

Name: _____

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.

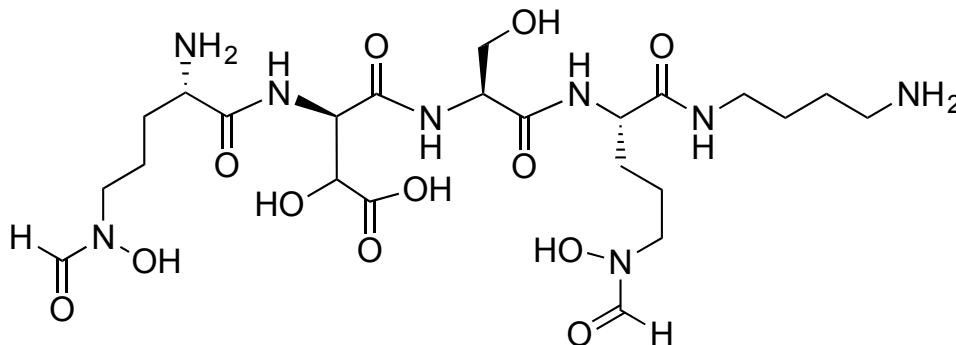
Write neatly – what cannot be read or deciphered will not be graded.

Question 1: _____ / **50 pts**

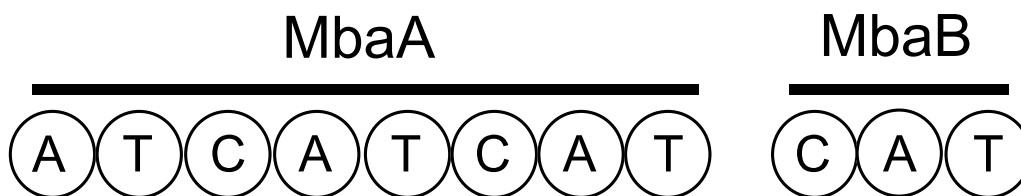
Question 2: _____ / **50 pts**

TOTAL: _____ / **100 pts**

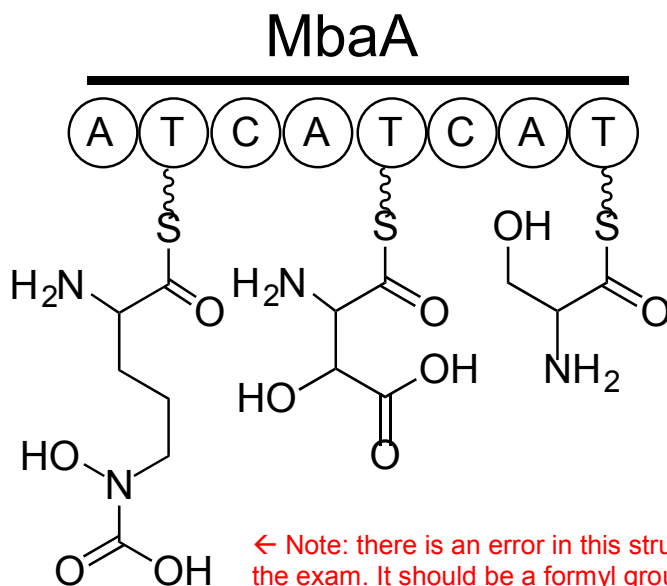
1. (50 pts total) *Burkholderia xenovorans* LB400 is an environmental bacterium that has the ability to degrade aromatic compounds, including environmental pollutants. In recent work, researchers discovered that *B. xenovorans* biosynthesizes and exports a non-ribosomal peptide when cultured under conditions 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.



