REGULATORY FUNCTION AND INTERDOMAIN COMMUNICATION OF GROUP II BIOTIN PROTEIN LIGASES

BY

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DISSERTATION

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Abstract

Biotin is a cofactor required for function of essential biotin dependent enzymes that are involved in metabolic processes including fatty acid biosynthesis, gluconeogenesis, and amino acid catabolism. In order for biotin to act as a cofactor it must first be covalently attached to the biotin dependent enzyme. Biotin protein ligase (BPL) catalyzes this covalent attachment in a two-step reaction. First BPL binds biotin and ATP to synthesize the intermediate biotinoyl-AMP, releasing pyrophosphate. In the second half reaction a conserved lysine residue from the biotin dependent enzyme acts as a nucleophile and attacks the mixed anhydride of biotinoyl-AMP to give the biotinylated protein and release of AMP. Microbial BPLs are classified into two groups. Both group I and group II BPLs have catalytic cores and C-terminal domains that are well conserved between the groups. Group II biotin protein ligases are characterized by the presence of an N-terminal DNA binding domain that functions in transcriptional regulation of the genes of biotin biosynthesis.

Escherichia coli BPL, called BirA, is the best-studied example of how an enzyme can also act as a transcriptional regulator of the biotin biosynthetic operon. Transcriptional repression of the *E. coli* biotin operon occurs when biotin acceptor proteins (biotin dependent enzymes) have been fully biotinylated thereby allowing BirA to accumulate biotinoyl-AMP in its active site. The presence of biotinoyl-AMP results in BirA dimerization and subsequent DNA binding that represses transcription of the biotin biosynthetic operon. Derepression of the biotin operon transcription occurs upon low biotin levels or increased levels of unmodified biotin acceptor proteins. Transfer of

accumulated biotinoyl-AMP to acceptor proteins results in monomeric BirA which is unable to bind the biotin operator. The N-terminal DNA binding domain of *E. coli* BirA is separated from the central domain by a linker region. It was proposed that *E. coli* BirA could be transformed into a group I BPL by simply removing the N-terminal DNA binding domain, where it would no longer have regulatory activity but would retain ligase activity. However, the *E. coli* BirA ligase activity was severely compromised when the N-terminal domain was removed, suggesting interdomain communication is required between the DNA binding domain and the central domain for normal ligase activity.

The *Bacillus subtilis* BPL, BirA, is also classified as a Group II BPL based on sequence predictions of an N-terminal helix-turn-helix motif and mutational alteration of its regulatory properties. We report evidence that *B. subtilis* BirA is a Group II BPL that regulates transcription at three genomic sites: *bioWAFDBI*, *yuiG* and *yhfUTS*. Moreover, unlike the paradigm Group II BPL, *E. coli* BirA, the N-terminal DNA binding domain can be deleted from *Bacillus subtilis* BirA without adverse effects on its ligase function. This is the first example of successful conversion of a Group II BPL to a Group I BPL with retention of full ligase activity.

The *Staphylococcus aureus* Group II BPL, BirA, has been reported to bind an imperfect inverted repeat located upstream of the biotin synthesis operon. DNA binding by other Group II BPLs requires dimerization of the protein, which is triggered by synthesis of biotinoyl-AMP (biotinoyl-adenylate), the intermediate in the ligation of biotin to its cognate target proteins. However, the *S. aureus* BirA was reported to dimerize and bind DNA in the absence of biotin or biotinoyl-AMP (85). These *in vitro* results argued that the protein would be unable to respond to the levels of biotin or

acceptor proteins and thus would lack the regulatory properties of the other characterized BirA proteins. We tested the regulatory function of the *S. aureus* protein using an *in vivo* model system and examined its DNA binding properties *in vitro* using electrophoretic mobility shift and fluorescence anisotropy analyses. We report that the *S. aureus* BirA is an effective regulator of biotin operon transcription and that the prior data can be attributed to artifacts of mobility shift analyses. We also report that deletion of the DNA binding domain of the *S. aureus* BirA results in loss of virtually all of its ligation activity.

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Finally, I would like to dedicate this thesis to my family. I would like to thank my family for their continued support. My parents have always prioritized education and for that I am very grateful. Without encouragement from my family this degree would not be possible.

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Chapter 1

Introduction

Physiological Importance of Biotin

Biotin is a cofactor for essential biotin dependent carboxylases, transcarboxylases, and decarboxylases that are involved in metabolic processes including fatty acid biosynthesis, gluconeogenesis, and amino acid catabolism (57). Biotin dependent carboxylases include acetyl-CoA carboxylase, pyruvate carboxylase, propionyl-CoA carboxylase, and 3-methylcrotonyl-CoA carboxylase. Acetyl-CoA carboxylase catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, the first committed step in fatty acid biosynthesis (Figure 1.1). Pyruvate carboxylase catalyzes the carboxylation of pyruvate to form oxaloacetate, to replenish oxaloacetate in the citric acid cycle. Propionyl-CoA carboxylase catalyzes the carboxylation of propionyl-CoA to form (S)-methylmalonyl-CoA, an important step in forming gluconeogenic intermediates from catabolism of fatty acids and amino acids. Methylcrotonyl-CoA carboxylase catalyzes the carboxylation of methylcrotonyl-CoA to form methylglutaconyl-CoA, a step in the pathway of leucine catabolism. Biotin is also a cofactor for transcarboxylase, which is involved in the synthesis of propionic acid by *Propionibacterium sherminii*. Biotin is also required for oxaloacetate decarboxylase, which converts oxaloacetate to pyruvate. Biotin dependent carboxylases typically have three subunits: biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP) (also called acceptor protein), and carboxyltransferase (CT) (5). Biotin is covalently attached to a conserved lysine residue of BCCP and acts as a swinging arm between BC and CT subunits (72). In the BC subunit, a carboxyl group is

transferred from a donor molecule to the nitrogen of the ureido moiety of biotin. Biotin transiently carries this carboxyl group to the CT subunit where it is transferred to the acceptor molecule (5).

Biotin is also associated with three other proteins: biotinidase, avidin and streptavidin. Biotinidase catalyzes the cleavage of biotin from proteins for reuse. Because humans are biotin auxotrophs, mutations in the gene encoding biotinidase can lead to multiple carboxylase deficiency (11). Supplementation of biotin alleviates these symptoms (11). Avidin is a biotin binding protein that is produced in the oviducts of birds, reptiles and amphibians. Avidin binds biotin with high affinity and specificity with a dissociation constant of $K_d = 10^{-15}$ M, one of the strongest non-covalent bonds known (50). The function of avidin is proposed to be a bacterial growth-inhibitor. Streptavidin, a similar protein with similar affinity for biotin, is produced by *Streptomyces avidinii* and thought to inhibit growth of competing bacteria (24). Both avidin and streptavidin are used extensively in biotechnology (53, 59, 67).

Biotin Biosynthesis

Biotin dependent enzymes are found across all three domains of life. Many bacteria, archaea, fungi and plants synthesize biotin, while other organisms are biotin auxotrophs, acquiring biotin from diet or microbiota. The complete biotin synthesis pathway has been determined in *E. coli* where the biotin biosynthetic operon is bi-directional and consists of *bioA* to the left, and *bioB*, *bioF*, *bioC*, and *bioD* to the right. A sixth gene, *bioH*, is also required for biotin synthesis and is found outside of this operon. BioC and BioH catalyze pimeloyl-ACP synthesis. BioC is a methyltransferase that

catalyzes methyl esterification of the methyl moiety of malonyl-acyl carrier protein (62). Malonyl-ACP methyl ester enters the fatty acid biosynthesis pathway and undergoes two rounds of elongation to give pimeloyl-ACP methyl ester (62). BioH cleaves the methyl ester, blocking further elongation in the fatty acid biosynthesis pathway, to give pimeloyl-ACP (62). BioF converts pimeloyl-ACP to 7-keto-8-aminopelargonic acid (KAPA). BioA converts KAPA to 7,8-diaminopelargonic Acid (DAPA). BioD converts DAPA to dethiobiotin (DTB) and BioB converts DTB to biotin. The later steps of biotin synthesis, BioFADB, are well conserved whereas pimelate synthesis is quite diverse. Some organisms that contain the *bioC* gene do not have a *bioH* gene but have a different gene that encodes an esterase responsible for cleaving pimeloyl-ACP methyl ester. For example, *Haemophilus influenzae* and *Campylobacter jejuni* have *bioG*; *Bacteroides fragilis* has a fusion of *bioG* and *bioC* (83). *Prochlorococcus marinus* has *bioK* (83), *Helicobacter pylori* has *bioV* (15), and *Francisella* species contain *bioJ* instead of *bioH* (43). *B. subtilis* lacks both *bioC* and *bioH* genes and instead has *bioW*, encoding a pimeloyl-CoA synthetase, and *bioI*, encoding a cytochrome P450 enzyme (63). Pimelate synthesis in *B. subtilis* is still not well understood.

Discovery of the Biotin Protein Ligase, BirA

In 1972 Campbell and coworkers isolated an *E. coli* mutant, *bir* (for biotin retention), which required high concentrations of biotin for growth. In a biotin auxotroph the *bir* mutant is deficient in biotin uptake and requires high concentrations of biotin (19). In a biotin prototroph the *bir* mutation causes derepression of the biotin operon leading to overproduction and excretion of biotin (19). By selecting for resistance to a biotin

analogue, Pai isolated a similar *E. coli* mutant, *bioR*, which derepressed transcription of the biotin operon at all biotin concentrations (69). The *bioR* mutant differed from the *bir* mutant in that the biotin requirement and biotin uptake were normal. Because of these distinct phenotypic differences, Pai proposed that these two mutations affected different genes.

In 1975 Eisenberg and coworkers compared 7,8-diaminopelargonic acid aminotransferase (BioA) and dethiobiotin synthetase (BioD) activities in *E. coli* wild type, *bioR* mutant, and *bir* mutant strains. At 5 μM biotin, a concentration that allows full transcriptional repression of the biotin operon in wild type, the *bir* mutant strain was 70% repressed while the *bioR* mutant strain was completely derepressed (42). This suggested that the *bir* mutant still had some regulatory function when the biotin uptake deficiency was overcome by a high biotin concentration but that the *bioR* mutant no longer had any regulation of the biotin operon. This raised further questions on how the original *bir* mutation (now designated *birA1*) was causing derepression of the biotin operon and inability to grow at normal biotin concentrations. Was the *bir* mutation a different alteration of the *bioR* gene or did it define a nearby gene that was somehow affecting BioR function?

To answer these questions, Campbell and coworkers mutagenized an *E. coli* biotin auxotroph strain (R901) and grew survivors at 80 nM biotin (20). These survivors were then screened for failure to grow at 2 nM biotin (20). From this method, several other *bir* mutations were isolated and classified into three groups: *birA,* linked to *argH* at 77 min*, birB*, linked to *metE* at 84 min*,* and *birC,* a double mutant of *birB* and another *metE* linked mutation (20). The *birA* mutants were found to be derepressed biotin operons

at biotin concentrations that cause repression in wild type and were also found to require elevated biotin concentrations for normal growth. The *birB* mutants were found to have a defect in biotin transport subsequently causing derepression of the biotin operon and the requirement for higher biotin concentrations for growth. The *birC* mutants were found to be double mutants with one mutation in *birB* and another temperature sensitive *metE*-linked mutation. Trying to determine why these mutants had growth defects at low biotin concentrations, they tested for the ability to catalyze the covalent attachment of labeled biotin to acetyl-CoA carboxylase and found that extracts from most of the *birA* mutants had no activity. Extracts from *birB* and *birC* mutants had wild type activity. They proposed that the *birA* gene product was responsible for both the covalent attachment of biotin to acceptor proteins and the regulation of the biotin operon. By 1981 it was clear that BirA has both of these activities after Barker and Campbell purified BirA and showed *in vitro* biotinylation of acetyl-CoA carboxylase and DNA binding activity to a biotin operon sequence (8, 10). BirB, now known as YigM, was later characterized as a membrane protein with ten transmembrane α-helices that is responsible for biotin transport (77).

Diversity of Biotin Protein Ligases

Genomic sequencing and comparative genomic analyses have identified biotin protein ligases (BPLs) in all three domains of life, which have been classified into four groups. All four groups contain the conserved catalytic and C-terminal domains but exhibit diversity in the N-terminal region. Group I BPLs are found in bacteria and archaea and only contain the conserved catalytic domain and the C-terminal domain (79).

Group II BPLs are found in bacteria and archaea and have an N-terminal helix-turn-helix DNA binding domain that is responsible for transcriptional regulation of the biotin operon (79). Group III BPLs are found in plants and have a small N-terminal domain that is responsible for organelle targeting (2, 93). Group IV BPLs are found in eukaryotes and have an extended N-terminal domain that does not resemble a DNA binding domain (68). The extended N-terminal domain of the human BPL is proposed to play a role in acceptor protein recognition (56). While most organisms have one BPL, *Francisella novicida*, *Clostridium acetobutuylicum*, *Lactococus lactis*, *Halobacterium sp.*, *Pyrococcus abyssii*, and *Pyrococcus furiosus* have a group I and a group II BPL (45, 79, 102). Differences in BPL dimerization have also been observed. The group I BPLs from *Mycobacterium tuberculosis* (65) and *Aquifex aeolicus* (94) are found to be primarily monomeric with or without biotinoyl-AMP bound in the active site. However, the group I BPL from *Pyrococcus horikoshii* is a dimer in the presence or absence of biotinoyl-AMP (6). The group II BPL, BirA, from *E. coli* is monomeric in the unliganded form and when biotin is bound. Dimerization is only observed when biotinoyl-AMP is active site bound (89).

The Biotin Protein Ligase Reaction

In order for biotin to act as a cofactor it must first be covalently attached to the biotin dependent enzymes. Biotin protein ligase catalyzes this essential covalent attachment in a two-step reaction (Figure 1.2). First BPL binds biotin and ATP to synthesize the biotinoyl-AMP intermediate, with release of pyrophosphate (29). In the second half reaction the ε-amino group of a conserved lysine residue from BCCP acts as a nucleophile and attacks the mixed anhydride of biotinoyl-AMP to give biotinylated

BCCP and the release of AMP (29). This reaction is extraordinarily specific as *E. coli* only has a single biotinylated enzyme and other organisms only contain up to five biotinylated proteins (29). The biotinylated lysine is part of a conserved AMKM tetrapeptide and acceptor proteins from other organisms are recognized and biotinylated by the *E. coli* BirA (29).

The biotin protein ligase reaction is useful in biotechnology for the labeling of proteins and peptides (37). Schatz developed small, unstructured peptides that are still recognized and biotinylated by *E. coli* BirA (82). Schatz peptides can be fused to a protein of interest, become biotinylated upon expression of *E. coli* BirA, and can be detected with avidin or streptavidin antibodies or purified with avidin or streptavidin columns (37). The biotin protein ligase reaction has also been adapted for proximity-dependent biotin identification (BioID) in eukaryotic cells (81). A promiscuous mutant version of *E. coli* BirA (R118G) is fused to a target protein and biotinylates proteins that interact with the target protein (30, 35, 81). The biotinylated proteins are then isolated with avidin or streptavidin columns and identified by mass spectrometry.

Transcriptional Regulation of the Biotin Operon

Biotin synthesis is expensive and therefore it is not surprising that organisms capable of biotin synthesis also have a mechanism to regulate this pathway. The *E. coli* biotin operon is a bidirectional operon with *bioA* transcribed to the left and *bioBFCD* transcribed to the right. Transcription is repressed in both directions when a high concentration of biotin is added to *E. coli* cultures (36). Transcription from both promoters is highest when an *E. coli* biotin auxotroph is starved for biotin in minimal

media (36). After these early transcription studies and isolation of the *bir* mutant, it appeared that BirA was a TrpR like repressor where it only binds DNA when bound with biotin. However, in 1979 Prakash and Eisenberg found that the co-repressor is not biotin but biotinoyl-AMP, the product of the first half reaction in the covalent attachment of biotin to acceptor proteins (75). In 1988, Cronan demonstrated that the level of repression of the *E. coli* biotin operon was sensitive to the level of apo acceptor protein (38). An increase in apo acceptor protein consumed BirA bound biotinoyl-AMP, causing dissociation of BirA from the biotin operator and derepression of the biotin synthesis genes (38). Dimerization and DNA binding studies by Streaker and Beckett found that *E. coli* BirA only efficiently dimerizes when biotinoyl-AMP is bound in the active site and that dimerization is required for DNA binding (89). These findings gave rise to the current model of transcriptional regulation of the *E. coli* biotin operon (Figure 1.3) (36). Under limiting biotin concentrations the small amount of biotinoyl-AMP synthesized by BirA is quickly attacked by the pool of apo AccB. Without biotinoyl-AMP bound in the active site, BirA cannot dimerize and therefore does not bind to the biotin operator. This allows transcription of the biotin operon in both directions. Under excess biotin concentrations the pool of AccB is sufficiently biotinylated allowing biotinoyl-AMP to accumulate in the BirA active site. This causes dimerization of BirA and subsequent binding to the biotin operator where transcription is repressed in both directions.

Comparative genomic analysis of organisms with group I BPLs, lacking the N-terminal DNA binding domain, have identified new transcriptional regulators of biotin synthesis and biotin transport genes. Alphaproteobacteria were found to have a GntR-type transcription factor, BioR, near biotin biosynthesis genes (80). BioR contains a

conserved helix-turn-helix DNA binding domain. Upstream regions of biotin biosynthesis genes were found to contain a palindromic consensus sequence called BIOR box (80). Electrophoretic mobility shift assays (EMSA) show that BioR binds BIOR box in a concentration dependent manner (44). Deletion of *bioR* showed a 1.5-fold increase in transcription of the biotin operon and overexpression of BioR showed a 10-fold decrease in biotin operon transcription indicating that BioR acts as a repressor (44). Transcription of the biotin operon decreases with increasing exogenous biotin concentrations indicating that regulation is biotin dependent (44). However, no regulatory ligand was found to be associated with BioR and it is still unclear how biotin is involved in regulation. Comparison of Actinobacteria genomes revealed genes encoding a transcriptional regulator from the TetR family, BioQ, are localized near biotin synthesis and transport genes (18). Palindromic 13-base pair BioQ DNA binding sites were also found upstream of biotin synthesis and biotin transport genes, and EMSAs show that *Corynebacterium glutamicum* BioQ does bind to these sites (18). Growth of *C. glutamicum* in excess biotin or biotin-limiting conditions only showed a moderate change in transcription of biotin related genes (18). Deletion of BioQ resulted in a large increase in transcription of biotin synthesis and biotin transport genes (18). However, the mechanism and regulatory ligand of BioQ are still unknown.

Two Competing Models for the *E. coli* BirA Molecular Switch

Weaver and coworkers argue that the molecular switch of BirA from transcriptional regulator to ligase enzyme is controlled by two competing protein-protein interactions (95). Trying to determine how BirA interacts with acceptor proteins, they

aligned the crystal structure of AccB with the crystal structure of biotin bound BirA so that the biotinylated lysine residue of AccB is positioned close to the biotin in BirA. Based on the alignment models, the β-sheet strand Lys122-Glu128 of AccB can form a parallel β-sheet with strand Val189-Lys194 of BirA to form a heterodimer. This same β-strand, Val189-Lys194, of BirA is found to form an antiparallel β-sheet with the same strand in a second BirA to form a homodimer. This model suggests that heterodimerization of BirA with AccB and homodimerization of BirA are mutually exclusive. Therefore when apo AccB concentration increases, heterodimerization with biotinoyl-AMP bound BirA occurs, preventing homodimerization and subsequent DNA binding. Heterodimerization of BirA with AccB allows transcription of biotin synthesis genes to sufficiently biotinylate AccB. When AccB synthesis is decreased, biotinoyl-AMP bound BirA is free to homodimerize and bind the biotin operon to prevent transcription of biotin synthesis genes.

Solbiati and Cronan argue that the molecular switch does not require extensive protein-protein interactions (87). They suggest that in the protein-protein interaction model the heterodimer between BirA and AccB must be long lived in order to outcompete homodimerization of BirA. Due to this long interaction it should be possible to directly detect the heterodimer. Since there has been no direct detection of the heterodimer Solbiati and Cronan argue that this is a major caveat to the protein-protein interaction model. In their experiment, Solbiati and Cronan show that the molecular switch from transcriptional regulator to ligase enzyme still occurs when they express small (14-15 residue) synthetic biotin acceptor proteins (Schatz peptides) that have extremely different sequences and structures from the natural acceptor protein. Many of

the AccB residues that are proposed to interact with BirA are missing in the Schatz peptides. In addition, no residue besides the biotinylated lysine is conserved in the AMKM tetrapeptide. Solbiati and Cronan propose that biotinoyl-AMP levels control the molecular switch. In this model, the switch is the removal of biotinoyl-AMP from the BirA active site by biotinylation of acceptor protein. The acceptor protein binds BirA, accepts the biotin molecule, and rapidly dissociates to leave BirA in the unliganded form preventing dimerization and subsequent DNA binding. Dimerization of BirA would only occur after the pool of acceptor proteins have been biotinylated, which allows biotinoyl-AMP to accumulate in the BirA active site.

The *E. coli* BirA N-Terminal DNA Binding Domain is Required for Ligase Activity

The crystal structure of *E. coli* BirA showed three distinct domains: N-terminal DNA binding domain, central catalytic domain, and a small C-terminal domain (Figure 1.4) (96). It appears that the N-terminal domain of *E. coli* BirA is well separated from the central domain by a linker region. Xu and Beckett proposed that *E. coli* BirA could be transformed into a group I BPL by simply removing the N-terminal DNA binding domain, where it would no longer have regulatory activity but would retain ligase activity (100). However, the ligase activity was severely compromised when the N-terminal domain was removed, suggesting interdomain communication is required between the DNA binding domain and the central domain for normal ligase activity (100).

Experiments investigating the role of the wing in the winged helix-turn-helix DNA binding domain also support the idea that interdomain communication is required for ligase activity. When the wing from the winged helix-turn-helix motif was deleted,

DNA binding activity was lost and surprisingly ligase activity was also compromised (26). Replacing the native wing with a wing from another helix-turn-helix transcription factor, OmpR, largely restored ligase activity but not DNA binding activity (26). This indicates that in the absence of DNA binding the wing is involved in active site organization. This also supports the idea that the BirA wing is required for specific interaction with the biotin operator. However, despite many attempts, there is no crystal structure of BirA bound to the operator and therefore the exact interaction is still unknown.

Function of *E. coli* BirA C-Terminal Domain

The C-terminal domain is found in all four BPL groups but the function has long been unknown. Experiments investigating the interaction between *E. coli* BirA and AccB have given us some clues. Mutant AccB having the substitutions E119K and E147K are poorly biotinylated by BirA (27). Complementary charge substitution mutations in BirA did not restore wild type levels of biotinylation. However, a mutant with a substitution in the C-terminal domain of BirA (K277E) was able to biotinylate the mutant AccB better than wild type BirA (27). This indicates that K277 may play a role in substrate recognition. Further mutational analysis found that a single amino acid substitution in the *E. coli* BirA C-terminal domain (R317E) was found to decrease affinity for ATP 25-fold and decrease ligase activity 10-fold (27). A mutant with a truncation of the C-terminal domain at I272 no longer had any ligase activity (27). However, it is unclear if the C-terminal truncation is defective in AccB interaction, synthesis of biotinoyl-AMP, or both.

Based on these data, the C-terminal domain is required for ligase activity and may play a role in binding ATP and interacting with acceptor protein.

Chakravartty and Cronan isolated mutations in *E. coli birA* that enhanced DNA binding activity. Surprisingly, one such mutant K267M mapped to the linker between the central domain and the C-terminal domain (25). Based on the crystal structure, this residue is solvent exposed and is not located near the dimer interface or the DNA binding domain but is somehow impacting operator binding. They propose that this substitution is interfering with interdomain communication (25). Based on these studies and those of N-terminal deletions, *E. coli* BirA has extensive interdomain communication between all three domains.

