In vitro and *in vivo* characterization of *Staphylococcus aureus* biotin protein ligase transcriptional repressor function.

by

Jiulia Nurannisa Satiaputra

B. Biotech (Honours)

A thesis submitted to the University of Adelaide, South Australia in fulfillment of the requirements for the degree of Doctor of Philosophy

Discipline of Biochemistry School of Molecular and Biomedical Sciences University of Adelaide South Australia

February 2017

Table of contents

Abbreviations

ACC	Acetyl CoA Carboxylase
AMP	Adenosine monophosphate
Apo	Unliganded enzyme
ATP	Adenosine triphosphate
AUC	Analytical Ultracentrifugation
BCCP	Biotin Carboxyl Carrier Protein
BirA	Biotin retention protein A (Biotin inducible repressor)
bp	Base pair
BPL	Biotin protein ligase
BSA	Bovine serum albumin
CAT	Chloramphenicol acetyltransferase
CPM	Count per minute
° C	Degree celcius
Da	Dalton
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribo Nucleic Qcid
DS-oligo	Double Stranded oligo
DTT	Dithiotheitol
*Ec*BioO	*Escherichia coli* biotin biosynthesis operon
*Ec*BirA	*Escherichia coli* Biotin retention protein A
*Ec*BPL	*Escherichia coli* Biotin protein ligase
EMSA	Electrophoretic Mobility Shift Assay

Holo	Ligand bound enzyme
HPLC	High performance liquid chromatography
*Hs*BPL	*Homo sapiens* Biotin protein ligase
K_d	Off rate
K_D	Dissociation constant
KDa	Kilo Dalton
K_i	Inhibition constant
K_m	Michaelis-Menten constant
K_R	Half-maximum repression
LB	Luria Broth
min	minute
M	Molar
MIC	Minimum Inhibitory Concentration
MS	Mass Spectroscopy
*Mt*BPL	*Mycobacterium tuberculosis* Biotin protein ligase
MW	Molecular weight
MWCO	Molecular weight cut-off
nESI-MS	native Electrospray Ionization-Mass Spectroscopy
PAGE	Polyacrylamide Gel Electrophoresis
PC	Pyruvate Carboxylase
PCR	Polymerase Chain Reaction
PDB	Protein data bank
PMSF	Phenylmethylsulfonylfluoride
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
rpm	Revolutions per minute

RNA	Ribonucleic acid
RNAP	RNA Polymerase
rRNA	ribosomal Ribonucleic Acid
RMSD	Root mean square deviation
*Sa*BirA	*Staphylococcus aureus* Biotin retention protein A
*Sa*BioO	*Staphylococcus aureus* Biotin biosynthesis operon
*Sa*BioY	*Staphylococcus aureus* BioY biotin transporter
*Sa*BPL	*Staphylococcus aureus* Biotin protein ligase
*Sa*PC	*Staphylococcus aureus* Pyruvate Carboxylase
SAXS	Small Angle X-ray Scattering
SDS	Sodium Dodecyl Sulphate
SEM	Standard error mean
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
WT	Wild-type

Abstract

Staphylococcus aureus is a versatile and potentially dangerous human pathogen. One of the traits of *S. aureus* that is crucial for its survival during pathogenesis is its ability to quickly adapt to changes in the microenvironment, including an ability to adapt to the limited availability of micronutrients such as biotin. Biotin is a co-factor required for important metabolic enzymes such as pyruvate carboxylase (PC) and acetyl CoA carboxylase (ACC). In certain bacteria like *S. aureus*, the protein that is responsible for managing biotin homeostasis is the biotin retention protein, BirA (also known as biotin protein ligase or BPL). BirA is a bi-functional protein that serves as both the enzyme responsible for protein biotinylation and a transcriptional repressor that regulates biotin biosynthesis and import.

Escherichia coli BirA (*Ec*BirA) has been well studied, however, less extensive studies have been performed on *S. aureus* BirA (*Sa*BirA). Whilst *Ec*BirA regulates transcription of the biotin biosynthesis operon (*bioO*), *Sa*BirA has multiple targets including *bioO*, the biotin transporter (*SabioY*) and genes involved in fatty acid synthesis (*SayhfS-SayhfT*). For both *Ec*BirA and *Sa*BirA, homodimerization is a pre-requisite for DNA binding and subsequent repressor activity. In the absence of protein requiring biotinylation, and when cellular demand for biotin is low, BirA will dimerize, bind to its target DNA and repress expression of biotin biosynthetic enzymes. Previous studies in our laboratory revealed clear differences between *Ec*BirA and *Sa*BirA. One of these differences is that dimerization and DNA binding of *Ec*BirA only takes place when the protein is in complex with the reaction intermediate biotinyl-5′-AMP (i.e. the holo-enzyme), whereas *Sa*BirA was able to dimerize and bind DNA in both the

holo (K_D^{2-1} = 29 μM, $K_{D\ DNA}$ = 108 nM) and non-liganded (i.e. apo) states (K_D^{2-1} = 30 μM, $K_{D\ DNA}$ = 649 nM).

I hypothesized that there are clear distinctions in the DNA binding interaction between *Sa*BirA and the well-studied *Ec*BirA. These differences allow *S. aureus* to elegantly orchestrate biotin synthesis and transport in response to external biotin availability. This study aims to define *Sa*BirA-regulated gene expression using *in vitro* and *in vivo* methods. In addition, the effect of extracellular biotin concentration on biotin uptake and gene expression in both *S. aureus* and *E. coli* were also investigated in this study. The result showed that within 30 minutes, biotin starved *S. aureus* could sense changes in exogenous biotin and responded with increased biotin uptake and down regulation of biotin synthesis (>100-fold). These rapid responses were not observed in *E. coli*.

Furthermore, the DNA-binding activity of *Sa*BirA was also probed *in vivo*. Since *S. aureus* is not naturally competent to transformation, it can be technically difficult to genetically manipulate this bacteria. To overcome this problem, reporter strains were constructed in *E. coli* containing chromosomally integrated *Sa*BirA and *Ec*BirA, as well as their target promoters fused to a *lacZ* reporter gene. Here I confirmed that birA from both bacteria are biotin-responsive transcription factors. Moreover, based on the dimerization constant of apo-*Sa*BirA (K_D^{2-1} = 30 μM) and apo-*Ec*BirA (K_D^{2-1} = 2 mM), and the predicted intracellular concentration of BirA (2nM – 100nM), it is estimated that these apo proteins are predominantly monomeric in growing cells. Therefore, mutant proteins with abolished *in vitro* dimerization ability were included as mimics of the monomeric apo-state, namely *Sa*BirA F123G and *Ec*BirA R119W . The results obtained from the *in vivo* assays showed that *Sa*BirA F123G repressed the target

promoters, whereas *Ec*BirA R119W was devoid of repressor activity. These results were confirmed *in vitro* by gel-shift assays. Cross-linking studies added further evidence that DNA promotes dimerization of *Sa*BirA F123G, but not *E. coli* R119W. *In vitro* analysis also revealed the affinity for DNA binding varies between *Sa*BirA-target promoters. This suggested a hierarchy of *Sa*BPL regulated genes, with the biotin biosynthesis operon being the most responsive to exogenous biotin concentration. Taken together, the outcomes from *in vivo* and *in vitro* analyses performed in this study have validated the hypothesis that *Sa*BirA uses different DNA binding mechanisms to *Ec*BirA. As a consequence, *Sa*BirA provides *S. aureus* with one avenue to adapt in response to its environment.

Finally, this study also investigated the role of a novel *Sa*BirA inhibitor, BPL199, as a co-repressor in DNA binding and its effect on gene transcription. Quantitative Real-Time PCR experiments revealed that BPL199 was able to act as a co-repressor to down-regulate expression of biotin-regulated genes *in vivo,* with similar kinetics as biotin. EMSA analysis showed that the affinity of *Sa*BPL:BPL199 for DNA binding was similar to that of the natural substrate, biotinyl-5′-AMP. This supported the proposal that BPL199 successfully mimics the action of biotinyl-5′-AMP in initiating transcriptional repression. In addition, a BPL199-resistant strain of *S. aureus* generated in our laboratory, was also investigated. DNA sequencing revealed a single point mutation in *Sa*BirA (D200E) that mapped within its dimerization interface. The ability of *Sa*BirA D200E to bind DNA, and down regulate gene expression, was subsequently addressed. The results indicated that *Sa*BirA D200E was compromised in the *Sa*BirA:DNA interaction *in vivo*. The most susceptible target was the *SabioY*

promoter, suggesting that increased transport of exogenous biotin is one mechanism that can be employed by the bacteria to overcome compounds that target BPL.

Thesis layout:

The thesis will be presented as a combination one published literature review, one manuscript to be submitted for publication as well as conventional chapters. Each manuscript will be a chapter with its own references. A general introduction and discussion will also be included to link together all the research conducted during this candidature. A publishing agreement with all co-authors involved with the work is also included.

Statement of Originality

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Jiulia Satiaputra and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship

06 - 02 - 2017

…… ……

Jiulia Satiaputra Date

Communications and presentations

Satiaputra, J., Soares da Costa, T.P., Yap, M.Y., Wilce, M.C.J., Perugini, M., Shearwin, K.E., Booker, G.W.& Polyak, S.W.(2014) "Transcription repressor function of *Staphylococcus aureus* biotin protein ligase", 39th *Lorne protein and structural biology conference.* Poster presentation.

Satiaputra, J., Soares da Costa, T.P., Yap, M.Y., Wilce, M.C.J., Perugini, M., Shearwin, K.E., Booker, G.W.& Polyak, S.W.(2014) "Transcription repressor function of *Staphylococcus aureus* biotin protein ligase", *Adelaide university school of biological sciences symposium*. Poster presentation.

Satiaputra, J., Shearwin, K.E., Booker, G.W.& Polyak, S.W., "Understanding transcription repressor function of *Staphylococcus aureus*: biotin homeostasis during pathogenesis"(2014), *Adelaide university student symposium*. Oral presentation.

