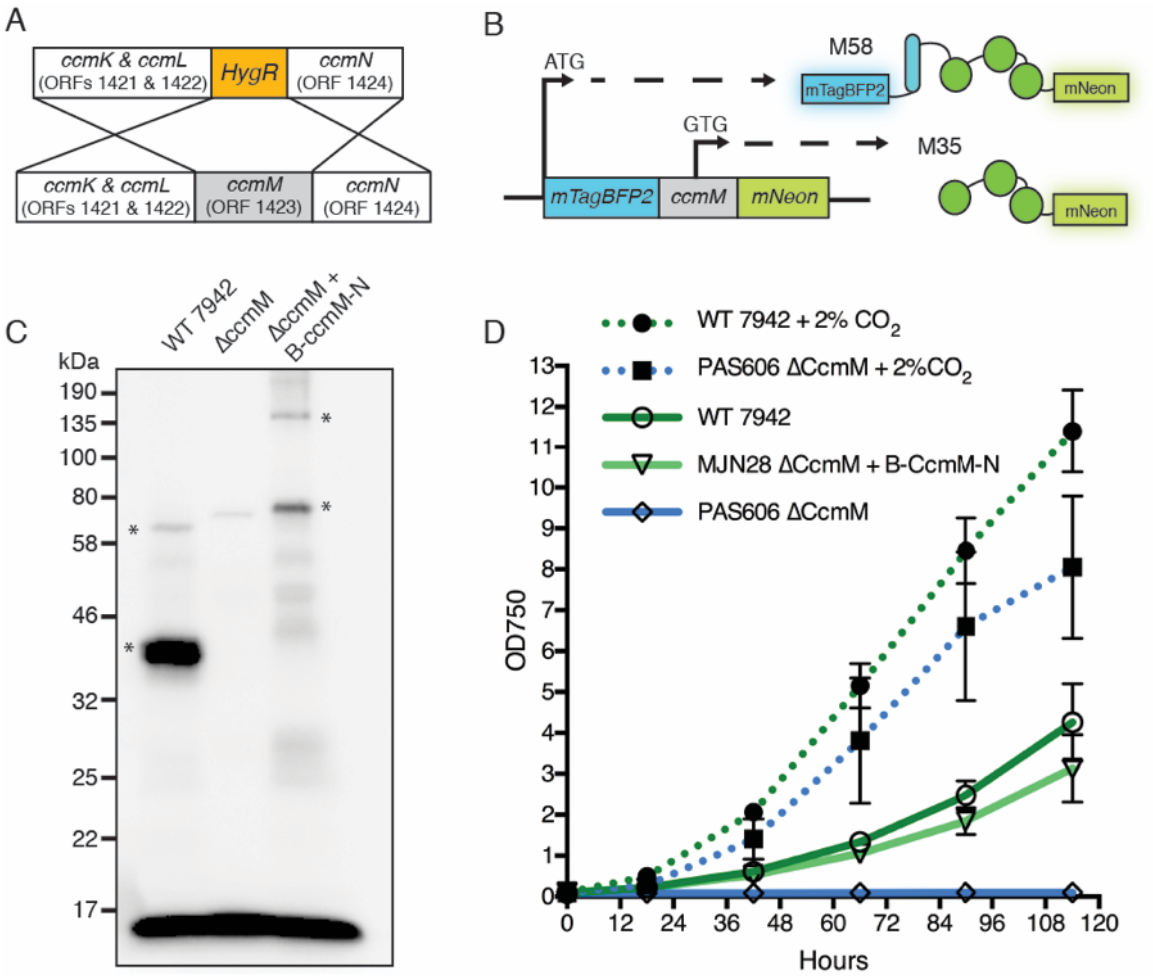



**Figure 2.** Fluorescently tagged *ccmM* integrated into the genome of a Δ*ccmM* knockout produces both CcmM isoforms and and restores growth in ambient air conditions. (A) The Δ*ccmM* knockout was made by integrating a hygromycin resistance marker into the genomic site of *ccmM*. (B) Diagram of fluorescently tagged *ccmM*. N- and C-terminal fusions of mTagBFP2 and mNeonGreen produces short and long isoforms with different fluorescent characteristics. This construct was integrated at Neutral Site II of the 7942 Δ*ccmM* genome to yield the Δ*ccmM* + *mTagBFP2-CcmM-mNeonGreen (+B-ccmM-N)* strain. (C) Anti-CcmM western blot of WT, Δ*ccmM*, and *+B-ccmM-N*. Full length M58 isoform of CcmM is ~58 kDa, while the truncated M35 is ~35 kDa. The fluorescent fusions of mTagBFP2-M58-mNeonGreen and M35-mNeonGreen have expected molecular weights of approximately 113 kDa and 63 kDa respectively. (D) Growth curves of WT 7942, Δ*ccmM*, and the *+B-ccmM-N* genetic complement. Dotted lines denote cultures grown with 2% supplemented $CO_2$. Solid lines denote cultures grown in ambient air conditions. n = 3 biological replicates, error bars = standard deviation.

