INVESTIGATING THE MECHANISM OF SUBSTRATE DELIVERY BY ADAPTOR PROTEINS DURING REGULATED PROTEOLYSIS IN BACTERIA

By

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To my sister: my mentor, my biggest support and my best friend since day one.

You inspire me each and every day to be a better human being.

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ABSTRACT

Regulated proteolysis is important for the timely removal of regulatory and signaling proteins. In the alphaproteobacteria *Caulobacter crescentus*, regulated proteolysis is particularly important for the asymmetric division of the cell into a swarmer and a stalk cell. During the swarmer to stalk cell transition, the regulatory protein CtrA is degraded by the protease ClpXP to initiate DNA replication. CtrA degradation requires the adaptor protein CpdR to prime the ClpX, as well as other adaptors RcdA and PopA. The stalk biogenesis regulator TacA also requires RcdA and CpdR primed ClpXP for cell cycle dependent degradation. The adaptor RcdA can also bind to and deliver two other substrates, CC2323 and CC3144. In this study, the binding site for RcdA within CC2323 was found to be present within the C-terminal end of the substrate. The study also looked at the conservation of the mechanism of substrate delivery by RcdA and CpdR in alphaproteobacteria domain, using the bacteria *Sinorhizobium meliloti*. It was found that although RcdA and CpdR from the bacteria can form an active adaptor complex, the mechanism of TacA delivery is not conserved. Furthermore, it was shown that CtrA degradation in *S.meliloti* requires a third adaptor that is currently unknown. This provides some insight into how bacteria have evolved to wire conserved mechanism to be species specific.

INTRODUCTION

Proteins are essential macromolecules within cells that are responsible for almost all cellular function, structure and maintenance. They are made up of amino acids that are strung together to make the primary structure of the protein. The protein then folds into a secondary, tertiary or quaternary structure. Proper folding of the protein is important for its function. In some cases, proteins are prematurely made, or the peptide is not folded properly and the presence of such damaged proteins can have toxic effect on the cell. Failure to degrade these proteins can result in complex diseases in organisms, such as Alzheimer's, Parkinson's and Huntington's disease.

Other than degrading improper proteins, cells also have to degrade regulatory proteins. Degradation of regulatory proteins ensure that their function is carried out at the appropriate time during the cell cycle or under the right condition. In many cancer cells, failure to degrade proteins that regulate cell cycle allows the cell to divide uncontrollably, which eventually lead to tumor formation. Protein degradation, therefore, provides a way of regulating protein levels within a cell, in addition to regulation by transcription and translation.

Because protein degradation is an irreversible process, the target protein needs to be degraded at the correct time. In bacteria, energy-dependent proteases selectively destroy such target proteins. The selectivity is achieved either by the inherent property of the protease to recognize the specific degradation signal

(degron) of the target or by the use of the auxiliary proteins called adaptor proteins.

In the alphaproteobacteria *Caulobacter crescentus*, regulated protein degradation plays an important role in maintaining the cell cycle and changing the morphology of the cell as it goes through the cycle. Cell division of this bacteria results in two distinct cell type, which can be clearly distinguished from each other. The cells can be easily synchronized so that the population starts as one single cell type, and their progression through cell cycle can be easily monitored, making it possible to monitor the change in protein levels throughout the cell cycle. For these reasons, *Caulobacter crescentus* is an ideal organism for studying regulated protein degradation.

Regulated protein degradation of certain proteins within this bacterium uses adaptor proteins. These adaptor proteins, along with the protease, are conserved among the class of alphaproteobacteria. Many of these alphaproteobacteria are plant symbionts, while some are pathogenic. Regulated protein degradation plays an important role in both symbiosis and pathogenesis, making these adaptor proteins and proteases important therapeutic drug targets.

The first part of this study looks at the interaction between an adaptor protein and its substrate in *Caulobacter crescentus*, involved in regulated protein degradation of the substrate. The second part of the study looks at the conservation of the mechanism of substrate delivery by the adaptor proteisn across the alphaproteobacteria domain. *Sinorhizobium meliloti* is used as the organism for this part of the study, because of its resemblances to *Caulobacter*

crescentus. Using the existing knowledge about *Caulobacter crescentus*, the mechanism of substrate delivery by adaptors is looked into in *Sinorhizobium meliloti*.

I. Cell cycle of C*aulobacter crescentus*

In the gram-negative alphaproteobacteria *Caulobacter crescentus*, regulated protein degradation plays a vital role in ensuring that DNA replication occurs only once during the asymmetric cell division of the bacteria. During the G1 phase of the cell cycle, the bacteria have motile swarmer morphology, with the presence of polar flagella and pili. During this time in the cell cycle, DNA replication cannot be initiated. Under favorable conditions, the swarmer cell transitions into an immotile stalked cell, where DNA replication can occur (S phase). For this transition to occur, many proteins, such as flagellar proteins and DNA replication inhibitor proteins have to be degraded. After the DNA replication initiation, the cell enters a pre divisional stage (G2 phase), where a new swarmer pole is created opposite the existing stalk. Upon complete cell division, a swarmer cell and a stalk cell are formed.

The DNA replication initiator protein DnaA, and the DNA replication inhibitor protein CtrA maintain the strict regulation of DNA replication in *C.crescentus*. DnaA binds to the origin of replication and requires ATP to activate DNA replication [1]. In the stalk cell, HdaA (homologous to DnaA) protein converts DnaA-ATP complex to DnaA-ADP complex, thus inactivating the DnaA protein[2]. CtrA can directly bind to the origin of replication and inhibit

replication[3]. CtrA level is maintained at the level of transcription, phosphorylation and degradation.

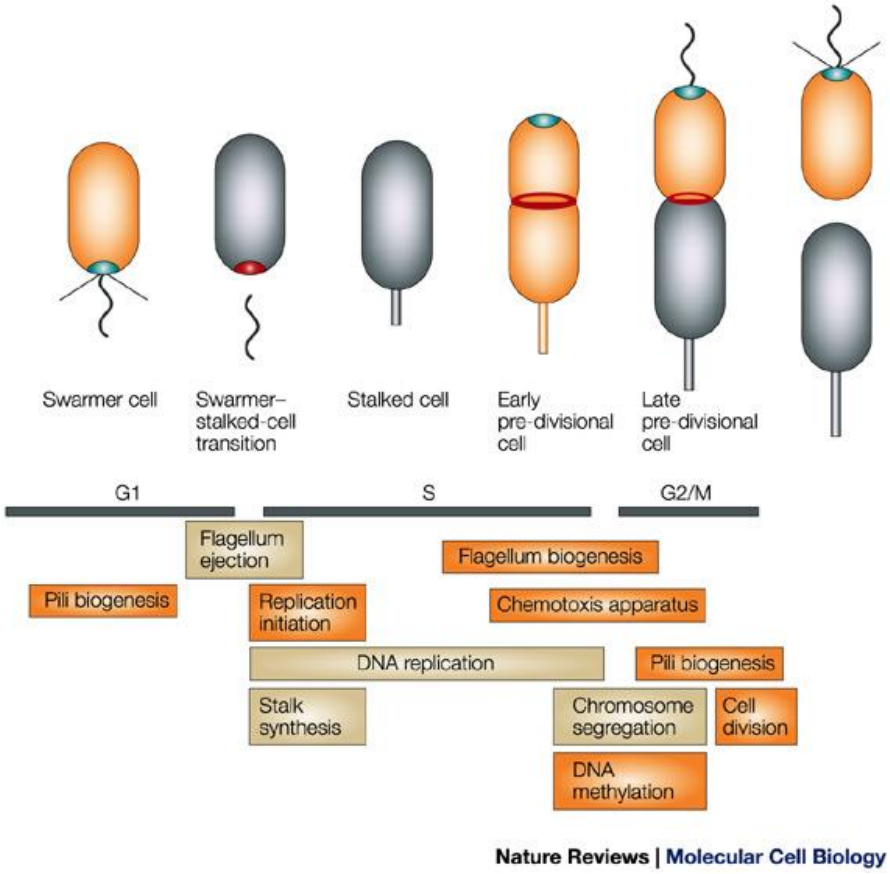


Figure 1: A schematic diagram of the asymmetric cell cycle of *Caulobacter crescentus*. Under favorable conditions, the motile swarmer cell differentiates into a stalk cell by replacing its flagellum with a stalk. During this transition, DNA replication is initiated. In the pre-divisional cell, DNA replication is completed, with the stalk cell dividing into a stalk and swarmer cell. Picture borrowed from Jensen et al. 2002.

II. Phosphorylation and localization of the master regulator CtrA

Phosphorylation of CtrA is dependent on the protein kinase DivL, response regulator DivK, two histidine kinases (DivJ and PleC) and the CcK-ChpT-CtrA phosphorelay system. In the pre-divisional cell, PleC functions as a DivK phosphatase at the new pole (swarmer cell), while DivJ acts as a DivK kinase at the old pole (stalk cell) [4,5]. Unphosphorylated DivK in the new swarmer cell allows the pseudokinase DivL to activate the CckA-ChpT-CtrA system [5]. The hybrid histidine kinase CckA is autophosphorylated, from which the phosphotransferase ChpT transfers the phosphoryl group either to CtrA or another protein CpdR [6,,8,9]. Phosphorylated CtrA binds to the origin of replication, preventing DNA replication, while phosphorylation of CpdR prevent degradation of CtrA by the protease ClpXP [3,7]. During swarmer to stalk cell transition, the phosphatase activity of CckA upon cyclic di-GMP binding is turned on allowing CtrA and CpdR to be dephosphorylated [10]. The dephosphorylated CpdR-mediated priming of the ClpXP protease then promotes degradation of CtrA with the help of additional adaptors (described in detail below) allowing initiation of DNA replication [14]. Thus, the level of active CtrA is regulated redundantly by multiple mechanisms including phosphorylation and degradation to tightly control replication and growth.

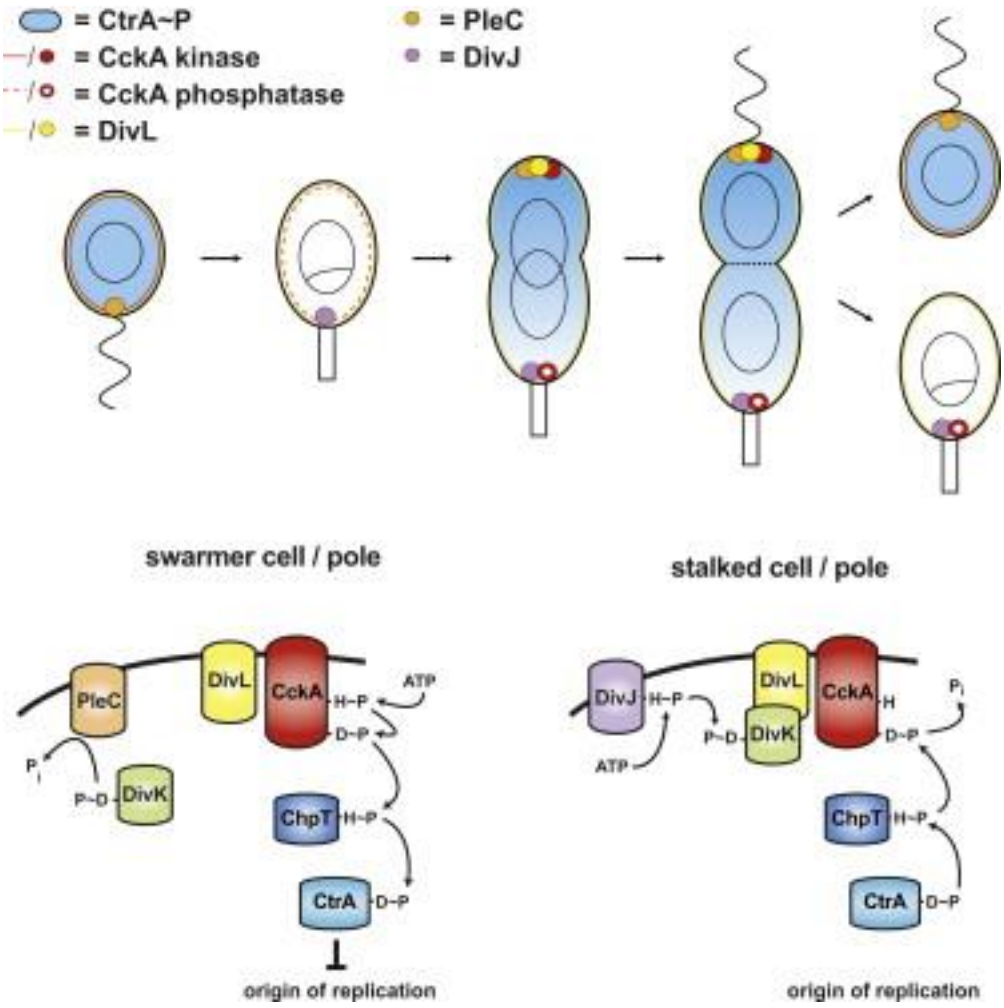


Figure 2: Localization of the histidine kinases PleC, CckA and DivJ, as well as of the protein kinase DivL and response regulator DivK, drives the phosphorylation of the master regulator CtrA at the correct stages of the cell cycle. At the swarmer pole of *C.crescentus*, CtrA is phosphorylated, allowing it to bind to the origin of replication in the DNA, preventing DNA replication. Phosphorylated CtrA also acts as a transcription factor for many genes. At the stalk pole, CtrA is unphosphorylated, which makes it a target for degradation. Without CtrA being bound to the origin of replication, the chromosome in the stalk cell can be replicated. Picture borrowd from Tsokos C. and Laub M. (2012).

III. Delivery of CtrA to the protease ClpXP by adaptor proteins

Regulated degradation of CtrA at the stalked pole is carried out by the ubiquitous ATP-dependent protease ClpXP during the G1-S phase [15,16]. Initially it was thought that the degradation of CtrA requires the auxiliary proteins CpdR, RcdA and PopA, as well as the protease, to be localized to the stalked pole[13,14,15]. However, current studies suggest that localization of RcdA is not necessary for degradation of CtrA, but rather the protein works as a proteolytic adaptor for CtrA degradation [18,19].

The small molecule, cyclic di-GMP (cdG), plays an important role in the localization of PopA and CtrA. When cdG level is high, it binds to the PleD like protein, PopA, sequestering PopA to the swarmer pole [17]. PopA, which can directly bind to RcdA independent of cdG, can then sequester RcdA to the pole[17]. PopA also directly binds to CtrA, in vitro, in presence of cdG and RcdA directly binds to CtrA to localize it to the pole [17,18].

ClpXP is localized at the pole independently of RcdA, but requires CpdR. Unphosphorylated CpdR can bind to ClpXP directly, sequestering it to the stalk pole [14]. Once the degradation machinery is assembled, CtrA degradation occurs. The last two residues of CtrA are important for its recognition by ClpX for degradation, whereas certain residues within the alpha-1 of the receiver domain of CtrA are important for its direct interaction with PopA [18,20].

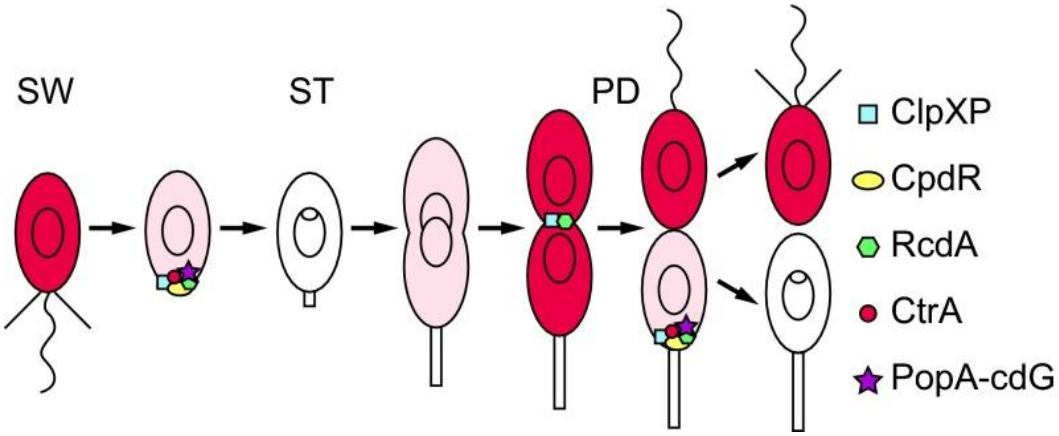


Figure 3: CtrA, protease and adaptor proteins localize to the stalk pole for degradation of CtrA. Closed circles represent non-replicating chromosome, whereas the theta structures represent chromosome replication occurring. The dark and light shade represents level of CtrA. In the swarmer cells, CtrA level is high with no localization of the degradation machinery, whereas in the stalk cell, CtrA level is zero with the degradation machinery localized to the pole for the degradation of CtrA. Picture borrowed from Smith et al. 2014

IV: The protease ClpXP

The protease ClpXP consists of an unfoldase (ClpX) and a peptidase (ClpP). Interaction between ClpX and ClpP occurs via the surface of the ClpX loops with the periphery of the ClpP barrel, and this interaction requires ATP [21]. The ability of ClpXP to degrade a wide range of diverse proteins enables it to be a key player in regulated protein degradation.