Satiaputra, J., Eijkelkamp, B., Shearwin, K.E., Booker, G.W.,Polyak, S.W.(2015) "The role of biotin protein ligase in regulating biotin synthesis in *Staphylococcus aureus*", *Adelaide university annual student symposium*. Oral presentation.

Satiaputra, J., Eijkelkamp, B., Shearwin, K.E., Booker, G.W.,Polyak, S.W.(2015) "The role of biotin protein ligase in regulating biotin-related gene expressions in *Staphylococcus aureus*", *Adelaide protein group meeting*. Poster presentation.

Satiaputra, J., Eijkelkamp, B., Shearwin, K.E., Booker, G.W.,Polyak, S.W.(2015) "The role of biotin protein ligase in regulating biotin-related gene expressions in *Staphylococcus aureus*", *International anti-microbial meeting*. Poster presentation

Satiaputra, J., Eijkelkamp, B., Shearwin, K.E., Booker, G.W.,Polyak, S.W.(2015) "The role of biotin protein ligase in regulating biotin-related gene expressions in *Staphylococcus aureus*", *Combio international conference*. Poster presentation

Satiaputra, J. Eijkelkamp, B., McDevitt, C., Shearwin, K.E., Booker, G.W.& Polyak, S.W.(2016) "Exploring the gene regulation mechanism in *Staphylococcus aureus "*, 41st *Lorne protein and structural biology conference.* Poster presentation.

Satiaputra, J. Eijkelkamp, B., McDevitt, C., Shearwin, K.E., Booker, G.W.& Polyak, S.W.(2016), "Defining molecular mechanism of transcriptional repressor of biotin protein ligase in Staphylococcus aureus", Australian Society of Medical Research annual meeting 2016, Oral presentation

Acknowledgement

I would like to thank my supervisor, Prof. Grant Booker for giving me the opportunity to join his laboratory and for the guidance and support he has given me throughout the last 3.5 years.

I would like to thank my supervisor, Dr. Steven Polyak for his support and guidance, his effort in reading my thesis drafts and manuscript. It was not an easy task and I would like thank you for all your hard work. I would also like to thank you for all the interesting conversation about TV shows (the bachelor in particular).

Thank you to Dr. Keith Shearwin, for all the help and support he has given me throughout my candidature. I really appreciate the time you spent to help me with my experiments (the longest meeting I had during my candidature was the "quick question" I had that ended up taking about four hours of your time) and the time you invested into the manuscript.

I would also like to thank Dr. Chris McDevitt and Dr. Bart Eijkelkamp for welcoming me into their lab and teaching me all I need to know about qPCR, and for all the advice and input to my project.

Thank you to my special friend Ashleigh Paparella, with whom I shared this PhD experience from day 1. Thank you for listening to all my whining, laugh with me (and sometimes let me laugh at you), for coming to the horror movies with me, Friday night dinners and shopping therapy.

Thank you to Louise Sternicki for helping me with my experiment, for being a truly amazing colleague and friend. Thank you for your companion in and outside the lab, for listening to the grumpy old me and giving me a (surprisingly) mature advices and baking all the nice treats for lab meeting.

Thank you to Dr. Kate Wegener for her support and companion. We are so lucky to have you in the lab Kate, I really appreciate your advices in getting me through the difficult time during this past 3.5 years, especially during thesis writing.

Thank you to past and current member of the Booker lab, Wanisa saelaeme, Al azhar, Wei Wei Tang, Andrew Thompson, Andrew Hayes, Okki Cho, David Haem, Zikai Feng for all the support and help they have given me, during my candidature.

Thank you to my Perth friends; Ellen Fortini, Carola Schwan, Karina Price, Nicole Kucera, Marlies Loescher, Lakshini, Fayth Good, Jenny Beumont and Joanne van vuuren for their never ending support.

Thank you to my best friends Novera, Cut fara, Risky, Ajeng, Fajar, Donny, Adinda, Nasreen, Mira and Imaina for their support and for cheering me up when things became stressful.

To Peter Greig, I would not have been able to do this without you. Times sure was tough for me during the last few months of writing but you made sure that I would be fine. Words cannot express how much your support means to me. Your constant encouragement was really what got me through those last few months, thank you so much and I hope I have made you proud.

Thank you to my parents, this degree would not be possible without their hard work, support and love. Thank you for believing in me and my decision to undertake this journey. I love you both very much.

To my grandma, I was half way through writing this thesis when you left us, I did not get the chance to show this thesis to you but I know you would be proud that I completed this thesis, as I promised you during our last conversation.

Chapter 1:

General introduction

1.1 *Staphylococcus aureus* and MRSA

S. aureus is a member of micrococcaceae family of bacteria with typical gold pigmentation as their distinctive attribute. As a consequence, they are often referred to as "golden staph". *S. aureus* is one of the most potentially dangerous human pathogens. *S. aureus* can quickly adapt to changes in their environment, allowing them to survive in a wide variety of niche microenvironments, to circumvent host immune responses and avoid antibiotic assault. *S. aureus* infection can cause severe disorders affecting skin, respiratory organs, soft tissues, bones, joints and the endovascular system. Life threatening *S. aureus* infection such as bacteremia, toxic shock syndrome, sepsis and endocarditis contribute to high mortality rates in hospitals and the wider community (reviewed in [1]). During 2014-2015, there were 1490 cases of bacteremia infection reported in Australia [2]. The United States Center for Disease Control have reported that there were 11285 death and 80461 severe infections related to *S. aureus* infections in 2013 in the United States [3]. In addition, a study conducted on 1000 hospitalized patients in the United States revealed that on average, *S. aureus* infection extends hospitalization by 3-times compared to patients without *S. aureus* [4].

Although *S. aureus* is naturally susceptible to a wide range of antibiotics, the bacteria have demonstrated a remarkable ability to evolve a variety of resistance mechanisms [5]. Methicillin resistant *Staphylococcus aureus* (MRSA) is a notorious nosocomial and community-acquired infection that has developed resistance to antibiotics such as β-lactams, quinolones and aminoglycosides [6, 7]. A report issued in 2013 by the Australian Government Department of Health suggested that the prevalence of MRSA infection is now as high as 19 percent in Australia [8]. One of the most common treatments for MRSA infection is vancomycin. In 1997 the US

Centre for Disease Control first reported vancomycin resistant MRSA in US patients [9]. Similar resistance profiles were also identified in Japan [9]. Since these first reports of the emergence of vancomycin resistance, cases from other countries such as Thailand [10], China [11] and more cases in the United States [12], have been reported. These findings strongly indicate the possibility of MRSA to acquire additional resistance in the future and highlight the need for new antibiotics to treat MRSA infection.

Moreover, it is undeniable that antibiotic resistance has become a major challenge for our public health care systems. Increased costs, longer hospitalization, as well as increased mortality rates are all attributed to antibiotic resistance [13]. One prevailing approach to combating antibiotic resistance is to develop new antibiotics that are not subject to existing resistance mechanisms. Despite the effort of major pharmaceutical companies and academic researchers to discover new classes of antibiotics, only four new classes of antibiotics have been launched in the last 40 years [14]. Most antimicrobials in the development pipeline are next generation analogues of existing scaffolds for which there are already pre-existing resistance mechanisms [15]. The discovery of new chemotherapies is underpinned by basic research into unexplored antibiotic drug targets that are not employed by current medicines. This thesis investigates the role of biotin in bacterial physiology with a view to exploiting this for the development of new antibiotics.

1.2 Biotin and biotin synthesis pathway

Biotin (also known as vitamin H or B7) is an important water-soluble micronutrient present throughout the living world. Biotin acts as a cofactor for

important biotin-dependent enzymes. In the prototypical bacteria *Escherichia coli,* there is a single biotin-dependent enzyme, namely acetyl CoA carboxylase (ACC). This essential enzyme catalyzes the carboxylation of acetyl CoA to malonyl CoA in the first committed step in fatty acid synthesis [16]. This metabolic pathway has been shown to be essential in a number of microorganisms (reviewed in [17]) and has been the subject to numerous antibiotic discovery projects (reviewed in [18]). As well as possessing ACC, *S. aureus* has a second biotin-dependent enzyme, pyruvate carboxylase (PC). PC is responsible for replenishing the TCA cycle with oxaloacetate that is synthesized by the carboxylation of pyruvate [19]. In humans, deficiencies in biotin availability, metabolism or adsorption result in serious illness [20]. Humans possess five biotin dependent enzymes: PC, two isozymes of ACC and two other enzymes responsible for amino acid metabolism, namely propionyl CoA carboxylase and Methylcrotonyl CoA carboxylase[21].

Biotin is an essential micronutrient required for *S. aureus* growth [22-25]. A study conducted by Gretler and co-workers [26] reporter that 38 out of 46 coagulase negative, clinical isolates of *S. aureus* had an absolute requirement for biotin in the growth media whereas 8 other strains failed to reach maximum growth in the absence of biotin. In addition, a study conducted by Mah and co-workers [27] suggested that biotin was essential in supporting the growth of *S. aureus* S-6 strain, when glutamic acid was used as a carbon source as opposed to glucose. This observation suggests that in its natural environment, when glucose may be limited, the ability of *S. aureus* to maintain the supply of biotin becomes critical for survival. Based on the findings outlined above, it is evident that biotin plays a significant role in *S. aureus* growth, and deprivation of biotin might be lethal to certain strains of *S. aureus*. The biotin utilization therefore serves as

potential targets for new antibiotic development against *S. aureus*. In addition, recent studies have shown a strong correlation between biotin availability and virulence in *Francisella novicida* [28, 29] and *E. coli* [30]. However, the role of biotin in *S. aureus* virulence has not yet been investigated.