Specific Aims of This Work

Before the studies in this thesis the regulatory function of group II BPLs had only been extensively studied in *E. coli* BirA. The entire model of group II BPL regulatory function has been based on *E. coli* BirA. Sequence alignments of *E. coli* BirA to other group II BPLs show low sequence identity and could potentially lead to differences in regulatory function. The *Bacillus subtilis* group II BPL, BirA, has 27% sequence identity to *E. coli* BirA and was found to play a biotin dependent role in regulation of the biotin operon (16, 17, 73). Unlike *E. coli*, three operons in *B. subtilis* are regulated by biotin (61). In contrast to *E. coli*, the *B. subtilis* biotin operon is unidirectional and contains *bioW* and *bioI* (16, 73). The other two operons include putative biotin transport genes (*yhfU* and *yuiG*) and putative fatty acid biosynthesis genes (*yhfT* and *yhfS*) (61). Also unlike *E. coli*, *B. subtilis* has three biotin dependent enzymes: acetyl-CoA carboxylase,

pyruvate carboxylase, and propionyl-CoA carboxylase (55). Could these additional factors influence transcriptional regulation of the biotin operon? This work looked to determine if other group II BPLs followed the *E. coli* model of transcriptional regulation of the biotin operon. I focused on several aspects of the regulatory model including dimerization, co-repressor identification, DNA binding, and response to increased levels of apo acceptor proteins. In addition, I examined if other group II BPLs require interdomain communication between the N-terminal DNA binding domain and the catalytic central domain for proper ligase activity or if this interdomain communication was specific to only *E. coli* BirA.

Figures

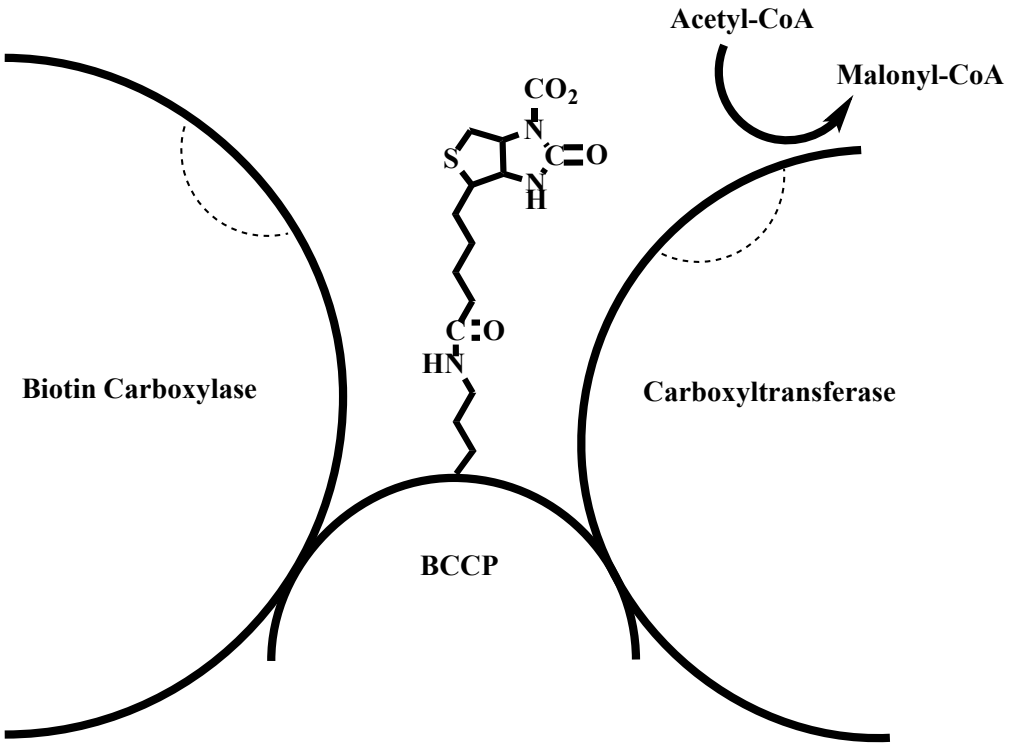

Figure 1.1. Role of biotin in acetyl-CoA carboxylase. Acetyl-CoA carboxylase has three subunits: biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), and carboxyltransferase (CT) (5). Biotin is covalently attached to a conserved lysine residue of BCCP and acts as a swinging arm between BC and CT (72). In the BC subunit, a carboxyl group is transferred from a donor molecule to the nitrogen of the ureido moiety of biotin (5). Biotin transiently carries this carboxyl group to the CT subunit where it is transferred to the acetyl-CoA to form malonyl-CoA (5). This is the first committed step of fatty acid biosynthesis.

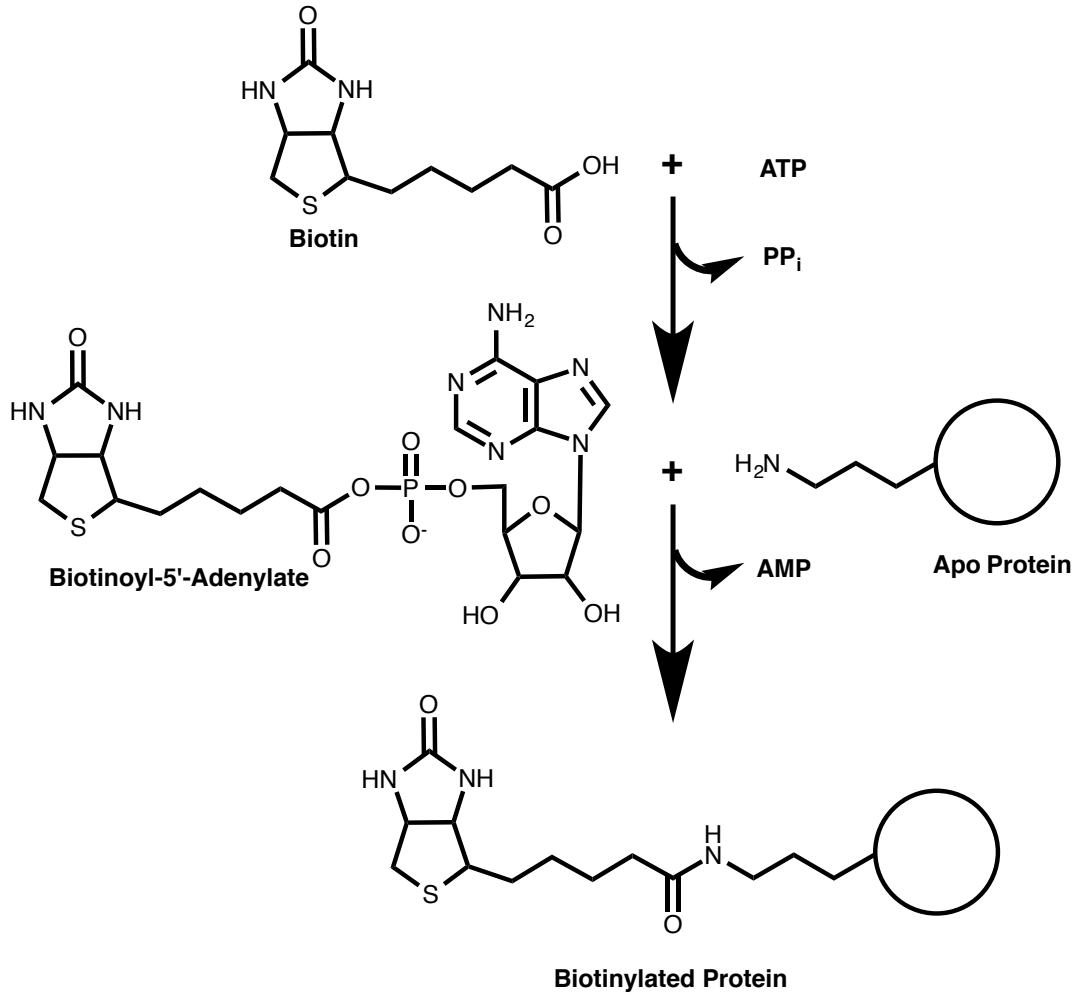

Figure 1.2. The Biotin protein ligase reaction. Biotin protein ligase catalyzes the covalent attachment of biotin to biotin dependent enzymes in a two step reaction. BPL binds biotin and ATP to synthesize the intermediate biotinoyl-AMP, releasing pyrophosphate. A conserved lysine residue from BCCP acts as a nucleophile and attacks the mixed anhydride of biotinoyl-AMP to give biotinylated BCCP and the release of AMP. Figure is from (54).

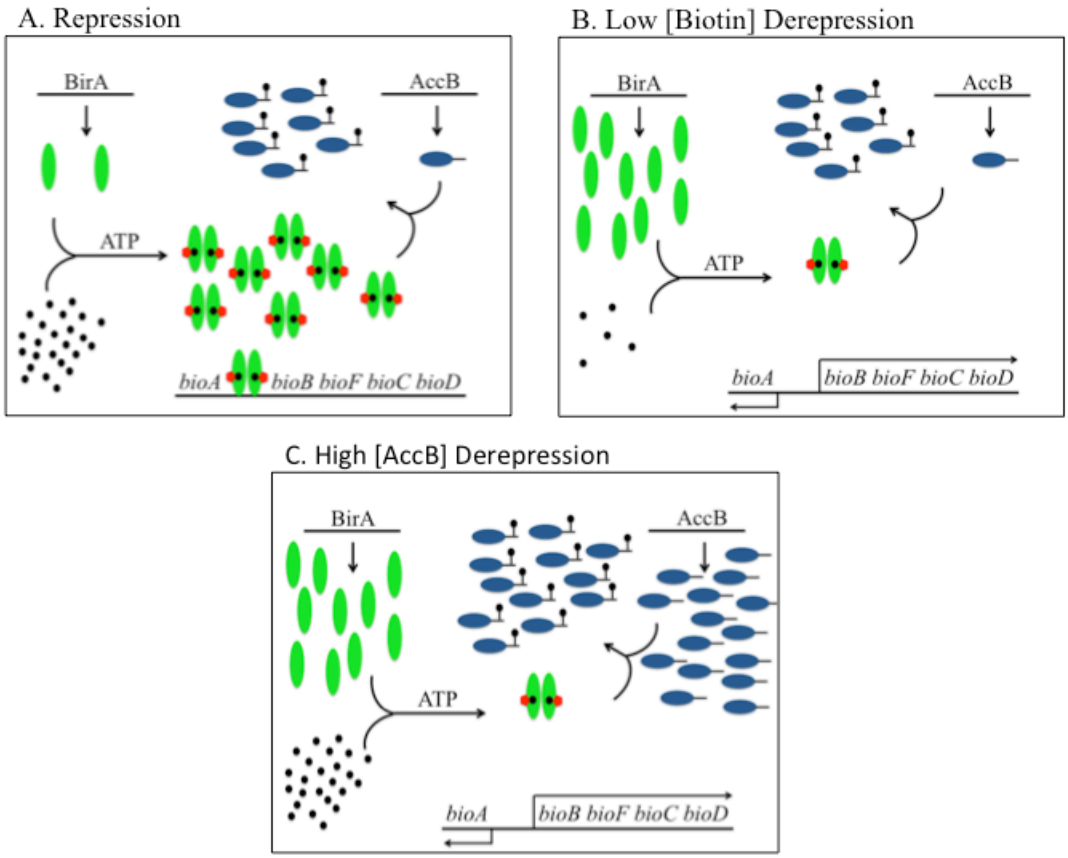

Figure 1.3. *E. coli* model of transcriptional regulation of the biotin operon (36).

A. Repression by BirA. Sufficient biotin is present that essentially all acceptor protein (AccB) is biotinylated. B. Derepression by limited biotin results in low levels of Bio-AMP such that BirA is unable to dimerize and exert repression. C. Derepression by an excess of AccB acceptor protein consumes the Bio-AMP resulting in low levels that preclude BirA dimerization and operator binding. Green ovals denote BirA, tailed blue ovals are AccB, black dots represent biotin, and black dots connected to small red pentagons denote biotinoyl-adenylate (Bio-AMP). Modified from reference (87).

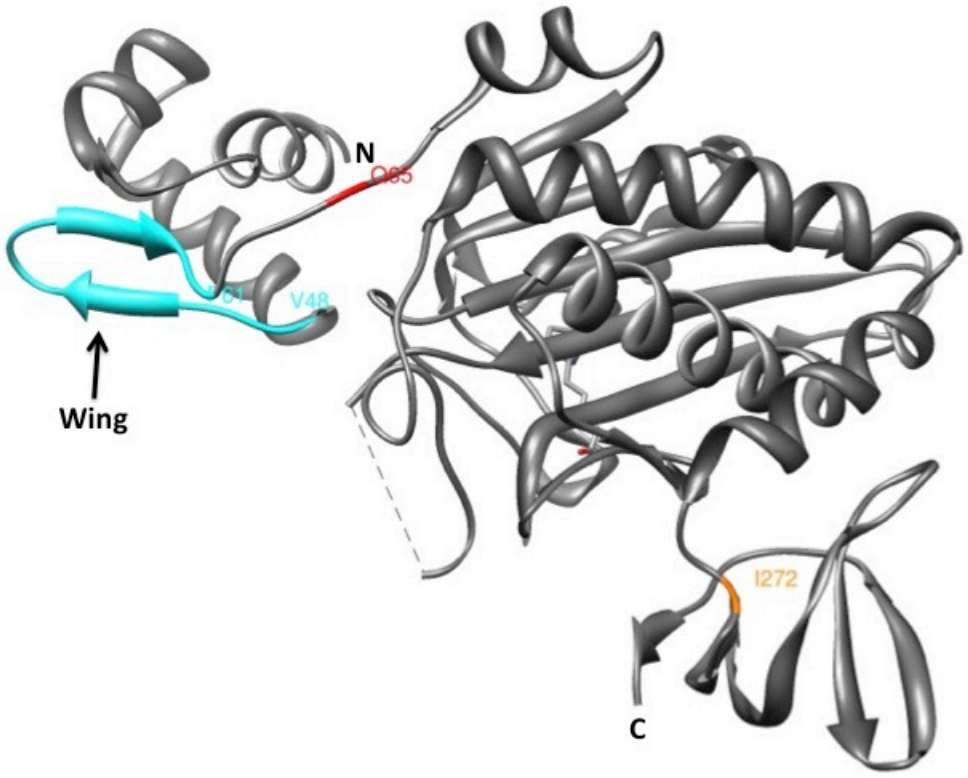

Figure 1.4. Structure of *E. coli* BirA. *E. coli* BirA has three distinct domains: N-terminal helix-turn-helix DNA binding domain, central catalytic domain, and a small C-terminal domain. This figure was created using Chimera (74) and Protein Data Bank file 1HXD (60). Deletion of the N-terminal domain, Δ2-65 (endpoint indicated in red), results in severely compromised ligase activity (100). Deletion of the wing from the helix-turn-helix in the N-terminal domain also compromises ligase activity (26). Truncation of the C-terminal domain at I272 (indicated in orange) results in no ligase activity (27). Interdomain communication between all three domains is required for proper ligase activity.

Chapter 2

Successful Conversion of the *Bacillus subtilis* BirA Group II Biotin Protein Ligase into a Group I Ligase[1]

Introduction

Biotin protein ligase (BPL) is required for the covalent attachment of biotin to biotin-dependent enzymes. This attachment proceeds in a two-step reaction. First, BPL binds both biotin and ATP to synthesize biotinoyl-5′-AMP (Bio-5′-AMP, also called biotinoyl-adenylate) with release of pyrophosphate (29). The ε-amino group of the conserved lysine residue of the acceptor protein acts as a nucleophile to attack the Bio-5′-AMP mixed anhydride bond to give covalently attached biotin plus AMP (Figure 1.2). Microbial BPLs are readily placed into two groups (79). Both groups have catalytic and C-terminal domains that show strong structural conservation (7, 12, 76, 94) whereas Group II BPLs are characterized by addition of an N-terminal helix-turn-helix (HTH) DNA binding domain that permits transcriptional regulation of the biotin synthetic genes. *E. coli* BirA, the paradigm for regulation of biotin biosynthesis, is the best studied Group II BPL. Transcriptional repression of the *E. coli* biotin operon occurs when BirA accumulates Bio-5′-AMP because all biotin acceptor proteins have been biotinylated (1, 38, 87). Bio-5′-AMP accumulation results in dimerization of BirA and subsequent DNA binding (13, 34). In all four *E. coli* BirA crystal structures (96-98) the HTH structure is spatially well removed from the other domains of the protein and thus deletion of the N-terminal DNA binding domain was expected to convert this Group II BPL into a fully

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functional Group I ligase. However, this was not the case: the resulting protein had severely compromised ligase activity (100). This was also true for ligases having smaller N-terminal deletions (25).

The sequences of other Group II BPLs suggest such proteins are found in γ-Proteobacteria, Bacilli, and Clostridii (79), although only the proteins from *E. coli* (12) and (very recently) *Staphylococcus aureus* (71) have been enzymatically characterized and crystallized. One of the Group II BPLs, *B. subtilis* BirA, has only 27% amino acid sequence identity to *E. coli* BirA (Figure 2.2). Despite this low sequence identity, *Bacillus subtilis birA* has been shown to complement the ligase activity of a temperature-sensitive *E. coli birA85* strain (17). Moreover, *B. subtilis birA* mutants show constitutive expression of a *bioW-lacZ* fusion, suggesting that BirA regulates biotin operon transcription (17). *B. subtilis* microarray data identified two additional transcripts, *yuiG* and *yhfUST*, regulated by biotin and BirA (61). Both YuiG and YhfU have strong sequence similarity to the structurally characterized BioY biotin transporter of *Lactococcus lactis* (14) and other well characterized energy-coupling factor (ECF) biotin transporters (52). All three transcripts have similar predicted BirA binding sites (61, 79) (Figure 2.3). *B. subtilis* has two known biotinylated proteins, pyruvate carboxylase (PyC) and the biotin carboxyl carrier protein (AccB) subunit of acetyl-CoA carboxylase (Figure 2.4). A third *B. subtilis* protein, biotin/lipoyl attachment protein (BLAP) encoded by the *yngHB* gene was found to be biotinylated and lipoylated when expressed in *E. coli* (39) although subsequently this protein was found not to be lipoylated by the *B. subtilis* enzymes that modify the known cognate lipoic acid acceptor proteins (32).

A major shortcoming of our picture of the enzymatic and *in vivo* regulatory activities of Group II BPLs is that it is based on a single example, *E. coli* BirA. For this reason we decided to study the enzymatic and regulatory properties of the BirA of *B. subtilis*, a bacterium that is evolutionarily diverse from *E. coli.* Moreover the amino acid sequence of *B. subtilis* BirA differs markedly from that of the *E. coli* protein and *in vivo B. subtilis* BirA biotinylates multiple proteins whereas *E. coli* BirA modifies only a single protein. Here we report that *B. subtilis* BirA is a Group II BPL that follows the *E. coli* model of regulation, but unlike the *E. coli* protein, *B. subtilis* BirA can be successfully converted into a fully active Group I BPL.

Materials and Methods

Strains chemicals and culture media.

The bacterial strains used were derivatives of *B. subtilis* 168 and *E. coli* K-12 (Table 2.1). The rich medium used to grow *E. coli* and *B. subtilis* was LB. The defined medium for *E. coli* was M9 salts supplemented with 0.5% glucose and 0.01% vitamin-free Casamino Acids (Difco) whereas the defined medium for *B. subtilis* was Spizizen salts (3) supplemented with 0.5% glycerol and 0.05% vitamin-free Casamino Acids (Difco) in addition to 0.01% each of tryptophan, tyrosine, isoleucine and phenylalanine. Antibiotics were used at the following concentrations (in μg ml^{-1}): sodium ampicillin, 100; kanamycin sulfate, 50; chloramphenicol, 25; tetracycline HCl, 12; erythromycin, 100; lincomycin, 12.5; streptomycin sulfate, 50 and spectinomycin sulfate, 50. The 15:1 mixture of ticarcillin disodium salt and potassium clavulanate (Research Products International) was used at 25 μg ml^{-1}. Oligonucleotides were purchased from Integrated

DNA Technologies. PCR amplification was performed using Taq polymerase (New England BioLabs) and *Pfu* polymerase (Stratagene) according to the manufacturer's specifications. DNA constructs were sequenced by ACGT, Inc. Reagents and chemicals were obtained from Sigma-Aldrich and Fisher, unless otherwise noted. New England BioLabs supplied restriction enzymes and T4 DNA ligase. Life Technologies provided SYBER Green I Nucleic Acid Gel stain and the 6% DNA Retardation Novex TBE Gels. Perkin Elmer provided [α-^{32}P]ATP (6,000 Ci/mmol). Analtech TLC Uniplates of microcrystalline cellulose matrix were purchased from Sigma-Aldrich.

Plasmids and plasmid constructions.

The plasmids used and constructed are given in Table. 2.2. The *B. subtilis birA* gene was amplified by PCR from *B. subtilis* strain 168 genomic DNA with primers SKH001 and SKH002 (all primers are given in Table 2.3) that added NdeI and XhoI sites. The product was digested with NdeI and XhoI and ligated into the same sites of pET19b, with an N-terminal hexahistidine tag to give pSKH001.

The C-terminal 86 residues of *B. subtilis* AccB (AccB-86) were amplified by PCR from *B. subtilis* strain 168 genomic DNA with primers SKH005 and SKH006 that added NcoI and XhoI sites. The product was digested with NcoI and XhoI and ligated into the same sites of pET-28 to give pSKH003 which encoded the untagged protein. The C-terminal 77 residues of *B. subtilis* PyC (PyC-77) were amplified by PCR from *B. subtilis* strain 168 genomic DNA with primers SKH007 and SKH009 and inserted into pET-28 by the same procedures.

B. subtilis BirA, Δ2-64 BirA, Δ2-66 BirA, Δ2-74 BirA, and Δ1-81 BirA were amplified by PCR from *B. subtilis* strain 168 genomic DNA with forward primers

SKH038, SKH036, SKH046, SKH048, SKH045, respectively, plus reverse primer SKH037. The primers added EcoRI and SalI sites. The products were digested with EcoRI and SalI and ligated into the same sites of pBAD322K, to give pSKH006, pSKH005, pSKH009, pSKH010 and pSKH011, respectively. The products were also ligated into the same sites of pBAD322Cm to give pSKH013, pSKH012, pSKH016, pSKH017 and pAKH018, respectively. *E. coli* BirA and Δ2-64 BirA were amplified by PCR from *E. coli* MG1655 genomic DNA with primers SKH049 and SKH043, and SKH042 and SKH043, respectively, thereby adding EcoRI and SalI sites. The products were digested with EcoRI and SalI and ligated into the same sites of pBAD322K, to give pSKH008 and pSKH007 respectively. The products were also ligated into the same sites of pBAD322Cm to give pSKH015 and pSKH014 respectively.

B. subtilis Δ2-64 BirA, Δ2-66 BirA, Δ2-74 BirA, and Δ1-81 BirA were amplified by PCR from *B. subtilis* strain 168 genomic DNA with forward primers SKH040, SKH047, SKH050, SKH044, respectively plus reverse primer SKH041. The primers added NdeI and XhoI sites. The products were digested with NdeI and XhoI and ligated into the same sites of pET-19b, adding an N-terminal hexahistidine-tag, to give pSKH019, pSKH020, pSKH021, and pSKH022, respectively.

A 500 base pair internal fragment of *B. subtilis bioW* was PCR amplified from *B. subtilis* strain 168 genomic DNA with primers SKH057 and SKH058 that added EcoRI and BamHI sites. The product was digested with EcoRI and BamHI and ligated into the same sites of pMUTIN4 to give pSKH023.

B. subtilis accB-86 was PCR amplified from strain 168 genomic DNA with primer SKH069 which added a HindIII and a strong RBS site

(AAGGAGGAAAAAATATG) plus primer SKH070 which contained an SphI site. The product was digested with HindIII and SphI and ligated into the same sites of pDR111 to give pSKH0024.