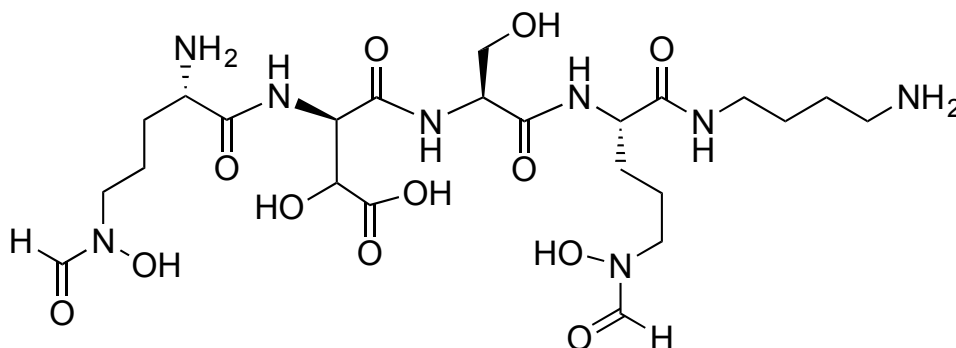
Individual domains of MbaA were overexpressed and purified. Biochemical characterization revealed the A domain selectivity as indicated below. ATP-PP_i exchange assays revealed that the non-proteinogenic amino acids L-Nδ-Nδ-formyl-ornithine and D-β-hydroxy-aspartate are activated by the A domains of 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?

3. (25 pts) The individual domains of MbaB were overexpressed in *E. coli* and purified as His₆-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**.

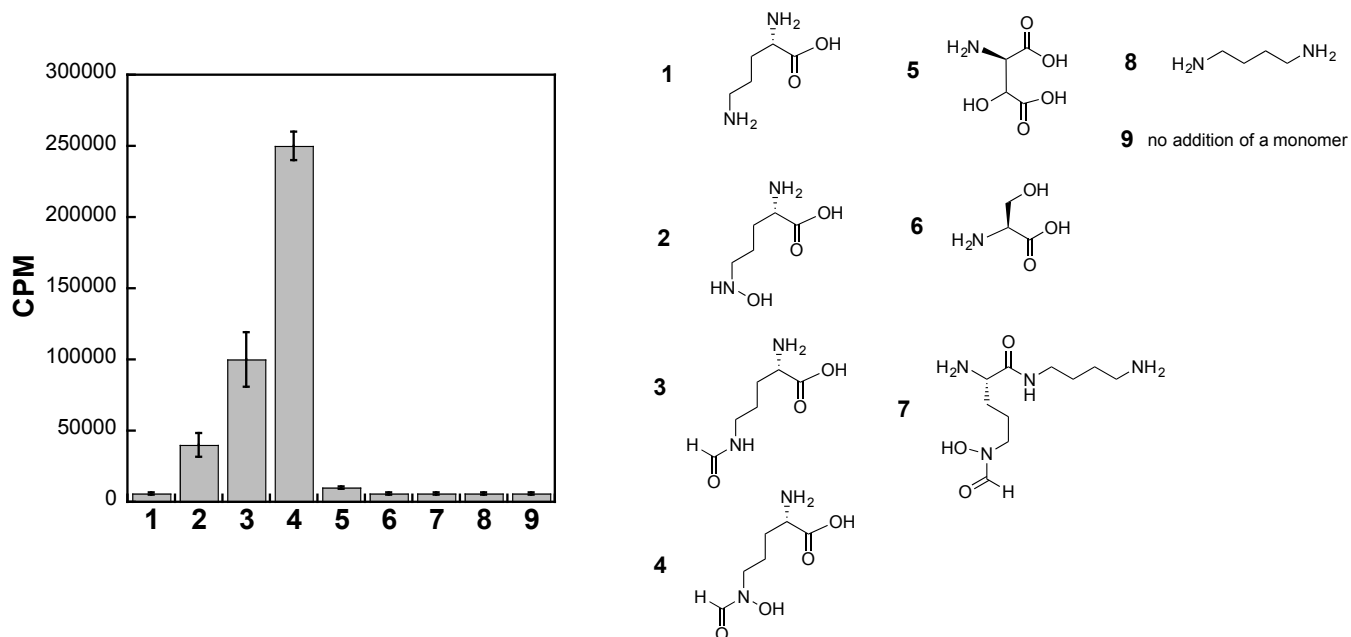


Figure 1. Results from ATP-PP_i exchange assays using His₆-MbaB and the substrates listed in the plot. The y-axis “cpm” (counts per minute) is a measure of ³²P incorporation into ATP. The reactions containing the A domain of MbaB, ATP, ³²PP_i and the substrate (indicated by number) were incubated in buffer and quenched all at the same time point.