Bacteria such as *E. coli* and *S. aureus* satisfy their biotin demand through two alternative mechanisms, *de novo* synthesis and import from exogenous sources through the action of a biotin transport system. On the other hand, humans and other mammals are auxotrophic for biotin due to the absence of a biotin biosynthesis pathway and rely solely on biotin uptake from dietary sources. This clear distinction between human and microbes suggests that biotin metabolic pathways are promising targets for new antibiotic discovery [31]. The biotin synthesis pathway is well characterized in *E. coli* and *Bacillius subtilis*, as described by Lin and Cronan [32]. The first stage involves the synthesis of a pimelate moiety, which provides most of the biotin carbon atoms. The reactions leading up to the production of pimelate are catalyzed by two different enzymes, encoded by the *bioC* and *bioH*. The second stage of the synthesis involves the assembly of the bicyclic rings as illustrated in **Figure 1.** The second stage of biotin synthesis starts with the conversion of pimelate thioester to 7-keto-8-aminopelargonic acid (KAPA) by the action of KAPA synthase that is encoded by the *bioF* gene. 7-keto-8-aminopelargonic acid is then transformed into 7,8-diamino-pelargonic acid (DAPA) by DAPA synthase encoded by *bioA*. Next, dethiobiotin (DTB) is then formed from DAN and CO_2 in a reaction catalyzed by DTB synthase, encoded by *bioD*. Finally, the thiophene ring of biotin is closed using S-adenosyl methionine in a reaction catalyzed by biotin synthase, encoded by *bioB*. In *bacteria*, these

enzyme-encoding genes are often clustered into an operon known as the biotin Operon or *bioO*.

In both *S. aureus* and *E. coli*, the regulation of the biotin operon is controlled by a bi-functional protein called Biotin retention protein or BirA (also known as biotin protein ligase or BPL). This protein also belongs to the family of biotin protein ligases that are responsible for the attachment of the biotin cofactor onto target enzymes such as PC and ACC. An introduction to BirA and BirA-regulated transcriptions is discussed in more detail in the next section and the review article attached to this chapter. Regulation of the biotin operon and BioY biotin transporter by BirA in *S. aureus* is the focus of this study.

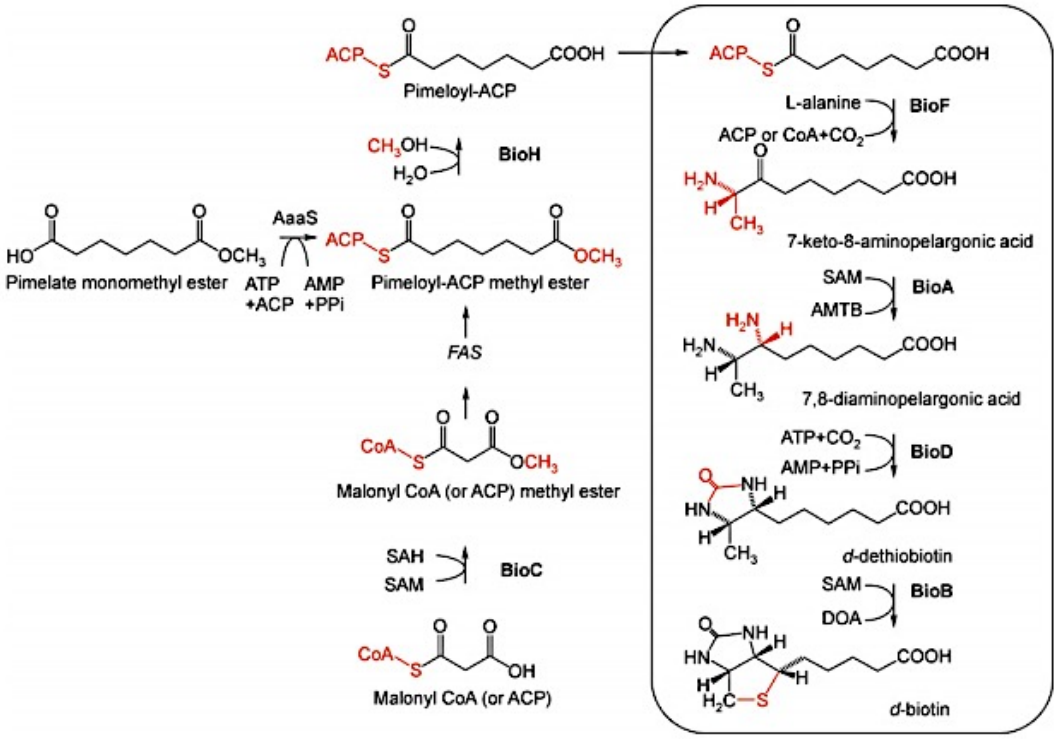


Figure 1: **Biotin synthesis pathway.**

Biotin is synthesized from pimelate thioester in a four-enzymes catalyzed reactions encoded by *bioF, bioA, bioD* and *bioB,* respectively. The figure is adapted from [31].

1.3 BirA regulates biotin biosynthesis operon and biotin transporter protein in *S. aureus*

Biotin biosynthesis is an expensive metabolic event. At least 4 gene products and an estimated 20 ATP equivalents are required to synthesize one biotin molecule [28]. Therefore, biotin biosynthesis needs to be stringently regulated. As mentioned above, in *S. aureus* and *E. coli* biotin synthesis is regulated through the action of the bifunctional protein, BirA. In *S. aureus*, bioinformatics analysis predicted that BirA also regulates the expression of a biotin transporter, BioY (*SabioY*), as well as two other genes involved in fatty acid metabolism, namely *SayhfS-SayhfT* (homologs of acetyl-CoA-acetyl transferase and Fatty acid-CoA ligase, respectively)[33]. However, this has not yet been demonstrated experimentally.

A detailed review of the relevant literature discussing the different classes of BirA, the bi-functional properties of class II BirA and the regulatory switch between the two functions is presented as a published manuscript and is attached to this chapter. This review provides the background literature about the BirA transcriptional regulator and importantly, highlights the differences between *Sa*BirA and the well-studied *Ec*BirA.

1.4 Targeting Biotin Protein Ligase for antibiotic development.

Targeting essential metabolic pathways present in bacteria is one well accepted approach to the discovery of new antibiotics [34]. In particular, vitamin biosynthesis pathways serve as attractive targets for new antibiotics. Vitamins such as thiamine, riboflavin and folic acid have all been the focus for antibiotic

development (reviewed in [35]). As biotin is an essential vitamin, targeting metabolic pathways where biotin is utilized as a co-factor would also serve as promising targets for antibiotic development.

Recent research in our group has focused on developing new antibiotics that target BirA from *S. aureus* [36, 37]. Chemical analogues of biotin, or the biotinylation reaction intermediate, biotinyl-5′-AMP [36-41], provide valuable starting points for target based drug discovery. This approach is promising, with one compound, BPL199, showing a K_i value of 2.4 nM and MIC of 0.50 μg/mL. A thorough characterization of BPL inhibitors as co-repressors of *Sa*BirA regulated gene expression, however, has not been reported. The current study will provide important knowledge on the effect of BPL199 on *Sa*BirA transcriptional repressor function.

Project aims and significance

The role of *Ec*BirA as a transcriptional regulator has been the subject of many studies over the years (reviewed in [42-44]). The molecular mechanism of DNA-binding and the regulatory switch between transcriptional repressor and enzyme function of *Ec*BirA have been well characterized. However, the function of *Sa*BirA as a transcriptional regulator has not been as well studied. Recent findings revealed that there are differences between the mechanism of dimerization observed in *Ec*BirA and the dimerization mechanism displayed by *Sa*BirA, and as a consequence, different DNA binding mechanism between the two proteins. Briefly, and as outlined in the attached review, it is known that for *Ec*BirA , dimerization is the pre-requisite to DNA binding and this dimerization is induced by the binding of the co-repressor, biotinyl-5′-AMP with a K_{Dim} = 1 -10 μM [45-48]. The apo- *Ec*BirA

exhibits weak dimerization activity with a K_{Dim} of 0.9 mM and is devoid of DNA binding activity [49, 50]. In contrast, a recent report has revealed that *Sa*BirA was able to dimerize and bind DNA in the absence of ligand [51], with the estimated K_D for DNA binding varying between 0.65-5.0 μM [51, 52]. These findings have given rise to the hypothesis that the two bacteria might exhibit subtly different ways of regulating biotin homeostasis, in response to environmental biotin. The ability of *S. aureus* to adjust to the changes in extracellular biotin availability, by orchestrating the intake of biotin via the transport protein and *de novo* synthesis, may contribute to the pathogen's rapid ability to adapt and, therefore, may play a crucial role during pathogenesis. This hypothesis warrants a more detail investigation into the mechanism of DNA-binding and biotin-regulated gene expression. In addition, understanding this transcriptional regulation would also contribute positively to the future design of antibiotics targeting *Sa*BirA , as favourable compounds would simultaneously inhibit BPL activity whilst also serving as co-repressors.

The aim of this study is to characterize the transcriptional repressor function of *Sa*BirA using both *in vivo* and *in vitro* methods and also to investigate the effect of *Sa*BirA inhibitor on gene regulation. The aims are presented in three experimental chapters in this thesis; a brief outline of each aim is presented below:

Aim 1: To develop an *in vivo* assay to analyze *Sa*BirA–DNA interaction within the context of cellular environment.

In order to study the cellular function of *Sa*BirA, it is important to be able to manipulate the *in vivo* environment and observe how the external stimuli affect interaction of *Sa*BirA with DNA. Ideally, genetically manipulated *S. aureus* should be utilized to accommodate this *in vivo* analysis. However, genetic manipulation of

S. aureus is challenging, as they possess a complex restriction modification barrier that constrains the uptake of foreign DNA, resulting in poor transformation efficiency. As an alternative, an *E. coli* reporter system was developed in order to facilitate the interaction of *Sa*BirA with DNA in an *In vivo* model. The construction of these reporter strains and the development of a biotin-regulated *β–galactosidase* expression assay are discussed in **chapter 3**. This system was also utilized in the characterization of *Sa*BirA inhibitor, BPL199, as discussed in **chapter 5**.

Aim 2: To Investigate the effect of biotin on *S. aureus* growth and characterization of *SaBirA* as a transcriptional repressor

The effect of biotin on the growth of *S. aureus* NCTC 8325, was investigated. In addition, characterization of *Sa*BirA as a transcriptional regulator was also investigated using the reporter strain system developed in **chapter 3,** in combination with other molecular techniques such as qPCR analysis, biotin uptake assay as well as *in vitro* DNA-binding analysis. This part of the study is presented in the form of a manuscript in **chapter 4**.