ClpX is active as a ring structure, that recognizes degradation tags (degrons) in a protein, unfolds the protein and translocates the unfolded protein to the peptidase, using ATP as the energy source. ClpX is able to recognize its substrates directly, or with the aid of adaptor proteins. The presence of flexible loops surrounding the entrance pore and containing an RKH sequence, as well as the N-terminal of the unfoldase, play an important role in direct recognition of substrates [21]. In some cases of degradation aided by adaptor proteins, once ClpX recognizes the degron on the substrate protein and starts pulling the substrate in for unfolding, the contact between the adaptor and the substrate protein is broken[21]. For RcdA-dependent degradation of substrates, the N-terminal domain of ClpX has to be primed by CpdR[22,23]. Unphosphorylated CpdR directly interacts with ClpX, priming the protease to recognize the disordered C-terminal tail of the adaptor RcdA[22,23].

The peptidase ClpP forma a barrel-like structure of two heptameric ClpP rings, inside which the active site is present [21]. The ring structure ensures that the local concentration of the active site is high, and that any substrate entering the

barrel can bind to several active sites at the same time, which efficiently degrades the substrate peptide [21].

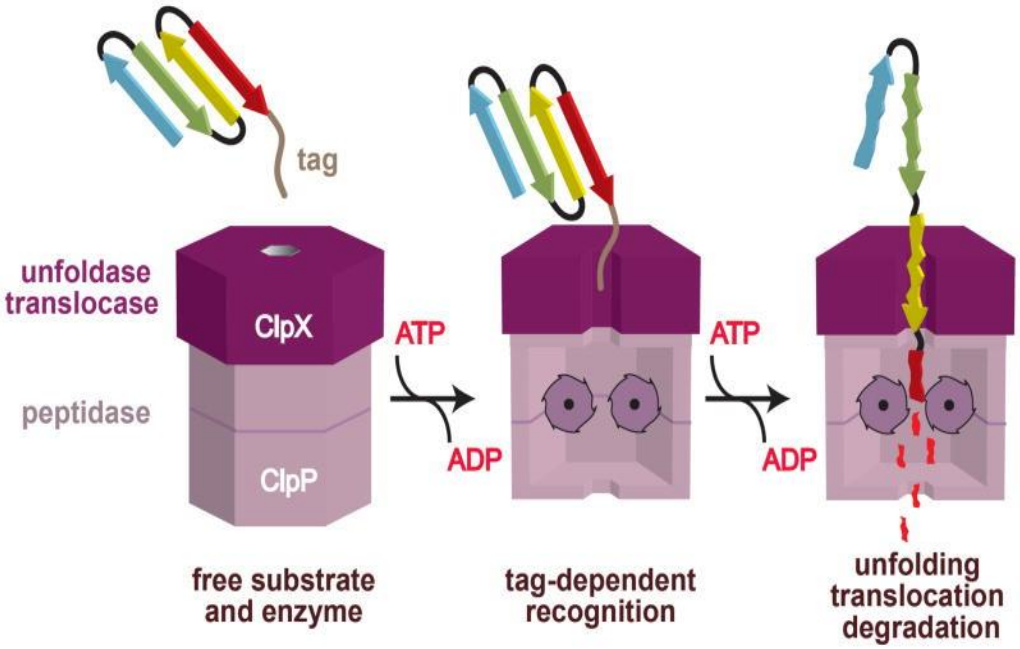


Figure 4: The protease ClpXP contains the unfoldase ClpX and the peptidase ClpP. ClpX can recognize its substrates with or without adaptors, unfold the substrate and translocate it to the peptidase chamber for degradation. Unfolding and degradation of the substrate requires ATP.

V: The adaptor RcdA

RcdA (Regulator of CtrA degradation) was identified to be important for CtrA degradation after it was established that the level of RcdA, as well as its localization pattern, coincided with CtrA proteolysis. Crystal structure of RcdA revealed the protein to form a crescent shaped dimer, with each monomer having three helices and a disorder C-terminus [19]. The protruding edges of RcdA contain conserved, charged residues, which along with conserved glutamic acid residues in the convex face of the protein, play an important role in RcdA and CtrA localization [19]. The disordered C-terminus of RcdA plays an important role in degradation of substrates. The C-terminal tail acts as a tethering motif for substrate delivery for CpdR-primed ClpXP, while residues upstream of the C-terminus form the substrate-binding site [23].

RcdA also acts as a proteolysis adaptor for the stalk biogenesis regulator TacA [23]. Although degradation of TacA by ClpXP was initially thought to be dependent on CpdR in vivo, recent data suggests that TacA degradation both in vivo and in vitro requires RcdA [23,24]. However, unlike CtrA degradation, TacA degradation does not require PopA. TacA can bind to both RcdA and RcdAΔC, however, degradation requires the C-terminal tail of RcdA to tether to a CpdR-primed ClpXP. [23]. RcdA can directly interact with the C-terminus DNA-binding domain of TacA, which also contains the degradation tag for TacA [23].

In addition to CtrA and TacA, two other substrates of RcdA exists in *C.crescentus*. The proteins of unknown function, CC2323 and CC3144, were found to bind directly to RcdA, as well as RcdAΔC [23]. The proteins could be

delivered to a CpdR-primed ClpXP by RcdA, but not by RcdAΔC suggesting that the mechanism of delivery by RcdA is same for TacA, CC2323 and CC3144 [23].

The substrates TacA, CC2323 and CC3144 might require the same proteolysis adaptor, however, it is unclear how all of them including PopA are binding to RcdA, when no obvious common sequence motif is shared among them. In the first part of this project, I tried to answer this question using the substrate CC2323. Using various biochemical methods, I truncated the substrate protein to find the region that could potentially bind to RcdA.

VI: The adaptors RcdA and CpdR are conserved across the alphaproteobacteria domain

The alpha proteobacteria domain consists of bacteria that are symbionts of plants (Rhizobia), pathogens (*Brucella*, *Rickettsia, Agrobacterium),* photosynthetic (*Rhodobacter*) and C1 metabolizing organism (*Methylobacterium)*[25]. In many of these bacteria, CtrA plays the role of master regulator, similar to *C.crescentus* [25]. Proteins regulating CtrA phosphorylation, localization and degradation are also conserved among this class of bacteria (Fig 22). While the protease ClpXP is completely conserved, the adaptor proteins RcdA and CpdR are conserved in some of the bacteria [25]. PopA is poorly conserved, although many of the bacteria contain the PopA ortholog PleD, while some of the bacteria contain both PopA and PleD [17]. We decided to see whether the mechanism of substrate delivery by the adaptors RcdA and CpdR is conserved across this class of bacteria. Using the nitrogen-fixing bacteria, *Sinorhizobium meliloti,* I addressed this question.

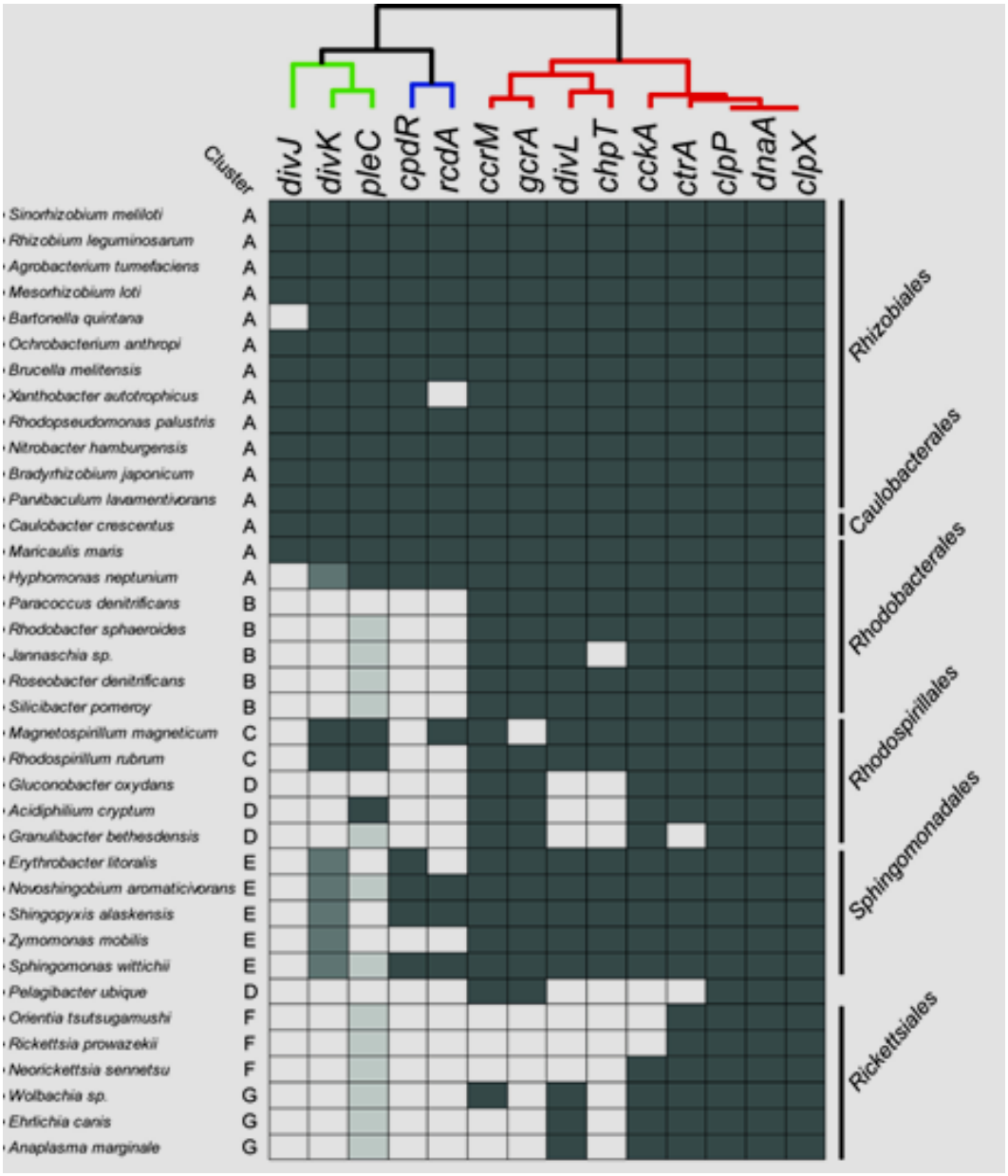


Figure 5: Conservation of proteins involved in cell cycle regulation of alphaproteobacteria. The protease ClpXP is completely conserved across the domain, the DNA replication inhibitor protein CtrA is highly conserved, while the proteins RcdA and CpdR are conserved in some of the bacteria.

VII: The plant bacteria *Sinorhizobium meliloti*

Sinorhizobium meliloti is an alpha-proteobacteria that can live freely or as a symbiotic partner of plant host legumes. In the free-living state, the bacteria divides asymmetrically, like *C.crescentus*. S.*meliloti* is taken up by host plant roots through infection threads, produced by the plants [26]. The bacteria travel through the infection thread and enter the plant cells, where they undergo differentiation into bacteroids [26]. During this differentiation, the cell stops dividing but DNA replication occurs several times [27]. Once cell division stops, nitrogen-fixation by the bacteroid is initiated.

S.meliloti CtrA shows remarkable similarity to *C.crescentus* CtrA. *S.meliloti* CtrA is also regulated by phosphorylation and its level fluctuates throughout the cell cycle, just like it does in *C.crescentus* [28]. However, unlike *C.crescentus*, where CtrA level is redundantly controlled, CtrA level in *S.meliloti* is possibly controlled by only degradation [28]. CtrA degradation in *S.meliloti* by ClpXP also requires the last three amino acids of CtrA, as well as RcdA and CpdR [28,29]. However, since *S.meliloti* does not have PopA, it is not clear how CtrA degradation occurs within the bacteria.

S.meliloti contains two homologs of CpdR, which share 42% amino acid similarity with each other [29]. CpdR1 and CpdR2 are 61% and 46% similar to the CpdR from *C.crescentus* [29]. CpdR1, like CpdR from *C.crescentus*, is responsible for polar localization of ClpX and *C.crescentus* CpdR can complement for *S.meliloti* CpdR1[29]. CpdR1 also plays an important role in symbiosis of the

bacteria with its plant host, differentiation into bacteroids, nitrogen fixation cell morphology [29].

The RcdA homologs between *S.meliloti* and *C.crescentus* share 46% similarity in the amino acid sequence, but in *S.meliloti* RcdA is an essential protein, unlike in *C.crescentus* [28]. Deletion of RcdA is lethal and the morphological defects are similar to cells lacking *cpdR1* or expressing a non-degradable version of CtrA [28].

In the second part of this study, I look at the conservation of the roles of RcdA and CpdR as adaptor proteins in *S.meliloti*, and whether the mechanism of substrate delivery by these adaptors are conserved in the bacteria.

MATERIALS AND METHODS

Cloning and restriction digestion

Truncated variants of CC2323 were constructed by designing primers and amplifying the desired region of CC2323 present in a plasmid. *S.meliloti* RcdA and TacA were amplified using plasmids as templates. *S.meliloti* CpdR1 was amplified using *S.meliloti* genomic DNA as template.

After running the PCR reaction, the mixture was run on a 1% DNA-Agarose gel and the bands visualized by UV. The band of interest was then cut out and the DNA extracted using the mini-prep protocol provided by BioBasics using EZ-10 columns. The extracted DNA was then cloned into pET23SUMO plasmid using Gibson reaction, by using 1uM:2uM plasmid:DNA ratio, 7.5uL of the Gibson mix and incubating the mixture at 50°C for an hour. pET23SUMO contains a Ampicillin resistant marker and adds a his-SUMO tag to the N-terminus of the construct that is inserted into the plasmid.

The Gibson mixture was then transformed into Top10 cells using the heat-shock technique. The cells were plated in LB/Amp plates overnight at 37°C. The next day, the plasmid was extracted from the cells and to check for successful ligation of the plasmid and DNA constructed, restriction digestion was carried out.

The restriction enzymes NdeI and XhoI were added to the extracted plasmid, and incubated at 37°C for 2-3 hours. NdeI cuts 300 bp upstream of the construct

inserted into the plasmid, while XhoI cuts right after where the construct ends. The digested mixture was then ran on a 1% DNA-Agarose gel, which was visualized under UV. The transformed plasmid was also sent out for sequencing to ensure that it contained the proper DNA sequence.

Protein production and purification

BL21(DE3)E.coli cells were then transformed with the cloned pET23SUMO by electrophoresis. Overnight culture of the cells were grown in Lysogeny broth (LB) and back diluted in 1:100 ratio to larger volume of LB containing Ampicillin. The cell culture was grown till the optical density 600 (OD_{600}) reached approximately 0.4, and protein production was induced with 0.4 mM IPTG for 3–5 hr. The cultures were then spinned down at 5000 rpm for 15 minustes. Pellets were resuspended in lysis buffer and frozen at -80 C until further use. Cells were lysed using a Microfluidizer system (Microfluidics, USA).

IPTG induction produced his-SUMO tagged protein that bound to Nickel in Ni-NTA column. 1ml of bed volume of Nickel was used for every 1 liter of culture grown. The Nickel was equilibrated with 4-5 column volumes of lysis buffer. Once the cell lysates were run over the column for affinity purification, the column was washed once with lysis buffer and again with lysis buffer containing 20mM imidazaol. The bound proteins were eluted out with elution buffer, where the high concentration of imidazole replaced the bound protein.

The eluted proteins were buffer-exchanged to lysis buffer only using PD-10 columns. 1uL:1mL of Ulp:protein was added to the buffer-exchanged protein for cleaving the his-SUMO tag, and left overnight at 4°C. The tag was separated out from the protein using the Ni-NTA column, where the untagged protein came out in the flow-through, while the his-SUMO tag bound to the column and eluted out when the column was washed with elution buffer. In certain cases, the eluted protein was run over a S200 column to separate out any bound his-SUMO tag.

Degradation assay

Reaction mixtures were set up in H-buffer with substrate, adaptors and protease at appropriate concentrations. The mixtures were incubated at 30°C for 5 minutes. ATP regeneration mix was added to initiate the assay. Samples were taken at the time points mentioned in results. The samples were either snap-freezed in liquid nitrogen or placed on ice to stop the reaction. The samples were then run on a 10% SDS-PAGE gel, using MOPS buffer. The gels were then stained with Coomaissie stain for 15 minutess, and destained overnight before visualization.

Trypsinization

0.1 mg/ml of trypsin was serial diluted to 0.0095 mg/ml. The varying concentration of trypsin were added to 20uM of full length CC2323. The digestion was allowed to go on for 15 minutes and the reaction was quenched by the addition of PMSF at room temperature. The reaction was set aside for half an

hour for the PMSF to fully inactivate the trypsin. The reaction mixture was run on SDS-PAGE gel, stained with Coomaissie stain for 15 minutes and destained overnight before visualization.

In vitro pull down assay

To make the Nickel bed in the column, Nickel+ethanol was taken and centrifuged at 700g for 2 mins to remove the ethanol. The Nickel resin was washed twice with water. 1uL:4uL of resin:H-buffer containing 20mM of imidazole was added and spinned overnight at 4°C overnight for equilibration. The next day, the mix was spinned down at 700g for 2 minutes to remove excess buffer.

