Name: _____KEY_____

5.08 Exam #2

This exam is worth **100 points**.

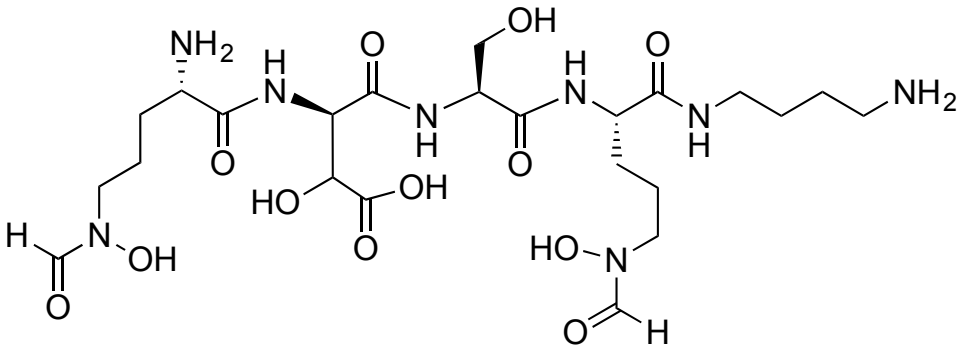
This exam contains **12 pages**.
Check that you have all pages before commencing the exam.
Read all questions, figure captions, and figures carefully.
Write neatly – what cannot be read or deciphered will not be graded.

Question 1: _____ / **50 pts**

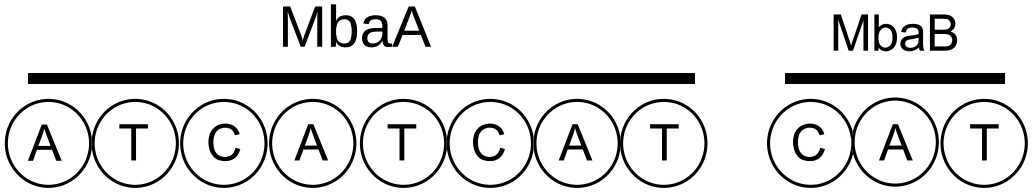
Question 2: _____ / **50 pts**

**TOTAL: _____ / 100 pts**

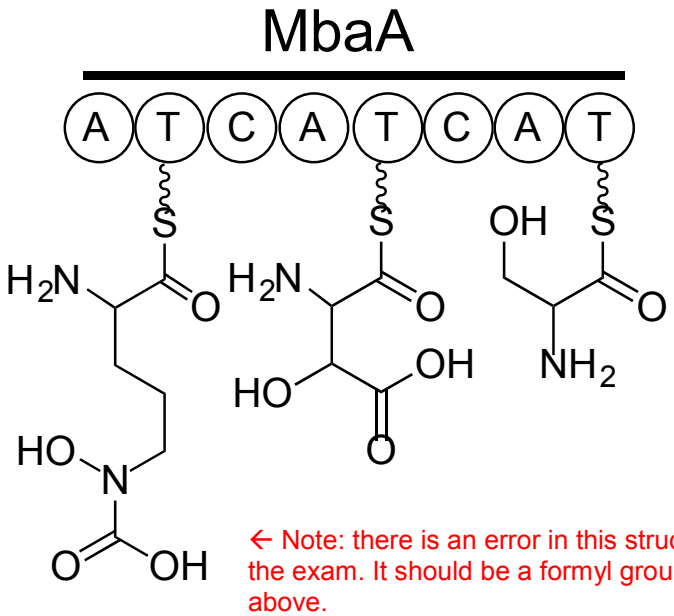
**1.** (**50 pts total**) *Burkholderia xenovorans* LB400 is an environmental bacterium that has the ability to degrade aromatic compounds, including environmental pollutants. In recent work, researchers discovered that *B. xenovorans* biosynthesizes and exports a non-ribosomal peptide when cultured under conditions of iron limitation (*PloS ONE* **2016**, ASAP). The chemical structure of this new metabolite revealed a number of non-proteinogenic amino acids and is shown below:



Bioinformatics analysis indicated that the NRPS responsible for synthesis of this molecule is composed of two proteins, MbaA and MbaB, shown below.