Aim 3: To investigate the effect of *SaBirA* inhibitor, BPL199 on DNA binding of *SaBirA* and gene regulation.

As discussed above, inhibitors designed to target *Sa*BirA have not been characterized with regards to their effect on DNA binding. In this part of the study, lead compound BPL199 was tested for its effect on *Sa*BirA's DNA binding function and consequently, its impact on gene regulation. In addition, a BPL199- resistant mutant generated in our laboratory was characterized. The *in vitro* and *in vivo* DNA

binding properties of this mutant were also investigated, to delineate the relationship between DNA binding activities and resistance mechanisms. Experimental methods and outcomes of this study are outlined in **chapter 5.**

Reference:

1. Voyich, J.M., et al., *Insights into mechanisms used by Staphylococcus aureus to avoid destruction by human neutrophils.* J Immunol, 2005. **175**(6): p. 3907-19.
2. *Bacteraemia in Australian public hospitals 2014-2015*. 2015, Australian government: Australian Institute of Health and Welfare. p. http://www.aihw.gov.au/WorkArea/DownloadAsset.aspx?id=60129554233.
3. *Antibiotic resistance threats in the United states, 2013.* . US department of Health and Human services (Centers for Disease Control and prevention).
4. Naber, C.K., *Staphylococcus aureus bacteremia: epidemiology, pathophysiology, and management strategies.* Clin Infect Dis, 2009. **48 Suppl 4**: p. S231-7.
5. Chambers, H.F., Deleo, F.R., *Waves of resistance: Staphylococcus aureus in the antibiotic era.* Nature reviews microbiology, 2009. **7**: p. 629-641.
6. Koyama, N., Inokoshi, J., Tomoda, H., *Anti-infectious agents against MRSA.* Molecules, 2012. **18**: p. 204-224.
7. Tomasz, A., *Multiple- Antibiotic-Resistant Pathogenic Bacteria.* The New England Journal of Medicine, 1994. **330**: p. 1247-1251.
8. *Responding to the threat of antimicrobial resistance: Australia's first national antimicrobial resistance strategy 2015-2019.* Departments of Health and Agriculture, Australian Government, June 2015. .
9. Hiramatsu, K., Hanaki, H., Ino, H., Yabuta., T., Oguri, T., Tenover, F.C., *Methicillin-resistant Staphylococcus ausreus clinical strain with reduced vancomycin susceptibility.* . J. Antimicrob. Chemother., 1997. **40**: p. 135-136.
10. Trakulsomboon, S., et al., *First report of methicillin-resistant Staphylococcus aureus with reduced susceptibility to vancomycin in Thailand.* J Clin Microbiol, 2001. **39**(2): p. 591-5.
11. Lu, J.J., et al., *Septic arthritis caused by vancomycin-intermediate Staphylococcus aureus.* J Clin Microbiol, 2005. **43**(8): p. 4156-8.
12. Walters M, L.D., Rasheed K, Albrecht, V, McAllister, S, Limbago B, Kallen A, *Investigation and Control of Vancomycin-resistant Staphylococcus aureus: A Guide for Health Departments and Infection Control Personnel.* . 2015, Division of Healthcare Quality Promotion Centers for Disease Control and Prevention: Atlanta, GA.
13. Chakravartty, V. and J.E. Cronan, *The wing of a winged helix-turn-helix transcription factor organizes the active site of BirA, a bifunctional repressor/ligase.* J Biol Chem, 2013. **288**(50): p. 36029-39.
14. Cooper, A., Shlaes, D, *Fix the antibiotics pipeline.* Nature, 2011. **472**: p. 32.
15. Payne, D.J., Gwynn, M.N., Holmes, D.J., and Pompliano, D.I., *Drugs for bad bugs: confronting the challenges of antibacterial discovery.* Nature reviews drug discovery, 2007. **287**(21): p. 21-40.
16. Thampy, K.G., *Formation of malonyl coenzyme A in rat heart. Identification and purification of an isozyme of A carboxylase from rat heart.* J Biol Chem, 1989. **264**(30): p. 17631-4.
17. Cronan, J.E., *A new pathway of exogenous fatty acid incorporation proceeds by a classical phosphoryl transfer reaction.* Mol Microbiol, 2014. **92**(2): p. 217-21.

18. Polyak, S.W., et al., *Structure, function and selective inhibition of bacterial acetyl-coa carboxylase.* Appl Microbiol Biotechnol, 2012. **93**(3): p. 983-92.
19. Jitrapakdee, S., Booker, G. W., Cassady, A. I. and Wallace, J. C., *Cloning, sequencing and expression of rat liver pyruvate carboxylase.* Biochem J, 1996. **316 (Pt 2)**: p. 631-7.
20. Mayende, L., Swift, R. D., Bailey, L. M., Soares da Costa, T. P., Wallace, J. C., Booker, G. W., Polyak, S. W., *A novel molecular mechanism to explain biotin-unresponsive holocarboxylase synthetase deficiency.* J Mol Med (Berl), 2012. **90**(1): p. 81-8.
21. Pendini, N.R., Bailey, L. M., Booker, G. W., Wilce, M. C., Wallace, J. C. and Polyak, S. W., *Microbial biotin protein ligases aid in understanding holocarboxylase synthetase deficiency.* Biochim Biophys Acta, 2008. **1784**(7-8): p. 973-82.
22. Knight, B.C., *The nutrition of Staphylococcus aureus; nicotinic acid and vitamin B(1).* Biochem J, 1937. **31**(5): p. 731-7.
23. Koser, S.A., Finkle, R.D, Dorfman, A., gordon, M.V and Sauders, F., *Studies on bacterial nutrition.* J. infectious Diseases, 1938. **62**: p. 209-218.
24. Sevag, M.G. and M.N. Green, *The Mechanism of Resistance to Sulfonamides: III. Pantothenic Acid and Tryptophane Metabolism: The Role of Pantothenic Acid in the Synthesis of Tryptophane by Staphylococcus aureus and the Effect of Vitamins on Tryptophane in Exercising Antagonism to Sulfonamides.* J Bacteriol, 1944. **48**(6): p. 631-8.
25. Porter, J.R., Pelczar, M.J., *The nutrition of Staphylococcus aureus the influence of biotin, BioS IIB and vitamin H on the growth of several strains.* Journal of bacteriology, 1941. **41**(2): p. 173-92.
26. Gretler, A.C., Mucciolo,P., Evans, J.B., Niven, JR., C.F., *Vitamin nutrition of staphylococci with special reference to their biotin requirements.* Journal of bacteriology, 1955. **70**(1): p. 44-49.
27. Mah, R.A., D.Y. Fung, and S.A. Morse, *Nutritional requirements of Staphylococcus aureus S-6.* Appl Microbiol, 1967. **15**(4): p. 866-70.
28. Feng, Y., et al., *A Francisella virulence factor catalyses an essential reaction of biotin synthesis.* Mol Microbiol, 2014. **91**(2): p. 300-14.
29. Napier, B.A., et al., *Link between intraphagosomal biotin and rapid phagosomal escape in Francisella.* Proc Natl Acad Sci U S A, 2012. **109**(44): p. 18084-9.
30. Yang, B., et al., *Enterohemorrhagic Escherichia coli senses low biotin status in the large intestine for colonization and infection.* Nat Commun, 2015. **6**: p. 6592.
31. Salaemae, W., Azhar, A., Booker, G. W., Polyak, S. W., *Biotin biosynthesis in Mycobacterium tuberculosis: physiology, biochemistry and molecular intervention.* Protein Cell, 2011. **2**(9): p. 691-5.
32. Brune, I., Gotker, S., Schneider, J., Rodionov, D. A. and Tauch, A., *Negative transcriptional control of biotin metabolism genes by the TetR-type regulator BioQ in biotin-auxotrophic Corynebacterium glutamicum ATCC 13032.* J Biotechnol, 2012. **159**(3): p. 225-34.
33. Rodionov, D.A., Mironov, A. A. and Gelfand, M. S., *Conservation of the biotin regulon and the BirA regulatory signal in Eubacteria and Archaea.* Genome Res, 2002. **12**(10): p. 1507-16.

34. Hughes, D. and A. Karlen, *Discovery and preclinical development of new antibiotics.* Ups J Med Sci, 2014. **119**(2): p. 162-9.
35. Mobegi, F.M., et al., *From microbial gene essentiality to novel antimicrobial drug targets.* BMC Genomics, 2014. **15**: p. 958.
36. Feng, J., et al., *Biotin Protein Ligase Is a Target for New Antibacterials.* Antibiotics (Basel), 2016. **5**(3).
37. Paparella, A.S., et al., *Structure guided design of biotin protein ligase inhibitors for antibiotic discovery.* Curr Top Med Chem, 2014. **14**(1): p. 4-20.
38. Soares da Costa, T.P., et al., *Selective inhibition of biotin protein ligase from Staphylococcus aureus.* J Biol Chem, 2012. **287**(21): p. 17823-32.
39. Soares da Costa, T.P., et al., *Biotin analogues with antibacterial activity are potent inhibitors of biotin protein ligase.* ACS Med Chem Lett, 2012. **3**(6): p. 509-14.
40. Tieu, W., et al., *Improved Synthesis of Biotinol-5'-AMP: Implications for Antibacterial Discovery.* ACS Med Chem Lett, 2015. **6**(2): p. 216-20.
41. Tieu, W., et al., *Heterocyclic acyl-phosphate bioisostere-based inhibitors of Staphylococcus aureus biotin protein ligase.* Bioorg Med Chem Lett, 2014. **24**(19): p. 4689-93.
42. Beckett, D., *Biotin sensing: universal influence of biotin status on transcription.* Annu Rev Genet, 2007. **41**: p. 443-64.
43. Beckett, D., *The Escherichia coli biotin regulatory system: a transcriptional switch.* J Nutr Biochem, 2005. **16**(7): p. 411-5.
44. Satiaputra, J., Shearwin, K.E., Booker, G.W., Polyak, S.W., *Mechanisms of biotin-regulated gene expressions in microbes.* Synthetic and Systems Biotechnology, 2016(1): p. 17-24.
45. Xu, Y. and D. Beckett, *Evidence for interdomain interaction in the Escherichia coli repressor of biotin biosynthesis from studies of an N-terminal domain deletion mutant.* Biochemistry, 1996. **35**(6): p. 1783-92.
46. Eisenstein, E. and D. Beckett, *Dimerization of the Escherichia coli biotin repressor: corepressor function in protein assembly.* Biochemistry, 1999. **38**(40): p. 13077-84.
47. Weaver, L.H., et al., *Corepressor-induced organization and assembly of the biotin repressor: a model for allosteric activation of a transcriptional regulator.* Proc Natl Acad Sci U S A, 2001. **98**(11): p. 6045-50.
48. Wood, Z.A., et al., *Co-repressor induced order and biotin repressor dimerization: a case for divergent followed by convergent evolution.* J Mol Biol, 2006. **357**(2): p. 509-23.
49. Kwon, K. and D. Beckett, *Function of a conserved sequence motif in biotin holoenzyme synthetases.* Protein Sci, 2000. **9**(8): p. 1530-9.
50. Streaker, E.D. and D. Beckett, *Coupling of site-specific DNA binding to protein dimerization in assembly of the biotin repressor-biotin operator complex.* Biochemistry, 1998. **37**(9): p. 3210-9.
51. Soares da Costa, T.P., et al., *Dual roles of F123 in protein homodimerization and inhibitor binding to biotin protein ligase from Staphylococcus aureus.* Mol Microbiol, 2014. **91**(1): p. 110-20.
52. Henke, S.K. and J.E. Cronan, *The Staphylococcus auresu group II biotin protein ligase birA is an effective regulator of biotin operon transcription and requires DNA binding domain for full enzymatic activity.* Molecular microbiology, 2016, vol.102(3), pp. 419-429.