1uM:2uM of bait:prey (CC2323:RcdAΔC) protein was taken and H-buffer (containing 20mM imidazole) was added to bring up the mixture to appropriate volumn. The mixture was then added to the pre-equilibrated Nickel resin and placed on rocker at 4°C for an hour. The mixture was spinned at 700g for 2 minutes. The supernatant was collected as the flow through. The column was washed with the buffer and spun at 350g for 1 minute to collect the wash. The bound proteins were eluted out using H-buffer containing 200mM of imidazole. After addition of the buffer, the mixture was incubated at 4°C for 5 minutes, and spun down at 700g for 5 minutes to collect the elution. The samples were then run on a SDS-PAGE gel, stained with Coomaissie stain for 15 minutes and destained overnight before visualization.

Sample preparation for mass-spectrometry and N-terminal sequencing

Mass spectrometry

Sample for mass spectrometry was produced using ZipTip. The ZipTip was prewet using wetting colution (50% methanol in TFA in Milli-Q water. The tip was equilibrated with equilibration solution (0.1%TFA in Milli-Q water) 3 times. The sample was aspirated and dispensed from the tip for 3 to 7 times.The tip was then washed with wash solution (0.1%TFA in Milli-Q water) 5 times. The bound sample was eluted out of the tip using elution solution (75% Methanol/0.1% TFA in Milli-Q water).

N-terminal sequencing:

The trypsin digested CC2323 was run on a SDS-PAGE gel. The gel was soaked in transfer (CAPS) buffer for a few minutes. Blotting papers were also soaked in the CAPS buffer for a minute. PVDF membrane was soaked in methanol for approximately 30 seconds and then soaked in the CAPS buffer for a minute. The gel and the membrane was placed in between the blotting papers and set into a transfer system for an hour. This transferred the protein from the gel to the membrane. The membrane was then stained with Ponceau stain for 15 minutes. The protein band of interest was then cut out from membrane and sent out for sequencing to the Tufts University Core Facility.

Buffer	Components	pH
Lysis buffer	50 mM Tris, 300 mM NaCl, 10% glycerol, 10mM imidazole and 5mM BME	8
Elution buffer	50 mM Tris, 300 mM NaCl, 10% glycerol, 300mM imidazole and 5mM BME	8
H-buffer	20mM HEPES, 100mM KCl, 10mM $MgCl_2$, 10% glycerol	7.5
CAPS buffer	2.21g CAPS, 500mL diH_20, 100mL MeOH (adjust to 1 liter with diH_20)	11

RESULTS

1. The C-terminus of CC2323 contains the binding site for RcdA

Two different approaches were taken to find the minimum region of CC2323 required for RcdA binding. In the first approach, two fragments of CC2323 were constructed, purified and used as substrate for degradation assay. The second approach consisted of trypsinization of CC2323 to obtain distinct fragments corresponding to the domains present in the protein. By using mass-spectrometry and biochemical methods, such as degradation assay, the fragment from trypsin digested CC2323 that could bind to RcdA could be identified.

(I.a) Construction and purification of two fragments of CC2323 to find the minimum binding region

In order to find the minimum binding region of CC2323 with RcdA, two fragments of the substrate protein were constructed. The first fragment contained the amino acid residues 2 to 146, and was termed as the N-terminus. The second fragment contained amino acid residues 147-377 and was termed as the C-terminus. The fragments were constructed and cloned into a pET23b vector that produced a his-SUMO tagged construct under the control of an IPTG inducible promoter. *E.Coli*cells were transformed with the vectors and protein production was induced on addition of IPTG (Fig 6).

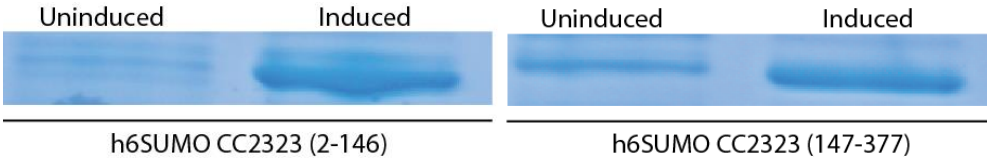


Figure 6: Induction assay for his-SUMO CC2323 (2-146) and his-SUMO CC2323 (147-377) indicate that the proteins were produced in the *E.Coli*cells. The uninduced samples were taken when the cell culture reached an OD_{600} of 0.4. IPTG was then added to the cultures and the induced samples were taken 1-2 hours afterwards. The samples were run on an SDS-PAGE gel and stained with Coomaissie stain for visualization. The thicker bands of the induced samples confirm that the proteins of interest were expressed in the cells.

His-SUMO CC2323 (2-146) and his-SUMO CC2323 (147-377) were purified from the cell lysates by affinity chromatography, before and after removing the his-SUMO tag (Fig 7); however, untagged version of CC2323 (147-377) could not be obtained due to the protein precipitating.

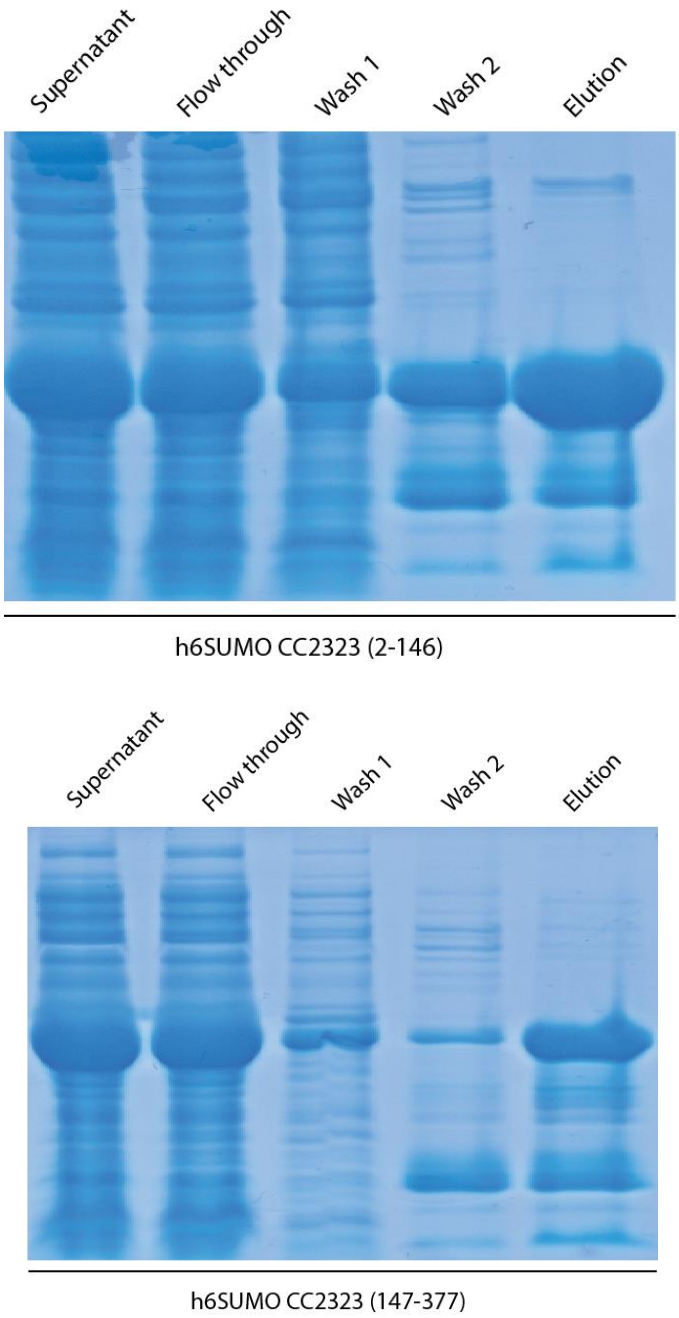


Figure 7: Purification of his-SUMO CC2323 (2-146) and his-SUMO CC2323 (147-377) from the cell lysates. The cell lysates from the different cells expressing different proteins of interest were run over the Nickel column (Supernatant) and the flow through was collected. The column was washed with lysis buffer only (Wash 1), lysis buffer containing 20mM imidazole (Wash 2). The proteins of interest bound to the column and were eluted out with elution buffer containing high amount of imidazole (Elution). The samples were run on a SDS-PAGE gel and stained with Coomaissie stain for visualization

(I.b) Degradation assay using the purified protein

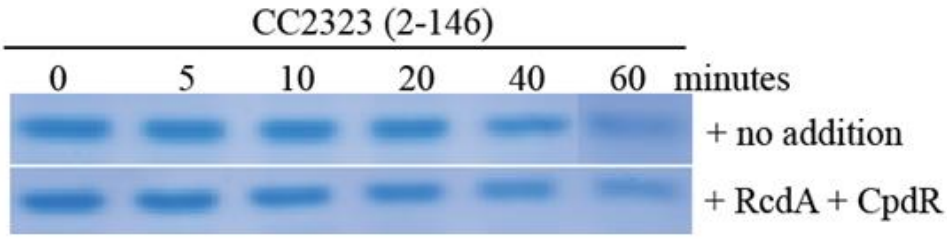
The purified proteins [CC2323 (2-146) and his-SUMO CC2323 (147-377)] were concentrated to appropriate concentration for Clp-XP mediated degradation assays. CC2323 (2-146) was seen to degrade both in presence and in absence of the adaptor proteins RcdA and CpdR (Figure8). This result showed that the fragment did not contain the RcdA binding site, since presence of that site would enhance the degradation of the fragment in presence of the adaptors. However, the fact that the fragment was degraded in both the experimental condition showed that the fragment contained within it a degradation tag, that directly interacted with the ClpXP, leading to similar degradation rate.

When his-SUMO tagged CC2323 (2-147) was used as the substrate for the degradation assay, the same pattern of degradation was seen: the substrate could be degraded both in presence and in absence of adaptors (Figure 9). However, the rate of degradation, compared to the untagged version of the fragment, was slower. It could be due to the his-SUMO tag interfering with the degradation tag of the substrate, which lowered the efficiency of the protease to degrade the substrate.

Degradation assay with his-SUMO CC2323 (147-377) showed slow degradation rate for both in presence and in absence of adaptors, compared to full length CC2323 (Figure 10 and 11). The rate of degradation was also similar in both conditions, indicating that this fragment too, did not contain the motif responsible for RcdA binding. However, since earlier experiments showed that the tag could interfere with degradation rates, it was not possible to reach a

definite conclusion. A control experiment was done with full length CC2323 to show that the adaptors and protease were active and functional.

(a)



(b)

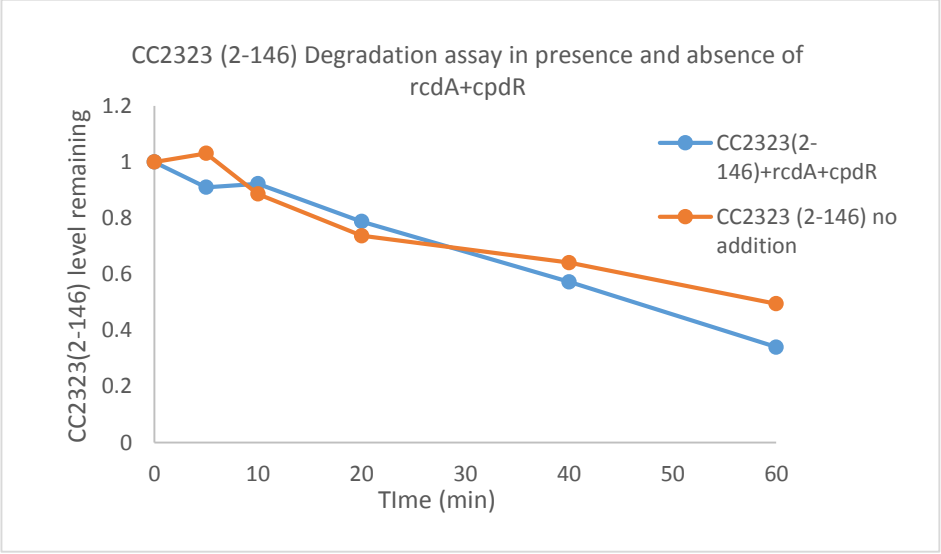
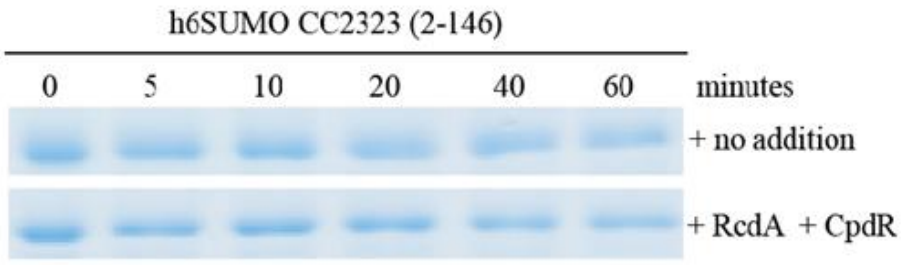


Figure 8: Degradation assay of CC2323 (2-146) indicates that the fragment does not contain the RcdA-binding site.Reaction mixture contained 1uM CC2323 (2-146), 1uM RcdA, 2uM CpdR, 0.4uM ClpP and 0.8uM ClpX.The reaction was started by adding ATP and samples were taken at time-points 0,5,10,20,40 and 60 minutes. (B)Quantification of the level of CC2323 (2-146) remaining with time. The substrate level was normalized to ClpP level.

(a)



(b)

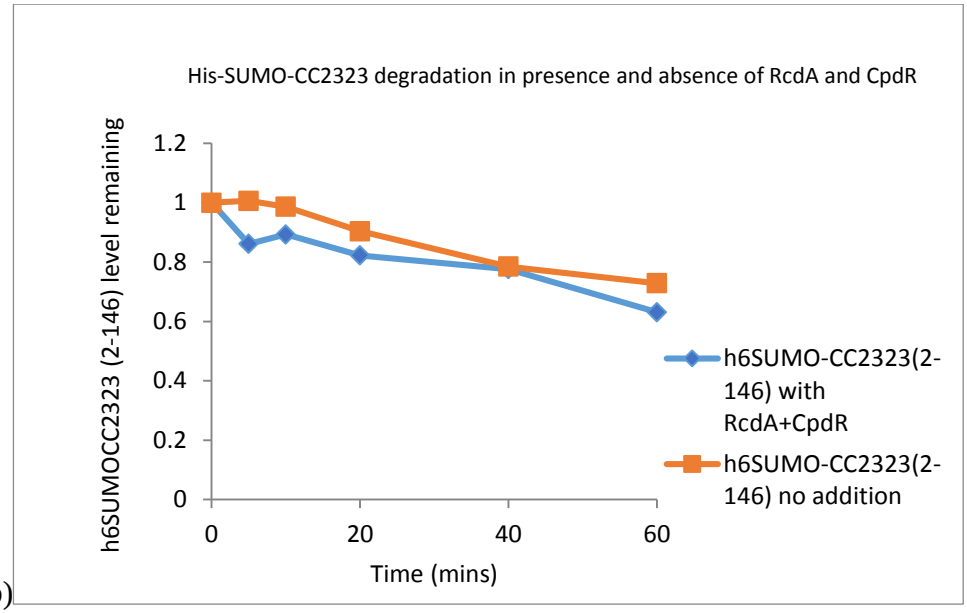
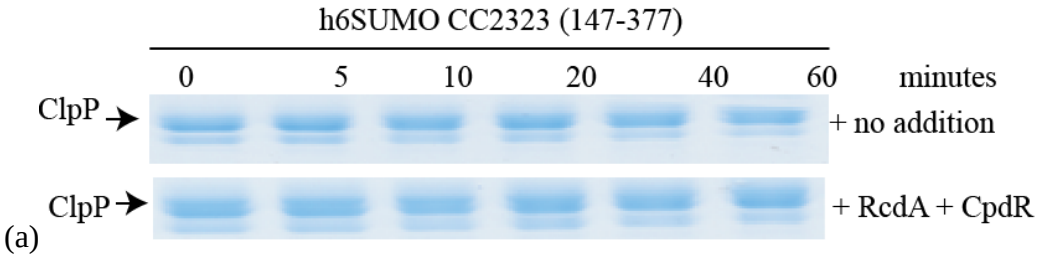
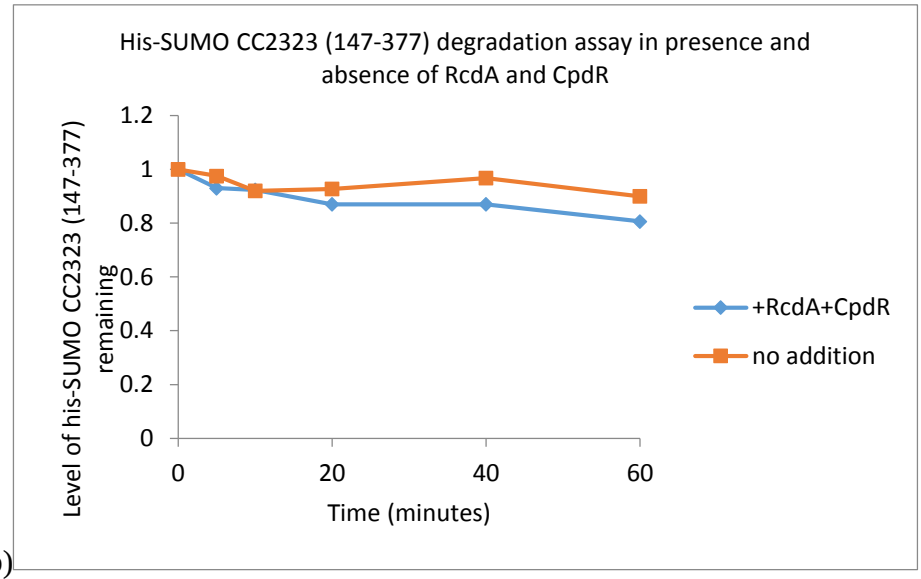


Figure 9: Degradation assay of h6-SUMO CC2323 (2-146) indicates that N-terminal of the fragment might contain a degradation tag that is recognized by ClpXP. Reaction mixture contained 1uM CC2323 (2-146), 1uM RcdA, 2uM CpdR, 0.4uM ClpP and 0.8uM ClpX.The reaction was started by adding ATP and samples were taken at time-points 0,5,10,20,40 and 60 minutes. (B) Quantification of the level of h6SUMO CC2323 (2-146) remaining with time. The substrate level was normalized to ClpP level.



