The Role of ParA/MinD ATPases in the Spatial Regulation of Bacterial Organelles

by

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To those who value this, and to those who benefit from it.

- Isaac Spiegel
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# Table of Contents

DEDICATION .................................................................................................................. ii

ACKNOWLEDGEMENTS ............................................................................................... iii

LIST OF FIGURES ........................................................................................................ viii

LIST OF TABLES ........................................................................................................... x

LIST OF ABBREVIATIONS .......................................................................................... xi

ABSTRACT .................................................................................................................... xii

CHAPTER 1: Introduction ............................................................................................... 1

Introduction to ParA/MinD ATPases ............................................................................. 1

Mechanistic Foundation of ParA/MinD ATPases ......................................................... 3

The Role of ParA in Chromosome Segregation ......................................................... 4

The Role of MinD in Divisome Positioning ............................................................... 8

The Role of McdA in Carboxysome Positioning ...................................................... 12

The Role of FlhG in Regulating Flagella Number .................................................... 14

The Role of ParC in Regulating Chemotaxis Clusters ........................................... 18

Dissertation Goals .................................................................................................... 21

References .................................................................................................................. 24

CHAPTER 2: Multiple ParA/MinD ATPases Separately Position Disparate Cargos in a Bacterial Cell ......................................................................................... 28

Abstract ..................................................................................................................... 28

Introduction ............................................................................................................... 30

Results ......................................................................................................................... 32

- A third of sequenced bacteria encode for multiple ParA/MinD family ATPases .......... 32
- Gene neighborhood analysis of A/D ATPases in *H. neapolitanus* implicate cellular cargos .......................................................... 33
- The ParAB system is required for chromosome segregation in *H. neapolitanus* ........ 35
- The MinCDE system aligns cell division at mid-cell in *H. neapolitanus* ................. 37
- The A/D ATPase, McdA, encoded in the carboxysome operon positions carboxysomes .......................................................... 38
- *Hn0716* is required for regulating flagella number and positioning ..................... 39
- *Hn0722* is required for chemotaxis cluster assembly and positioning .................... 40
- Cargo positioning is not directly controlled by A/D ATPases encoded at distant loci ........................................................................ 41
- Deletion of *parA, minD*, or *flhG* results in anucleate cells via three different mechanisms .................................................................. 42
- Anucleate cells inherit carboxysomes ...................................................................... 43
- Deletion of *parA, minD* or *flhG* influence chemotaxis cluster assembly ............ 44
- A/D ATPases have unique interfaces that confer cargo-positioning specificity ....... 45
Discussion and Future Directions ................................................................. 47
  Encoding multiple A/D ATPases is a shared feature across prokaryotes ................. 48
  Complementation confirms the role of A/D ATPases in directly regulating a specific cellular cargo ................................................................. 49
  5 A/D ATPases, 5 Cargos, 1 Cell ................................................................. 51
  Coordinated cargo positioning throughout the cell cycle ........................................ 53
  Investigating the dynamics of A/D ATPases ..................................................... 54
  Partner proteins and specificity determinants linking an A/D ATPase to its cargo .......... 56
  Minimal Autonomous Positioning Systems ....................................................... 57

Summary .............................................................................................................. 58

References .......................................................................................................... 58

Figures ............................................................................................................... 61

Supplementary Figures ....................................................................................... 72

Supplementary Tables .......................................................................................... 88

CHAPTER 3: The McdAB System Positions α-carboxysomes in Proteobacteria .. 89

Abstract .............................................................................................................. 89

Introduction .......................................................................................................... 90

Results and Discussion ....................................................................................... 93
  McdA- and McdB-like proteins distribute carboxysomes in H. neapolitanus ............. 93
  Without the McdAB system, clustered carboxysomes are nucleoid excluded to the cell poles ................................................................. 97
  α-McdB is targeted to α-carboxysomes and interacts with α-McdA ......................... 100
  Defining the conserved features of α-McdAB proteins .......................................... 101
  α-McdAB systems are widespread among α-carboxysome-containing proteobacteria ................................................................. 102
  Cyanobacterial α-carboxysomes likely originated from a proteobacterium lacking α-mcdA within their cso operon ............................................. 105
  α-McdAB systems are distinct from the β-McdAB systems of cyanobacteria ........... 107
  All McdB types show LLPS activity, but with different minimal oligomeric units ...... 109
  McdAB systems are not restricted to carboxysome BMCs ..................................... 111

Future directions ................................................................................................. 112
  The McdAB system can redistribute carboxysome aggregates .............................. 112

Summary .............................................................................................................. 114

References .......................................................................................................... 114

Figures ............................................................................................................... 120

Supplementary Figures ....................................................................................... 129

CHAPTER 4: Coordinated Spatial Regulation of a Flagellum and Chemotaxis Cluster in the Bacterium Halothiobacillus neapolitanus .............................................. 135

Introduction .......................................................................................................... 135

Results ............................................................................................................... 137
  FlhG positions the de novo assembly of a single flagellum at the new pole of the aflagellate daughter cell immediately following division in H. neapolitanus ......................... 137
  ParC positions the de novo assembly a single chemotaxis cluster immediately proximal to the new pole of the daughter cell lacking a chemotaxis cluster in H. neapolitanus ......................... 138
  Flagella spatial organization by FlhG also influences chemotaxis cluster assembly and positioning ................................................................. 139
  The flagellum and chemotaxis cluster share the same cell pole, but are adjacent to one another. 139
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1-1</td>
<td>ParA/MinD ATPases share many mechanistic features</td>
<td>4</td>
</tr>
<tr>
<td>Figure 2-1</td>
<td>ParA/MinD-like ATPases are widespread in bacteria.</td>
<td>62</td>
</tr>
<tr>
<td>Figure 2-2</td>
<td>Each A/D ATPase in <em>H. neapolitanus</em> positions a specific cargo.</td>
<td>64</td>
</tr>
<tr>
<td>Figure 2-3</td>
<td>Cargo positioning is not directly controlled by A/D ATPases encoded at distant loci.</td>
<td>66</td>
</tr>
<tr>
<td>Figure 2-4</td>
<td>Anucleate cells form via three different mechanisms.</td>
<td>68</td>
</tr>
<tr>
<td>Figure 2-5</td>
<td>Anucleate cells inherit carboxysomes.</td>
<td>69</td>
</tr>
<tr>
<td>Figure 2-6</td>
<td>Deletion of <em>parA, minD, or flhG</em> influences chemotaxis cluster assembly.</td>
<td>70</td>
</tr>
<tr>
<td>Figure 2-7</td>
<td>A/D ATPases have unique interfaces that confer cargo-positioning specificity.</td>
<td>71</td>
</tr>
<tr>
<td>Figure S2-1</td>
<td>Flanking Genes (FlaGs) analysis shows conservation among A/D ATPase gene neighborhoods.</td>
<td>73</td>
</tr>
<tr>
<td>Figure S2-2</td>
<td><em>Hn2335</em> is required for chromosome segregation in <em>H. neapolitanus</em>.</td>
<td>75</td>
</tr>
<tr>
<td>Figure S2-3</td>
<td><em>Hn1364</em> is required for cell division positioning.</td>
<td>76</td>
</tr>
<tr>
<td>Figure S2-4</td>
<td>Carboxysome positioning is determined by McdA, the ParA/MinD-like ATPase encoded in the carboxysome operon.</td>
<td>77</td>
</tr>
<tr>
<td>Figure S2-5</td>
<td><em>Hn0716</em> is required for regulating flagella position and copy number.</td>
<td>78</td>
</tr>
<tr>
<td>Figure S2-6</td>
<td><em>Hn0722</em> is required for chemotaxis cluster positioning.</td>
<td>79</td>
</tr>
<tr>
<td>Figure S2-7</td>
<td>AlphaFold2 structural predictions for the A/D ATPases of <em>H. neapolitanus</em>.</td>
<td>80</td>
</tr>
<tr>
<td>Figure S2-8</td>
<td>Complementation confirms the role of A/D ATPases in regulating specific cellular cargos.</td>
<td>81</td>
</tr>
<tr>
<td>Figure S2-9</td>
<td>It remains unclear whether the conjugation operon in <em>H. neapolitanus</em> is active.</td>
<td>83</td>
</tr>
<tr>
<td>Figure S2-10</td>
<td>All five cargos can be labelled simultaneously.</td>
<td>85</td>
</tr>
<tr>
<td>Figure S2-11</td>
<td><em>mcdA</em> can be tagged in the internal loop.</td>
<td>86</td>
</tr>
<tr>
<td>Figure S2-12</td>
<td>N-terminal tag on MinD results in asymmetrical division.</td>
<td>87</td>
</tr>
<tr>
<td>Figure 3-1</td>
<td>Overview of α- and β-carboxysome composition, operon structure, and prevalence in proteobacteria.</td>
<td>120</td>
</tr>
<tr>
<td>Figure 3-2</td>
<td>An McdAB system positions α-carboxysomes.</td>
<td>121</td>
</tr>
<tr>
<td>Figure 3-3</td>
<td>Aggregated α-carboxysomes are nucleoid excluded.</td>
<td>122</td>
</tr>
<tr>
<td>Figure 3-4</td>
<td>α-McdB loads onto α-carboxysomes and interacts with α-McdA.</td>
<td>124</td>
</tr>
<tr>
<td>Figure 3-5</td>
<td>Conserved features among α-McdAB proteins within cso operons.</td>
<td>125</td>
</tr>
<tr>
<td>Figure 3-6</td>
<td>McdAB systems are widespread among carboxysome-containing proteobacteria.</td>
<td>126</td>
</tr>
<tr>
<td>Figure 3-7</td>
<td>α-carboxysome evolution among proteobacteria and α-cyanobacteria.</td>
<td>127</td>
</tr>
<tr>
<td>Figure 3-8</td>
<td>Similarities and differences among all known McdA and McdB proteins.</td>
<td>128</td>
</tr>
<tr>
<td>Figure S3-1</td>
<td>Aggregation of α-carboxysomes caused a local cell bulging morphology in the absence of α-McdA or α-McdB.</td>
<td>129</td>
</tr>
<tr>
<td>Figure S3-2</td>
<td>WT and ΔmcdB cells treated with cephalaxin caused cell elongation, but no significant increase in cytoplasmic space due to added chromosomal content.</td>
<td>130</td>
</tr>
<tr>
<td>Figure S3-3</td>
<td>MSA of identified α-McdA sequences from proteobacteria.</td>
<td>131</td>
</tr>
<tr>
<td>Figure S3-4</td>
<td>MSA of identified α-McdB sequences from proteobacteria.</td>
<td>132</td>
</tr>
<tr>
<td>Figure S3-5</td>
<td>Carboxysome redistribution on the nucleoid is dependent on McdAB.</td>
<td>134</td>
</tr>
<tr>
<td>Figure 4-1</td>
<td>A single flagellum forms at the new pole of the daughter cell lacking a flagellum.</td>
<td>145</td>
</tr>
<tr>
<td>Figure 4-2</td>
<td>A chemotaxis cluster forms at the new pole of the daughter cell lacking a chemotaxis cluster.</td>
<td>146</td>
</tr>
<tr>
<td>Figure 4-3</td>
<td>Flagella positioning influences the spatial regulation of chemotaxis clusters.</td>
<td>147</td>
</tr>
<tr>
<td>Figure 4-4</td>
<td>The flagellum and chemotaxis cluster share the same cell pole and are immediately adjacent to one another.</td>
<td>148</td>
</tr>
</tbody>
</table>
Table 1-1  Previously investigated ParA/MinD ATPases in bacteria  2
Table 1-2  The Par system is critical for proper chromosome segregation and inheritance.  5
Table 1-3a The Min system drives positioning of the divisome at mid-cell  10
Table 1-3b Alternative A/D ATPase systems for positioning the divisome  11
Table 1-4 McdA controls carboxysome positioning and foci number  13
Table 1-5 FlhG typically affects flagella number and motility without affecting flagella localization  16
Table 1-6 ParC operon typically affects chemotaxis cluster positioning and foci number  20
Table S2-1 ParA/MinD family ATPase hits across the bacterial domain.  88
Table S2-2 Amino acids comprising the interfaces of ParA/MinD family ATPases in *H. neapolitanus* predicted to be important specificity determinants  88
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δgene#</td>
<td>Knock-out or deletion of indicated gene#</td>
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<tr>
<td>A/D ATPase</td>
<td>ParA/MinD ATPase</td>
</tr>
<tr>
<td>AF2</td>
<td>AlphaFold2</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BMC</td>
<td>Bacterial microcompartment</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>CbbS</td>
<td>Ribulose bisphosphate carboxylase, small subunit</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>FlaGs</td>
<td>Flanking genes</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>GNA</td>
<td>Gene neighborhood analysis</td>
</tr>
<tr>
<td>ICE</td>
<td>Integrative conjugative element</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase pairs</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
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<tr>
<td>LLPS</td>
<td>Liquid-liquid phase separation</td>
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<tr>
<td>M</td>
<td>Molar</td>
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<tr>
<td>mL</td>
<td>Milliliter</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mNG</td>
<td>Monomeric NeonGreen</td>
</tr>
<tr>
<td>MSA</td>
<td>Multiple sequence alignment</td>
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<tr>
<td>mTq</td>
<td>Monomeric Turquoise2</td>
</tr>
<tr>
<td>MTS</td>
<td>Membrane targeting sequences</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCC</td>
<td>Pearson correlation coefficient</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>SEC-MALS</td>
<td>SizeExclusion Chromatography–Multiple Angle Laser Light Scattering</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
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</table>
ABSTRACT

In eukaryotes, linear motor proteins govern intracellular transport and organization. In bacteria, where linear motors are absent, the ParA/MinD (A/D) family of ATPases spatially organize an array of genetic- and protein-based cellular cargos. ParA is well known to segregate plasmids and chromosomes, as is MinD for its role in divisome positioning. Less studied is the growing list of ParA/MinD-like ATPases found across prokaryotes and involved in the spatial organization of diverse protein-based organelles, such as Bacterial Microcompartments (BMCs), flagella, chemotaxis clusters, and conjugation machinery. Given the fundamental nature of these processes in both cell survival and pathogenesis, it is unsurprising that the positioning of these cargos has been independently investigated to varying degrees in several organisms. However, it remains unknown whether multiple A/D ATPases can coexist and coordinate the positioning of such a diverse set of fundamental cargos in the same cell. If so, what are the mechanistic commonalities, variation, and specificity determinants that govern the positioning reaction for each cargo? Here, we find that over a third of sequenced bacteria encode multiple A/D ATPases. Among these bacteria, we identified several human pathogens as well as the experimentally tractable organism, *Halothiobacillus neapolitanus*, which encodes seven A/D ATPases. We directly demonstrate that five of these A/D ATPases are each dedicated to the spatial regulation of a single cellular cargo: the chromosome, the divisome, the carboxysome BMC, the flagellum, and the
chemotaxis cluster. We identify putative specificity determinants that allow each A/D ATPase to position its respective cargo. Finally, we show how the deletion of one A/D ATPase can have indirect effects on the inheritance of a cargo actively positioned by another A/D ATPase, stressing the importance of understanding how organelle trafficking, chromosome segregation, and cell division are coordinated in bacterial cells. Together, our data show how multiple A/D ATPases coexist and function to position a diverse set of fundamental cargos in the same bacterial cell.
CHAPTER 1: Introduction

Introduction to ParA/MinD ATPases

In bacteria, a widespread family of ParA/MinD (A/D) ATPases spatially organize plasmids, chromosomes, and an array of protein-based organelles, many of which are fundamental to cell survival and pathogenesis. By far the two best studied ATPases, and family namesake, are ParA involved in plasmid partition and chromosome segregation\(^1\)–\(^{17}\), and MinD involved in divisome positioning\(^18\)–\(^{27}\). Less studied is the growing list of A/D ATPases, widespread across prokaryotes, involved in the spatial regulation of diverse protein-based organelles, such as Bacterial Microcompartments (BMCs)\(^{28}\)–\(^{30}\), flagella\(^{31}\)–\(^{43}\), chemotaxis clusters\(^{44}\)–\(^{47}\), and conjugation machinery\(^{48}\) (Table 1-1). Mispositioning of these cargos have been found to result in decreased survival, motility, growth rate, and overall cell fitness, stressing the importance of understanding the mechanistic details that underlie each system.

Given the fundamental nature of these cargos to cell survival and pathogenesis, it is unsurprising that these systems have been independently investigated to varying degrees in several prokaryotes. However, it remains to be understood just how many A/D ATPase positioning systems can coexist in a single organism, how bacteria
spatiotemporally coordinate the positioning of such a diverse set of fundamental cargos in the same cell, or what unique specificity determinants link each A/D ATPase to its respective cargo type. This gap in knowledge largely exists because A/D-based positioning systems are typically studied independently of one another and in bacteria with few A/D ATPases (Table 1-1).

**Table 1-1: Previously investigated ParA/MinD ATPases in bacteria.** ParA/MinD ATPases have been shown to be important for cargo localization in a number of bacteria.
In the following sections, I summarize mechanistic commonality shared among all A/D ATPases, I explore mechanistic aspects specific to each A/D ATPase, and then I discuss the gaps in our knowledge of these positioning ATPases that my dissertation research fills.

Mechanistic Foundation of ParA/MinD ATPases

Despite the diversity of cargo, A/D ATPases share several structural and mechanistic similarities. The hallmark of the A/D ATPase family is a ‘deviant’ Walker A motif (KGGxxK[S/T]) (Figure 1-1A), which is required for ATP binding and hydrolysis49,50. Upon ATP-binding, ParA and MinD proteins form ATP-sandwich homodimers51–53 (Figure 1-1B). ParA dimerization forms a positively-charged interface for non-specific DNA binding54,55, while MinD dimerization aligns two membrane targeting sequences (MTS) for membrane binding56,57. Dimerization also forms a binding site for an ATPase-activating partner protein52,58. Thus, despite the cargos being so diverse, A/D ATPases share several features: (i) all form ATP-sandwich dimers, (ii) dimerization forms an interface for binding a positioning matrix, and (iii) dimerization also forms a binding site for a cognate partner protein that connects an ATPase to its cargo and stimulates its release from the positioning matrix.

Although A/D ATPases share several key mechanistic similarities, the phenotypes associated with each A/D ATPase type varies by cargo. In the next five sections, I will summarize the currently known roles of A/D ATPases in the spatial regulation of five
different cargos: chromosome, divisome, carboxysome, flagellum, and chemotaxis cluster. These five cargos and their A/D ATPases are the subject of my thesis work. As I move through each cargo section, I will emphasize the mechanistic similarities and differences associated with each A/D ATPase. My goal is to introduce you to the broad field of A/D ATPase-mediated spatial regulation and organelle positioning in bacteria before I address some of the gaps in knowledge with my thesis work. To start, ParA is one of the best-studied A/D ATPases, responsible for chromosome and plasmid segregation.

The Role of ParA in Chromosome Segregation

Chromosome segregation is driven by the tripartite system consisting of ParA, ParB and parS. ParB loads onto a centromere-like site, called parS, to form a massive complex on the chromosome near the origin of replication (OriC)$^2$. ParA interacts with this ParB-parS complex to facilitate the proper positioning of the replicated origins into

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Figure 1-1: ParA/MinD ATPases share many mechanistic features. (A) ParA/MinD ATPases are part of the P-loop superfamily of ATPases. A/D ATPases are distinguished from the superfamily by a signature N-terminal lysine (red). (B) ParA/MinD ATPases bind ATP and dimerize. This changes the conformation to allow for surface interaction. Interaction with the cargo and partner protein stimulates ATP hydrolysis and release of the ATPase from the surface. (Red = A/D ATPase, Yellow = ATP, Blue = cargo and partner protein.)
future daughter cells, either at the cell poles (as in *Caulobacter crescentus*, *Vibrio cholerae* Chromosome I, and *Corynebacterium glutamicum*) or near the cell center (as in *Bacillus subtilis* and *Vibrio cholerae* Chromosome II). Without ParABS, DNA is asymmetrically inherited, resulting in anucleate and polyploid cells, and reduced cell fitness or death\(^{49,50}\). In some organisms, such as *C. crescentus* and *Mycobacterium tuberculosis*, the Par system is essential and deletion mutants could not be obtained. In the following table, I summarize the phenotypes of *parA* mutations in different organisms:

<table>
<thead>
<tr>
<th>Organism</th>
<th>Wild-type positioning</th>
<th>Mutant positioning</th>
<th>Phenotype summary:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacillus subtilis</strong> (^{59})</td>
<td><img src="image1" alt="Wild-type" /></td>
<td><img src="image2" alt="Mutant" /></td>
<td>WT: During vegetative growth, origins are localized near the center of the nucleoid (shown). During sporulation, origins are localized to the polar edges of the nucleoid (not shown). &lt;br&gt;(\Delta soj): &lt;br&gt;- Anucleate cells &lt;br&gt;- Mispositioning and missegregation of the origin</td>
</tr>
<tr>
<td><strong>Caulobacter crescentus</strong> (^{4,5})</td>
<td><img src="image3" alt="Wild-type" /></td>
<td><img src="image4" alt="Mutant" /></td>
<td>WT: unipolar ParB focus in young cells. As the cells prepare to divide, ParB foci become bipolar. &lt;br&gt;ParA over-expression: &lt;br&gt;- Anucleate cells &lt;br&gt;- Elongated cells &lt;br&gt;- Less cells with division sites &lt;br&gt;- ParB foci are diffuse</td>
</tr>
<tr>
<td><strong>Corynebacterium glutamicum</strong> (^{6})</td>
<td><img src="image5" alt="Wild-type" /></td>
<td><img src="image6" alt="Mutant" /></td>
<td>WT: unipolar ParB focus in young cells. As the cells prepare to divide, ParB foci become bipolar. &lt;br&gt;(\Delta parA): &lt;br&gt;- Anucleate cells &lt;br&gt;- Mispositioning of ParB foci &lt;br&gt;- Slower growth &lt;br&gt;- Larger variation in cell length</td>
</tr>
</tbody>
</table>

Red: DNA stain (DAPI)  <br>Green: Origin (TetR-CFP)  <br>Yellow arrow: Central origin  <br>White arrow: Polar origin  <br>Blue: DNA stain (DAPI)  <br>White arrow: anucleate cell  <br>Red: membrane stain
<table>
<thead>
<tr>
<th>Organism</th>
<th>WT: most cells had a single ParB focus at ~37% of the cell length. Cells with two foci had them at the quarter positions.</th>
<th>WT: most cells had two ParB foci, each near the quarters of the cell. Cells with a single focus had them asymmetrically positioned at ~41% of the cell length.</th>
<th>( \Delta \text{parA} ):</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>ParAB are essential for growth</td>
<td></td>
<td>- Anucleate cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Slower growth</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Larger variation in cell length</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Mispositioning of the ori</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- More likely to have more or less than two ParB foci/cell</td>
</tr>
<tr>
<td><em>Mycolicibacterium smegmatis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
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<tr>
<td><em>Pseudomonas putida</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces coelicor</em></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 1-2: The Par system is critical for proper chromosome segregation and inheritance.

**Bacillus subtilis**
- Accession #: WP_003219244.1; During vegetative growth, origins are localized near the center of the nucleoid. Deletion of soj resulted in anucleate cells and mispositioning of the origin.
- **Caulobacter crescentus**
- Accession #: YP_002519242.1; parA deletion is lethal. ParA over-expression resulted in anucleate cells, elongated cells, less cells with division sites, and diffuse ParB foci.
- **Corynebacterium glutamicum**
- Accession #: WP_003855308.1; In young cells, ParB foci were localized to a single pole. As the cells prepare to divide, ParB foci become bipolar. Deletion of parA resulted in greater variation in cell length, anucleate cells, mispositioning of ParB foci, and changes to the number of ParB foci/cell.
- **Mycobacterium tuberculosis**
- Accession #: WP_003400180.1; Most cells had a single ParB focus at ~37% of the cell length. Cells with two foci had them at the quarter positions. ParA is essential for growth and cannot be deleted.
- **Mycolicibacterium smegmatis**
- Accession #: WP_011731642.1; Most cells had two ParB foci, each near the quarters of the cell. Cells with a single focus had them asymmetrically positioned at ~41% of the cell length. Deletion of parA resulted in anucleate cells, slower growth, larger variation in cell length, mispositioning of the origin, and changes to the number of ParB foci/cell.
- **Pseudomonas aeruginosa**
- Accession #: NP_254250.1; Cells had a single ParB focus at mid-cell or two foci at the quarter positions. Deletion of parA resulted in mispositioning of the origin, anucleate cells, and slower growth.
- **Pseudomonas putida**
- Accession #: WP_003253182.1; Chromosomes are properly segregated into daughter cells. Deletion of parA resulted in anucleate cells and slowed growth.
- **Streptomyces coelicor**
- Accession #: WP_003975045.1; Chromosomes are segregated into uniform compartments. Deletion of parA resulted in mis-segregation of chromosomes into non-uniform compartments.

**Vibrio cholerae**
- Accession #: WP_000516546.1; Vibrio cholerae has 2 chromosomes. For chromosome 1, young cells had a single unipolar focus. As the cells prepared to divide, ParB foci became bipolar. Deletion of parA resulted in mispositioning of ParB foci and filamentous cells.
- **Vibrio cholerae ChrI**
- Accession #: WP_000817997.1; Vibrio cholerae has 2 chromosomes. For chromosome 2, cells normally had two foci, each near the quarters of the cell. Deletion of parA resulted in mispositioning of parB foci, loss of chromosome 2, inviable CHUB cells, and slowed growth. Images are reprinted with permission.

Despite all that we know regarding the spatial organization of chromosomes across a variety of bacteria, many questions remain regarding how chromosome segregation is coordinated with other fundamental processes. For example, as I show here in this thesis, many bacteria encode multiple A/D based positioning systems. What are the
specificity determinants that allow the chromosome to be specifically positioned by ParA? How is chromosome segregation coordinated with organelle trafficking, especially when many of these organelles use the nucleoid as a positioning matrix? It has been difficult to investigate this coordination because there is little overlap in the organisms used for studying the chromosome, the divisome, and the protein-based organelles shown to require an A/D ATPase for positioning. In the next section, we will discuss the other well-studied A/D ATPase, MinD, and its role in positioning the divisome.

The Role of MinD in Divisome Positioning

Many bacteria divide by binary fission using a complex called the divisome. Proper positioning of the divisome at mid-cell ensures that when a cell divides, both daughter cells are roughly equal in length. The divisome is composed of dozens of proteins, but the main structural element is an ancestral tubulin homolog, FtsZ. FtsZ assembles into a complex called the Z-ring, which serves as a scaffold for downstream divisome proteins. In many bacteria, Z-ring formation at mid-cell is directed by the Min system. Removal of the Min system typically results in aberrant divisions at the cell pole, producing anucleate “minicells” and filamentous cells. Thus, proper positioning of the divisome is critical to cell survival. The Min system has been largely studied in *E. coli* and *B. subtilis*, but more recently has been investigated in other organisms. Below, I summarize the salient similarities and differences among Min systems (Table 1-3a).

In *E. coli* 19-21, before the advent of modern genetic tools, researchers discovered a strain that produced many miniature anucleate cells19. Two decades later, it was
determined that the mutated locus encodes for three proteins: MinC, MinD, and MinE\textsuperscript{20}. MinD is an ATPase in the ParA/MinD family. MinD binds the membrane and recruits MinC, which inhibits Z-ring formation. MinE stimulates MinD ATPase activity and release from the membrane\textsuperscript{52,60}, resulting in MinCD oscillations that ultimately inhibit division everywhere but at mid-cell\textsuperscript{61}. Without the Min system, division occurs at any nucleoid-free region\textsuperscript{62}, producing anucleate mini-cells, the products of polar divisions. The Min system has since been discovered to serve similar roles in other organisms.