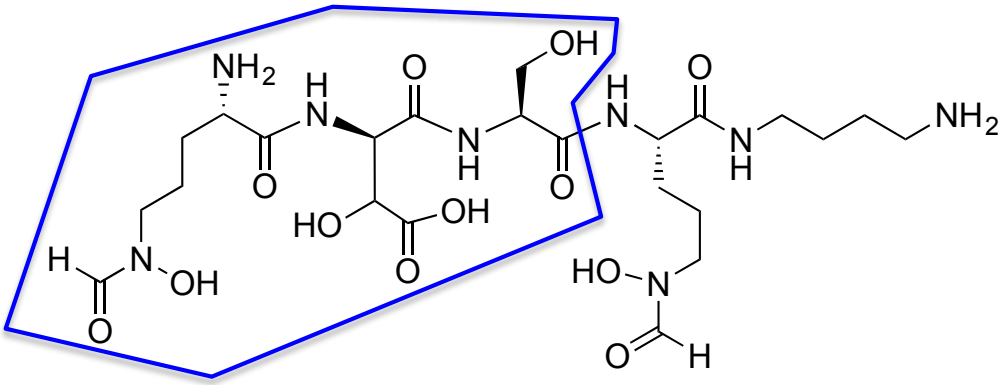

Individual domains of MbaA were overexpressed and purified. Biochemical characterization revealed the A domain selectivity as indicated below. ATP-$PP_i$ exchange assays revealed that the non-proteinogenic amino acids L-*N*δ-*N*δ-formyl-ornithine and D-β-hydroxy-aspartate are activated by the A domains of the loading module and the first elongation module, respectively. The A domains also transfer these monomers to the respective T domains. The A domain of the third module activates and loads L-serine.




← Note: there is an error in this structure, and was caught during the exam. It should be a formyl group as shown in the structure above.

Answer the following questions:

**1. (5 pts)** On the basis of McbA characterization, circle the portion of the molecule that is biosynthesized by this protein. Do so on the structure below and not on the prior page.



**2. (5 pts)** Characterization of MbaA implicates MbaB as the termination module. What is unusual about this termination module?

There is no TE (thioesterase) domain to catalyze release of the product.

**3. (25 pts)** The individual domains of MbaB were overexpressed in *E. coli* and purified as $His_6$-fusion proteins. ATP-$PP_i$ exchange assays were performed to investigate the substrate selectivity of the A domain of MbaB. The results are shown below in **Figure 1**.

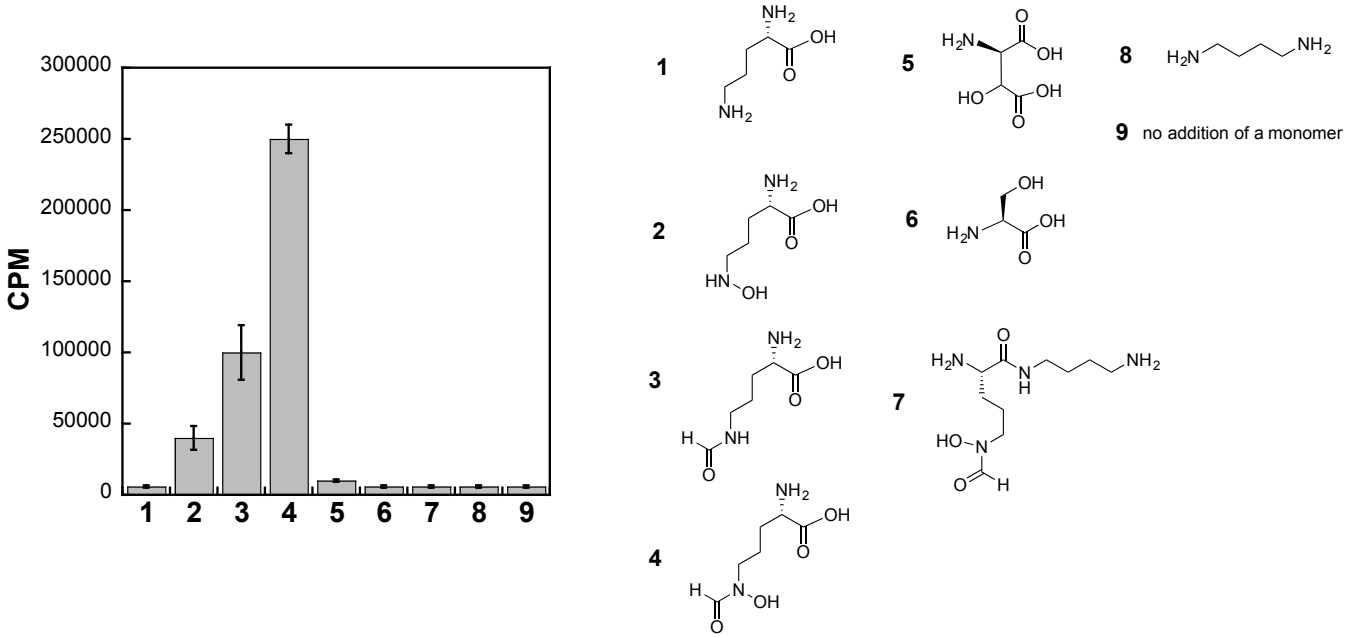
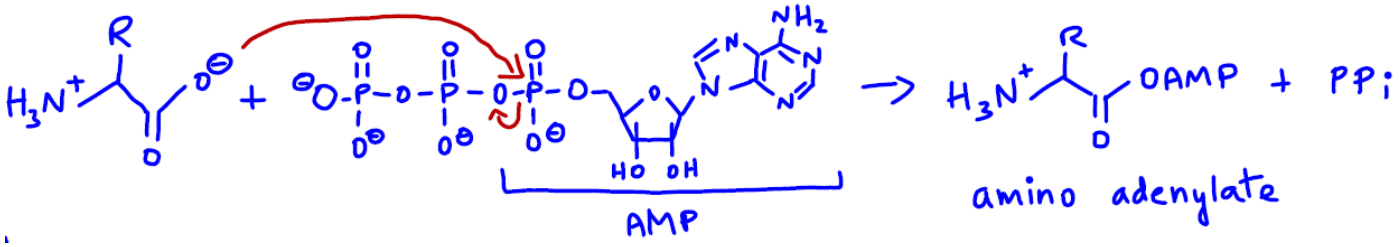