Bacillus subtilis strain construction.

B. subtilis competent cell preparation and transformation were carried out as described by Dubnau and Davidoff-Abelson (41). To create a *bioW-lacZ* chromosomal fusion, pSKH023 was transformed into strain 1A330 creating strain SKH001. Single crossover integration into *bioW* was verified by PCR and sequencing. To construct an IPTG inducible chromosomal copy of *accB-86*, strain SKH001 was transformed with linearized pSKH024 creating strain SKH002. Double crossover integration into *amyE* was verified by the amylase production screen of Harwood and Cutting (51). Integration was also verified by PCR and by sequencing with primers SKH065 and SKH066, SKH067 and SKH075 and SKH073 and SKH074.

Structural modeling and sequence alignment.

The *B. subtilis* AccB biotin attachment domain was identified by InterProScan (101) and structural modeling to *E. coli* AccB-87 crystal structure (PDB 1A6X) using Swiss-Model automated mode (4, 58, 70). The *B. subtilis* PyC biotin attachment domain was identified by InterProScan (101) and structural modeling to the *Staphylococcus aureus* pyruvate carboxylase biotin attachment domain crystal structure (3HBL) using Swiss-Model as above. *B. subtilis* BirA N-terminal deletions were determined by modeling to *S. aureus* BirA crystal structure (4DQ2) as a template using Swiss-Model automated mode as above. The final image was created using UCSF Chimera package (74). Sequence alignments were created using the Clustal Omega

(http://www.ebi.ac.uk/Tools/msa/clustalo/) and the output was processed by ESPript 3.0 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) to generate the final figure (49).

In vivo complementation.

Two *E. coli birA* strains were tested. In the more straightforward test strain BM4092 (9) was transformed with either plasmid pSKH001 or pSKH002 followed by selection for transformants by plating on LB supplemented with ampicillin or kanamycin, respectively. These strains were grown at 37°C on M9 minimal plates (66) with 25 μM 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal) and varying concentrations of biotin (1.6 nM, 4.1 nM, 41 nM, 4.1 μM, or 41 μM) (9). Note that gene expression from T7 promoter based multi-copy plasmids in the absence of T7 RNA polymerase has been shown to be equivalent to that of a single copy plasmid (88). Derivatives of strain BM4092 expressing the mutant BirAs encoded by kanamycin resistant plasmids pSKH005, pSKH006, pSKH007, pSKH008, pSKH009, pSKH010 or pSKH011 were similarly obtained and tested.

Strain VC618 which carries a deletion of the chromosomal *birA* gene was transformed with pSKH012, pSKH013, pSKH014, pSKH015, pAKH016, pSKH017, or pSKH018 and transformants were selected on LB plates supplemented with ampicillin and chloramphenicol at 30°C. These strains were cured of the temperature sensitive plasmid VC18 which expresses the *Saccharomyces cerevisiae* Bpl1 ligase by growth at 42°C on the defined medium supplemented with chloramphenicol and biotin. Loss of the temperature-sensitive plasmid was indicated by ampicillin sensitivity (26). These strains were then grown at 37°C on M9 minimal plates (66) with 25 μM X-gal and varying concentrations of biotin as above (9).

Protein purification.

For purification of the wild type and *B. subtilis* N-terminally deleted BirA Proteins *E. coli* strain BL21 (λ DE3) was transformed with pSKH001, pSKH019, pSKH020, pSKH021, or pSKH022. The strains were grown at 37°C in LB medium supplemented with ticarcillin-clavulanate to an OD_{600} of 0.8 and induced by addition of IPTG to 1 mM for an additional 6 h at 37°C. Cells were centrifuged and resuspended in lysis buffer which was 50 mM Tris-HCl, 500 mM NaCl, 0.1 mM tris(2-carboxyethyl)phosphine (TCEP), 10 mM imidazole, 5% glycerol, pH 8.0). The cells were lysed by passage through a French pressure cell and the lysate was centrifuged. The supernatant was added to Ni NTA beads (Qiagen) and incubated for 30 min. The mixture was added to a disposable 10 ml polypropylene column (Pierce) and washed with three column volumes of wash buffer (50 mM Tris-HCl, 500 mM NaCl, 0.1 mM TCEP, 60 mM imidazole, 5% glycerol, pH 8.0). BirA was eluted in 1 ml fractions with elution buffer (50 mM Tris-HCl, 500 mM NaCl, 0.1mM TCEP, 250 mM imidazole, 5% glycerol, pH 8.0). Fractions were subjected to SDS-PAGE to determine purity. Pure fractions were combined and dialyzed against storage buffer (50 mM Tris-HCl, 500 mM NaCl, 0.1 mM TCEP, 5% glycerol, pH 8.0). Aliquots were flash frozen and stored at -80°C.

The *B. subtilis* acetyl-CoA carboxylase biotin carboxyl carrier protein biotin attachment domain (AccB-86) was purified from *E. coli* strain BL21 (λ DE3) transformed with pSKH003. The strain was grown at 37°C in LB medium supplemented with kanamycin to an OD_{600} of 0.8 and induced by addition of IPTG to 1 mM for an additional 4 h at 30°C. The cells were centrifuged and resuspended in starting buffer (20 mM Tris-HCl, 1 mM NaCl, 0.1 mM TCEP, 5% glycerol, pH 8.0). The cells were lysed

by passage through a French pressure cell. The lysates were centrifuged and the supernatant was subjected to 60% isopropanol precipitation followed by anion exchange chromatography using a HiTrap Q FF column (GE Healthcare) and fast liquid chromatography (AKTA) (28, 78).

The *B. subtilis* pyruvate carboxylase biotin attachment domain (PyC-77) was purified *E. coli* strain BL21 (λ DE3) transformed with pSKH004. The strain was grown at 37°C in LB medium supplemented with kanamycin to an OD_{600} of 0.8 and induced by addition of IPTG to 1 mM for an additional 4 h at 30°C. The cell were harvested by centrifugation and resuspended in starting buffer (50 mM sodium acetate, 100 mM NaCl, 0.1 mM TCEP, 5% glycerol, pH 4.9). The cells were lysed by passage though a French pressure cell and centrifuged. The supernatant was subjected to cation exchange chromatography using HiTrap SP FF column (GE Healthcare) and fast liquid chromatography (AKTA). PyC-77 was eluted by a NaCl gradient. Fractions were subjected to SDS-PAGE and fractions containing PyC-77 were combined and dialyzed against 50 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer (pH 7.5) containing 500 mM NaCl and 0.1 mM TCEP. To further purify PyC-77 the protein was subjected to Superdex-75 size exclusion chromatography. Eluted fractions were analyzed by SDS-PAGE to determine purity. Fractions containing pure PyC-77 were combined, dialyzed against 50 mM HEPES buffer (pH 7.5) containing 500 mM NaCl, 0.1 mM TCEP, and 5% glycerol, flash frozen and stored at -80°C.

Plasmid pQC026C a derivative of vector pET28b encoding a terminal hexahistidine tagged BLAP was transformed into *E. coli* strain BL21 (λ DE3). The strain was grown in LB medium to an OD_{600} of 0.8 and induced by addition of IPTG to 1 mM

for an additional 4 h. BLAP was purified by Ni^{+2} affinity chromatography as previously described (32). The C-terminal hexahistidine tagged Δ2-65 BirA was purified as previously described (25).

Electrophoretic Mobility Shift Assay (EMSA) of DNA binding.

The predicted *B. subtilis* BirA binding sites upstream of the coding sequences of *bioO*, *yhfU* and *yuiG* were PCR amplified from *B. subtilis* 168 genomic DNA with primers SKH014 and SKH015, SKH016 and SKH017 and SKH018 and SKH019, respectively. A DNA fragment containing one half-site of *B. subtilis bioO* was similarly amplified with primers SKH034 and SKH035. *E. coli bioO* was amplified with primers SKH026 and SKH027 from MG1655 genomic DNA. Negative control DNA (*blap*) was amplified from *B. subtilis* 168 genomic DNA with primers SKH028 and SKH029. All DNA fragments were 125 bp in length. The PCR products were sized on a 1.8% agarose gel and purified using a QIAquick PCR Purification Kit (Qiagen). DNA concentrations were determined at OD_{260} by using a NanoDrop 2000c. Purified BirA was incubated with the small nucleophile hydroxylamine at neutral pH to cleave any Bio-5′-AMP bound in the active site (33) and then dialyzed against storage buffer. The reaction contained 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 50 mM NaCl, 10% glycerol, 40 nM DNA and various concentrations of BirA (500 nM, 250 nM, 125 nM, 62.5 nM, 31.25 nM, 15.6 nM), 1 mM ATP, 1 mM $MgCl_2$, and 1 μM biotin (26). The binding reactions were incubated at room temperature for 30 min and then loaded into a 6% DNA retardation gel (Invitrogen). The gel was run in 0.5X TBE at 100 V for 85 min. The gel was stained with SYBR Green I nucleic acid gel stain (Invitrogen) and visualized using Bio-Rad Chemidoc XRS and Quantity One software.

Chemical cross-linking of BirA.

Purified BirA was dialyzed against 50 mM HEPES buffer (pH 7.5) containing 500 mM NaCl, 0.1 mM TCEP and 5% glycerol. BirA (30 μM) was incubated with or without biotin (0.1 mM) and ATP (0.1 mM) at room temperature for 30 min. Various concentrations of ethylene glycol bis[succinimidylsuccinate] (EGS) (0.125, 0.25, 0.375, 0.5 mM) were added and incubated at room temperature for 30 min. SDS-loading dye was added and samples were heated to 99°C for 5 min. Samples were loaded on 4-20% gradient SDS polyacrylamide gels (Bio-Rad) and run at 110 v for 1 h.

Mass spectrometry.

Purified acceptor proteins AccB-86, PyC-77, and BLAP (10 μM), with or without incubation with BirA (0.5 μM), ATP (3 mM), and biotin (250 μM), were dialysed against 2 mM ammonium acetate, dried under a stream of nitrogen and subjected to electrospray mass spectrometric analysis (28).

Bio-5′-AMP synthesis assays.

The assays contained 50 mM Tris-HCl buffer (pH 8.0), 5.5 mM $MgCl_2$, 100 mM KCl, 0.1 mM TCEP, 10 μM ATP, 25 μM biotin, 2.5 μM BirA, 0.1 μM [α-^{32}P]ATP and with or without 50 μM AccB-86, PyC-77, or BLAP for a total reaction mixture of 20 μl (25, 26). The reaction mixtures were incubated at room temperature for 30 min. A portion of each reaction mixture (1 μl) was spotted on cellulose thin-layer chromatography (TLC) plates and developed in isobutyric acid-NH_4OH-water (66:1:33) (75). The thin-layer chromatograms were dried for 10 h and exposed to a phosphorimaging screen and visualized using a Fujifilm FLA-3000 Phosphor Imager and Fujifilm Image Gauge software.

β-Galactosidase assays.

Cultures were grown overnight in defined medium containing 1.6 nM biotin. The cultures were diluted to an OD_{595} of 0.2 in defined media containing various concentrations of biotin (1.6, 4, 20, 40, 80, 400 nM, 4 μM) and grown to OD_{595} of 0.8 and induced with 1 mM IPTG for an additional 2 h. β-Galactosidase activity was determined as described by Harwood and Cutting for *Bacillus* following permeabilization with lysozyme (51).

Results

B. subtilis BirA is a Group II BPL that binds three operator sites.

To test the relative affinities of the predicted *B. subtilis* BirA binding sites we purified the protein (Figure 2.2) and performed electrophoretic mobility shift assays (EMSAs) on DNA fragments containing the three sites (Figure 2.3A). Full dependence of binding on ATP and biotin required treatment of the protein with neutral hydroxylamine to remove Bio-5′-AMP accumulated in the active site during expression in *E. coli*. With the treated BirA binding of the biotin biosynthetic operator (*bioO*) was observed only in the presence of both biotin and ATP (Figure 2.3B). BirA also bound the *yhfU* and *yuiG* operators only in the presence of biotin and ATP (Figure 2.3C, 2.3D). Although the three binding sites have slightly different DNA sequences (Figure 2.3A), analysis of binding over a range of BirA concentrations showed that the three sites had very similar binding affinities (Figure 2.3E). *B. subtilis* BirA failed to show non-specific DNA binding (Figure 2.3F) as assayed by use of a fragment from the coding sequence of the *yngHB* gene. BirA preparations that had not undergone hydroxylamine treatment showed some interaction

with *bioO* in the absence of biotin and ATP (Figure 2.3G). BirA did not interact with a site that was composed of only one of the *B. subtilis bioO* inverted repeats suggesting that the form of BirA active in DNA binding is a dimer (Figure 2.3H). *B. subtilis* BirA interacted only very weakly with the *E. coli bioO* DNA site (Fig 2.3I).

B. subtilis BirA biotinylates three cognate proteins and the reactions proceed via Bio-5′-AMP.

Each of the acceptor proteins, AccB-86, PyC-77 and BLAP (Figure 2.4A), was purified after high-level expression in *E. coli* (Figure 2.2). In the first two cases the N-terminal halves of the proteins were deleted to avoid protein aggregation during purification (the deleted segments are responsible for interaction with other proteins of the enzyme complexes and play no role in biotinylation). Electrospray ionization mass spectrometry results matched the theoretical masses of the apo forms of the three proteins and showed that the preparations were free of the biotinylated forms (Figure 2.4B). When the apo forms of AccB-86, PyC-77 and BLAP were incubated with ATP, biotin and purified *B. subtilis* BirA and subsequently analyzed by mass spectrometry, mass values very similar to the theoretical values for biotinylated forms of all three acceptor protein were obtained (Figure 2.4B). In the presence of α-^{32}P-labeled ATP and biotin *B. subtilis* BirA formed labeled Bio-5′-AMP**.** Upon addition of any of the three acceptor proteins (AccB-86, PyC-77, or BLAP) the Bio-5′-AMP intermediate was no longer detected and AMP accumulated which indicated transfer of biotin to each of the acceptor proteins (Figure 2.4C). In conclusion, we have experimentally verified the bioinformatic analyses of the genes regulated by *B. subtilis* BirA and the proteins modified by its ligase activity.

B. subtilis BirA does not require the N-terminal DNA binding domain for normal ligase activity**.**

The *in vitro* data presented above indicate that *B. subtilis* BirA behaves in a manner that strongly parallels that of *E. coli* BirA despite the low sequence similarity of the two proteins. However, *E. coli* BirA has recently been shown to undergo extensive inter-domain communication that is required for full ligase activity (25). These observations confirm and extend those of Xu and Beckett (100) and demonstrate that the *E. coli* BirA N-terminal domain plays a role in organizing the active site of BirA (25). Specifically, the wing of the winged HTH structure interacts with the ligase active site biotin binding loop and acts to organize the active site to give high affinity binding of biotin and Bio-5′-AMP (25).

To determine if another Group II BPL, that of *B. subtilis*, requires such communication of the catalytic and N-terminal domains for full ligase activity, we constructed genes encoding several N-terminal *B. subtilis* BirA deletion proteins. The deletion endpoints were based on structural modeling of *B. subtilis* BirA based on the crystal structure of *S. aureus* BirA (PDB 4DQ2) (71) (Figure 2.5). BirA deletions Δ2-63 and Δ2-65 eliminated the predicted N-terminal domain whereas BirA deletions Δ2-74 and Δ1-81 also cut into the predicted central catalytic domain. Complementation assays using the *E. coli birA1* mutant strain BM4092 and the *E. coli* Δ*birA* deletion strain VC618 were used to test the ligase activities of the *B. subtilis* BirA N-terminally deleted proteins. These assays showed that upon expression of the *B. subtilis* Δ2-63 BirA, Δ2-65 BirA and wild type proteins in strain BM4092 all three BPLs supported growth equally well on medium containing 1.6 nM biotin (the minimal level allowing growth of *E. coli*)

(Figure 2.6A). These results indicated that the ligase activities of these two deletion proteins were essentially normal. In contrast, upon expression of the *E. coli* BirA lacking its amino terminus (Δ2-65 BirA), growth of the transformed strain required a biotin concentration that was 1000-fold greater. The complementation activities of the other two *B. subtilis* deletion proteins, BirA Δ2-74 and BirA Δ1-81 were either partially (BirA Δ2-74) or totally (BirA Δ1-81) compromised (Figure 2.6A). To ensure that residual activity of the host BirA1 protein did not play a role in growth restoration we also performed complementation of the Δ*birA E. coli* strain VC618 and obtained a similar complementation pattern (Figure 2.6B).

The ligase activities of the purified *B. subtilis* BirA N-terminal deletion proteins (Figure 2.2) were also tested by *in vitro* biotinylation assays. As expected from the complementation results the Δ2-63 and Δ2-65 BirA proteins showed Bio-5′-AMP synthesis and biotin transfer activities indistinguishable from those of the wild type protein (Figure 2.7). In contrast the Bio-5′-AMP synthetic abilities of the Δ2-74 and Δ1-81 BirAs were significantly reduced relative to wild type BirA. However, the biotin transfer activity of the Δ2-74 BirA was comparable to wild type levels. Biotin transfer by the Δ1-81 BirA was significantly reduced compared to the wild type protein (Figure 2.7). In agreement with prior work (25, 100) the *E. coli* Δ2-65 BirA was significantly reduced in Bio-5′-AMP synthesis and in biotin transfer (Figure 2.7).

Bacillus subtilis BirA dimerizes in the presence of biotin and ATP.

Dimerization is required for DNA binding of *E. coli* BirA and dimerization is dependent on biotin and ATP binding. To determine the oligomerization state of *B. subtilis* BirA, purified BirA was incubated with EGS in the presence or absence of biotin

and ATP. Chemical crosslinking indicated that BirA forms a dimer only in the presence of both biotin and ATP (Figure 2.8A). Dimerization was also observed for the N-terminally truncated Δ2-65 BirA (Figure 2.8B). Note that the characterized Group I BPLs can either be dimeric as is the *Pyrococcus horikoshii* protein (7, 40) or monomeric as are the *Mycobacterium tuberculosis* (76), *Aquifex aeolicus* (94) and *Propionibacterium freudenreichii* subsp. *shermanii* (84) BPLs.

Bacillus subtilis BirA follows the *E. coli* BirA regulatory model.

In *E. coli* regulation of the biotin biosynthetic gene transcription depends not only on the concentration of biotin but also the levels of unbiotinylated AccB (1, 38, 87). This is an important attribute because biotin is only active in central metabolism when it is protein bound. Hence, the rate of biotin operon transcription is sensitive not only to the intracellular concentration of biotin, but also to the supply of the proteins to which the biotin must be attached. Moreover, accumulation of the unmodified protein increases the rate of biotin synthesis thus ensuring replacement of the biotin consumed in protein modification.

To determine if this regulatory facet also applies to another Group II BPL, we constructed a *B. subtilis* strain that could be used to monitor regulation of biotin operon transcription upon alteration of the levels of the AccB-86 acceptor protein. This strain contains the *bioB141* mutation rendering it a biotin auxotroph, a *bioW-lacZ* transcriptional fusion and an ectopic IPTG-inducible gene encoding AccB-86. This strain was grown in chemically defined media containing various concentrations of biotin. Upon induction of AccB-86 expression with IPTG β-galactosidase assays showed that

expression of the biotin operon was dereprepressed at biotin concentrations that normally cause repression (Figure 2.9) in a manner similar to that seen in *E. coli* (1, 38, 87).

Discussion

B. subtilis BirA complements the ligase activity of *E. coli* BirA mutant strain BM4092, but fails to complement the regulatory function of *E. coli* BirA. This latter result is not surprising since *E. coli* and *B. subtilis* BirAs bind different operator sequences and have different spacing of the palindromic elements. We have shown that *B. subtilis* BirA is a Group II BPL that binds equally well to the three operators predicted by others (Figure 2.3). Binding is observed only in the presence of both biotin and ATP indicating that Bio-5′-AMP is the regulatory ligand. Interestingly, EMSA showed that *B. subtilis* BirA weakly binds *E. coli bioO* (Figure 2.3I), suggesting some distant relationship between the operators. Similar to *E. coli*, dimerization of *B. subtilis* BirA occurs only in the presence of biotin and ATP, a further indication that Bio-5′-AMP is the regulatory ligand (Figure 2.8A). The *B. subtilis* Δ2-65 BirA also formed dimers indicating that the N-terminal domain is not required for dimerization (Figure 2.8B).

Deletions of the N-terminal DNA binding domain of *E. coli* BirA result in proteins having only very weak ligase activities indicating inter-domain interactions are required for full ligase activity (25, 100). These interactions have been shown to occur between the wing of the winged HTH domain and the biotin-binding loop of the catalytic domain. Deletion of only the fourteen-residue wing has as drastic an effect as deletion of the entire N-terminal domain, but was largely restored by insertion of a foreign wing of

similar structure (25). Moreover, a mutation within the wing can restore function to proteins having mutant biotin binding loops (25).

Although the HTH domain of our modeled *B. subtilis* BirA structure includes a wing, the structure is not required for full ligase activity. Both the Δ2-63 and Δ2-65 BirAs lack the entire N-terminal domain but performed the ligase partial reactions as well as the wild type protein both *in vivo* and *in vitro* (Figure 2.7). Deletions that entered the predicted catalytic core of the protein resulted in either compromised (Δ2-74 BirA) or highly defective (Δ1-81 BirA) proteins (Figure 2.7). The Bio-5′-AMP synthetic ability of the Δ2-74 BirA was significantly decreased, although the protein transferred the biotin moiety normally suggesting that the putative α-helix near residue 74 may be involved in stabilizing biotin and ATP or Bio-5′-AMP binding. Although *B. subtilis* Δ1-81 BirA failed to replace the *E. coli* ligase *in vivo* (Figure 2.6), it retained weak Bio-5′-AMP synthesis and biotin transfer activities (Figure 2.7) suggesting that the loop predicted near residue 81 is required for efficient binding of biotin and ATP. These data indicate that unlike *E. coli*, *B. subtilis* BirA does not require an intact N-terminal DNA binding domain for full ligase activity. Instead, the first α-helix and loop of the modeled central domain seem to be important in binding biotin and ATP.

E. coli BirA is a highly dynamic protein as shown both by physical (91, 92, 99) and mutational (26) analyses. In the latter case mutations in the catalytic and C-terminal domains as well as in the HTH domain can result in super-repressor phenotypes. These proteins are BirAs that repress *bio* operon expression even at biotin concentrations that normally fail to repress transcription. Although *E. coli* BirA has been studied for over 30 years (8, 75) and four different crystal structures have been solved, the details of how the

protein binds its operator site remain unknown. Despite much effort in several laboratories no diffraction grade crystals of the operator DNA with liganded *E. coli* BirA have been obtained. This could be due to competing interactions of the N-terminal domain with the catalytic domain and operator giving a mixture of molecular species that preclude crystallization. If so, the apparent lack of such interactions in *B. subtilis* BirA may allow crystallization of this protein with its operator DNA.

The BLAP protein was biotinylated by *B. subtilis* BirA *in vitro* suggesting that BLAP may be the biotin carboxyl carrier protein for a biotin-dependent enzyme. The genes that neighbor *yngHB*, the BLAP encoding gene, are annotated as propionyl-CoA carboxylase subunits. However, a recent report suggests that these genes may comprise a methylcrotonyl-CoA carboxylase involved in leucine degradation (55).

Tables

Table 2.1. Bacterial strains used in this chapter.

Strain	Relevant Genotype or Description	Reference or Derivation
B. subtilis		
168	*trpC2*	Lab collection
1A330	*aroG932 bioB141*	BGSC
SKH001	1A330 *bioW::lacZ*	This study
SKH002	SKH001 *amyE*:: P spac *accB-86*	This study
E. coli		
MG1655	*E. coli* K-12 wild type	Lab collection
DH5α	*Δ(argF-lacZ)U169 glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Lab collection
BM4092	[*araD139]B/r, Δ(argF-lac)169, λ-, TP(bioF-lacZ)501, flhD5301, Δ(fruK-yeiR)725(fruA25), relA1, rpsL150(strR), rbsR22, birA1, Δ(fimB-fimE)632(::IS1), deoC1*	Barker and Campbell, 1980
BL21 λ (DE3)	*ompT, hsdSB (rB–, mB–), dcm, gal, λ(DE3)*	Novagen
VC618	MG1655 *Δ lacZY, bioF::lacZY, birA::Km^R,* pVC18	Chakravartty and Cronan, 2012
VC832	BL21 λ (DE3), pVC36	Chakravartty and Cronan, 2013

Table 2.2. Plasmids used in this chapter.