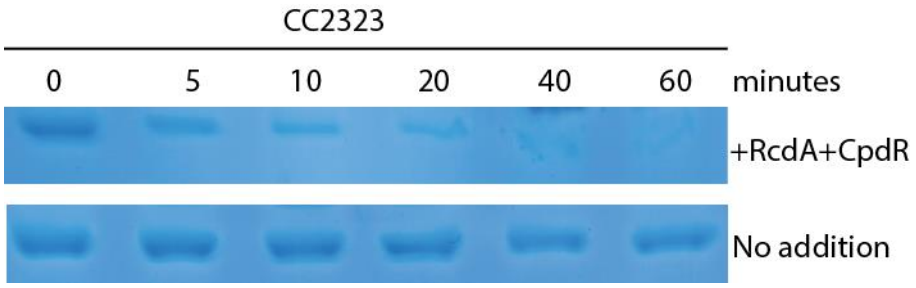
(a)



(b)

Figure 10: Degradation assay of h6-SUMOCC2323 (147-377) indicates that the fragment does not contain the RcdA-binding site. Reaction mixture contained 1uM CC2323 (2-146), 1uM RcdA, 2uM CpdR, 0.4uM ClpP and 0.8uM ClpX.The reaction was started by adding ATP and samples were taken at time-points 0,5,10,20,40 and 60 minutes.(B) Quantification of the level of h6S-SUMO CC2323 (147-377) remaining with time shows slow degradation of the substrate protein. The substrate level was normalized to ClpP level.

(a)



(b)

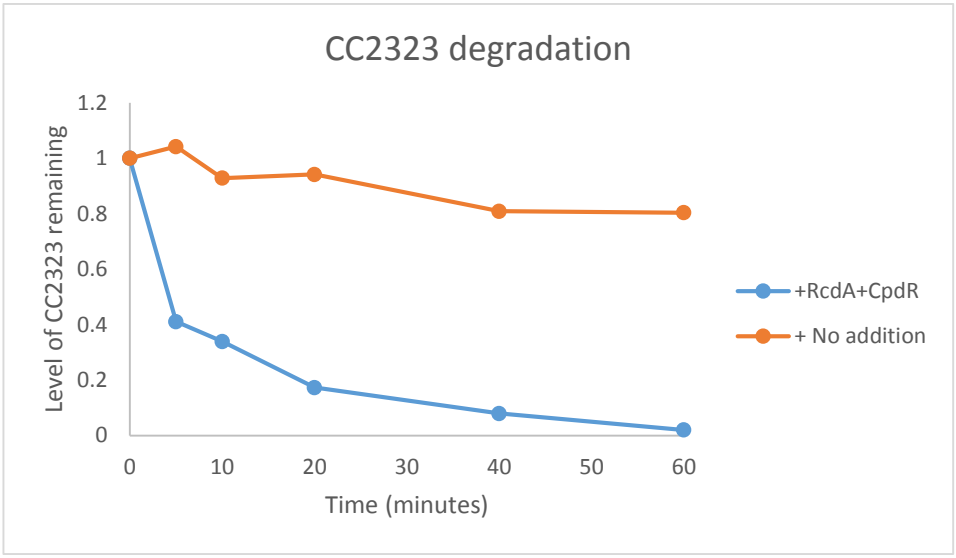


Figure 11: Degradation of full length CC2323 as a control shows that the adaptors and protease is active. (A) The degradation was carried out using 0.2uM ClpX, 0.4uM ClpP, 1uM RcdA, 2uM CpdR and 1uM CC2323. (B) Quantifications for CC2323 shows that the rate of degradation is much higher in presence of the adaptors. CC2323 level was normalized to ClpP levels.

(II.a) Tyrpsinization of CC2323 to find the minimum binding region

CC2323 was treated with trypsin of various concentrations, in order to find the best concentration at which distinct fragments of CC2323 could be obtained. Digesting CC2323 with 0.05 mg/ml of trypsin produced the best result, giving six distinct fragments. The digested CC2323 was then sent out for mass-spectrometry to identify the fragments.The size of five of the six fragments could be obtained from mass spectrometry, while one of the fragments did not produce any signal.

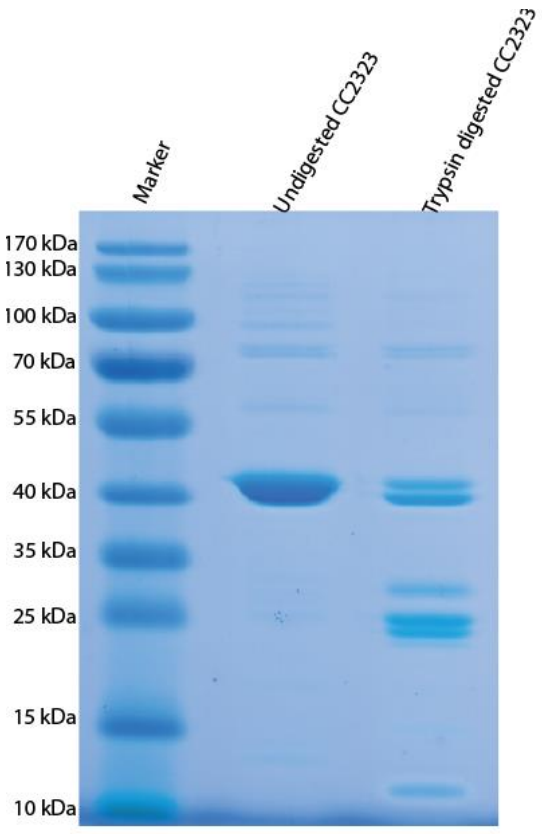
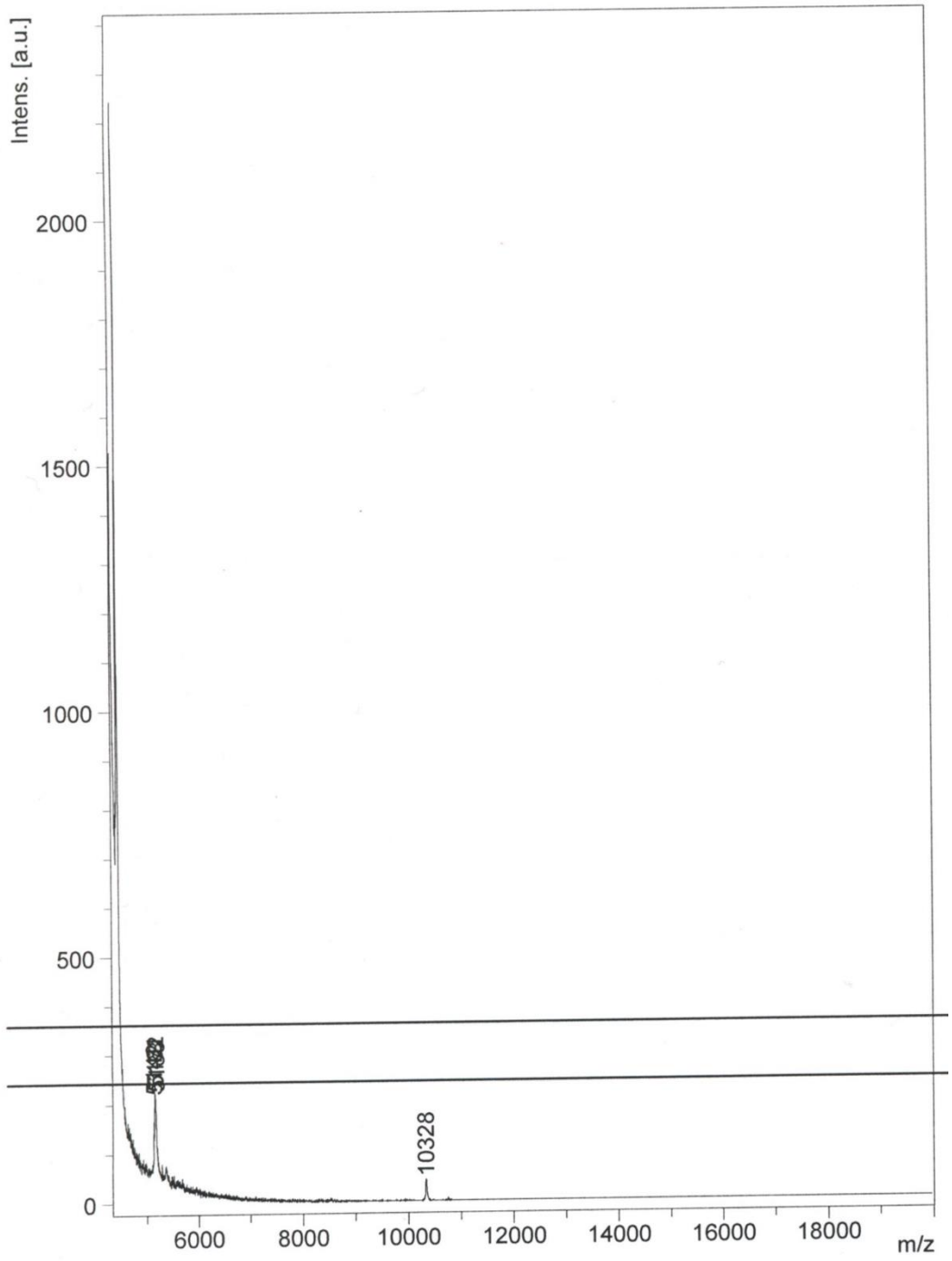


Figure 12: Trypsin digestion of CC2323 with 0.05 mg/ml of trypsin produced six distinct fragments. Tryspin was added to 20 uM CC2323 and the digestion was allowed to go on for 15 minutes. The reaction was quenched with 2mM PMSF for 30 minutes. The reaction mixture was then run on a SDS-PAGE gel and stained with Coomaissie stain for visualization.

(II.b) Mass-spectrometry results



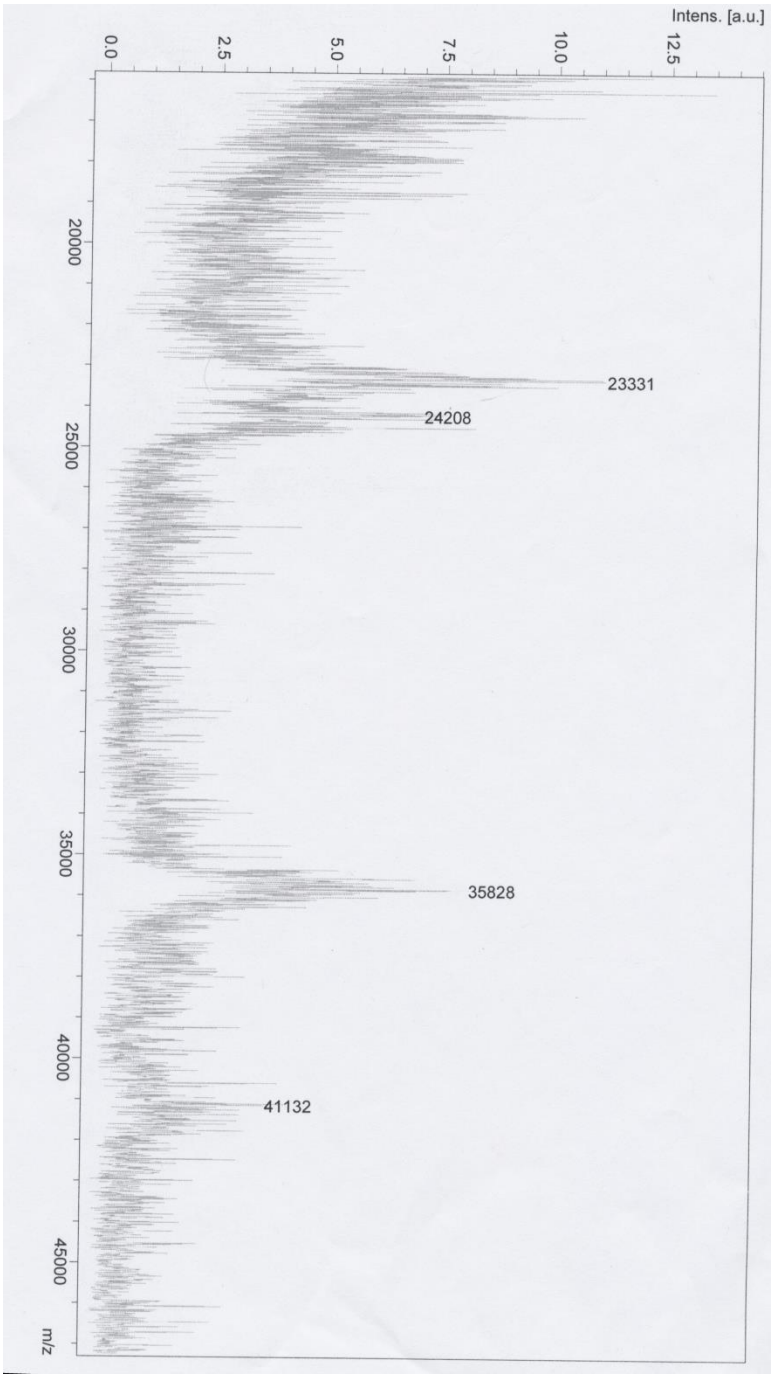


Figure 13: Mass spectrometry result of trypsinized CC2323 gives the size of four of the fragments. The highest sized fragment is the same as full length CC2323, representing undigested CC2323. The second fragment runs at around 35 kDa, while the fragment lower than that did not produce any signal in the mass spectrometry. The next two fragments are of approximately same size and the lowest fragment is of 10 kDa.

(II.c) In vitro pull down assay of CC2323 with his-RcdA

In order to find which of the fragment resulting from digestion contained the region required for RcdA binding, an in vitro pull down assay was carried out using a Nickel column (Fig 14). His6 RcdAΔC was able to bind to the column due to the tag interacting with the Nickel. When trypsin digested CC2323 alone was run over the column, none of the fragments bound to the column due to the lack of tag. However, when the CC2323 and his6 RcdAΔC were mixed together and run over the column, all the fragments of CC2323 bound indirectly to the column, by interacting with the his6 RcdAΔC, suggesting that all the fragments had the RcdA binding motif.

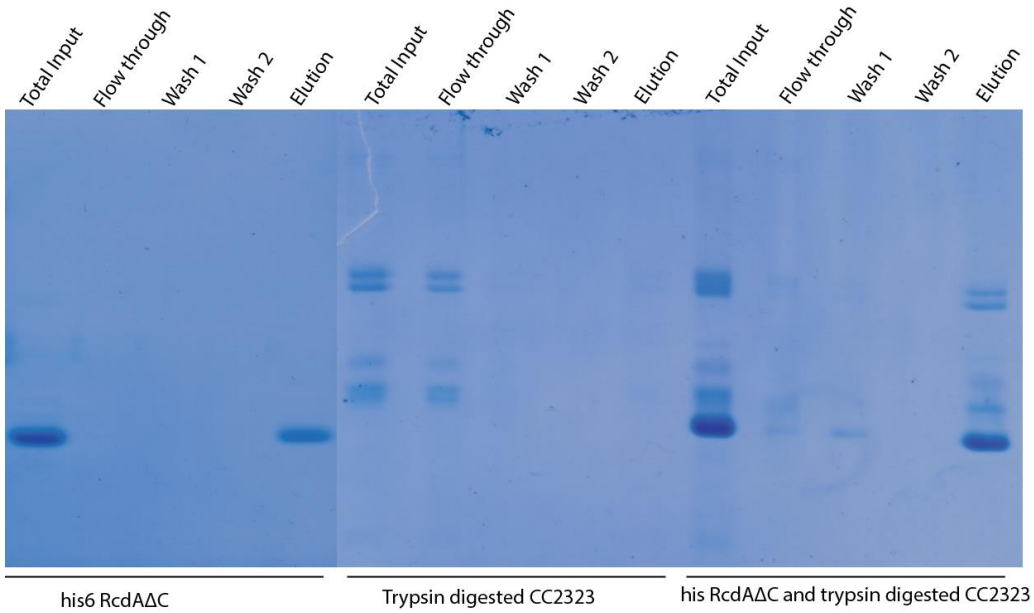


Figure 14: In vitro pull down results suggest that all the fragments obtained from trypsin digestion of CC2323 can bind to RcdA. His6-RcdAΔC was used as the bait protein, which bound to the Nickel column due to the tag, and the fragments of CC2323 that had the binding region of RcdA, bound to his6-RcdAΔC, and as a result, to the column as well. The bound proteins were eluted out by using H-buffer containing high imidazole concentration.