**Figure 1.** Results from ATP-$PP_i$ exchange assays using $His_6$-MbaB and the substrates listed in the plot. The y-axis "cpm" (counts per minute) is a measure of $^{32}P$ incorporation into ATP. The reactions containing the A domain of MbaB, ATP, $^{32}PP_i$ and the substrate (indicated by number) were incubated in buffer and quenched all at the same time point.

**(i)** How do A domains activate monomers? Show the reaction mechanism.

A domains are adenylation domains that activate monomers as adenylates for subsequent transfer to T domains. The chemistry is shown below:




OK to have included transfer to the T domain in the answer, but not necessary for full credit.
OK to have a base on the A domain deprotonate the amino acid, or to present as shown above.
Correct structure of ATP required for full credit.

**(ii)** Is the result for **monomer 8** expected or unexpected? Briefly explain your reasoning.

This question requires that you think about the A domain substrate and chemistry. It activates amino acids and aryl acids. We see no activity of the A domain of MbaB towards monomer 8. This result is expected because 1,4-diaminobutane has no $–CO_2H$ group, so an amino adenylate cannot be formed.

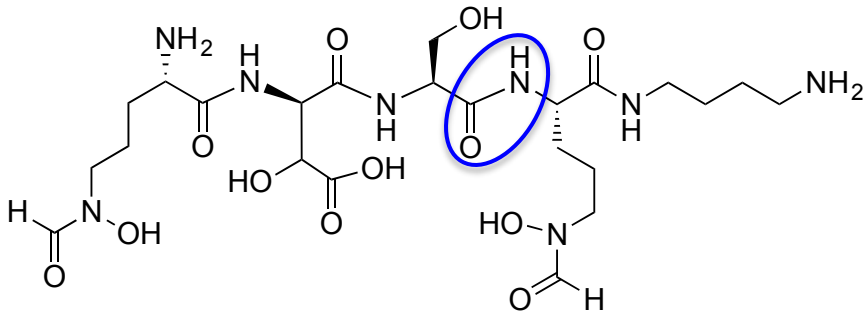
**(iii)** What do we learn about the substrate specificity of the A domain of MbaB from the data in **Figure 1**? Provide three conclusions that summarize the data completely.

The ATP/$PP_i$ exchange assay tells us about what amino acid monomers are selected and activated by a given A domain. The observed counts per minute (CPM) for the different samples are as follows:

4 > 3 > 2 >1 ~ 5 – 9

1. The A domain of MbaB selects and activates several monomers with preference 4 > 3 > 2.
2. There is negligible activity with monomers 1 and 5-8 (9 is a no monomer control). Therefore MbaB does not activate these monomers. These trends can be correlated to structural features (e.g. modification of the amino group), but these details were not required for full credit.
3. Monomer 4 appears to be the preferred substrate of MbaB.

**(iv)** On the basis of these data, what amide bond does MbaB form? Circle the bond on the structure below.



**(v)** Do the C domains of NRPS assembly lines perform covalent or non-covalent catalysis?

Non-covalent catalysis.