Plasmid	Relevant Genotype or Description	Reference or Derivation
pET19b	T7 promoter expression vector, AmpR	Novagen
pSKH001	pET19b encoding N-terminal hexahistidine tagged *B. subtilis* BirA, AmpR	This study
pET28b	T7 promoter expression vector, KanR	Novagen
pSKH003	pET28 encoding accB-86	This study
pSKH004	pET28 encoding pyc-77	This study
pQC026	pET28b encoding C-terminal Hexahistidine tagged BLAP	[23]
pBAD322K	Medium copy expression vector, KanR	[48]
pSKH005	pBAD322K encoding *B. subtilis* 64-325	This study
pSKH006	pBAD322K encoding *B. subtilis* WT BirA	This study
pSKH007	pBAD322K encoding *E. coli* BirA 66-321	This study
pSKH008	pBAD322K encoding *E. coli* WT BirA	This study
pSKH009	pBAD322K encoding *B. subtilis* 66-325	This study
pSKH010	pBAD322K encoding *B. subtilis* 75-325	This study
pSKH011	pBAD322K encoding *B. subtilis* 82-325	This study
pBAD322Cm	Medium copy expression vector, CmR	[48]
pSKH012	pBAD322Cm encoding *B. subtilis* 64-325	This study
pSKH013	pBAD322Cm encoding *B. subtilis* WT BirA	This study
pSKH014	pBAD322Cm encoding *E. coli* BirA 66-321	This study
pSKH015	pBAD322Cm encoding *E. coli* WT BirA	This study
pSKH016	pBAD322Cm encoding *B. subtilis* 66-325	This study
pSKH017	pBAD322Cm encoding *B. subtilis* 75-325	This study
pSKH018	pBAD322Cm encoding *B. subtilis* 82-325	This study
pSKH019	pET19b encoding N-terminal hexahistidine tagged BirA 64-325, AmpR	This study
pSKH020	pET19b encoding N-terminal hexahistidine tagged BirA 66-325, AmpR	This study
pSKH021	pET19b encoding N-terminal hexahistidine tagged BirA 75-325, AmpR	This study
pSKH022	pET19b encoding N-terminal hexahistidine tagged BirA 82-325, AmpR	This study
pMUTIN4	spoVG-*lacZ*, ermR	[49]
pDR111	SpcR, 5'-*amyE*, 3'-*amyE*, p*hyper-spank*	G. W. Ordal
pSKH023	pMUTIN4 encoding *B. subtilis bioW* internal 500 bp	This study
pSKH024	pDR111 encoding *B. subtilis accB-86*	This study

Table 2.3. Oligonucleotide primers used in this chapter.

Oligo-nucleotide	Description	Sequence
SKH001	BirA F NdeI	GAGTGGCTGAACATATGCGGTCAAC
SKH002	BirA stop R XhoI	GGCTTGTACCCTCGAGTTAGCCCAATTC
SKH005	accB F NcoI	CCATGGAAAAGCAAGATGAGAATCTGCATAAA
SKH006	accB stop R XhoI	CTCGAGCTTACTCCGCTTTTACAAGAAATAGAGG
SKH007	pyc F NcoI	CCATGGAACGGACAAATCCAAGCCAC
SKH009	pyc stop R XhoI	CTCGAGTTTATGCTTTTTCAATTTCAAGGAGC
SKH014	bioO F	GATCCTTTCTTCTATTGACAGAAAC
SKH015	bioO R	CGC CCTTTCACTGATAACTGAAGAAC
SKH016	yhfU F	ACCATCAAAAACCGGTCTGCCATAC
SKH017	yhfU R	CCAAAAAGTAATCAAATATGGTTATAC
SKH018	yuiG F	ATTGATCGGACTGTCTTGTT
SKH019	yuiG R	CCCTTAGGTTGACATACACA
SKH026	EC bioO F	GATATGGCGTTGGTCAAAGGCAAG
SKH027	EC bioO R	GGGGCTTCTCCAAAACGTGTTTTTTG
SKH034	bioO half F	GAAAAAGACCGTTTTGTGTG
SKH035	bioO half R	ATTCAAAGGTTAACAATTAGAATATATTATTCTCTCCTG
SKH028	Non 125 F	GCATGACGGTTAGCATACAAATGGCAG
SKH029	Non 125 R	AACGATCGGGATTTCCATTTTCATCGATTC
SKH036	BirA 64-325 EcoRI F	ACTGCGAATTCACCATGAAACCCGGAAAACTCAGTGAAAGCG
SKH037	BirA SalI R	ACTGAGTCGACTTAGCCCAATTCGATATCGGCAG
SKH038	BirA EcoRI F	CTGACGAATTCACCATGCGGTCAACATTAAGAAAAGACC
SKH040	BirA 64-325 NdeI F	CAGTCCATATGAAACCCGGAAAACTCAGTGAAAG
SKH041	BirA BamHI R	ACTGGGATCCTTAGCCCAATTCGATATCG
SKH042	EC BirA 65-321 EcoRI F	ACTGCGAATTCACCATGCAGTTACTTAATGCTAAACAGATATTG
SKH043	EC BirA SalI R	CAGTCGTCGACTTATTTTTCTGCACTACGCAG
SKH044	BirA 82-325 NdeI F	ACGTACATATGGGCCAGCATCTTATTTACCATG
SKH045	BirA 82-325 EcoRI F	AGCTAGAATTCACCATGGGCCAGCATCTTATTTAC

Table 2.3. (cont.)

SKH046	BirA 66-325 EcoRI F	ACTGCGAATTCACCATGGGAAAACTCAGTGAAAGCG
SKH047	BirA 66-325 NdeI F	CAGTCCATATGGGAAAACTCAGTGAAAGCG
SKH048	BirA 75-325 EcoRI F	ACTGCGAATTCACCATGTTTGGATTAAAAACGGAAG
SKH049	EC BirA EcoRI F	ACTGGAATTCACCATGAAGGATAACACCGTGCCAC
SKH050	BirA 75-325 NdeI F	CAGTCCATATGTTTGGATTAAAAACGGAAG
SKH057	bioW 500 F	ACGTGAATTCCATACAGTCAATGCTTTATTAG
SKH058	bioW 500 R	GACTGGATCCCTTACCCGCAACATAGCCTG
SKH065	ycgB F	CTTACAGAAGAGCGGTAAAAGAAGAAATAAAAAAG
SKH066	lacI R	CCGTCTCACTGGTGAAAAGAAAAAC
SKH067	ldH R	CATTGCTTTTTCTTTATTTACATCAATGACCACAA
SKH069	RBS accb-86 F	ACGGTCGACAAGGAGGAAAAAATATGGAAGCACCAAAGCAAGATG
SKH070	accb-86 R	GACGCATGCTTACTCCGCTTTTACAAGAAATAGAGGTTGTC
SKH073	pDR111 F Seq	CTCGAGGGTAAATGTGAGCACTCAC
SKH074	pDR111 R Seq	GAAAGTATTACATATGTAAGATTTAAATGCAACCG
SKH075	specR F	TGAATCTTCTCCATTAGAACATAGGGAGAG
MM1	5' BioW F	CGATCCTTTCTTCTATTGACAGAAACAGG
MM2	LacZ R	GGTGTAGATGGGCGCATCGTAAC
MM3	pSPAC F	CTACACAGCCCAGTCCAGACTATTCGG
MM4	3' BioW R	ATGGCGTCATCTAGTTCTTTTTTGCGG

Figures

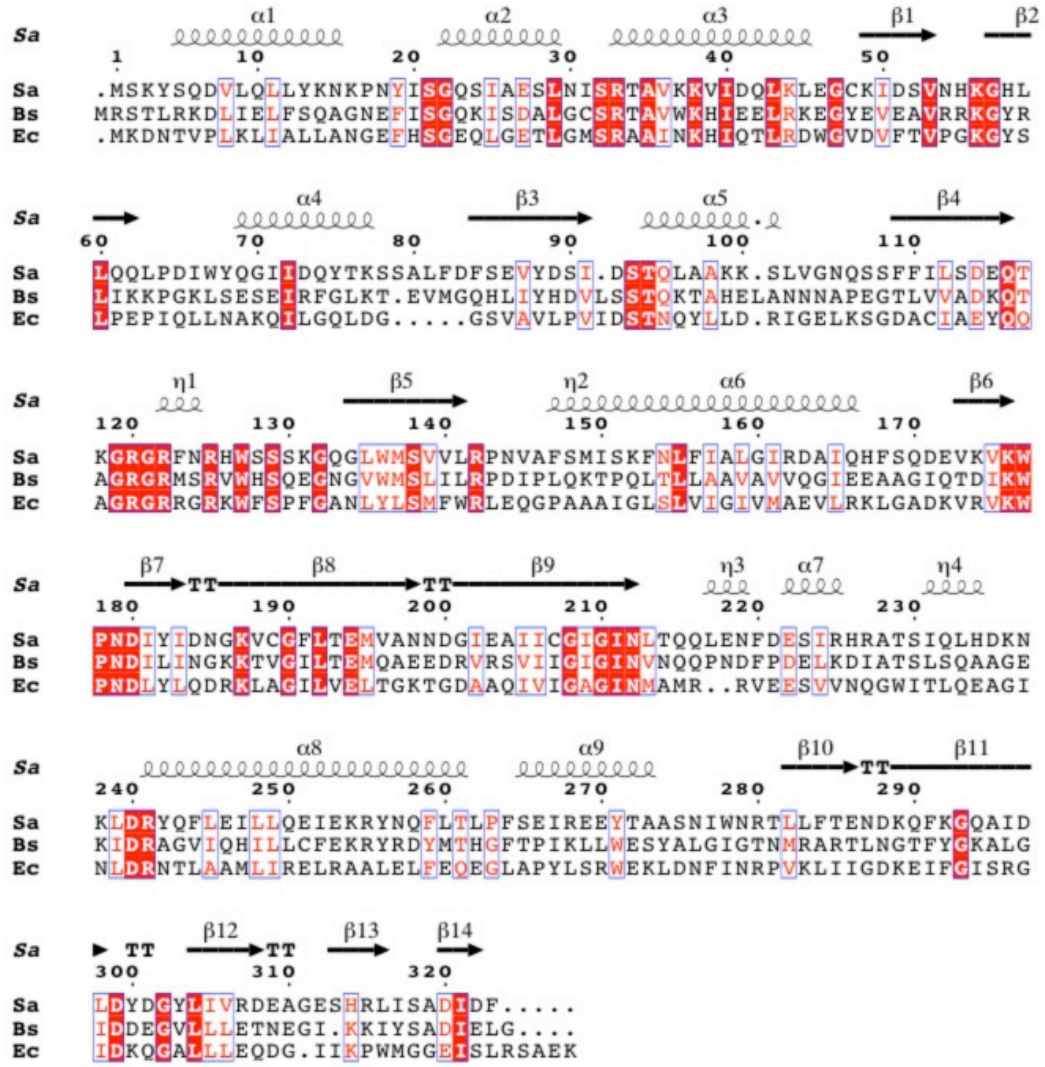

Figure 2.1. Sequence alignments of *S. aureus* BirA, *B. subtilis* BirA and *E. coli* BirA. *B. subtilis* BirA has 31% amino acid identity to *S. aureus* BirA and 27% amino acid identity to *E. coli* BirA. Conserved residues are in white text and highlighted in red and similar residues are in red text and boxed in blue. The *S. aureus* BirA secondary structure (PDB: 4DQ2) (71) is shown above the amino acid sequence.

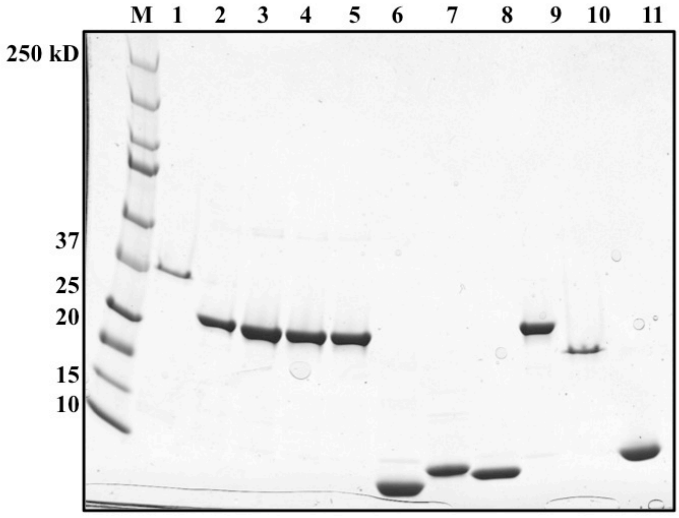

Figure 2.2. Purification of the wild type and N-terminal deletion BirA proteins and the biotin acceptor proteins. The proteins were purified as described in Experimental Procedures and subjected to SDS-electrophoresis on a 4-20% polyacrylamide gel (from BioRad). M: molecular weight strandards (Precision Plus Protein Standard Kaleidoscope from BioRad). Lane 1: *B. subtilis* N-terminally hexahistidine-tagged BirA (38.9 kDa). Lanes 2-5. *B. subtilis* N-terminally hexahistidine-tagged Δ2-63 BirA (31.8 kDa), Δ2-65 BirA (31.6 kDa), Δ2-74 BirA (30.4 kDa) and Δ1-81 BirA (29.7 kDa), respectively. Lanes 6-11 are the *B. subtilis* acceptor proteins AccB-86 (9.4 kDa), PyC-77 (8.3 kDa) and biotin lipoyl attachment protein (BLAP) (8.73 kDa). Lane 10 is *E. coli* C-terminal hexahistidine-tagged BirA. Lane 11 is *E. coli* C-terminal hexahistidine tagged Δ2-65 BirA (29.18 kDa) and lane 11 is *E. coli* AccB-87.

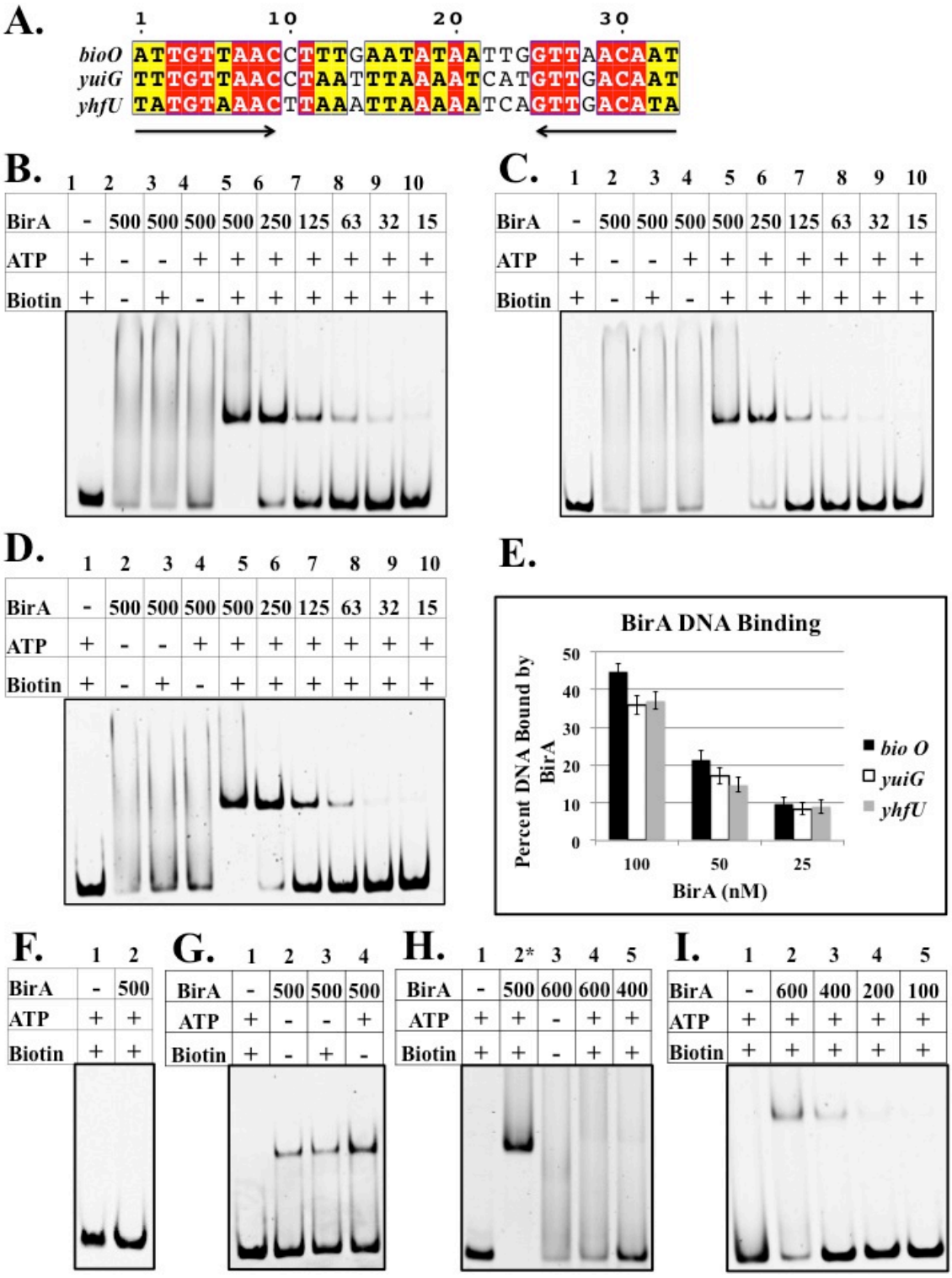

Figure 2.3. Sequence alignments of *B. subtilis* BirA DNA binding sites and electrophoretic mobility shift assay of DNA binding by BirA. A. *B. subtilis* has three predicted BirA DNA binding sites: 5' UTR of the *bioWAFDBI* operon, 5' UTR of *yuiG*,

Figure 2.3. (cont.)

and 5' UTR of the *yhfUTS* operon. Conserved residues are highlighted in red and similar residues are highlighted in yellow. **B.-D.** *B. subtilis* BirA binding to *bioO,* the *yuiG* operator and the *yhfU* operator, respectively. Note that only in the presence of biotin and ATP is binding observed. **E.** Quantitation of DNA binding by BirA (Quantity One software). The results show the average of three independent experiments, and the error bars denote standard error of the mean. **F.** BirA binding to non-operator DNA (a 125 bp internal fragment of the *yngHB* gene that encodes BLAP). **G.** BirA binding to *bioO* without hydroxylamine treatment. Bio-5′-AMP accumulates in the active site during expression in *E. coli* and survives purification of BirA. **H.** *B. subtilis* BirA binding to a half site of the inverted repeat of *B. subtilis bioO.* Note lane 2 is positive control full-length *bioO.* **I.** *B. subtilis* BirA binding to *E. coli bioO*. A collection of all putative BirA binding sites in diverse bacteria can be found in the RegPrecise database (http://regprecise.lbl.gov/RegPrecise/).

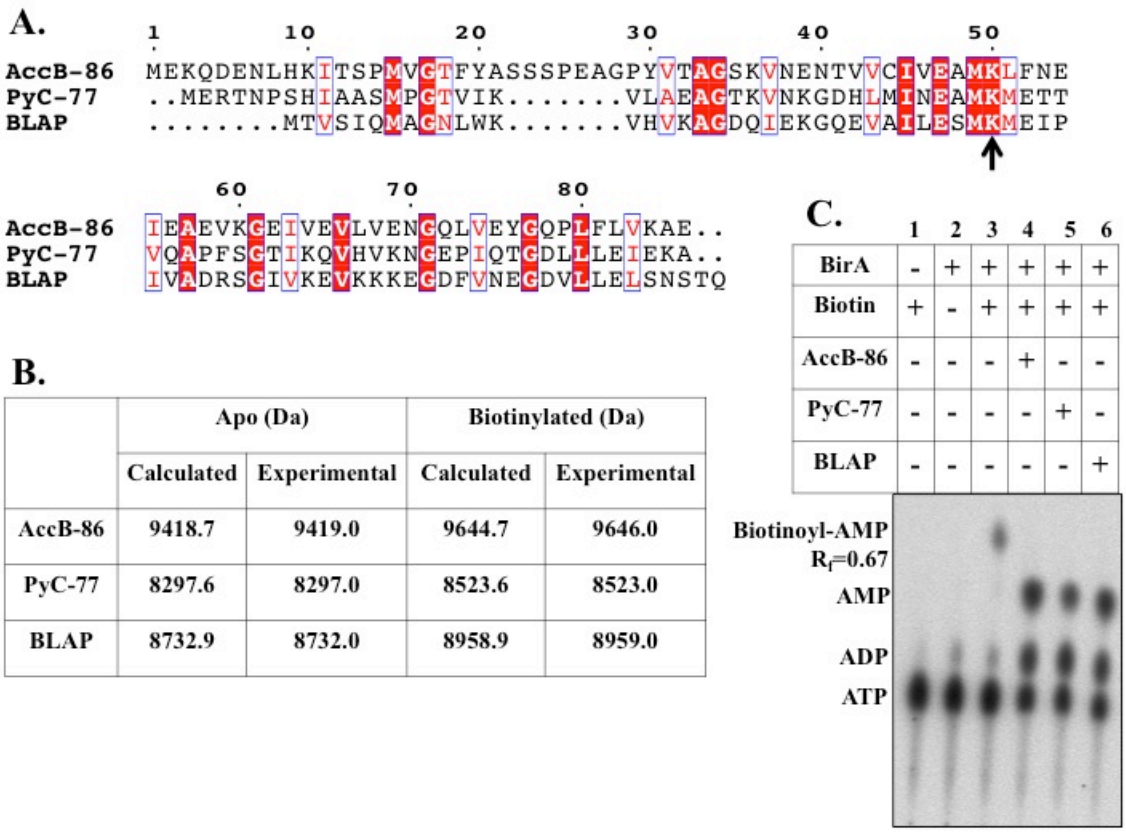

Figure 2.4. *In vitro* biotinylation of the *B. subtilis* biotin acceptor proteins. A. Sequence alignment of the *B. subtilis* biotinylated proteins. Conserved residues are in white text and highlighted in red and similar residues are in red text and boxed in blue. The black arrow indicates the conserved lysine residue that becomes biotinylated. **B.** Mass spectrometry values for purified acceptor proteins AccB-86, PyC-77, and BLAP. **C.** Thin layer chromatographic analysis of *B. subtilis* BirA ligase reaction: synthesis of Bio-5′-AMP and transfer of biotin to AccB-86, PyC-77, and BLAP.