(II.d) Degradation assay of trypsinized CC2323

Degradation assay of trypsinized CC2323 indicated that the fragment at 27 kDa was able to directly bind to RcdA and be delivered to the CpdR primed ClpXP, in the same way that full length CC2323 is degraded[14] (Fig 15). The fragment was not degraded in any of the control reactions (in presence of only RcdA or only CpdR or when no adaptors were added). In order to identify the residues present in the fragment of interest, an N-terminal sequencing was done. Sequencing result indicated that the fragment starts from the 134th residue of CC2323, and covered all the residues after that. Based on these results, it was concluded that the binding motif of CC2323 with RcdA lies somewhere in the C-terminus (Fig 16).

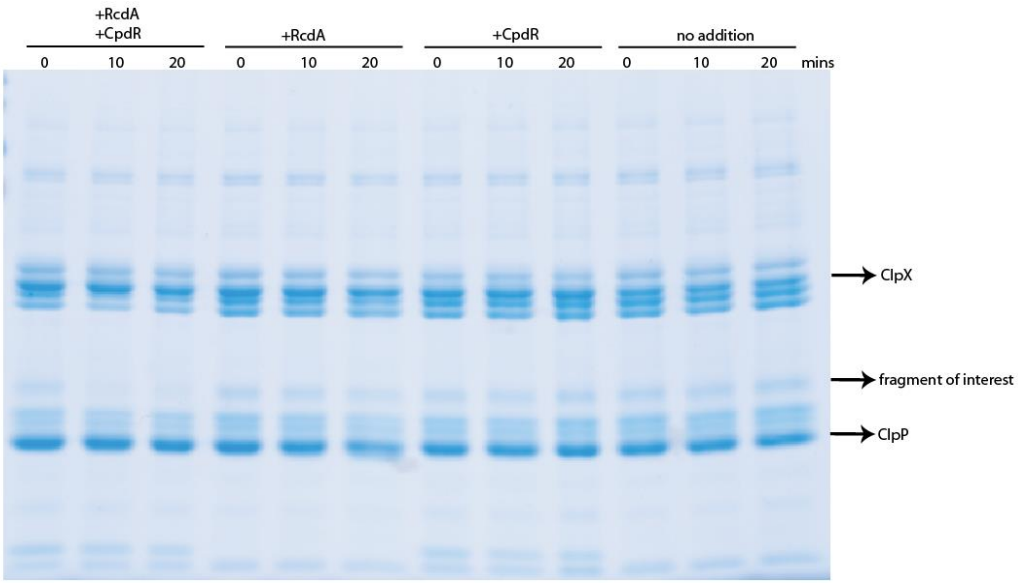


Figure 15: Degradation assay of trypsin digested CC2323 resulted in one of the fragments degrading in a RcdA and CpdR dependent manner by ClpXP, suggesting that the fragment contains the binding motif required by RcdA to bind to the full length protein to deliver it to a CpdR primed ClpXP. The degradation assay used 0.2 uMClpX, 0.4uM ClpP, 1uM RcdA, 2uM CpdR. 50% of the degradation reaction mixture was trypsin digested CC2323.

MATTRAALTDADIRMLVKGATPDERALAAHKLCRSIDRSVLSEEEREVA
HDILRVMAADAAELVRRAMAVTLKNSLALPPDVANRLARDVESVSLPIIS
FSPVFTDTDLAEIVKVGGPVRQMAVAKRPKLSSKITTLLVEQGTEDVVA
TVCANDNARFSEVSLQKALDRFAKSEQVLQAVAYRSALPLAVTERLIDM
VGEQLRDHILTSHALSPERTMELILGATERATIDLVDQAGRAADPKAFVA
HLNKMGRLSPSLILRALAHGHMSFFEWAVAELAGVPHHRTWLMIHDAGP
LGLKAICERAGLPSRLYSAFRAGVDAFHGLEFDGGAHDRERFQEHMIQRF
LTSSHIASREDSEYLLERMDRSASKRRAQSA*

Figure 16: The highlighted residues within the CC2323 were found to be the N-terminal starting residues for the fragment of interest. The fragments starts at the 134th residue of the full length CC2323 and covers all the way to the last residues.

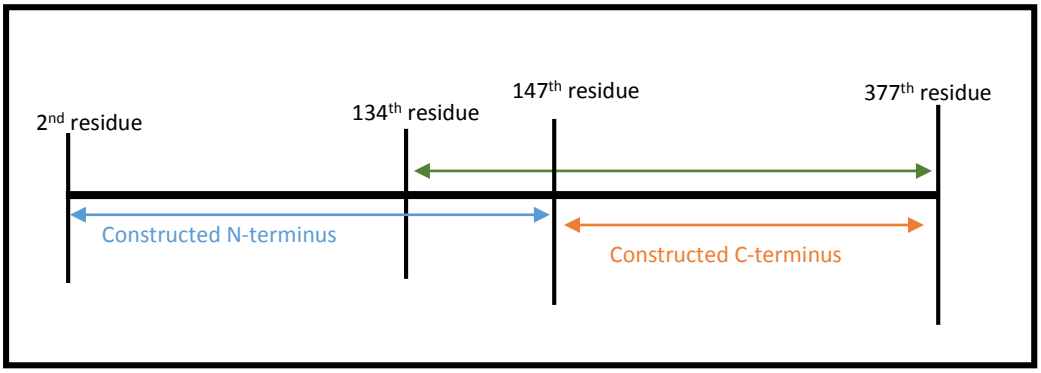


Figure 17: A schematic diagram of the residues contained within the fragments of CC2323. The constructed N-terminus contain the residues 2-146, C-terminus contains 147-377 and the fragment of interest obtained from N-terminal sequencing contains the residues 134 to 377. Since the constructed fragments contain residues overlapping with the fragment of interest from N-terminus sequencing, it is possible that the RcdA binding site was chopped off when the constructed fragments were made.

2. Mechanism of TacA delivery by adaptors is different for *C.crescentus* and *S.meliloti*

Using the model known for TacA degradation in *C.crescentus, S.meliloti* TacA degradation was carried out using the same adaptors. The results indicate that the mechanism of TacA delivery for degradation is different in the two bacteria.

(I.a) Construction and purification of *S.meliloti* Rcda, CpdR and TacA

In order to investigate where *S.meliloti* RcdA and CpdR could also form an adaptor complex to deliver TacA, degradation assay was carried out. *S.meliloti* CpdR1, RcdAand TacA were constructed and cloned into a vector that produced a his-SUMO tagged construct (Fig 18 and Fig 19). *E.Coli* cells were transformed with the vectors and protein production was induced on addition of IPTG (Fig 20).

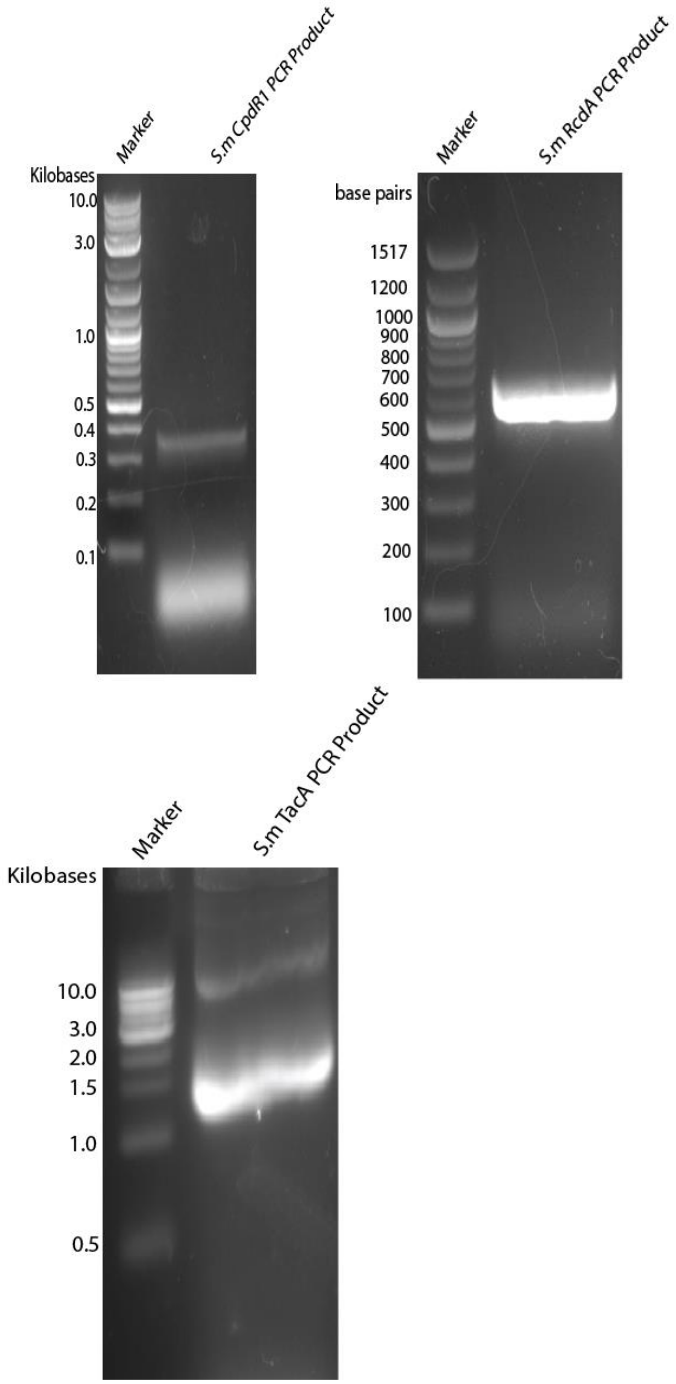


Figure 18: PCR amplification of *S.meliloti* CpdR1, RcdA and TacA. The PCR reaction was carried out using genomic DNA as template for CpdR1, and plasmid templates for RcdA and TacA. The reaction mixtures were run on a 1% agarose gel and all the reactions showed bands of correct size. DNA from the bands were then extracted using mini-prep protocol.

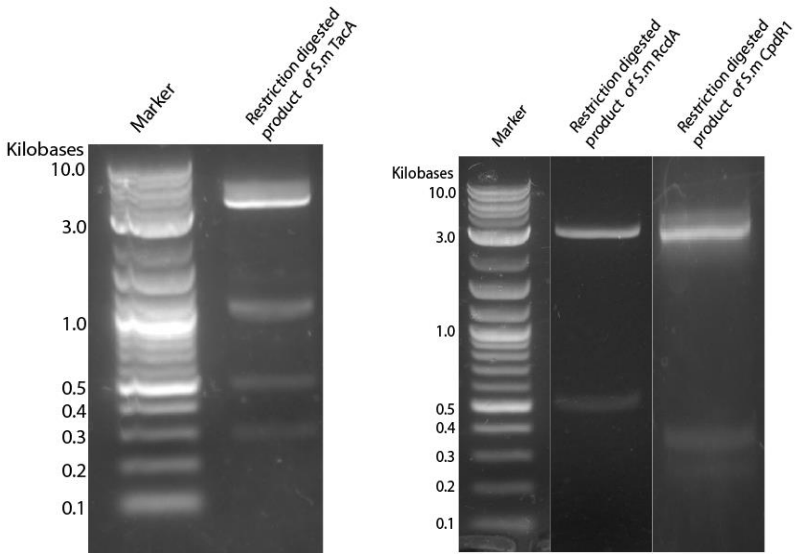


Figure 19: Restriction digestion analysis of transformed pET23b plasmid. The amplified PCR products were transformed into empty pET23b plasmid by Gibson assembly. Restriction digestion of the transformed plasmid by NdeI and XhoI enzymes showed a drop in the band size, indicating that the transformations were successful. Transformed plasmid runs at around 4kB, and the lower bands are representative of the amplified PCR product within the transformed plasmid.

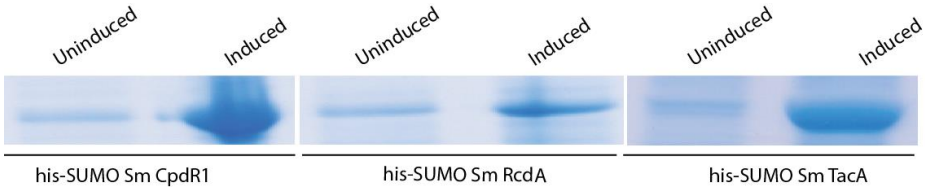


Figure 20: Induction assays for his-SUMO *S.meliloti* CpdR1, RcdA and TacA indicate that the proteins were produced in the *E.Coli* cells. The uninduced samples were taken when the cell culture reached an OD_{600} of 0.4. IPTG was then added to the cultures and the induced samples were taken 1-2 hours afterwards. The samples were run on an SDS-PAGE gel and stained with Coomaissie stain for visualization. The thicker bands of the induced samples confirm that the proteins of interest were expressed in the cells

The proteins were purified by affinity chromatography, both before and after removing the his6-SUMO tag (Fig 21 and 22). An uncleaved version of *S.meliloti* TacA could not be obtained due to the protein precipitating on cleaving. The proteins obtained were concentrated to appropriate concentration.

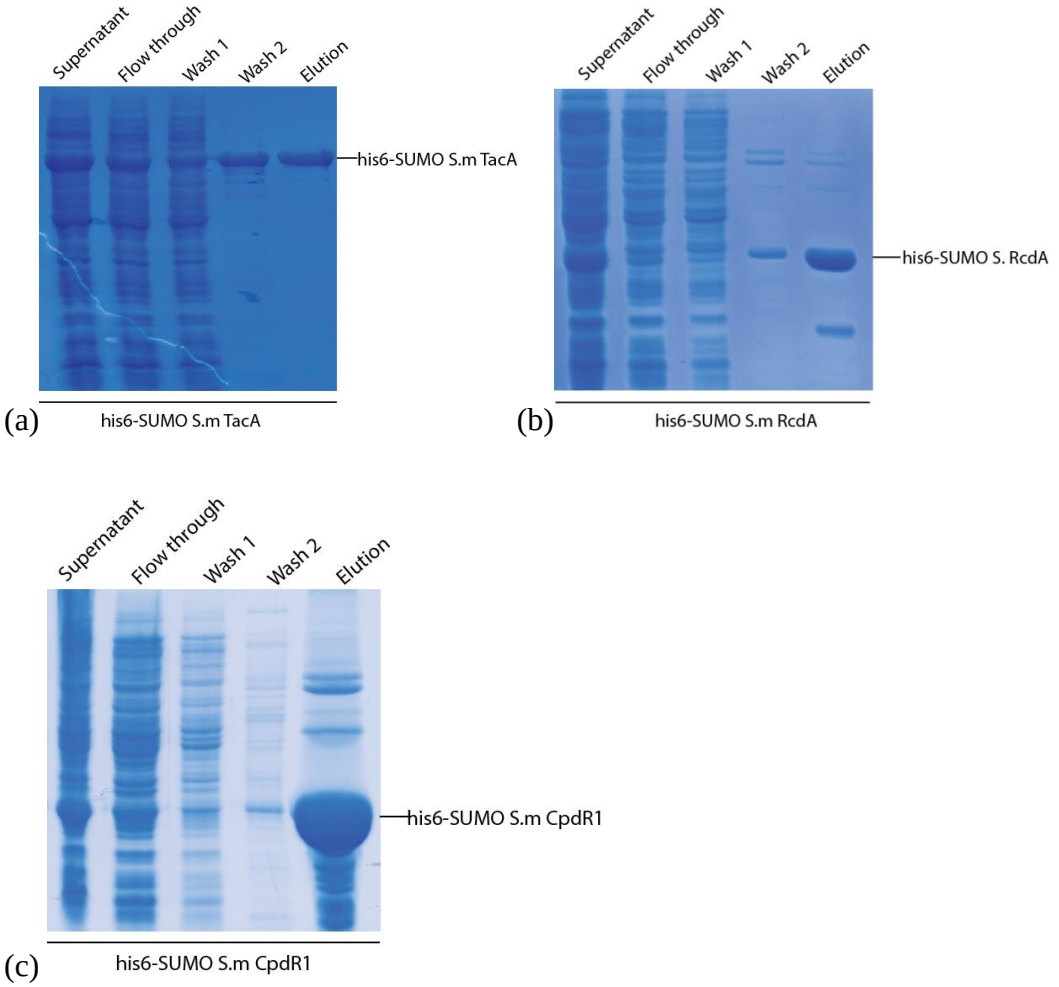


Figure 21: Affinity chromatography of his6-SUMO S.m TacA (a), his6-SUMO S.m RcdA (b) and his6-SUMO S.m CpdR1 (c). The cell lysates from the different cells expressing different proteins of interest were run over the Nickel column (Supernatant) and the flow through was collected. The column was washed with lysis buffer only (Wash 1), lysis buffer containing 20mM imidazole (Wash 2). The proteins of interest bound to the column and were eluted out with elution buffer containing high amount of imidazole (Elution).