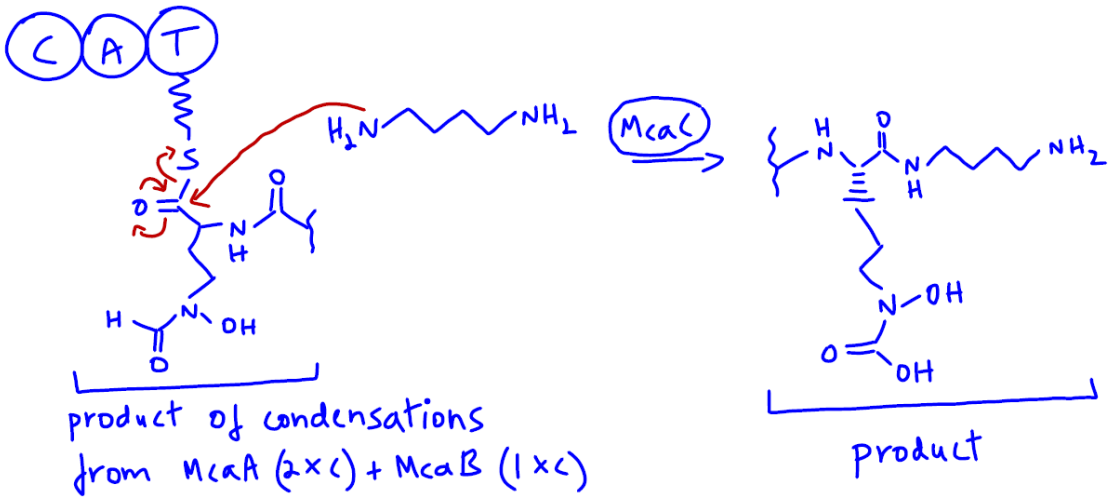
(**vi**) Why does nature use thioesters in NRPS assembly lines?


The leaving group is another aspect, but less important as discussed in lecture.

**4. (10 pts**) At this point, the researchers were scratching their heads about the 1,4-diaminobutane moiety in the siderophore and how termination occurs, and performed further bioinformatics analyses. This effort revealed a gene encoding a single-domain protein upstream of the *mbaA* and *mbaB* genes. They named this gene *mbaC*, and they overexpressed and purified the MbaC protein as a $His_6$-fusion protein.

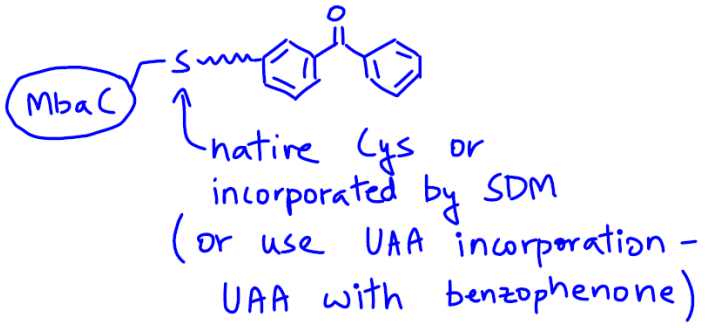
Incubation of all of the MbaA domains, all of the MbaB domains, identified monomers, ATP, 1,4-diaminobutane and MbaC resulted in formation of the metabolite, which was identified by liquid chromatography and mass spectrometry. When MbaC was omitted from the assay, no product was detected. Provide an explanation for this result that highlights the role of MbaC. Be sure to justify your explanation using your knowledge of assembly line biosynthesis and chemistry. A depiction of the relevant part of the assembly line, chemical structures and mechanism is appropriate.

MbaC catalyzes a condensation reaction between 1,4-diaminobutane and the elongated chain, which results in product release. The chemistry is shown below:



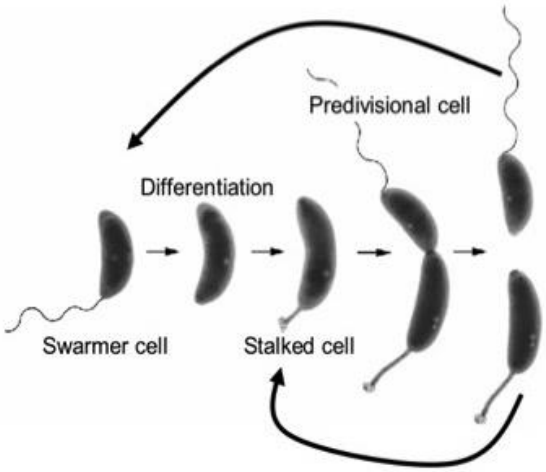
**5. (5 pts)** Having thought about this work, you hypothesize that MbaC interacts with one or more components of the NRPS, and you decide to test your hypothesis by performing a cross-linking experiment. Briefly explain the design of your experiment, including what type of cross-linker will you employ and why.

I would use a specific heterobifunctional cross-linker with a photoreactive group like a benzophenone. Thus, we can control placement of the cross-linker on MbaC and control the reactivity of the linker by irradiation with light.




Limitations of non-specific homo-bifunctional cross-linkers were discussed in recitation #5. This answer received partial credit (or full-credit with follow-up experiments). Even without information about how how two proteins interact, one can be specifically modified at one location (or, even better, many locations) by incorporating a site-specific Cys residue by site-directed mutagenesis or UAA incorporation (e.g. benzophenone) and the cross-linking experiments performed.

