IDENTIFICATION OF A NOVEL REGULATOR OF STALK SYNTHESIS IN
CAULOBACTER CRESCENTUS

BY

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A thesis submitted to the Graduate School-Camden

Rutgers, the State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Master of Science

Graduate Program in Computational and Integrative Biology

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January 2018
Prokaryotes exist in diverse morphologies in nature. The shape of the cell offers the cell the ability to adapt to the selective pressures of its environment. Bacterial shape is a function of its cell wall composition. Our current knowledge of bacterial cell wall synthesis is generally limited to rod shaped cells. To address the question of how special morphological features, aid different bacterial cells in survival, we use Caulobacter crescentus as a model organism. Caulobacter crescentus has a polar cellular extension called a stalk that elongates dramatically in response to phosphate starvation. Stalk synthesis is a highly regulated process of unidirectional extension of the cell envelope. The stalk also serves as a cell polarity marker during the asymmetric cell division in Caulobacter crescentus. The phenomenon of stalk localization is governed by a polar localization complex; however, the mechanism of stalk synthesis remains unknown. The current work aims at characterizing a novel penicillin binding protein (PBP), CC_2105 that may have a function in the regulation of stalk synthesis.
Acknowledgements

This work would not have been possible without the guidance of my advisor Dr. Eric Klein. I thank you for your dedication to teaching, patience, valuable advice and your warm reception when I first arrived. Your enthusiasm and curiosity for learning, and your humbleness to science inspire me each day. Special thanks to my committee members Dr. Nir Yakoby and Dr. Benedetto Piccoli, for guiding me towards the best of my abilities. Thank you also to Dr. Grace Brannigan for your advice and support during my time in Rutgers- Camden.

Thank you to my former and current fellow lab members for their support. Thanks to all my extended family of friends at Rutgers for being there with me through my good and tough times: Sudha, Alessio, Cody, Nastassia, Julia, Kate, Vaani, Steve, Srobona. My roommates Smruthi and Andrea, deserve special thanking for being there for me. I also thank every other member of the CCIB, and my friends in India Mahesh, Rupesh, Harsha, Raghu, Vamsi, Mrudula who have supported me all along.

Lastly, and most importantly, my loving family- mom (Sobha Revur), dad (Dr. Prasad Revur) and my loving sister Sreeja Revur, who continue to believe in me and who stand by my choices, no matter how good or bad they are. Thanks for being so supportive of me and my ambitions and dreams. Mom and dad, your continuous efforts towards learning something new even at this age (not that you are too old) is hope and inspiration for me that I will go back to graduate school someday. Thank you for being such wonderful family! Also thanks to my family here in the US- aunts Ratna and Latha, uncles Ramu and Mahesh, cousins Neha, Sid and Krishna for watching out for me and helping me.
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Background

Bacteria exist in a wide variety of shapes and sizes in nature. These unicellular organisms have evolved into varied sizes and shapes to meet the requirements of their environment [1]. The size and shape of these cells have been known to influence the survival fitness factors including motility, predation, nutrient uptake, etc [1]. Some bacteria change their morphologies based on the environmental morphogens available. Certain pathogens like *L. pneumophila* take multiple morphologies for several functions during its lifetime, before infecting the host [2]. Other bacteria grow filaments (e.g., *Actinomyces israelii*) or stalks (e.g., *Caulobacter* and *Rhodomicrobium*) in response to nutritional restriction [3]. The changing morphologies and the development of extracellular appendages like filaments, stalks or motility factors serve as a gain of function for the bacteria. Therefore, understanding the extracellular structures and their mechanisms would help understand the evolutionary advantages of a bacterial shape.

The cell wall of the bacterial cells defines the morphology of a cell. Peptidoglycan is the fundamental unit of a bacterial cell wall [4]. The varied shapes of different bacteria are an outcome of different peptidoglycan compositions and their different structural arrangements [5]. The peptidoglycan layer of a cell offers the cell its integrity, mechanical strength, flexibility and helps maintain the osmotic pressure [6]. Based on the number of layers of peptidoglycan present in the cell, the bacterial cells are classified broadly into gram-positive bacteria and gram-negative bacteria [7]. The penicillin binding proteins aid in the process of assembling the peptidoglycan sub-units in the bacterial cells [8, 9]. These enzymes have a beta-lactamase domain in common and can bind to the broad spectrum antibiotic penicillin [10]. The PBP’s oversee trans-glycosylation, trans-peptidase and
endopeptidase functions, and are classified into high and low molecular weight PBP’s[6]. Bacterial cells have multiple PBP’s which have overlapping yet specific functions [11]. The molecular mechanism of cell wall synthesis is well established in rod-shaped bacteria. However, in case of the oddly shaped cells with special appendages, the understanding of molecular mechanisms of cell wall synthesis remains unknown. To partly address this, we employ *Caulobacter crescentus*, a crescent-shaped bacterium with a special appendage, “the stalk” as a model organism.