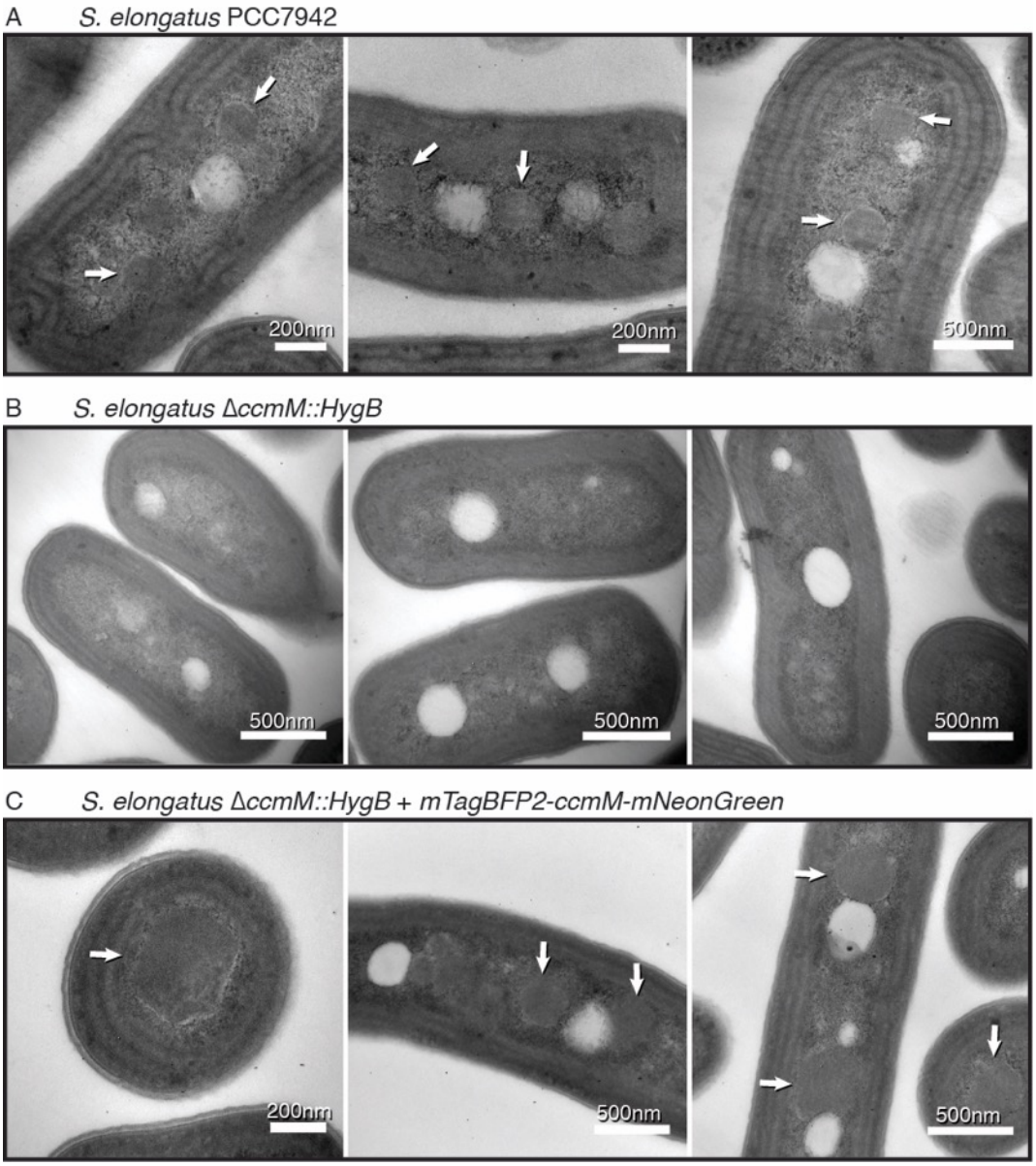
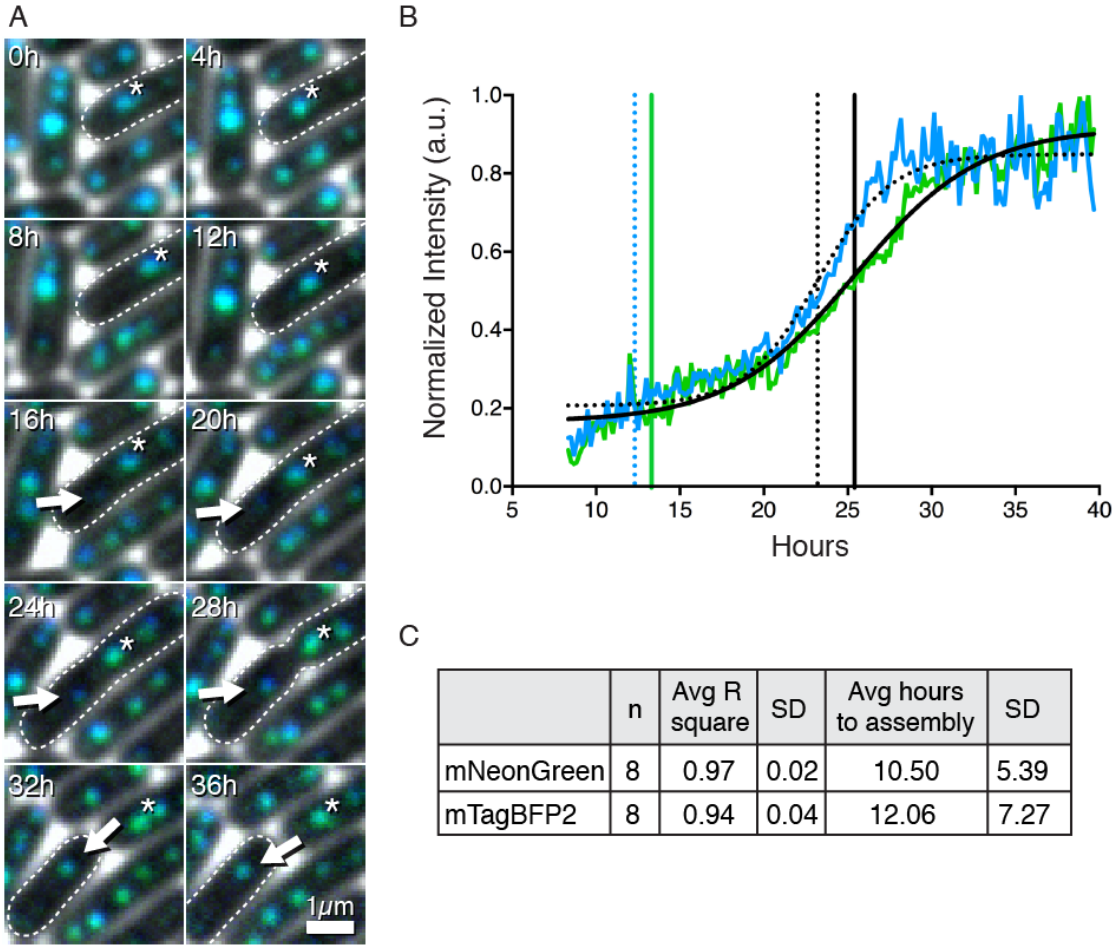