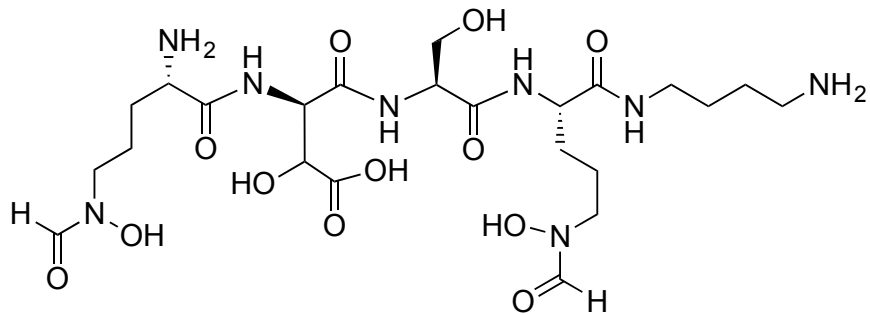
5.08 Spring 2016 Exam 2

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

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

(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.

(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?

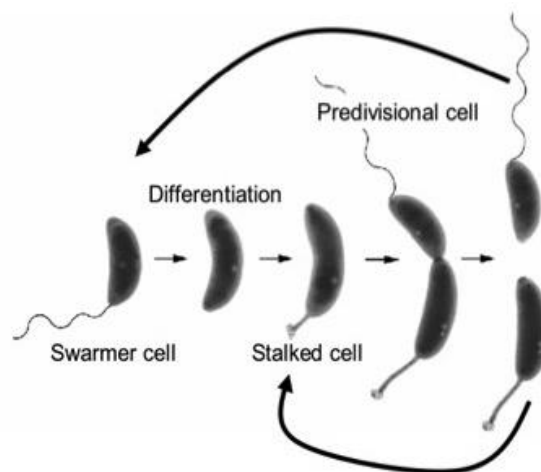
(vi) Why does nature use thioesters in NRPS assembly lines?

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 His₆-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.

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.

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*.

© [Kathleen Ryan](#), University of California, Berkeley. All rights reserved. This content is excluded from our Creative Commons license. For more information, see <https://ocw.mit.edu/help/faq-fair-use>.

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 $\Delta rcdA$ (cannot express functional RcdA) and $\Delta cpdR$ (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**.