\textit{B. subtilis}\textsuperscript{22–24} also has MinCD, but does not have MinE. Instead, a protein called DivIVA functions in an analogous role. DivIVA localizes to the poles and anchors MinCD. Therefore, the MinCD system in \textit{B. subtilis} does not oscillate like the MinCD system found in \textit{E. coli}. Despite being mechanistically different, the systems in \textit{E. coli} and \textit{B. subtilis} serve similar functions. Without this system, division septa in \textit{B. subtilis} are also irregularly positioned, resulting in anucleate mini-cells. Min systems investigated in other model organisms share many features with the Min systems found in \textit{E. coli} and \textit{B. subtilis}, but each system has distinct characteristics.

\textit{Vibrio cholerae}\textsuperscript{25}, \textit{Helicobacter pylori}\textsuperscript{26}, and \textit{Synechococcus elongatus}\textsuperscript{27} have Min systems that are homologous to that of \textit{E. coli} and/or \textit{B. subtilis}. However, the mechanistic and/or phenotypic details vary in all cases. In \textit{V. cholerae}, MinCD oscillates from pole-to-pole, like \textit{E. coli}, but loss of MinCD does not result in a division defect unless the chromosome is mispositioned. In \textit{H. pylori}, Min dynamics have not yet been investigated, but asymmetric positioning of the divisome resulted in filamentous cells without anucleate mini-cells. In \textit{S. elongatus}, both MinE and a DivIVA-like protein, called CdV3, are necessary and do not serve redundant roles. Although all five Min systems
discussed thus far are homologous, the mechanistic and phenotypic differences emphasize the importance of investigating how A/D ATPases function in diverse organisms.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Wild-type positioning</th>
<th>Mutant positioning</th>
<th>Phenotype summary:</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
<td><strong>WT</strong>: mid-cell division</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>ΔminD</strong>:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Asymmetric division</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Mini-cells</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td></td>
<td></td>
<td><strong>WT</strong>: mid-cell division</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>ΔminD</strong>:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Asymmetric division</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Mini-cells</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td></td>
<td></td>
<td><strong>WT</strong>: Z-ring at mid-cell.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>ΔminD</strong>:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- No changes to localization of Z-ring</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- No cell defects EXCEPT when chromosome arrangement is perturbed.</td>
</tr>
<tr>
<td><em>Helicobacter pylori</em></td>
<td></td>
<td></td>
<td><strong>WT</strong>: mid-cell division</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>ΔminD</strong>:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Asymmetrical division</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Filamentous cells, but no anucleate mini-cells</td>
</tr>
<tr>
<td><em>Synechococcus elongatus</em></td>
<td></td>
<td></td>
<td><strong>WT</strong>: Z-rings at mid-cell, mid-cell division</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>ΔminD</strong>:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- FtsZ is mispositioned</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Asymmetric division</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Mini-cells</td>
</tr>
</tbody>
</table>

Table 1-3a: The Min system drives positioning of the divisome at mid-cell. *Escherichia coli*: Accession # NP_415693.1; In WT, cells divide at mid-cell. In ΔminD, cells divide asymmetrically, resulting in mini-cells. *Bacillus subtilis*: Accession # WP_004398624.1; In WT, cells divide at mid-cell. In ΔminD, cells divide asymmetrically, resulting in mini-cells. *Vibrio cholerae*: Accession # WP_000086775.1; FtsZ (red) localized at the pole in young cells and at mid-cell when a cell prepares to divide. ΔminCD barely altered the localization of ftsZ. *Helicobacter pylori*: Accession # WP_001019069.1; In WT, cells divide at mid-cell. In ΔminD, cells divide asymmetrically, resulting in filamentous cells but not anucleate mini-cells. *Synechococcus elongatus*: Accession # WP_011242956.1; WT cells
(red) typically position FtsZ (white) at mid-cell resulting in mid-cell divisions; In $\Delta\text{minD}$, ftsZ was mispositioned and cell sizes were more variable. Images are reprinted with permission.

Despite the prevalence of Min systems, some organisms like *Caulobacter crescentus*\(^63\) and *Myxococcus xanthus*\(^64,65\) have alternate A/D-based systems for divisome positioning (Table 1-3b). The mechanistic details of these systems vary substantially from Min systems. Briefly, in *C. crescentus*, MipZ (an A/D ATPase) localizes to the poles via interaction with ParB and inhibits Z-ring formation by directly interacting with FtsZ. In *M. xanthus*, PomZ (an A/D ATPase) accumulates at mid-cell and functions to positively regulate Z-ring formation. The evolution of diverse analogous systems to serve the same function stresses the importance of proper divisome positioning.

<table>
<thead>
<tr>
<th>Alternative A/D ATPase systems for positioning the divisome (not MinD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Organism</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
</tbody>
</table>
| *Caulobacter crescentus*\(^63\) | ![Wild-type positioning](image) | ![Mutant positioning](image) | **WT**: division occurs near mid-cell to produce a slightly larger stalk cell and slightly smaller swarmer cell  
**MipZ depletion:**  
- Mispositioned Z-ring  
- Elongated stalk cells and mini swarmer cells |
| *Myxococcus xanthus*\(^64,65\) | ![Wild-type positioning](image) | ![Mutant positioning](image) | **WT**: constriction sites at mid-cell in longer cells but not shorter cells  
**$\Delta\text{pomZ}$:**  
- Cells are less likely to form a Z-ring.  
- Z-ring formation happens in cells of all lengths.  
- Mini-cells and filamentous cells |

Table 1-3b: Alternative A/D ATPase systems for positioning the divisome. *Caulobacter crescentus*: Accession # YP_002517619.1; MipZ depletion results in aberrant division patterns with elongated stalk cells and mini swarmer cells. *Myxococcus xanthus*: Accession # WP_026113902.1; In WT, constriction sites are located at mid-cell. In $\Delta\text{pomZ}$, very few cells formed a Z-ring. When there was a ring, it was found along the entire cell length in cells of varying length. These division defects resulted in mini-cells and filamentous cells. Images are reprinted with permission.
Despite all that we know regarding the spatial organization of divisomes across a variety of bacteria, many questions remain regarding how division is coordinated with other fundamental processes in bacteria. For example, as I show here in this thesis, many bacteria encode multiple A/D based positioning systems. What are the specificity determinants that allow the divisome to be specifically positioned by MinD? How is the organization of other fundamental processes influenced by the positioning and licensing of cell division? How is cell division positioning coordinated with chromosome segregation and organelle trafficking? It has been difficult to investigate this coordination because there is little overlap in the organisms used for studying the divisome, the chromosome, and the protein-based organelles shown to require an A/D ATPase for positioning. In the next three sections, I describe the A/D ATPases involved in organelle trafficking in bacteria, starting with arguably the most important organelle on Earth called the carboxysome.

The Role of McdA in Carboxysome Positioning

Bacterial microcompartments, or BMCs, are large icosahedral protein-based organelles that encapsulate sensitive metabolic reactions to provide microbes with distinct growth advantages. BMCs are in 29 bacterial phyla and ~20% of sequenced bacterial genomes. Since BMCs play critical roles in carbon fixation, carbon source utilization, and pathogenesis, their functions are of great ecological, biotechnological, and medical interest. Despite their importance, little is known about how BMCs are spatially regulated in the cell.
The model BMC is the carbon-fixing carboxysome found in photosynthetic cyanobacteria and some chemoautotrophic proteobacteria\textsuperscript{29,68}. Carboxysomes encapsulate the enzyme Rubisco with its CO\textsubscript{2} substrate, significantly increasing enzymatic efficiency. It was recently found that an A/D ATPase, termed Maintenance of carboxysome distribution protein A (McdA), is widespread in cyanobacteria and required for spacing carboxysomes on the nucleoid along with its partner protein McdB\textsuperscript{28,30} (Table 1-4). In the absence of the McdAB system, carboxysomes in the cyanobacterium \textit{Synechococcus elongatus} are no longer positioned on the nucleoid; instead, carboxysomes aggregate and form clusters of carboxysomes within nucleoid-free regions of the cell\textsuperscript{28}. Carboxysome clustering resulted in slowed growth rate, cell elongation, and asymmetric cell division\textsuperscript{69}, stressing the importance of carboxysome positioning to cell fitness.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Wild-type positioning</th>
<th>Mutant positioning</th>
<th>Phenotype summary:</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Synechococcus elongatus} \textsuperscript{28,30}</td>
<td>![Image]</td>
<td>![Image]</td>
<td><strong>WT</strong>: many carboxysomes positioned along the length of the nucleoid.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>\textbf{\Delta mcdA}: large carboxysome aggregates that are nucleoid-excluded.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- TEM confirms that carboxysome aggregates are large clusters of assembled carboxysomes.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Carboxysome clustering resulted in slower growth rate.</td>
</tr>
</tbody>
</table>

\textbf{Table 1-4: McdA controls carboxysome positioning and foci number.} \textit{Synechococcus elongatus}: Accession \textit{WP_011244571.1}; \textbf{WT} have many carboxysomes. \textbf{\Delta mcdA} cells have large carboxysome foci. TEM confirms that carboxysome aggregates are large clusters of assembled carboxysomes. Images are reprinted with permission.

The carboxysome positioning system has only been investigated in the cyanobacterium \textit{S. elongatus} and many questions remain to be answered. Is the McdAB system conserved in proteobacteria that encode for carboxysomes that are both
structurally and phyletically distinct from that of cyanobacteria? If so, how similar are these systems to each other? Given that some organisms have multiple A/D based positioning systems, what are the specificity determinants that link McdB-bound carboxysomes to McdA? Since carboxysomes are positioned on the nucleoid, how does carboxysome positioning coordinate with the process of DNA segregation? Given that \textit{S. elongatus} has multiple chromosome copies, it is challenging to explore this coordination in this cyanobacterium. In my thesis, we identify a model capable of addressing all these gaps in knowledge.

The Role of FlhG in Regulating Flagella Number

Flagella are external filamentous structures that allow a bacterium to be motile. Bacteria vary in flagella location, number, and pattern. Although flagellation pattern can vary dramatically between organisms, it is nonetheless a highly regulated process that is conserved across generations. Defects in flagellation pattern can result in detrimental changes to motility. Motility is critical to chemotaxis, biofilm formation, pathogenesis, and colonization. Therefore, a fundamental understanding of flagella regulation is also of great medical interest.

Some flagellation patterns occur stochastically\textsuperscript{70}, while others rely on cell-pole landmarks\textsuperscript{71,72}; moreover, many bacteria encode for an A/D ATPase (called FlhG, FleN, MotR, or YlxH) in their flagellar operon\textsuperscript{31–43}. Although the role of \textit{flhG} is not yet fully understood, deletion of \textit{flhG} typically results in an increase in flagella number with a corresponding decrease in motility (\textit{Table 1-5}). There are several exceptions: 1) In \textit{Bacillus subtilis}, flagella bundled together in tufts and motility was unaffected\textsuperscript{31}. 2) In
Helicobacter pylori, flagella were lost\textsuperscript{34}. 3) In the unusual case of Campylobacter jejuni, cell division defects have been observed, in addition to the typical changes to flagella number and motility\textsuperscript{32}.

The mechanism behind FlhG-based flagellar regulation has not yet been elucidated. In earlier studies, it was noted that deletion of FlhG resulted in hyper-flagellation\textsuperscript{36}, leading to the hypothesis that FlhG is responsible for regulating flagellar number. Meanwhile, it was noted that interruption of FlhF, a GTP-binding protein located immediately upstream of FlhG, resulted in the lack of flagella or the random localization of flagella\textsuperscript{73}, suggesting that FlhF is responsible for regulating flagellar production and location. Interestingly, FlhF overexpression resulted in hyper-flagellation at the pole\textsuperscript{40,73}, similar to an FlhG deletion, suggesting that a fine balance between FlhF and FlhG is what determines flagella location and number. This hypothesis is supported by the observation that deletion of FlhF and FlhG resulted in positive and negative changes to the transcriptional activity of flagellar genes, suggesting that FlhF positively regulates transcription while FlhG negatively regulates transcription\textsuperscript{42}.

More recent studies show that FlhF and FlhG interact. FlhF was found to localize to the poles. The model is one where FlhF localizes to the pole to determine location and production of flagella\textsuperscript{41}, while FlhG interacts with FlhF as a negative regulator. In this model, more FlhG (due to FlhF deletion or FlhG overexpression) would result in the lack of FlhF localization to the pole, and thus reduced flagella. More FlhF (due to FlhG deletion or FlhF overexpression), on the other hand, would result in more FlhF at the pole and thus hyper-flagellation. However, there does not appear to be a universal mechanism for FlhF and FlhG.
The above observations were made in polar flagellates of *Pseudomonas* and *Vibrio* species. However, it has been suggested that the different FlhG-based mechanisms can also contribute to various other flagellation patterns. Compared to the polar flagellates discussed above, there are subtle differences in the roles of *flhF* and *flhG* in the peritrichous organism, *B. subtilis*. Deletion of *flhG* did not result in an increase in flagellar number, but instead resulted in an aggregation of flagellar bodies. Additionally, deletion of *flhF* resulted in mislocalized flagellar basal bodies that concentrated at the poles, as opposed to the sides of wild-type cells. This change in localization, however, did not seem to affect swimming or swarming. Like polar flagellates, the stoichiometric ratio between FlhF and FlhG is important as a mutant overexpressing FlhF resembled a ∆*flhG* mutant and overexpression of both proteins resembled WT. It seems as though FlhG antagonizes FlhF, but the mechanism remains unclear.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Wild-type positioning</th>
<th>Mutant positioning</th>
<th>Phenotype summary:</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em>&lt;sup&gt;31&lt;/sup&gt;</td>
<td><img src="image1.png" alt="Wild-type" /></td>
<td><img src="image2.png" alt="Mutant" /></td>
<td>WT: peritrichous&lt;br&gt;&lt;br&gt;∆<em>flhG</em>:  - Flagella bundled together as tufts.&lt;br&gt;- Flagella are more likely to be found near the poles&lt;br&gt;- Motility/swarming was not affected.</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em>&lt;sup&gt;32,33&lt;/sup&gt;</td>
<td><img src="image3.png" alt="Wild-type" /></td>
<td><img src="image4.png" alt="Mutant" /></td>
<td>WT: mono- or amphitrichous&lt;br&gt;&lt;br&gt;∆<em>flhG</em>:  - &gt;1 flagellum per pole&lt;br&gt;- Decreased motility&lt;br&gt;- Cell division defects. (<em>C. jejuni</em> does not have a Min system.)</td>
</tr>
<tr>
<td><em>Helicobacter pylori</em>&lt;sup&gt;34&lt;/sup&gt;</td>
<td><img src="image5.png" alt="Wild-type" /></td>
<td><img src="image6.png" alt="Mutant" /></td>
<td>WT: 2-3 polar flagella&lt;br&gt;&lt;br&gt;∆<em>flhG</em>: (aka <em>ylxH</em>)&lt;br&gt;- No flagella&lt;br&gt;- Decreased motility</td>
</tr>
<tr>
<td>Species</td>
<td>WT Description</td>
<td>ΔflhG Effects</td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
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<td>-------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>WT: single polar flagellum</td>
<td>∆flhG: (aka fleN)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Tuft of 3-6 flagella</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Changes in motility</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas putida</em></td>
<td>WT: 3-4 flagella</td>
<td>∆flhG: (aka fleN or motR)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Tuft of 13-18 flagella</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Decreased motility</td>
<td></td>
</tr>
<tr>
<td><em>Shewanella oneidensis</em></td>
<td>WT: single polar flagellum</td>
<td>∆flhG:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Increased flagellar number</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Decreased motility</td>
<td></td>
</tr>
<tr>
<td><em>Shewanella putrefaciens</em></td>
<td>WT: single polar flagellum</td>
<td>∆flhG:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Increased flagellar number (2-16)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Decreased motility</td>
<td></td>
</tr>
<tr>
<td><em>Vibrio alginolyticus</em></td>
<td>WT: single polar flagellum</td>
<td>∆flhG:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Increased flagellar number (≈15x)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Decreased motility</td>
<td></td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td>WT: single polar flagellum</td>
<td>∆flhG:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Increased flagellar number (8-10 on one or both poles)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Decreased motility</td>
<td></td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em></td>
<td>WT: single polar flagellum</td>
<td>∆flhG:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Increased flagellar number (2+)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Decreased motility</td>
<td></td>
</tr>
</tbody>
</table>

Table 1-5: FlhG typically affects flagella number and motility without affecting flagella localization. *Bacillus subtilis*: Accession # WP_010886507.1; WT have peritrichous flagella; ΔflhG have flagella bundled together as tufts. Accurate filament counts could not be obtained because filaments were too numerous and had the tendency to form bundles. *Campylobacter jejuni*: Accession # WP_002851985.1; WT are mono- or amphitrichous; ΔflhG have more than one flagellum per pole. *Helicobacter pylori*: Accession # WP_001064464.1; WT cells typically have 2-3 polar flagella. Δyhh cells have no flagella. *Pseudomonas aeruginosa*: Accession # WP_003083064.1; WT cells typically have a single polar flagellum; ΔfleN cells have tuft of 3-6 flagella. Campos-Garcia found that ΔmotR resulted in greater migration in chemotaxis plates. Dasgupta found that ΔfleN resulted in no migration on chemotaxis plates. *Pseudomonas putida*: Accession # WP_003254408.1; WT cells typically have a polar bundle of 3-4 flagella; ΔfleN/ΔmotR cells have a tuft of 13-18 flagella. *Shewanella oneidensis*: Accession # WP_011073096.1; WT cells have a single polar flagellum; ΔflhG are multi-flagellate. *Shewanella putrefaciens*: Accession # WP_011919601.1; WT cells are monotrichous; ΔflhG cells have 2-16 flagella. *Vibrio alginolyticus*: Accession # WP_213890071.1; WT cells have a single polar flagellum; ΔflhG cells are hyperflagellated with ~15 flagella. *Vibrio cholerae*: Accession # WP_000182546.1; WT cells have a single polar flagellum; ΔflhG have 8-10 flagella at one pole or multiple flagella on
Despite flagella positioning having been investigated in several organisms of diverse flagellation patterns, many questions remain to be answered. What is the mechanism underlying the control of flagella location and number? How does this differ in organisms of varying flagellation patterns? Given that some organisms have multiple A/D based positioning systems, what are the specificity determinants that allow flagella to be positioned by FlhG? Since flagellar apparatuses are positioned on the membrane, how is flagella localization coordinated with the process of cell division? In most organisms investigated, deletion of FlhG does not have a strong effect on cell division. However, in *Campylobacter jejuni*, cell division defects have also been observed. How does this system vary from that of other polar flagellates? Can we use these models to predict deletion phenotypes in other organisms?

Since flagella are necessary for chemotaxis, it is possible that cross-talk exists in the spatial organization of flagella and chemotaxis clusters. Intriguingly, several bacteria also encode an A/D ATPase for chemotaxis cluster positioning in the cell. Below, I discuss the role of A/D ATPases in regulating chemotaxis clusters in bacteria.

### The Role of ParC in Regulating Chemotaxis Clusters

Bacteria use flagella to move towards favorable conditions via chemotaxis. Directing motility are large hexagonal arrays comprised of chemoreceptors, an adaptor protein (CheW), and a kinase (CheA). Being motile and chemotactic is a survival advantage for many bacteria. Therefore, daughter cells need to not only be flagellate, but must also
inherit a chemotaxis cluster on division to rapidly sense and respond to the environment. Several mechanisms to control both the number and positioning of chemotaxis clusters have evolved—stochastic self-assembly (E. coli)\textsuperscript{74}, cell-pole landmarks (C. crescentus)\textsuperscript{75}, and the use of A/D ATPases (Rhodobacter sphaeroides, Vibrio cholerae, Vibrio parahaemolyticus)\textsuperscript{44-47}. Deletion of the A/D ATPase (called ParC in Vibrio species or PpfA in R. sphaeroides) results in mispositioning of chemotaxis cluster foci, reduction in foci number, and reduction in swarming (Table 1-6).

In R. sphaeroides\textsuperscript{44,45}, newly formed cells typically have a cytoplasmic chemotaxis cluster at mid-cell. As the cell prepares to divide, the clusters migrate to the quarter positions, which will become the mid-cell after cell division. This pattern is reminiscent of plasmid partitioning systems, which use the nucleoid as a positioning matrix. Indeed, it was found that PpfA diffusely coats the nucleoid, but is enhanced near the chemotaxis cluster. In the absence of ppfA, a cell never had more than one chemotaxis cluster, clusters were more randomly distributed in the cell, and clusters were more mobile\textsuperscript{44,45}. The distribution of soluble chemotaxis clusters differ from those of transmembrane ones.

In V. cholerae\textsuperscript{46} and V. parahaemolyticus\textsuperscript{47}, the transmembrane chemotaxis clusters are found as a single unipolar cluster in shorter cells or bipolar foci in longer cells. The bipolar localization allows each daughter cell to inherit a chemotaxis cluster at their old pole upon cell division. The unipolar and bipolar localizations of chemotaxis clusters are preceded by similar localization patterns in ParC. In the absence of ParC, chemotaxis clusters are more likely to be absent or mislocalized. Although the localization of these clusters vary, the outcome is similar.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Wild-type positioning</th>
<th>Mutant positioning</th>
<th>Phenotype summary:</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhodobacter sphaeroides</em> 44,45</td>
<td><img src="image1.png" alt="Location of: 1 focus" /> 2 foci</td>
<td><img src="image2.png" alt="Location of: 1 focus" /> 2 foci</td>
<td><strong>WT</strong>: single chemotaxis focus at mid-cell or two foci at the quarter positions</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td><strong>ΔparC:</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Cells never have more than one focus.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Foci are more randomly positioned</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em> 46</td>
<td><img src="image3.png" alt="Location of" /></td>
<td></td>
<td><strong>WT</strong>: young cells have a single unipolar focus. As cells prepare to divide, they have bipolar foci.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>ΔparC:</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- More likely to have zero foci.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- More likely to have non-polar foci.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Reduction in swarming</td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em> 47</td>
<td><img src="image4.png" alt="Location of" /></td>
<td></td>
<td><strong>WT</strong>: young cells have a single unipolar focus. As cells prepare to divide, they have bipolar foci.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>ΔparC:</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- More likely to have zero foci.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- More likely to have non-polar foci.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Reduction in swarming</td>
</tr>
</tbody>
</table>

Table 1-6: ParC typically affects chemotaxis cluster positioning and foci number. *Rhodobacter sphaeroides*: Accession # WP_002720193.1; WT cells have a single focus at mid-cell or two foci at the quarter positions. ΔparC cells never have more than a single focus. This focus is more randomly positioned in the cell and more mobile. *Vibrio cholerae*: Accession # WP_000637668.1; WT cells have a single unipolar focus. As the cell gets ready to divide, a second focus forms on the opposite pole of the cell. ΔparC cells are more likely to have non-polar foci or no foci at all. ΔparC mutant exhibited a reduction in swarming ability. *Vibrio parahaemolyticus*: Accession # WP_005479466.1; WT cells have a single unipolar focus. As the cell gets ready to divide, a second focus forms on the opposite pole of the cell. ΔparC cells are more likely to have non-polar foci or no foci at all. ΔparC mutant exhibited a reduction in swarming ability. Images are reprinted with permission.

Much of this research remains in its infancy. In the examples shown above, localization of the A/D ATPase precedes that of the chemotaxis cluster. Although the location of the chemotaxis cluster varies, in all cases, localization allowed daughter cells to inherit a cluster. Given that some organisms have multiple A/D based positioning systems, what are the specificity determinants that allow chemotaxis clusters to be
positioned by ParC? Since chemotactic machinery are positioned on the nucleoid or membrane, how is cluster localization coordinated with the processes of DNA segregation and cell division? Since flagella and chemotaxis clusters communicate, does their assembly and spatial organization show crosstalk, independencies, and/or coordination? These questions remain to be investigated because the model organisms used to investigate these positioning events are different. In my thesis work, I investigate how organelle trafficking reactions are coordinated with each other and the processes of DNA segregation, and cell division.

The research discussed in the above sections serve as a foundation for my thesis work. I have identified and discussed some of the gaps in knowledge. A common knowledge gap to all cargos is their coordinated positioning throughout the cell cycle and the specificity determinants that define each system. This knowledge gap motivated my thesis project, where I identified and investigated an organism with multiple A/D ATPases, *H. neapolitanus*. Below, I describe my thesis goals and accomplishments using this organism that I have developed as an ideal model organism to study the ParA/MinD family of ATPases.

**Dissertation Goals**

As described above, the ParA/MinD family of ATPases have important roles in positioning of a variety of cargos in the cell such as the chromosome, divisome, carboxysome, flagellum, and chemotaxis cluster. Much of this research is still in its infancy and many questions remain to be answered. How widespread are ParA/MinD ATPases in bacteria? Why do some organisms require an A/D ATPase for positioning a
particular fundamental cargo, while others position their cargos stochastically, or by other active mechanisms? Can an organism have multiple ParA/MinD ATPases for multiple disparate cargos? If so, what are the specificity determinants that govern these interactions? What are the defining characteristics of each ParA/MinD-like system? How are these systems coordinated with each other throughout the cell cycle? Since A/D ATPases function in minimal self-organizing systems, can we take advantage of these systems to move things in the cell for synthetic biology applications? The research described in my thesis directly answers or sheds light on many of these fundamental questions relating to subcellular organization in bacteria.

In Chapter 2, I identify a plethora of organisms with multiple A/D ATPases. One, called *Halothiobacillus neapolitanus*, has seven putative A/D ATPases for seven disparate putative cargos. The abundance of A/D ATPases, along with its genetic tractability and slow-growing nature, make *H. neapolitanus* an excellent candidate for investigating the spatiotemporal regulation of multiple cargos and probing the specificity determinants that govern these interactions. I start by demonstrating how each A/D ATPase in *H. neapolitanus* are dedicated to the positioning and faithful inheritance of a specific cargo type: chromosome, divisome, carboxysome, flagellum, and chemotaxis cluster. I further show how the deletion of one A/D ATPase can have indirect effects on the inheritance of a cargo actively positioned by another A/D ATPase, stressing the importance of understanding how organelle trafficking, chromosome segregation, and cell division are coordinated in bacterial cells. We conclude this study by identifying putative specificity determinants that allow each A/D ATPase to position its respective cargo. Together, our study probes mechanistic commonality and variation in the most
widespread ATPase family used in the spatial regulation of diverse cellular cargos across prokaryotes, and all within a single cell.

In Chapter 3, we dive more deeply into the carboxysome positioning system (McdAB) and its prevalence in carboxysome-containing proteobacteria. Carboxysomes are anabolic bacterial microcompartments that encapsulate the enzymes responsible for carbon fixation in cyanobacteria and proteobacteria, thus maximizing the efficiency of this critical metabolic process. Carboxysomes can be categorized into two large categories: α- and β-. Although functionally similar, α- and β-carboxysomes differ in composition, structure, and phylogeny. It was previously shown that β-cyanobacteria contain an McdAB system for carboxysome positioning. Here, we demonstrate that, although structurally and phylogenetically distinct from β-carboxysomes in β-cyanobacteria, α-carboxysomes found in proteobacteria also possess McdAB systems as spatial regulators. We further show that this McdAB system is widespread among α-carboxysome-containing proteobacteria and that α-cyanobacteria that lack an McdAB system likely inherited an α-carboxysome operon from a proteobacterium encoding the mcdAB system at a distant locus. Together, this study demonstrates that McdAB is a cross-phylum two-component system necessary for positioning both α- and β-carboxysomes.