**2. (50 pts total)** *Caulobacter crescentus* is a Gram-negative bacterium that has a dimorphic lifestyle. It must transition from a swarmer cell to a stalked cell during its life cycle as shown in the image below. **The transition from swarmer cell to stalked cell is driven by the degradation of key regulatory proteins** that include **TacC** (a developmental regulator) and **CtrA** (a transcription factor). This question examines the role of ClpXP and adaptors in protein degradation during the *C. crescentus* cell cycle (*Cell* **2015**, *163*, 419-431).




**Dimorphic cell cycle of *C. crescentus.***



Prior studies revealed that the **degradation of TacC and CtrA depends on a protein named CpdR that functions as an adaptor for ClpXP**. CpdR functions differently from SspB, the adaptor protein we discussed in class that recognizes the ssrA tag. CpdR itself does not bind a condemned protein substrate readily. Instead, it binds to ClpX and effectively "prepares" ClpX for engagement with select substrates. Binding of CpdR to ClpX results in some conformational change and formation of a "recruitment interface" where certain condemned

substrates can bind. Other biological studies have identified additional protein factors that are important for cell-cycle dependent protein degradation in *C. crescentus*; however, many mechanistic details about how these additional proteins function to modulate protein degradation by ClpXP is unclear. The experiments below consider one of these proteins named **RcdA**.

In **experiment #1**, cultures of either wild-type *C. crescentus* or a mutant strain were synchronized such that all of the cells were at the same point in the cell cycle. The mutants are Δ*rcdA* (cannot express functional RcdA) and Δ*cpdR* (cannot express functional CpdR). Equal numbers of swarmer cells were released into fresh culture medium and the culture was allowed to grow. Aliquots of the culture were taken at different time points corresponding to different stages in the cell cycle. The cells were lysed and the lysates were separated by SDS-PAGE and probed with anti-TacA, anti-CtrA, anti-McpA and anti-ClpP antibodies. TacA, CtrA, and McpA are proteins that are known substrates for ClpXP. The resulting data are shown in **Figure 1**.

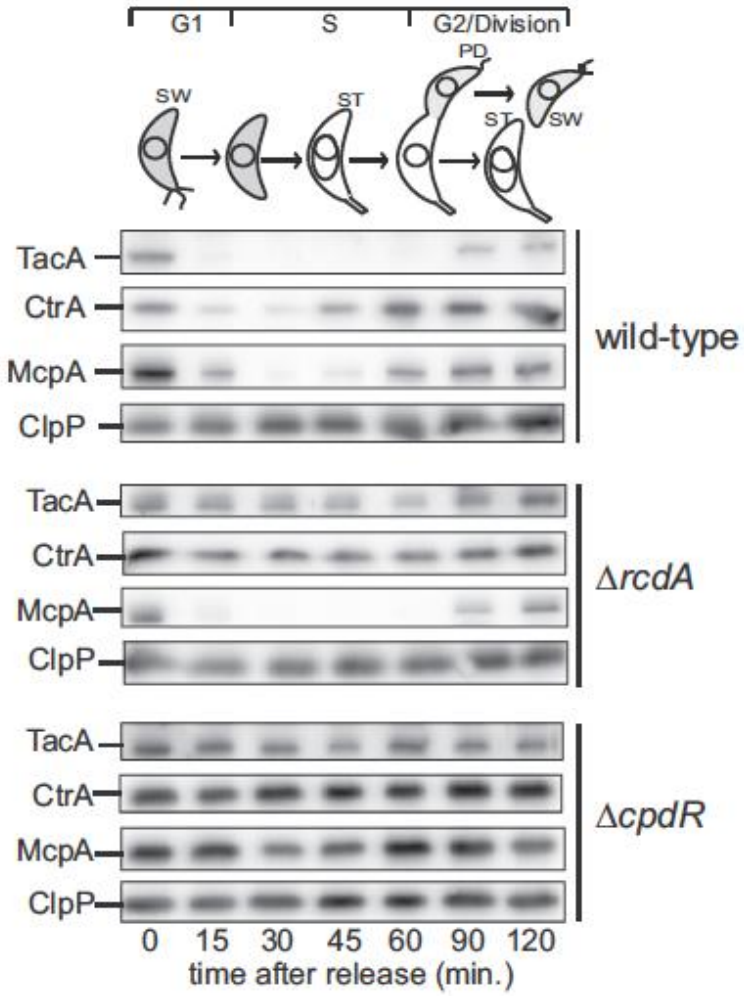
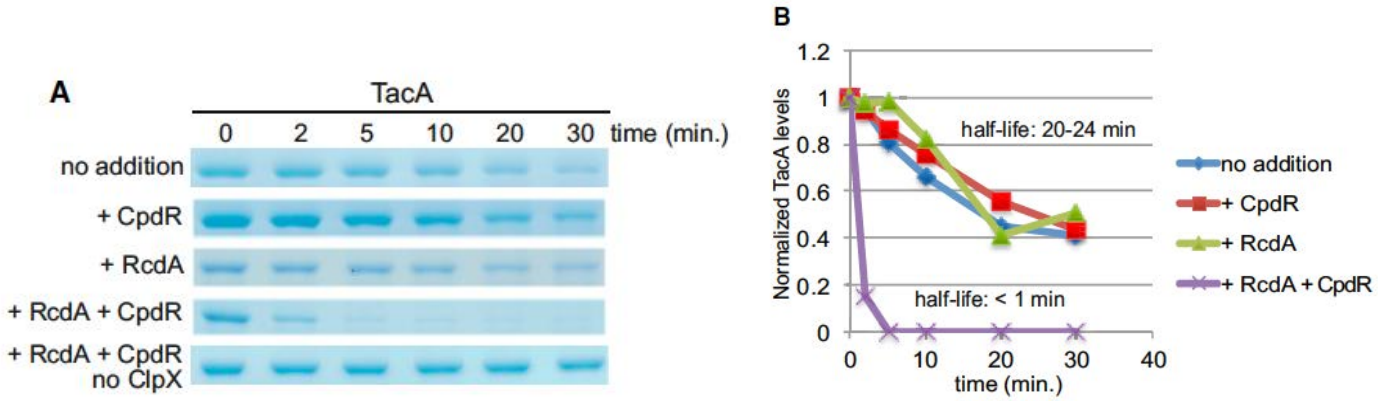