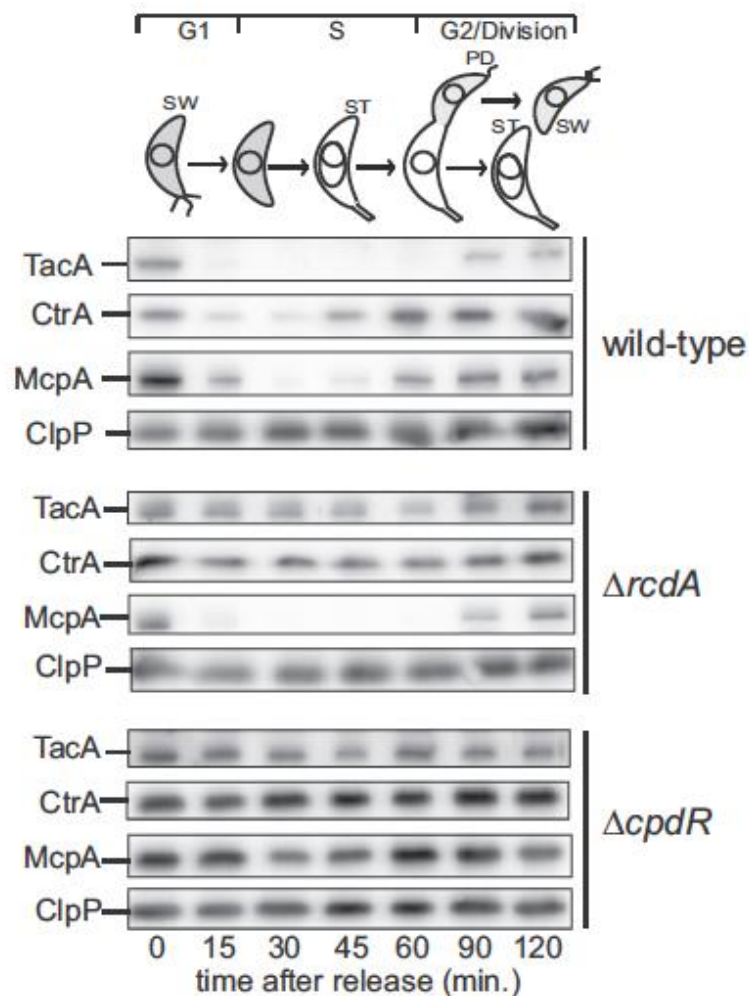
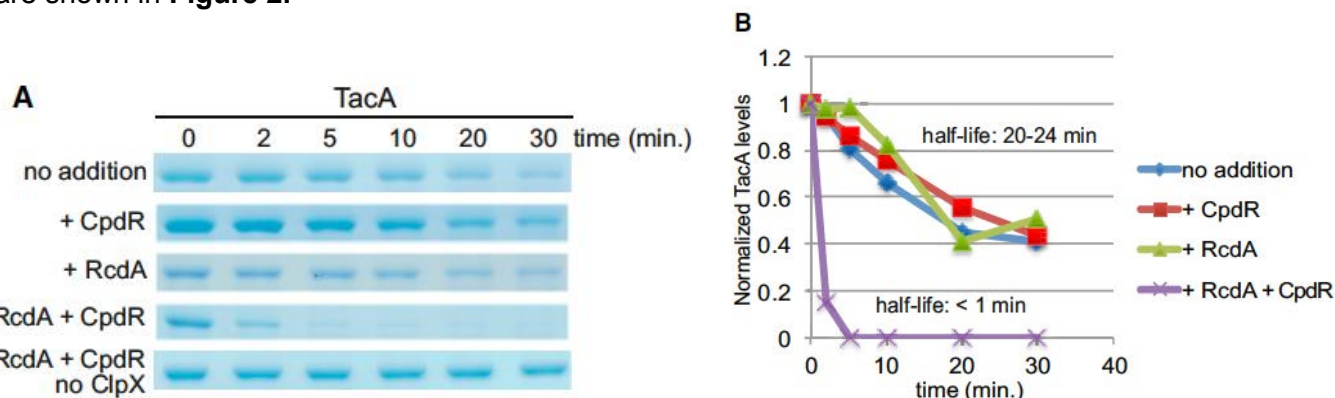


Figure 1. Cell-cycle dependent levels of the ClpXP substrates TacA, CtrA and McpA.

Abbreviations: SW = swarmer cell, ST = stalk cell, PD = predivisional 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**.



© Elsevier. K.K. Joshi, M. Bergé, et al. "An Adaptor Hierarchy Regulates Proteolysis during a Bacterial Cell Cycle." *Cell*. 2015 Oct 8; 163(2): 419–431. All rights reserved. This content is excluded from our Creative Commons license. For more information, see <https://ocw.mit.edu/help/faq-fair-use>.

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

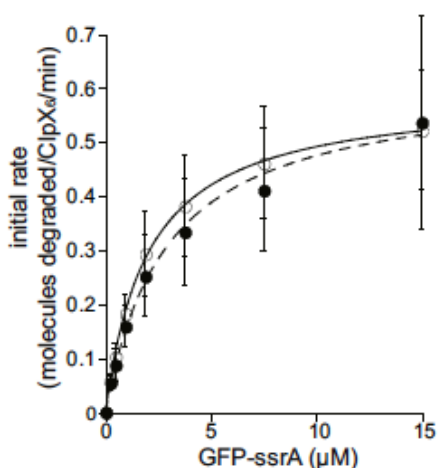
Figure 2. Degradation of TacA by ClpXP in the absence and presence of CpdR and/or RcdA.

(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 ClpX₆, 0.8 μM ClpP₁₄, 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 ClpX₆, 0.8 μM ClpP₁₄, 2 μM CpdR, 1 μM RcdA. An ATP regeneration system was included in this assay so ATP is not limiting.