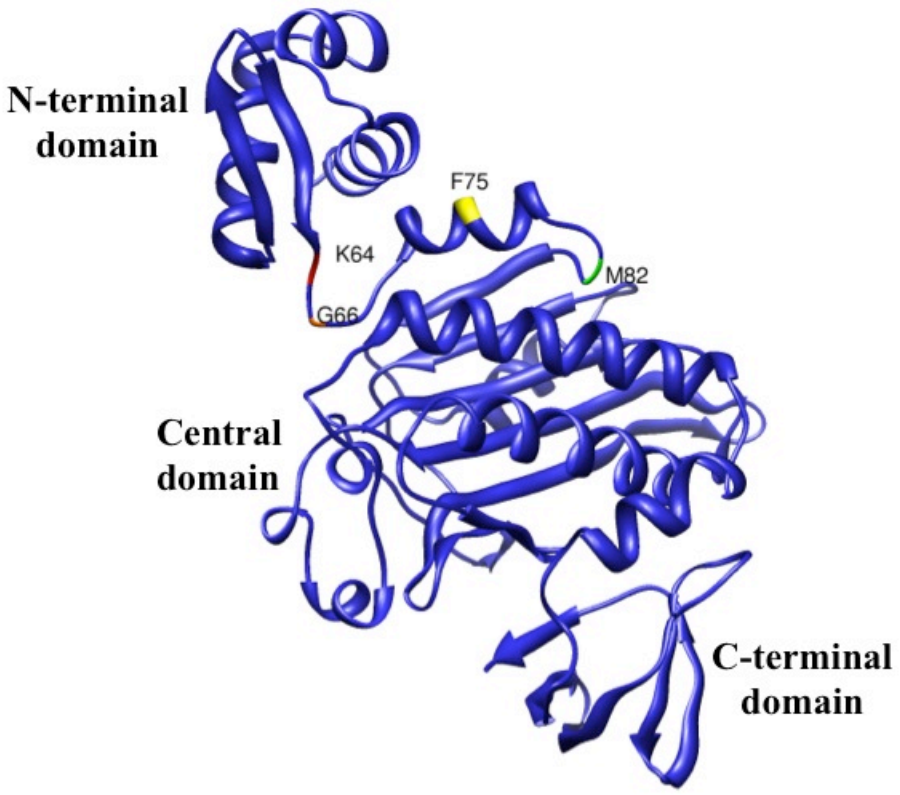

Figure 2.5. *B. subtilis* BirA modeled using the *S. aureus* BirA crystal structure (PDB 4DQ2) (71) as template. The UCSF Chimera package (74) was used to create the image. Residues corresponding to the N-terminal deletion end points are given. The domains of the modeled protein are indicated.

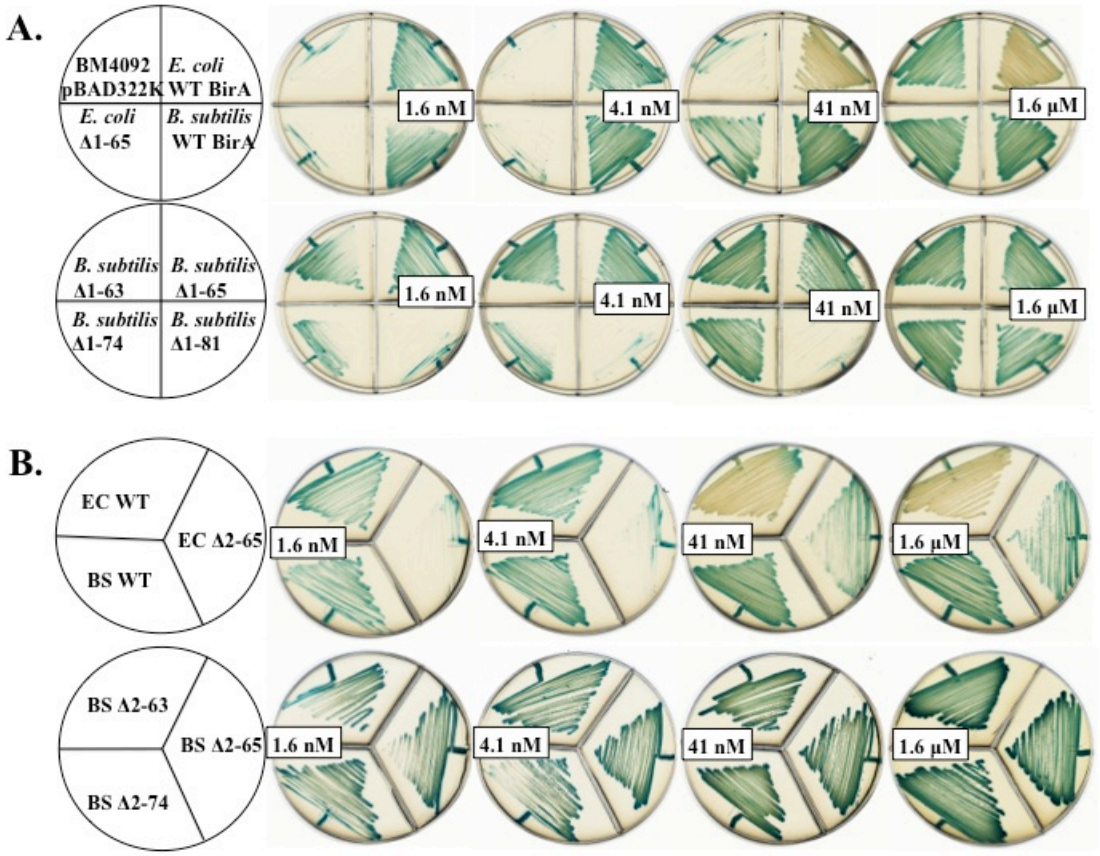

Figure 2.6. Complementation of *E. coli* strains by expression of the BirA N-terminal deletion proteins. A. Complementation of *E. coli* BirA mutant strain BM4092. **B.** Complementation of *E. coli ΔbirA* strain VC618. Strains were grown on M9 minimal medium containing different biotin concentrations (1.6 nM, 4.1 nM, 41 nM and 1.6 µM) and X-gal. The blue color indicates transcription of *bioF-lacZ* fusion. The white colonies indicate transcriptional repression of the biotin operon by BirA binding at *bioO*. Note that *B. subtilis* wild type BirA does not complement the regulatory function of *E. coli* BirA and thus gives blue colonies.

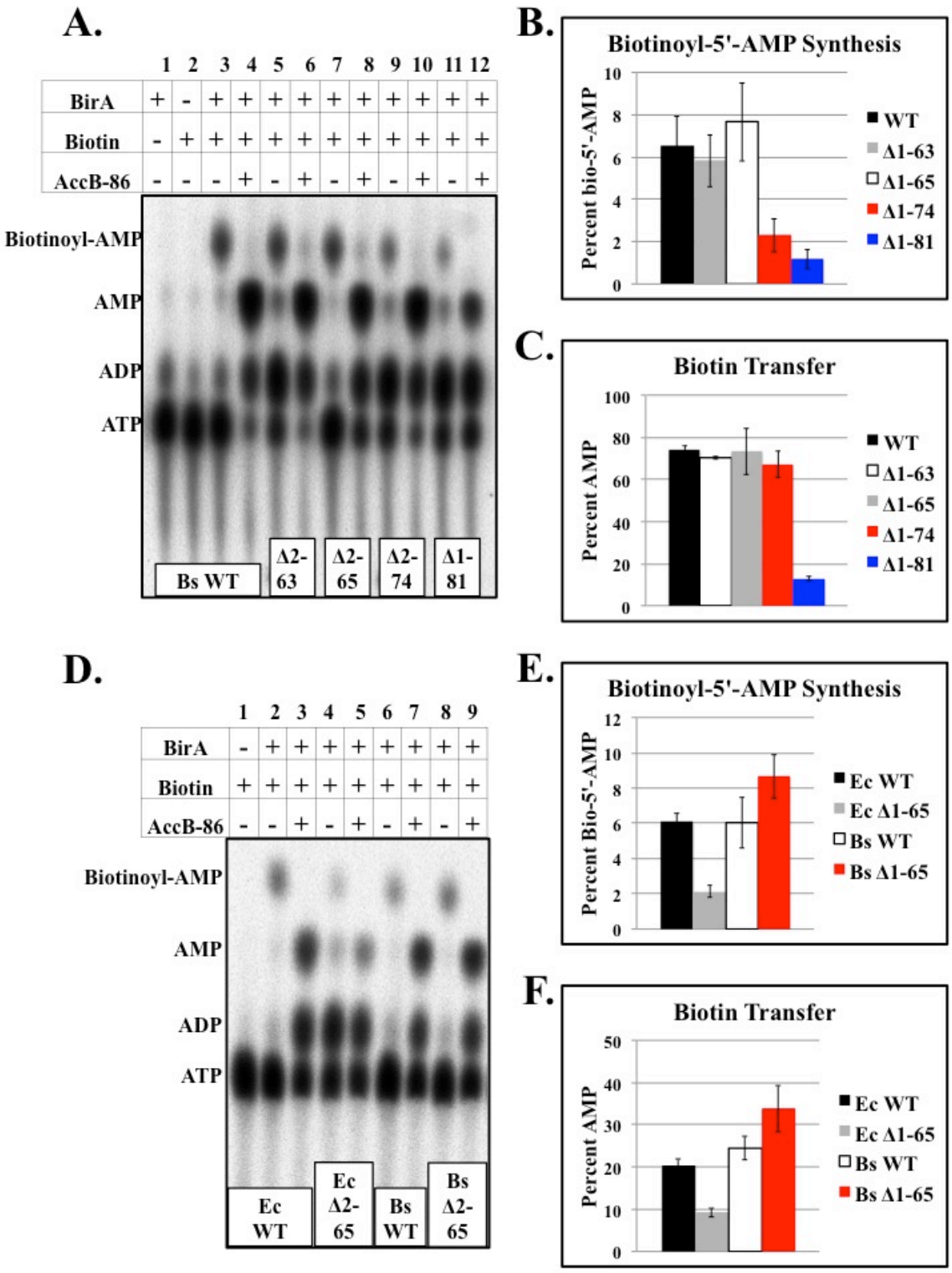

Figure 2.7. *In vitro* biotinylation analyses of the BirA N-terminal deletion proteins. **A.** Thin layer chromatographic analysis of wild type *B. subtilis* BirA and *B. subtilis* BirA N-terminal deletions with and without the addition of acceptor protein AccB-86. The *B.*

Figure 2.7. (cont.)

subtilis wild type BirA and the *B. subtilis* Δ2-63, Δ2-65, Δ2-74 and Δ1-81 BirA deletion proteins were assayed as given on the figure. **B.** Quantitation of Bio-5′-AMP synthesis by wild type *B. subtilis* BirA and the *B. subtilis* BirA N-terminal deletion proteins. The results show the average of three independent experiments, and the error bars denote standard error of the mean. **C.** Quantitation of biotin transfer to AccB-86 by wild type *B. subtilis* BirA and *B. subtilis* BirA N-terminal deletions. The results show the average of three independent experiments, and the error bars denote standard error of the mean. **D.** Thin layer chromatographic analysis of wild type *E. coli* BirA, *E. coli* Δ2-65 BirA, wild type *B. subtilis* BirA, and *B. subtilis* Δ2-65 BirA. **E.** Quantitation of Bio-5′-AMP synthesis by wild type *E. coli* BirA, *E. coli* Δ2-65 BirA, wild type *B. subtilis* BirA, and *B. subtilis* Δ2-65 BirA. The results show the average of three independent experiments, and the error bars denote standard error of the mean. **F.** Quantitation of biotin transfer to *E. coli* AccB-87 or *B. subtilis* AccB-86 by wild type *E. coli* BirA, *E. coli* Δ2-65 BirA, wild type *B. subtilis* BirA and *B. subtilis* Δ2-65 BirA. The results show the average of three independent experiments, and the error bars denote standard error of the mean.

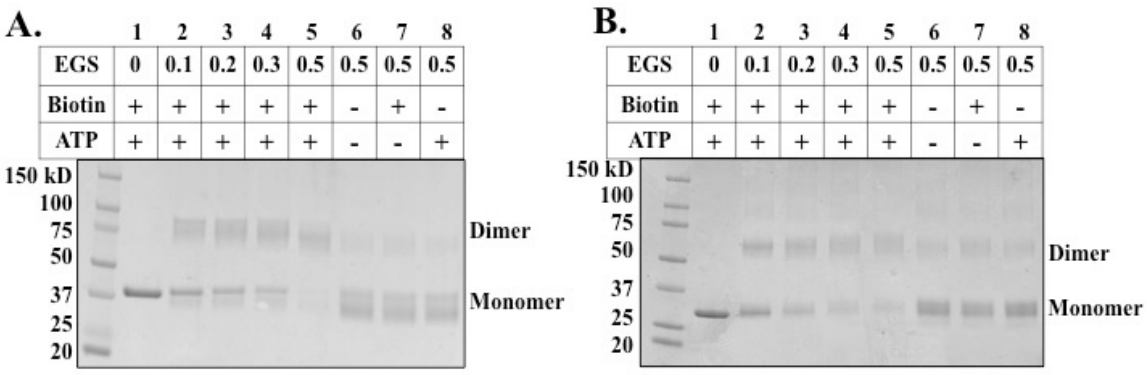

Figure 2.8. Chemical crosslinking of the *B. subtilis* wild type and Δ2-65 BirA proteins. A. Wild type BirA. **B.** The Δ2-65 BirA. Note that efficient dimer formation requires the presence of both biotin and ATP. The EGS concentrations are given in mM.

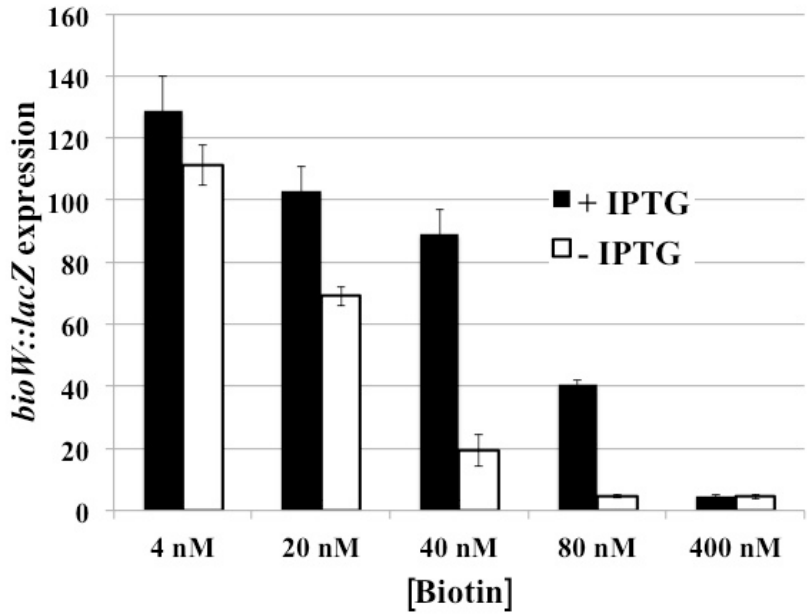

Figure 2.9. β-Galactosidase assays of the effect of AccB-86 levels on *bioO*-dependent transcription. The *bioW::lacZ* spac *accB-86* strain SKH002 was grown in defined medium supplemented with the indicated concentrations of biotin plus or minus addition of IPTG to induce synthesis of the AccB-86 acceptor protein. The results are the average of three independent experiments and the error bars denote standard error of the mean.

Chapter 3

The *Staphylococcus aureus* Group II Biotin Protein Ligase BirA is an Effective Regulator of Biotin Operon Transcription and Requires the DNA Binding Domain for Full Enzymatic Activity[2]

Introduction

Biotin protein ligase (BPL) is an essential enzyme that catalyzes covalent attachment of biotin to biotin dependent enzymes in a two step reaction (Figure 3.1A). In the first half reaction, BPL binds both biotin and ATP to synthesize biotinoyl-AMP (Bio-AMP) and pyrophosphate. The mixed anhydride bond of Bio-AMP then undergoes nucleophilic attack by the ε-amino group of the conserved lysine residue of the acceptor protein resulting in covalently attached biotin and free AMP (Figure 3.1A). Microbial BPLs are classified into two groups. Both Group I and Group II BPLs have well conserved catalytic cores and C-terminal domains. Group II BPLs (generally called BirAs) are classified based on the presence of an N-terminal helix-turn-helix DNA binding domain that is lacking in the Group I enzymes. The *E. coli* BirA is the best studied example of how an enzyme can also act as a transcriptional regulator of the biotin biosynthetic operon (12, 34). Transcriptional repression of the *E. coli* biotin operon occurs when biotin acceptor proteins have been fully biotinylated thereby allowing BirA to accumulate Bio-AMP in its active site. The presence of Bio-AMP results in BirA dimerization and subsequent DNA binding that represses transcription of the biotin biosynthetic operon (Figure 3.1B). Derepression of the biotin operon transcription occurs upon increased levels of unmodified biotin acceptor proteins (Figure 3.1D) (1, 38, 87) or

[2] This chapter was published in Molecular Microbiology (2016) 102(3):417-429.

low biotin levels (9) (Figure 3.1C). Transfer of accumulated Bio-AMP to acceptor proteins results in monomeric BirA which is unable to bind the biotin operator. The BirA of the distantly related bacterium *Bacillus subtilis* closely follows the *E. coli* transcriptional regulation model (17, 54).

The putative *S. aureus* BirA contains an N-terminal winged helix-turn-helix DNA binding domain. However, the protein was recently reported to dimerize in the absence of either biotin or Bio-AMP with a Kd of 29 μM (85), a concentration much lower than analogous preparations of *E. coli* BirA (90). Moreover, it was reported that *S. aureus* BirA bound the operator sequence in the absence of biotin and Bio-AMP leading to a question of the biological function of the protein. The reported electrophoretic mobility shift assays (EMSAs) showed that the unliganded *S. aureus* BirA bound the operator DNA with only a 6-fold lower affinity than the protein that bound the Bio-AMP regulatory ligand (Kd of 649 ± 43 nM vs. Kd of 108 ± 6 nM) (85). This high level of DNA binding activity in the absence of biotin or Bio-AMP should preclude *S. aureus* BirA from being an effective regulator of biotin operon transcription since DNA binding would occur without regard for the cellular levels of biotin and Bio-AMP. Thus, even if the cellular concentration of biotin was low and engenders a requirement for biotin biosynthesis (and/or biotin transport), operator binding would result in transcriptional repression of the biotin biosynthesis operon (and of the biotin transporter, *bioY*). Therefore, the *S. aureus* BirA would hinder rather than expedite cellular responses to biotin deficiency. To test whether or not the *S. aureus* BirA had this seemingly perverse activity, we utilized the fact that the *S. aureus* BirA DNA binding sites *bioO* and *bioY* are very similar to that of the previously studied *B. subtilis bioO* operator (Figure 3.3A). We

replaced the well-characterized *B. subtilis birA* gene (17, 54) with that encoding the *S. aureus* BirA and assayed regulation by the levels of biotin and acceptor protein. Since these experiments indicated that the *S. aureus* BirA was an efficient regulator *in vivo*, we reconsidered the EMSA results and utilized fluorescence anisotropy to test DNA binding in free solution.

Finally we tested the role of the *S. aureus* BirA DNA binding domain in the enzymatic activity of the protein. We expected that, like the *B. subtilis* protein (54), deletion of the N-terminal domain would not affect ligase activity. To our surprise deletion of the DNA binding domain resulted in severely decreased ligation activity as was previously observed with *E. coli* BirA (25, 100).

Materials and Methods

Strains, plasmids, chemicals and culture media.

All *B. subtilis* strains were derivatives of strain 168 whereas the *E. coli* strains were derivatives of strains B and K-12 (Table 3.1). The plasmids used and constructed are given in (Table 3.2). The rich medium for growth of *E. coli* and *B. subtilis* was LB broth. The defined medium for *E. coli* was M9 salts supplemented with 0.5% glucose or 0.5% glycerol and 0.01% vitamin-free Casamino Acids (Difco) whereas the defined medium for *B. subtilis* was Spizizen salts supplemented with 0.5% glycerol and 0.05% vitamin-free Casamino Acids (Difco) plus 0.01% each of tryptophan, tyrosine, isoleucine and phenylalanine. Antibiotics were used at the following concentrations (in μg/ml): kanamycin sulfate, 50; chloramphenicol, 25; erythromycin sulfate, 1; lincomycin, 12.5 and spectinomycin, 100. Oligonucleotides were purchased from Integrated DNA

Technologies. PCR amplification was performed using Phusion high fidelity DNA polymerase (New England BioLabs) according to manufacturer protocols. DNA constructs were sequenced by ACGT, Inc. Reagents and chemicals were obtained from Sigma-Aldrich and Fisher, unless otherwise noted. New England BioLabs supplied restriction enzymes and T4 DNA ligase. Life Technologies provided SYBER Green I Nucleic Acid Gel stain and the 6% DNA Retardation Novex TBE Gels.

Plasmid constructions.

The *S. aureus birA* gene was amplified by PCR from *S. aureus* strain Newman genomic DNA with primers SKH076 and SKH077 (Supporting information Table S3) that contained PciI and XhoI sites. The product was digested with PciI and XhoI and ligated into the NcoI and XhoI sites of pET28b resulting in plasmid pSKH025 that encoded the protein with a C-terminal hexahistidine tag.

The 500 bp immediately downstream of *B. subtilis birA* was PCR amplified from *B. subtilis* strain 168 genomic DNA using primers SKH102 and SKH103 (Supporting information Table S3) that, respectively, contained SalI and XhoI sites. The product was digested with SalI and XhoI and ligated into plasmid pDG780 digested with the same enzymes to give pDG780-panB. The last 500 base pairs of *B. subtilis cca* was PCR amplified from *B. subtilis* strain 168 genomic DNA with primers SKH104 and SKH105 (Supporting information Table S3) that contained a BamHI site and an overlapping *S. aureus birA* sequence, respectively. *S. aureus birA* was PCR amplified from *S. aureus* strain Newman genomic DNA with primers SKH106 that contained the *B. subtilis cca* overlap and SKH107 that contained an EcoRI site. The last 500 base pairs of *B. subtilis cca* and *S. aureus birA* were assembled by overlap PCR using primers SKH104 and

SKH107 (Table 3.3). The product was digested with BamHI and EcoRI and ligated into pDG780-panB digested with the same enzymes to give pSKH031.

The plasmids encoding the SaBirA N-terminal domain deletions were constructed as follows. *S. aureus* wild type *birA*, Δ2-65 *birA*, Δ2-74 *birA*, and Δ2-81 *birA* constructs were amplified by PCR from *S. aureus* strain Newman genomic DNA using forward primers SKH076, SKH078, SKH079, SKH080, respectively, plus reverse primer SKH081. The primers contained PciI and SalI sites, respectively. The products were digested with PciI and SalI and ligated into the NcoI and SalI sites of pBAD322Cm, to give pSKH026, pSKH027, pSKH028, and pSKH029 respectively. *S. aureus* Δ48-63 *birA* was amplified by PCR from *S. aureus* strain Newman genomic DNA with forward primer SKH076 containing a PciI site and reverse primer SKH097 which contained an overlapping sequence and forward primer SKH098 which contained the complement of the SKH097 overlap and reverse primer SKH080 containing a SalI site. The two fragments were fused by overlap extension PCR using primers SKH076 and SKH080 (Table 3.3). The product was digested with PciI and SalI and ligated into the NcoI and SalI sites of pBAD322Cm, to give pSKH030.

Bacillus subtilis strain constructions.

B. subtilis competent cell preparation and transformation were carried out as described by Dubnau and Davidoff-Abelson (41). Strains SKH001 and SKH002 were transformed with either linearized pSKH026 to replace the *B. subtilis birA* with *S. aureus birA* with a kanamycin cassette to give strains SKH003 and SKH004, respectively. The integration event was verified by PCR and sequencing of the PCR product with primers SKH134 and SKH135.

Purification of *S. aureus* BirA.