In Chapter 4, we start to explore the interplay between flagella positioning and the spatial regulation of chemotaxis clusters. In these studies, we discovered that both flagella and chemotaxis clusters form at the new pole of the daughter cell lacking both structures. We observed that these positioning reactions are coordinated, and that chemotaxis cluster assembly at the new pole is dependent on flhG, the A/D ATPase
that directly positions the flagellum. We also observed that while flagella and chemotaxis clusters localized to the same pole, they do not appear to be in direct physical contact. The molecular players responsible for this crosstalk between the spatial regulation of the flagellum and chemotaxis cluster will be a subject of future research.

Altogether, my findings advance the new field of organelle trafficking in bacteria. A/D ATPases are widespread in bacteria, occurring in more than 95% of organisms. Our investigation has allowed us to investigate the coordination of multiple A/D ATPases in the same cell, identify putative specificity determinants in each system, and demonstrate the widespread occurrence of similar positioning systems.

References

CHAPTER 2: Multiple ParA/MinD ATPases Separately Position Disparate Cargos in a Bacterial Cell

This chapter is based on the following preprint: Lisa T. Pulianmackal, Jose Miguel I. Limcaoco, Keerthikka Ravi, Sinyu Yang, Jeffrey Zhang, Mimi K. Tran, Matthew J. O'Meara, Anthony G. Vecchiarelli. “Multiple ParA/MinD ATPases coordinate the positioning of disparate cargos in a bacterial cell”. bioRxiv 2022.06.09.495121, doi: https://doi.org/10.1101/2022.06.09.495121. At the time of writing, this article has not yet undergone a full review process. For tables and movies, see the above article.

Abstract

In eukaryotes, linear motor proteins govern intracellular transport and organization. In bacteria, where linear motors are absent, the ParA/MinD (A/D) family of ATPases spatially organize an array of genetic- and protein-based cellular cargos. ParA is well known to segregate plasmids and chromosomes, as is MinD for its role in divisome positioning. Less studied is the growing list of ParA/MinD-like ATPases found across prokaryotes and involved in the spatial organization of diverse protein-based organelles, such as Bacterial Microcompartments (BMCs), flagella, chemotaxis clusters, and conjugation machinery. Given the fundamental nature of these processes in both cell
survival and pathogenesis, the positioning of these cargos has been independently investigated to varying degrees in several organisms. However, it remains unknown whether multiple A/D ATPases can coexist and coordinate the positioning of such a diverse set of fundamental cargos in the same cell. If so, what are the mechanistic commonalities, variation, and specificity determinants that govern the positioning reaction for each cargo? Here, we find that over a third of sequenced bacteria encode multiple A/D ATPases. Among these bacteria, we identified several human pathogens as well as the experimentally tractable organism, *Halothiobacillus neapolitanus*, which encodes seven A/D ATPases. We directly demonstrate that five of these A/D ATPases are each dedicated to the spatial regulation of a single cellular cargo: the chromosome, the divisome, the carboxysome BMC, the flagellum, and the chemotaxis cluster. We identify putative specificity determinants that allow each A/D ATPase to position its respective cargo. Finally, we show how the deletion of one A/D ATPase can have indirect effects on the inheritance of a cargo actively positioned by another A/D ATPase, stressing the importance of understanding how organelle trafficking, chromosome segregation, and cell division are coordinated in bacterial cells. Together, our data show how multiple A/D ATPases coexist and function to position a diverse set of fundamental cargos in the same bacterial cell. With this knowledge, we anticipate the design of minimal autonomous positioning systems for natural- and synthetic-cargos in bacteria for synthetic biology and biomedical applications.
Introduction

Actin filaments, microtubules, and the linear motor proteins that walk along them, are well known for spatial organization in eukaryotic cells. In bacteria, however, where linear motor proteins are absent, a widespread family of ParA/MinD (A/D) ATPases spatially organize plasmids, chromosomes, and an array of protein-based organelles, many of which are fundamental to cell survival and pathogenesis. By far the two best studied ATPases, and family namesake, are ParA involved in plasmid partition and chromosome segregation (Baxter and Funnell, 2014; Jalal and Le, 2020), and MinD involved in divisome positioning (Lutkenhaus, 2007). Less studied is the growing list of A/D ATPases, widespread across prokaryotes, involved in spatially regulating diverse protein-based organelles, such as Bacterial Microcompartments (BMCs) (MacCready et al., 2021; Savage et al., 2010), flagella (Schuhmacher et al., 2015b, 2015a), chemotaxis clusters (Ringgaard et al., 2011; Thompson et al., 2006), and conjugation machinery (Atmakuri et al., 2007).

Despite the cargos being so diverse, A/D ATPases share a number of features: (i) all form ATP-sandwich dimers (Shan, 2016), (ii) dimerization forms an interface for binding a positioning matrix - the nucleoid for ParA-like ATPases (Hester and Lutkenhaus, 2007; Kiekebusch et al., 2012) or the inner membrane for MinD-like ATPases (Hu and Lutkenhaus, 2003; Szeto et al., 2002), and (iii) dimerization also forms a binding site for a cognate partner protein that connects an ATPase to its cargo and stimulates its release from the positioning matrix. For example, in chromosome segregation, the ParA partner is ParB, which loads onto a centromere-like site, called parS, to form a massive complex on the chromosome near the origin of replication (OriC) (Jalal and Le, 2020).
This ParB-parS complex locally stimulates ParA ATPase activity and nucleoid release, which generates ParA gradients on the nucleoid. Segregation ensues as sister chromosomes chase nucleoid-bound ParA gradients in opposite directions (Vecchiarelli et al., 2010). Therefore, unlike the mitotic-spindle apparatus used in eukaryotic chromosome segregation, prokaryotes use a fundamentally different mode of spatial organization - A/D ATPases make waves on biological surfaces to position their respective cargos.

Chromosome segregation, cell division positioning, and organelle trafficking reactions have been independently investigated to varying degrees in several prokaryotes. Yet, it remains unknown how many A/D ATPases can be encoded in a single bacterium to position multiple disparate cargos, or how bacteria spatiotemporally coordinate the positioning of such a diverse set of fundamental cargos in the same cell. Here, we find that a third of sequenced bacteria encode for multiple A/D ATPases. Among these bacteria, we identified several human pathogens as well as the non-pathogenic and experimentally tractable organism, *Halothiobacillus neapolitanus* (*H. neapolitanus* hereafter), with seven putative A/D ATPases. Neighborhood analysis of the A/D ATPase genes in *H. neapolitanus* implicate several putative cargos, six of which are already known to be positioned by an A/D ATPase in other bacteria. The tractability and number of A/D ATPases make *H. neapolitanus* a valuable tool for investigating how bacteria coordinate the processes of chromosome segregation and cell division with organelle trafficking – a well-studied question in eukaryotic cells that remains unaddressed in prokaryotes.
Additionally, the mechanistic variations and specificity determinants that govern the positioning of such a diverse set of cellular cargo also remain unclear. This is because A/D-based positioning reactions are typically studied independently of one another and in model bacteria with few A/D ATPases. Here we use genetics and cell biology to assign five of the A/D ATPases in *H. neapolitanus* to their cargos. Our findings show that each ATPase is directly dedicated to the positioning and faithful inheritance of a specific cargo type. We then show how the deletion of one A/D ATPase can have indirect effects on the inheritance of disparate cargos positioned by other A/D ATPases via defects in DNA replication, chromosome segregation, and/or cell division. We also, for the first time, provide evidence that flagella positioning influences the spatial regulation of chemotaxis clusters. Finally, we identify putative sequence- and structural-determinants that uniquely link each A/D ATPase to a specific cargo, ultimately allowing these related ATPases to coexist and function in the same cell. Together, our study probes mechanistic commonality and variation in the most widespread ATPase family used in the spatial regulation of diverse cellular cargos across prokaryotes, and all within a single cell.

Results

A third of sequenced bacteria encode for multiple ParA/MinD family ATPases

It is unclear how many ParA/MinD (A/D) family ATPases can be encoded in a single organism to position multiple disparate cargos in the same cell. To answer this question, we performed an extensive tBLASTn analysis using a consensus protein sequence, generated from well-studied A/D ATPases, as the query (see methods). As already
established (Koonin, 1993), we found that ~ 95% of bacteria from the NCBI Reference Sequence (RefSeq) database encode for at least one A/D ATPase (Table S2-1). These hits were binned by bacterial species, which were then ordered by the number of A/D hits. From this initial list, we found many bacterial genomes encoding 10 to 20 A/D ATPases. However, these bacteria with the most A/D ATPases had their genomes encoded on multiple plasmids and chromosomes, each of which encode its own ParA-based DNA segregation system (Tilly et al., 2012). For this study, we were specifically focused on understanding how multiple A/D ATPases coexist and coordinate the positioning of disparate cargos in the same cell. Therefore, we further filtered our dataset to identify bacteria encoding multiple A/D ATPases, but only one chromosome and no stable plasmids (Table S2-1). Even after accounting for bacteria with genomes encoded on multiple genetic elements, our bioinformatic analysis revealed that more than a third of sequenced bacteria encode multiple A/D ATPases (Figure S2-1A, Table S2-1).

Gene neighborhood analysis of A/D ATPases in H. neapolitanus implicate cellular cargos

We next set out to determine how multiple A/D ATPases can coexist in the same cell to position disparate cargos. To address this question, we identified an organism from our list of bacteria encoding multiple A/D ATPases (Table S2-1). Among the top 1% of bacteria (encoding six or more A/D ATPases), we identified several human pathogens as well as the non-pathogenic and experimentally tractable organism, H. neapolitanus - a slow-growing, sulfur-oxidizing chemoautotroph that encodes seven putative A/D
ATPases on one chromosome (Figure 2-1B). Spatial regulation by A/D ATPases has largely been studied in fast-growing model bacteria. We intentionally chose a slow growing bacterium (6 hr doubling time) because the infrequent DNA segregation and cell division events allowed for larger observation windows and a more direct view into the dynamics of organelle trafficking. The tractability, slow-growth rate, and abundance of A/D ATPases made *H. neapolitanus* an ideal choice for further study.

To determine whether the A/D ATPase hits in *H. neapolitanus* were indeed spatial regulators, we performed a gene neighborhood analysis (GNA) to identify putative cargos (Figure 2-1B). GNA allows us to infer function because A/D ATPase genes are often encoded near cargo-associated loci. For example, the ParAB chromosome segregation system is typically encoded near OriC (Livny et al., 2007). Strikingly, the putative cargos we identified using this approach includes the chromosome and all known protein-based cargos of the A/D ATPase family (Lutkenhaus, 2012; Vecchiarelli et al., 2012). While spatial regulation of these diverse cargos has been individually studied in many different model bacteria, their coordinated positioning by multiple A/D ATPases has never been investigated together in one organism.

As a second line of bioinformatic evidence linking each A/D ATPase to a specific cargo, we investigated the conservation of the A/D ATPase gene neighborhoods using FlaGs (Flanking Genes) analysis (Saha et al., 2021). FlaGs analysis predicts functional associations by taking a list of NCBI protein accessions as input and clusters neighborhood-encoded proteins into homologous groups. Homologs of each A/D ATPase in *H. neapolitanus* were identified using BLASTp, and top hits were used as input for FlaGs analysis (Figure S2-1). The analysis shows strong conservation of the
A/D ATPase gene neighborhoods across multiple bacterial phyla, further implicating the putative cargos. Due to the limited data on A/D ATPases associated with conjugation (Atmakuri et al., 2007) or nitrogen metabolism, we excluded these hits from further investigation in this study.

With the remaining five A/D ATPases, we performed a multiple sequence alignment against ParA/MinD family members that have been previously established to position a cellular cargo (Figure 2-1C). Conveniently, each A/D ATPase in H. neapolitanus clustered with a specific family member known to position a specific cellular cargo in other bacteria – the chromosome (ParA (Jalal and Le, 2020)), the divisome (MinD (Raskin and De Boer, 1999)), the carboxysome (McdA (MacCready et al., 2021; Savage et al., 2010)), the flagellum (FlhG (Schuhmacher et al., 2015b, 2015a)) and the chemotaxis cluster (ParC (Ringgaard et al., 2011, 2014; Thompson et al., 2006)). These data provide a third line of evidence that further implicates the putative cargos identified by our GNA. We next sought to directly identify the role of each A/D ATPase in positioning the cargos implicated by bioinformatics.

The ParAB system is required for chromosome segregation in H. neapolitanus

Chromosome segregation prior to cell division is critical for cellular survival. In most bacteria, faithful chromosome segregation and inheritance are mediated by a ParAB system encoded near OriC (Livny et al., 2007). Without ParAB, DNA is asymmetrically inherited, resulting in anucleate and polyploid cells, and reduced cell fitness or death (Jalal and Le, 2020). In H. neapolitanus, there is a putative parAB system encoded near OriC (Figure 2-2A). To image chromosome segregation, the ParB homolog encoded
downstream of the putative *parA* gene (*Hn2335*) was fused to the fluorescent protein monomeric Neon Green (mNG). ParB-mNG was observed as one or two puncta per cell (Figure 2-2B, left). Population analysis showed that shorter cells had a single focus at mid-cell, whereas longer cells had two foci at the quarter positions of the cell (Figure 2-2B, left). When the putative *parA* gene (*Hn2335*) was deleted, ParB-mNG foci were randomly positioned regardless of cell length (Figure 2-2C), or completely absent in 25% of cells (Figure S2-2D). When present, ParB foci were also significantly brighter compared to that of WT (wild-type) cells (Figure S2-2E). The data suggest that replicated chromosomes were no longer faithfully segregated, resulting in anucleate and polyploid cells.

We then performed time-lapse microscopy of ParB-mNG foci and SYTOX-stained nucleoids to observe chromosome segregation in real time. Newborn WT cells have a single ParB focus at mid-cell, which then splits into two foci that bidirectionally segregate towards the quarter positions of the growing cell (Figure S2-2F and Movie 2-1A). Foci positioning at the cell quarters was maintained, which then became the mid-cell position of each daughter cell following division. Faithful chromosome segregation and inheritance were lost when the putative *parA* gene (*Hn2335*) was deleted (Figure S2-2G and Movies 2-1B). Many cells had a single large ParB focus at a cell pole that did not split and all DNA was concentrated into a single daughter cell upon division. These polyploid daughters continued to divide, while the anucleate daughters were no longer viable. These data confirmed that the increased ParB foci intensities in the deletion mutant represents an increase in chromosome copy number in these cells. In
summary, the A/D ATPase encoded by *Hn2335* is required for faithful chromosome segregation (Figure 2-2D) and we will henceforth refer to this protein as ParA.

The MinCDE system aligns cell division at mid-cell in *H. neapolitanus*

Proper positioning of the divisome ensures that when a cell divides, both daughter cells are roughly equal in length. Without the Min system, division occurs at any nucleoid-free region (Yu and Margolin, 1999), producing anucleate minicells, the products of polar divisions. Our bioinformatic analyses showed that the protein encoded by *Hn1364* is a MinD homolog, and immediately downstream of this gene in the same operon is a gene encoding a MinE homolog (Figure 2-2E). To determine if *Hn1364* was indeed involved in divisome positioning, we analyzed dividing cells and identified their constriction sites relative to cell length (Figure 2-2F-G). WT cells divided close to mid-cell (Figure 2-2F and Figure S2-3D) while the Δ*Hn1364* cells divided asymmetrically (Figure 2-2G and Figure S2-3E). Population analysis of dividing cells identified mid-cell constriction sites in 97% of WT cells compared to only 41% of Δ*Hn1364* cells (Figure S2-3F and Movie 2-2). In addition to single asymmetric division events, 9% of dividing cells in the Δ*Hn1364* mutant population formed multiple division sites simultaneously along the cell length (Figure S2-3G and Movie 2-2C). The unequal divisions resulted in greater variation in cell length (Figure S2-3H). Overall, our findings show that *Hn1364* is critical for positioning the divisome at mid-cell (Figure 2-2H) and we will henceforth refer to this protein as MinD.
The A/D ATPase, McdA, encoded in the carboxysome operon positions carboxysomes

Bacterial microcompartments, or BMCs, are large icosahedral protein-based organelles that encapsulate sensitive metabolic reactions to provide prokaryotes with distinct growth advantages (Kerfeld et al., 2018). Despite their importance, little is known about how BMCs are spatially regulated. The model BMC is the carbon-fixing carboxysome found in cyanobacteria and proteobacteria (Turmo et al., 2017). It was recently found that an A/D ATPase, we termed Maintenance of carboxysome distribution protein A (McdA), is widespread among carboxysome-containing bacteria, including H. neapolitanus (Figure 2-2I) (Maccready and Vecchiarelli, 2021). McdA spaces carboxysomes on the nucleoid along with its partner protein, McdB (MacCready et al., 2018, 2021). To demonstrate its requirement for carboxysome positioning, we visualized carboxysomes by labelling the small subunit of the encapsulated Rubisco enzyme (cbbS) with mTurquoise2 to form CbbS-mTQ. As previously shown, in WT cells, carboxysomes are distributed down the cell length (Figure 2-2J). In the deletion mutant (ΔHn0912), carboxysome aggregates form a large polar focus at one or occasionally both poles (Figure 2-2K). These data are consistent with our previous observations using TEM, which showed that foci in the mutant population represent an aggregation of assembled carboxysomes (MacCready et al., 2021).

We extend our previous findings here with long-term time-lapse microscopy. In WT cells, carboxysomes are dynamically positioned along the cell length across multiple generations (Figure S2-4F and Movie 2-3A). In the deletion mutant, carboxysome aggregates were stagnant (Figure S2-4G and Movie 2-3B). Together, our findings show that the A/D ATPase encoded within the carboxysome operon, we termed McdA,
is essential for distributing carboxysomes across the cell length and ensuring organelle homeostasis following division (Figure 2-2L).

**Hn0716** is required for regulating flagella number and positioning

Flagella are external filamentous structures that allow for bacterial motility. Bacteria vary in flagella location, number, and pattern. Many bacteria encode for an A/D ATPase called FlhG in their flagellar operon, which is essential for diverse flagellation patterns in many bacteria (Schuhmacher et al., 2015b), yet the mechanisms remain unclear. Deletion of flhG typically results in changes in flagella number, location, and motility. Our bioinformatic analysis suggests that Hn0716 within the flagella operon encodes an FlhG homolog (see Figure 2-1C). To determine if Hn0716 is involved in flagellar spatial regulation, we first visualized a mNG fusion of FliN, which is a component of the flagellar basal body that assembles at the cytoplasmic face of the membrane (Figure 2-2M) (Chang et al., 2020). WT cells had a single mNG-FliN focus at the extreme cell pole (Figure 2-2N). In ΔHn0716 cells, FliN foci were no longer faithfully positioned (Figure 2-2O) and cells were more likely to have multiple foci (Figure S2-5D). The foci in ΔHn0716 cells were also lower in fluorescence intensity compared to WT (Figure S2-5E). These data suggest that Hn0716 is required for positioning flagellar machinery at a single pole in *H. neapolitanus*.

We next set out to determine whether these alterations to FliN positioning affected cell motility. Motility assays found that ΔHn0716 cells were not motile (Figure S2-5F). Loss of motility could be due to flagellar mispositioning, a loss of flagella, or hyper-flagellation. To image flagella, we engineered flagellin<sup>T185C</sup>, which allows for fluorescent
labeling of flagella via the addition of a cysteine-reactive maleimide stain to the media (Kühn et al., 2018). We found that WT H. neapolitanus cells are monotrichous, with a single polar flagellum emanating from the FliN focus (Figure S2-5G). ΔHn0716 cells also had flagella emanating from FliN foci, however, the cells were hyper-flagellated, with multiple flagella often bundled together as tufts, emanating from several FliN foci. We conclude that Hn0716 is required for regulating flagella number and position in H. neapolitanus (Figure 2-2P) and we will henceforth refer to this protein as FlhG.

Hn0722 is required for chemotaxis cluster assembly and positioning

Directing bacterial motility are large hexagonal arrays called chemotaxis clusters, comprised of chemoreceptors, an adaptor protein (CheW), and a kinase (CheA). Several mechanisms have evolved to control both the number and positioning of chemotaxis clusters, including the use of A/D ATPases (called ParC in Vibrio species or PpfA in R. sphaeroides) (Ringgaard et al., 2011; Roberts et al., 2012). Deletion of the A/D ATPase alters chemotaxis cluster number and positioning in cells, which results in a reduction in swarming motility. Our bioinformatics analysis showed that the protein encoded by Hn0722 is a ParC homolog within the chemotaxis operon of H. neapolitanus (see Figure 2-1C). To determine if Hn0722 is important for spatially regulating chemotaxis clusters, we first imaged chemotaxis clusters by fusing mNG to CheY (Figure 2-2Q). CheY is a response regulator that is phosphorylated by CheA, and has previously been shown to colocalize with chemotaxis clusters in E. coli (Kentner and Sourjik, 2009; Sourjik and Berg, 2000). Consistent with electron micrographs of chemotaxis clusters in H. neapolitanus (Brieger et al., 2009), CheY-mNG formed a
single focus immediately proximal to one cell pole in ~ 85% of WT cells (Figure 2-2R, Figure S2-6D). However, when Hn0722 was deleted, the CheY-mNG signal was diffuse in ~ 80% of the cell population (Figure 2-2S, Figure S2-6D). In the ~ 20% of ΔHn0722 cells that had a CheY-mNG focus, the foci were significantly lower in intensity (Figure S2-6E). Together, we conclude that Hn0722 is required for chemotaxis cluster assembly and positioning in H. neapolitanus (Figure 2-2T) and we will henceforth refer to this protein as ParC.

Cargo positioning is not directly controlled by A/D ATPases encoded at distant loci

We have thus far provided direct evidence showing that five A/D ATPases position five disparate cellular cargos in H. neapolitanus (Figure 2-2). We next asked if each of the five positioning reactions occurred independently from each other, or whether they exhibited cross-talk. To answer this question, we individually deleted each A/D ATPase in every cargo-labeled background strain (Figure 2-3). We found carboxysome (Figure 2-3C) and flagella (Figure 2-3D) positioning were largely unaffected by the deletion of distant A/D ATPases. Intriguingly, chromosome (Figure 2-3A), divisome (Figure 2-3B), and chemotaxis cluster (Figure 2-3E) positioning were all influenced in ΔflhG cells, albeit with intermediate phenotypes when compared to deleting the dedicated A/D ATPase (Figure 2-3, bold boxes). Together our data show that each A/D ATPase is dedicated to the positioning of a specific cargo type, and not directly involved in the positioning of other cargos. However, our data at the cell-population level also unveiled potential coordination, crosstalk, and/or interdependencies among certain positioning reactions. In the next three sections, we dissect how the deletion of one A/D ATPase
can indirectly affect the positioning and inheritance of cellular cargos positioned by another A/D ATPase.

Deletion of \textit{parA}, \textit{minD}, or \textit{flhG} results in anucleate cells via three different mechanisms. We have shown how the deletion of \textit{parA} results in a significant fraction of anucleate cells because ParA is directly required for chromosome segregation and inheritance following cell division (Figure 2-4A). Deleting \textit{minD} or \textit{flhG} also resulted in anucleate cells, but the mechanism was different in each case. In \textit{ΔminD} cells, chromosome positioning (Figure 2-4B) and ParB foci intensities (Figure 2-4C) were similar to that of WT, showing that chromosome segregation was still active. Instead, it was divisome mispositioning in \textit{ΔminD} cells and subsequent asymmetric cell division (Figure 2-4D) that indirectly caused asymmetric chromosome inheritance and anucleate cell formation.

In \textit{ΔflhG} cells, chromosome positioning (Figure 2-4B) and ParB foci intensities (Figure 2-4C) were also similar to that of WT, again suggesting that chromosome segregation was unaffected. But, divisome mispositioning was not as severe as \textit{ΔminD} cells (Figure 2-4DE), suggesting that anucleate cells were forming via a third distinct mechanism. Intriguingly, \textit{ΔflhG} cells were less likely to have two ParB foci compared to WT cells (Figure 2-4A). Instead, pre-divisional cells still had a single ParB focus (Figure 2-4B) with intensities that suggested the presence of only a single chromosome copy (Figure 2-4C). Time-lapse microscopy confirmed that ParB foci positioning was actively maintained (Movie 2-4). Therefore, anucleate \textit{ΔflhG} cells are likely formed due
to defects in chromosome replication and/or premature cell division; a question of future study.

Together, the findings emphasize the importance of probing the functional relationships of A/D ATPases with each other and the bacterial cell cycle, when occupying the same organism.

Anucleate cells inherit carboxysomes

McdA uses the nucleoid as a positioning matrix for distributing carboxysomes (MacCready et al., 2018, 2021). Therefore, it was surprising that in the ΔparA mutant population, anucleate cells retained carboxysomes (Figure 2-5A). To determine if anucleate cells synthesized carboxysomes de novo or carboxysomes were somehow still inherited following division, we performed time-lapse microscopy on ΔparA cells with fluorescent carboxysomes (Figure 2-5B and Movie 2-5A). Intriguingly, anucleate cells indeed inherited carboxysomes, but in the most unexpected fashion. In dividing ΔparA cells that failed to split their ParB-mNG foci, carboxysomes in the to-be-anucleate cell bundled up immediately adjacent to the division plane. Carboxysome bundling was coincident with the chromosome spooling action that occurred at the invaginating septum just prior to complete division and asymmetric chromosome inheritance (see Figure S2-2G and Movie 2-1B). After septation was complete, the massive carboxysome bundle was explosively liberated from the new pole of the anucleate cell, resulting in multiple free-diffusing carboxysome foci (Figure 2-5B and Movie 2-5A). Anucleate cells harboring carboxysomes did not divide further. We found that anucleate cells in the ΔminD (Figure 2-5C and Movie 2-5B) and ΔflhG (Figure 2-
5D and Movie 2-5C) cell populations also inherited carboxysomes via the same mechanism. Together the data show how carboxysome trafficking and distribution by McdA are dependent upon faithful chromosome segregation. However, anucleate cells can still inherit carboxysomes in parA, minD, or flhG deletion strains because carboxysomes are scraped off of missegregated chromosomes that are spooled through the invagination site during septation. We speculate that all mesoscale cargos using asymmetrically inherited nucleoids as a positioning matrix would show the same mode of inheritance.

Deletion of parA, minD or flhG influence chemotaxis cluster assembly

The A/D ATPase that positions chemotaxis clusters in Rhodobacter sphaeroides uses the nucleoid as its positioning matrix (Roberts et al., 2012). Therefore, we suspected that A/D ATPase deletions resulting in anucleate cells would indirectly impact the spatial regulation of chemotaxis clusters in these strains. Indeed, we found that parA, minD, and flhG deletion strains, all of which form anucleate cells (see Figure 2-4A), had a corresponding increase in cells lacking chemotaxis clusters (Figure 2-6A), and when cells had foci, they were notably dimmer compared to WT (Figure 2-6B).

It is important to note that while ΔparA and ΔminD strains exhibited moderate effects on chemotaxis cluster assembly, deletion of flhG was more severe (Figure 2-6AB). Given that chemotaxis clusters communicate with the flagellum to move the bacterium towards favorable conditions, we hypothesize that chemotaxis cluster assembly and organization in H. neapolitanus is regulated by flagellum positioning. Interestingly, this effect was not reciprocal, as deletion of parC had no effect on flagella foci number
(Figure 2-6C) or flagella basal body intensity (Figure 2-6D). Identifying the molecular players responsible for this crosstalk in the spatial regulation of the flagellum and chemotaxis cluster is a subject of future work. Together, our data demonstrate interdependencies in how A/D ATPases coordinate the positioning of protein-based organelles with each other as well as the processes of DNA segregation and cell division.