**Caulobacter crescentus**

*Caulobacter crescentus* is a ubiquitously found aquatic bacterium. This crescent-shaped bacterium is classified as a gram-negative alpha proteobacterium with a stalk [12]. The asymmetrically dividing bacteria display different appendages like the stalk, flagellum, and pilus, during its lifecycle [12]. The stalk of *Caulobacter* has a holdfast, rich in the polysaccharide, which allows it to stick to a surface in the aquatic microenvironment [12, 13].

The crescent shape is maintained by the *creS* gene producing the protein crescentin [14]. The curved shape of the cell with the immobilized stalk offers a buoyant advantage from the flow [15]. It has been shown that the curved shape enables these cells to arc towards the direction of flow and enhances surface colonization [16].

The cell cycle of *Caulobacter crescentus* is dimorphic (Figure 1). The stalked cell elongates and generates a flagellum and pili on end opposite to the stalk. The pre-divisional cell holds a stalk at one pole and flagella and pili on the other pole. After cell division, the cell with the flagella swarms away and matures eventually into the stalked
cell before re-entering the cell cycle. The other daughter cell with the stalk continues with the next round of cell division, immediately [17].

**Figure 1- Lifecycle of Caulobacter crescentus:**

A depiction of dimorphic cell cycle in *Caulobacter crescentus* [18]

The cell cycle in the *Caulobacter* is highly regulated at multiple levels. The control of DNA synthesis, segregation of proper cellular machinery, maintaining the cell polarity are all critical during the cell division. CtrA is a key regulator of replication, that binds to Cori (*Caulobacter* origin of replication) to repress the process of replication, when phosphorylated [19, 20]. The process of replication is initiated by DnaA, that remains in higher concentrations during the process of replication [21]. Maintaining the polarity of the cell marks the key to determining the fate of the future daughter cell [22]. A set of histidine kinase two-component system DivJ, PleC localized at the two different polar ends, act on a single response regulator DivK during the cell division [23, 24] (Figure 8 a,b). While the gradient of DivK phosphorylated and un-phosphorylated states are generated the cell division complex comes to play in quickly sealing the cell into two compartments with varying concentration of DivK [25, 26]. The polar markers PopZ, SpmX, PodJ play a key role defining the cell polarity [27-29]. The polar marker popZ, that localizes at the base of the stalk is known to regulate the cell cycle by influencing the chromosome segregation [30-34]. Overall, the stalk plays a key role in marking the cell polarity and the cell division.

The stalk of *Caulobacter crescentus* exhibits a phenomenon of dramatically long stalks when deprived of phosphate in the environment [35, 36]. It has been predicted that this elongation of the stalk helps in increasing the surface-volume ratio of the cell thus helping
in greater nutrient diffusion [15, 37]. Other hypothesized functions include the advantage of reaching phosphate from beyond the micro-environment and also gain a survival advantage in a co-localized colony [38]. Stalk elongation is regulated independently of the cell cycle through phosphate transport genes \textit{pst} and regulon \textit{phoB}, in the phosphate-starved environment [36].

The stalk of \textit{Caulobacter} is a very thin extension of cell envelope comprising of the outer and the inner membranes, S-layer, peptidoglycan and periplasm and membrane proteins [12, 39]. The size of the stalk is only \textasciitilde{} 100-150 nm in diameter [40]. The stalks are also known to have crossbands which were originally predicted to be made up of peptidoglycan, synthesized only during cell cycle [41, 42]. Lately, the crossbands were found to be made up of stalk specific protein complex – StpA-D, which generate a membrane diffusion barrier between the stalk and the cell body [43].

The synthesis of the stalk is highly regulated and is unidirectional. Previous studies on the factors influencing the stalk synthesis show that the polar localization molecules, the cytoskeletal proteins and the PBPs function in concert for the stalk synthesis in \textit{C. crescentus}. MreB, an actin homolog is known to aid the cell elongation and has been observed at the stalked poles. [14]. Rod A functions along with MreB in maintaining the cell shape [44]. Divakaruni (2007), showed that MreB and FtsZ are essential for the stalk morphogenesis, while Rod A and MreC were found to be essential for the cell wall synthesis. Rod A, Mre C are known to function along with PBP 2 [44, 45]. Mutants of stalk polar markers like spmX, popZ are also known to affect the stalk localization and synthesis [33, 46]. PBP 3 is known to respond to ftsZ during cell division [47]. Although much is
known about the factors influencing the stalk localization and the synthesis, the precise molecular mechanism involved in the stalk synthesis remains unknown.