**Figure 1.** Cell-cycle dependent levels of the ClpXP substrates TacA, CtrA and McpA.
Abbreviations: SW = swarmer cell, ST = stalk cell, PD = predivisonal cell. G1, S and G2/Division are different points in the cell cycle.

In **experiment #2**, the researchers focused on the degradation of TacA by ClpXP using *in vitro* assays with purified proteins. The effect of CpdR and RcdA on ClpXP-catalyzed degradation of TacA was examined. Degradation assays were performed by combining ClpXP, ATP, TacA, and CpdR and/or RcdA, and these reactions were quenched various time points. TacA degradation was monitored by SDS-PAGE. The resulting data are shown in **Figure 2.**

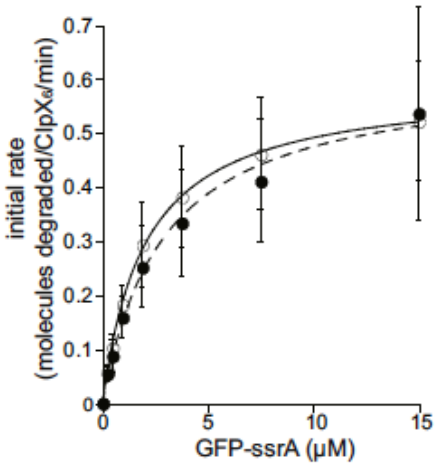





**Note: "no addition" means that CpdR and RcdA are not added. ClpXP is present.**

**Figure 2.** Degradation of TacA by ClpXP in the absence and presence of CpdR and/or RcdA.
**(A)** SDS-PAGE analysis. The bands in the gel are full-length TacA.
**(B)** Quantification of the gel shown in panel A.
Concentrations employed: 1 μM TacA, 0.4 μM $ClpX_6$, 0.8 μM $ClpP_{14}$, 2 μM CpdR, 1 μM RcdA. An ATP regeneration system was included in this assay so ATP is not limiting.

In **experiment #3**, the same type of experiment was performed except that GFP-ssrA was used as a substrate. A steady-state kinetic analysis was performed and the resulting data are shown in **Figure 3.**




| | | $V_{max}$ (molecules degraded / $ClpX_6$ / min) | $K_m$ (μM) |
|---|---|---|---|
| ○ | RcdA + CpdR | 0.59 ± 0.13 | 2.05 ± 0.08 |
| ● | No addition | 0.61 ± 0.23 | 2.86 ± 0.52 |

**Note: "no addition" means that CpdR and RcdA are not added. ClpXP is present.**

**Figure 3.** Degradation of GFP-ssrA (varying concentrations) by ClpXP in the absence and presence of CpdR and RcdA. Plot of initial rate versus the GFP-ssrA concentration. The error bars indicate the standard deviation from the mean.
Concentrations employed: 0.4 μM $ClpX_6$, 0.8 μM $ClpP_{14}$, 2 μM CpdR,1 μM RcdA. An ATP regeneration system was included in this assay so ATP is not limiting.

Subsequently, the researchers determined that RcdA and TacA form a protein-protein complex (experiments/data not shown, but size-exclusion chromatography was used). RcdA has a disordered C-terminal region and the researchers questioned whether this region of RcdA is important.