A/D ATPases have unique interfaces that confer cargo-positioning specificity

We have experimentally demonstrated that multiple A/D ATPases coexist and function in the same cell to position multiple disparate cargos. We also showed that A/D-based positioning is cargo specific. A/D ATPases have been shown to form very similar sandwich dimer structures (Dunham et al., 2009; Leonard et al., 2005; Schumacher et al., 2012; Wu et al., 2011), and AlphaFold2 (AF2) predictions suggest this is also the case for all five A/D ATPases of *H. neapolitanus* studied here (Figure S2-7). The structural similarities suggest there are conserved interfaces unique to each A/D ATPase that provide specificity, linking an A/D ATPase to its particular cargo.

Using AF2 and Rosetta predictions, we determined the A/D ATPase structures from *H. neapolitanus* and identified the putative interaction interfaces that provide specificity to each positioning reaction. There are three interfaces on an A/D ATPase that confers specificity: 1) the dimerization interface, 2) the interface for interaction with its positioning matrix (nucleoid or membrane), and 3) the interface for interaction with its partner protein, which ultimately links the ATPase to its cargo. We identified these three
interfaces for all five of the A/D ATPases in *H. neapolitanus* (Figure 2-7) and predict key residues required for these associations (Table S2-2).

Specificity at the dimer interface restricts A/D ATPases to homodimerization, and thereby allows each A/D ATPase to function independently in the same cell without cross-interference (Figure 2-7A). The putative residues required for homodimerization of the five A/D ATPases in *H. neapolitanus* are provided in Table S2-2, Tab 1.

ParA-like ATPases use the nucleoid and MinD-like ATPases use the inner membrane for cargo positioning. ParA, McdA, and ParC have basic residues at their C-termini for non-specific binding to nucleoid DNA, whereas MinD and FlhG have membrane targeting sequences (MTSs) for membrane binding (Figure 2-7B). We identified the predicted residues required for each A/D ATPase to associate with their respective positioning matrix (Table S2-2, Tab 2).

Cargo specificity for A/D ATPases comes from interacting with a partner protein that either associates with the cargo, or is a physical component of the cargo itself. Partner proteins have a stretch of amino acids enriched in charged residues at the N-terminus that exclusively interacts with its A/D ATPase, while the rest of the partner protein is dedicated to cargo association (Schumacher et al., 2021). We generated docking models of each A/D ATPase with an N-terminal peptide from its partner protein (Figure 2-7C). The peptides were defined as the first 30 residues of the putative partner protein from the N-terminus. The peptide docking simulations identified several putative residues that are key to system specificity between an A/D ATPase and its partner protein (Table S2-2, Tab 3).
Finally, we performed in silico alanine-scanning mutagenesis across all residues comprising the interacting interface of the N-terminal peptides of partner proteins docked onto their cognate A/D ATPase (Table S2-2, Tab 4). The resulting ΔΔG values identify the extent to which each residue contributes to the stability of the partner protein interaction with its cognate A/D ATPase. Importantly, our in silico alanine-substitution simulations identify the residues experimentally shown to be important for docking the Escherichia coli MinE peptide onto the MinD dimer (Park et al., 2011, 2012; Wu et al., 2011) (Figure S2-7C). Together, the in silico data provide a roadmap for strategic mutagenesis and mechanistic probing of the specificity determinants involved in bacterial chromosome segregation, cell division positioning, and protein-based organelle trafficking by A/D ATPases across prokaryotes.

Discussion and Future Directions

The study of A/D ATPases has focused on a specific cargo of a certain biological process, and largely in model organisms that encode only one or two A/D ATPases. In these studies, two questions are typically posed: How does a specific cargo find its correct position, and how does this position change over time? Here, our focus was on the positioning systems, rather than a specific cargo-type or biological process, and as such, is the first systems biology approach to address how multiple A/D ATPases coordinate the positioning of diverse cargos in the same cell.
Encoding multiple A/D ATPases is a shared feature across prokaryotes

Over a third of all sequenced bacterial genomes encode multiple A/D ATPases (Figure 2-1A), with some bacteria encoding more than 10. Interestingly, although most bacteria have the same fundamental cargos, not all use dedicated A/D-based positioning systems for each cargo. For example, many of the cellular cargos we found here to be positioned by A/D ATPases in certain bacteria, like \textit{H. neapolitanus}, are not actively positioned in others, like \textit{E. coli}. What necessitates an A/D ATPase for positioning a certain cellular cargo in one bacterium and not in another remains an open question. There does, however, seem to be a limit to the number of A/D ATPases that a bacterium can encode.

A/D ATPases are also encoded in archaeal genomes (Leipe et al., 2002), but little is known about their roles in subcellular organization. A recent study showed that archaeal species across several phyla, Euryarchaeota in particular, encode multiple A/D ATPases (Nußbaum et al., 2020). Several of these species contained more than a dozen, including \textit{H. volcanii} with 13 A/D ATPases, four of which are MinD-homologs. Strikingly, all four MinD homologs were not required for cell division, but one (MinD4) stimulated the formation of chemotaxis arrays and the archaella, which is the functional equivalent of the bacterial flagellum. This study stresses the importance of experimentally linking A/D ATPases to their cellular cargos as we show here.
Complementation confirms the role of A/D ATPases in directly regulating a specific cellular cargo

*H. neapolitanus* encodes for seven A/D ATPases. We directly demonstrated that five of these A/D ATPases are each dedicated to the spatial regulation of a single cellular cargo: the chromosome, the divisome, the carboxysome BMC, the flagellum, and the chemotaxis cluster. Deletion of each A/D ATPase resulted in: (i) chromosome missegregation in the ΔparA strain, (ii) divisome mispositioning and asymmetric cell division in the ΔminD strain, (iii) carboxysome aggregation in the ΔmcdA strain, (iv) misregulation of flagellar location and number in the ΔflhG strain, and finally, (v) disruption in the assembly of chemotaxis clusters in the ΔparC strain. As our gene neighborhood analysis revealed (see Figure 2-1), these A/D genes are within or near the genes encoding the positioned cargo. We therefore set out to complement each deletion mutant to confirm that their phenotypes are indeed a consequence of deleting the positioning ATPase and not due to polar effects.

There are several challenges to complementing A/D ATPase deletion mutants. For example, it is known that both the absolute level and relative ratio of A/D ATPase and partner protein must be precise to maintain WT positioning of the cargos (Mohl et al., 1997). To fine tune expression, an IPTG-inducible pTrc promoter was used to generate inducible constructs that were inserted into a neutral site in the chromosome, instead of expressing from a plasmid, which also allowed for growth in the absence of antibiotics. Since inducible constructs have not yet been tested in *H. neapolitanus*, we first determined the dynamic range of expression by using a fluorescent focus-forming protein in the cell (mNG-mcdB in ΔmcdA) (MacCready et al., 2021), induced with IPTG.
concentrations ranging from 0.25 µM to 1 M. We quantified the fluorescence intensity of the foci and determined the dynamic range to be 0.25 µM – 50 µM. Within this range of IPTG concentration, we were able to achieve increasing levels of protein expression by changing concentration and induction time. IPTG concentrations above 50 µM did not yield higher levels of protein. With the tools to precisely induce our proteins in *H. neapolitanus*, we next induced each A/D ATPase independently.

To date, we have successfully complemented 3 of the 5 mutants. Upon induction of ParA in the ΔparA strain, ParB foci positioning and intensities were returned to the WT phenotype (Figure S2-8A). Induction of MinD in the ΔminD strain returned cell division positioning back to mid-cell (Figure S2-8B). In the ΔflhG strain, induction of FlhG caused FliN foci to be restored in number and fluorescence intensity similar to that of wildtype (Figure S2-8C). We have yet to complement the ΔparC or ΔmcdA strains with suitable expression constructs of their respective ATPases.

Importantly, several of these A/D ATPase genes overlap with upstream and/or downstream genes. Interruption of the co-transcription of genes could provide one explanation for our inability to complement our ΔparC or ΔmcdA strains. The *mcdA* gene, for example, overlaps with the downstream gene, *mcdB*. Therefore, we expressed both *mcdA* and *mcdB* from the inducible exogenous locus, as described above. In this expression setup, we observed complementation - carboxysome aggregates were redistributed as individual carboxysomes across the length of the cell (Figure S2-8D).

The final A/D ATPase involved in chemotaxis positioning and assembly, *parC*, also overlaps with its neighboring genes. In ongoing studies, we will complement by
reintroducing parC in the exogenous locus along with its neighboring genes. I anticipate that the diffuse CheY signal will be restored to polarly positioned foci (Figure S2-8E). Altogether, these data confirm that the mispositioning phenotypes observed are the result of deleting the positioning A/D ATPase for each cargo.

5 A/D ATPases, 5 Cargos, 1 Cell

Our bioinformatics also identified a 6th A/D ATPase gene in the *H. neapolitanus* genome. *Hn1669* is an A/D ATPase that shows homology to VirC1 and is located near *trb* genes, which encode conjugation machinery components (Figure 2-9A). A single study has shown that the VirC1 ATPase, encoded on the Ti plasmid of Agrobacterium *tumefaciens*, is involved in recruiting the conjugative Ti plasmid to the Type IV secretion machine at the cell poles (Atmakuri et al., 2007). Intriguingly, in *H. neapolitanus*, the VirC1 homolog is encoded on an Integrative Conjugative Element (ICE) and the downstream gene from this A/D ATPase encodes a ParB homolog (*Hn1670*). We hypothesized that the ParB homolog demarcates the ICE locus during conjugation. It is attractive to speculate that the VirC1 homolog and its downstream ParB homolog are involved in transporting and positioning the ICE locus to the Type IV secretion machinery at the cell pole for conjugation.

ParB proteins bind onto and around a DNA-binding site called *parS*, which demarcates the origin region for chromosome segregation by ParA. As shown in Figure S2-2, ParB-mNG binding onto and around *parS* results in punctate foci. Given that *Hn1670* encodes a ParB homolog, we hypothesized that it may also have a DNA-binding site in the ICE element, which upon binding would result in an observable focus.
To test for DNA binding activity of Hn1670 under normal growing conditions, we natively tagged both N- and -C termini of Hn1670. We observed some very faint signal in both the N-terminal (Figure 2-9B) and the -C terminal fusions (Figure 2-9C). It is currently unclear whether either of these fusions are functional and representative of Hn1670 localization in the cell. However, it is important to note that the faint signal was unsurprising because our normal growing conditions may not induce conjugation.

We next attempted to induce conjugation using a variety of stressors: low temperature (18ºC), lower thiosulfate concentrations, and high salt concentrations. To determine if conjugation was being induced, I designed a simple reporter assay (Figure 2-9D), placing LacZ under the promoter of the conjugation operon. Expression of the operon would turn colonies blue when plated on X-gal. I spotted cells on plates with varying thiosulfate and salt concentrations, and incubated at 30ºC or 18ºC (Figure 2-9E). Colonies remained white under all conditions tested. As a positive control, LacZ was placed under the parAB promoter, and in this case, colonies turned blue. Determining whether the ICE is active and if so, what conditions trigger conjugation are areas of continued research.

In the absence of a trigger for conjugation, I next attempted chemical induction of the conjugation operon. First, I expressed a second fluorescent copy of Hn1670 under an IPTG-inducible promoter (mNG-ParB2). If this ParB-like protein exhibits DNA binding activity, a future experiment would be to induce the entire operon. One could then delete the A/D ATPase gene Hn1669 (VirC1) to observe for any changes in the positioning of ParB2 foci over the nucleoid region of the cell. However, the trigger to ICE
excision and conjugation would remain unclear. Importantly, deletion of *Hn1669* did not affect the localization of other cargos (Figure 2-9F).

We generate mutants in *H. neapolitanus* by simply washing the cells in water, adding DNA, and allowing the cells to recover overnight. In our Hn1669 deletion strain we do not observe a difference in transformation efficiency. Therefore, gene *Hn1669*, does not seem to be required for transformation or cell competency.

**Coordinated cargo positioning throughout the cell cycle**

We were able to visualize the localization of five cargos in *H. neapolitanus* using a combination of phase contrast microscopy and epifluorescence. During these studies, we found that each A/D ATPase is dedicated to the positioning of a specific cargo type, and not directly involved in the positioning of other cargos. However, our data at the cell-population level also unveiled potential coordination, crosstalk, and/or interdependencies among certain positioning reactions. To observe relative cargo positioning throughout the cell cycle, we labelled all cargos together in a single strain using the following fluorescent fusions and filter sets:

<table>
<thead>
<tr>
<th>Cargo</th>
<th>Fluorescent fusion</th>
<th>Color / Filter set</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosome</td>
<td>ParB-mApple</td>
<td>TexasRed</td>
</tr>
<tr>
<td>Divisome</td>
<td>--none--</td>
<td>(Phase contrast only)</td>
</tr>
<tr>
<td>Carboxysome</td>
<td>CbbS-mTq</td>
<td>CFP (can also see w/ Dapi)</td>
</tr>
<tr>
<td>Flagella</td>
<td>JF650-HaloTag-FliN</td>
<td>Cy5</td>
</tr>
<tr>
<td>Chemotaxis</td>
<td>CheY-mNG</td>
<td>YFP (also GFP)</td>
</tr>
</tbody>
</table>

We chose the fluorescent proteins mApple, mNeonGreen, and mTurquoise2 because their spectra are well-separated thus preventing signal bleed-though during multi-channel imaging. Unfortunately, a bright far-red fluorescent protein has yet to be identified. However, there are a number of far-red dyes that can be conjugated to
HaloTagged fusion proteins. We fused FliN to the HaloTag as means to label the flagella base-plate by conjugating to the Janelia Fluor far-red dye JF650. With all five cargos labelled (Figure 2-10), we can image how organelle trafficking, DNA segregation, and cell division are coordinated with each other, all in the same cell. Future studies will also involve making single deletion mutants to determine interdependencies among positioning reactions.

Investigating the dynamics of A/D ATPases

A/D ATPase dynamics vary with cargo and organism. Visualizing these dynamics can help us elucidate the mechanisms that underlie each positioning reaction. For example, *E. coli* MinD forms dynamic oscillations on the membrane for divisome positioning (Wu et al., 2011; Hu et al., 2002), and ParA forms dynamics gradients on the nucleoid to position chromosomes and plasmids (Yamaichi et al., 2007). We attempted to label the A/D ATPases of *H. neapolitanus*, but were met with difficulties outlined below.

Like ParA, the Vecchiarelli group found that McdA from the cyanobacterium *S. elongatus* forms dynamic oscillations on the nucleoid to equally space carboxysomes down the cell length in response to its partner protein McdB (MacCready et al., 2018, 2020; Savage et al., 2010). McdA dimerizes and binds nonspecifically to the nucleoid. McdB acts as an adaptor by binding to carboxysomes and linking the carboxysome cargo with its positioning ATPase on the nucleoid. McdB-bound carboxysomes interact with and stimulate the release of McdA from the nucleoid. As a result, McdA is removed in the vicinity of carboxysomes, resulting in robust McdA oscillations on the nucleoid.
McdA dynamics in *S. elongatus* are dependent on carboxysome copy number (MacCready et al., 2018). With three or fewer carboxysomes in the cell, McdA was evenly distributed on the nucleoid except for a depletion zone immediately surrounding each carboxysome. In the presence of four or more carboxysomes, McdA oscillatory behavior returned. Therefore, McdA oscillations in *S. elongatus* are dependent on the presence of numerous McdB-bound carboxysomes. Given the large number of carboxysomes in a *H. neapolitanus* cell, we hypothesize that McdA would be dynamic; perhaps with even faster oscillations than what has been observed in *S. elongatus*. Unfortunately, labeling either terminus resulted in carboxysome aggregation, similar to the ∆mcdA phenotype (Figure S2-11A-E). AlphaFold2 structures of McdA from *H. neapolitanus* revealed an internal loop that may support the insertion of a fluorescent protein without affecting function (Figure S2-11A,F). Indeed, insertion of the fluorescent protein photoactivatable monomeric Cherry (PAmCherry) in this loop does not result in carboxysome aggregation (Figure S2-11E). Instead carboxysomes are distributed in the cell, similar to that of WT. Studies are currently underway to observe the dynamics of McdA in *H. neapolitanus* using this internal fluorescent fusion construct.

MinD dynamics have been investigated in organisms such as *E. coli*, *B. subtilis*, and *S. elongatus*. It has been observed to undergo oscillations (*E. coli* and *S. elongatus*) or tethering (*B. subtilis* and *S. elongatus*). Given the similarity of our system to that of *E. coli*, we expect *H. neapolitanus* MinD to oscillate. Unfortunately, mNG-minD fusions with varying linker lengths resulted in division defects, similar to the ∆minD strain (Figure S2-12A-B). Studies are currently underway to insert an internal fluorescent fusion, similar to that used for internally labelling McdA above.
The final three A/D ATPases (ParA, FlhG, and ParC) have yet to be labelled with a fluorescent protein. ParA is important for proper chromosome segregation and inheritance. In some systems, ParA localizes to the poles (as in *V. cholerae* ChrI, *C. glutamicum*, or *M. smegmatis*), while in other systems, ParA oscillates rapidly from pole-to-pole (as in *V. cholerae* ChrII). We anticipate that ParA in *H. neapolitanus* will adopt one of these two patterns. FlhG is important for regulating flagella number and location. In polar flagellates, FlhG has been shown to localize to the poles (Kusumoto et al., 2008). Although we have not yet tried to label FlhG in *H. neapolitanus*, we anticipate that it will also localize to the poles. ParC is important for chemotaxis cluster assembly and positioning. ParC is expected to interact with the nucleoid. However, chemotaxis cluster positioning in *H. neapolitanus* has not fully been elucidated. Chemotaxis clusters in young cells appear to be more dynamic, perhaps alluding to the possibility that *de novo* cluster assembly is positioned on the nucleoid. In older cells, chemotaxis clusters appear to snap onto the membrane, resulting in a less diffusive focus. How chemotaxis clusters assemble, potentially on the nucleoid, and then anchor to the membrane is an exciting avenue for future research.

Partner proteins and specificity determinants linking an A/D ATPase to its cargo

While A/D ATPases share sequence, structural, and biochemical commonalities, the partner proteins linking these ATPases to their cargos are extremely diverse. Due to this diversity, the partner protein has not always been identified, and as a result, many A/D ATPases are called ‘orphans’ (Lutkenhaus, 2012). The extreme diversity is largely due to the partner proteins providing the specificity determinants linking an A/D ATPase to
its cognate cargo. Despite their extreme diversity, data across the field supports the idea that partner proteins interact with and stimulate their A/D ATPases via a shared mechanism. Partner proteins involved in plasmid partition and chromosome segregation, as well as those required for positioning BMCs, flagella, chemotaxis clusters, and the divisome have all been shown, or suggested, to interact with their A/D ATPase via a positively charged and disordered N-terminus (Schumacher et al., 2021). Our in silico analysis of A/D ATPase dimers docked with N-terminal peptides of their partner proteins demonstrates how specific cargos are assigned, and how these related positioning systems coexist and function in the same cell without cross-interference.

Minimal Autonomous Positioning Systems

Going forward, we aim to use H. neapolitanus as a model to define the general mode of transport shared among the entire A/D ATPase family and to determine how positioning reactions are altered for disparate cargos. These findings are significant because A/D ATPases spatially organize essentially all aspects of bacterial cell function. An additional future direction is to experimentally verify the specificity determinants we identified here for each partner protein and cargo, and leverage this knowledge in the rational design of positioning systems in bacteria. These contributions are expected to be significant because minimal self-organizing systems are vital tools for synthetic biology (Schwille and Diez, 2009). We aim to design Minimal Autonomous Positioning Systems (MAPS) consisting of a positioning ATPase and their partner-protein N-terminal peptide as a “luggage tag” to be used as spatial regulators for natural- and synthetic-cargos in heterologous bacteria.
Summary

We have observed in *H. neapolitanus* the positioning reactions of five A/D ATPases, each dedicated to the spatial regulation of a distinct cellular cargo. This makes *H. neapolitanus* an ideal model for investigating cross-talk, coordination, and interdependencies among these systems. Future research will also experimentally probe the specificity determinants predicted in this chapter that govern each system.

References


Figures
Figure 2-1: ParA/MinD-like ATPases are widespread in bacteria. (A) 95% of sequenced bacterial genomes encode for at least one A/D ATPase and more than a third encode for multiple. Each spike represents a bacterial species and spike length indicates the number of unique A/D hits per bacterium. (B) *H. neapolitanus* encodes for seven putative ParA/MinD-like positioning systems. GNA implicates the putative cargos associated with each putative A/D ATPase. (C) MSA of each A/D ATPase from *H. neapolitanus* against experimentally-verified ParA/MinD family members further implicates the putative cargos identified by GNA. Each of the putative A/D ATPases in *H. neapolitanus* cluster with family members shown to position the indicated cellular cargos (orange) in other bacteria.
Figure 2-2: Each A/D ATPase in *H. neapolitanus* positions a specific cargo. (A-D) *Hn2335* is required for chromosome segregation. (A) *Hn2335* is found near *OriC* and has a ParB-homolog encoded downstream. (B, C) The origin region of the chromosome was tagged by labelling the ParB homolog. Light red: 1 focus/cell; dark red: 2 foci/cell. Short WT cells had a single focus at mid-cell, whereas longer cells had two foci at the quarter positions. Δ*Hn2335* cells displayed random positioning of ParB foci regardless of cell length. (D) Cartoon diagrams depict chromosome segregation in WT and Δ*Hn2335* cells. (E-H) *Hn1364* is required for divisome positioning. (E) *Hn1364* is found upstream of *minE*. (F, G) Divisome positioning was determined by the location of constriction sites. Each dot on the graph represents one identified constriction site. In WT cells, constriction sites were at mid-cell. In Δ*Hn1364*, constriction sites were found across the cell length. (H) Cartoon diagrams depict cell division in WT and Δ*Hn1364* cells. (I-L) Carboxysome positioning is determined by *McdA*. (I) *Hn0912* is found near genes encoding carboxysome shell proteins and RuBisCO. (J, K) Carboxysomes were visualized by labelling the small subunit of the Rubisco enzyme, *cbbS*. In WT cells, carboxysomes are distributed along the cell length. In Δ*mcdA*, carboxysomes formed large polar foci at one or both poles. (L) Cartoon diagrams depict carboxysome distribution in WT and Δ*Hn0912* cells. (M-P) *Hn0716* is required for regulating flagella position and copy number. (M) *Hn0716* is found near flagella-associated genes. (N, O) Flagella localization was visualized by labelling a component of the flagellar basal body, *fliN*. WT cells had a single polar FliN focus. In Δ*Hn0716* cells, FliN foci were more randomly positioned along the cell length. (P) Cartoon diagrams depict flagella localization and number in WT and Δ*Hn0716* cells. (Q-T) *Hn0722* is required for chemotaxis cluster positioning. (Q) *Hn0722* is found near chemotaxis-associated genes. (R, S) Chemotaxis clusters were visualized by labelling the response regulator, *cheY*. WT cells had a single CheY polar focus. Δ*Hn0722* mutant cells typically had no CheY foci. (T) Cartoon diagrams depict chemotaxis clusters in WT and Δ*Hn0722* cells. (All images) Scale bar: 2 μm. (All graphs) Cells were analyzed and quantified using MicrobeJ. On the x-axis, cells are sorted by increasing cell length. The y-axis represents the distance from mid-cell in microns; the center horizontal line equates to a distance of zero from mid-cell. Each dot represents where a focus was found along the length of the cell. For flagella, chemotaxis, and carboxysome graphs, the cell pole closest to a focus was oriented at the top; foci in the bottom half of the graph indicate the presence of a second focus. Graph axes for chromosome, carboxysome, flagella, and chemotaxis-labelled mutants: x-axis range (cell length): 0.8 to 2.1 μm; y-axis range (distance from mid-cell): -1.1 to 1.1 μm. X-axis of ΔflhG cells is 0.5 to 1.8 μm. Graph axes for divisome: x-axis range (cell length): 1.5 to 3.0 μm; y-axis range (distance from mid-cell): -1.5 to 1.5 μm.
**Figure 2-3: Cargo positioning is not directly controlled by A/D ATPases encoded at distant loci.** Each of the five cellular cargos were fluorescently tagged as indicated (Left): (A) chromosome (ParB-mNG), (B) divisome (invagination site), (C) carboxysomes (CbbS-mTQ), (D) flagella (mNG-FliN), and (E) chemotaxis clusters (CheY-mNG). The “Label Only” column shows the WT positioning of each of the fluorescent cargos. Deletion of an A/D ATPase resulted in the mislocalization of only its specific cargo (bolded rectangles). Graph axes for chromosome, carboxysome, flagella, and chemotaxis-labelled mutants: x-axis range (cell length): 0.8 to 2.1 μm; y-axis range (distance from mid-cell): -1.1 to 1.1 μm. All x-axes of ΔflhG cells are 0.5 to 1.8 μm. Graph axes for divisome: x-axis range (cell length): 1.5 to 3.0 μm; y-axis range (distance from mid-cell): -1.5 to 1.5 μm.
Figure 2-4: Anucleate cells form via three different mechanisms. (A) Deletion of the A/D ATPase required for chromosome (parA), divisome (minD), or flagellar (flhG) positioning resulted in anucleate cells (grey boxes). (B) Only ΔparA cells had mispositioned ParB foci. Cells with a single ParB focus are red. Cells with two ParB foci are black. ΔflhG cells maintained a single ParB focus in long cells, suggesting a DNA replication defect. (C) ParB foci are brighter only in ΔparA cells. (D) Deletion of minD or flhG resulted in divisome mispositioning. Constriction sites were considered “mid-cell” when found within 5% of the cell center along the long axis. (E) Deletion of flhG resulted in an intermediate effect on divisome positioning when compared to ΔminD.
Figure 2-5: Anucleate cells inherit carboxysomes. (A) Carboxysomes are present in anucleate cells (dashed circle). (B) Time-lapse microscopy shows that carboxysomes in ΔparA cells are bound to the nucleoid, but are inherited in anucleate cells via release from the extruded chromosome. Carboxysomes are also inherited in (C) ΔminD anucleate cells and (D) ΔflhG anucleate cells via the same mechanism. (All videos) White arrows highlight carboxysome bundling and subsequent release. Scale bar: 1 μm.
Figure 2-6: Deletion of parA, minD, or flhG influences chemotaxis cluster assembly. Deletion of parA, minD, or flhG influences chemotaxis cluster (A) number and (B) assembly. Only deletion of flhG influenced flagella (C) number and (D) assembly. **** p-value < 0.0001.
Figure 2-7: A/D ATPases have unique interfaces that confer cargo-positioning specificity. (A) Homodimer structures of the A/D ATPases in *H. neapolitanus* were generated using AlphaFold2 (cyan). Putative residues for homodimer specificity are highlighted magenta. (B) Dimers from (A) are oriented over their positioning matrix – nucleoid DNA or membrane are highlighted red. (C) Dimer structures docked with the N-terminal interacting peptide from putative partner proteins (orange), which confer cargo specificity. Predicted residues critical for this association are space-filled cyan on the ATPase and orange on the partner protein in the zoom.
Supplementary Figures
**Figure S2-1: Flanking Genes (FlaGs) analysis shows conservation among A/D ATPase gene neighborhoods.**

(A) The A/D ATPase gene involved in chromosome segregation (*parA*) is typically found a few genes downstream of the origin of replication (red cone) and upstream of a *parB* gene. 

(B) The A/D ATPase gene involved in divisome positioning (*minD*) is typically between genes encoding MinC and MinE. *minC* in *H. neapolitanus* was found elsewhere in the genome. 