The peptidoglycan of the stalk is synthesized during the cell division and also during the elongation of stalk due to phosphate limiting stimulus. The initial studies on PBPs of *Caulobacter crescentus* identified high and low molecular weight PBPs [48, 49]. The well-characterized PBP’s in *Caulobacter* are the bifunctional paralogues bPBPs, PBP 1A, PBP C, PBP X, PBP Y and PBP Z, that have redundant functions [50, 51]. It is also known that different PBP’s contribute to specific cross-links to the peptidoglycan [50]. A double deletion mutant of ΔpbpC ΔpbpX causes a reduction in the stalk length in low phosphate conditions in *Caulobacter crescentus* [51]. Recent studies have shown that the deletion of PBP glycosyltransferase paralogs, in combination or alone except for PBP Z, *Caulobacter* mutants were still able to generate stalks under low phosphate conditions [51]. This may be explained by the contribution of redundant functions of other paralogues. Given that the evidence that the stalk morphologies are not affected in the absence of PBP’s individually, it is likely that the stalk PBPs involved in stalk synthesis are yet to be identified. We aim to identify the stalk specific proteins.

**Figure 2- PBP’s of the stalk:**

7.5% SDS PAGE of Penicillin binding proteins isolated from CB15 WT form (a) PYE cultures, (b) HIGG cultures and stalks of (c) CB15 WT or (d) CB15NY106 from HIGG

Koyasu et al., (1983) had earlier reported that the stalk of *Caulobacter* exhibited a unique set of PBP’s that were absent in the cell body fraction (Figure 2). Based on this experiment, penicillin-binding protein was previously isolated among the PBP extracts corresponding
to the stalks of *Caulobacter crescentus*, and the protein was identified as CC_2105 using mass-spectrometry (Klein, E- unpublished). The current study aims at characterizing CC_2105 with a hypothesis that *CC_2105 may have a role in the stalk synthesis in Caulobacter crescentus*. 
Materials and Methods

Bacterial strains and growth conditions:

The wildtype strain NA1000, and the mutant clones of CC_2105 were grown in Peptone-Yeast-Extract (PYE) media [12] or M2G media (Minimal media) at 30°C. To generate the long stalk phenotype, cells were grown under limited phosphate conditions in Hutners-imidazole-glucose-glutamate (HIGG) media [52] containing 1µM phosphate at 30°C. Appropriate inducers 0.3% glucose, 0.3% xylose, 0.5mM vanillate were used along with antibiotic tetracycline (1 µg/ml).

Molecular cloning:

The clones generated during this study include CC2105 ΔCT (C-terminus deletion), DS1, DS2, DS3, DS4. Standard protocols were used for transformation as described previously [53]. The mCherry clones were generated with an inducible promoter as previously described [54].

Microscopy methods:

1µl of live cells were collected and placed on 1% agarose gel pads made in appropriate media (HIGG/PYE). After letting the surface of the pads dry, coverslips were placed. Light and fluorescence microscopy images were recorded with Nikon TiE microscope equipped with Zyla sCMOS camera. The brightness and contrast of the images were adjusted using ImageJ software.
# Table 1 - List of strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Construction</th>
<th>Reference or Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Caulobacter crescentus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA 1000</td>
<td>Wild type (CB15 -synchronize-able strain)</td>
<td></td>
<td>Evinger and Agabain, 1977</td>
</tr>
<tr>
<td>ΔCC_2105</td>
<td>NA 1000 ΔCC_2105</td>
<td>Deletion of endogenous cc2105 from NA1000</td>
<td>Dr. Klein unpublished</td>
</tr>
<tr>
<td>ΔCT</td>
<td>NA 1000 CC_2105 Δ 535-712</td>
<td>Deletion of c-terminus domain corresponding to a.a 535-712 from endogenous cc2105 in NA1000 using pNPTS138</td>
<td>Current study</td>
</tr>
<tr>
<td>NTOE</td>
<td>NA1000 Pxy:: Pxy CC_2105 (1-534)-tetR</td>
<td></td>
<td>Dr. Klein unpublished</td>
</tr>
<tr>
<td>2105OE</td>
<td>NA1000 Pvan:: Pvan CC_2105 tetR</td>
<td></td>
<td>Dr. Klein unpublished</td>
</tr>
<tr>
<td>DS1</td>
<td>NA1000ΔCC_2105 Pxy:: Pxy CC_2105 mcherry tetR</td>
<td>Transformation of ΔCC_2105 with pXCHYC-5 carrying CC_2105</td>
<td>Current study</td>
</tr>
<tr>
<td>DS2</td>
<td>NA1000 Pxy:: Pxy CC_2105 mcherry tetR</td>
<td>Transformation of NA1000 with pXCHYC-5 carrying CC_2105</td>
<td>Current study</td>
</tr>
<tr>
<td>DS3</td>
<td>NA1000 ΔCC_2105 pXCHYC -5</td>
<td>Transformation of ΔCC_2105 with pXCHYC-5</td>
<td>Current study</td>
</tr>
<tr>
<td>DS4</td>
<td>NA1000 pXCHYC-5</td>
<td>Transformation of NA1000 with pXCHYC-5</td>
<td>Current study</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17-1</td>
<td>E. coli 294::RP4-2(Tc::Mu) (KM::Tn7)</td>
<td></td>
<td>Liss,L.R 1987</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>pXCHYC-5</td>
<td>Integrative plasmid with C-terminus red fluorescent protein m-cherry fusion - controlled by a Xylose promoter, tetR</td>
<td></td>
<td>Thanbichler et al., 2007</td>
</tr>
<tr>
<td>pNPTS138</td>
<td>SacB containing suicidal vector used for double homologous recombination, KanR</td>
<td></td>
<td>Alley, M.R.K, unpublished</td>
</tr>
</tbody>
</table>
All clones were screened using antibiotic selection and confirmed using colony-PCR followed by gel electrophoresis. All the primers used are listed in Table 2.

Table 2- List of Primers used in this work:

<table>
<thead>
<tr>
<th>Clone generated</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔCT</td>
<td>EK 668 TACTAAGCTTGACGGCAAAAACCATCTTCTCG</td>
</tr>
<tr>
<td></td>
<td>EK671 TACTGAATTCGATCAAGGGCTATGCCTTCC</td>
</tr>
<tr>
<td>DS1, DS2</td>
<td>EK 260 TACTCATATGACGTCGCCTCCAGGTCAGG</td>
</tr>
<tr>
<td></td>
<td>EK 261 TACTGAGCTCGGCTCCAGGAGCAGG</td>
</tr>
</tbody>
</table>
Results

Mutants of CC_2105:

The architecture of CC_2105 predicts a signal peptide at the N-terminus, a beta-lactamase domain, and four transmembrane helices at the C-terminus (Figure 2 a). The homology modeling predicts a transpeptidase function for the penicillin-binding protein CC_2105 owing to the beta-lactamase domain in the N terminus. To assess the role of CC_2105 we generated a series of mutants with varying functional domains of CC_2105. The mutants include:

ΔCC_2105: a complete cc2105 deletion mutant,

CC2105ΔCT: a C-terminus deletion mutant (Δ536-712) lacking the four transmembrane domains,

NTOE: an N-terminus Overexpression CC_2105 (1-535) under a xylose-inducible promoter, tetR and

CC_2105 OE: a complete CC_2105 overexpression clone under a vanillate inducible promoter, tetR.

An N terminus deletion could not be generated, as the deletion of the signal peptide would affect the proper localization of the protein in the membrane.

All mutants exhibited normal phenotypes comparable to that of the wildtype (WT) when grown in PYE media at 30°C (Figure 3 b), confirming that CC_2105 is not an essential gene. The redundant functions of multiple penicillin-binding proteins of Caulobacter crescentus possibly compensate for the loss of a functional CC_2105 [51].
Figure 3 - Mutants of CC_2105:

a) Illustration of domains of CC_2105 with, N-terminus signal peptide, a transpeptidase domain, four transmembrane spanning regions at the C-terminus. (b-g) Mutants were grown in PYE media for 24 hours, with appropriate inducers and antibiotics (b)WT, (c) ΔCC_2105, (d) CC2105ΔCT, (e) NTOE- 0.3% Glucose, (f) NTOE- 0.3% Xylose, (g) 2105 OE-0.5mM Vanillate.

Effect of CC_2105 on stalks:

To test whether CC_2105 has a role in the stalk synthesis, the mutants, and the wildtype cells were grown in 1μM phosphate supplemented HIGG media and the phenotypes were observed (Figure 4). Surprisingly, the deletion mutant ΔCC_2105 had a second stalk phenotype at 72 hours. Interestingly the C-terminus deletion mutant mirrored the same double stalk phenotype as the complete deletion (Figure 4 c, h). The overexpression mutants did not show any phenotypical effects on the stalk and retained a single stalk phenotype like the WT (Figure 4 d, e, f).
The double stalk phenotype development was closely monitored among these mutants every 24 hours. Interestingly, at 48 hours a very small population of cells in the deletion mutants start to exhibit the second stalk.