**Figure 3.** Transmission electron micrographs show the formation of carboxysomes in Δ*ccmM* cells complemented with fluorescently tagged *ccmM*. (A-D) Carboxysomes (white arrows) in wild type PCC7942 cells show characteristic hexagonal geometry. Diameters of WT carboxysomes labeled in micrographs range from 150 - 280 nm with an average of 180 ± 33nm (N=???). (E-H) Micrographs of the Δ*ccmM* knockout show the absence of carboxysomes. (I-L) Integration of the fluorescently tagged *ccmM* construct *B-ccmM-N* into the Δ*ccmM* knock out rescues the formation of apparently normal carboxysomes. These carboxysomes had geometry similar to that observed in wild type cells. Carboxysomes with tagged CcmM appeared to range more widely in size than in wild type cells with an average diameter of 319 ± 92nm (N=???). Some (J & L) were of typical size (200nm in diameter), while others (K) are larger at 300-330 nm. Larger carboxysomes were also observed, like that seen in (I), which approaches 600nm at its widest point.




C

| | n | Avg R square | SD | Avg hours to assembly | SD |
|---|---|---|---|---|---|
| mNeonGreen | 8 | 0.97 | 0.02 | 10.50 | 5.39 |
| mTagBFP2 | 8 | 0.94 | 0.04 | 12.06 | 7.27 |

**Figure 4.** CcmM isoforms co-assemble at the site of new carboxysomes. (A) Merged montage of carboxysome biogenesis in the Δ*ccmM* + *mTagBFP2-ccmM-mNeonGreen* gene complement strain shows simultaneous co-localization of both blue (mTagBFP2-M58) and green (M58-mNeonGreen / M35-mNeonGreen) particles. White arrows indicate the new carboxysome. White asterisk indicates the parent carboxysome. (B) Channel specific intensity plots of carboxysome biogenesis depicted in panel A reveal sigmoidal-like dynamics in assembly timing in M35 and M58 CcmM isoforms. Vertical blue and green lines denote point at which automatic particle tracking first identified the new carboxysome. Tracks were extended backwards to measure background by manually measuring spot at or near the new particle (Methods). Four-parameter logistic equations were fit to each track; black solid or dotted vertical lines denote half-max values of each channel. (C) Statistics of sigmoidal curves fit to 8 measured biogenesis events find similar assembly dynamics in both green and blue channels. Assembly time was measured from first time point of automatic tracking to first value $\geq$ 0.8 on normalized intensity plot.