E. coli BL21 Star (DE3) was transformed with pSKH025. The strain was grown at 37°C in LB medium supplemented with kanamycin to an OD_{600} of 0.8 and expression was induced by addition of IPTG to 1 mM. Following growth for an additional 12 h at 30°C the cells were recovered by centrifugation and suspended in lysis buffer. Lysis buffer was 50 mM HEPES, 250 mM NaCl, 0.1 mM tris(2-carboxyethyl)phosphine (TCEP), 10 mM imidazole and 5% glycerol (pH 7.5). The cells were lysed by passage through a French pressure cell and the lysate was centrifuged to remove unbroken cells and cellular debris and the supernatant was added to Ni NTA beads (Qiagen) and incubated for 30 min before adding to a disposable 10 ml polypropylene column. The column was washed with three column volumes of wash buffer (lysis buffer containing 60 mM imidazole). BirA was eluted in 1 ml fractions with elution buffer (lysis buffer containing 250 mM imidazole). The fractions were analyzed by SDS-PAGE to determine purity. Pure fractions were combined and dialyzed against storage buffer (lysis buffer lacking imidazole). Aliquots were flash frozen and stored at -80°C. The SaBirA and SaBirA(ATP) samples were prepared by incubation of a 1:1 molar ratio of ATP and protein plus 1 mM $MgCl_2$ to convert biotin to biotinoyl-AMP. The SaBirA(btn) sample was prepared by incubating purified protein with a 1:1 molar ratio of biotin and 1 mM $MgCl_2$ to ensure any ATP in the solution would be converted to biotinoyl-AMP and could be removed by treatment with 0.2 M neutral hydroxylamine followed by dialysis against storage buffer. SaBirA was also prepared by incubating the as purified protein with a 5-fold molar excess of purified *B. subtilis* AccB-86 plus a 2-fold molar excess of

ATP and 1 mM $MgCl_2$. The protein was then purified using Qiagen Ni-NTA spin columns. Purification of *B. subtilis* AccB-86 was performed as previously described (54).

Electrophoretic Mobility Shift Assay (EMSA) of DNA binding.

The *B. subtilis* BirA DNA binding site upstream of *bioO* was PCR amplified from *B. subtilis* 168 genomic DNA with primers SKH014 and SKH015. Negative control DNA (*blap*) was amplified from *B. subtilis* 168 genomic DNA with primers SKH028 and SKH029. The *S. aureus* BirA binding site upstream of *bioO* was PCR amplified from *S. aureus* strain Newman genomic DNA with primers SKH099 and SKH100. All DNA fragments were 125 bp in length. The PCR products were sized on a 1.8% agarose gel and purified using a QIAquick PCR Purification Kit (Qiagen). DNA concentrations were determined at OD_{260} by using a NanoDrop 2000c. The DNA binding reaction contained 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 50 mM NaCl, 10% glycerol, 40 nM DNA, the indicated concentrations of BirA, 1 mM ATP, 1 mM $MgCl_2$, and 1 μM biotin or were modified to the solution conditions used by Soares da Costa and coworkers (85). These were 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10% glycerol, 40 nM DNA, indicated concentrations of BirA, 5 μM ATP, 1 mM $MgCl_2$, and 5 μM biotin (Figure 3.6D). The binding reactions were incubated at room temperature for 30 min and then loaded into a 6% DNA retardation gel. The gel was run in 0.5X TBE buffer at 100 V for 1 hour and 25 min. The gel was stained with SYBR Green I nucleic acid gel stain and visualized using Bio-Rad Chemidoc XRS and Quantity One software.

Thin layer chromotographic biotinylation assay.

The *in vitro* biotinylation assays were performed as previously reported (54). Reactions contained 50 mM Tris-HCl buffer (pH 8.0), 5.5 mM $MgCl_2$, 100 mM KCl, 0.1

mM TCEP, 10 μM ATP, 25 μM biotin, 2.5 μM BirA, 0.1 μM [α-^{32}P] ATP and with or without 50 μM AccB-86 for a total reaction mixture of 20 μl. The reaction mixtures were incubated at room temperature for 30 min. A portion of each reaction mixture (1 μl) was spotted on cellulose thin-layer chromatography (TLC) plates and developed in isobutyric acid-NH_4OH-water (66:1:33) (75). The thin-layer chromatograms were dried for 10 h and exposed to a phosphorimaging screen and visualized using a Fujifilm FLA-3000 Phosphor Imager and Fujifilm Image Gauge software.

β-Galactosidase assays.

SKH003 was grown overnight in defined media containing 1.6 nM biotin, kanamycin, erythromycin and lincomycin. SKH004 was grown overnight in defined media containing 1.6 nM biotin, kanamycin, erythromycin with lincomycin, and spectinomycin. Cultures were diluted to OD_{595} of 0.2 in defined media containing various concentrations of biotin (4, 20, 40, 80, 400 nM) and grown to an OD_{595} of 0.8 and then induced with 1 mM IPTG for an additional 2 h. β-Galactosidase activity was determined as described by Harwood and Cutting (51) following permeabilization of the cells with lysozyme.

Biotin bioassays.

E. coli strain NRD25 (31) was grown overnight in defined media supplemented with chloramphenicol and 1 nM biotin. The cells were recovered by centrifugation at 15,000x*g* for 5 min and washed 4 times with 1 ml of M9 medium. The cells were re-suspended in 1 ml of glucose M9 medium and sub-cultured into 100 ml of glucose M9 minimal media lacking biotin and containing 5 units of avidin. These cultures were incubated at 37°C for 5 h, centrifuged at 15,000x*g* for 5 min and the cell, pellets washed

5 times with 1 ml of M9 medium. The cells were then suspended in 1 ml M9 and added to 150 ml of minimal media agar containing the redox indicator 2,3,5-triphenyl tetrazolium chloride (0.1%, w/v) (64). Six ml of the agar mixture was added to sectored petri dishes. A sterile 6 mm paper disc was applied to the top of the agar and spotted with 10 µl of biotin standards or denatured protein samples. Protein samples were denatured by heating to 99°C for 20 minutes and centrifuged at 15,000x*g* for 5 min. The supernatant was collected and used to spot the paper discs. The pmol indicated were calculated using the protein concentrations before denaturation. The plates were incubated at 30°C overnight. Growth of strain NRD25 was visualized as a deposit of red formazan.

Assay of DNA binding by fluorescence anisotropy.

The sense strand of the 33 base pair *S. aureus* BirA binding site upstream of *bioO* was synthesized by IDT to include a 5'-fluorescein-label. The labeled sense strand (SKH162) and unlabeled antisense strand (SKH163) oligonucleotides were suspended in annealing buffer (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM DTT) and annealed by mixing 1:1 molar ratio and heating to 95°C for 5 minutes and slowly cooled. The annealed DNA was purified by PAGE to remove any single stranded DNA. The DNA concentration was determined at OD_{260} by using a NanoDrop 2000c. DNA binding reactions contained 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10% glycerol, 2 nM DNA, and various concentrations of BirA (10 nM to 40 µM). The SaBirA(btn) samples contained 200 µM biotin, SaBirA(ATP) samples contained 400 µM ATP and 1 mM $MgCl_2$ and the BirA(Bio-AMP) samples contained 200 µM biotin, 200 µM ATP and 1 mM $MgCl_2$. Buffer containing the fluorescein-labeled DNA was added to a black 96 well microplate (Molecular Devices). The indicated amount of a BirA sample was added to

the wells in a final reaction volume of 20 µl. Reactions were incubated at room temperature for 30 minutes. Anisotropy values were measured using the fluorescence polarization function of the Analyst HT Plate Reader (Molecular Devices) at the High-throughput Screening Facility, School of Chemical Sciences at the University of Illinois. GraphPad Prism 4 software was used for analysis of DNA binding.

Complementation tests.

Complementation of strain BM4092 (9) transformed with plasmids pSKH26, pSKH27, pSKH28, pSKH29 or pSKH30 was tested as described previously (54). The strains were grown at 37°C on glycerol M9 minimal plates (66) containing chloramphenicol and various concentrations of biotin (4 nM, 40 nM, 400 nM) (9). Strain BM4092 carrying pSKH30 was also grown with 25 µM 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal).

DNA sequence alignments.

Sequence alignments were obtained using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and the output was processed by ESPript 3.0 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) to generate the final figure (49).

Results

S. aureus BirA binds the *B. subtilis bioO* operator and modifies the *B. subtilis* acceptor *in vitro.*

For simplicity we will call the *S. aureus* BPL SaBirA where SaBirA will be used in the formal sense as the primary translation product lacking biotin and Bio-AMP. The SaBirA form containing biotin is called SaBirA(btn), that containing ATP as

SaBirA(ATP) and that containing Bio-AMP as SaBirA(Bio-AMP). Note that Mg++ is always present in the latter two cases.

To assess the possibility that the *B. subtilis bioO* strain constructed previously (54) could be used to test *in vivo* regulation by SaBirA, we asked if SaBirA is capable of binding *B. subtilis bioO.* The protein was purified (Figure 3.2) and electrophoretic mobility shift assays (EMSAs) were performed (Figure 3.3B). SaBirA bound the *B. subtilis bioO* binding site in the presence of biotin and ATP (e.g., as SaBirA(Bio-AMP) whereas low levels of DNA binding were observed for SaBirA and SaBirA(btn) samples which suggested that some of the protein had the biotinoyl-AMP intermediate bound in the active site. SaBirA(ATP) bound the operator almost as well as SaBirA(Bio-AMP) suggesting that the untreated, as purified, BirA had biotin bound in the active site. This was surprising because biotin was reported to readily dissociate from SaBirA (86). A similar co-purification of biotin with the protein was previously observed with *B. subtilis* BirA (54). We also assayed SaBirA for the ability to biotinylate AccB-86, the C-terminal biotin accepting domain of *B. subtilis* AccB (54), by transfer of biotin from Bio-AMP assayed by thin layer chromatography (Figure 3.3C) and by mass spectral analysis of AccB-86 biotinylation (Figure 3.4). The ability of SaBirA to function with the *B. subtilis* components indicated that the regulatory properties of the *S. aureus* protein could be tested with this system.

In vivo SaBirA responds to biotin and acceptor protein levels.

To assay the regulatory properties of SaBirA we modified the *B. subtilis* strain used previously (54) by replacement of the *B. subtilis birA* with the *S. aureus birA.* The strain also contained a *bioB141* mutation that causes biotin auxotrophy plus a *bioW-lacZ*

fusion that allows measurement of *bio* operon transcription by β-galactosidase assays. The new strain was grown in defined media supplemented with kanamycin, erythromycin, licomycin and various concentrations of biotin. As the biotin concentration increased the level of *bioW-lacZ* transcription decreased indicating SaBirA mediated transcriptional repression of the biotin operon in response to biotin levels (Figure 3.5A). We also tested regulation of the biotin operon by SaBirA in response to the level of the *B. subtilis* AccB-86 acceptor protein. This required a modification of the SaBirA-*B. subtilis* strain by introduction of an ectopic IPTG-inducible gene encoding the AccB-86 acceptor. This strain was grown as above with various concentrations of biotin. Upon induction of AccB-86 production β-galactosidase assays showed that expression of the biotin operon became derepressed at biotin concentrations that normally give repression (Figure 3.5B). These results parallel those seen in *E. coli* and *B. subtilis* upon overproduction of acceptor proteins (1, 26, 38, 54, 87). Therefore, the SaBirA protein is a fully functional regulatory ligase which raised the question of how to explain the conflicting *in vitro* results reported by Soares da Costa and coworkers (85)

In vitro DNA binding of *S. aureus* BirA to *S. aureus bioO*.

The as purified SaBirA bound the *S. aureus bioO* probe in EMSA analysis without additions of biotin or ATP (Figure 3.6A). The as purified SaBirA plus either biotin or ATP also bound the probe. This could not be attributed to non-specific DNA binding activity because a probe of unrelated sequence showed no shifts (Figure 3.6A). These data indicated that as purified SaBirA had acquired biotin from the *E. coli* host and converted at least some to Bio-AMP. To rid the protein of these ligands we incubated the as purified SaBirA with ATP and $MgCl_2$ to convert any biotin to Bio-AMP. We then

treated the protein with neutral hydroxylamine to cleave any biotinoyl-AMP to give AMP plus biotinoyl hydroxamate. Following this treatment SaBirA no longer bound the operator DNA in the presence or absence of biotin (Figure 3.6B). However DNA binding activity was still observed for the treated SaBirA in the presence of ATP, a surprising result in that biotin was shown to be required for binding of ATP-Mg^{++} by SaBirA (86). Incubation with an excess of the AccB-86 acceptor protein failed to prevent the ATP-dependent shift (Figure 3.6C). These results argued that either biotin was still bound in the SaBirA active site or that it was not required for the observed mobility shift. We tested these hypotheses by assay of the protein samples for biotin using a sensitive bioassay that can detect as little as 1 pmol of biotin.

Samples of the as purified and treated SaBirA were denatured and centrifuged. The supernatants were spotted on paper disks in sectored minimal plates containing a redox indicator and the *E. coli* biotin auxotroph NRD25 which contains a complete deletion of the *bio* operon (Figure 3.7). As expected the as purified SaBirA samples contained biotin (or Bio-AMP which would be hydrolyzed to biotin in the protein denaturation step). This was surprising because prior workers reported a rapid off rate for biotin as measured by surface plasmon resonance analyses (86). In contrast the treated SaBirA contained no detectable biotin or Bio-AMP even when large amounts of the protein were assayed. As a control we incubated the treated SaBirA with an equimolar concentration of biotin. Upon denaturation and bioassay the expected level of biotin was detected indicating that the protocol did not interfere with the release of biotin into the supernatant. The bioassays confirmed that incubation of SaBirA with ATP and $MgCl_2$ prior to neutral hydroxylamine treatment effectively removed biotin from the as purified

SaBirA preparations. This raised the question why the EMSA analyses detected DNA binding activity for the SaBirA(ATP) when no biotin was present. One possibility was that binding was an artifact of the EMSA analyses (21, 22, 46-48).

To test this possibility we turned to a florescence anisotropy assay that allowed DNA binding to be measured in free solution. SaBirA, SaBirA(btn), SaBirA(ATP) and SaBirA(Bio-AMP) samples were prepared from the as purified protein as described in Experimental Procedures. These preparations were then assayed for DNA binding activity by EMSA using the buffer conditions given by Soares da Costa and coworkers (85) (Figure 3.6D). The DNA binding activities of four SaBirA protein samples were tested with a fluorescein end-labeled 33 base pair *S. aureus bioO* binding site. The changes in anisotropy were plotted against the protein concentrations to obtain binding curves and the data were analyzed using the non-linear curve fitting function of GraphPad Prism 4 to calculate Kd values (Figure 3.8). The SaBirA and SaBirA(ATP) samples had very high Kd values of 5.284 ± 0.23 μM and 7.841 ± 1.1 μM, respectively, values that differed greatly from the SaBirA Kd value of 649 ± 43 nM reported from EMSA data by Soares da Costa and coworkers (85). Unexpectedly, SaBirA(btn) had the appreciably lower binding Kd of 182.8 ± 10.97 nM. As expected, SaBirA(Bio-AMP) sample had the lowest Kd. A value of 83.1 ± 4.2 nM was found which is similar to that previously derived from EMSA data (85). Note that in the case of *E. coli* BirA, a major component of the 2000-fold difference in operator binding of the BirA:Bio-AMP complex versus the unliganded protein is that the unliganded BirA has essentially no dimerization ability (equilibrium dimerization constant of 1−2 mM) (90). In contrast, unliganded SaBirA has an equilibrium dimerization constant of 29 μM (85).

If unliganded *E. coli* BirA had the dimerization ability of SaBirA a rough calculation suggests that the difference in binding affinities of the unliganded and Bio-AMP liganded proteins would be comparable to those of SaBirA and SaBirA(Bio-AMP).

SaBirA requires N-terminal domain sequences for full ligase activity.

E. coli BirA was recently shown to require specific interdomain interactions for full ligase activity (25). The wing of the winged HTH structure interacts with the biotin binding loop of the ligase active site and acts to organize the active site to give high affinity binding of biotin and biotinoyl-AMP (25). Unlike *E. coli*, *B. subtilis* BirA does not require an intact N-terminal DNA binding domain for full ligase activity (54). We previously modeled the *B. subtilis* BirA structure on the SaBirA BirA crystal structure (PDB 4DQ2) because among the biotin ligase proteins of known structure that protein had highest amino acid sequence identity (31%) to *B. subtilis* BirA. Based on this modeling we expected that deletions of the SaBirA N-terminal domain would retain ligase activity as did N-terminal domain deletion derivatives of the *B. subtilis* protein (54). To test this expectation we constructed N-terminal deletions of three different lengths as previously done for *B. subtilis* BirA (54). SaBirA deletion Δ2-65 eliminated the N-terminal domain whereas SaBirA deletions Δ2-74 and Δ1-81 cut into the central domain (Figure 3.9).

Complementation assays using the *E. coli birA1* strain BM4092 (9) were used to test the ligase activity of the three SaBirA N-terminal deletion proteins. All three N-terminal deleted *S. aureus* BirAs failed to restore ligase activity to the *E. coli* mutant strain at low biotin concentrations (Figure 3.10A). However at higher biotin concentrations the Δ2-65 construct allowed markedly better growth than the empty vector indicating that the

protein was expressed and stable. To determine if, like *E. coli* BirA, the wing of the HTH structure was important for active site organization we constructed a Δ48-63 wing deletion and tested the activity of this protein by complementation of the *E. coli birA* mutant strain. The wing deletion protein failed to complement ligase activity at low biotin concentrations although at higher biotin concentrations expression of the protein largely restored ligase activity (Figure 3.10B) as seen with the *E. coli* wing deletion BirA (25). As expected from the differing operator sequences, the blue colonies formed by the wild type SaBirA indicated that the protein was unable to regulate *E. coli bio* operon transcription.

Discussion

The BirA proteins of *E. coli* and *B. subtilis* provide a simple, yet sophisticated, regulatory system that monitors both intracellular biotin supply and the levels of enzyme proteins that require biotinylation for activity (1, 12, 34, 54). This is accomplished by use of the intermediate in the ligase reaction, Bio-AMP, as the regulatory ligand. Only when the active site is occupied by Bio-AMP do the proteins efficiently dimerize and thereby acquire the ability to bind their cognate operator sequences. Soares da Costa and coworkers (85) reported the surprising result that SaBirA dimerizes and binds its operator in the absence of substrates/ligands with an affinity only 6-fold lower than the affinity of the Bio-AMP complex. This is in contrast to the *E. coli* BirA where the operator binding affinity of the unliganded protein is 2000-fold lower than the Bio-AMP complex as measured by nuclease footprinting (90). Such sensitive methods have yet to be applied to *B. subtilis* BirA, but the available data argue that the Bio-AMP complex has an affinity

for its operators at least 50-fold greater than the unliganded protein. Physiologically the ability of unliganded SaBirA to bind its operator would result in decreased *bio* operon transcription and transport even when biotin was limiting. Moreover, the cell would be unable to respond to unmodified acceptor protein levels and central metabolism would be compromised. Indeed, the unliganded protein could behave much as a dominant negative mutant protein by blocking binding of SaBirA(Bio-AMP) to the operator.

Our *in vivo* data demonstrate that this perverse scenario does not occur. SaBirA is a competent regulatory protein that responds to both biotin supply and the levels of apo acceptor protein (Figure 3.5). These results raised the question of how the results of Costa and coworkers (85) could be rationalized. The short answer is that EMSA analysis of interaction of SaBirA with its operator is rife with artifacts. For example we found that ATP-Mg^{++} elicits operator binding in the absence of biotin (Figure 3.6) although surface plasmon resonance measurements indicate that the protein cannot bind ATP-Mg^{++} under these conditions (86). The reported binding of unliganded SaBirA to its operator (86) also seems an artifact of their EMSA conditions since we could detect no binding in our EMSA analyses (Figure 3.6) and only very weak binding by fluorescence anisotropy (Figure 3.8).

Although EMSA analyses are often considered straightforward, it is well documented that EMSA conditions can stabilize interactions that are weak in solution (47, 48). Three overlapping mechanisms have been put forth to rationalize such results. These are interactions with the gel matrix that stabilize protein-DNA affinities, the cage effect and sequestration. The latter two mechanisms have received experimental verification. The cage effect is the inability of dissociated molecules to diffuse

sufficiently far away from one another to prevent reassociation (21, 22). This effect has been demonstrated for LacI repressor-operator interactions (46, 47). Sequestration is the decreased solution volume accessible to the protein and DNA which increases the effective concentrations of the components thereby promoting interaction. Sequestration has been demonstrated for interaction of the *E. coli* cyclic AMP receptor protein with its binding site on the Lac promoter (48). The opposite effects have also been reported. The *E. coli* tryptophan (TrpR) repressor binds its operator at pH 8.3 as shown by nuclease footprinting although no gel shift was seen at this pH (23).

The binding measurements we obtained in free solution accurately reflect the mechanism of the regulatory system *in vivo*. Indeed, the behavior of the regulatory system *in vivo* must be the litmus used to judge the relevance of *in vitro* measurements. The binding curves obtained by fluorescence anisotropy indicated that SaBirA and SaBirA(ATP) bind DNA much more weakly than SaBirA(Bio-AMP). However, SaBirA(btn) binding was comparable to that of SaBirA(Bio-AMP) with only a modest decrease in DNA binding affinity. This interesting result suggests that biotin alone may be sufficient for dimerization and DNA binding and play a role in the regulatory mechanism. However, the finding that high level production of an acceptor protein derepresses transcription at high biotin concentrations argues against this suggestion because SaBirA(Bio-AMP) is required for protein modification. We view the SaBirA(btn) dimerization as facilitating Bio-AMP synthesis and dimerization by raising the local concentration of biotin-bound monomer and structuring the ATP binding site. Efficient operator binding in the presence of biotin has not been observed for *E. coli* BirA. Relative to the Bio-AMP complex the biotin complex binds the operator 100-fold

(75) or 146-fold (90) less tightly. Much of this difference is due to the weak dimerization elicited by biotin binding (90). In EMSA analyses neither *B. subtilis* BirA (54) nor SaBirA (Figure 3.6) showed significant operator binding in the presence of biotin. A possible explanation is that due to its charge biotin may electrophorese away from the protein.

Contrary to our expectations deletion of SaBirA N-terminal domain sequences gave results that paralleled those of the analogous *E. coli* BirA deletions (25, 100) rather than those of the more highly sequence and taxonomically related *B. subtilis* BirA (54). Therefore, SaBirA like *E. coli* BirA requires interdomain communication for full ligase activity. It would be interesting to compare the *S. aureus* and *B. subtilis* BirA regulatory efficiencies under conditions where each protein has the same level of expression and bind its cognate operator.

Tables

Table 3.1. Strains used in this chapter.

Strain	Relevent Genotype or Description	Reference or Derivation
B. subtilis		
1A330	*aroG932, bioB141*	Bacillus Genetic Stock Center
SKH001	1A330, *bioW::lacZ ermR*	(54)
SKH002	SKH001 *amyE*:: P spac *accB*-86 spcR	(54)
SKH003	SKH001, *B. subtilis birA::S. aureus birA kanR*	This study
SKH004	SKH002, *B. subtilis birA::S. aureus birA kanR*	This study
E. coli		
BL21 Star (DE3)	*ompT hsdSB rne131 (DE3)*	Novagen
NRD25	MC1061, *bioABFCD::CmR*	(31)
BM4092	*Δ(argF-lac)169 TP(bioF-lacZ)501*	(9)

Table 3.2. Plasmids used in this chapter.

Plasmid	Relevent Genotype or Description	Reference or Derevation
pET28b	T7 promoter expression vector, KanR	Novagen
pSKH025	pET28b encoding S. aureus BPL	This study
pBAD322Cm	copy control expression vector, CmR	Cronan, 2006
pSKH026	pBAD322Cm encoding WT S. aureus BPL	This study
pSKH027	pBAD322Cm encoding Δ2-65 S. aureus BPL	This study
pSKH028	pBAD322Cm encoding Δ2-74 S. aureus BPL	This study
pSKH029	pBAD322Cm encoding Δ2-81 S. aureus BPL	This study
pSKH030	pBAD322Cm encoding Δ48-63 S. aureus BPL	This study
pDG780	Kanamycin-resistance cassette for Bacillus subtilis, BGSCID: ECE93	Bacillus Genetic Stock Center
pDG780-panB	pDG780 with 500 bp after B. subtilis BirA	This study
pSKH031	pDG780-panB encoding last 500 bp cca and S. aureus BPL	This study

Table 3.3. Oligonucleotide primers used in this chapter.