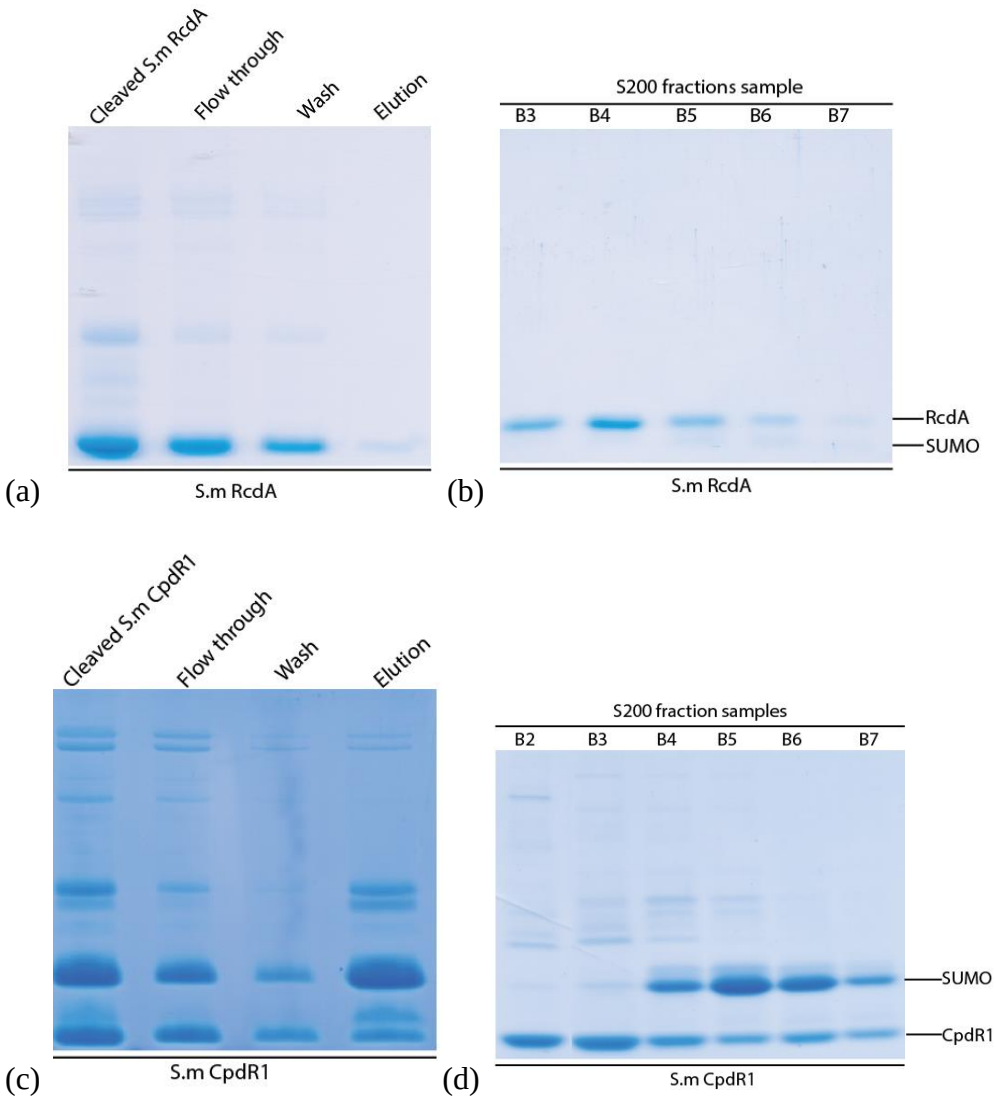
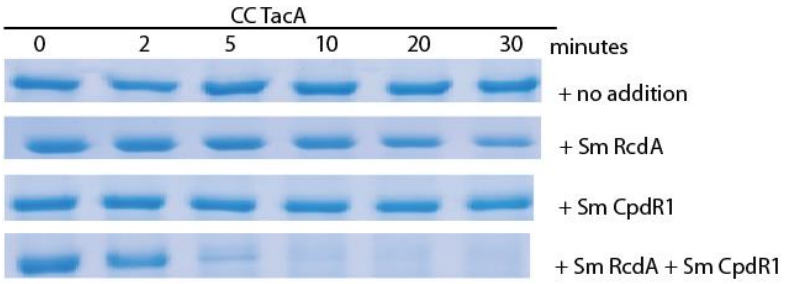


Figure 22: Purification of S.m RcdA and S.m CpdR1 after SUMO cleavage by Ulp enzyme. The his-SUMO tag was cleaved off from the proteins of interest by overnight Ulp treatment. The proteins were purified by affinity chromatography using Nickel column and size exclusion chromatography using S200 column. Nickel purification of S.mRcdA was followed by a run on S200 (b) to separate out the RcdA from any SUMO tag. Similarly, cleaved S.m CpdR1 was first purified by Nickel column (c), followed by S200 run (d). The fractions from S200 run containing only the proteins of interest were pooled together and concentrated.

(I.b) *C.crescentus* TacA can to delivered to ClpXP by *S.meliloti* RcdA and CpdR

To test whether RcdA and CpdR from *S.meliloti* could interact with each other and form an active adaptor complex, a degradation assay was carried out using *C.crescentus* TacA as the substrate (Fig 23). *C.crescentus* TacA was seen to be degraded in presence of *S.meliloti* RcdA and CpdR in the same way that degradation occurs when *C.crescentus* RcdA and CpdR are present. This results indicate that the RcdA and CpdR within both the bacteria are able to form an adaptor complex that can deliver *C.crescentus* TacA to the ClpXP.

(a)



(b)

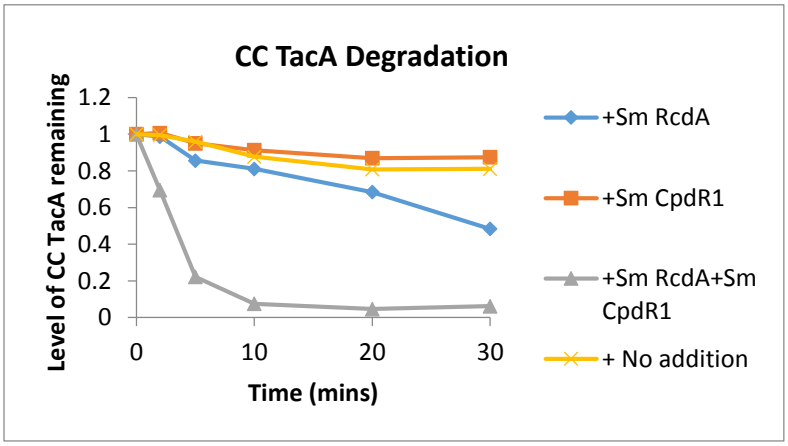
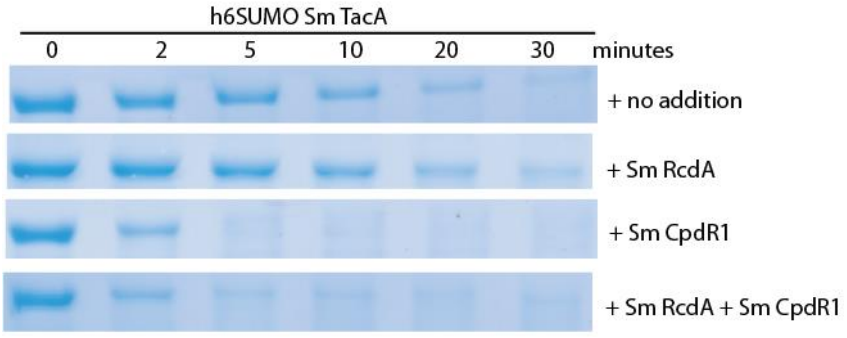


Figure 23: CC TacA is degraded by ClpXP in the presence of both Sm RcdA and Sm CpdR. This degradation pattern is similar to the one seen when adaptors from *C.crescentus* is used, showing the conservation of the role of the adaptors. (a)Degradation assay was carried out with 1uM of CC TacA, 1uM of Sm RcdA, 1uM of Sm CpdR1, 0.2uM of ClpX and 0.4uM of ClpP. (b)Quantification of his-SUMO Sm TacA degradation assay. The substrate level was normalized to ClpX levels.

(I.c) *S.meliloti* CpdR is sufficient for delivery of *S.meliloti* TacA for degradation by ClpXP

Once it was established that RcdA and CpdR from *S.meliloti* can work together to deliver *C.crescentus* TacA, it was assumed that *S.meliloti* TacA would also be delivered to ClpXP by the RcdA and CpdR from the bacteria. However, degradation assay suggests that *S.meliloti* CpdR is sufficient to deliver the TacA, suggesting that the mechanism of TacA delivery is not conserved in *S.meliloti* and *C.crescentus* (Fig 24).

(a)



(b)

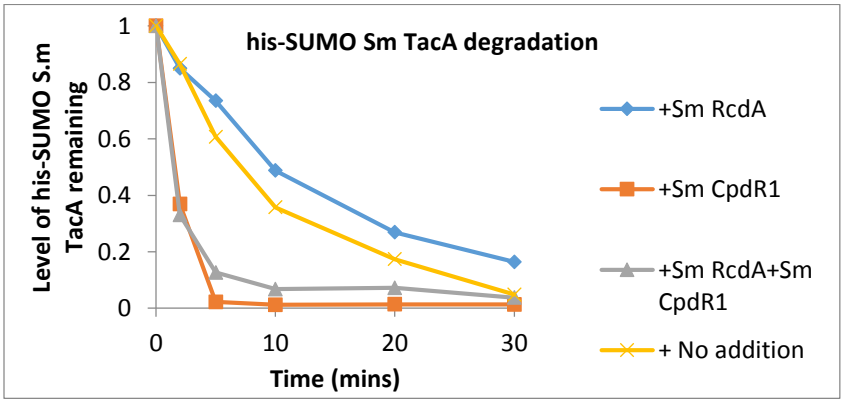
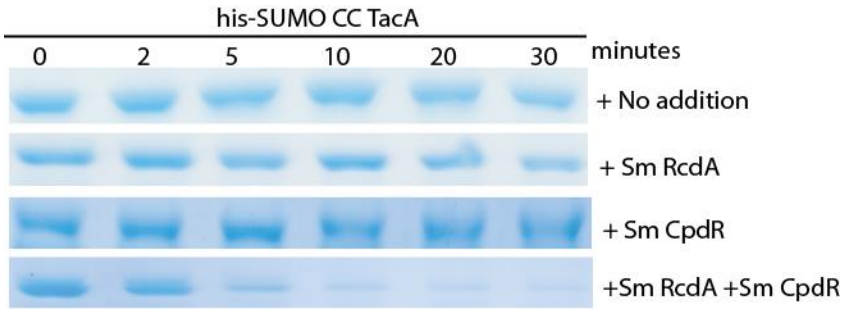


Figure 24: His-SUMO Sm TacA can be degraded by ClpXP in the presence of Sm CpdR1 alone. This mechanism of substrate delivery is different from the one seen when CC TacA is used as the substrate. (a)Degradation assay was carried out with 1uM of his-SUMO Sm TacA, 1uM of Sm RcdA, 1uM of Sm CpdR1, 0.2uM of ClpX and 0.4uM of ClpP. (b) Quantification of his-SUMO Sm TacA degradation assay. The substrate level was normalized to ClpX levels.

However, since the *S.meliloti* TacA had the tag, it could also be possible that the tag interfered with the degradation assay result. A control experiment was done with his-SUMO tagged *C.crescentus* TacA, to check whether the tag was directly interacting with CpdR1 to deliver the substrate. However, the tagged *C.crescentus* TacAwas seen to degrade in the same pattern as the untagged one, implying that the tag does not interfere with the degradation assay (Fig 25).

(a)



(b)

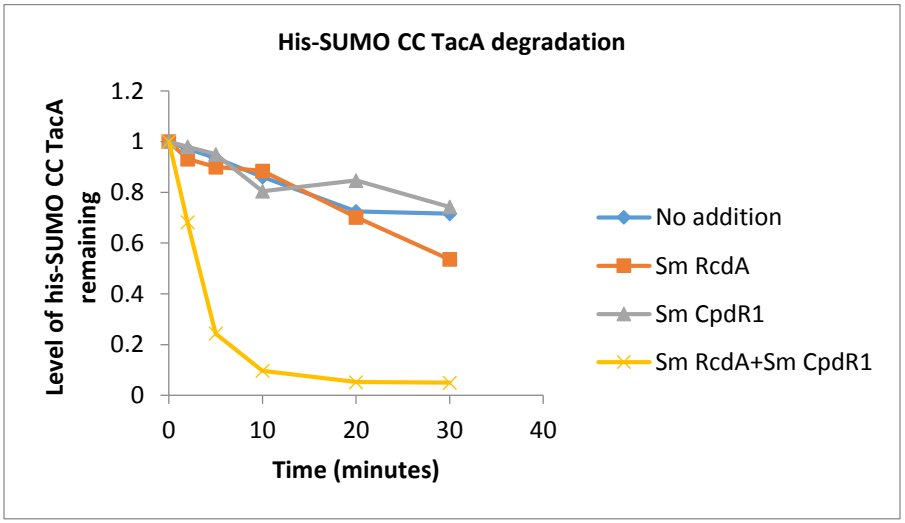
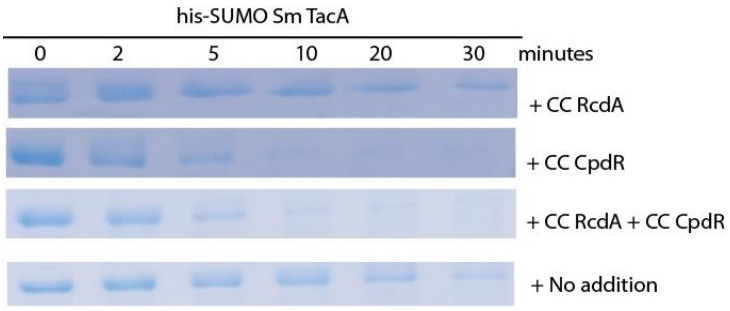


Figure 25: Degradation assay of his-SUMO *C.crescentus* TacA follows the same pattern of degradation as untagged *C.crescentus* TacA. This result indicates that the his-SUMO tag does not directly interact with the adaptor CpdR for enhanced degradation of the substrate. (A) The assay was carried out using 0.2uM of ClpP, 0.4uM of ClpX, 1uM of RcdA, 1uM of CpdR1 and 1uM of TacA. (B) Quantifications of his-SUMO CC TacA degradation assay. The substrate level was normalized to ClpP levels.

(I.d) *C.crescentus* CpdR is also sufficient for delivery of *S.meliloti* TacA for degradation by ClpXP

Degradation assay of his-SUMO *S.meliloti* TacA with adaptors from *C.crescentus* also suggests that CpdR alone is sufficient to deliver the substrate, suggesting that the *S.meliloti* TacA might be directly interacting with CpdR. A control experiment was done using his-SUMO *C.crescentus* TacA to ensure that the tag was not interfering with the results. His-SUMO *C.crescentus* TacA required both RcdA and CpdR, indicating that the tag was not involved in interacting with CpdR.

(a)



(b)

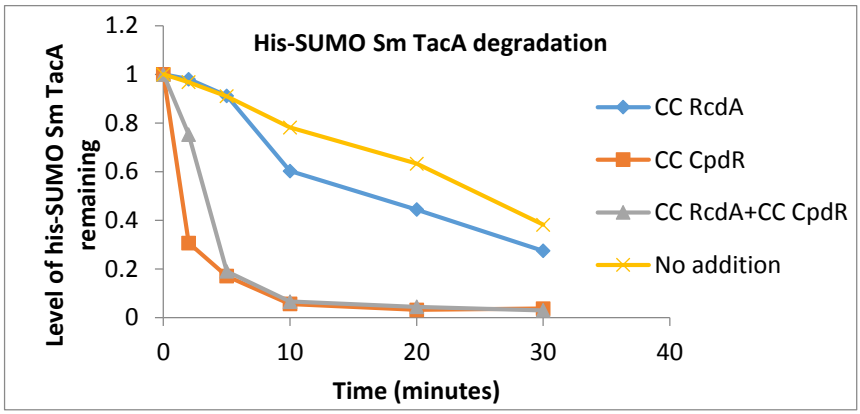


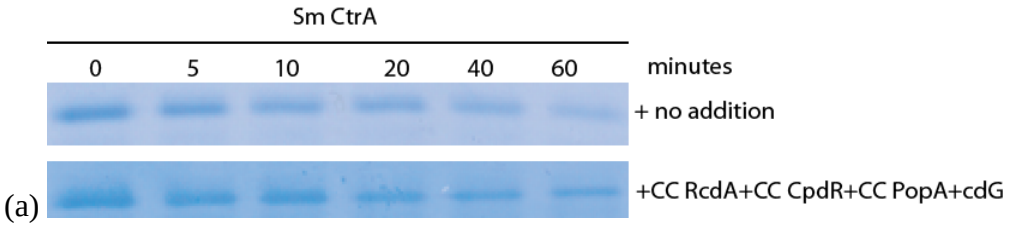
Figure 26: Degradation assay of his-SUMO *S.meliloti* TacA shows that *C.crescentus* CpdR is sufficient for the delivery of the substrate. (A) The assay was carried out using 0.2uM of ClpP, 0.4uM of ClpX, 1uM of RcdA, 1uM of CpdR1 and 1uM of TacA. (B) Quantifications of his-SUMO Sm TacA degradation assay. The substrate level was normalized to ClpP levels.