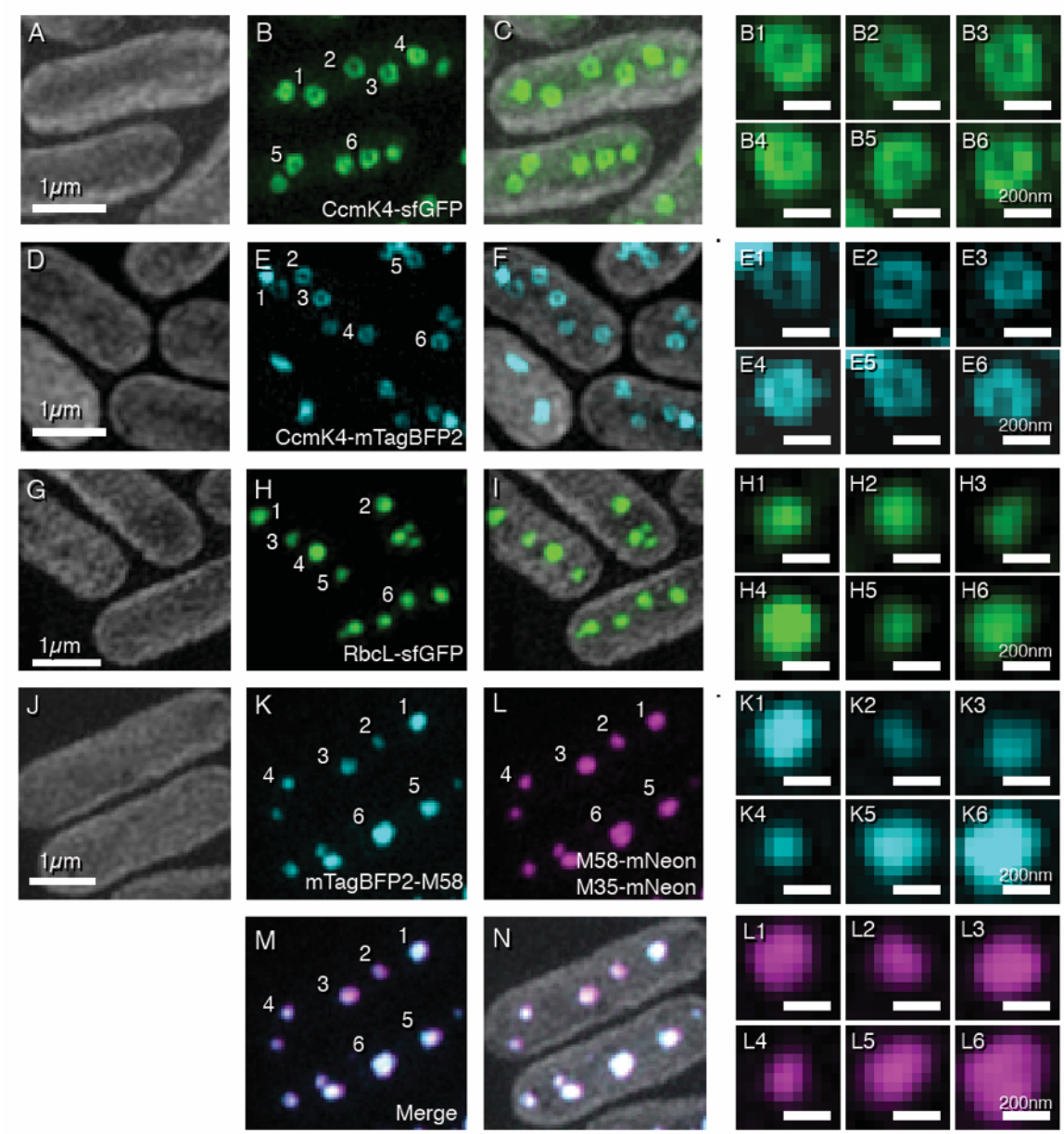
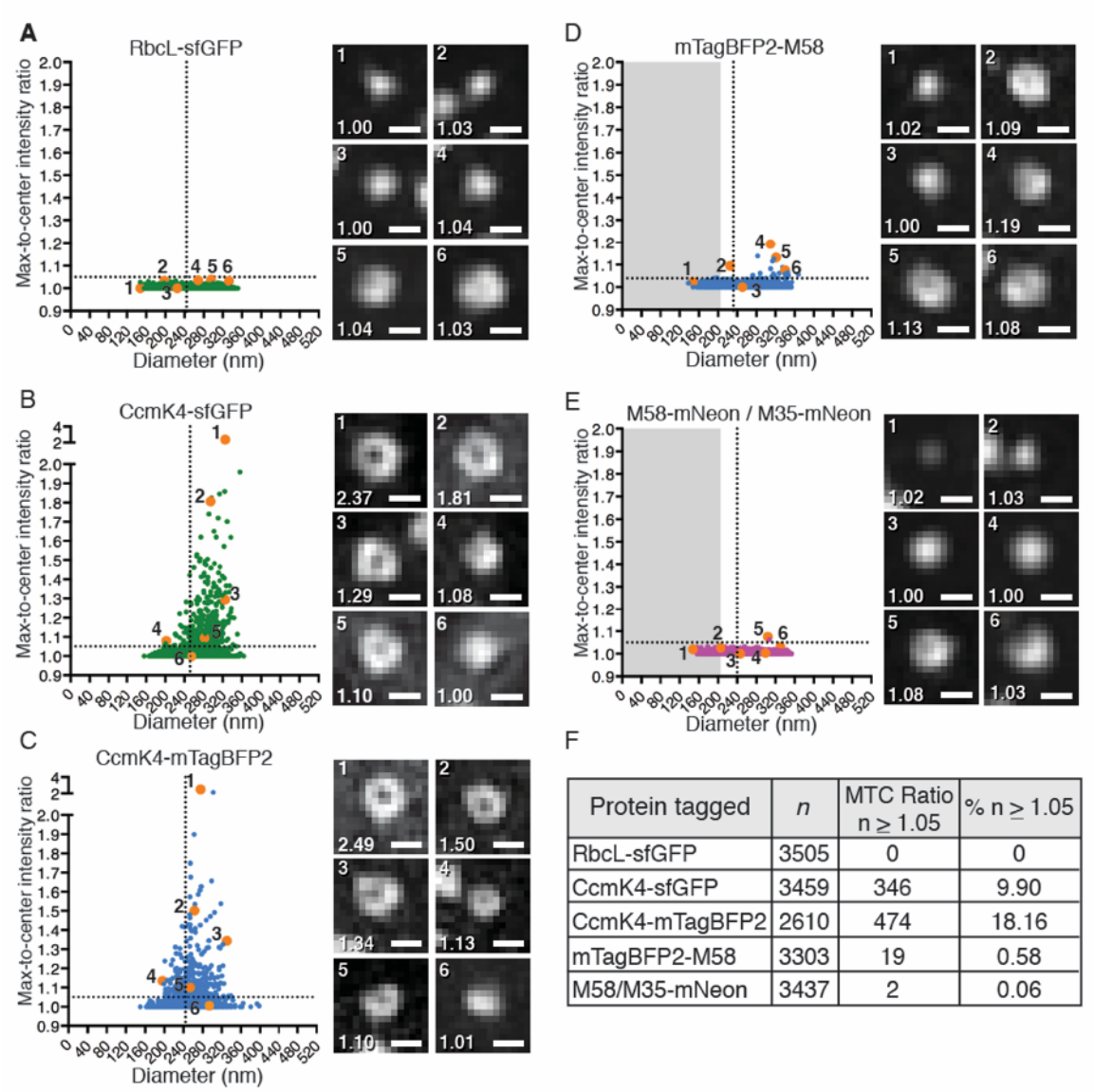