Oligo-nucleotide	Description	Sequence
SKH014	Bs bioO F	GATCCTTTCTTCTATTGACAGAAAC
SKH015	Bs bioO R	CGC CCTTTCACTGATAACTGAAGAAC
SKH028	Non 125 F	GCATGACGGTTAGCATACAAATGGCAG
SKH029	Non 125 R	AACGATCGGGATTTCCATTTTCATCGATTC
SKH076	SaBPL F PciI	GTACATGTCAAAATATAGTCAAGATGTAC TTCAATTACTC
SKH077	SaBPL R XhoI	CCAGTCTCGAGAAAATCTATATCTGCACTA ATTAAACGGT
SKH078	SaBPL F delta 2-65 BspHI	AGTCATGATTTGGTATCAAGGTATAATAG
SKH079	SaBPL F delta 2-74 PciI	GTACATGTATACAAAAAGTTCTGCTTTG
SKH080	SaBPL F delta 2-81 PciI	GTACATGTTTGATTTTAGTGAAGTATACG
SKH081	SaBPL R SalI	ACGTGTCGACTTAAAAATCTATATCTGCAC
SKH097	SaBPL 1-141 R overlap	AAATATCTGGGCCTCCCTCTAACTTTAATT GGTC
SKH098	SaBPL 190-972 F overlap	GTTAGAGGGAGGCCCAGATATTTGGTATC AAGG
SKH099	Sa bioO F	TACTGAGAAGTTTAAATTCATGTTTTCTTG
SKH100	Sa bioO R	TCGTACTTGTAATAAAAATCCTCATAAAA ATAAC
SKH102	PanB 500 F SalI	ACGTGTCGACTAATGTGTTGGTACAAGCC CGTTGA
SKH103	PanB 500 R XhoI	ACGTACTCGAGCGGCATATCTGTCACAAT AAAGG
SKH104	cca 500 F BamHI	ACGTGGATCCTTTTATCATAAACGAGAAA ACCTG
SKH105	cca 500 R SaBPL overlap	ATATATTTTGACATGTCTTCAGCCACTCCT CTATGTG
SKH106	SaBPL F cca overlap	GTGGCTGAAGACATGTCAAAATATATAAA AGATGTAC
SKH107	SaBPL R EcoRI	ACGTGAATTCTTAAAAATCTATATCTGCAC TAATTAAAC
SKH134	SaBPL seq F	TTGTGACAGGGAAGCTCTCC
SKH135	SaBPL seq R	CCTCATCCTCTTCATCCTCTTCG
SKH162	6-F sense Sa bioO	56-FAM/AATGTAAACTTATTAATTATAAAAGT TTACATT
SKH163	Antisense Sa bioO	AATGTAAACTTTTATAATTAATAAGTTTAC ATT

Figures

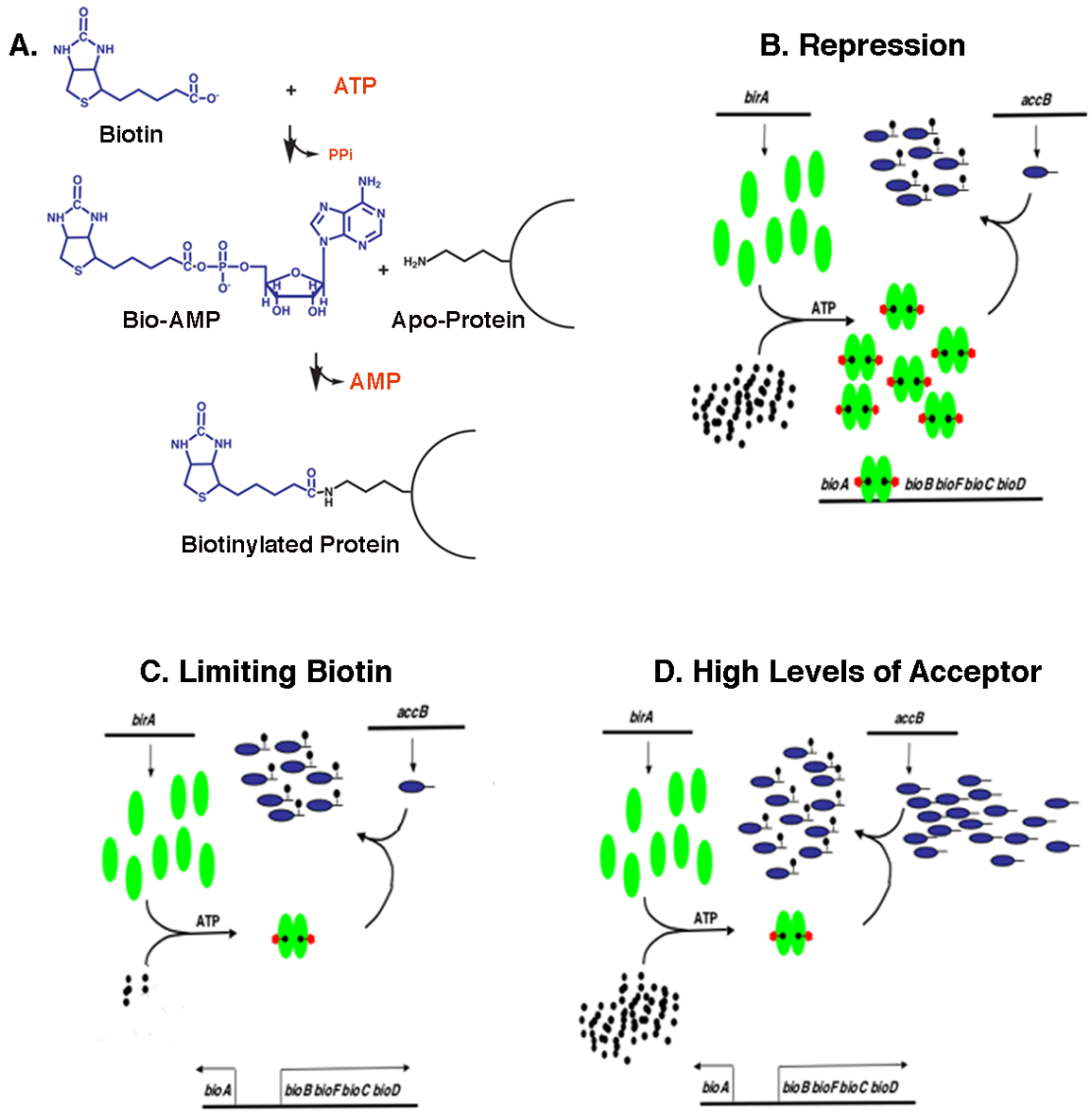

Figure 3.1. The biotin protein ligase reaction, current model of *E. coli* BirA regulation. A. BirA binds both biotin and ATP to synthesize biotinoyl-AMP with release of pyrophosphate. The mixed anhydride bond of biotinoyl-AMP then undergoes nucleophilic attack from the ε-amino group of the conserved lysine residue of the acceptor protein resulting in covalently attached biotin and free AMP. **B.** Repression. Sufficient biotin is present and essentially all acceptor protein (AccB) is biotinylated. **C.** Limited biotin results in low levels of Bio-AMP such that BirA is unable to dimerize and

Figure 3.1. (cont.)

exert repression. **D.** An excess of AccB acceptor protein consumes the Bio-AMP resulting in low levels that preclude dimerization and repression. Green ovals denote BirA, tailed blue ovals are AccB, black dots represent biotin, and black dots with red pentagons denote biotinoyl-adenylate (Bio-AMP).

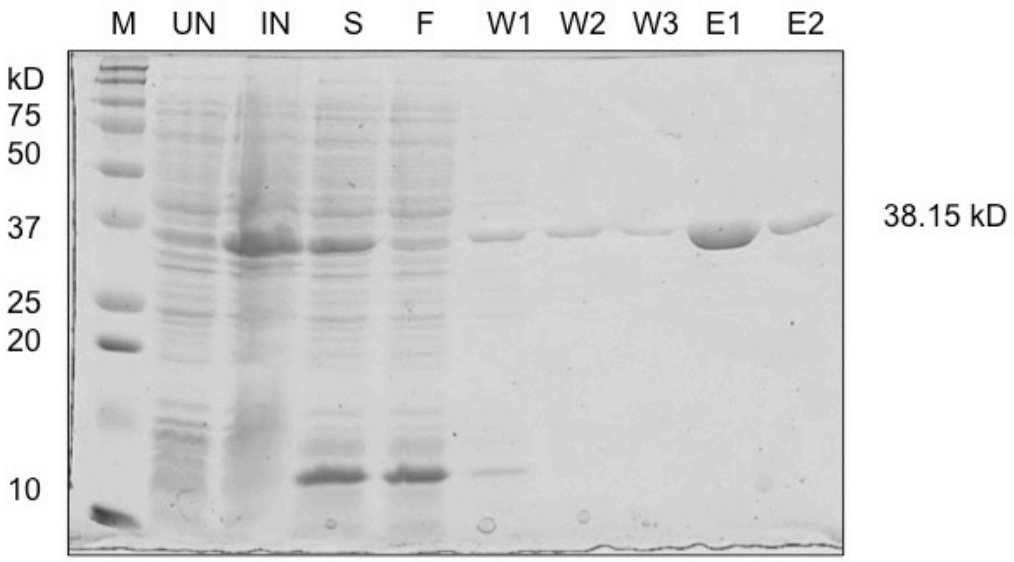

Figure 3.2. Purification of *S. aureus* C-terminal His-tagged BirA. The SaBirA was purified as described in Experimental Procedures and subjected to SDS-electrophoresis on a 12% polyacrylamide gel. M: molecular weight standards (Precision Plus Protein Standard Kaleidoscope from BioRad), UN: uninduced whole cell lysate, IN: induced whole cell lysate, S: clarified lysate, F: flow though, W1: wash fraction one, W2: wash fraction 2, W3: wash fraction 3, E1: elution fraction 1, E2: elution fraction 2.

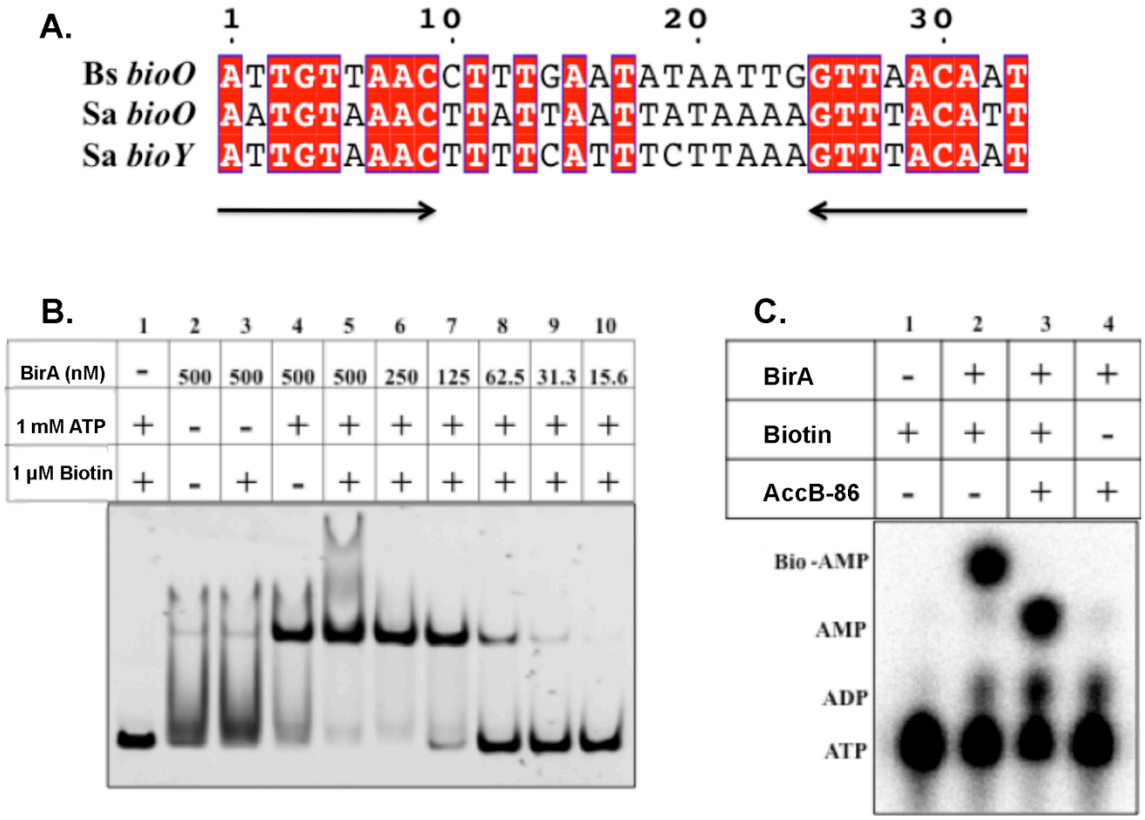

Figure 3.3. *S. aureus* BirA binds *B. subtilis bioO* and modifies AccB-86. A. Sequence alignments of *B. subtilis bioO*, *S. aureus bioO*, and *S. aureus bioY* DNA binding sites. The SaBirA DNA binding sites are almost identical to the *B. subtilis bioO* binding site. Only a single base in each *B. subtilis bioO* half site differs from those of both *S. aureus* operators. Conserved nucleotides are in white text and highlighted in red. Half site binding regions are indicated with the black arrows. **B.** EMSA showing *S. aureus* BirA binding to *B. subtilis bioO*. Note the small shift seen for BirA only and BirA with biotin only samples and the large shift seen for the BirA with ATP only sample. **C.** *In vitro* biotinylation thin layer chromatographic assay of biotin transfer from *S. aureus* BirA to the *B. subtilis* acceptor protein AccB-86. Transfer is detected by the production of AMP (Figure 3.1A) plus consumption of ATP.

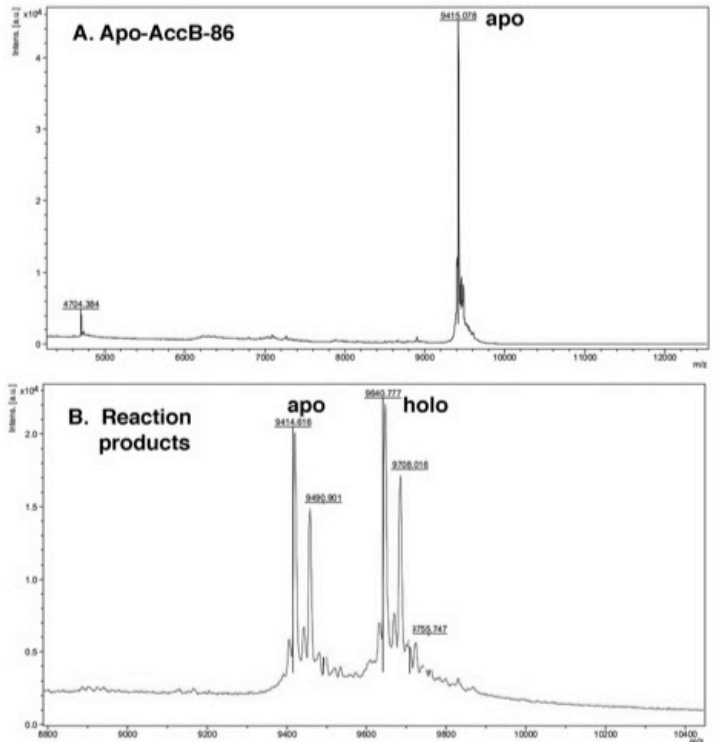

Figure 3.4. MALDI Mass spectral analysis of the SaBirA ligase catalyzed biotinylation of *B. subtilis* AccB-86. Panel A shows the purified AccB-86. The reaction of Panel B did not go to completion. The peaks upstream of the apo and holo are adducts formed by salts of the reaction mixture (AccB-86 is an unusually acidic protein). The calculated and determined masses for apo AccB-86 were 9418.7 and 9415.1, respectively, whereas the calculated and determined masses for holo AccB-86 were 9644.7 and 9640.8, respectively.

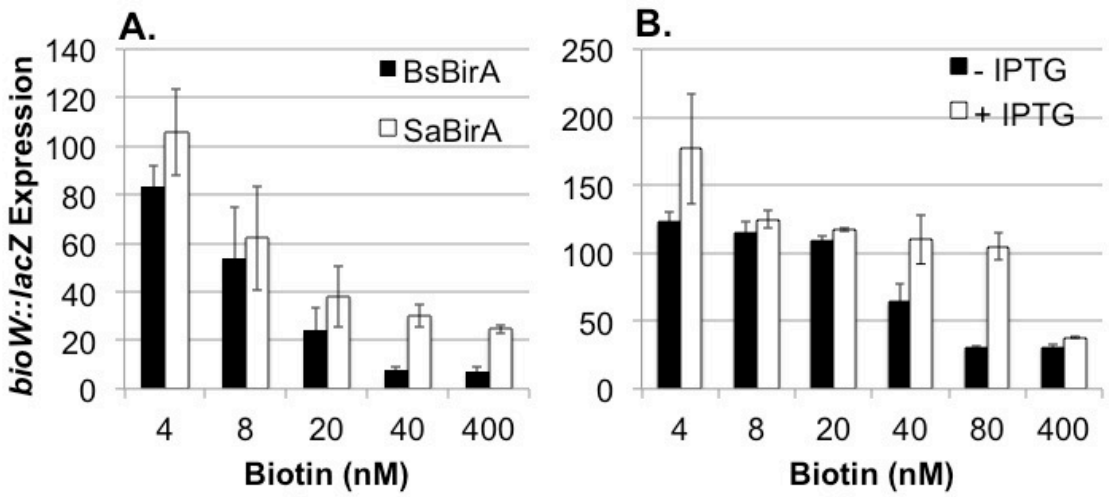

Figure 3.5. *S. aureus* BirA responds to biotin and apo acceptor protein levels. A. β-Galactosidase assays of the effects of biotin concentration on *bioO*-dependent transcription**.** The biotin requiring strain was grown in defined medium supplemented with the indicated concentrations of biotin. Error bars denote SEM**. B.** β-Galactosidase assays of the effect of *B. subtilis* AccB-86 expression on *bioO*-dependent transcription**.** The strain was grown in defined medium supplemented with the indicated concentrations of biotin in the presence or absence of IPTG. Note the increase in some biotin concentrations in panel B. Error bars denote SEM**.**

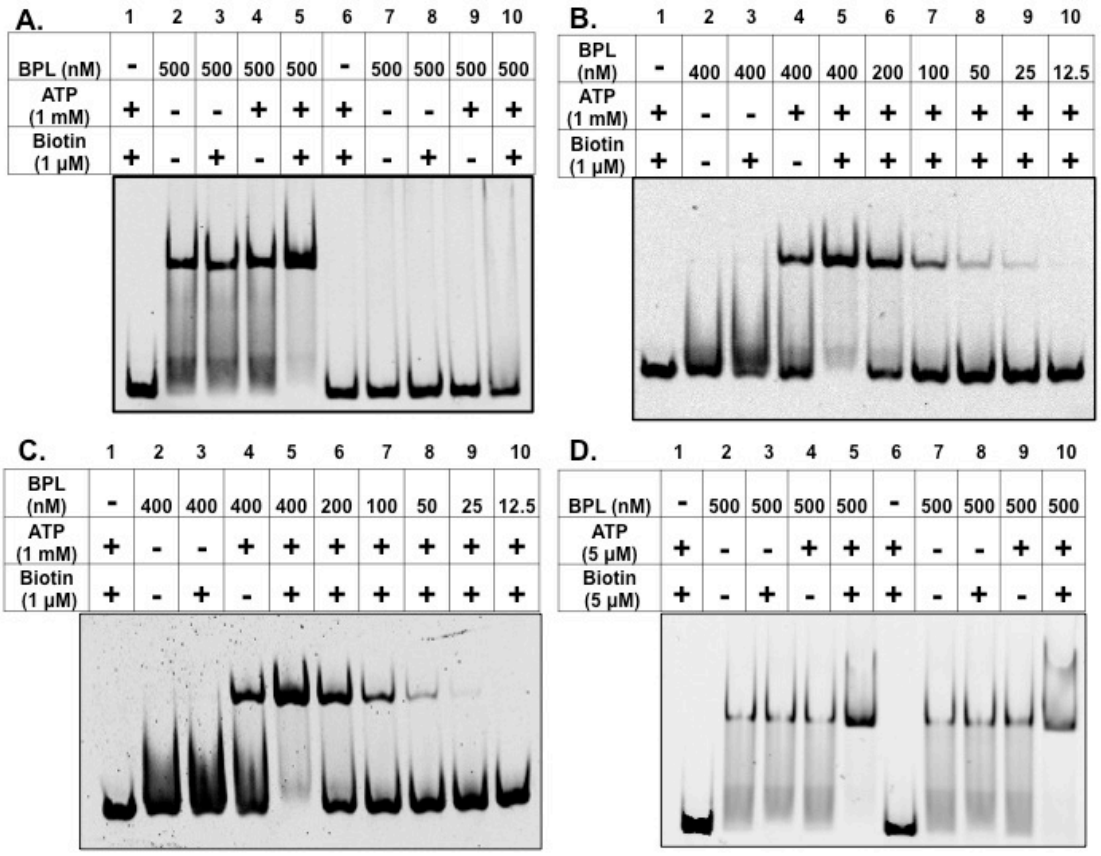

Figure 3.6. Electrophoreteic Mobility Shift Assay of SaBirA DNA binding to *S. aureus bioO.* **A.** SaBirA binding to *S. aureus bioO* (lanes 1-5) or to a nonspecific DNA sequence (lanes 6-10). **B.** SaBirA was incubated with ATP and then treated with neutral hydroxylamine to remove any biotinoyl-AMP intermediate. **C.** SaBirA was incubated with ATP and *B. subtilis* AccB-86 to remove any biotin or Bio-AMP from the active site. **D.** SaBirA was incubated with either biotin (lanes 2-5) or ATP (lanes 7-10) and then treated with neutral hydroxylamine to remove any intermediate formed. Note the binding conditions were modified to those used by Soares da Costa and coworkers (85) (the composition of the gels utilized was not reported).

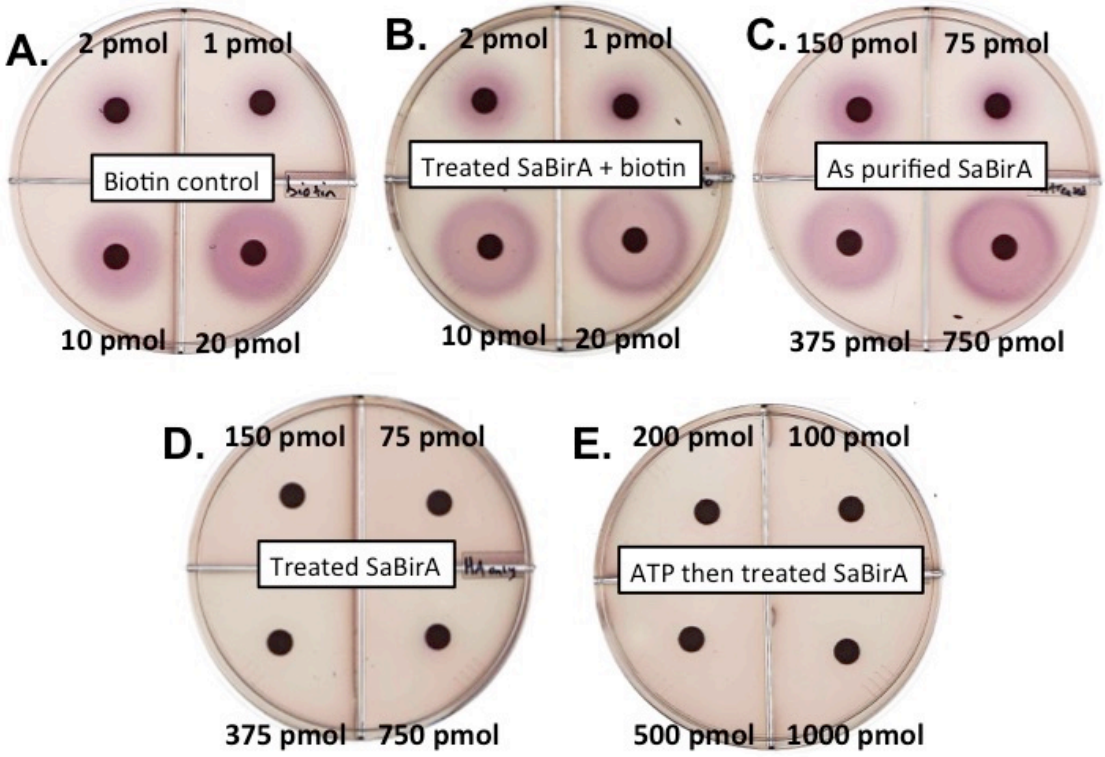

Figure 3.7. Treatment with hydroxylamine effectively removes Bio-AMP from SaBirA. A. Biotin standards. **B.** Recovery of biotin from a mixture of biotin and hydroxylamine-treated SaBirA. **C.** The as purified SaBirA contains either biotin or Bio-AMP. **D. - E.** After incubation without (D) or with ATP plus MgCl2 (E) the samples were treated with neutral hydroxylamine followed by hydroxylamine removal. The protein samples were then denatured and the supernatants assayed for biotin. In the absence of ATP any bound biotin present would retain its free carboxyl group and be active in the bioassay whereas the biotin moiety of Bio-AMP (either formed from bound biotin in the presence of ATP or bound to the as purified protein) would be inactive in the bioassay due to its blocked carboxyl group. The lack of bioassay activity in the sample lacking ATP indicates the absence of bound biotin. Hence, the bound biotin must be in the form of Bio-AMP. Excepting the biotin standards plate the pmol values given correspond to the amount of protein denatured.