Statement of Authorship

Title of Paper	Mechanism of biotin-regulated gene expression in microbes
Publication Status	☒ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Satiaputra, J., Shearwin, K.E., Booker, G.W & Polyak, S.W. (2016), "Mechanism of biotin-regulated gene expression in microbes", Synthetic and System Biotechnology (I):17-24

Principal Author

Name of Principal Author (Candidate)	Jiulia Satiaputra		
Contribution to the Paper	Compiled the materials for the review article and took the primary role in writing the manuscript and preparing figures.		
Overall percentage (%)	90		
Certification:	This invited review article is an original work I wrote during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	08/12/16

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Keith Shearwin		
Contribution to the Paper	Helped evaluate and review the manuscript		
Signature		Date	8/12/2016

Name of Co-Author	Grant Booker
Contribution to the Paper	Helped evaluate and review the manuscript
Signature	Date 12/12/2016

Name of Co-Author	Steven Polyak
Contribution to the Paper	Was approached by the editor for this invited review. Supervised the development of writing and was the corresponding author.
Signature	Date 8/12/2016.

Synthetic and Systems Biotechnology 1 (2016) 17–24

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: keaipublishing.com/synbio

Mechanisms of biotin-regulated gene expression in microbes

J. Satiaputra [a], K.E. Shearwin [a], G.W. Booker [a,b], S.W. Polyak [a,b,*]

[a] Department of Molecular and Cellular Biology, School of Biological Science, University of Adelaide, North Tce, Adelaide, South Australia 5005, Australia
[b] Centre for Molecular Pathology, University of Adelaide, North Tce, Adelaide, South Australia 5005, Australia

ARTICLE INFO

Article history:
Received 17 August 2015
Received in revised form 8 January 2016
Accepted 10 January 2016
Available online

ABSTRACT

Biotin is an essential micronutrient that acts as a co-factor for biotin-dependent metabolic enzymes. In bacteria, the supply of biotin can be achieved by *de novo* synthesis or import from exogenous sources. Certain bacteria are able to obtain biotin through both mechanisms while others can only fulfill their biotin requirement through *de novo* synthesis. Inability to fulfill their cellular demand for biotin can have detrimental consequences on cell viability and virulence. Therefore understanding the transcriptional mechanisms that regulate biotin biosynthesis and transport will extend our knowledge about bacterial survival and metabolic adaptation during pathogenesis when the supply of biotin is limited. The most extensively characterized protein that regulates biotin synthesis and uptake is BirA. In certain bacteria, such as *Escherichia coli* and *Staphylococcus aureus*, BirA is a bi-functional protein that serves as a transcriptional repressor to regulate biotin biosynthesis genes, as well as acting as a ligase to catalyze the biotinylation of biotin-dependent enzymes. Recent studies have identified two other proteins that also regulate biotin synthesis and transport, namely BioQ and BioR. This review summarizes the different transcriptional repressors and their mechanism of action. Moreover, the ability to regulate the expression of target genes through the activity of a vitamin, such as biotin, may have biotechnological applications in synthetic biology.

1. Introduction

Biotin (vitamin H or B7) is an important micronutrient that functions as a cofactor for biotin-dependent enzymes.[1] These include the biotin-dependent carboxylases, decarboxylases and transcarboxylases, all of which are found in the microbial world. In the prototypical bacteria *Escherichia coli*, there is a single biotin-dependent enzyme, namely acetyl CoA carboxylase, that catalyzes the first committed step in the fatty acid biosynthesis pathway.[2,3] Other examples of biotin-dependent enzymes commonly found in prokaryotes include pyruvate carboxylase responsible for replenishing the TCA cycle with oxaloacetate,[4] and propionyl CoA carboxylase required for the metabolism of certain amino acids and fatty acids.[5] Micro-organisms, plants and some fungi are able to synthesize biotin *de novo* as well as importing it from their environment through the action of a biotin transport system. In contrast, humans and other mammals are biotin auxotrophs and rely solely on uptake from external sources, such as intestinal microflora or the diet.[6] This genetic difference in biotin metabolism between humans and microbes provides potential drug targets for new antibiotic discovery (reviewed[7]). The biotin synthesis pathway is well characterized in *E. coli* and *Bacillus subtilis* and has recently been reviewed.[8] In many bacteria the genes that encode the biotin biosynthetic enzymes are often clustered into an operon known as the *bio* operon.[9] Briefly, the synthetic pathway commences with L-alanine and S-adenosyl-L- methionine being introduced into pimeloyl-ACP by the activities of 7-keto-8-aminopelargonic acid synthase (encoded by *bioF*) and 7,8-diaminopelargonic acid synthase (encoded by *bioA*), respectively, to generate 7,8-diaminopelargonic acid. Dethiobiotin synthetase (encoded by *bioD*) and biotin synthase (encoded by *bioB*) then catalyze the closure of the ureido and thiophane heterocycles, respectively, liberating biotin.

The *de novo* synthesis of biotin is metabolically costly, requiring 20 equivalents of ATP for each molecule of biotin and the activities of at least 4 metabolic enzymes.[10] Therefore, transcriptional regulation of the biotin biosynthetic enzymes needs to be

Abbreviations: BCCP, biotin carboxyl carrier protein; BirA, biotin retention protein A; BPL, biotin protein ligase; *Ec*BirA, *Escherichia coli* BirA; *Sa*BirA, *Staphylococcus aureus* BirA.

* Corresponding author. Department of Molecular and Cellular Biology, School of Biological Sciences, University of Adelaide, South Australia 5005, Australia. Tel.: +61 8 8313 6042; fax: +61 8 8313 4362.
E-mail address: steven.polyak@adelaide.edu.au (S.W. Polyak).

Peer review under responsibility of KeAi Communications Co., Ltd.

http://dx.doi.org/10.1016/j.synbio.2016.01.005

tightly controlled. In the model bacteria *E. coli*, the balance of biotin demand versus supply is maintained through the action of the biotin retention protein A (BirA); a bi-functional protein that is not only a transcriptional repressor but also serves as the biotin ligase that catalyzes the attachment of biotin onto the biotin-dependent carboxylases. In other microorganisms, such as *Corynebacterium glutamicum* and *Agrobacterium tumefaciens*, there is no BirA homolog to regulate biotin synthesis and transport. Instead, alternative DNA-binding proteins perform this function, namely BioQ and BioR respectively. The mechanisms by which BirA, BioQ and BioR regulate biotin biosynthesis and transport will be discussed in this review.

2. BirA is a bi-functional protein

BirA serves as both a transcriptional repressor and the enzyme responsible for protein biotinylation (outlined in Fig. 1). As both biotin ligase and transcriptional repressor activities are intimately linked, we provide an overview of both functions as background for the reader to understand the sophistication of this elegant system. Protein biotinylation is achieved through a conserved, two-step reaction mechanism that is catalyzed by biotin protein ligase (BPL) in all organisms. In the first partial reaction biotin and ATP are required to form biotinyl-5′-AMP that serves as both the reaction intermediate for protein biotinylation and corepressor for transcriptional regulation. The BirA: biotinyl-5′-AMP (holo) enzyme can then adopt one of two different fates. When the cellular demand for biotin is low holo BirA can dimerize and bind DNA where it functions as the transcriptional repressor of the biotin biosynthesis operon, thereby inhibiting the synthesis of more biotin. In contrast, in the presence of substrate requiring biotinylation the holo BirA functions as a biotin ligase. Here BPL recognizes and binds to a biotin carboxyl carrier protein (BCCP) present in the receiving enzyme that contains the lysine residue targeted for biotinylation.[11] Protein biotinylation is an example of a post-translational modification that is performed with exquisite specificity. For example, the *E. coli* biotin ligase (BirA) modifies just one of the >4000 different proteins in the bacterial cell.[12] Moreover, the biotin cofactor is covalently attached onto the side chain of one single, specific target lysine residue present in the active site of biotin-dependent enzymes. BPLs from a wide variety of species are able to modify BCCP from unrelated organisms,[13–15] highlighting how highly conserved both the catalytic mechanism and the protein:protein interactions between enzyme and substrate have remained throughout evolution. The possible mechanisms through which BirA can switch between its two functions are described later in this review.