(C) The A/D ATPase gene involved in carboxysome distribution (*mcdA*) is typically found near carboxysome shell components.

(D) The A/D ATPase gene involved in flagella positioning (*flhG*) is typically found near core components of the flagellar apparatus.

(€) The gene neighborhoods of conjugation operons are not well conserved. However, the A/D ATPase gene involved in spatially organizing conjugation (*virC1*) is typically found upstream of a ParB-like protein. The conjugation operons in *Methylovorans glucosetrophicus* and *P. bacteriovorus spreentiae* were found on plasmids pMsip01 and pI3WSM5005, respectively.

(G) Gene neighborhood of A/D ATPase genes associated with nitrogen metabolism are not well conserved. Only one other organism had similar neighboring genes as *H. neapolitanus*. 
Figure S2-2: Hn2335 is required for chromosome segregation in H. neapolitanus. 
(A) Hn2335 is found near the origin of replication (OriC) and has a ParB-homolog encoded immediately downstream. The genomic location of Hn2335 suggests it encodes for the chromosome segregation ParA ATPase. (B) The chromosome origin of replication was tagged by labelling the ParB homolog with mNeonGreen to form ParB-mNG. Population analysis of foci localization: Cells were analyzed and quantified using MicrobeJ. On the x-axis, cells are organized according to increasing cell length. The y-axis represents the distance from mid-cell (μm). The foci on the graphs represent where the ParB foci are found along the length of the cell. Light green: 1 focus/cell; dark green: 2 foci/cell. Short WT cells had a single ParB focus at mid-cell, whereas longer cells had two foci at the quarter positions. Scale bar: 2 μm (C) ΔHn2335 mutant cells displayed random positioning of ParB foci regardless of cell length. Scale bar: 2 μm (D) WT cells had 1-2 foci. 25% of ΔHn2335 cells had no foci. (E) ΔHn2335 cells had much brighter ParB foci compared to WT. Wilcoxon test p-value < 0.0001 (F) Newborn WT cells have a single ParB focus at mid-cell. Foci are then faithfully segregated to the quarter positions prior to division. Green: ParB foci; Magenta: SytoxBlue stain. Scale bar: 1 μm (G) In ΔHn2335, faithful foci positioning and segregation is lost. Because both ParB foci remain on the left-hand side of the dividing cell, the cell on the right becomes anucleate following cell division. Green: ParB foci; Magenta: SytoxBlue nucleoid stain. Scale bar: 1 μm
Figure S2-3: *Hn1364* is required for cell division positioning. (A) *Hn1364* is found directly upstream of *minE*. *minC* is also present elsewhere in the genome. Therefore, the MinCDE system is present and likely involved in divisome positioning in *H. neapolitanus*. (B, C) Divisome positioning was determined by the location of constriction sites. Cells were analyzed and quantified using MicrobeJ. For each mutant, ~300 dividing cells were analyzed for the location of their constriction sites relative to mid-cell. On the x-axis, cells are organized according to increasing cell length. The y-axis represents the distance from mid-cell (μm). Each dot on the graph represents one identified constriction site. In this density plot, light green represents high density and dark green represents low density. In WT cells, constriction sites were found close to mid-cell. In Δ*Hn1364*, constriction sites could be found throughout the length of the cell. Scale bar: 2 μm (D) WT cells had constriction sites at mid-cell (green arrows). Scale bar: 2 μm (E) Δ*Hn1364* cells were more likely to divide asymmetrically at non-mid-cell locations (green arrows). Multiple division sites could also be found simultaneously on the same cell. Scale bar: 2 μm (F) 97% of WT cells divided at mid-cell. Only 41% of Δ*Hn1364* cells divided at mid-cell. Constriction sites were considered “mid-cell” when they were found within 5% of the cell center along the long axis. (G) WT cells only had one division site per cell at any given time. In Δ*Hn1364*, 9% of dividing cells had multiple division sites per cell. (H) Mutant cells displayed greater variability in cell size. Wilcoxon test p-value < 0.0001 (I) *Hn1364* is critical for positioning the divisome at mid-cell in *H. neapolitanus*. 
Figure S2-4: Carboxysome positioning is determined by McdA, the ParA/MinD-like ATPase encoded in the carboxysome operon. (A) Hn0912 encodes McdA and is found near genes encoding carboxysome shell proteins and RuBisCO. (B) Carboxysomes were visualized by labelling the small subunit of the Rubisco enzyme (cbbS) with mTurquoise2 to form CbbS-mTurquoise2. Population analysis of foci localization: Cells were analyzed and quantified using MicrobeJ. On the x-axis, cells are organized according to increasing cell length. The y-axis represents the distance from mid-cell (μm). The foci on the graphs represent where the foci are found along the length of the cell. In WT cells, carboxysomes are distributed across the cell length. Scale bar: 2 μm (C) In ΔmcdA, carboxysomes formed a large polar focus at one or both poles. The pole of the cell that is closest to a focus is oriented to the top. Foci in the bottom half of the graph indicate a second focus. Scale bar: 2 μm (D) Carboxysome foci count varied dramatically between WT and mutant. In WT, 95% of cells had three or more foci, compared to only 5% of mutant cells. Instead, the vast majority of mutant cells had one or two carboxysome foci at the cell poles. Additionally, ~ 5% of the mutant population had no foci, which was never observed in WT cells. (E) Fluorescence intensity analysis of the foci revealed that, although the mutant population had fewer foci, the foci were significantly brighter. Wilcoxon test p-value < 0.0001 (F) In WT, carboxysomes are dynamically positioned along the cell length throughout the cell cycle and across multiple generations. Scale bar: 2 μm (G) In ΔmcdA, carboxysome aggregates were stagnant throughout the cell cycle and across multiple generations. Scale bar: 2 μm
Figure S2-5: *Hn0716* is required for regulating flagella position and copy number. (A) *Hn0716* is found near flagella-associated genes, suggesting it is involved in positioning flagella. (B) Flagella localization was visualized by labelling a component of the cytoplasmic ring of the flagellar basal body (*fliN*) with mNeonGreen to form mNG-FliN. Population analysis of foci localization: Cells were analyzed and quantified using MicrobeJ. On the x-axis, cells are organized according to increasing cell length. The foci on the graphs represent where the foci are found along the length of the cell. The pole of the cell that is closest to the FliN focus is oriented to the top. Foci under the mid-cell mark represent a second focus in the cell. WT cells typically had a single polar FliN focus. Scale bar: 2 μm (C) In Δ*Hn0716* cells, FliN foci were more randomly positioned along the cell length. Scale bar: 2 μm (D) 85% of WT cells had a single focus whereas only 45% of Δ*Hn0716* cells had a single focus. Also, instead of having a single polar focus, mutant cells were 3.5 times more likely to have two or more foci and 2.5 times more likely to have no foci at all. (E) When FliN foci were present in Δ*Hn0716* cells, the foci were much dimmer than those of WT cells. Wilcoxon test p-value < 0.0001 (F) Δ*Hn0716* was not motile in motility assays. (G) WT cells had a single polar flagellum next to the FliN focus. Δ*Hn0716* cells typically had multiple flagella, often as tufts, emanating from multiple FliN foci. Scale bar: 2 μm
Figure S2-6: *Hn0722* is required for chemotaxis cluster positioning. (A) *Hn0722* is found near chemotaxis-associated genes, suggesting its involvement in positioning chemotaxis clusters. (B) Chemotaxis clusters were visualized by labelling the response regulator (*cheY*) with mNeonGreen to form CheY-mNG. Population analysis of foci localization: Cells were analyzed and quantified using MicrobeJ. On the x-axis, cells are organized according to increasing cell length. The foci on the graphs represent where the foci are found along the length of the cell. The pole of the cell that is closest to the CheY focus is oriented to the top. Foci under the mid-cell mark represent a second focus in the cell. WT cells had a single CheY polar focus. Scale bar: 2 μm (C) Δ*Hn0722* mutant cells typically had no CheY foci. Scale bar: 2 μm (D) 87% of WT cells had one focus whereas only 19% of Δ*Hn0722* cells had a single focus. (E) When Δ*Hn0722* cells did have a detectable focus, the foci were much dimmer in fluorescence intensity compared to WT. Wilcoxon test p-value < 0.0001 (F) *Hn0722* is critical for chemotaxis cluster assembly and positioning in *H. neapolitanus*. 
Figure S2-7: AlphaFold2 structural predictions for the A/D ATPases of H. neapolitanus. (A) AlphaFold2 predicted models of A/D ATPases in H. neapolitanus overlaid showing similar sandwich homodimers. ParA (magenta), McdA (gray), ParC (cyan), MinD (salmon), and FlhG (green). (B) Predicted A/D ATPase structures from H. neapolitanus (purple) overlaid with experimentally determined homologs (gray) from the Protein Data Bank (PDB). ParA, McdA, ParC, MinD, and FlhG were overlaid with PDB ID #’s 5U1G, 6NOP, 5U1G 3QPL, and 4R23 respectively. ATP-binding pockets are zoomed in below - the indicated signature lysine defines the A/D ATPase family and interacts with the γ-phosphate of the ATP molecule (orange) bound to the opposing monomer. (C) In silico alanine-substitution simulations identify the H. neapolitanus MinE residues (orange) experimentally shown to be important for docking the Escherichia coli MinE peptide (green) onto the MinD dimer in cyan (PDB ID 3Q9L). Predicted H. neapolitanus MinD dimer is shown overlaid in cyan.
Figure S2-8: Complementation confirms the role of A/D ATPases in regulating specific cellular cargos. (A) **Chromosome:** WT cells had a single focus at mid-cell or two foci at the quarter positions. ΔparA cells displayed random positioning of ParB foci. After 6 hours of 50 µM IPTG induction, chromosome positioning returned to WT. (B) **Divisome:** In WT cells, constriction sites were at mid-cell. In ΔminD, constriction sites were found across the cell length. After 6 hours of 50 µM IPTG induction, constriction sites returned to mid-cell. (C) **Flagella:** WT cells had a single polar FliN focus. In ΔflhG, FliN foci were dim and more randomly positioned along the cell length. Leaky expression (without IPTG induction) of flhG was sufficient to restore foci positioning and intensity. (D) **Carboxysomes:** In WT cells, carboxysomes are distributed along the cell length. In ΔmcdA, carboxysomes formed large polar foci at one or both poles. After 3 hours of 50 µM IPTG induction, carboxysomes were redistributed throughout the cell.
(E) Chemotaxis: WT cells had a single CheY polar focus. ΔparC mutant cells typically had no CheY foci. After 3 hours of 50 µM IPTG induction, CheY foci were restored.
Figure S2-9: It remains unclear whether the conjugation operon in *H. neapolitanus* is active. (A) Conjugation machinery operon. Hn1669, the A/D ATPase,
is shown in orange. Neighboring genes are shown in grey. Downstream of \textit{Hn1669} is \textit{Hn1670}, a ParB-like protein. (B) An N-terminal fusion of \textit{Hn1670}, the downstream ParB-like protein, resulted in foci in some cells. (C) A C-terminal fusion of \textit{Hn1670}, the downstream ParB-like protein, resulted in foci in some cells but also diffuse signal in many cells. (D) The promoter in front of the conjugation operon was placed in front of LacZ for an assay. (E) A variety of conditions were tested to induce the conjugation operon. The conditions tested are represented by the orange dots. (F) Deletion of \textit{Hn1669} did not affect the positioning of other cargos.
Figure S2-10: All five cargos can be labelled simultaneously. Each cargo is visualized using the following fusion proteins and filter sets: 1) Chromosome: ParB-mApple, TexasRed filter, 2) Carboxysome: CbbS-mTq, CFP filter, 3) Chemotaxis: CheY-mNG, YFP filter, 4) Flagella: HaloTag-FliN, Cy5 filter, and 5) Divisome: no fluorescent fusion, phase contrast only.
Figure S2-11: *mcdA* can be tagged in the internal loop. (A) AlphaFold2 structures of McdA revealed a loop (magenta) as a potential labelling site. (B) In WT, carboxysomes are distributed along the cell length. (C) In Δ*mcdA*, carboxysomes aggregate at the poles. (D) Attaching the fluorescent protein, PAmCherry, on the N-terminus results in carboxysome aggregates, similar to Δ*mcdA*. (E) Attaching the fluorescent protein, PAmCherry, on the C-terminus results in carboxysome aggregates, similar to Δ*mcdA*. (E) AlphaFold2 predictions suggest that inserting PAmCherry between amino acids 59 and 60 in *mcdA* may not compromise structure or function. Carboxysomes in this strain resemble that of WT.
Figure S2-12: N-terminal tag on MinD results in asymmetrical division. (A) mNG was fused to MinD using a short 6 amino acid (GSGSGS) linker. (B) mNG was fused to MinD using a longer 12 amino acid (GSGSGSGSGSGS) linker. Orange arrow: invagination. Scale bar: 2 µm.
Supplementary Tables

**Table S2-1:** ParA/MinD family ATPase hits across the bacterial domain.

**Table S2-2:** Amino acids comprising the interfaces of ParA/MinD family ATPases in *H. neapolitanus* predicted to be important specificity determinants for homodimerization (Tab 1), positioning matrix association (Tab 2), and partner protein binding (Tab 3). Tab 4 shows *in silico* alanine-substitution mutagenesis and the resulting ΔΔG values for all residues comprising the N-terminal peptides of partner proteins docked onto their cognate A/D ATPase.
CHAPTER 3: The McdAB System Positions α-carboxysomes in Proteobacteria

This chapter is based on the following publication: Joshua S. MacCready*, Lisa Tran*, Joseph L. Basalla, Pusparanee Hakim, Anthony G. Vecchiarelli. “The McdAB system positions α-carboxysomes in proteobacteria”. Molecular Microbiology 2021 February 26, doi: https://doi.org/10.1111/mmi.14708. *Joshua S. MacCready and Lisa Tran share co-first authorship. For movies, see the above article.

Abstract

Carboxysomes are protein-based organelles essential for carbon fixation in cyanobacteria and proteobacteria. Previously, we showed that the cyanobacterial nucleoid is used to equally space out β-carboxysomes across cell lengths by a two-component system (McdAB) in the model cyanobacterium Synechococcus elongatus PCC 7942. More recently, we found that McdAB systems are widespread among β-cyanobacteria, which possess β-carboxysomes, but are absent in α-cyanobacteria, which possess structurally and phyletically distinct α-carboxysomes. Cyanobacterial α-carboxysomes are thought to have arisen in proteobacteria and then horizontally transferred into cyanobacteria, which suggests that α-carboxysomes in proteobacteria
may also lack the McdAB system. Here, using the model chemoautotrophic proteobacterium *Halothiobacillus neapolitanus*, we show that an McdAB system distinct from that of β-cyanobacteria operates to position α-carboxysomes across cell lengths. We further show that this system is widespread among α-carboxysome-containing proteobacteria and that cyanobacteria likely inherited an α-carboxysome operon via horizontal gene transfer from a proteobacterium lacking the *mcdAB* locus. These results demonstrate that McdAB is a cross-phylum two-component system necessary for positioning both α- and β-carboxysomes. The findings have further implications for understanding the positioning of other protein-based bacterial organelles involved in diverse metabolic processes.

Introduction

Bacterial Microcompartments (BMCs) are large cytosolic protein-based organelles that encapsulate sensitive metabolic processes to provide microbes with a distinct environmental growth advantage (Kerfeld *et al.*, 2018). Diverse in structure and function, BMCs have been identified across 29 bacterial phyla and ~20% of all sequenced bacterial genomes (Axen *et al.*, 2014), so their functions are of great ecological, evolutionary, biotechnological, and medical interest. Despite their prevalence and importance in bacterial metabolism, little is known about how BMCs are spatially regulated in the cell. The model BMC is the carboxysome, which is named for its involvement in carbon fixation. Carboxysomes are classified as either α or β depending on the type of Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) they encapsulate; α-carboxysomes contain Rubisco form 1A, β-carboxysomes contain
Rubisco form 1B, and both carboxysome types possess a phyletically distinct set of protein components necessary for encapsulation (Figure 3-1AB) (Rae et al., 2013). Since Rubisco can utilize either CO$_2$ or O$_2$ as a substrate, driving either the Calvin-Benson-Bassham cycle (CO$_2$) or the wasteful process of photorespiration (O$_2$), encapsulation of Rubisco and carbonic anhydrase within a selectively permeable protein shell generates a high internal CO$_2$ environment within carboxysomes that minimizes photorespiration (Price and Badger, 1989; Kaplan et al., 1991; Badger et al., 1998; Tcherkez et al., 2006). Through this mechanism, carboxysomes contribute to greater than 35% of global carbon fixation through atmospheric CO$_2$ assimilation (Cohen and Gurevitz, 2006; Kerfeld and Melnicki, 2016).

In our previous study, we identified and characterized the two-component McdAB system (Maintenance of Carboxysome Distribution protein A and B) required for maintaining the equidistant positioning of multiple carboxysomes along the nucleoid in the model rod-shaped cyanobacterium Synechococcus elongatus PCC 7942 (Maccready et al., 2018). We found that McdA, a ParA-type ATPase, non-specifically bound DNA in the presence of ATP. McdB, a novel small disordered protein, localized to carboxysomes and stimulated the ATPase activity of McdA, removing McdA from the nucleoid. Once removed, McdA then recycled its nucleotide and rebound the nucleoid at locations with the lowest concentration of McdB. The local removal of McdA created depletion zones in the vicinity of McdB-bound carboxysomes that subsequently evolved into emergent global oscillations of McdA. McdB-bound carboxysomes therefore generate and exploit dynamic McdA gradients to move in a directed and persistent manner towards increased concentrations of McdA on the nucleoid. Since we found that...
carboxysomes clustered in the absence of the McdAB system, we concluded that its Brownian-ratchet based distribution mechanism functioned primarily as an “anti-aggregation” system while simultaneously ensuring proper inheritance of carboxysomes.

More recently, we found that two distinct types of McdAB systems (Type 1 and Type 2) exist among β-cyanobacteria, which possess β-carboxysomes (MacCready et al., 2020). Surprisingly however, we also found that α-cyanobacteria, which possess α-carboxysomes, completely lack the McdAB system. We previously reported that many anoxygenic photosynthetic proteobacteria as well as chemoautotrophic bacteria (Figure 3-1C), also possess α-carboxysomes and potentially encode for McdA and McdB within their carboxysome (cso) operon (Figure 3-1D) (Maccready et al., 2018). Interestingly, α-carboxysomes are thought to have arisen in proteobacteria and were subsequently horizontally transferred into cyanobacteria, resulting in the distinct α-cyanobacterial lineage. Thus, it remains unclear whether proteobacterial α-carboxysomes are positioned by the McdAB system and whether this system evolved prior to or after horizontal transfer into cyanobacteria.

Here, we identify a novel McdAB system that positions α-carboxysomes in proteobacteria. In the sulfur-oxidizing chemoautotrophic proteobacterium *Halothiobacillus neapolitanus* c2, we show that uniform distributions of α-carboxysomes are lost in the absence of either McdA or McdB. As shown for the β-McdAB system in *S. elongatus*, we found that *H. neapolitanus* α-McdA and α-McdB interact with each other, and that α-McdB co-localizes with α-carboxysomes in vivo. Furthermore, we found that α-McdAB systems are widespread among α-carboxysome-containing proteobacteria,
some possessing two distinct copies of α-McdB. As we showed previously for β-McdB proteins, purified *H. neapolitanus* α-McdB undergoes liquid-liquid phase separation (LLPS) *in vitro*. Despite this shared LLPS activity, we found that different McdB types had different minimal oligomeric units *in vitro*. β-McdB Type 1 forms a hexamer, β-McdB Type 2 forms a dimer, and α-McdB remains a monomer. These results further our understanding of how different McdB proteins interface with their cognate McdA and carboxysome cargo. Collectively, we reveal a widespread McdAB system required for positioning structurally and phyletically distinct α-carboxysomes in proteobacteria and provide insights into the evolution of α-carboxysomes between cyanobacteria and proteobacteria. The findings have broad implications for understanding the subcellular organization of protein-based organelles in bacteria.

Results and Discussion

McdA- and McdB-like proteins distribute carboxysomes in *H. neapolitanus*

We began this study by testing the hypothesis that the McdA-like coding sequence that we previously identified within proteobacterial *cso* operons functioned to distribute α-carboxysomes in the cell (*Figure 3-1D*) (Maccready *et al.*, 2018). We also tested whether the small coding sequence following the putative *mcdA* gene encoded a functional homolog of cyanobacterial McdB. To explore this, we used the sulfur-oxidizing chemoautotroph *Halothiobacillus neapolitanus* c2 (hereafter *H. neapolitanus*), which has been established as a model system for studying α-carboxysomes in proteobacteria (Shively *et al.*, 1973; Cannon and Shively, 1983; Holthuijzen *et al.*, 1987; Schmid *et al.*, 2006; Dou *et al.*, 2008; Rae *et al.*, 2013; Cai *et al.*, 2015; Oltrogge *et al.*,...
First, in *H. neapolitanus*, we fused the native gene that encodes the small subunit of Rubisco (CbbS) with the fluorescent protein mTurquoise2 to form CbbS-mTQ (Goedhart *et al.*, 2012). The CbbS-mTQ signal appeared as several dynamic and overlapping foci (Movie 3-1), which from a single image, looked like a nearly homogeneous signal across the length of individual *H. neapolitanus* cells (Figure 3-2A). In *S. elongatus*, fluorescent-labeled carboxysomes are sufficiently separated from one another to be resolved (Savage *et al.*, 2010; Cameron *et al.*, 2013; Maccready *et al.*, 2018). In general, *H. neapolitanus* carboxysomes are greater in number (4 to 18) and smaller (40 to 200 nm diameter) compared to the fewer (3 to 5) and larger (150 to 400 nm) β-carboxysomes of *S. elongatus* (Cannon *et al.*, 2001; Schmid *et al.*, 2006; Savage *et al.*, 2010; Rae *et al.*, 2013; Maccready *et al.*, 2018; Sun *et al.*, 2019). Therefore, we interpret this data as α-carboxysomes being distributed across the cell length (Figure 3-2A), but the smaller size of α-carboxysomes combined with their high-copy number precludes single carboxysome resolution using traditional fluorescence microscopy imaging of *H. neapolitanus* cells, which are also relatively small (0.5 x 1.5 µm). We therefore performed Transmission Electron Microscopy (TEM) to determine if this CbbS-mTQ signal in wild-type cells indeed represented assembled and distributed carboxysomes. Consistent with our interpretation of the fluorescent imaging, we frequently found that α-carboxysomes were distributed over the nucleoid region of the cell and down the medial axis (Figure 3-2B). The distribution of fluorescent-labelled carboxysomes in our TEM images are very similar to those shown previously by other groups imaging native carboxysomes in *H. neapolitanus* cells (Shively *et al.*, 1973; Cannon and Shively, 1983; Schmid *et al.*, 2006; Dou *et al.*, 2008; Rae *et al.*, 2013).
Next, using this CbbS-mTQ α-carboxysome reporter, we individually deleted our candidate \textit{mcdA} or \textit{mcdB} genes. In the absence of either \textit{mcdA} or \textit{mcdB}, the homogenous distribution of α-carboxysomes down the cell length was lost (Figure 3-2CE and Movie 3-1). Instead, the majority of cells had a single high-intensity CbbS-mTQ focus at one pole (~76\% of n=210 cells for ∆\textit{mcdA} and ~66\% of n=220 cells for ∆\textit{mcdB}) (Figure 3-2G). Additionally, 7\% of ∆\textit{mcdA} and 5\% of ∆\textit{mcdB} cells displayed two foci at opposing cell poles. The remaining cells (17\% of ∆\textit{mcdA} and 29\% of ∆\textit{mcdB}) lacked a CbbS-mTQ focus entirely, suggesting that these cells did not inherit α-carboxysomes after division and that the carboxysomes have yet to be assembled \textit{de novo}. We performed TEM on the ∆\textit{mcdA} and ∆\textit{mcdB} strains to determine if these massive foci of CbbS-mTQ represented assembled carboxysomes or amorphous protein aggregates of carboxysome components. In the absence of either \textit{mcdA} or \textit{mcdB}, the vast majority of cells (~75\% of n=214 cells) had a single cluster of multiple assembled carboxysomes (Figure 3-1DF). Once again, the TEM assisted in our interpretation of the fluorescent imaging. Now that we have identified the McdAB systems of α and β carboxysomes, from this point forward, we distinguish the two systems according to the type of carboxysome being distributed: α-McdAB systems position proteobacterial α-carboxysomes and β-McdAB systems position cyanobacterial β-carboxysomes.

Our identification of an α-McdAB system for the positioning of proteobacterial α-carboxysomes is intriguing on multiple fronts. First, α-carboxysomes are typically much smaller and more numerous than β-carboxysomes in cells (Cannon \textit{et al.}, 2001; Schmid \textit{et al.}, 2006; Rae \textit{et al.}, 2013; Sun \textit{et al.}, 2019). Therefore, given that \textit{H. neapolitanus}
cells are also smaller (0.5 x 1.5 microns) than *S. elongatus* cells (1.3 x 3 microns), it could have been reasoned that α-carboxysomes would rely on random diffusion for their inheritance and distribution in the cell; whereas the larger size and lower copy number of cyanobacterial β-carboxysomes necessitate an active positioning system. However, since we found that assembled α-carboxysomes aggregated towards the polar region of cells in the absence of α-McdA or α-McdB, reminiscent of β-carboxysomes in the absence of β-McdA or β-McdB (Maccready *et al.*, 2018), we conclude that all McdAB systems actively position carboxysomes to not only ensure proper inheritance following cell division, but to primarily function as an anti-aggregation system for protein-based organelles that have the capacity to self-associate. Our findings provide an explanation as to why α- and β-carboxysomes, when heterologously expressed in the absence of their cognate McdAB systems, undergo extreme aggregation in other bacterial species or in plant chloroplasts (Bonacci *et al.*, 2012; Lin *et al.*, 2014; Baumgart *et al.*, 2017; Long *et al.*, 2018). It is very likely that adding the two-component McdAB system will solve the aggregation issue that arises when carboxysome components are expressed in these heterologous hosts.

In addition to α- and β-carboxysome size and quantity being significantly different between *S. elongatus* and *H. neapolitanus*, so is their nucleoid biology. While newborn *H. neapolitanus* cells are monoploid (Desmarais *et al.*, 2019), *S. elongatus* cells are polypoid; harboring as many as 10 copies of their chromosome (Chen *et al.*, 2012). Some β-cyanobacteria that we have previously shown to encode an McdAB system, such as *Synechocystis sp.* PCC 6803, can have over 50 chromosome copies (Zerulla *et al.*, 2016). Since McdA uses the nucleoid as a matrix for positioning McdB-bound
carboxysomes, our findings show that McdAB systems can function on nucleoids with drastically different chromosome contents. It remains to be determined how chromosome content influences McdA dynamics and carboxysome positioning on the nucleoid. It has been shown that the copy numbers of both carboxysomes and chromosomes are regulated in cyanobacteria by environmental growth conditions (Sun et al., 2019; Ohbayashi et al., 2019). It is possible that expression levels of the McdAB system may also be regulated by environmental cues, such as nutrient availability.

Without the McdAB system, clustered carboxysomes are nucleoid excluded to the cell poles.