In **experiment #4**, the researchers overexpressed and purified $His_6$-RcdA-ΔC, a RcdA variant that lacks 19 residues from the C-terminus, and used this protein in studies of TacA degradation by ClpXP. The resulting data are shown in **Figure 4.**

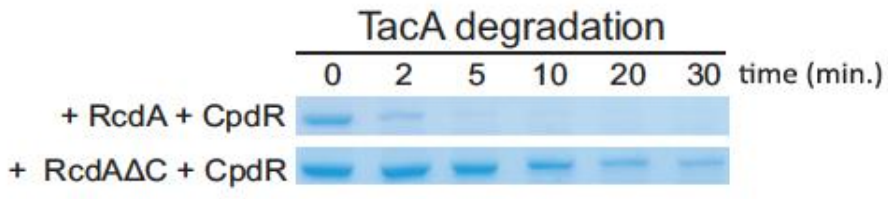



**Figure 4.** Degradation of TacA by ClpXP in the absence and presence RcdA or RcdA-ΔC. The bands in the SDS-PAGE gels are full-length TacA.
Concentrations employed: 1 μM TacA, 0.4 μM $ClpX_6$, 0.8 μM $ClpP_{14}$, 2 μM CpdR, 1 μM RcdA. An ATP regeneration system was included in this assay so ATP is not limiting.



In **experiment #5**, they used $His_6$-tagged RcdA-ΔC in pull-down experiments to determine whether this variant binds TacA. $His_6$-RcdA-ΔC and TacA were incubated alone or together with Ni-NTA resin at 4 °C for 1 hour. Then, the resin was transferred to a column and washed twice with buffer (20 mM HEPES, 100 mM KCl, pH 7.5) containing 20 mM imidazole. Then, the resin was washed with 200 mM imidazole. The fractions were analyzed by SDS-PAGE and these data are shown in **Figure 5.**

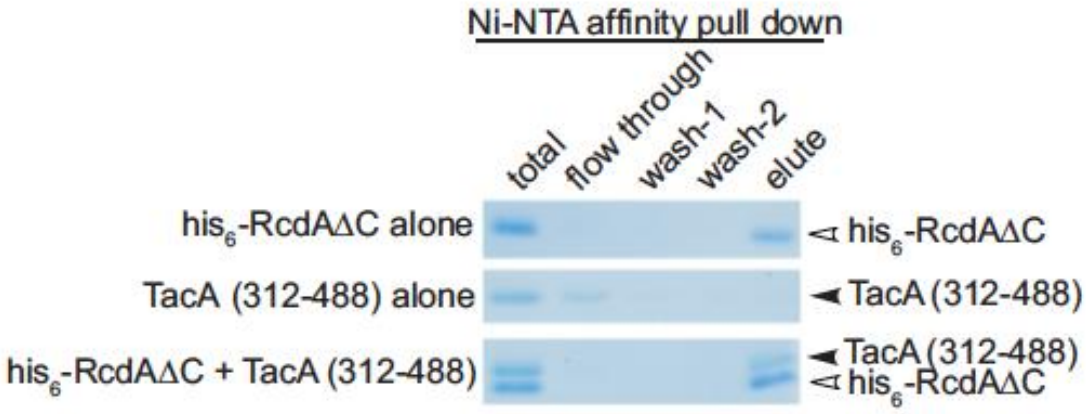
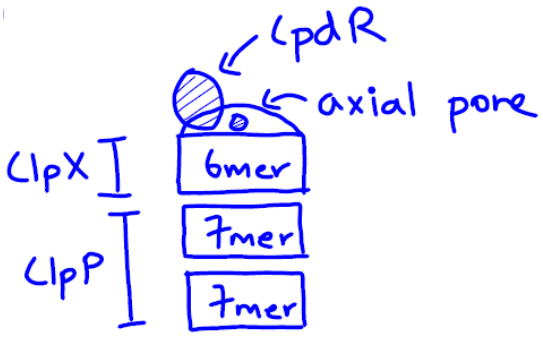



**Figure 5.** Assays to examine the interaction of TacA with RcdA-ΔC. The factions were analyzed by SDS-PAGE and the identities of the bands are indicated on the right.

5.08 Spring 2016 Exam 2

Answer the following questions:

1. (**5 pts**) Draw a cartoon of the protein degradation machine ClpXP and, based on the information provided in this question, indicate how CpdR interacts with the machine. Include the oligomeric states of ClpX and ClpP in your answer.




2. (**3 pts**) What three amino acids compose the catalytic triad of ClpP?

Serine, histidine and aspartic acid.

3a. (**5 pts**) What do we learn from the data in **Figure 1** (**experiment #1**) about cell-cycle dependent levels of TacA, CtrA, McpA and ClpP in wild-type *C. crescentus?*

The data in Figure 1 show:

ClpP → The level of ClpP is relatively constant throughout the cell cycle, with slightly elevated levels at the G2/division stage.