**Figure 5.** SIM of fluorescently tagged carboxysomes resolves the ring of the outer shell, and an absence of shell-like localization of either RuBisCO or CcmM. (A-C) SIM of CcmK4-mTagBFP2 resolves the carboxysome shell as a hollow ring, while (D-F) SIM of RbcL-sfGFP produces solid particles. (G-K) SIM of Δ*ccmM* + *mTagBFP2-ccmM-mNeonGreen* produces RuBisCO-like, apparently solid particles. M35/M58-mNeonGreen particles are pseudocolored magenta. Numbers identify individual carboxysomes and correspond to magnified panels on the right.




| Protein tagged | *n* | MTC Ratio n ≥ 1.05 | % n ≥ 1.05 |
|---|---|---|---|
| RbcL-sfGFP | 3505 | 0 | 0 |
| CcmK4-sfGFP | 3459 | 346 | 9.90 |
| CcmK4-mTagBFP2 | 2610 | 474 | 18.16 |
| mTagBFP2-M58 | 3303 | 19 | 0.58 |
| M58/M35-mNeon | 3437 | 2 | 0.06 |

**Figure 6.** Analysis of 3D-SIM data. Using radially averaged intensity profiles of fluorescent particles in 3D-SIM data, maximum intensity to center (MTC) intensity ratios can measure the degree to which a fluorescent object is resolved as a ring. (A) Analysis of RbcL-sfGFP 3D-SIM data found no objects with a max-to-center ratio (MTC) greater than 1.04, with an average ratio of 1.002 ± 0.005 and average particle size of 244 ± 45 nm marked by the vertical line. An upper ratio limit of 1.05, below which objects are likely to be solid, was calculated by adding two standard deviations to the RbcL-sfGFP max ratio. The 1.05 MTC cutoff is marked by a horizontal line on all plots. (B) Size versus intensity ratio plot of CcmK4-sfGFP shows a population of particles (345, 9.97%) with MTC ratios ≥ 1.05. (C) Plot of CcmK4-mTagBFP2 shows a two-fold increase in the fraction of ring-like particles compared to the GFP tagged version (474, 18.16%). (D) Analysis of particles in the blue channel of the Δ*ccmM* ± *mTagBFP2-CcmM-mNeonGreen* strain, representing M58, finds only a few particles with noticeable ring-like appearance (19, 0.58%), with the vast majority of particles appearing solid. Based on the size

of smallest detected ring in the tagged shell strains (196nm) and a shell thickness of 5-6nm, the minimum diameter at which ring-like shapes can be detected is calculated as 220nm; this is marked in gray. Ring-like particles within this region are unlikely to be resolved due to their small size. (E) Plot of particles in the green channel of Δ*ccmM* ± *mTagBFP2-CcmM-mNeonGreen* strain, representing M58-mNeonGreen and M35-mNeonGreen are predominantly solid with only 2 ring-like structures identified (2, 0.06%). (F) Table summarizing analysis statistics. In microscopy panels, scale bars are 200nm, and numbers denote MTC ratio.