Macromolecular crowding of the cytoplasm in combination with the nucleoid acting as a formidable diffusion barrier have been shown to be responsible for the polar localization of several large intracellular bodies in bacteria (Straight et al., 2007; Winkler et al., 2010), such as plasmids lacking an active positioning system (Erdmann et al., 1999; Ringgaard et al., 2009). Without a ParA-system, plasmids that were once distributed equally along the nucleoid length, become nucleoid ‘excluded’ – the passive effect of the nucleoid as a diffusion barrier to mesoscale bodies in the cytoplasm (Planchenault et al., 2020). Therefore, it seems that ParA-based positioning systems, such as McdAB, not only overcome the nucleoid as a diffusion barrier, but exploit it as a matrix for the movement and positioning of their mesoscale intracellular cargos.

*H. neapolitanus* is a halophile capable of growing under extreme hyperosmotic conditions where the cell membrane pushes against the compacted nucleoid (see Figure 3-2D and F), so that the nucleoid forms a barrier in the cell that can even restrict
the diffusion of GFP (Van Den Bogaart et al., 2007; Konopka et al., 2009). We therefore asked if the polar localization of carboxysome clusters in the ΔmcdA and ΔmcdB strains is the result of nucleoid exclusion. To explore this question, we used 4′,6-diamidino-2-phenylindole (DAPI) to stain the nucleoids of WT, Δα-mcdA, and Δα-mcdB H. neapolitanus strains encoding the CbbS-mTQ α-carboxysome reporter. In wild-type cells, carboxysome foci were confined within the nucleoid signal (PCC = 0.80, n=128 cells) (Figure 3-3A). The finding is consistent with our previous report that the McdAB system uses the nucleoid as a matrix to position carboxysomes (Maccready et al., 2018). In Δα-mcdA (PCC = 0.36, n=436 cells) or Δα-mcdB (PCC = 0.33, n=135 cells) strains, α-carboxysome foci were nucleoid excluded at the cell poles (Figure 3-3BC). Intriguingly, the nucleoids of both the ΔmcdA and ΔmcdB strains are shifted away from midcell in the opposing direction of the carboxysome cluster. The data suggests that the polar cluster of carboxysomes is large and dense enough to interfere with nucleoid placement in the cell. In some instances, the carboxysome clusters grew large enough to locally expand and deform the rod-shaped morphology of the cell itself (Figure S3-1).

In the deletion strains, it was unclear whether the nucleoid-excluded carboxysomes interacted to form aggregates at the cell poles or whether they simply accumulated at the poles, but without actually sticking to each other. To address this question, we sought to increase the cytoplasmic space by elongating cells with cephalexin treatment. However, the additional chromosomal content in these elongated cells prevented us from determining if carboxysomes aggregated in a manner that was independent of nucleoid exclusion (Figure S3-2). We next tried the gyrase-inhibitor ciprofloxacin to condense the nucleoid, which significantly increased the cytoplasmic
space in the cell (Figure 3-3D-I). In WT cells, carboxysomes remained colocalized with the compacted nucleoid after ciprofloxacin treatment (Figure 3-3D-E). In the ΔmcdA and ΔmcdB strains, carboxysomes remained aggregated despite the significant increase in diffusible cytoplasmic space (Figure 3-3F-I). Altogether, these data show that the McdAB system functions to both position carboxysomes on the nucleoid and to prevent their aggregation.

Upon their discovery, it was frequently noted that the carboxysomes of cyanobacteria and chemoautotrophs are commonly associated with the nucleoid region of the cell (Gantt and Conti, 1969; Shively et al., 1970; Wolk, 1973; Shively et al., 1973). Our work shows that, for both groups of bacteria, it is the McdAB system that is responsible for this association. We also find that in the absence of the α-McdAB system, nucleoid positioning and cell morphology are altered by the carboxysome aggregate, which likely has drastic physiological consequences. While α- and β-carboxysome aggregation does not result in a high CO₂-requiring phenotype (Maccready et al., 2018; Desmarais et al., 2019), we recently found that β-carboxysome aggregation in S. elongatus results in slower growth, cell elongation, asymmetric cell division, and altered cellular levels of Rubisco (Rillema et al., 2020). It was also recently found that inactive carboxysomes are degraded at polar regions of a cyanobacterial cell (Hill et al., 2020). How the polar localization of carboxysome aggregates, from a lack of an McdAB system, perturbs carboxysome function and turnover remains unclear. A future direction will be the study of α- and β-McdAB systems under varying biotic and abiotic conditions to understand the impact of carboxysome mispositioning and aggregation on cellular physiology.
α-McdB is targeted to α-carboxysomes and interacts with α-McdA

We recently showed that β-McdB acts as an adaptor between β-McdA on the nucleoid and β-carboxysomes in the cyanobacterium S. elongatus. To determine whether H. neapolitanus α-McdB is targeted to α-carboxysomes, we expressed a second copy of α-McdB as a fusion to the fluorescent protein mNeonGreen (mNG) (Shaner et al., 2013), producing mNG-α-McdB, in our native CbbS-mTQ strain. Consistent with mNG-β-McdB loading onto β-carboxysomes in S. elongatus (Maccready et al., 2018), we found that mNG-α-McdB colocalized with the CbbS-mTQ signal of H. neapolitanus α-carboxysomes (PCC = 0.81; n=173 cells) (Figure 3-4A and Movie 3-2). Due to the diffuse nature of the α-carboxysome fluorescence signal, as described above, we took advantage of our finding that α-carboxysomes cluster to form bright and clearly resolved puncta at the cell poles without α-McdA present. In our Δα-mcdA deletion strain, mNG-α-McdB clearly colocalized with α-carboxysome aggregates (PCC = 0.90; n=355 cells) (Figure 3-4B and Movie 3-3). The data shows that α-McdB associates with α-carboxysomes, and α-McdA is not required for this association.

Finally, as we did for the β-McdAB system in S. elongatus, we sought to determine if α-McdA and α-McdB of H. neapolitanus self-associate and directly interact with each other by performing a Bacterial-2-Hybrid assay (B2H) in E. coli. Consistent with ParA-family members forming dimers (Schumacher, 2007), including a β-McdA-like homolog (Schumacher et al., 2019), α-McdA was positive for self-association (Figure 3-4C). Also like the β-McdAB system of S. elongatus, α-McdA directly interacts with α-McdB. Surprisingly however, we found that H. neapolitanus α-McdB did not self-
associate in our B2H assay, whereas *S. elongatus* β-McdB strongly self-associates (Maccready et al., 2018). It is difficult to draw firm conclusions on the lack of McdB self-interaction because the T18/T25 domains may sterically hinder the interaction, or influence protein stability. Therefore, we subsequently performed Size Exclusion Chromatography – Multiple Angle Laser Light Scattering (SEC-MALS) and found that purified α-McdB indeed remains a monomer in solution (Figure 3-4D). This difference in α- and β-McdB self-association has implications for understanding how McdB proteins are recruited to structurally distinct α- and β-carboxysomes as well as how McdBs interact with their cognate McdA ATPase on the nucleoid.

Defining the conserved features of α-McdAB proteins

We recently found that McdAB systems are widespread across β-cyanobacteria, which possess β-carboxysomes (MacCready et al., 2020). A surprising finding in this search was that McdAB systems were absent in α-cyanobacteria, which possess α-carboxysomes. Thus, our finding here of an α-McdAB system that positions α-carboxysomes in *H. neapolitanus* was unexpected. We next sought to determine how widespread this system was among α-carboxysome-containing proteobacteria. Given the amino acid sequence diversity we previously found among cyanobacterial β-McdA proteins, and especially for β-McdB proteins, we first searched for α-McdAB systems by performing neighborhood analyses within and around proteobacterial α-carboxysome operons. Using BlastP, we identified α-carboxysome-containing proteobacteria within NCBI and JGI IMG databases using α-carboxysome components CsoS2, CsoS4A, or CsoS4B as queries (see Figure 3-1D). Next, we searched around each α-carboxysome
operon in these organisms to identify α-mcdAB-like genes. We classified positive hits for α-mcdA as proteins containing the deviant-Walker-A box, which defines the ParA family of ATPases (Koonin, 1993), as well as Walker A’ and Walker B boxes. We classified positive hits for α-mcdB as a small coding sequence immediately following the α-mcdA gene.

Alignment of the amino acid sequences of all α-McdA hits identified through our analyses revealed a high percentage of similarity (~53% pairwise identity), but also revealed three regions of conservation among α-McdA proteins not present among classical ParA-type proteins (Figure 3-5A and Figure S3-3). While α-McdA proteins were largely conserved, α-McdB proteins displayed extreme diversity. This was also true for cyanobacterial β-McdAB systems (MacCready et al., 2020). All α-McdB hits also shared many of the general sequence features present in β-McdB proteins, including: (i) a well-conserved charged N-terminus, (ii) an invariant C-terminal tryptophan residue (all ended with the amino acid sequence V(I)WPD), (iii) low hydrophobicity, (iv) biased amino acid composition, and (v) intrinsic disorder (Figure 3-5B and Figure S3-4).

α-McdAB systems are widespread among α-carboxysome-containing proteobacteria

We previously found that many β-cyanobacteria had mcdA and mcdB genes at distant loci from the operons encoding carboxysome components (MacCready et al., 2020). Our identification of highly conserved regions that are specific to α-McdA proteins, along with the conserved features among α-McdB proteins, allowed us to re-search proteobacterial genomes to identify more α-McdA and α-McdB genes that did not fall within or near the α-carboxysome operon. Across 250 α-carboxysome-
containing proteobacterial genomes, we identified 228 α-McdA sequences (~91% of genomes analyzed) and 278 α-McdB sequences (100% of genomes analyzed) (Figure 3-6A). These results present a similar trend to what we previously found in several β-cyanobacteria - β-McdB can exist without the presence of β-McdA (“orphaned” McdBs) and β-McdB proteins greatly varied in length (51 – 169 amino acids) (Figure 3-6A). Interestingly, 12% of the genomes analyzed contained a second α-mcdB gene (see McdB sequences labelled as “Special Orphan” in Figure S3-4). Orphaned McdB paralogs lack the charged N-terminus conserved in McdB proteins that are encoded adjacent to the mcdA gene. Aside from this distinction, both McdB paralogs are predicted to be completely disordered and both have the invariant V(I)WPD sequence present across all α-McdB proteins identified thus far. To our knowledge, this is the first example of two cognate ParA partner protein paralogs potentially involved in the same process.

In total, we found three possible genomic arrangements of α-mcdA and α-mcdB genes: (i) α-mcdA and α-mcdB were both within the α-carboxysome operon (Figure 3-6B), (ii) only α-mcdB was found within the operon (Figure 3-6C), or (iii) one orphan α-mcdB gene was within the operon, and a second α-mcdB gene was present at a distant locus but next to α-mcdA (Figure 3-6D). Consistent with our identification of orphan McdB proteins, we have recently found that McdB in S. elongatus plays an important, but currently unclear, role in carboxysome function outside of its role in positioning carboxysomes with McdA (Rillema et al., 2020). Indeed, ParA-partner proteins involved in the positioning of other protein-based cargos, such as chemotaxis clusters (Ringgaard et al., 2011; Roberts et al., 2012; Alvarado et al., 2017), are also key
functional components of the cargo itself. We note however that 76% of genomes without α-mcdA were still incomplete, therefore it is still possible that an unidentified α-mcdA gene is present and next to the additional α-mcdB gene in these genomes.

Overall, we found that α-McdAB systems are present across most major taxonomic classes and orders of proteobacteria; largely present in the class γ-proteobacteria (60% of genomes) and order Chromatiales (45% of genomes) (Figure 3-6E). Unlike cyanobacteria, which perform oxygenic photosynthesis, the metabolisms of α-carboxysome-containing proteobacteria can greatly vary (see Figure 3-1C). We found α-McdAB systems in nitrite, ammonia, and iron utilizers, as well as biocathode electroautotrophs that can acquire energy by taking up electrons from electrodes, while using CO₂ as an inorganic carbon source and terminal electron acceptor (Nevin et al., 2010) (Figure 3-6E). However, α-McdAB systems were primarily found in purple sulfur bacteria (38% of genomes), which perform anoxygenic photosynthesis, and sulfur oxidizing chemoautotrophs, such as *H. neapolitanus* (33% of genomes). Purple sulfur bacteria use sulfide and hydrogen as an electron donor, whereas purple non-sulfur bacteria utilize organic compounds (Madigan and Jung, 2009). Interestingly, the size and quantity of β-carboxysomes in cyanobacteria are directly linked to not only CO₂ availability, but also light intensity and quality (Sun et al., 2016; Rohnke et al., 2018; Sun et al., 2019; Rilléma et al., 2020; Rohnke et al., 2020). Given the prevalence of McdAB systems in sulfur utilizers, it is intriguing to speculate that sulfur-limitation will also alter α-carboxysome size, number and/or distribution in *H. neapolitanus*. 
Cyanobacterial α-carboxysomes likely originated from a proteobacterium lacking α-mcdA within their cso operon.

It is largely believed that α-carboxysomes emerged in proteobacteria and then were horizontally transferred into cyanobacteria; creating the distinct phylogenetic clade of α-cyanobacteria (Badger et al., 2002; Badger and Price, 2003; Marin et al., 2007; Badger and Bek, 2008; Rae et al., 2013). However, following our recent study (MacCready et al., 2020), why α-cyanobacteria lack the McdAB system remained an outstanding question. In an attempt to better understand α-carboxysome evolution among α-cyanobacteria and proteobacteria, we constructed a Maximum Likelihood phylogenetic tree inferred using a concatenation of the major α-carboxysome components CbbL, CbbS, CsoS3, CsoS4A, and CsoS4B (see Figure 3-1D). We note that the major carboxysome component CsoS2 was omitted from this analysis due to its intrinsically disordered nature (Oltrogge et al., 2020), which has relaxed selection due to the lack of structural constraint.

Recall that we found α-carboxysome-containing proteobacteria encoding two α-McdB genes, where one α-mcdB gene is orphaned within the cso operon without an adjacent α-mcdA gene, and the other is encoded next to α-mcdA at a distant locus away from the cso operon (see Figure 3-6D). Intriguingly, we found that α-carboxysomes from proteobacteria with two copies of α-mcdB formed a single clade (Figure 3-7A). This clade was more closely related to α-cyanobacterial α-carboxysomes than to proteobacterial α-carboxysomes that have both α-mcdA and α-mcdB encoded within the cso operon. Indeed, while H. neapolitanus possess both α-mcdA and α-mcdB within their cso operon (Figure 3-7B), many other proteobacterial species, such as
*Thiohalospira halophila* DSM 15071, only possess an orphaned \( \alpha-mcdB \) within their *cso* operon; \( \alpha-mcdA \) and the second \( \alpha-mcdB \) gene are encoded at a distant locus (Figure 3-7C). Thus, our phylogenetic tree suggests that \( \alpha \)-cyanobacteria, such as *Cyanobium gracile* PCC 6307, likely horizontally inherited a *cso* operon from a proteobacterium that encoded \( \alpha-mcdA \) and a second \( \alpha-mcdB \) gene away from the *cso* operon (Figure 3-7D). While it is possible that \( \alpha \)-cyanobacteria inherited the orphan \( \alpha-mcdB \) gene present within the proteobacterial *cso* operon, it is likely that this protein no longer provided a fitness advantage in the absence of \( \alpha-McdA \), or the second \( \alpha-McdB \) protein, and was therefore lost.

Our finding that McdAB systems are widespread in \( \beta \)-cyanobacteria and proteobacteria, but completely absent in \( \alpha \)-cyanobacteria raises the question: What is different about \( \alpha \)-carboxysomes in \( \alpha \)-cyanobacteria that makes active distribution by the McdAB system unnecessary? In general, \( \beta \)-carboxysomes are significantly larger in diameter (\( \beta \)-carboxysome of *Spirulina platensis* is > 500 nm) than proteobacterial \( \alpha \)-carboxysomes (\( \alpha \)-carboxysome of proteobacterium *Nitrobacter agilis* ~ 120 nm), and of the limited number of \( \alpha \)-carboxysomes studied in \( \alpha \)-cyanobacteria, these are typically the smallest (*Prochlorococcus marinus* ~ 90 nm) (Rae *et al.*, 2013). \( \alpha \)-carboxysome copy number in the cell is also typically much higher. This inverse correlation of carboxysome size and copy number is also true for plasmids. High-copy plasmids are typically smaller, whereas low-copy plasmids are larger (Planchenault *et al.*, 2020).

Intriguingly, it was recently found across the sequenced genomes of enterobacteria that ParA-based plasmid partition systems are present in larger plasmids (>25 kb) and lacking in smaller plasmids (Planchenault *et al.*, 2020). All plasmids larger than 180 kb
encoded a ParA-based partition system without exception. Therefore it is attractive to speculate that, like for small plasmids, the α-carboxysomes of α-cyanobacteria may rely on their high-copy number and smaller size for inheritance, which would make the McdAB system dispensable in these organisms. In line with this reasoning, not a single active distribution system for encapsulin nanocompartments that are even smaller in diameter (~ 30 nm) has yet to be identified.

α-McdAB systems are distinct from the β-McdAB systems of cyanobacteria

Given the quantity of α- and β-McdAB systems that we have identified across cyanobacteria and proteobacteria, we next sought to identify similarities and differences among these subtypes. We previously identified two distinct β-McdAB systems exclusive to β-cyanobacteria, with Type 2 systems being more ancestral and more abundant than Type 1 (MacCready et al., 2020). Here, we found that α-McdA proteins share more features with β-McdA Type 2 proteins, including: (i) the presence of the signature lysine residue in the deviant-Walker A box, which defines the ParA family but is absent in β-McdA Type 1, (ii) a conserved central tryptophan residue in region 1, (iii) a highly similar Walker B box, and (iv) greater conservation in region 4 (Figure 3-8A). This further demonstrates the divergence of Type 1 β-McdA proteins (present in S. elongatus), which lack the signature lysine residue in the deviant-Walker A box and possesses a large mid-protein extension (MacCready et al., 2020).

Next, we examined the similarities and differences among all McdB amino acid sequences identified. ParB proteins that connect plasmid or chromosomal cargo to their cognate ParA ATPase use a charged N-terminal region to interact with ParA and
stimulate its ATPase activity (Baxter and Funnell, 2014; Badrinarayanan et al., 2015). We found that all McdB types encoded next to their cognate mcdA gene also possess conserved highly-charged N-termini (Figure 3-8B). But in proteobacteria where the second α-mcdB gene is orphaned in the cso operon, this highly-charged N-terminus is absent (Figure 3-8B). Given this observation, we designate α-McdB proteins with the charged N-terminal region and encoded downstream of the α-mcdA gene as Type 1, and α-McdB proteins lacking the charged N-terminus and orphaned in the cso operon as Type 2. It remains to be determined if the charged N-terminus of McdB proteins is indeed responsible for interaction with McdA. Further dividing α- and β-McdB proteins, we find that α-McdB proteins lack the predicted coiled-coil region and mid-protein glutamine-rich stretches conserved among β-McdB proteins (Figure 3-8B). Despite these differences among subtypes, all McdB proteins possess an invariant C-terminal tryptophan residue. This amino acid is intriguing because many proteins involved in the assembly of viral- or phage-capsids also contain a tryptophan at their C-terminus (Deeb, 1973; Skoging and Liljeström, 1998; Tsuboi et al., 2003; Komla-Soukha and Sureau, 2006; Marintcheva et al., 2006; Johnson et al., 2020). Given the capsid-like icosahedral structure of the carboxysome, it is attractive to speculate that the C-terminal tryptophan residue plays a role in McdB recruitment to carboxysomes.

Our finding that many proteobacteria possess an additional distinct copy of α-McdB was intriguing. We have yet to identify a cyanobacterial species with two mcdB genes (MacCready et al., 2020). The need for two α-McdB proteins is not clear, but an absence of the charged N-terminus that is conserved in the McdB paralog suggests a role in modulating carboxysome interactions with McdA on the nucleoid. These α-McdB
paralogs may work together in modulating interactions with α-McdA, possibly tuning the stimulation of McdA ATPase activity and release from the nucleoid. For instance, both McdB copies likely associate with carboxysomes, but only the paralog with the charged N-terminus would interact with α-McdA. Therefore the relative ratio of these McdB paralogs would dictate the extent to which carboxysomes associate with McdA on the nucleoid. Paralog interactions regulating each other’s function is not uncommon. One such example is FtsZ paralogs (FtsZ1 and FtsZ2) involved in archaeal cell division (Liao et al., 2020) and chloroplast division (Chen et al., 2018). In the case of chloroplasts, these paralogs assemble into heteropolymers at mid-chloroplast to define the division site. FtsZ1 increases FtsZ2 turnover, which is thought to facilitate FtsZ2 remodeling during chloroplast constriction (TerBush and Osteryoung, 2012). In this way, FtsZ1 functions as a regulator of FtsZ2 activity and is a highly conserved feature throughout photosynthetic lineages (TerBush et al., 2018). Elucidating how α-McdB paralogs influence carboxysome function and organization is an exciting avenue of future research.

All McdB types show LLPS activity, but with different minimal oligomeric units. A conserved feature across all McdB types we identified is intrinsic disorder, but to varying degrees (Figure 3-8C). While β-McdB Type 1 proteins are on average 41% disordered and β-McdB Type 2 proteins are on average 64% disordered, α-McdB proteins are significantly more disordered at ~ 95%. This dramatic difference in disorder for α-McdB proteins is likely due to the lack of the predicted coiled-coil found in both β-McdB types (see Figure 3-8B). In addition to intrinsic disorder, all McdB proteins share
low complexity regions with repetitive and biased amino acid compositions, and low hydrophobicity. All these features are hallmarks of proteins that can undergo Liquid-Liquid Phase Separation (LLPS) (Alberti et al., 2019). LLPS refers to the ability of biomolecules, such as protein, to separate in solution to form a condensed liquid phase with material properties distinct from those of the surrounding dilute phase. We have previously shown that purified representatives from both types of β-McdB proteins (β-McdB Type 1 from *S. elongatus* and β-McdB Type 2 from *Synechococcus sp. PCC 7002*) can undergo LLPS *in vitro* (MacCready et al., 2020). We purified *H. neapolitanus* α-McdB and found that it too has LLPS activity, forming droplets with liquid-like behaviors such as the fusion of two adjacent droplets into one (Figure 3-8D, Movie 3-4). We conclude that LLPS is a conserved activity across all McdB proteins, but it remains to be determined how McdB LLPS activity plays a role in carboxysome positioning and function.

The fact that β-McdB proteins have a predicted coiled-coil, while α-McdB proteins are almost completely disordered, suggests that β-McdB proteins form oligomers. We performed Size Exclusion Chromatography – Multiple Angle Laser Light Scattering (SEC-MALS) to determine the oligomeric state of purified α- and β-McdB proteins. We found that *S. elongatus* Type 1 β-McdB formed a hexamer, *Synechococcus sp. PCC 7002* Type 2 β-McdB formed a dimer, and consistent with lacking a predicted coiled-coil, *H. neapolitanus* α-McdB remained a monomer (Figure 3-8E). These findings are consistent with our B2H data, where β-McdB of *S. elongatus* strongly self-associates (MacCready et al., 2018), whereas *H. neapolitanus* α-McdB showed no self-association (see Figure 3-4C). We conclude that the predicted coiled-coil domains exclusive to β-
McdB proteins are likely required for oligomerization and are important for β-carboxysome positioning and function, whereas α-McdB proteins function as monomers.

A future study will determine how differences in oligomeric state influence the ability of McdB proteins to undergo LLPS.

McdAB systems are not restricted to carboxysome BMCs

Lastly, an outstanding question is whether the McdAB system is restricted to carboxysome BMCs. While carboxysomes are the only known anabolic BMC, several other catabolic BMCs exist (Kerfeld et al., 2018), including: (i) EUT (Ethanolamine UUtilization microcompartment), (ii) PDU (1,2-Propanediol Utilization microcompartment), (iii) GRM (Glycyl Radical enzyme-containing Microcompartment), (iv) PVM (Planctomycetes and Verrucomicrobia Microcompartment), (v) ETU (ETHanol-Utilizing microcompartment), (vi) RMM (Rhodococcus and Mycobacterium Microcompartment), (vii) MUF (Metabolosome of Unknown Function microcompartment), (viii) MIC (Metabolosome with Incomplete Core microcompartment), (ix) SPU (Sugar Phosphate Utilizing microcompartment), and (x) BUF (BMC of Unknown Function) (Figure 3-8F). In four such examples, we find McdB- and/or McdA-like sequences within or neighboring these BMC operons (Figure 3-8F). In cases where McdB-like sequences were observed, all possess a C-terminal aromatic residue (tyrosine instead of tryptophan) within the last 4 amino acids. As we detailed above, a C-terminal tryptophan residue is invariant across all carboxysome-associated
McdB proteins we have identified to date (Figure 3-8B), and may play a role in McdB association with their cognate BMC.

Collectively, our results show that α-McdAB systems are widespread among α-carboxysome containing proteobacteria and function as an anti-aggregation mechanism to ensure proper distribution and inheritance of α-carboxysomes following cell division. Our results have important implications for understanding the evolution and function of diverse McdA and McdB proteins as it relates to structurally and phyletically distinct α- and β-carboxysomes, and also have much broader implications for understanding the equidistant positioning of diverse catabolic BMCs across the bacterial domain.

Future directions
The McdAB system can redistribute carboxysome aggregates

In WT cells, carboxysomes are distributed over the nucleoid region along the cell length. However, in the ΔmcdA strain, carboxysomes form nucleoid-excluded aggregates at one or both cell pole(s). When the carboxysome positioning system was expressed from an exogenous locus in the ΔmcdA strain, carboxysome distribution over the nucleoid region of the cell was restored (Figure S2-8D). Interestingly, we noticed that some cells showed carboxysome aggregates pulled onto the nucleoid region of the cell (1 hour post-induction) prior to the carboxysome aggregates being fully distributed (3 hours post-induction) (Figure S3-5A). The data suggests that in addition to positioning newly assembled carboxysomes, the McdAB system can counter the self-aggregating nature of carboxysomes. That is, the McdAB system can rip aggregates apart, and then redistribute individual carboxysome across the cell length. We conclude
that the McdAB is not only an anti-aggregation system for self-assembling cargos, it can also distribute pre-formed carboxysome aggregates.

To observe carboxysome redistribution by the McdAB system, we ran time-lapse microscopy under the same induction conditions (50 µM IPTG). We observed that carboxysomes were either first dragged onto the nucleoid before being separated into individual carboxysomes (Figure S3-5B) or carboxysomes were individually plucked from the "mother" aggregate (Figure S3-5C). We hypothesize that these differences correlate with the size of the mother aggregate. Smaller aggregates have a sufficiently small radius such that allows them to be first dragged onto the nucleoid before being ripped apart. Larger aggregates on the other hand have too large a radius to get pulled into the nucleoid region of the cell and therefore remain at the pole. In this regime, the "mother" aggregate remained at the pole, and only individual “daughter” carboxysomes are sufficiently small enough to get distributed over the nucleoid by the McdAB system. If this were true, there should be a correlation between carboxysome foci intensity and restoration behavior, which is a question for future study.

Alternatively, it is possible that lower concentrations of McdA and/or McdB are insufficient for dragging the entire carboxysome aggregate onto the nucleoid. In this case, low McdAB levels may only be sufficient to pluck off individual carboxysomes. At sufficiently higher McdAB levels, the entire aggregate may only then be dragged onto the nucleoid. This phenotypic variation is expected given the cell-to-cell variability of protein expression. In order to correlate McdAB levels to the observed phenotypes, we must be able to quantify McdAB at the cellular level. Unfortunately, both McdA and McdB have proven tricky to fluorescently label; attempts to label either protein have
resulted in carboxysome aggregation. To circumvent this obstacle, we plan to express a diffuse fluorescent protein in the same operon as an indirect measure of the amount of McdAB in each cell. A diffuse fluorescent reporter protein will give us the relative protein expression levels among the population, allowing us to correlate expression levels with recovery phenotype.