All BPLs contain a conserved 2-domain catalytic core responsible for biotinyl-5′-AMP synthesis and protein biotinylation.[16] The greatest divergence between the BPLs is in their N-terminal regions (see Fig. 2A). Class I BPLs are composed only of the conserved catalytic module that is required for protein biotinylation. Hence, these are mono functional enzymes. X-ray crystal structures of Class I BPLs have been reported for *Mycobacterium tuberculosis*[19] and *Pyrococcus horikoshii*.[21] In contrast, the Class II BPLs are truly bi-functional having both biotin ligase and transcriptional repressor activities due to an N-terminal DNA binding domain. BirA from *E. coli* is the most extensively studied representative of a Class II BPL, having been the subject of structural, genetic and biophysical studies (reviewed[22,23]).

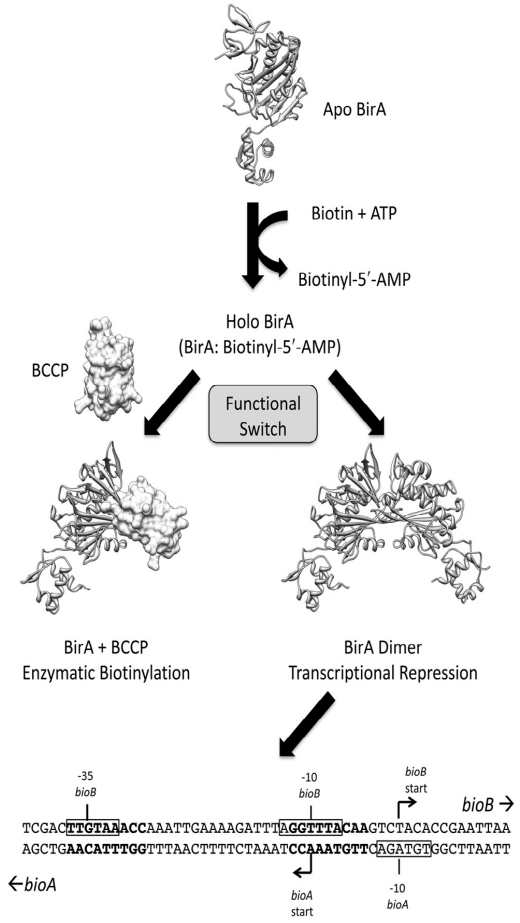


Fig. 1. Bifunctional BirA from *Escherichia coli*. The schematic shows the two alternative functions for the protein. The *bioO* sequence from the biotin biosynthetic operon is shown below, with the BirA binding sites in bold text and the –10 and –35 sequences boxed.

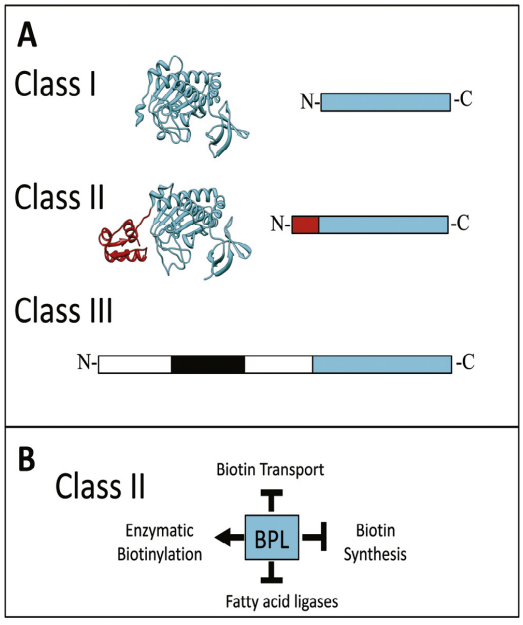


Fig. 2. Biotin Protein Ligase. (A) The relative sizes of the three structural classes of BPLs are shown. The conserved catalytic region is depicted in blue, the DNA binding domain of Class II enzymes in red and the proof reading domain in human BPL is boxed black.[17,18] The structures of BPLs from *M. tuberculosis* [PDB 3RUX[19]] and *E. coli* [PDB 2EWN[20]] are highlighted. (B) Schematic overview showing the single protein model of protein biotinylation and transcriptional regulation in Class II BPLs.

Several recent reports of the homolog from *S. aureus* (*Sa*BirA) have appeared in the literature and provide some interesting points of difference to the prototypical *Ec*BirA that will be elaborated further below. Genetic knockout studies performed on both *E. coli* and *S. aureus*, among other bacteria, have demonstrated that BirA is an essential gene product.[19,24–26] Therefore, biotin ligases serve as promising targets for new antibacterials.[27–29] Interestingly, a recent study reported that *Francisella novicida* expresses both a Class I and Class II BPL.[30] In this bacterium the Class I enzyme functions as the biotin ligase whereas the Class II homolog is a transcriptional regulator essential for virulence in mouse infection models. However, this is exceptional as most bacteria only possess one BPL equivalent. The Class III BPLs, such as those found in mammals and certain eukaryotes, contain an extended N-terminal region making them at least twice the size of the Class II BPLs (Fig. 2A). This extension bears no sequence or functional similarity to the Class II DNA binding domain. Recent mutagenic, genetic and biophysical studies have demonstrated that the N-terminal extension contains a 'proof reading' activity to ensure that only the appropriate enzymes are selected for protein biotinylation.[17,18]

3. Structural biology delineates the bifunctional activities of class II BPLs

The X-ray crystal structures of bifunctional Class II BPLs from *E. coli* and *S. aureus* reveal that the proteins are composed of three discrete domains. The N-terminal domain is a winged helix-turn-helix motif required for DNA binding. The central and C-terminal domains form the catalytic core of the enzyme that share high sequence homology with BPLs across all three kingdoms of life. The central domain of BirA is composed of α-helices and β-strands whereas the C-terminal domain is composed of antiparallel β-sheets.[31] Although the function of the C-terminal domain has not been elucidated, it is believed that this region of the enzyme contributes to the binding of the BCCP substrate.[32] The catalytic site of the enzyme is located in the central domain, where biotinyl-5′-AMP synthesis and biotinyl-transfer occur.[33] Structural biology has provided further insights into the BPL catalyzed reaction. The adenylation of biotin proceeds in a sequential manner whereby biotin binds first to the enzyme followed by ATP, and its subsequent hydrolysis produces biotinyl-5′-AMP.[20] Comparisons of the crystal structures of *Ec*BirA in its unliganded (ie apo) and holo forms show that important conformational changes accompany ligand binding. Here the biotin-binding loop (amino acids 110–128) undergoes a disordered-to-ordered transition that closes over the biotin-binding pocket. This conformational change positions the side chain of a key tryptophan residue (Trp123) in the active site necessary for π–π stacking interactions with the adenylate moiety of ATP.[20] Amino acids within this loop are also required for protein dimerization and, consequently, DNA binding.

Surprisingly, the N-terminal helix-turn-helix domain of *Ec*BirA is required for both catalytic function and DNA binding. Removal of the first 64 amino acids (Δ1–64) resulted in a truncated enzyme that had reduced affinity for biotin and biotinyl-5′-AMP.[34] Hence, long-range interactions through the protein are believed to help stabilize the conformational changes associated with ligand binding. This observation was supported by recent studies by Chakravartty and Cronan that showed an *E. coli* 'delta wing' (Δ48–61) mutant strain resulted in the accumulation of ADP due to the hydrolysis of ATP.[35] These cells exhibited slow growth under low biotin conditions. When the DNA binding domain from an unrelated protein, OmpR, was fused onto the N-terminus of *Ec*BirA the chimeric protein restored growth of the *E. coli* delta wing strain in minimal media containing low biotin concentrations. The accumulation of ADP was also no longer observed. The authors propose that the wing in the helix-turn-helix structure is needed to stabilize the biotin-binding loop.[35] Interestingly, in a recent study on the BirA from *B. subtilis*, deletion of the N-terminal region did not compromise enzyme activity *in vitro*[36] suggesting that the role of the N-terminus in assisting catalysis is not conserved among all Class II BPLs. Indeed the recombinant expression of truncated *B. subtilis* BirA was able to complement a strain of *E. coli* expressing the N-terminally deleted *Ec*BirA (Δ1–64).[36]

The N-terminal DNA-binding domains of both *E. coli* and *S. aureus* BirAs recognize specific palindromic sequences present in the operator site, *bio*O, upstream of the biotin biosynthesis operon. Bioinformatics analysis predicted that *Sa*BirA is also responsible for regulating expression of the biotin transporter *bioY* and fatty acid biosynthetic enzymes *yhfT* and *yhfS* in *S. aureus* (summarized in Fig. 2B).[9] The difference in target gene regulation between *E. coli* and *S. aureus* suggests that *Sa*BirA is solely responsible for maintaining biotin levels within the bacteria by regulating expression of both biotin synthesis and transport proteins, as well as contributing to fatty acid synthesis through the transcriptional regulation of *yhfT* and *yhfS* and activation of acetyl CoA carboxylase. This is in contrast to *E. coli* where the BirA recognition sequence is only present in the promoter of the biotin biosynthesis operon. Consequently, we propose that BirA regulated gene expression is potentially more responsive to environmental stimuli in *S. aureus* than the bacterial model *E. coli*.