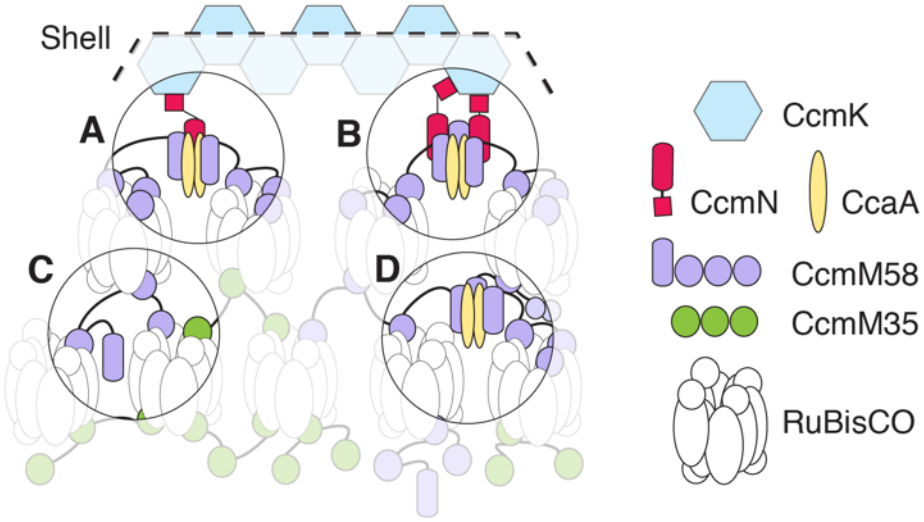



**Figure 7.** Mode of possible structural and functional roles of CcmM in the *S. elongatus* beta-carboxysome. (A-B) Full-length CcmM may form trimeric complexes through N-terminal interactions, which most likely include CcaA. Based on structural similarity between N-terminal domains of CcmN and CcmM, CcmN may be directly displacing units of CcmM in this complex or binding the timer to coordinate exterior shell recruitment (Kinney *et al.,* 2012). (C-D) We find evidence supporting a model in which full-length CcmM58 is also present within the core of the carboxysome, which may involve single units of CcmM58 coordinating multiple RuBisCO enzymes as in (C) or may mean that CcmM58 is forming trimeric complexes within the interior.

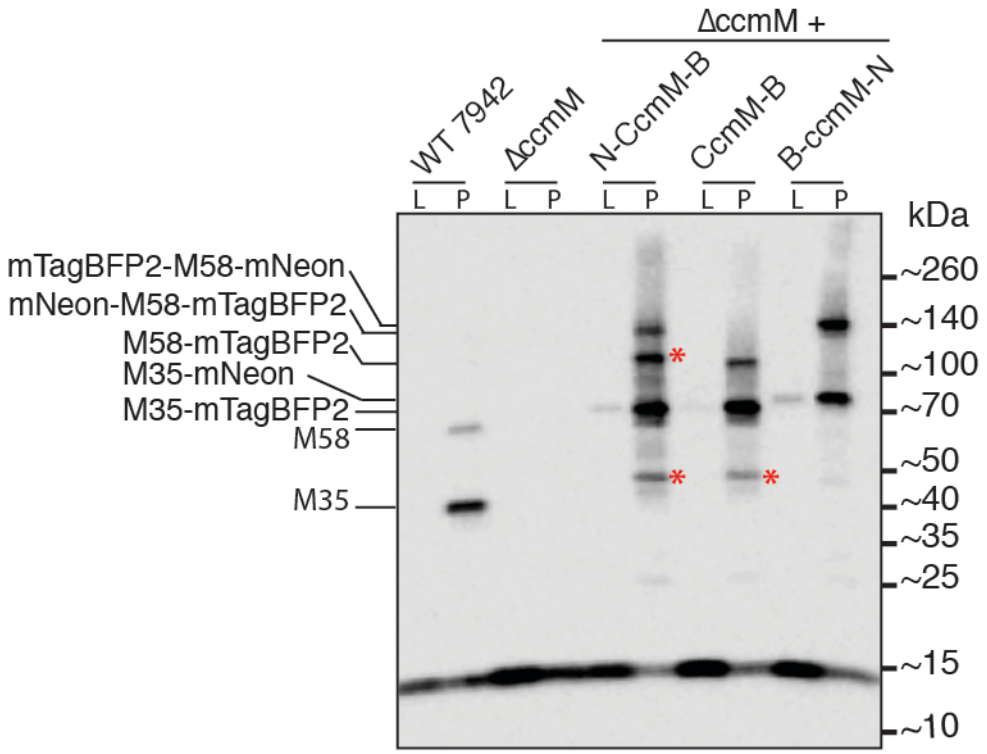



**Figure S1.** Protein degradation in variant *ccmM* gene complements. Western blot analysis of Δ*ccmM* + *mNeonGreen-ccmM-mTagBFP2*, and Δ*ccmM* + *ccmM-mTagBFP2* revealed prominent unexpected bands (marked by red asterix) that could be indicative of protein degradation in the cell. The Δ*ccmM* + *mTagBFP2-ccmM-mNeonGreen* strain did not show similar unexpected bands. L = cell lysate and P = post-lysis pellet.