![Image](image_url)

**Figure 4- Effect of CC_2105 mutants on stalk phenotype:** Clones of CC_2105 and WT grown in HIGG media with 1µM Phosphate, after 72 hours at 30°C. Appropriate inducers and antibiotics were added to the cultures. a) WT, b) ΔCC_2105, c) CC2105ΔCT, d) NTOE- 0.3% Glucose, e) NTOE- 0.3% Xylose, f) 2105 OE-0.5mM Vanillate; zoomed images of the double stalk phenotypes ( g- ΔCC_2105), ( h- CC2105ΔCT). The black arrows indicate the development of a second stalk.

The average double stalk percentage observed in the deletion mutants was 13-fold more than WT cells, at 72 hours (Figure 5 b, Table 3). The two-tailed student t-test, conducted for individual mutants with the WT revealed that there is a significant increase in the double stalk phenotype observed among the deletion mutants ΔCC_2105 (p-value <0.05) and CC_2105ΔCT (p-value< 0.05) (Figure 5 b, Table 3). The ectopic stalk was observed at the flagellar pole, which continues to elongate from the time of appearance. Around 120 hours the deletion mutants show double stalk phenotype five times more than the WT (Figure 5...
- a, b). Notably, the mutants had a smaller cell body and a pear-shaped morphology (Figure 4 - g, h).

The overexpression mutants, NTOE, 2105 OE, appeared healthy and exhibited a single stalk phenotype while the cell body and shape comparable to the WT, at 72 and 120 hours (Figure 4, d, e, f). The percentage of double stalks observed in these mutants remain as low as that observed in the WT (Figure 5 b, Table 3).

Table 3: Average Double stalk observed at 72 hours and 120 hours.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Average % DS 72 hours</th>
<th>SD-72 hours</th>
<th>p-Value 72 hours</th>
<th>Average % DS 120 hours</th>
<th>SD-120 hours</th>
<th>p-Value 120 hours</th>
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<tbody>
<tr>
<td>WT</td>
<td>3.46</td>
<td>0.98142</td>
<td></td>
<td>15.9766</td>
<td>10.1840</td>
<td></td>
</tr>
<tr>
<td>ΔCC_2105</td>
<td>46.5266</td>
<td>6.22725</td>
<td>0.00898</td>
<td>77.821</td>
<td>6.29758</td>
<td>0.00367</td>
</tr>
<tr>
<td>CC_2105ΔCT</td>
<td>47.0533</td>
<td>3.88969</td>
<td>0.00250</td>
<td>79.7896</td>
<td>2.41154</td>
<td>0.00945</td>
</tr>
<tr>
<td>NTOE 2105</td>
<td>3.52666</td>
<td>5.16259</td>
<td>0.97524</td>
<td>9.32433</td>
<td>6.16043</td>
<td>0.482304</td>
</tr>
<tr>
<td>Glu</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTOE 2105</td>
<td>1.08333</td>
<td>0.32293</td>
<td>0.06376</td>
<td>6.66266</td>
<td>2.14158</td>
<td>0.3242</td>
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<td>Xyl</td>
<td>2.10333</td>
<td>0.89749</td>
<td>0.223131</td>
<td>5.921667</td>
<td>1.43411</td>
<td>0.296534</td>
</tr>
<tr>
<td>2105 OE</td>
<td></td>
<td></td>
<td></td>
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</table>
Figure 5- The double stalk phenotype:
a) Double stalk phenotype observed in ΔCC_2105, CC2105ΔCT after 72 hours and 120 hours respectively when grown in HIGG media. WT cells are used as a control exhibiting a single stalk. b) Graph representing the percentage double stalks observed at 72 hours and 120 hours among the mutants of CC_2105. The data represents an average of three biological replicates, and the error bars indicate standard deviation.

CC_2105 rescue:

To confirm that the double stalk phenotype is a direct effect of the deletion of the predicted transpeptidase, CC_2105 was reintroduced in the deletion mutants to observe the reverse effect. An integrative plasmid with xylose inducible CC_2105 (pX CC_2105 mChy -tetR) was cloned into ΔCC_2105 (DS1) and WT cells (DS2). As a control, empty plasmids (pXCHYC-tetR) were transformed into ΔCC_2105 (DS3) and WT (DS4).