3. Degradation of *S.meliloti* CtrA requires a third, unknown adaptor

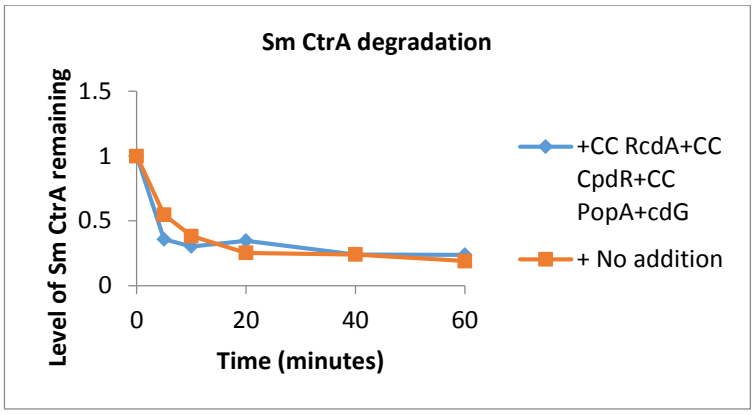
Degradation of *C.crescentus* CtrA requires the adaptors RcdA, CpdR and PopA, along with the small molecule cdG. *S.meliloti* does not contain a PopA, and therefore, degradation assays using various combination of adaptors from *C.crescentus* and *S.meliloti* were carried out to find the adaptor complex required to degrade *S.meliloti* CtrA.

(I.a) Adaptors from *C.crescentus* cannot enhance degradation of *S.meliloti* CtrA

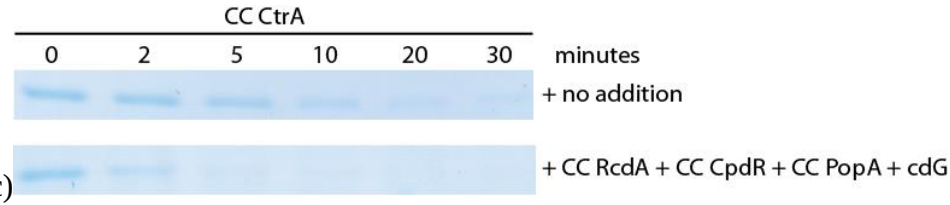
Degradation assay of *S.meliloti* CtrA with RcdA, CpdR and PopA from *C.crescentus* showed that the CtrA degraded in the same rate as when no adaptors are present, indicating that the adaptors from *C.crescentus* cannot interact with the *S.meliloti* CtrA to deliver the CtrA to ClpXP. A control experiment was done with *C.crescentus* CtrA which showed that the adaptor complex was active and able to deliver substrate, proving that there was no problem with the adaptor and protease complex itself (Figure 27).



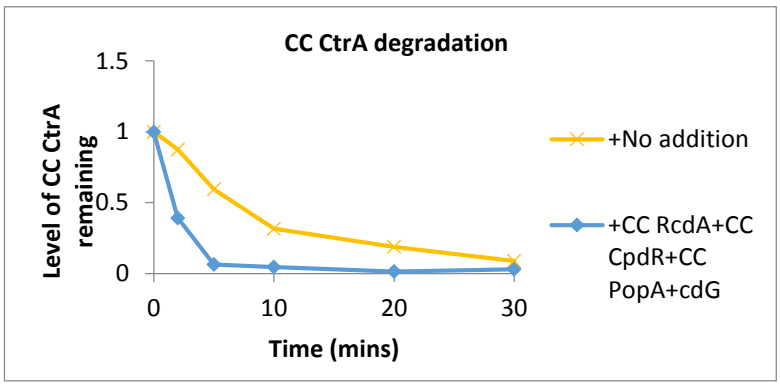
(a)



(b)



(c)



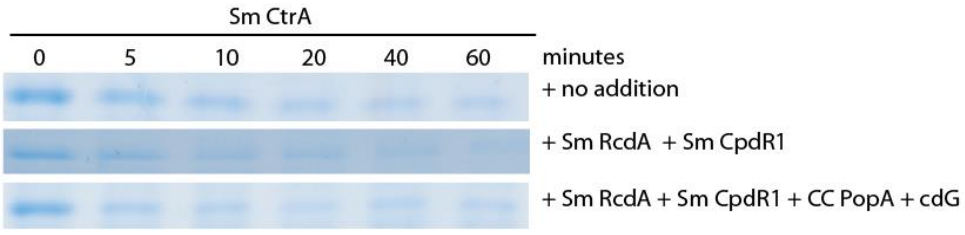
(d)

Figure 27: Degradation assay of *S.meliloti* CtrA shows that *C.crescentus* adaptors cannot deliver the substrate to ClpXP. (A) The assay was carried out using 0.4uM of ClpP, 0.8uM of ClpX, 1uM of RcdA, 1uM of CpdR1 and 1uM of CtrA. (B) Quantifications of Sm CtrA degradation assay. The substrate level was normalized to ClpP levels. (C) Degradation assay of *C.crescentus* CtrA using the same adaptors show that the adaptor complex is active.(D) Quantifications of CC CtrA degradation assay. The substrate level was normalized to ClpP levels.

(II.b) *S.meliloti* adaptors, along with *C.crescentus* PopA, cannot enhance degradation of *S.meliloti* CtrA

Since *S.meliloti* does not have PopA, *C.crescentus* PopA was used in a degradation assay of *S.meliloti* CtrA, along with *S.meliloti* RcdA and CpdR. The mixture of adaptor complex also could not enhance the degradation of CtrA (Figure 28). The experiment was also carried out with *C.crescentus* CtrA, where the degradation was not enhanced either, showing that the adaptor complex is not active (Figure 29). The result also shows that *C.crescentus* PopA is not able to interact with *S.meliloti* RcdA, thus leading to the inefficiency of degrading the substrate.

(a)



(b)

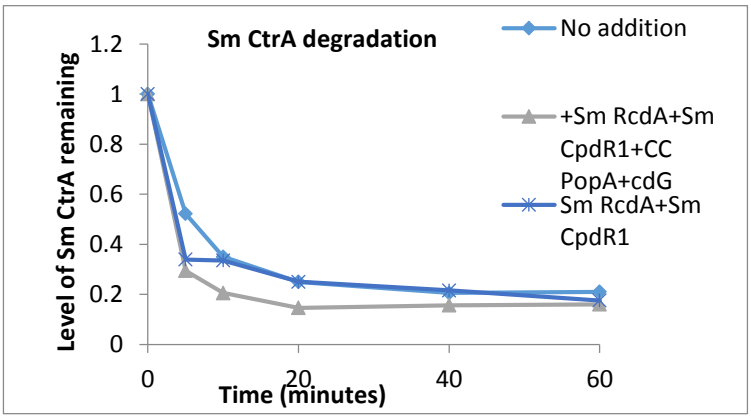
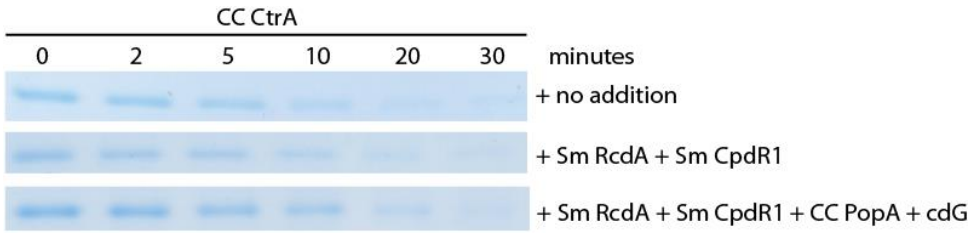


Figure 28: *S.meliloti* CtrA cannot be degraded by an adaptor complex consisting of adaptors from both species of bacteria. The rate of degradation for the CtrA in presence and absence of adaptors were same. (A) The assay was carried out using 0.4uM of ClpP, 0.8uM of ClpX, 1uM of RcdA, 1uM of CpdR1 and 1uM of CtrA.

(B) Quantifications of Sm CtrA degradation assay. The substrate level was normalized to ClpP levels.

(a)



(b)

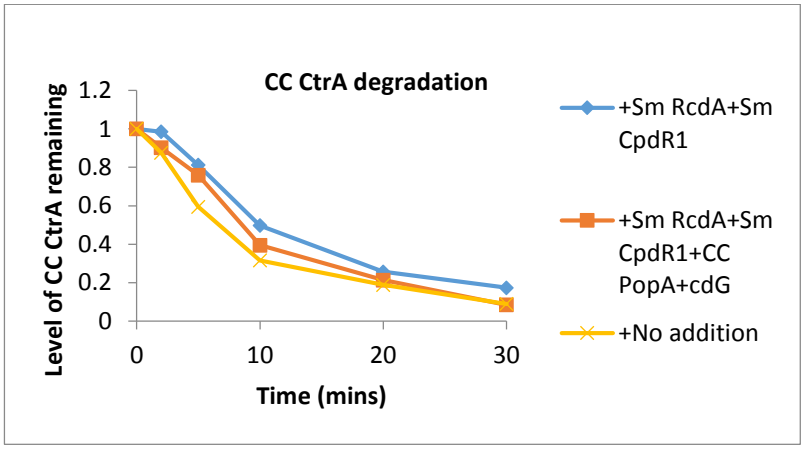
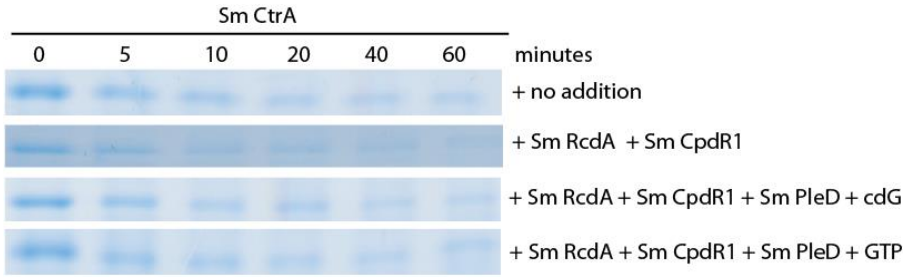


Figure 29: Degradation of *C.crescentus* CtrA was not enhanced by adaptors from *S.meliloti* and *C.crescentus*, showing that the adaptor complex is not active. Since previous results have shown that *S.meliloti* RcdA and CpdR can form an adaptor complex, it can be inferred that the C.crescentus PopA cannot interact with *S.meliloti* RcdA to form an active complex. (A) The assay was carried out using 0.2uM of ClpP, 0.4uM of ClpX, 1uM of RcdA, 1uM of CpdR1 and 1uM of CtrA. (B) Quantifications of CC CtrA degradation assay. The substrate level was normalized to ClpP levels.

(II.C) PleD, a PopA homolog, cannot enhance degradation of *S.meliloti* CtrA

Degradation assay of *S.meliloti* CtrA was also carried out using all *S.meliloti* components. A PopA homolog, PleD, was used in the assay to see whether it acted in the same way as PopA for degradation of CtrA. The rate of degradation of CtrA in presence and absence of the adaptor components showed that PleD was not the third adaptor required for *S.meliloti* CtrA degradation.

(a)



(b)

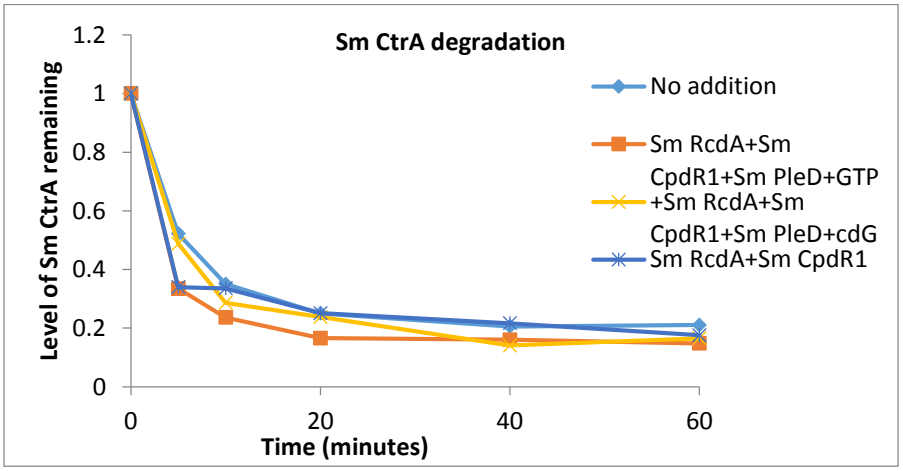


Figure 30: PleD, an ortholog of PopA in *S.meliloti*, does not play a role in the degradation of *S.meliloti* CtrA. Degradation assay rates for CtrA in presence and in absence of components from *S.meliloti* are the same. (A) Degradation assay was carried out using 0.4uM ClpX, 0.8uM ClpP, 1uM RcdA, 2uM CpdR1, 1uM PleD and 20mM cdG/GTP. (B) Quantifications for the level of CtrA remaining, normalized to ClpP levels.

Protein	Adaptors present	Half-life (minutes)
CC2323	RcdA and CpdR	9
	No adaptor	130
CC2323 (2-146)	RcdA and CpdR	46
	No adaptor	56
His-SUMO CC2323 (2-146)	RcdA and CpdR	86
	No adaptor	101
His-SUMO CC2323 (147-377)	RcdA and CpdR	176
	No adaptor	471
His-SUMO *S.meliloti* TacA	Sm RcdA only	14
	Sm CpdR1 only	1
	Sm RcdA and CpdR1	1
	No adaptor	11
His-SUMO *S.meliloti* TacA	CC RcdA only	5
	CC CpdR only	2
	CC RcdA and CpdR	3
	No adaptor	6
S.meliloti CtrA	Sm RcdA and Sm CpdR1	9
	Sm RcdA, Sm CpdR1, CC PopA, cdG	1
	Sm RcdA, Sm CpdR1, Sm PleD, cdG	11
	Sm RcdA, Sm CpdR1, Sm PleD, GTP	4
	CC RcdA, CC CpdR, CC PopA, cdG	3
	No adaptor	14

Table 1: The table above shows the half-lifes of substrate proteins obtained from degradation assay. The half –lifes were calculated by finding the equation of the line obtained from the assay and calculating g the time at which the concentration of the substrate reaches 50%.

DISCUSSION

1. C-terminus of CC2323 contains the binding site for RcdA

RcdA can directly bind to three degradation substrates: TacA, CC2323 and CC3144. The substrates contain a binding site for RcdA and a degron for recognition by ClpXP. The RcdA binding allows faster deilvery of the substrates to a CpdR-primed ClpXP for degradation . The initial approach to finding the RcdA binding site in CC2323 did not produce any definite result. The constructed CC2323 (2-146) fragment was constructed in such a way that it contained an artificial degron, so that if the RcdA binding motif was present in the fragment, it would degrade at the same rate as the full length CC2323, in presence of the adaptors. However, the constructed N-terminus was degraded by ClpXP in vitro both in presence and in absence of adaptors. It is possible that the degron present within the constructed fragment, allowed the fragment to directly interact with ClpXP, thus surpassing the need for proteolytic adaptors. Since the rate of degradation was not enhanced in presence of the adaptors, it was concluded that the fragment probably did not contain the RcdA binding site.

When a his-SUMO tagged version of the fragment was used as a substrate for degradation assay, the rate of degradation was slower than the untagged version of the fragment. A his-SUMO tag is a N-terminal tag, which means that the fragment of interest had its N-terminus blocked. Since the his-SUMO N-terminus fragment had lowered degradation rate, it can be inferred that the tag was either blocking the degron, making it more difficult for the ClpXP to

recognize the substrate, or the tagged protein was being folded in such a way that the degron was inaccessible to the protease. The result seen with the tagged protein was consistent with the previous result, confirming that the constructed N-terminus fragment of CC2323 did not contain the RcdA binding motif.

An untagged version of the constructed C-terminus could not be obtained. The degradation assay results using the his-SUMO tagged protein showed similar degradation rate in presence and in absence of adaptors, showing that this fragment too did not contain the RcdA binding motif. Moreover, results with constructed N-terminal fragment showed that the his-SUMO tag can interfere with degradation results, and since the C-terminus fragment was tagged, it is difficult to conclude that the result is representative of the untagged C-terminus fragment. It could be possible that the tag was causing the fragment to fold in such a way that the RcdA binding site was being covered, preventing enhanced degradation in presence of the adaptors.