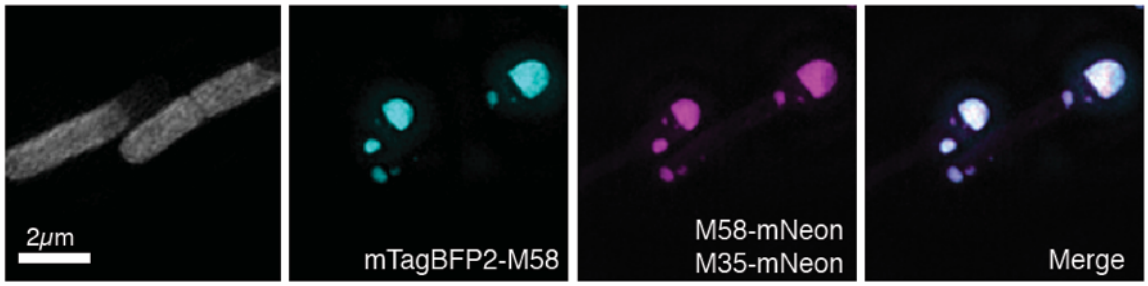
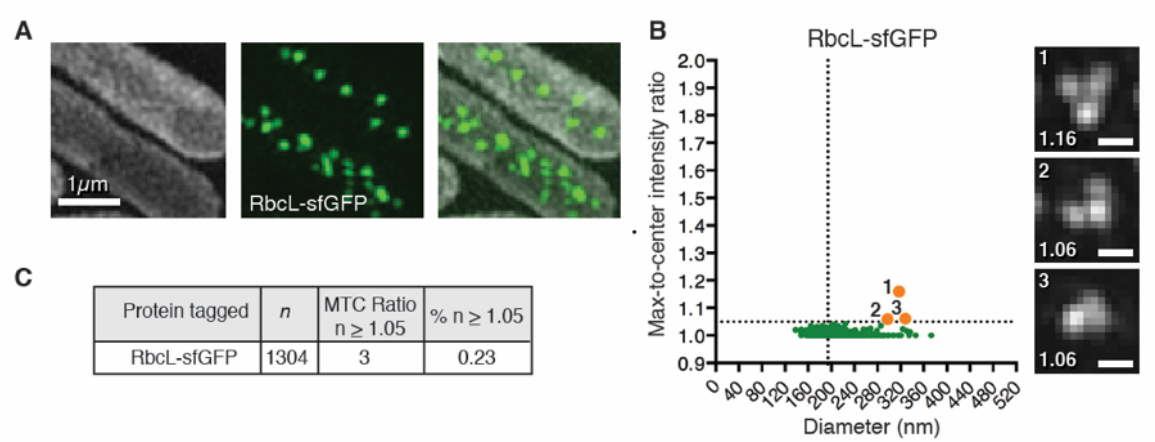



**Figure S2.** Induction of the P*trc*::Δ*ccmM* + *mTagBFP2-ccmM-mNeonGreen* in *S. elongatus* leads to protein aggregation**.** SIM images of Δ*ccmM* + *mTagBFP2-ccmM-mNeonGreen* cells induced with 50μM IPTG show large aggregations of fluorescent signal near the cell polls.




| Protein tagged | *n* | MTC Ratio n ≥ 1.05 | % n ≥ 1.05 |
|---|---|---|---|
| RbcL-sfGFP | 1304 | 3 | 0.23 |

**Figure S3.** Uninduced cells expressing RbcL-sfGFP still produce solid particles. Qualitative data and image analysis results of PCC7942 RbcL-sfGFP cells without any IPTG induction show similar incidence of low MTC ratio solid particles compared to cells induced with 50μM IPTG. Three particles were identified as ring-like with MTC ratios > 1.05, but were confirmed to be three particle clusters incorrectly analyzed as single objects by our analysis program.

| Strain | Tag | IPTG (μM) | # Imgs | Refractive Index | Wiener Filter | RIH (477) | RIH (528) | RIH (541) | RIH (608) |
|---|---|---|---|---|---|---|---|---|---|
| DFS724 | RbcL-sfGFP | 50 | 31 | 1.518 | 0.01 | | 12.74 ± 1.63 | | 2.78 ± 0.66 |
| AHC134 | CcmK4-sfGFP | - | 31 | 1.517 | 0.01 | | 13.15 ± 1.78 | | 3.53 ± 1.08 |
| MJN31 | CcmK4-mTagBFP2 | 0 | 29 | 1.517 | 0.01 | 8.28 ± 1.84 | | | 2.77 ± 0.96 |
| MJN28 | mTagBFP2-CcmM-mNeon | 0 | 47 | 1.517 | 0.01 | 9.45 ± 2.80 | | 12.58 ± 2.51 | 3.10 ± 0.84 |

**Tables S1**. Structured Illumination Microscopy reconstruction scores. Reconstructed Intensity Histogram scores (RIH) were averaged across all image sets for each strain used for image analysis. Scores of 6-12 are considered good, and greater than 12 excellent. Columns with RIH scores are channel specific. Channel 608 captured the red auto-fluorescence of cells which was not used for any analysis. Error = Standard deviation.