When induced with xylose, the DS1 clones showed a loss of double stalk phenotype and recovery of WT morphology. The phenotypes of DS1 clones and DS2 clones were the same (Figure 6 a). However, the DS1 clones retained the double stalk phenotype like ΔCC_2105, when repressed with glucose, confirming the absence of leaky induction (Figure 6 a). Empty plasmids pXCHYC-5 were also parallely transformed into ΔCC_2105 (DS3) and
WT (DS4), to ensure that the plasmid did not have any effect on the double stalk phenotype. As expected the DS3 mutants exhibited a double stalk phenotype in the presence of both xylose and glucose, confirming that expression of CC_2105 is essential for the rescue (Figure 6 a).

The average double stalks observed at 72 hours in ΔCC_2105 expressing CC_2105 (DS1-0.3% Xylose) was as low as 1.9 ± 0.9 %, comparable to WT 0.52 ± 0.7% (p-value > 0.05). Similarly, 46.76 ± 9% DS1 glucose repression cells exhibited a double stalk phenotype nearly equivalent to the ΔCC_2105 mutant cells 48.9 ± 10 % (p > 0.05). A student t-test comparison of ΔCC_2105 and DS1 -0.3% xylose, shows a significant difference in the average double stalk phenotype (p <0.05) (Figure 6 b). Thus CC_2105 expression significantly influenced the recovery from double stalk phenotype.

Since the deletion of CC_2105 does not affect the length of stalk formed but rather affects the localization of stalks, CC_2105 may not be involved in stalk synthesis as earlier hypothesized. The absence of ectopic stalk synthesis in overexpression, and the second stalk phenotype in deletion mutants together, indicates a possible role of CC_2105 in stalk regulation.

**Localization of CC_2105:**

Understanding the localization of CC_2105 in the cell is critical in understanding the possible function in the regulation of stalk synthesis. To serve this purpose the recovery clone DS1, DS2 was constructed with an m-Cherry tag at the C-terminus of CC_2105. When induced with xylose we observe that CC_2105-mchy localizes all along the cell and the stalk (Figure 7 A-A’, B-B’). The empty vector clones DS3, DS4 exhibited fluorescence
only in the cell body and not the stalks, confirming CC_2105 is localized in the stalks and the cell body as predicted (Figure 6 C’, D-D’).

![Image of Figure 6 showing expression patterns]

**Figure 6- CC_2105 recovery causes loss of double stalk phenotype:**

a) The top two panels represent the expression of CC_2105 in ΔCC_2105 (DS1), under a xylose inducible promoter pXyl CC_2105-mcherry-tetR, induced with 0.3% xylose and 0.3% glucose. The bottom two panels represent the expression of CC_2105 in WT (DS2), under a xylose inducible promoter pXyl CC_2105-mcherry-tet, induced with 0.3% xylose and 0.3% glucose, used as a control.

b) Graph representing an average percentage of double stalk phenotypes observed in the wild-type (WT), ΔCC_2105, and the (DS1) ΔCC_2105: pXyl CC_2105- mcherry-tetR

All cells were grown in HIGG media with 1 µM phosphate, and appropriate inducers and antibiotics added.

The localization of CC_2105 in the stalk was an interesting result, considering its absence does not affect the synthesis of the stalk. The recovery of crescent shape with the expression of CC_2105 in the ΔCC_2105 mutant and the uniform localization of CC_2105 along the cell body suggest that, apart from stalk localization, CC_2105 directly or indirectly influences the peptidoglycan synthesis in the cell body. Given the new insights on
differentiated functions of PBP 1B as trans-glycosylase and transpeptidase at different conditions, in *E. coli*, we speculate whether CC_2105 has distinct functions in the stalk and the cell body [55]. This may be regulated by an unknown protein interaction with CC_2105. Alternatively, CC_2105 may have another functional domain that remains to be identified.

**Figure 7- CC_2105 localization studies via CC_2105-mcherry expression:**

Figures A-D correspond to fluorescent images showing CC_2105-mCherry expression. A’-D’ represent the light microscope images of A-D respectively. (A, A’)- ΔCC_2105 mutant cells expressing CC_2105-mCherry under a Xylose inducible promoter (DS -1), (B, B’)-WT mutant cells expressing CC_2105-mCherry under a Xylose inducible promoter (DS-2), (C, C’)- The expression of Empty plasmid –pXCHYC-5 when induced with Xylose, in ΔCC_2105 mutant (DS- 3) [CONTROL], (D, D’)- The expression of Empty plasmid –pXCHYC-5 when induced with Xylose, in WT (DS -4) [CONTROL]. All cells were grown for 72 hours in 1uM phosphate supplemented HIGG media and induced with 0.3 % xylose.
Future directions and discussion

Previous studies on the PBP’s of Caulobacter have addressed the functions of various glycosyl transferases. It has been well established that the PBP’s of Caulobacter have redundant functions. Lately, one of the well characterized PBPs – PBPC was shown to localize in the stalk of Caulobacter. Although it is known that PBP C when deleted along with PPBP X causes a shortening of stalk in low phosphate conditions, the exact role of PBP-C in the stalk synthesis remains unclear. The relationship between the stalk and the PBP’s is not well studied in Caulobacter. The current study throws light on the possible involvement of PBPs in stalk regulation.