TacA → TacA is most abundant in the swarmer cells (G1) and in the G2/division stage where PD, ST and SW cells occur. It appears that TacA expression is associated with swarmer cells.

CtrA → CtrA is detected throughout the cell cycle, and is highest during G2/division and lowest during the transition between SW → ST.

McpA → This protein is most abundant in swarmer cells, and is absent in stalk cells.

Together, we see that there are subtle differences in cell-cycle dependent expression levels for TacA, CtrA and McpA.

3b. (**5 pts**) What do we learn from the data in **Figure 1** (**experiment #1**) about the role of CpdR?

When CpdR is knocked out, there is no or negligible change (i.e. reduction) in the levels of TAcA, CtrA and McpA at any point in the cell cycle. Thus, CpdR is required for those proteins to be degraded in the cell-cycle dependent manner. (The McpA levels might be somewhat reduced at 30 min and 120 min, but some further experiments are needed to confirm.)

3c. (**5 pts**) What do we learn from the data in **Figure 1** (**experiment #1**) about the role of RcdA?

When RcdA is knocked out, there is negligible effect on McpA levels compared to wild-type. In contrast, we see that TacA and CtrA levels remain approximately constant throughout the cell cycle, which is in marked contrast to the wild-type. These data indicate that RcdA expression is required for cell-cycle dependent degradation of TacA and CtrA, but not for McpA.

4. **(5 pts**) What do we learn from the data in **Figure 2** (**experiment #2**) about the role of RcdA?

This experiment probes the effect of the known adaptor CpdR and RcdA on ClpXP-catalyzed degradation of TacA. We observe that +RcdA alone or +CpdR alone has negligible effect on the ClpXP-catalyzed degradation of TAcA, whereas the combination of CpdR and RcdA enhances the degradation of TacA. Thus, it appears that CpdR and RcdA work together to enhance the degradation of TacA.

5a. (**2 pts**) Why was **experiment #3** performed?

To determine whether the observations in Experiment #2 are specific or general towards other proteins as well. This experiment also gives insight into whether CpdR and/or RcdA is an anti-adaptor for ssrA-tagged proteins (blocks or inhibits degradation rather than enhancing degradation).

5b. (**5 pts**) What can we conclude from the data in **Figure 3 (experiment #3)**?

The combination of CpdR and RcdA has no effect on degradation of the non-native/model substrate GFP-ssrA. Thus, they do not service all substrates destined for degradation by ClpXP, and do not block degradation of this ssrA-tagged protein.

6. (**5 pts**) From your professor's point of view, an important piece of information is missing from **Figure 4.** What is missing and why would this data be helpful in interpreting the results from **experiment #4**?

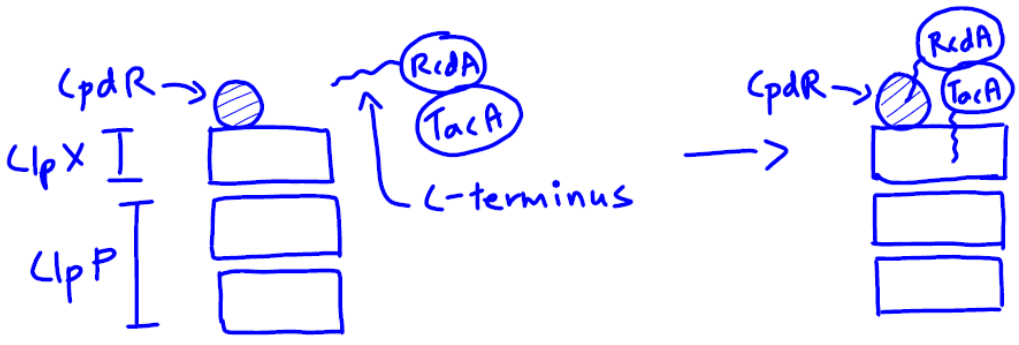
It would be helpful to see a -RcdA, +CpdR control. It is difficult to conclude whether RcdAΔC has no activity or lower activity than full-length RcdA without the data from the -RcdA, +CpdR control shown in conjunction.

7. (**5 pts**) What do we learn from the data in **Figure 5** (**experiment #5)**?

TacA and RcdAΔC form a complex; thus, the 19 residues at the C-terminus of RcdA are not required for the formation of the complex.

8. (**5 pts**) Using the information provided in this question and your analysis/interpretation of the data, provide a model for the function of RcdA in ClpXP-mediated degradation of TacA.

CpdR is an adaptor protein that interacts with ClpX, as described in the text. RcdA forms a complex with TacA (independent of the RcdA C-terminus) and interacts with CpdR or ClpXP *via* its C-terminus. This model with the C-terminus of RcdA interacting with CpdR is depicted below:




A proposal for the role of the RcdA C-terminus was required for full credit.

END OF EXAM

# 5.08J Biological Chemistry II

Spring 2016