Summary

Little is known about how BMCs are spatially regulated in the cell. In this chapter, I demonstrate that, although structurally and phyletically distinct from β-carboxysomes in β-cyanobacteria, α-carboxysomes found in proteobacteria also possess McdAB systems as spatial regulators. We further show that McdAB positioning systems are widespread in carboxysome-containing bacteria.

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Figures

Figure 3-1: Overview of α- and β-carboxysome composition, operon structure and prevalence in proteobacteria. (A) Cartoon illustration of internal reactions (left) and known components (right) of β-carboxysomes and (B) α-carboxysomes. (C) α-carboxysome-containing proteobacteria display diverse metabolic capacities. (D) *H. neapolitanus* α-carboxysome operon. Red = Rubisco or Rubisco associated, green = CsoS2 which mediates Rubisco/shell interaction, blue = carbonic anhydrase, purple = putative *mcDA* gene, yellow = putative *mcDB* gene, dark grey = shell component, light grey = hypothetical protein (Hyp). Gene colors are matched to the proteins shown in panel B and functionally-equivalent proteins for β-carboxysomes in panel A.
**Figure 3-2: An McdAB system positions α-carboxysomes.** (A) The carboxysome reporter CbbS-mTQ is distributed in WT *H. neapolitanus* cells. Scale bar: 2 µm. (B) Electron micrograph showing α-carboxysomes confined to the nucleoid in WT cells. Scale bar: 200 nm. (C) Homogenous distribution of α-carboxysomes is lost in the absence of McdA. Scale bar: 2 µm. (D) Polar localization of assembled α-carboxysomes in the absence of McdA (yellow arrow). Scale bar: 200 nm. (E) Homogenous distribution of α-carboxysomes is lost in the absence of McdB. Scale bar: 2 µm. (F) Polar localization of assembled α-carboxysomes in the absence of McdB (yellow arrow). Scale bar: 200 nm. (G) Quantification of carboxysome distributions. In ∆mcdA (n = 210 cells), 76% of cells had a single polar focus, 7% displayed two foci at opposing poles, and 17% lacked a focus. In ∆mcdB (n = 220 cells), 66% of cells had a single polar focus, 5% displayed two foci at opposing poles, and 29% lacked a focus.
Figure 3-3: Aggregated α-carboxysomes are nucleoid excluded. (A) The carboxysome reporter CbbS-mTQ colocalizes with the DAPI-stained nucleoid in WT *H. neapolitanus* cells. In (B) ΔmcdA and (C) ΔmcdB mutants, aggregated α-carboxysomes are nucleoid excluded. PCC values were calculated from n ≥ 100 cells per cell population. (D) In WT cells, carboxysomes colocalize with the DAPI-stained nucleoid. In ∆mcdA (F) and ∆mcdB (H) strains, aggregated carboxysomes are excluded from the DAPI-stained nucleoid. After ciprofloxacin treatment of ∆mcdA (G) and ∆mcdB (I) strains, the nucleoid condenses and carboxysomes remain aggregated despite the increased cytoplasmic space. Scale bar: 2 µm.
Figure 3-4: α-McdB loads onto α-carboxysomes and interacts with α-McdA. (A) mNG-McdB (magenta) colocalizes with the carboxysome reporter CbbS-mTQ (cyan) in WT H. neapolitanus. (B) In the absence of McdA, McdB strongly colocalizes with carboxysome aggregates. PCC values were calculated from n ≥ 100 cells per cell population. Scale bar: 2 µm. (C) Bacterial-2-Hybrid (B2H) analysis of α-McdA and α-McdB. α-McdA was positive for self-association. α-McdA directly interacts with α-McdB. α-McdB did not self-associate. B2H image is representative of 3 independent trials. (D) SEC-MALS plot for H. neapolitanus α-McdB; monomer MW = 10 kDa.
Figure 3-5: Conserved features among α-McdAB proteins within cso operons. (A) Features conserved or distinct (boxed) among identified α-McdA proteins encoded within the cso operon of α-carboxysome-containing proteobacteria compared to classical ParA-type proteins involved in the positioning of diverse cargoes. The deviant-Walker A (blue), A’ (red), and B (purple) boxes, are conserved among all ParA family proteins. ParA-type proteins shown: *Escherichia coli* phage P1 ParA (plasmid partitioning—YP_006528) (Abeles *et al*., 1985), *Escherichia coli* F plasmid SopA (plasmid partitioning—NP_061425) (Mori *et al*., 1986), *Caulobacter crescentus* ParA (chromosome segregation—AAB51267) (Mohl and Gober, 1997), *Caulobacter crescentus* MipZ (cell-division positioning—NP_420968) (Thanbichler and Shapiro, 2006), *Rhodobacter sphaeroides* PpfA (chemotaxis cluster distribution—EGJ21499) (Roberts *et al*., 2012), and *Bacillus subtilis* Soj (chromosome segregation—NP_391977) (Marston and Errington, 1999). (B) General features of α-McdB proteins encoded within the cso operon of α-carboxysome-containing proteobacteria. Percent composition of the amino acids alanine (A), proline (P), lysine (K), serine (S), and threonine (T) are presented to illustrate the strong bias for these amino acids (center). All α-McdB proteins identified are highly hydrophilic across the entire primary sequence (bottom).
Figure 3-6: McdAB systems are widespread among carboxysome-containing proteobacteria. (A) Table highlighting the prevalence and genomic context of all identified α-McdAB sequences within α-carboxysome-containing proteobacteria. (B) Genomic arrangement when α-McdAB are encoded within the cso operon. (C) Genomic arrangement when only α-McdB is encoded within the cso operon. (D) Genomic arrangement when one copy of α-McdB is encoded within the cso operon and a second copy of α-McdB is encoded next to α-mcdA at a distant locus. (E) α-McdAB systems are widely distributed among proteobacterial taxonomic classes (left), orders (center) and metabolisms (right).
Figure 3-7: α-carboxysome evolution among proteobacteria and α-cyanobacteria. (A) Inferred phylogeny of α-carboxysome-containing proteobacteria and α-cyanobacteria. Line colors: α-McdAB found within cso operon (black), only α-McdB found within cso operon (red), and neither α-McdAB found within cso operon (blue). Black dot represents >70% bootstrap support (500 replicates). Green asterisk represents a shared cso operon ancestor among α-cyanobacteria and proteobacteria that lacks the α-mcdA gene. (B) Genomic arrangement of the H. neapolitanus cso operon. (C) Genomic arrangement of the T. halophila cso operon. (D) Genomic arrangement of the C. gracile cso operon, which lacks an McdAB system.
Figure 3-8: Similarities and differences among all known McdA and McdB proteins. (A) Features conserved or distinct among α-McdA (green), β-McdA Type 1 (red), β- McdA Type 2 (blue), and diverse classical ParA-type proteins. All Walker-boxes are well-conserved among all classic ParA family proteins. ParA-type proteins shown: Escherichia coli phage P1 ParA (plasmid partitioning—YP_006528) (Abeles et al., 1985), Escherichia coli F plasmid SopA (plasmid partitioning—NP_061425) (Mori et al., 1986), Caulobacter crescentus ParA (chromosome segregation—AAP51267) (Mohl and Gober, 1997), Caulobacter crescentus MipZ (cell-division positioning—NP_420968) (Thanbichler and Shapiro, 2006), Rhodobacter sphaeroides PpfA (chemotaxis cluster distribution—EGJ21499) (Roberts et al., 2012), and Bacillus subtilis Soj (chromosome segregation—NP_391977) (Marston and Errington, 1999). (B) α-McdB proteins lack the predicted central coiled-coil and glutamine-rich regions found in β-McdB proteins. α- McdB Type 2 proteins lack the charged N-terminus conserved in all other McdB types. (C) PONDR disorder scatter plot for all McdB protein types. (D) DIC microscopy images showing purified H. neapolitanus McdB undergoes LLPS in vitro in the presence of the crowders PEG or Ficoll. Droplets exhibit liquid-like properties such as fusion (yellow arrows). (E) SEC-MALS plot for a representative β-McdB Type 1 (S. elongatus McdB; monomer MW = 17 kDa), β-McdB Type 2 (Synechococcus sp. PCC 7002 McdB; monomer MW = 21 kDa), and α-McdB (H. neapolitanus McdB; monomer MW = 10 kDa). (F) McdA/B-like sequences genomically neighbor BMC components across diverse microbes.
Figure S3-1: Aggregation of α-carboxysomes caused a local cell bulging morphology in the absence of (A) α-McdA or (B) α-McdB. Scale bar: 2 µm.
Figure S3-2: WT and ΔmcdB cells treated with cephalexin caused cell elongation, but no significant increase in cytoplasmic space due to added chromosomal content. (A) WT and (B) ΔmcdB cells with CbbS-mTQ labelled carboxysomes (cyan) and DAPI-stained nucleoids (red). (C) WT and (D) ΔmcdB cells treated with cephalexin caused cell elongation, but no significant increase in cytoplasmic space due to added chromosomal content. Scale bar: 2 µm.
Figure S3-3: MSA of identified α-McdA sequences from proteobacteria
Figure S3-4: MSA of identified α-McdB sequences from proteobacteria. α-McdB proteins designated as “Special Orphan” are encoded alone in cso operon (without the mcdA gene upstream), but there is an additional α-McdB paralog encoded next to α-McdA elsewhere in the genome.
Figure S3-5: Carboxysome redistribution on the nucleoid is dependent on McdAB. (A) mcdAB genes were placed under the pTrc promoter. 1 hour after IPTG induction (50 µM), carboxysomes were observed to be over the nucleoid of the cell. 3 hours after IPTG induction, carboxysomes were distributed along the cell length. Scale bar: 2 µm (B) Sometimes, large carboxysome aggregates were dragged onto the nucleoid before being separated into smaller carboxysome foci. The white arrow indicates the carboxysome aggregate. Scale bar: 1 µm (C) Sometimes carboxysome aggregates remained at the poles while individual carboxysomes are plucked from the larger aggregate. The white arrow indicates the polar carboxysome aggregate. Scale bar: 1 µm
CHAPTER 4: Coordinated Spatial Regulation of a Flagellum and Chemotaxis Cluster in the Bacterium *Halothiobacillus neapolitanus*

Introduction

Bacteria vary in flagella location, number, and pattern. Many bacteria encode for an A/D ATPase called FlhG in their flagellar operon, which is essential for diverse flagellation patterns, yet the mechanism remains unclear. In most polar flagellates, deletion of *flhG* resulted in changes to flagellar number but not positioning \(^1\text{–}^{13}\) (Table 1-5); \(\Delta\text{flhG}\) cells were hyperflagellated at the pole. In *H. neapolitanus*, however, FlhG is important for both flagella number and positioning. In the absence of FlhG, flagella were bundled together as tufts in non-polar regions of the cell, resulting in decreased motility. Interestingly, deletion of *flhG* also resulted in cell division defects (Figure S2-5). The differences between an *flhG* deletion in *H. neapolitanus* compared to that of other organisms suggest a unique mechanism of interaction. These mechanisms currently remain unexplored.

Chemotaxis clusters are also important for motility. In *Vibrio* species, unipolar chemotaxis clusters localized to both poles in mature cells, allowing each daughter cell to inherit a chemotaxis cluster\(^{14,15}\). Deletion of *parC* resulted in mislocalization of foci,
leading to defects in inheritance (Table 1-6). The data in Chapter 2 suggests that chemotaxis cluster inheritance in *H. neapolitanus* varies from organisms previously studied. Firstly, chemotaxis clusters did not exhibit bipolar localization. Instead, most cells, regardless of cell length, had a single unipolar focus, suggesting that cells did not immediately inherit a chemotaxis cluster. Secondly, in the absence of ParC, CheY signal was diffuse, suggesting that ParC may also play a role in chemotaxis cluster assembly. The mechanism that underlies chemotaxis cluster assembly and inheritance in *H. neapolitanus* remains to be studied.

Interestingly chemotaxis cluster number and fluorescence intensity were affected by the deletion of *flhG* (Figure 2-6). In this chapter, we explore the inheritance of both the flagellum and chemotaxis throughout the cell cycle. This preliminary data sheds light on the potential crosstalk during the spatial regulation of these two systems. I found that, immediately following cell division, a new flagellum and new chemotaxis cluster are assembled at the new pole of the daughter cell lacking these structures. The other daughter cell inherits the “old” flagellum and chemotaxis cluster. In previously investigated organisms, both of these cargos assemble prior to division and are localized to the old poles before cell division. The localization of these cargos to the new pole after cell division is a novel observation for both flagella and chemotaxis clusters, providing further evidence that flagella and chemotaxis clusters in *H. neapolitanus* are positioned via a distinct unstudied mechanism.
Results

FlhG positions the *de novo* assembly of a single flagellum at the new pole of the aflagellate daughter cell immediately following division in *H. neapolitanus*.

In *H. neapolitanus*, deletion of *flhG* resulted in hyper-flagellation at non-polar regions of the cell (Figure S2-5), leading us to conclude that *flhG* is critical for regulating flagella number and positioning. This was surprising because in other polar flagellates investigated to date, deletion of *flhG* affected flagella number but not location (Table 1-5). This difference in phenotype suggests a novel mechanism of action for *flhG* and led us to ask the following questions: when do cells acquire a new flagellum and what is the role of *flhG* in determining flagella position and number? To answer these questions, I used time-lapse microscopy to visualize a mNG fusion of FliN, which is a component of the flagellar basal body that assembles at the cytoplasmic face of the membrane.

As previously shown, WT cells had a single mNG-FliN focus at the extreme cell pole (Figure 2-2N, Figure S2-5). Surprisingly, the new FliN focus formed immediately following division, but only on the new pole of the aflagellate daughter cell (Figure 4-1A-B). This observation is contrary to what has been observed in other monotrichous model organisms. In Δ*flhG*, FliN foci formed at the new pole in only 24% of dividing cells (Figure 4-1B). The remaining cells did not form a focus (33%), formed a focus at the old pole (29%), or formed a focus in a nonpolar region of the cell (14%) (Figure 4-1B-C). The role of *flhG* in localizing the new flagellum to the new pole of a bacterium is an uninvestigated and novel phenomenon.
ParC positions the *de novo* assembly a single chemotaxis cluster immediately proximal to the new pole of the daughter cell lacking a chemotaxis cluster in *H. neapolitanus*.

In *H. neapolitanus*, chemotaxis clusters also localized near a single pole, similar to the localization of the flagellum (Figure 2-2). However, the polar localization of chemotaxis clusters is different from the systems that have previously been investigated (Table 1-6). In *Vibrio* species, as cells mature and prepare to divide, unipolar foci become bipolar foci prior to division, thus allowing both daughter cells to inherit a chemotaxis cluster\(^{14,15}\). In *H. neapolitanus*, essentially all cells, even long ones about to divide, had a single focus (Figure 2-2R), suggesting a different mode of chemotaxis cluster inheritance. To address this gap in knowledge, I used time-lapse microscopy to visualize CheY, a response regulator that has been shown to localize to chemotaxis clusters\(^{14,16,17}\).

As previously shown, WT cells had a single CheY-mNG focus proximal to one cell pole (Figure 2-2R, Figure S2-6). Shortly after cell division, the new CheY focus formed immediately adjacent to the new pole of the newborn cell (Figure 4-2A), contrary to what has been observed in other model organisms. This novel localization of a chemotaxis cluster was surprising, but reminiscent of the observations made for flagella positioning in *H. neapolitanus*. In ΔparC, CheY signal was diffuse in ~80% of the cells (Figure 4-2B), with a small proportion of cells still forming CheY foci, albeit transient. The similar localization patterns of flagella and chemotaxis clusters led us to investigate a possible crosstalk in their spatial regulation.
Flagella spatial organization by FlhG also influences chemotaxis cluster assembly and positioning.

Both the flagellum and chemotaxis cluster were found to localize to the new pole of the daughter cell lacking these structures shortly after cell division. Although flagella and chemotaxis clusters are involved in the same biological function, chemotaxis, no studies thus far have demonstrated a relationship in the spatial organization of these two cargos. However, the unexpected localization of both cargos to the new pole led us to investigate whether the A/D ATPase-based positioning of these cargos occurred independently of each other or whether they exhibited interdependency. We found that flagella localization was unaffected by the deletion of parC (Figure 4-3AB). However, chemotaxis cluster assembly and localization to the new pole was negatively affected by the deletion of flhG (Figure 4-3CD). My data suggests a role for FlhG, flagellum assembly, and/or flagellum positioning in the assembly of chemotaxis clusters, but the mechanism remains unknown.

The flagellum and chemotaxis cluster share the same cell pole, but are adjacent to one another.

Given the genetic association of ΔflhG cells having negatively affected chemotaxis cluster assembly and positioning, and the fact that both structures occupied the same pole, we set out to determine the order of assembly and relative positioning of these two structures using time-lapse microscopy. We could not spatiotemporally resolve exactly when a division event completed in H. neapolitanus using phase contrast imaging. However, we could resolve the start of invagination, and we therefore used this event to
monitor the time-to-assembly for both structures. Flagellum assembly and positioning at the new pole occurred at what seems to be immediately after cell division (Figure 4-3A,C), or ~28 minutes (n = 13 cells) after the start of invagination. Chemotaxis cluster assembly and positioning, on the other hand, was significantly slower, ~87 minutes (n = 13 cells) after the start of invagination. Together, we find that the flagellum quickly assembles following division in the aflagellate daughter, followed by a slower assembly of the chemotaxis cluster at the same pole. However, it is important to note that these mutants were independently imaged on different days, making the timing vulnerable to variation.

To investigate the relative positioning and coordinated timing of these cargos, I labelled both in the same strain. In cells that only have a single focus, 100% of those foci are FliN and not CheY, supporting our previous hypothesis that assembly of the flagellum precedes that of the chemotaxis cluster. In cells that had foci for both cargos, these foci were localized to the same cell pole in 96% of cases (n=103). Despite being localized to the same cell pole, the two structures rarely overlap (Figure 4-4AB). Instead, the flagellum is found at the immediate cell pole, whereas the chemotaxis cluster is polar-adjacent (Figure 4-4ABC). In future studies, time-lapse microscopy will be used to track the assembly of both structures throughout the cell cycle to better pinpoint the time to assemble each structure. We will then set out to determine the molecular players involved in the crosstalk between the A/D-based positioning of these two cellular cargos, both involved in chemotaxis and cell motility.
Discussion and Future Directions

Why the new pole?

*H. neapolitanus* cells have a single mNG-FliN focus at the extreme cell pole. We observed via time-lapse microscopy that the new FliN focus forms at the new pole of only the aflagellate daughter cell, while the other daughter inherits the old flagellum (*Figure 4-1A*). This was surprising because almost all monotrichous bacteria studied to-date synthesize new flagella at the old pole opposite the flagellated pole prior to division. There is only one published example of this occurring in Magnetotactic bacteria of the gammaproteobacterial class\(^\text{18}\). Lefèvre *et al.* hypothesized that placing the flagellum at the septum circumvents the need to reprogram their motility apparatus to align with their magnetic polarity. However, this organism was not genetically tractable and was therefore not subject to further research. Here, we have identified an organism that is not magnetotactic, is genetically tractable, and has no immediately obvious reason for maintaining flagellar polarity. A future direction will be to investigate the mechanisms driving this currently unstudied mode of polarity.

ParC directs the *de novo* assembly of the chemotaxis cluster at a single nucleoid pole.

Most CheY foci remain relatively static near the cell pole. However, during their assembly, CheY foci appear more dynamic. These mobile foci eventually transitioned into more stagnant state, as if they became anchored into the membrane at a certain point in the assembly process. I hypothesize that chemotaxis clusters are assembled on the nucleoid and subsequently anchored to the membrane. Importantly, I labelled CheY, which may localize to chemotaxis clusters later, masking the dynamics at the start of
chemotaxis cluster assembly. Future studies will test the exciting hypothesis that chemotaxis clusters potentially assemble on the nucleoid and then transfer onto the membrane when ready for chemotactic signaling.

Flagella positioning influences the spatial regulation of chemotaxis clusters

Similar to flagellum positioning, the chemotaxis cluster assembled and localized to the new cell pole of the daughter lacking these structures (Figure 4-2A), while the other daughter cell inherits both structures from the mother. This was surprising because polar chemotaxis clusters in Vibrio species localize to both old poles prior to division, and both daughters inherit these structures. The similar localization patterns of flagella and chemotaxis clusters led us to question whether the assembly of chemotaxis clusters is linked to flagella positioning.

Through a series of deletion mutants, we found that chemotaxis clusters were affected by a deletion of flhG but flagella were not affected by a deletion of parC. Consistently, I also find that the flagellum quickly assembles first following division, whereas the chemotaxis cluster assembles later and more slowly. The findings suggest the spatiotemporal regulation of the flagellum has upstream control of chemotaxis cluster assembly. It is well known that chemotaxis clusters direct cell motility by controlling the direction of flagellar rotation. However, to our knowledge, this is the first study showing crosstalk in the spatial regulation of flagella and chemotaxis clusters. Future studies will determine the molecular players involved in the crosstalk between A/D-based positioning of these two cellular cargos both involved in cell motility.
How does flhG affect cell division?

In addition to defects in flagella positioning and chemotaxis cluster assembly, ΔflhG cells also exhibited a strong defect in cell division and DNA replication (see Figure 2-4). In Campylobacter jejuni, cell division defects have also been observed in ΔflhG cells, in addition to changes to flagella number and motility\(^2\). It is attractive to speculate that since the new flagellum is positioned on the new pole, near the site of cell division machinery, the spatial regulation of one could indirectly affect the other.

References

Figure 4-1: A single flagellum forms at the new pole of the daughter cell lacking a flagellum. (A) WT cells had a single FliN focus at the extreme cell pole. Shortly after cell division, a new focus formed on the new pole of the new cell. Filled green arrows indicate new foci at the new pole. (B) In WT (n=55), 100% of dividing cells formed the new focus at the new pole. In ΔflhG (n=21), the new focus formed at the new pole 24% of the time. The remaining cells did not form a focus (33%), formed a focus at the old pole (29%), or formed a focus in a nonpolar region of the cell (14%). When cells had multiple foci, the analysis was done according to the brightest focus. (C) In ΔflhG, FliN foci were no longer faithfully positioned. New foci formed randomly in the new cell. Empty white arrows indicate missing foci where foci should have been found. Scale bar: 1 µm
Figure 4-2: A chemotaxis cluster forms at the new pole of the daughter cell lacking a chemotaxis cluster. (A) WT cells had a single CheY focus near the extreme cell pole. Shortly after cell division, a new focus formed on the new pole of the new cell. Filled green arrows indicate new foci at the new pole. (B) In ΔparC, CheY signal was diffuse. Scale bar: 1 µm
Figure 4-3: Flagella positioning influences the spatial regulation of chemotaxis clusters. (A) WT cells had a single FliN focus at the extreme cell pole. Shortly after cell division, a new focus formed on the new pole of the new cell. (B) ParC deletion did not affect flagella positioning. ΔparC cells had a single FliN focus at the extreme cell pole. Shortly after cell division, a new focus formed on the new pole of the new cell. (C) WT cells had a single CheY focus near the extreme cell pole. Shortly after cell division, a new focus formed on the new pole of the new cell. (D) FlhG is required for chemotaxis cluster assembly and positioning. In ΔflhG, CheY signal was typically diffuse. For all panels: filled green arrows indicate new foci at the new pole while empty white arrows indicate missing foci where foci should have been found. Scale bar: 1 μm
Figure 4-4: The flagellum and chemotaxis cluster share the same cell pole and are immediately adjacent to one another. (A) Chemotaxis clusters and flagella localize to the same pole. (B) Diagram representing the colocalization of both chemotaxis clusters and flagella foci to the same pole. Scale bar: 2 μm
CHAPTER 5: General Discussion and Future Directions

General discussion

The ParA/MinD (A/D) family of ATPases spatiotemporally regulates a growing list of diverse mesoscale complexes critical to fundamental processes in prokaryotes, including cell growth and division, DNA segregation, motility, and conjugation. Therefore, understanding how A/D ATPases coordinate the positioning of cellular cargos at the correct location at the correct time is key to understanding bacterial cell function. Using H. neapolitanus as a non-pathogenic model, our findings strongly support the idea that each ATPase is dedicated to the positioning of a specific cellular cargo. Importantly, our model also revealed the dependency of protein-based organelle trafficking on the faithful segregation of the chromosome as well as the positioning of cell division at mid-cell.

Despite the need to understand the coordinated positioning of cellular cargos, one reason why this coordination remains uninvestigated is because the study of subcellular organization in prokaryotes has largely been “cargo-centric”. A prime example is the carboxysome BMC. Despite being studied since 1950, their A/D-based positioning system was only recently identified. This gap remained because the study of BMCs has focused on assembly, structure, function, and re-purposing for synthetic biology. Moreover, the study of A/D ATPases is often focused on a specific cargo of a certain
biological process. In these studies, two questions are typically posed: How does a specific cargo find its correct position, and how does this position change over time. Here, our focus was on the positioning systems, rather than a specific cargo-type or biological process, and as such, is the first systems biology approach to address how multiple A/D ATPases coordinate the positioning of diverse cargos in the same cell.

Another reason why the coordinated positioning of multiple cargos remains largely unstudied because there is little overlap in the organisms used for studying the chromosome, the divisome, and the protein-based organelles shown to require an A/D ATPase for positioning. Prior to our study, most organisms investigated only had 1-2 confirmed A/D ATPase positioning systems. In Chapter 2, we identify a plethora of organisms with multiple putative A/D-based positioning systems. We chose an organism that had five A/D ATPase for five putative disparate cargos and verified the roles of all five A/D ATPases. As such, our study will serve as a foundation for investigating the coordinated positioning of diverse cargoes.

In summary, how these positioning reactions coordinate with each other and the timing of the DNA segregation and cell division is entirely unstudied. The data provided here are the first towards a systems biology view of spatial organization encompassing the fundamental processes of chromosome segregation, cell division, and protein-based organelle trafficking in prokaryotes mediated by the ParA/MinD family of ATPases.

Future directions

ParA deletion had a direct impact on chromosome segregation but the resulting asymmetric chromosome inheritance also led to indirect defects in the positioning of
carboxysomes. Previous studies in *S. elongatus* demonstrated that carboxysomes used the nucleoid as a matrix for positioning. Therefore, it was unsurprising that carboxysome positioning was affected in anucleate cells. However, *S. elongatus* is not a model used for studying chromosome inheritance. Our model, which has both systems, allowed us to witness the strength and permanence of carboxysome interaction with DNA. Future studies will investigate the mechanisms underlying the role of McdB in positioning carboxysomes on DNA. Observations from the experiments in Chapters 2 and 3 provide invaluable clues that can inform these mechanistic studies.

We also identified a previously uncharacterized epistatic relationship in flagella positioning by FlhG and its downstream influence on the spatial regulation of the chemotaxis cluster by ParC. It is well known that chemotaxis clusters direct cell motility by controlling the direction of flagellar rotation. However, to our knowledge, this is the first study showing crosstalk in the spatial regulation of flagella and chemotaxis clusters. While exploring this possible interaction further, we discovered that both the new flagellum and the new chemotaxis cluster are positioned at the new pole of the same cell lacking these structures shortly after division. The localization of these cargos to the new pole after cell division is a novel observation for both flagella and chemotaxis clusters. Future studies will determine the molecular players involved in the crosstalk between A/D-based positioning of these two cellular cargos both involved in cell motility.