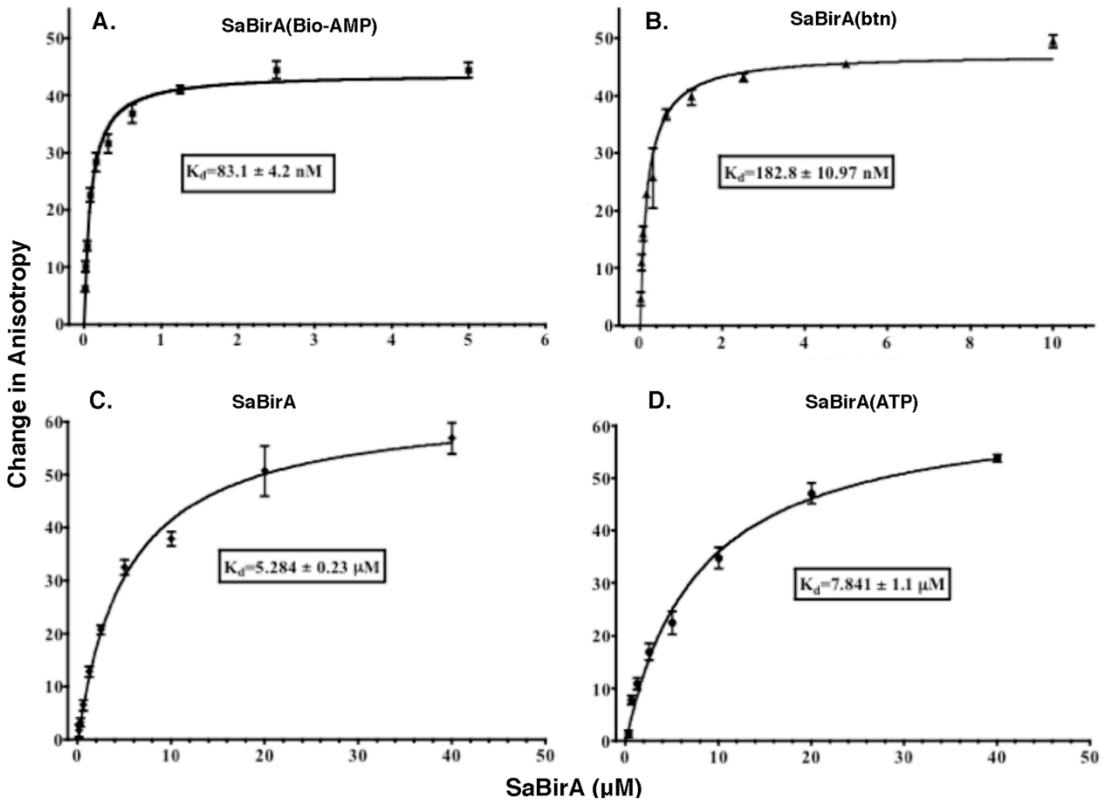

Figure 3.8. Binding isotherms of SaBirA binding to *S. aureus bioO* obtained by fluorescence anisotropy. A. SaBirA(Bio-AMP) gave a dissociation constant of 83.1 ± 4.2 nM. **B.** SaBirA(btn) gave a dissociation constant of 182.8 ± 10.97 nM. **C.** SaBirA gave a dissociation constant of 5.284 ± 0.23 µM. **D.** SaBirA(ATP) gave a dissociation constant of 7.841 ± 1.1 µM. Note the differing abscissa protein concentrations.

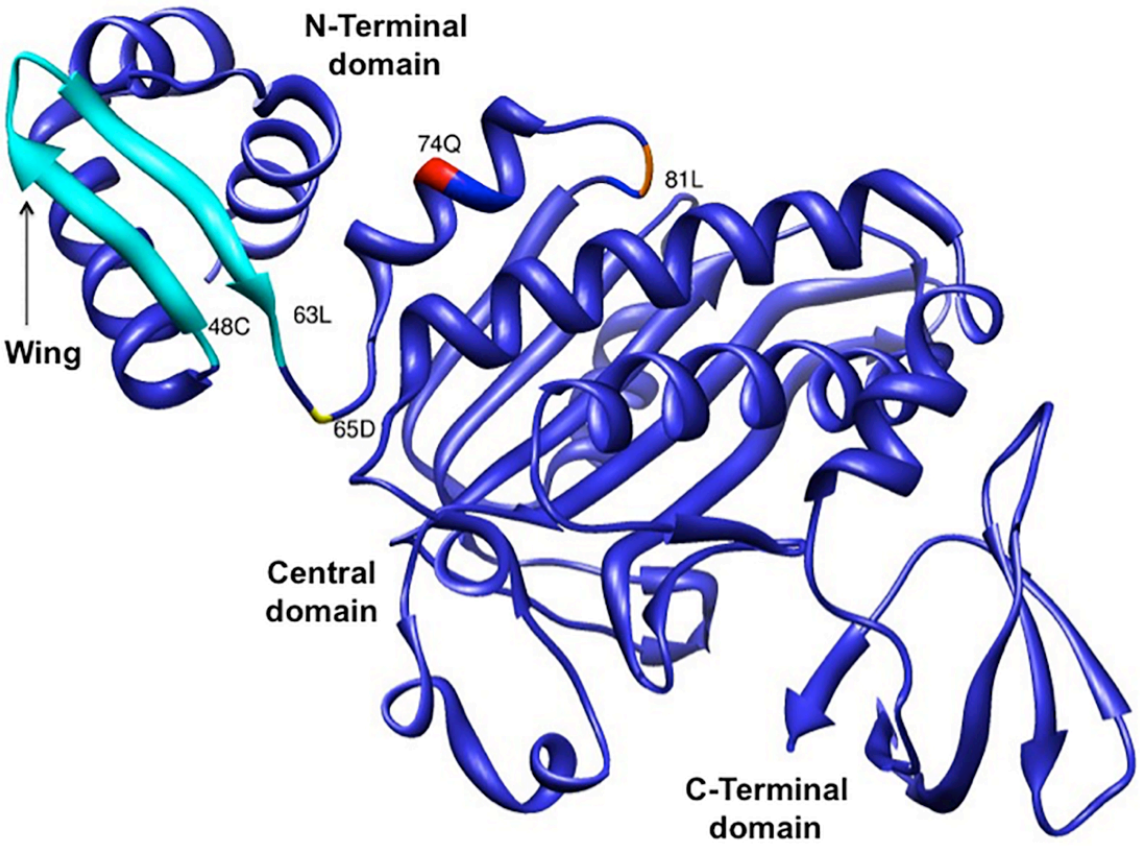

Figure 3.9. *S. aureus* BirA N-terminal deletions. The wing that was deleted (Δ48-63) is shown in cyan. The end point of N-terminal deletion Δ2-65 is shown in yellow. The end point of N-terminal deletion Δ2-74 is shown in red whereas that of N-terminal deletion Δ2-81 is shown in orange. The image was created from the PDB file (4DQ2) using UCSF Chimera (74).

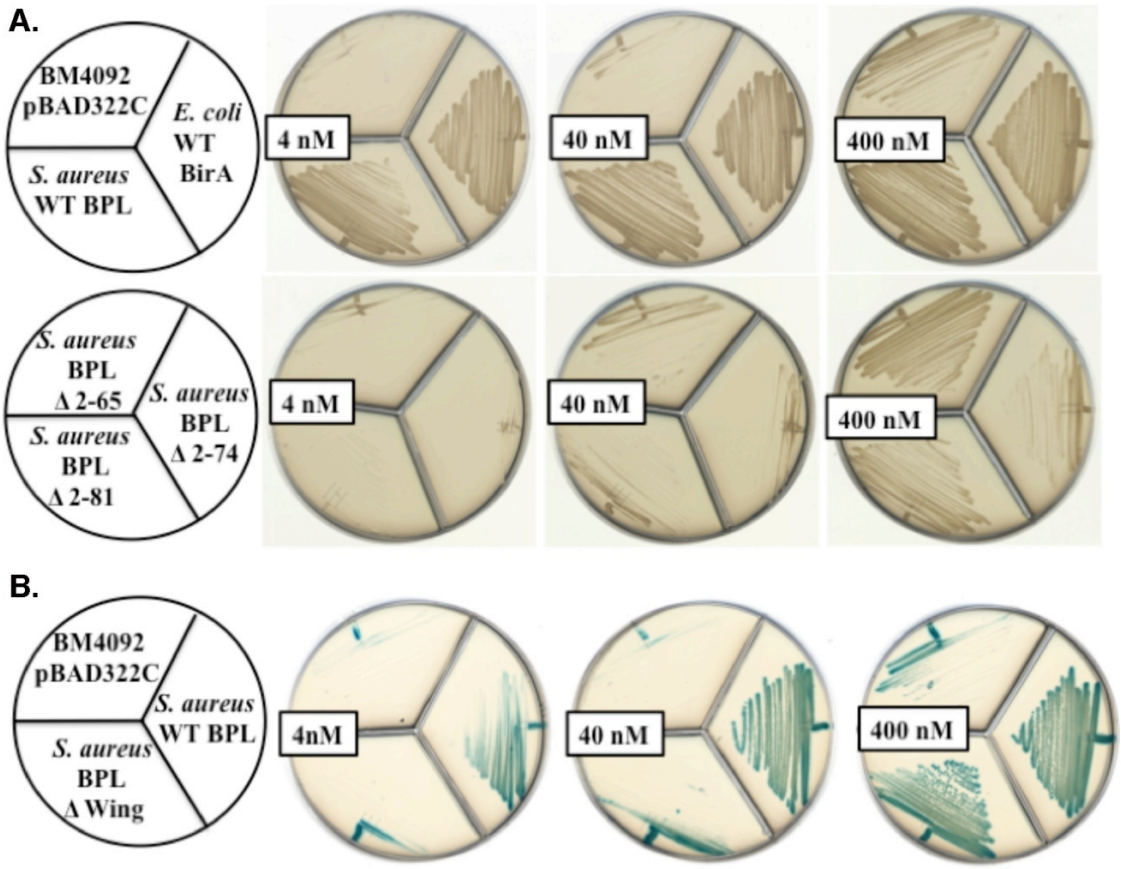

Figure 3.10. Growth of *E. coli* strain BM4092 supported by expression of *S. aureus* BirA and its N-terminal deletions. A. Strains were grown on glycerol M9 minimal media with biotin (4 nM, 40 nM or 400 nM). **B.** Strains were grown on glycerol M9 minimal media with biotin (4 nM, 40 nM or 400 nM) and X-gal. Glucose represses the arabinose promoter to give a low level of expression consistent with the low expression of BirA proteins. The blue color indicates derepressed transcription of the *bioF-lacZ* fusion. Note that *S. aureus* wild type BirA does not complement *E. coli* BirA regulatory function.

Chapter 4

Conclusions

Lessons Learned from *Bacillus subtilis*

The objective of this thesis was to investigate other group II BPLs to determine if they follow *E. coli* BirA regulatory properties and interdomain communication characteristics. Before this study, other group II BPLs were predicted based on the helix turn helix motif in the N-terminal domain. The regulatory properties of these predicted group II BPLs were untested but expected to follow the *E. coli* BirA model of transcriptional regulation of the biotin operon. However, the predicted group II BPLs have low sequence identity to *E. coli* BirA, have multiple predicted DNA binding sites, have different biotin synthetic genes, and contain multiple biotin dependent enzymes. This study examined other examples of group II BPLs to determine if these differences add a layer of complexity to the regulatory function.

The *E. coli* BirA requires the wing of the helix turn helix motif of the N-terminal DNA binding domain for proper ligase activity. This requirement is surprising for two reasons. First, the crystal structure of the *E. coli* BirA indicates that the N-terminal domain is separated from the catalytic central domain by a linker region. Second, group I BPLs do not have an N-terminal DNA binding domain and still have adequate ligase activity. This study looked to determine if other group II BPLs require interdomain communication between the N-terminal DNA binding domain and the central catalytic domain for proper ligase activity.

In Chapter 2, I studied the group II BPL from *B. subtilis*. Purified *B. subtilis* BirA binds to the three predicted DNA binding sites only in the presence of both biotin and ATP. This indicates that the co-repressor is the intermediate biotinoyl-AMP, as seen for *E. coli* BirA. Similar to *E. coli* BirA, *B. subtilis* BirA only dimerizes significantly in the presence of both biotin and ATP. In *E. coli*, homodimerization of BirA is required for DNA binding to occur. *In vivo* transcriptional analysis of the biotin operon indicates that *B. subtilis* BirA responds to both biotin concentration and apo biotin acceptor protein levels. These two characteristics are fundamental to the *E. coli* BirA regulatory model. Although the *B. subtilis* BirA has low sequence identity to *E. coli* BirA, has three DNA binding sites, and has three biotin acceptor proteins, *B. subtilis* BirA follows the *E. coli* BirA regulatory model.

I also studied the role of the N-terminal DNA binding domain in the ligase activity of *B. subtilis* BirA. Using a structural model of *B. subtilis* BirA based on the crystal structure of *S. aureus* BirA, I determined end point residues for N-terminal deletions. Ligase activity was determined for the N-terminal deletions first by a complementation assay using an *E. coli* strain with a BirA mutant. Complementation was observed for several of the N-terminal deletions. Purification of these N-terminal deletions and *in vitro* biotinylation assays further showed that the *B. subtilis* BirA could successfully be converted into a group I BPL. Unlike *E. coli*, this indicates that the *B. subtilis* BirA does not require interdomain communication between the N-terminal DNA binding domain and the central catalytic domain for proper ligase activity.

Lessons Learned from *Staphylococcus aureus*

In Chapter 3, I studied the group II BPL from *S. aureus*. It was reported that *S. aurues* BirA could dimerize and bind DNA in the absence of biotin and ATP. This was surprising since *E. coli* and *B. subtilis* group II BPLs require both biotin and ATP for dimerization and subsequent DNA binding. Without the requirement of biotin and ATP binding for dimerization and DNA binding, *S. aureus* BirA would not be an effective regulator of the biotin operon. Repression of the biotin operon would occur without regard for the biotin concentration or the level of apo acceptor protein. Using the biotin operon transcriptional test strain I created for *B. subtilis*, I was able to test *S. aureus* BirA regulation *in vivo*. *S. aureus* BirA does respond to both biotin concentration and increased levels of apo acceptor protein, similar to *E. coli* and *B. subtilis* BirA. It was then important to determine why *S. aureus* BirA DNA binding activity was observed in the absence of biotin *in vitro* in EMSA. To determine if the EMSA gel matrix was giving *in vitro* artifacts we decided to use a solution-based method. Using fluorescence anisotropy I was able to generate DNA binding curves for *S. aureus* BirA in the presence and absence of biotin and ATP. These binding curves corroborate the results obtained from the *in vivo* transcriptional test strain. Surprisingly, the biotin alone had a similar DNA binding affinity compared to biotinoyl-AMP. This result was not seen for *S. aureus* BirA using EMSA and biotin only DNA binding has not been observed for *E. coli* or *B. subtilis* BirA. However, fluorescence anisotropy has not been used to determine DNA binding for *E. coli* or *B. subtilis* BirA in the presence of only biotin.

I also tested the role of the *S. aureus* BirA DNA binding domain in the enzymatic activity of the protein. Because the crystal structure of *S. aureus* was used to model the *B.*

subtilis BirA, we expected that deletion of the N-terminal domain would not affect ligase activity. To our surprise, deletion of the DNA binding domain resulted in severely decreased ligation activity as was previously observed with *E. coli* BirA. Furthermore, deletion of the wing from the helix-turn-helix motif in the DNA binding domain also severely decreased ligase activity. This indicates that like *E. coli* BirA, the wing is playing a role in active site organization.

Future Directions

Future work will investigate the two annotated biotin protein ligases from *Clostridium acetobutylicum* (79). Sequence alignments of *C. acetobutylicum* BPLs Ca 0212 and Ca 0589 to *E. coli* BirA show that Ca 0212 is a group II BPL, BirA, that is lacking the conserved C-terminal domain and that Ca 0589 is a group I BPL (Figure 4.1). Three Ca 0212 DNA binding sites have been predicted based on sequence alignments and are found near putative biotin biosynthetic genes or putative biotin transport genes (Figure 4.2) (79). Future work will determine if both *C. acetobutylicum* BPLs have ligase activity and if Ca 0212 has regulatory function as predicted by sequence alignments. It is unclear why *C. acetobutylicum* would need two BPLs, thus it will be interesting to determine if each protein has evolved specialized functions. Previous work showed that the two *F. novicida* BPLs have distinct roles (45). BplA, a group I BPL, is the more efficient enzyme that is required for pathogenicity, whereas the main role of the other BPL, BirA, is to prevent wasteful biotin synthesis (45).

Figures

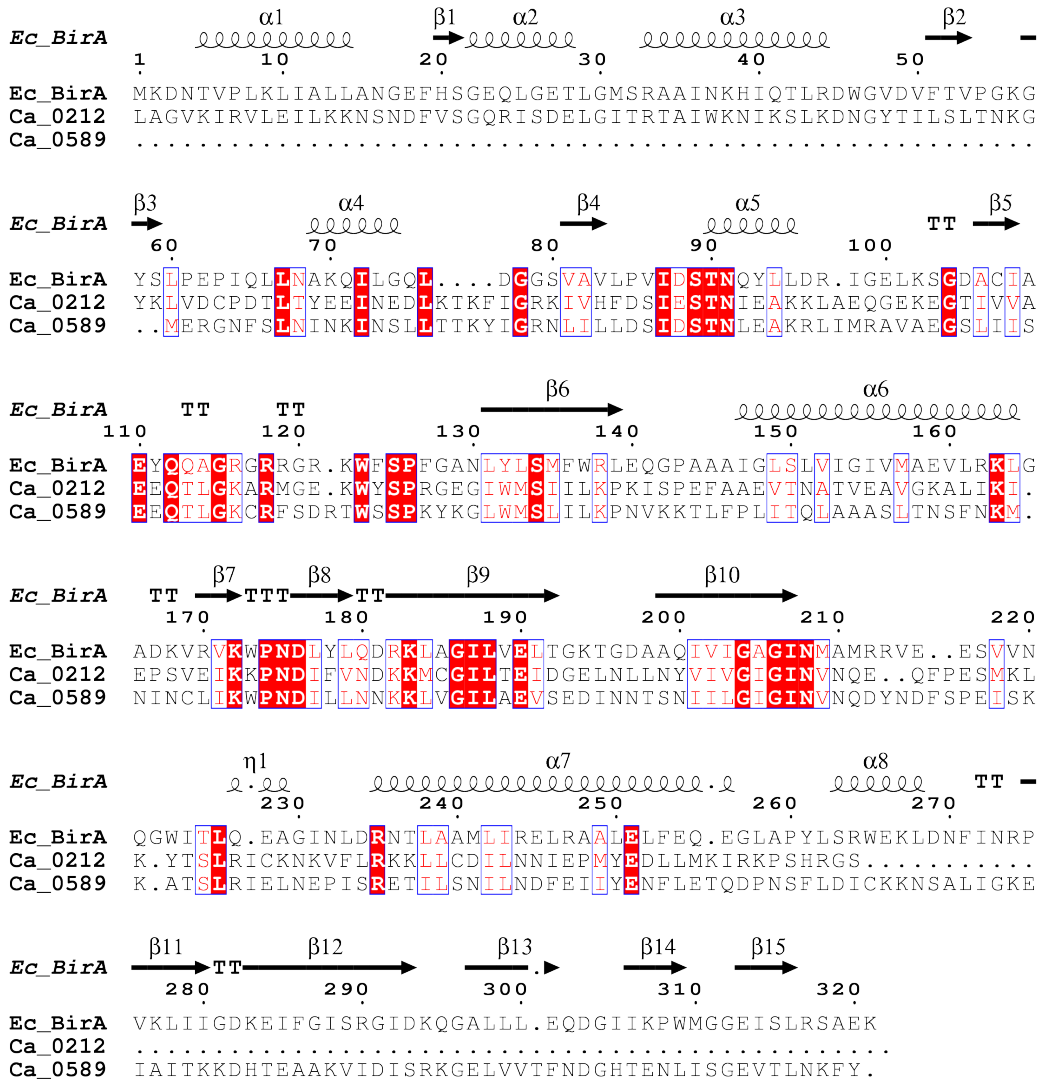

Figure. 4.1. Sequence alignments of *E. coli* BirA, *C. acetobutylicum* BPL 0212, and *C. acetobutylicum* BPL 0589. *C. acetobutylicum* BPL 0212 (Ca 0212) has 25% sequence identity to *E. coli* BirA and 40% sequence identity to *C. acetobutylicum* BPL 0589 (Ca 0589). Ca 0589 has 22% sequence identity to *E. coli* BirA. Conserved residues are in white text and highlighted in red and similar residues are in red text and boxed in blue. The *E. coli* BirA secondary structure (PDB: 1HXD) is shown above the amino acid

Figure 4.1. (cont.)

sequence. Note that Ca 0212 is missing the conserved C-terminal domain and Ca 0589 is missing the N-terminal DNA binding domain.

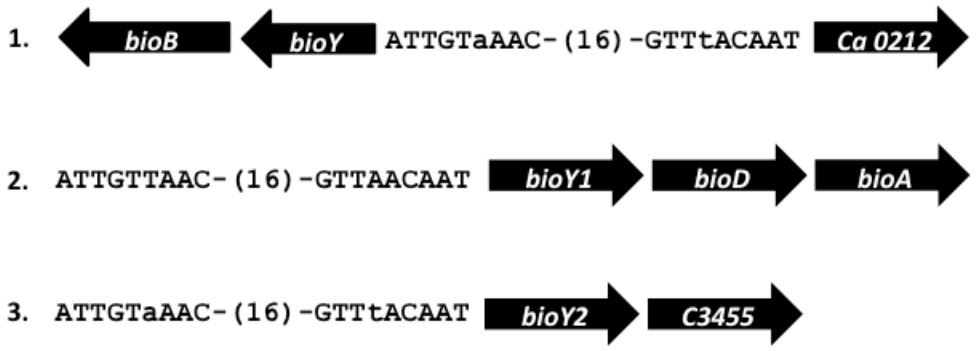

Figure 4.2. Predicted *C. acetobutylicum* BPL 0212 DNA binding sites. *C. acetobutylicum* BPL 0212 has three predicted DNA binding sites near putative biotin biosynthetic genes or putative biotin transport genes. Note that one binding site is located near *bpl Ca 0212* indicating self-regulation may occur.

Chapter 5

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