4. BirA dimerization is intimately linked to DNA binding

Homodimerization of *Ec*BirA is a prerequisite for DNA binding. The binding of BirA to *bioO* is a co-operative event involving two BPL subunits and two *bioO* operator half-sites (Fig. 1).[37,38] The more stable the homodimer, the greater the affinity for DNA.[39,40] Mutation of amino acids that reside in the dimer interface of BirA results in loss of DNA binding activity.[41] Sedimentation equilibrium studies have revealed the dimerization constant (K_D) of apo BirA is greater than 1 mM[39] and, thus, apo BirA is not likely to dimerize at the physiological concentrations present inside the bacterial cell which have been estimated at <10 molecules per cell.[42] Similarly, biotin-bound BirA exhibits weak dimerization with a K_D of 0.9 mM.[39] In contrast, biotinyl-5′-AMP enhances dimerization free energy by –4.0 kcal/mol yielding a K_D of 1 – 10 μM,[39,43,44] suggesting that the co-repressor acts as an allosteric activator to dimer assembly and DNA binding.[33]

The crystal structures of both *Ec*BirA and *Sa*BirA show the dimers assemble in a side-by-side anti-parallel arrangement such that the two N-terminal HTH motifs are aligned for DNA binding.[45,46] An X-ray structure of any Class II BPL in complex with DNA has not yet been reported. However, molecular modeling studies propose that the N-terminal domain from one subunit of *Ec*BirA binds to the major groove of the double helix while the other subunit binds to the minor groove.[47] Mutation of amino acids Ser-32, Arg-33 and Ala-34 in the DNA-binding α-helices abolishes DNA binding and results in loss of repression activity.[48] In *E. coli*, two face-to-face promoters drive expression of the *bio* operon. The recognition sequence for *Ec*BirA (*bioO*) is an inverted repeat that is located in between the two promoters, at the –35 and –10 sites of the operator sequence (Fig. 1). Circular permutation analysis suggests that the double stranded DNA might be bent when in complex with *Ec*BirA.[47] On the other hand, small angle X-ray scattering analysis performed on the *Sa*BirA:*SabioO* complex proposed that the DNA does not bend for this species.[46] Hence, the footprint observed on the DNA is likely smaller for *Sa*BirA than *Ec*BirA.

5. Co-repressor induces BPL dimerization

Upon binding of the biotinyl-5′-AMP co-repressor, five loops located within the central domain of *Ec*BirA undergo a

disorder-to-order transition, with three of these loops (amino acids 110–128, 140–146 and 193–199) located in the dimerization interface.[41] When dimerization occurs, an extended intermolecular β-sheet is formed involving residues 189–195 in the central domain.[49] Following the structural changes induced by biotin, the biotin-binding loop encases the co-repressor and is stabilized through a network of hydrophobic interactions[50] as well as direct hydrogen bonding interactions involving R118.[49] Binding of the co-repressor leads to the ordering of the ATP-binding loop (residues 212–223) and better packing of the biotin-binding loop, which supports bonding interactions that stabilizes the dimer.[20] Therefore, the biotin-binding loop must fold before dimerization.[49] Direct interactions between the two monomers involve amino acids found in the loops, namely R118, R119 and D197.[49]

A recent study involving random mutagenesis to generate super-repressor mutants in *E. coli* identified an amino acid substitution with stronger DNA-binding to *bioO* than wildtype, namely G154 to aspartate.[40] Interestingly, this amino acid is neither located in the helix-turn-helix motif nor the dimerization interface. This suggests that other residues within the central domain but located outside of the dimer interface can participate in long-range interactions that stabilize the dimer, resulting in tighter binding to DNA. No structural data for this mutant has yet been reported to fully understand these long-range bonding interactions. Likewise, a recent study focused on G142 that is present in the dimer interface but that does not directly contribute to dimerization.[51] Substitution of G142 with alanine altered the structure of the 140–146 loop, and this in turn prevented the 193–199 loop from undergoing the disordered-to-ordered transition through long-range interactions. Together these studies highlight the importance of long-range allosteric interactions on dimerization and DNA binding.

In *Sa*BirA, ligand binding induces similar conformational changes in the loops that are located at the dimerization interface. Like *Ec*BirA, the biotin-binding loop in this interface (residues 118–129) undergoes a disorder-to-order transition to facilitate the interaction between the two-dimer subunits.[46] The dimerization interfaces of biotinyl-5′-AMP bound *Ec*BirA and *Sa*BirA are illustrated in Fig. 3. The subunits are connected by an analogous intermolecular β-sheet interaction as observed for *Ec*BirA, but the dimer is stabilized by additional intersubunit contacts.[46] Of particular note is F123 that forms a hydrophobic interaction with the side chain of D200 from the opposing monomer (Fig. 3A).[52] In EcBirA the homologous amino acid is R119 that forms a hydrogen bond with D197 on the partner subunit (Fig. 3B). Interestingly, substitution of R119 with an aromatic amino acid (R119W) has been shown to strongly disrupt homodimerization.[44] Analytical ultra centrifugation studies on *Sa*BirA revealed that replacing the F123 with either glycine or arginine abolished dimerization even in the presence of biotin, highlighting a role for this aromatic residue in homodimer assembly.[52]

Recent studies on *Sa*BirA dimerization indicate that the non-liganded form of the enzyme is also able to dimerize at low concentrations with a K_D of 29 ± 1.8 μM. This is a sharp contrast to apo *Ec*BirA, which only dimerizes at millimolar concentrations.[39,43,44] Apo-*Sa*BirA dimer was also shown to bind DNA in an electrophoretic mobility shift assay with K_D = 649 ± 43 nM, which is only 6-fold weaker than the binding of the holo-enzyme (K_D = 108 ± 6.0 nM).[52] Hence, allosteric regulation of the BirA switch in *S. aureus* may be more complex than originally thought. These data highlight key differences between *E. coli* and *S. aureus* BirAs that may have important physiological consequences that impact the bacteria's ability to sense their surroundings and adapt to the niche microenvironments they inhabit.

6. Switching between enzymatic and repressor functions of BPL

Both the biotin ligase and transcriptional repressor activities of BPL are critical for cell metabolism and survival. Therefore, controlling the switch between these two mutually exclusive functions is likely important for virulence. This raises a key question; how does the enzyme switch between enzymatic and DNA binding modes? One hypothesis proposed by Weaver and co-workers[53] is based on the observation that *Ec*BirA utilizes the same β sheet for both homodimerization and the interaction with the substrate BCCP (Fig. 4). A co-complex of the enzyme with BCCP has not yet been crystallized with a Class II BPL, but has been achieved with the Class I enzyme from *P. horikoshii*.[21] The model proposes that when there is an excess of non-biotinylated substrate, holo-BirA will preferentially bind BCCP, thereby preventing BirA homodimerization. Alternatively, when the concentration of BCCP is low, holo-BirA will accumulate and homodimerize, leading to DNA binding and subsequent repression of transcription. Therefore, in this model, the regulatory switch between the enzymatic and transcriptional repressor functions of BirA is governed by competing protein:protein

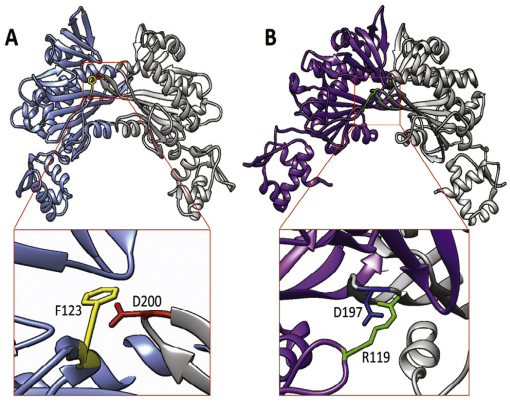


Fig. 3. Intersubunit contacts of BirA. The structures of dimeric holo BirA from (A) *S. aureus* [PDB 3RIR[46]] and (B) *E. coli* [PDB 2EWN[20]] are shown, with one subunit colored while the other subunit is in gray ribbon. Key amino acids in the dimerization interface are highlighted.

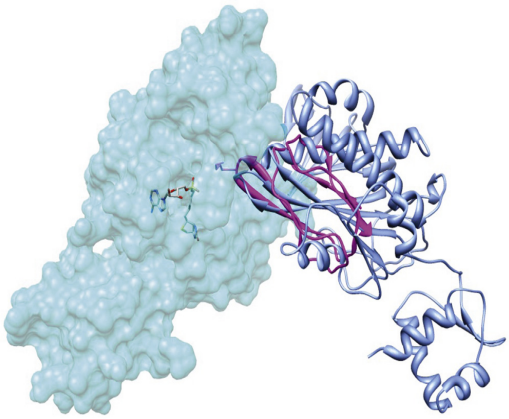


Fig. 4. Competing protein:protein interactions. The structure of holo *E. coli* BirA is shown with one subunit in space filled mode and the other in blue ribbon [PDB 2EWN[20]]. The BCCP substrate bound to BirA (pink ribbon) has been modeled using the BPL:BCCP complex from *P. horikoshii* [PDB 2EJG[32]] with UCSF Chimera software.[54]

interactions and the intracellular concentration of non-biotinylated BCCP.[46,53] Recent DNase I footprint studies performed in the presence of BCCP provide support for this model.[55]

In contrast, Solbiati and Cronan[56] proposed an alternative mechanism whereby biotinyl-5′-AMP is the key regulator of alternative protein:protein interactions. These authors argue that in order to compete with homodimerization, the BirA:BCCP complex must be strong and long lived in the cell. However, the enzyme-substrate interaction is believed to be transient as there has been no evidence to support a stable BirA:BCCP complex. Furthermore, these authors showed that a small 14 amino acid synthetic peptide was effective at de-repressing a *Ec*BirA regulated reporter construct *in vivo*.[56] Given the small size of this biotin-accepting substrate, extensive protein–protein interactions were not required to effectively disrupt *Ec*BirA binding to DNA. The authors propose that the regulatory switch between the two functions is, therefore, the removal of the biotinyl-5′-AMP co-repressor from the active site of *Ec*BirA rather than the competing protein-protein interactions.

A study conducted by Pendini and colleagues suggests that the level of the apo-BCCP is likely to be the key switch between the two mutually exclusive functions, at least in *S. aureus*.[46] Small-angle X-ray scattering data showed that in the absence of BCCP, *Sa*BPL formed a homodimer that was receptive to binding DNA. However, when apo-BCCP was included in the same reaction mixture, DNA binding activity was disrupted. These authors highlight that X-ray crystal structures demonstrated that upon the removal of biotin from the enzyme's catalytic site, the dimerization interface is destabilized by the conformational changes in the biotin-binding loop. Presumably this mechanism allows for the release of BCCP following biotinylation.