© Elsevier. K.K. Joshi, M. Bergé, et al. "An Adaptor Hierarchy Regulates Proteolysis during a Bacterial Cell Cycle." *Cell*. 2015 Oct 8; 163(2): 419–431. All rights reserved. This content is excluded from our Creative Commons license. For more information, see <https://ocw.mit.edu/help/faq-fair-use>.

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 His₆-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**.

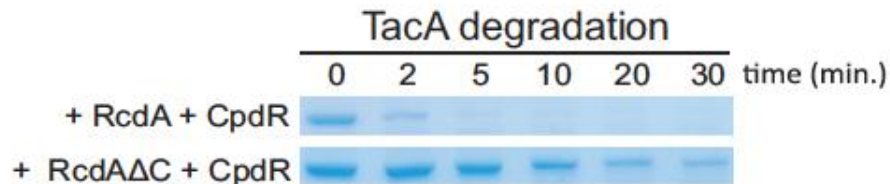


Figure 4. Degradation of TacA by ClpXP in the absence and presence RcdA or RcdA-ΔC. The bands in the SDS-PAGE gels are full-length TacA.

Concentrations employed: 1 μM TacA, 0.4 μM ClpX₆, 0.8 μM ClpP₁₄, 2 μM CpdR, 1 μM RcdA. An ATP regeneration system was included in this assay so ATP is not limiting.

© Elsevier. K.K. Joshi, M. Bergé, et al. "An Adaptor Hierarchy Regulates Proteolysis during a Bacterial Cell Cycle." *Cell*. 2015 Oct 8; 163(2): 419–431. All rights reserved. This content is excluded from our Creative Commons license. For more information, see <https://ocw.mit.edu/help/faq-fair-use>.

In **experiment #5**, they used His₆-tagged RcdA-ΔC in pull-down experiments to determine whether this variant binds TacA. His₆-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**.

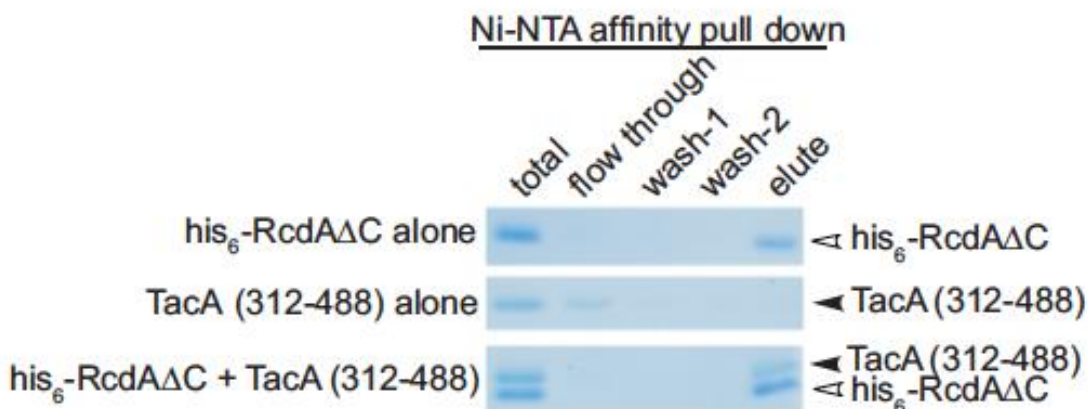


Figure 5. Assays to examine the interaction of TacA with RcdA-ΔC. The fractions were analyzed by SDS-PAGE and the identities of the bands are indicated on the right.

© Elsevier. K.K. Joshi, M. Bergé, et al. "An Adaptor Hierarchy Regulates Proteolysis during a Bacterial Cell Cycle." *Cell*. 2015 Oct 8; 163(2): 419–431. All rights reserved. This content is excluded from our Creative Commons license. For more information, see <https://ocw.mit.edu/help/faq-fair-use>.

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?

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*?

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

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

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

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

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

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**?

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

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.

MIT OpenCourseWare
<https://ocw.mit.edu>

5.08J Biological Chemistry II
Spring 2016

For information about citing these materials or our Terms of Use, visit: <https://ocw.mit.edu/terms>