Going forward, we aim to use *H. neapolitanus* as a model to define the general mode of transport shared among the entire A/D ATPase family and to determine how positioning reactions are altered for disparate cargos. These findings are significant because A/D ATPases spatially organize essentially all aspects of bacterial cell
function. We plan to experimentally verify the specificity determinants we identified in Chapter 2 for each partner protein and cargo, and leverage this knowledge in the design of positioning systems for both natural- and synthetic-cargos in bacteria. These contributions are expected to be significant because minimal self-organizing systems are vital tools for synthetic biology (Schwille and Diez, 2009). For example, carboxysomes are positioned by the A/D ATPase McdA (MacCready et al., 2018), and several other BMC operons encode McdA homologs (MacCready et al., 2021). Many groups are repurposing BMCs for the production of fuels, therapeutics, and chemicals (Jakobson et al., 2018; Li et al., 2020; Yeates and Bobik, 2018). These synthetic BMCs assemble in heterologous hosts, but form nucleoid-excluded aggregates with low chemical production, likely because these BMCs are not co-expressed with their positioning systems. We aim to design Minimal Autonomous Positioning Systems (MAPS) consisting of A/D ATPases and their partner-protein N-terminal peptides to be used as spatial regulators for natural- and synthetic-cargos in heterologous bacteria.
APPENDIX

Methods

tBLASTn analysis. tBLASTn analysis was done using a ParA/MinD consensus sequence, as a query against RefSeq Representative genomes database with max target sequences as 5000 and E value threshold at 0.0001. Sequences were filtered for those that shared sequence homology and had one of the identified putative cargo genes, confirmed using webFlaGs (https://pubmed.ncbi.nlm.nih.gov/32956448/). The consensus query was generated using COBOLT ("https://pubmed.ncbi.nlm.nih.gov/17332019/").

Flag analysis. A few representative genomes were selected to display gene neighborhood conservation. Identification of replication origins (OriC’s) were performed using Ori-Finder (https://bmcbioinformatics.biomedcentral.com/articles/10.1186/1471-2105-9-79). FlaGs analysis figure was generated using Gene Graphics (https://katlabs.cc/genegraphics/).

Multiple sequence alignment. Sequences were aligned using Clustal Omega. The resulting tree was imported into iTOL to generate an unrooted tree. (Accession numbers: Hn2335 – ACX97145.1; Hn1364 – ACX96198.1; Hn0912 – ACX95755.1; Hn0716 – ACX95565.1; Hn0722 – ACX95571.1; Hn1669 – ACX96495.1; Hn0255 – ACX95118.1)

Media and growth conditions. All mutants described in this study were constructed using WT Halothiobacillus neapolitanus (Parker) Kelly and Wood (ATCC® 23641™) purchased from ATCC. Cultures were grown in ATCC® Medium 290: S-6 medium for Thiobacilli (Hutchinson et al., 1965) and incubated at 30°C, while shaken at 130 RPM in air supplemented with 5% CO₂. Strains were preserved frozen at −80°C in 10% DMSO.

Construct designs and cloning. All constructs were generated using Gibson Assembly and verified by sequencing. Fragments for assembly were synthesized by PCR or ordered as a gBlock (IDT). Constructs contained flanking DNA that ranged from 750 to 1100 bp in length upstream and downstream of the targeted insertion site to promote homologous recombination into target genomic loci. Cloning of plasmids was performed in chemically competent E. coli Top10 or Stellar cells (Takara Bio).
Making competent cells in *H. neapolitanus* C2. Competent cells of *H. neapolitanus* were generated as previously reported. In short, 1 L of culture was grown to an OD of 0.1-0.15. Cultures were harvested by centrifugation at 5,000xg for 20 minutes at 4°C. Pellets were resuspended and washed twice with 0.5 volumes of ice-cold nanopore water. All wash centrifugation steps were performed at 3,000xg for 30 minutes at 4°C. The resulting pellet after washing was resuspended in 1x10⁻³ volumes of ice-cold nanopore water. These competent cells were used immediately or frozen at -80°C for future use. Frozen competent cells were thawed at 4°C before use.

Transformation in *H. neapolitanus* C2. 50-100 µL of competent cells were mixed with 5 µL plasmid DNA (1-5 µg) and incubated on ice for 5 minutes. This mixture was then transferred to a tube containing 5 mL ice-cold S6 medium without antibiotics and incubated on ice for 5 minutes. Transformations were recovered for 16-36 hours, while shaken at 130 RPM, at 30°C, in air supplemented with 5% CO₂. Clones were selected by plating on selective medium with antibiotics. Colonies were restreaked. Restreaked colonies were verified for mutation by PCR.

Native fluorescent fusions. For the native fluorescent fusion of ParB-mNG, mNG-FliN, and CheY-mNG, the sequence encoding the fluorescent protein mNeonGreen (mNG) was attached to the 3' or 5' region of the native coding sequences, separated by a GSGSGS linker. For the native fluorescent fusion of Cbbs-mTQ, the sequence encoding the fluorescent protein mTurquoise (mTQ) was attached to the 3' region of the native coding sequence, separated by a GSGSGS linker. A kanamycin resistance cassette was inserted before the gene for N-terminal tags or after the gene for C-terminal tags. When necessary, the promoter was duplicated. The mutant was selected by plating on S6 agar plates supplemented with 50 µg/mL of kanamycin. All fusions were verified by PCR.

Deletion mutants. For deletions of *Hn2335, Hn1364, Hn0912, Hn0722,* and *Hn0911,* the genes were replaced with a spectinomycin resistance cassette, followed by a duplicated promoter for the downstream gene. Deletion of *Hn0716* was obtained by codon-optimizing the downstream gene and inserting the spectinomycin resistance cassette after this codon-optimized gene. Mutants were selected by plating on S6 agar plates supplemented with 50 µg/mL of spectinomycin. All mutations were verified by PCR.

Microscopy. All live-cell microscopy was performed using exponentially growing cells. 3-5 µL of cells were dropped onto a piece of 2% UltraPure agarose + S6 pad and imaged on a Mantek dish. All fluorescence and phase contrast imaging were performed using a Nikon Ti2-E motorized inverted microscope controlled by NIS Elements software with a SOLA 365 LED light source, a 100X Objective lens (Oil CFI Plan Apochromat DM Lambda Series for Phase Contrast), and a Photometrics Prime 95B Back-illuminated sCMOS camera or a Hamamatsu Orca Flash 4.0 LT + sCMOS camera. ParB-mNG, mNG-FliN, and CheY-mNG were imaged using a “GFP” filter set (C-FL GFP, Hard Coat, High Signal-to-Noise, Zero Shift, Excitation: 470/40 nm [450-490 nm], Emission: 525/50nm [500-550nm], Dichroic Mirror: 495 nm). Cbbs-mTQ

**Long-term microscopy.** For multigenerational time-lapse microscopy, 1.5% UltraPure agarose + S6 pads were cast in 35-mm glass-bottom dishes. Dishes were preincubated at 30°C in 5% CO2 for at least 24 hr. 4 µl of exponentially growing cells were spotted onto the agar pad. Temperature, humidity, and CO₂ concentrations were controlled with a Tokai Hit Incubation System. NIS Elements software was used for image acquisition. Cells were preincubated in the stage top for at least 30 minutes before image acquisition. Videos were taken at one frame per 2.5-5 minutes for a duration of 12-24 hours.

**Image analysis.** Several fields of view were captured for each mutant. All fluorescence channels were subjected to background subtraction on Fiji with a rolling ball radius of 50 µm. Background-subtracted fluorescence images were merged with phase contrast images to create composites used for image analysis. Image analysis including cell identification, quantification of cell length, foci localization, foci number, foci fluorescence intensity, and identification of constriction sites were performed using Fiji plugin MicrobeJ 5.13I (Schindelin et al., 2012; Ducret et al., 2016). Cell perimeter detection and segmentation were done using the rod-shaped descriptor with default threshold settings at a tolerance of 56. Maxima detection parameters were individually set for each cargo. For ParB-mNG (cargo = chromosome) foci detection, tolerance and z-score were both set to 100. For CheY-mNG (cargo = chemotaxis) foci detection, tolerance was set to 150 and z-score was set to 100. For mNG-FliN (cargo = flagella) foci detection, tolerance was set to 710 and z-score was set to 69. For CbbS-mTQ (cargo = carboxysome) foci detection, point detection was used instead of foci detection, and tolerance was set to 1010. Results were manually verified using the experiment editor, and non-segmented cells were cut using a particle cutter. Associations, shape descriptors, profiles, and localization were recorded for each strain. Localization graphs were automatically generated through MicrobeJ. Fluorescence intensity graphs and foci number count graphs were made in GraphPad Prism (GraphPad Software, San Diego, CA, [www.graphpad.com](http://www.graphpad.com)).

**Nucleoid staining for live imaging.** Cells were harvested by centrifugation at 5,000 g for 5 minutes. Following centrifugation, the cells were washed in PBS, pH 7.4. The resulting cell pellet was resuspended in 100 µL PBS and stained with SytoxBlue at a final concentration of 500 nM. The samples were incubated in the dark, at room temperature for 5-10 minutes. Stained cells were directly loaded onto an S6 agar pad that had been infused with 500 nM SytoxBlue.
**Carboxysome count.** Eleven frames were captured over the course of 2 minutes. Carboxysomes were counted in each frame. The highest number counted per cell was used for carboxysome count.

**Motility assay.** Motility assays were run in S6 in 0.4% agar. Cells were grown on a plate of S6 medium. Individual colonies were inoculated into tubes or plates of motility media and incubated at 30°C in air supplemented with 5% CO₂. Tubes and plates were checked daily for motility for 2 weeks.

**Cysteine-labelling of flagellin.** *H. neapolitanus* flagellin was identified using a BLAST homology search with Hag from *Bacillus subtilis* as the query. Only a single flagellin gene was found. Several threonine and serine residues were considered for cysteine mutagenesis. Residues were mutated using the Q5 site-directed mutagenesis kit. Clones were verified for cysteine mutation by sequencing.

**Flagella stain.** Alexa Fluor 594 C₅ maleimide dye was resuspended in DMSO to the working concentration of 10 mM. *H. neapolitanus* cultures were grown to an OD of 0.1-0.2. The cultures were adjusted to a pH of 7.0 using PBS, pH 11.7. Cells at an adjusted pH of 7.0 were then stained with Alexa Fluor 594 C₅ maleimide dye at a final dye concentration of 100 µM. Staining cultures were incubated overnight at 4°C. The stained cells were washed 4+ times in PBS, pH 7.4. All centrifugation steps were performed at 5,000xg for 3 minutes.

**Statistical analysis.** For all population analyses, we used a non-parametric Wilcoxon test using GraphPad Prism.

**Movie editing.** Movies were cropped using Fiji. Movies were stabilized using the multi-channel hyperstack alignment plug-in called HyperStackReg. Time stamp and scale bar annotations were added using Fiji. Arrows and pauses were added using Adobe Premier Pro.

**Structure prediction and peptide docking using AlphaFold2 and Rosetta.** For MinD and McdA, we generated the N-terminus peptide-ATPase docking models using the CollabFold implementation of AlphaFold2 (*Jumper et al., 2021*; *Mirdita et al., 2022*). The N-terminal peptides were defined as the first 30 residues of the putative partner protein from the N-terminus. Multiple sequence alignments were constructed using MMseqs2. For each peptide-ATPase pair, we generated five structures with the default CollabFold/AlphaFold2 hyperparameters saved for the number of recycles for each model being increased to 12. As default to AlphaFold2, the structures were energetically minimized with AMBER using the Amber99sb force field. We selected docked peptide models based on the pLDDT scores of the binding interface residues and similarity to previously resolved ParA-like ATPase/partner-protein crystal structures.

For ParA, FlhG, and ParC we generated docked peptide models using Rosetta’s FlexPepDock protocol (*Raveh et al., 2011*). First, we equilibrated the ATPase homodimer structures generated from AlphaFold2 to the ref2015_cart_cst Rosetta force
field with the FastRelax full-atom refinement protocol with cartesian coordinate space minimization using the lbfgs_armijo_nonmonotone minimizer. To preserve the position of the backbone atoms predicted by AlphaFold2, a backbone atom coordinate constraint was added. For this initial step, we generated 20 trajectories with the lowest scoring structure being used for the peptide docking step. We used the score3 with the docking_cen.wts_patch and the REF2015 force fields for the low-resolution and high-resolution docking steps of the FlexPepDock protocol, respectively. For each putative partner protein/ATPase pair, we simulated 50,000 docking trajectories. The top two thousand trajectories defined by the lowest energetics were then clustered using Calibur (Li and Ng, 2010). We then chose the final models based on cluster information, energetics, and mechanistic plausibility. From the final docked models chosen from the Rosetta and AlphaFold2 simulations, we sought to identify key binding residues through an in-silico alanine mutation scan and ΔΔG calculations. We iteratively mutated all interface residues of the docked peptide and calculated the ΔΔG using the FlexDDG protocol in Rosetta with the talaris2014 forcefield (Barlow et al., 2018). In FlexDDG, for each mutation, the backbone and side chain conformations were sampled 35,000 times using Rosetta's Monte Carlo backrub method. At every 2,500 sample interval the ΔΔG of mutation was calculated. The final reported ΔΔG is the average of 35 such trajectories.

(Accession numbers: ParA partner protein (ParB) - ACX97144.1; MinD partner protein (MinE) - ACX96199.1; McdA partner protein (McdB) - ACX95754.1; FlhG partner protein (FliA) - ACX95566.1; ParC partner protein (CheW) - ACX95572.1). The first 30 amino acids from the N-terminus of each partner protein were docked onto the dimer structures in silico using AlphaFold2 (MinD, McdA) or Rosetta (ParA, FlhG, ParC). Binding interface was defined by those residues that shared a minimum of 1 Angstrom² of surface area.

**Complementation of mutants.** Cells were grown to an OD of 0.1. They were induced with 0.25, 1, 5, 10, 50 µM IPTG for up to 6 hours. They were imaged for complementation at various time points.

- Divisome: 50 µM IPTG for 6 hours.
- Carboxysome: 50 µM IPTG for 3 hours.
- Flagella: 0 µM IPTG, leaky expression.
- Chromosome: 50 µM IPTG for 3 hours.
- Chemotaxis: 50 µM IPTG for 3 hours.

**HaloTag staining.** JaneliaFluor646 far-red dye was resuspended in DMSO to a working concentration of 5 µM. Cells were harvested at an OD of 0.1-0.2 and resuspended in S6. Washed cells were then stained with JaneliaFluor646 at a final concentration of 250 nM for 3 hour at 30°C. Cells were washed 5x in PBS. All centrifugation steps were performed at 5,000xg for 5 minutes.

**Exogenous Expression of mNG-McdB.** The sequence encoding the fluorescent protein mNeonGreen (mNG) was attached to the 5’ region of the mcdB coding sequence, separated by a GSGSGS linker. The gene was codon-optimized, placed under the expression of a P_{trc} promoter, and inserted into a neutral site, located
between genes Hn0933 and Hn0934. Mutants were selected by plating on S6 agar plates supplemented with 25 µg/mL chloramphenicol. The insertion was verified by PCR. Exogenous expression of mNG-McdB was not induced. All images obtained resulted from leaky expression of the Prc promoter. Exogenous expression images of mNG-McdB was compared back to an empty vector control, which showed no fluorescence signal.

**Ciprofloxacin and Cephalexin treatments.** Cells were collected at an OD of 0.05-0.1 and treated with 50 µM ciprofloxacin or with 8 µM cephalexin for 24 hours. Cells were then stained with 2 µg/mL DAPI for 15 minutes and imaged.

**Transmission Electron Microscopy.** Log cultures of *H. neapolitanus* were pelleted and fixed overnight at 4°C in 2.5% glutaraldehyde in 0.1 M Sorensen’s buffer (pH 7.4). After several washes in 0.1 M Sorensen’s buffer, cells were post-fixed overnight at 4°C in 1% osmium tetroxide in 0.1 M Sorensen’s buffer. Cells were washed until the solution became clear. Cells were then immobilized in 1% agarose and sliced thinly into 0.5 mm slices. Slices were dehydrated in an increasing series of ethanol or acetone (30-100%) washes for 10 minutes each. Slices were infiltrated with Embed812 resin (25% increments for 1-16 hours each at room temperature). A final incubation in full strength resin was performed at room temperature under vacuum. Cells were then embedded in blocks, incubated under vacuum overnight, and transferred to a 60°C oven to polymerize for 1-2 days. Thin sections of approximately 50 nm were obtained by using a Leica UC7 Ultramicrotome. Sections were post-stained with 7% uranyl acetate for 10 minutes, Reynold’s lead citrate for 5 minutes, and visualized on an JEOL 1400-plus transmission electron microscope equipped with a XR401 AMT sCMOS camera.

**Bacterial Two-Hybrid Analysis.** N-terminal T18 and T25 fusions of McdA and McdB were constructed using plasmid pKT25, pKNT25, pUT18C and pUT18, sequence-verified and co-transformed into *E. coli* BTH101 (Karimova et al., 1998). Several colonies of T18/T25 cotransformants were isolated and grown in LB medium with 100 µg/mL ampicillin, 50 µg/mL kanamycin and 0.5 mM IPTG overnight at 30°C while shaking at 225 RPM. Overnight cultures were spotted on indicator Xgal plates supplemented with 100 µg/mL ampicillin, 50 µg/mL kanamycin and 0.5 mM IPTG. Plates were incubated at 30°C up to 48 hr before imaging.

**α-McdAB Homolog Search and Neighborhood Analysis.** Identification of all α-carboxysome containing proteobacteria within the NCBI and JGI databases was performed via BlastP using the α-carboxysome components CsoS2, CsoS4A, or CsoS4B as queries. Neighborhood analyses for McdA- and McdB-like sequences were then carried out within these resulting genomes by manually searching 4000 bp upstream and downstream of the cso operon. Multiple sequence alignment for identified putative McdA proteins was performed using MAFFT 1.3.7 under the G-INS-I algorithm (Katoh and Standley, 2013), whereas the E-INS-I algorithm was used for McdB proteins due to long gaps caused by intrinsic disorder. Subsequent identification of McdA- and McdB-like sequences away from the cso operon was performed via BlastP using highly conserved regions/features identified from the multiple sequence alignments.
**α-McdB Sequence Analysis.** Coiled-coil predictions for α-McdB proteins were performed using DeepCoil (Ludwiczak *et al*., 2019). α-McdB protein disorder predictions were conducted using PONDR with the VL-XT algorithm (Romero *et al*., 1997; Li *et al*., 1999; Romero *et al*., 2001). Analysis of α-McdB hydrophobicity was performed using ProtScale using the Kyte and Doolittle scale (Kyte and Doolittle, 1982; Gasteiger *et al*., 2005).

**Phylogenetic Inference.** Ortholog sequences for *H. neapolitanus* CbbL (Hneap_0922), CbbS (Hneap_0921), CsoS3 (Hneap_0919), CsoS4A (Hneap_0918), and CsoS4B (Hneap_0917) were obtained via BlastP for each proteobacterium and cyanobacterium. Multiple sequence alignments for each protein sequence were performed using MAFFT 1.3.7 (Katoh and Standley, 2013) under the G-INS-I algorithm and BLOSUM62 scoring matrix. The five resulting alignments were then concatenated into one alignment using Geneious 11.1.5 (https://www.geneious.com). Regions of low conservation within the resulting alignment were removed using gBlocks 0.91 b (Castresana, 2000; Talavera and Castresana, 2007). A phylogenetic tree was then estimated with maximum likelihood analyses using RAxML 8.2.11 (Stamatakis, 2014) under the LG+Gamma scoring model of amino acid substitution. Bootstrap values were calculated from 500 replicates.

**Expression and Purification of McdB Homologs.** All McdB proteins were expressed with an N-terminal His-SUMO tag off a pET11b plasmid. The expression plasmids were transformed into competent BL21-Al cells. Expression cultures were grown in 2 L of LB + carbenicillin (100 µg/mL) at 37°C to an OD600 of 0.6. Cultures were then induced with final concentrations of IPTG at 1 mM and L-arabinose at 0.2% (w/v) and allowed to grow for 4 hours at 37°C. Cultures were pelleted and resuspended in 80 mL lysis buffer (50 mM Tris-HCl pH 8.5, 300 mM KCl, 5 mM 2-Mercaptoethanol (BME), 0.05 mg/mL lysozyme, 0.05 µL/mL benzonase, protease inhibitor) on ice. Cells were lysed using a tip sonicator at 50% power with 10s on/20s off cycles for 6 minutes at 4°C. Lysates were spun down at 35,000 x g at 4°C, and supernatants loaded onto 5 mL HP HIS-TRAP columns (GE Healthcare Life Sciences) equilibrated in buffer A (50 mM Tris-HCl pH 8.5, 300 mM KCl, 5 mM BME, 20 mM imidazole). Proteins were eluted using a 5-100% gradient of buffer B (50 mM Tris-HCl pH 8.5, 300 mM KCl, 5 mM BME, 500 mM imidazole) via an AKTA pure system (GE Healthcare Life Sciences). Peak fractions were pooled and diluted in buffer A to a final imidazole concentration < 100 mM. Purified His-Ulp1 (300 µL) was added and reactions were incubated at 30°C to cleave the His-SUMO tag. Reactions were then passed over 5 mL HP HIS-TRAP columns to remove both the His-SUMO tag and His-Ulp1 enzyme. Flow-through was concentrated and passed over an SEC column (HiLoad 16/600 Superdex 200 pg; GE Healthcare Life Sciences) equilibrated in buffer C (50 mM Tris-HCl pH 8.5, 150 mM KCl, 5 mM BME, 10% glycerol) using an AKTA pure system. Peak fractions were pooled, flash frozen with liquid N2, and stored at -80°C.

**Microscopy of McdB Phase Separation in vitro.** Images were taken of 450 µM McdB in a buffer consisting of 20 mM HEPES (pH 7.0) and 100 mM KCl, with or without the
addition of either 15% (w/v) PEG-8000 or Ficoll-400 as indicated. All samples were left to incubate for two hours prior to imaging at room temperature. Imaging was performed using 16 well CultureWells (Grace BioLabs). Wells were passivated by overnight incubation in 5% (w/v) Pluronic acid (Thermo-Fischer), and washed thoroughly with the corresponding buffer prior to use. Imaging of McdB droplet formation was performed using a Nikon Ti2-E motorized inverted microscope (60× DIC objective and DIC analyzer cube) with a Transmitted LED Lamp house and a Photometrics Prime 95B Back-illuminated sCMOS Camera. Image and video analyses were performed using Fiji v 1.0.

**Size-Exclusion Chromatography with Multi-Angle Light Scattering (SEC–MALS).** For each McdB homolog analyzed, 500 µL of sample at 1.5 mg/mL was passed over a SEC column (Superdex 200 Increase 10/300 GL; GE Healthcare Life Sciences) at a flow rate of 0.15 mL/min in buffer (50 mM Tris-HCl pH 8.5, 150 mM KCl, 5 mM BME) at 4 °C. Following SEC, the samples were analyzed using an A280 UV detector (AKTA pure; GE Healthcare Life Sciences), the DAWN HELEOS-II MALS detector (Wyatt Technology) and the OptiLab rEX refractive index detector (Wyatt Technology). The data were analyzed to calculate mass using ASTRA software (Wyatt Technology). Bovine serum albumin was used as the standard for calibration.
Supplemental Movie Legend

**Movie 2-1: Hn2335 is required for chromosome segregation in H. neapolitanus.**

(A) Time-lapse microscopy of ParB-mNG foci (green) and SYTOX-stained nucleoids (magenta) showing newborn WT cells with a single ParB focus at mid-cell, which then splits into two foci that bidirectionally segregate towards the quarter positions of the growing cell. Foci positioning at the quarters of the cell was maintained, which then became the mid-cell position of each daughter cell following division. (B) In the ΔHn2335 (ΔparA) mutant, faithful chromosome segregation and inheritance were lost, resulting in polyploid cells that continued to divide and non-viable anucleate cells. Arrow highlights the invagination-dependent spooling of the chromosome immediately prior to complete septation. Phase Contrast (blue) shows cell perimeter. Videos accelerated ~2,400 x real time.

**Movie 2-2: Hn1364 is required for cell division positioning.** (A) Time-lapse microscopy shows WT cells dividing at mid-cell. (B) ΔHn1364 (ΔminD) cells divide asymmetrically. (C) ΔminD cells occasionally undergo multiple divisions simultaneously along the cell length. Phase Contrast (blue) shows cell perimeter. Arrows highlight division sites. Videos accelerated ~2,400 x real time in (A, B) and ~19,000 x real time in (C).

**Movie 2-3: Carboxysome positioning is determined by McdA, the A/D ATPase encoded in the carboxysome operon.** (A) Time-lapse microscopy of fluorescent-labelled carboxysomes (green) in dividing WT cells shows that carboxysomes are dynamically positioned along the cell length throughout the cell cycle and across multiple generations. (B) In ΔHn0912 (ΔmcdA) cells, carboxysome aggregates were static at the cell poles throughout the cell cycle and across multiple generations. Phase Contrast (blue) shows cell perimeter. Videos accelerated ~2,400 x real time.

**Movie 2-4: Chromosome positioning is still actively maintained in ΔflhG.** Time-lapse microscopy of ParB-mNG foci (green) in a ΔflhG mutant shows that chromosome segregation remains functional. But, anucleate cells form because longer cells only have a single chromosome, suggesting a defect in chromosome replication. Phase Contrast (blue) shows cell perimeter. Video accelerated ~2,400 x real time.

**Movie 2-5: Carboxysomes are inherited in anucleate cells.** Time-lapse microscopy of fluorescent-labelled carboxysomes (green) in (A) ΔparA, (B) ΔminD, and (C) ΔflhG strains showing that carboxysomes can be inherited in anucleate cells. Carboxysomes in the to-be-anucleate cell bundled up immediately adjacent to the division plane (green arrows). Carboxysome bundling was coincident with chromosome extrusion through the invaginating septum just prior to complete division and asymmetric chromosome inheritance. After septation, the carboxysome bundle was explosively liberated from the anucleate cell pole, resulting in multiple freely diffusible carboxysome foci. Anucleate cells harboring carboxysomes did not divide further. Phase Contrast (blue) shows cell perimeter. Videos accelerated ~2,400 x real time.
Movie 3-1: Carboxysomes aggregate and mislocalize to the cell poles in the absence of McdA or McdB. Time-lapse fluorescence microscopy (1 frame/min) of wild-type, ΔmcdA, and ΔmcdB cell lines of *H. neapolitanus* with CbbS-mTQ labelled carboxysomes (cyan) merged with the Phase Contrast channel showing cell perimeters. Video accelerated 100 x real time.

Movie 3-2: α-McdB localizes with dynamic carboxysomes. Time-lapse fluorescence microscopy (1 frame/min) of wild-type *H. neapolitanus* cells with CbbS-mTQ labelled carboxysomes (cyan) and α-mNG-McdB (magenta) merged with the Phase Contrast channel showing cell perimeters (far right panel). Video accelerated 100 x real time.

Movie 3-3: α-McdB remains localized to static carboxysome aggregates in the absence of McdA. Time-lapse fluorescence microscopy (1 frame/min) of Δ α-mcdA *H. neapolitanus* cells with CbbS-mTQ labelled carboxysomes (cyan) and α-mNG-McdB (magenta) merged with the Phase Contrast channel showing cell perimeters (far right panel). Video accelerated 100 x real time.

Movie 3-4: Purified *H. neapolitanus* α-McdB forms liquid-like droplets in vitro. Time-lapse DIC microscopy (2 frames/second). Video accelerated 3.5 x real time. The sample contains 450 µM α-McdB in buffer consisting of 100 mM KCl, 20 mM HEPES pH 7, and 15% PEG8000.