The second approach to finding the binding motif provided a promising result. Trypsin digestion cleaves a protein after every lysine and arginine residue, except when followed by proline. Since the trypsin digestion of CC2323 was carried out for a short time, the trypsin was able to cleave after the lysine and arginine residues that are exposed in the interdomain space. Therefore, it can be inferred that the six distinct fragments obtained from the trypsinization of CC2323 corresponds to domains found within the full length protein.

Mass spectrometry results suggested that the fragment running at the largest size in the gel is the same exact size of full length CC2323, meaning that

the fragment represents undigested CC2323. The fragment right below it was around 5kDa smaller, meaning that the fragment had lost some of the residues from the full length CC2323. The third highest largest fragment did not produce any signal in mass spectrometry, while the next two were of almost same size, and the smallest one had a weight of approximately 10 kDa.

In vitro pull down assay of the trypsin digested CC2323 with RcdAΔC showed that all the fragments could bind to the adaptor, while the degradation assay showed that only one of the fragment could bind to RcdA and be delivered to the protease. This means that the fragment had both the binding motif for RcdA, as well as the degron for ClpX to recognize the substrate. It is possible that the all the other fragments produced from trypsin digestion had lost the degron tag and therefore could not be degraded.

N-terminal sequencing result showed that the fragment of interest had the 134th residue of the full-length protein as its first residue. Since it is known that the last two residues of CC2323 are important for degradation, it can be concluded that the fragment also contained the last two amino acids. This mean that the fragment contained the last 199 residues of the full length CC232.

The constructed C-terminus fragment of CC2323 contained all the 199 residues that are present within the fragment of interest that was sent for N-terminal sequencing. Therefore, the constructed C-terminus fragment should have degraded in a RcdA-CpdR dependent manner. It is highly likely that the his-SUMO tag was blocking the RcdA-binding site and/or the degron, which is why no enhancement of degradation was observed in presence of adaptors. It might

also be possible that the construction of the two fragmnets was carried out in such a way that the RcdA binding site was cleaved and both the constructed fragments had parts of the binding sites, which were not sufficient for enhanced degradation.

Based on the trypsinization results, it can be concluded that the RcdA binding motif of CC2323 lies within the last 199 residues of the protein. Further studies are required to find the minimum binding region required and to see whether the binding site shares any similarity with the binding site within TacA.

2. Mechanism of TacA delivery for degradation is different in *C.crescentus* and *S.meliloti*

Degradation assay with *C.crescentus* TacA showed that *S.meliloti* RcdA and CpdR1 can interact with each other and form an active adaptor complex. However, when *S.meliloti* TacA was used as a substrate for degradation, *S.meliloti* CpdR1 was sufficient for delivery of the TacA. The rate of degradation of *S.meliloti* TacA when RcdA alone was present was same as when no adaptors were added, while the rates were the same for when CpdR1 alone was present and when both RcdA and CpdR1 was present, meaning that the enhanced degradation was due to CpdR1. This result is completely different from the one seen for *C.crescentus* TacA, indicating that although RcdA and CpdR1 in *S.meliloti* can act as adaptors and form an adaptor complex, just like they do in *C.crescentus*, the mechanism by which the adaptors deliver substrate is different in *C.crescentus* and *S.meliloti*.

Since the *S.meliloti* TacA was his-SUMO tagged, the unexpected results could have been due to the tag directly interacting with CpdR1, thus surpassing the need for RcdA interaction. However, when a his-SUMO tagged *C.crescentus* TacA was used as a substrate for degradation assay, the substrate was not degraded in presence of CpdR1 alone, showing that the his-SUMO tag was possibly not responsible for the results seen with *S.meliloti* TacA.

The his-SUMO *S.meliloti* TacA was also used for degradation in presence of RcdA and CpdR from *C.crescentus*. Similar to the result seen with *S.meliloti* adaptors, the TacA was degraded in a CpdR dependent manner.

This result confirmed that *S.meliloti* TacA is a direct substrate of CpdR, rather than RcdA.

The TacA in *S.meliloti* is an ortholog of *C.crescentus* TacA, and the both are under the control of a conserved genetic circuit [30]. The exact function of TacA in *S.meliloti* is not yet known but it is assumed that it acts as a RNA polymerase sigma-54 factor, like in *C.crescentus*. However, it is possible that the *S.meliloti* TacA might actually have a different function, and its regulation is different from that in *C.crescentus*, making it a direct substrate for CpdR1, rather than RcdA.

Future studies need to be done with an untagged version of *S.meliloti* TacA to confirm that CpdR1 is the only adaptor required for TacA degradation. In vivo degradation of TacA should also be monitored in a CpdR1 deleted strain of *S.meliloti*, which should show no or slow degradation of TacA, if the in vitro model presented here is correct.

3. Degradation of *S.meliloti* CtrA requires a third, unknown adaptor

Previous studies have shown that *S.meliloti* RcdA and CpdR1 are required for CtrA degradation [28]. In vitro degradation assay suggests that RcdA and CpdR1 together are not sufficient to deliver *S.meliloti* CtrA for degradation. Since *C.crescentus* CtrA degradation requires the third adaptor PopA for degradation, it is possible that *S.meliloti* CtrA also requires a third adaptor. However, since *S.meliloti* does not contain PopA, *C.crescentus* PopA was used in the degradation assay to see if enhanced degradation could occur. The degradation assay was carried out with both *C.crescentus* CtrA and *S.meliloti* CtrA and none of them were degraded rapidly in presence of *S.meliloti* RcdA, CpdR1 and *C.crescentus* PopA.

The results with adaptors from both bacteria shows that *S.meliloti* RcdA cannot interact with *C.crescentus* PopA. PopA directly binds to RcdA in *C.crescentus* for CtrA degradation, and the degradation assay with TacA suggested that RcdA and CpdR1 from *S.meliloti* can form an complex. Therefore, there must be a defect in the interaction between *S.meliloti* RcdA and *C.crescentus* PopA that does not allow an active adaptor complex to be formed for CtrA degradation.

PleD, an ortholog of PopA, that is present in *S.meliloti* was also used in degradation assay to see whether it acts as the third proteolytic adaptor. cdG, which is a small molecule required by PopA, was also used in the assay, as well as GTP. However, the assay did not produce a positive result, indicating that the third adaptor for CtrA degradation is unknown.

Since CtrA degradation is essential in *S.meliloti*, it is important to find the adaptors that aid in the degradation process. Immunoprecipitation of CtrA can be done to find the proteins that bind to CtrA and mass spectrometry can be carried out to identify the proteins. The identified proteins can then be used in degradation assay to find the missing third adaptor. It will be interesting to see whether this adaptor has any resemblance to PopA, which will provide some insights into how the class of alphaproteobacteria has wired the mechanism of cell cycle regulation to be species-specific.

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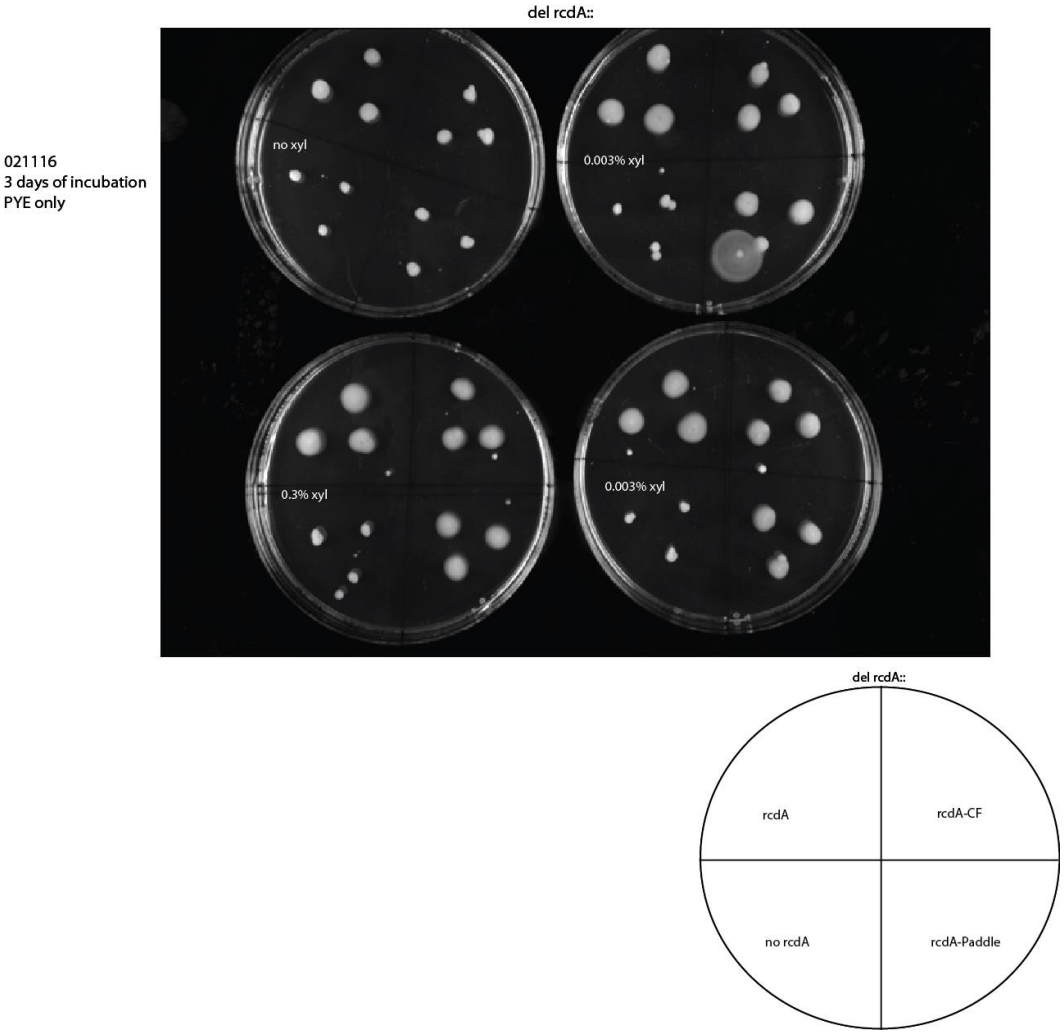
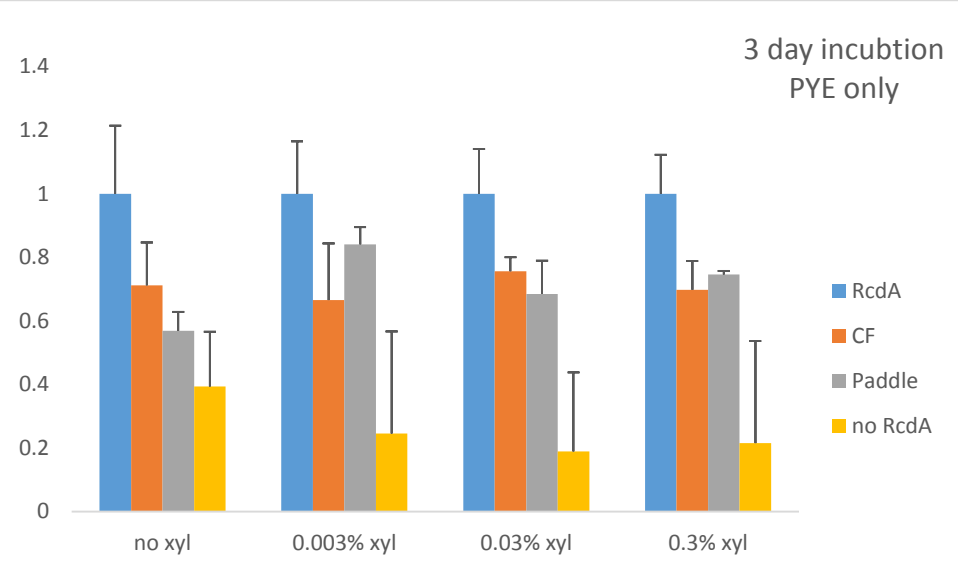
APPENDIX 1:

(A)



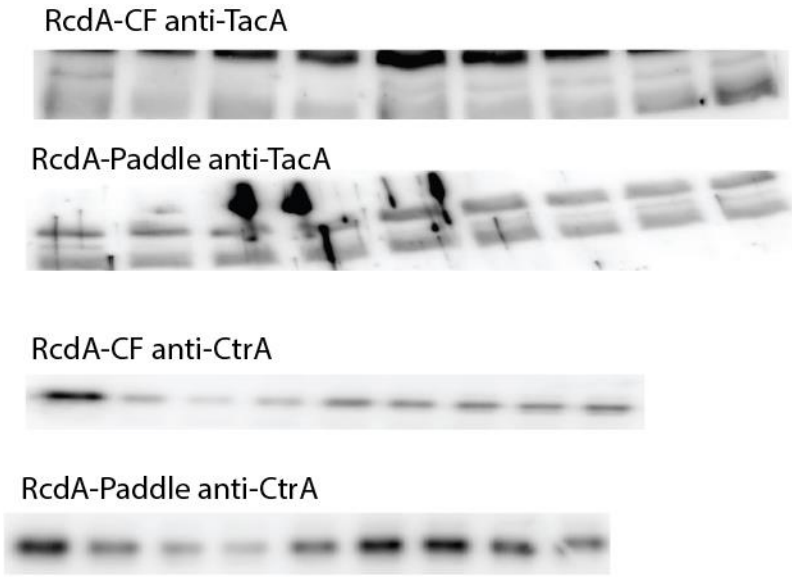
Colonies for RcdA-CF and RcdA-Paddle are smaller than colonies for RcdA at lower concentration of xylose.

(B) Quantification of colony sizes for RcdA mutants



APPENDIX 2:

In vivo degradation assay of TacA in RcdA-CF and RcdA-Paddle backgrounds suggests that TacA can interact with the mutant RcdA to be delivered for degradation. CtrA degradation was used as a control.

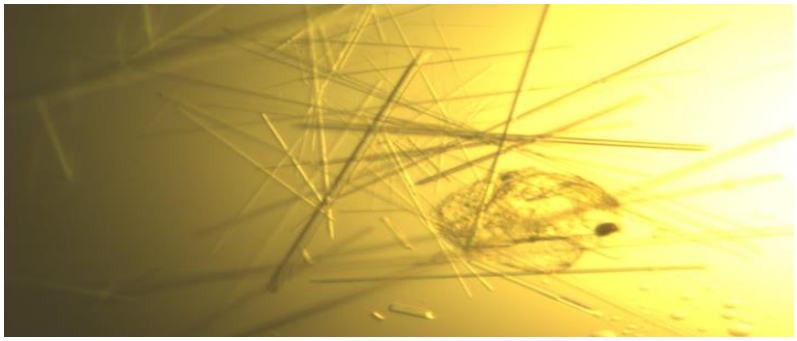


APPENDIX 3:

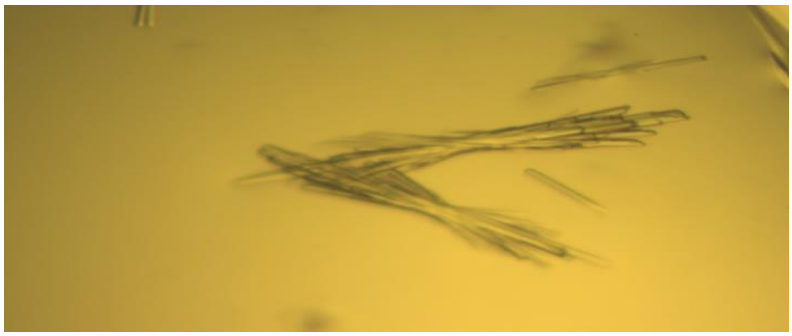
(a) Crystallization tray setup for h6RcdA-TacA DBD complex

1M Na formate 0.1M Tris pH 8.5	1.2M Na formate 0.1M Tris pH 8.5	1.4M Na formate 0.1M Tris pH 8.5	1.6M Na formate 0.1M Tris pH 8.5	1.8M Na formate 0.1M Tris pH 8.5	2.0M Na formate 0.1M Tris pH 8.5
1M Na formate 0.1M Hepes pH 7.5	1.2 M Na formate 0.1M Hepes pH 7.5	1.4M Na formate 0.1M Hepes pH 7.5	1.6M Na formate 0.1M Hepes pH 7.5	1.8M Na formate 0.1M Hepes pH 7.5	2.0M Na formate 0.1M Hepes pH 7.5
1M Na formate 0.1M Tris pH 8.5	1.2M Na formate 0.1M Tris pH 8.5	1.4M Na formate 0.1M Tris pH 8.5	1.6M Na formate 0.1M Tris pH 8.5	1.8M Na formate 0.1M Tris pH 8.5	2.0M Na formate 0.1M Tris pH 8.5
1M Na formate 0.1M Hepes pH 7.5	1.2M Na formate 0.1M Hepes pH 7.5	1.4M Na formate 0.1M Hepes pH 7.5	1.6M Na formate 0.1M Hepes pH 7.5	1.8M Na formate 0.1M Hepes pH 7.5	2.0M Na formate 0.1M Hepes pH 7.5

(b) Crystals of h6RcdA-TacA DBD complex



Crystallization condition: 1.8M Na and 0.1M tris pH 8.5



Crystallization condition: 2.0M Na and 0.1M Hepes pH 7.5