A

| Welch's ANOVA | Degrees of freedom | $F_s$ | *P* |
|---|---|---|---|
| Among groups | 4.00 | 160.48 | 6.51 x 10-132 |
| Within groups | 7377.44 | | |

B

| Games-Howell test | Mean difference | q | > $q_{crit}$ 5.484 (α=0.001) |
|---|---|---|---|
| CcmM-mNeon vs CcmK4-sfGFP | 0.020465 | 22.20 | Significant |
| CcmM-mNeon vs CcmK4-mTagBFP2 | 0.033914 | 26.34 | Significant |
| CcmM-mNeon vs mTagBFP2-CcmM | 0.001283 | 9.56 | Significant |
| CcmM-mNeon vs RbcL-sfGFP | 0.000050 | 0.57 | Not significant |
| CcmK4-sfGFP vs CcmK4-mTagBFP2 | 0.013449 | 8.51 | Significant |
| CcmK4-sfGFP vs mTagBFP2-CcmM | 0.019182 | 20.69 | Significant |
| CcmK4-sfGFP vs RbcL-sfGFP | 0.020515 | 22.26 | Significant |
| CcmK4-mTagBFP2 vs mTagBFP2-Ccm | 0.032631 | 25.27 | Significant |
| CcmK4-mTagBFP2 vs RbcL-sfGFP | 0.033964 | 26.38 | Significant |
| mTagBFP2-CcmM vs RbcL-sfGFP | 0.001333 | 10.05 | Significant |

852
853 **Table S2.** Statistical test results of particle MTC ratio comparison.
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| Strain or Plasmid | Genotype | Resistance | Reference |
|---|---|---|---|
| *E-coli strains* | | | |
| DH5-alpha | Host strain for plasmid construction (PAS644) | | |
| DH10-beta | Host strain for plasmid construction (PAS641, PAS642, PAS643) | | |
| Turbo | Host strain for plasmid construction (PAS645) | | |
| *S. elongatus strains* | | | |
| PCC7942 | Wild-type *Synechococcus elongatus,* ATCC 33912 | | |
| Δ*ccmM* | PCC7942 Δ*ccmM*::*HygR* knock out | HygB | |
| PAS646 | PCC7942 *Ptrc:CcmK4-mNeon* integrated in NS2 | Kan | This work |
| PAS647 | PCC7942 Δ*CcmM::HygR + Ptrc:mNeon-CcmM-mTagBFP2* integrated in NS2 | HygB, Kan | This work |
| PAS648 | PCC7942 Δ*CcmM::HygR + Ptrc:CcmM-mTagBFP2* integrated in NS2 | HygB, Kan | This work |
| PAS649 | PCC7942 Δ*CcmM::HygR + Ptrc:mTagBFP2-CcmM-mNeon* integrated in NS2 | HygB, Kan | This work |
| PAS650 | PCC7942 *Ptrc:CcmK4-mTagBFP2* integrated in NS2 | Kan | This work |
| PAS651 | PCC7942 *Papca:CcmK4-mNeon* integrated in NS1 | Sp | Chen et al., 2013 |
| PAS652 | PCC7942 *Ptrc:RbcL-sfGFP* integrated in NS2 | Kan | Savage et al., 2010 |
| *Plasmids* | | | |
| pAM2314 | *S. elongatus* Neutral Site I integration vector | Sp | Mackey et al., 2007 |
| pAM1579 | *S. elongatus* Neutral Site II integration vector | Kan | Mackey et al., 2007 |
| pDFS21 | derivative of pAM1579 with *trc* promoter from pTRC99 and *lacI* | Kan | Savage et al., 2010 |
| pAHC134 | P*apca*::*ccmK4-sfGFP* | Sp | Chen et al., 2013 |
| pDFS724 | P*trc*::*RbcL-sfGFP* | Kan | Savage et al., 2010 |
| PAS641 | P*trc::mNeonGreen-CcmM-mTagBFP2* cloned into pDFS21 at XbaI and NotI sites | Kan | This work |
| PAS642 | P*trc::CcmM-mTagBFP2* cloned into pDFS21 at XbaI and NotI sites | Kan | This work |
| PAS643 | P*trc::mTagBFP2-CcmM-mNeonGreen* cloned into pDFS21 at XbaI and NotI sites | Kan | This work |
| PAS644 | *Ptrc::CcmK4-mNeonGreen* cloned into pDFS21 at XbaI and NotI sites | Kan | This work |
| PAS645 | *Ptrc::CcmK4-mTagBFP2, mTagBFP2* cloned in place of *mNeonGreen* in pDFS21 | Kan | This work |

856
857 **Table S3.** Strains and plasmids