The lack of stalk phenotype in the over-expression mutants in either PYE media growth conditions and the HIGG media with limited phosphate conditions deflected the original hypothesis that CC_2105 has a role in the stalk synthesis. If the hypothesis were true, we would have expected to see a greater stalk synthesis among the overexpression mutants. Similarly, the deletion should have caused a loss of stalk phenotype, if the initial hypothesis were true. However, in the absence of CC_2105, the cell generates two stalks, doubling the stalk synthesis. These results indicate that CC_2105 may be a regulator of the stalk.

The similarity of the double stalk phenotype observed in ΔCT demonstrates that the C-terminus domain is important for the function of CC_2105. The C-terminus may be essential either to offer stability for the function of the protein or may have a possible role in other protein interaction. The exact role of the C-terminus domain needs to be examined.
The loss of cell shape and decreased size of the cell body in the deletion mutants, suggest that the transpeptidase domain of CC_2105 may be active and is essential for the proper cell structure, apart from stalk localization regulation. Additionally, the localization of CC_2105 in the cell body adds to this rationale. It has been shown that due to redundancy of PBP’s the cells retain their phenotype and grow similar to the WT, however the peptidoglycan composition of individual PBP mutants vary [50]. These results also indicate that at a molecular level the overexpression and the deletion of CC_2105 may have an impact on the peptidoglycan architecture.

The double stalk phenotype poses the obvious question of cell polarity. Polarity is highly defined and regulated in \textit{Caulobacter}. The stalked pole and the flagellar pole are specified by discrete polar complexes during each cell division [22, 28, 56]. The stalked cell pole is defined by a complex popZ-SpmX- DivJ, localized at the stalked pole (Figure 8 a). Any perturbation to this complex leads to a cell division anomaly in the cells [56, 57]. Cell division constrictions are not a characteristic of the double stalked mutants, ruling out the possibility of mislocalization of stalk-base complex. Each cell pole hosts one component of the two-component system, which acts on a single response element DivK (Figure 8 b). DivK gets phosphorylated at the stalked pole and diffuses to the swarmer pole during cytokinesis to get de-phosphorylated [58]. The gradient of DivK phosphorylation in each daughter cell at the time of cell division dictates the cell fate.

During cell division, the flagellated pole is defined by PodJ, recruiting PleC, a phosphotase which in turn associates with flagella and pili regulating elements [59]. PodJ isoforms, and unphosphorylated DivK govern the swarmer cell maturation state [58, 60]. PodJ exists as two isoforms PodJ L (Long or complete) with the N terminus end at the cytoplasm and the
C terminus end towards the periplasm, and PodJ S, a shorter form of Pod L, without the periplasmic C-terminus [61] (Figure 9).

**Figure 8- PodJ isoform transitions during cell division:**
a) PodJ L (Yellow) to PodJ S (Blue) conversion during cell division and compartmentalization of un-phosphorylated DivK in the swarmer cells in dividing cells and maturation into stalked cells after cytokinesis. b) A network of protein interactions at opposite ends of a cell, defining cell polarity. PodJ recruits PleC which further regulates the flagellar synthesis. The stalked pole base is recognized by a pop-Z SpmX-DivJ complex.

At the onset of cytokinesis, PerA cleaves the PodJ L at the periplasmic end, leaving the inner membrane and the cytoplasmic end intact- PodJ S [62]. Simultaneously, the unphosphorylated DivK is trapped in the swarmer cells due to the compartmentalization during cytokinesis. On the completion of cell division, the swarmer cell retracts the pillus and sheds the flagella to develop a stalk at the same pole. Eventually the PodJ S is degraded by matrix-metallo-proteases, and a new PodJ L is generated at the next cell division, at the new pole [63].
PodJ L is stabilized by the 54-amino acid peptidoglycan binding domain on the C-terminus. In the absence of PodJ ΔPG domain, PodJ L is quickly cleaved to PodJ S [61, 64]. Previous studies have confirmed that the PodJ L to PodJ S isoform transformation happens at the onset of cytokinesis [62]. During this study a mutant *divK*<sub>D90G</sub> when depleted of FtsZ, exhibited a second stalk phenotype as observed in our mutant ΔCC_2105. The *divK*<sub>D90G</sub> mutant can skip the cytokinesis signal triggering premature cleavage of PodJ L to PodJ S, while FtsZ depletion inhibits cell division preventing compartmentalization of unphosphorylated DivK. Together, the mutation and depletion create a pseudo-microenvironment of newly divided swarmer cell within the cell, triggering early stalk maturation in the cells, giving rise to a second stalk.

![Figure 9](image)

**Figure 9- PodJ isoforms of C. crescentus:**
A depiction of PodJ isoform conversion at the flagellated pole, starting at PodJ L- the pre-divisional cell (left) converted into PodJ S upon cytokinesis, cleaved by the action of PerA (center), and degradation of PodJ S by Matrix-metallo-proteases after the differentiation of the swarmer cell into the stalked cell (right).

This interesting parallel in the second stalk phenotype directed our attention towards a polarity variation on the flagellar pole in ΔCC_2105. The appearance of the second stalk at the flagellated pole nearly after 72 hours of growth, and the non-uniform cell shapes and stalk lengths is a puzzling phenomenon. With the evidence that premature PodJ cleavage
in conjunction with lack of cell compartmentalization triggers premature stalk formation at the flagellar pole; I hypothesize that in ΔCC_2105 mutant, PodJ is prematurely cleaved.

In a low phosphate media, *Caulobacter crescentus* is known to undergo cell division for a few cell cycles before eventually pausing the cell division, and turning on the stalk elongation. During this phase, the cell continues to synthesize long stalks and inhibits FtsZ, a microtubule homologue that orchestrates separation at the mid-cell during cell division. In our deletion mutant, although PodJ is prematurely cleaved, the presence of FtsZ and the quick cell division during the first few cycles in low phosphate conditions masks the second stalk phenomena. However, when an individual cell decides to stop dividing and switches to the stalk elongation mode, the prematurely cleaved PodJ S isoform, and the FtsZ depletion together promote early stalk maturation at the flagellar pole leading to the second stalk phenotype in low phosphate conditions. The switch from cell division to stalk elongation mode, in low phosphate conditions fits with the time lag observed for the second stalk phenotype. Also, in high phosphate conditions, since cell division is rapid and FtsZ is not depleted, the second stalk phenotype remains masked in the mutants. (Figure 10 b).

In case of wild type cells growing in low phosphate conditions, the stability of PodJ L resists the stalk maturation even in the absence of cell compartmentalization. Similarly, when grown in high phosphate conditions, the rate of cell division and quick compartmentalization, leads to maturation of the stalk in the new daughter cell just as in the mutants. (Figure 10 a)
Figure 10- Proposed mechanism of double stalk synthesis in ΔCC_2105:

a) Mechanism of PodJ L to PodJ S transition and DivK compartmentalization during high phosphate and low phosphate conditions in WT cells.
b) Mechanism of premature PodJ L to podJ S transition and DivK compartmentalization during high phosphate and low phosphate conditions in ΔCC_2105.

Thus, the stalk maturation at the flagellar pole requires the two independent events -PodJ isoform transformation and cell compartmentalization to occur simultaneously. PodJ is a conserved polarity defining protein among alpha-proteobacteria. Recent studies have shown that PodJΔl deletions in Agrobacterium tumefaciens lead to ectopic polar growth (ectopic peptidoglycan synthesis), and failure to recognize new and old cell poles [65]. Deletion of PodJ1, a PodJ L isoform in Sinorhizobium meliloti, also leads to cell polarity issues and ectopic growth [66]. The ectopic growth and disoriented cell polarity remain as a characteristic feature of PodJ L deletion, across alpha-proteobacteria, adding evidence to the proposed hypothesis.
This hypothesis can be tested by measuring the levels of PodJ isoforms in the WT and the deletion mutants ΔCC_2105 in low phosphate conditions, in time course. Parallely, the levels of FtsZ may be tested to confirm the coordinative effect of the two events. However, the role of CC_2105 in this mechanism remains unclear.

Previous studies have shown that individual bi-functional PBP’s generate distinct muropeptide structures in *Caulobacter crescentus* [50]. The cell morphology defects in the ΔCC_2105 mutant and the predicted function of CC_2105 as a transpeptidase suggest a possible change in the peptidoglycan composition. Taken together, a plausible reason for Pod J instability in the ΔCC_2105 mutant may be due to the disrupted interaction of the PodJ peptidoglycan binding domain, due to altered peptidoglycan structure.
References