7. Regulation of biotin biosynthesis and biotin transport in organisms with class I BPL

In organisms containing Class I BPLs and no BirA homolog, such as *α-proteobacteria*, the transcriptional regulation of biotin biosynthesis has to be fulfilled by other proteins. A comparative genome study revealed co-localization of biotin biosynthetic genes with a recognition sequence for a GntR-type transcription factor called BioR in many *α-proteobacterial* genomes.[57] Additionally, a protein belonging to the TetR family of transcription factors, BioQ, has recently been identified as the key player in the regulation of biotin biosynthesis in *Mycobacterium smegmatis*[58] and biotin transport in *Corynebacterium glutamicum*.[59] The target genes regulated by BioQ and BioR are summarized in Fig. 5A. As these two proteins have only been identified recently, they have not yet been as extensively studied as BirA. For both BioR and BioQ, the repressor function of both proteins appears to be independent of biotin, and no ligands have yet been identified for these transcription factors.[58] A two-protein model involving BPL together with either BioQ or BioR has been proposed as a possible mechanism for biotin sensing and regulation in bacteria containing Class I BPLs (Fig. 5B).[58] This model assumes that there is cross talk between the biotin sensor (BPL) and the transcriptional regulator (BioQ/R). However, the molecular details supporting this model still require experimental validation.

8. BioR mediated gene expression

Bioinformatic analysis of genomic sequences from *α-proteobacteria* suggested that biotin synthesis in these bacteria is regulated by BioR.[57] It was also observed that the DNA recognition sequence for BioR was found upstream of the *bioY* gene biotin transporter in several other α-proteobacteria such as *M. loti*, *B. melitensis*, *Silicibacter* sp. TM1040 and *S. pomeroyi*. The BioR recognition sequence also co-localized with *bioR* genes in certain organisms such as *Mesorhizobium loti*, *Brucella melitensis*, *Bradyrizhobium japonicum*,

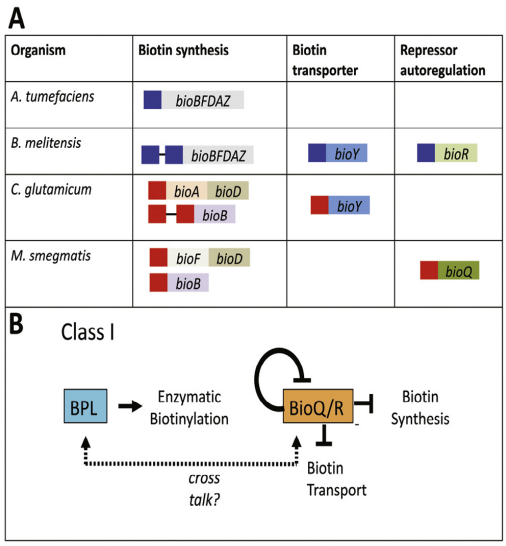


Fig. 5. Summary of BioR and BioQ transcriptional regulation. (A) The genes and metabolic pathways regulated by BioR (blue boxes) and BioQ (red boxes) are shown. Each box represents an individual operator. (B) Schematic overview showing the two-protein model of protein biotinylation and transcriptional regulation in Class I BPLs.

Silicibacter pmeroyi and *Rhodobacder sphaeroides*, suggesting autoregulation – a feature not observed with BirA/BPL. However this autoregulation is not completely conserved as the BioR recognition sequence was not present upstream of the *bioR* gene in *Agrobacterium tumefaciens*. Moreover, in *A. tumefaciens*, BioR does not control the expression of the BioY biotin transporter protein. Fig. 6 outlines the localization of BioR recognition sequence in different *α-proteobacteria*.

Feng and co-workers further investigated the role of BioR in regulating biotin synthesis by conducting a series of electromobility gel shift assays.[10] In addition to binding the recognition sequence in its own genome, BioR from *A. tumefaciens* was able to bind DNA probes with sequences derived from *B. japonicum*, *R. sphaeroides* and *B. melitensis*. In addition, the *B. melitensis* BioR was able to repress expression of *A. tumefaciens bioB in vivo*.[10] These data support the hypothesis that the BioR:operator interaction is well conserved in *α-proteobacteria*. It also revealed that the expression of the biotin operon (*bioBFDAZ*) in wildtype *A. tumefaciens* was 10–15 fold lower relative to a BioR knockout when the bacteria were grown in high (1 μM) biotin media. This observation further validates the role of BioR as a transcriptional repressor.[10]

In *B. melitensis*, the BioR recognition sequence is located upstream of the genes encoding both BioR and the BioY biotin transporter (Fig. 6). Two BioR sites are also present in the *bioBFDAZ* operon, indicating a complex regulatory network of biotin metabolism involving BioR.[10] Electromobility gel shift assays also confirmed that *B. melitensis* BioR was able to bind to a DNA probe containing the BioY promoter sequence, providing the first evidence that BioR can mediate expression of the biotin transporter in these bacteria.[10] In contrast, *A. tumefaciens* BioR does not regulate biotin transport and only binds weakly to the recognition site present within the coding region of *bioB*, *in vivo*.[10] As a consequence, the amount of biotin produced by *A. tumefaciens* is greater than their minimum growth requirement, which presumably is beneficial for survival in their environment.[10]

A search for a ligand and co-repressor of BioR concluded that biotin is not the natural ligand.[10] Complete removal of biotin from the protein preparations had no effect upon DNA-binding *in vitro*. Conversely, the hypothesis that biotin can serve as a dissociation factor that disrupts DNA-BioR complex was also tested. Again, the

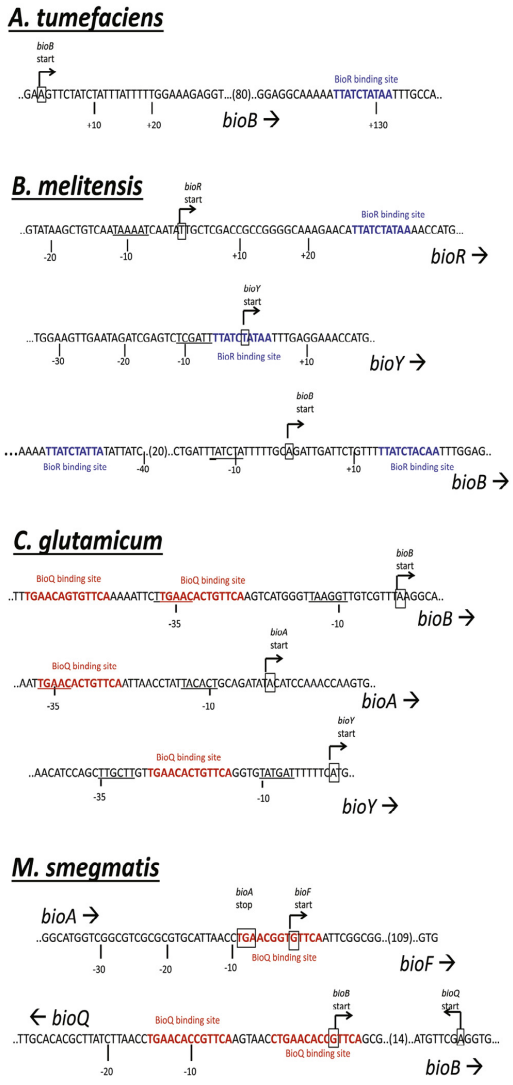


Fig. 6. BioR and BioQ. Sequences of the binding sites for BioR and BioQ are shown. Transcription start sites are boxed, and –35 and –10 sequences are underlined. Sequences of the binding sites for BioR (blue) and BioQ (red) are colored.

addition or removal of biotin had no effect on DNA-binding activity. Various biotin metabolites were also tested and none showed evidence of being a ligand for BioR. Hence, further studies are required to define the links between biotin sensing by BPL and downstream control by BioR in the two protein model of biotin-regulated gene expression.

9. BioQ mediated gene expression

In *Corynebacterium glutamicum*, the biotin synthesis pathway is incomplete, thereby rendering the bacteria biotin auxotrophic. However, the expression of the *bio* genes is proposed to be controlled by a transcription factor belonging to the TetR protein family, namely BioQ. Although the TetR family of regulators has been well characterized, none have previously been shown to regulate biotin synthesis.[60] Bioinformatic analysis of the *C. glutamicum* genome revealed co-localization of the *bioQ* coding region with the biotin biosynthetic genes (Fig. 6).[59] The presence of the BioQ recognition sequence in the promoter of the *bioQ* gene suggests auto-regulation of the transcription factor (Fig. 6). In addition, the same regulatory sequence is also found upstream of the *bioY* biotin transporter, suggesting BioQ regulates its expression. This is crucial for biotin auxotrophic *C. glutamicum* that relies on supply of the micronutrient from the external environment. In addition, the study showed that increasing the biotin concentration in the growth media only had modest repression on the transcription of the biotin operon in this organism.[59]

In contrast to *C. glutamicum*, BioQ is believed to regulate expression of the transporter genes in *Mycobacterium smegmatis*. The recognition sites are also localized upstream of the *bioF* and *bioQ/B* genes suggesting regulation of biotin biosynthesis and autoregulation by BioQ in this species (Fig. 6).[58] Tang et al. also showed that BioQ binding sites are also found in other mycobacterium species such as *M. abcessus*, *M. gilvum*, *M.JLS*, *M. massiliense*, *M. rhodesiae* and *M. vanbaalenii*, but not in the clinically important human pathogen *M. tuberculosis*.[58] The same study also confirmed the role of BioQ as a functional repressor. Increased mRNA levels of *bioF*, *bioB* and *bioD* were measured in a ΔbioQ mutant strain of *M. smegmatis* compared to the wildtype parent. LacZ-based reporter assays using the *bioFD* promoter also showed expression was highly increased in ΔbioQ strain whereas no LacZ activity was observed in the wildtype strain.[58] In addition, increasing levels of biotin in the growth media resulted in decreasing expression of biotin biosynthesis genes *bioF*, *bioD* and *bioB* for the wildtype bacteria whereas there was no significant change in the Δ*bioQ* strain. These findings underline the biotin sensing ability of BioQ.

10. Potential biotechnological applications

Synthetic biology facilitates us to better understand life through the dissection then reconstruction of complex biological systems.[61] At the heart of this endeavor are engineered genetic circuits that allow us to dissect the interplay between genes, proteins, cells and systems. Synthetic biology is currently used to deliver valuable bioproducts and therapeutic molecules, such as fine chemicals, peptides, proteins and antibodies. For example, most monoclonal antibodies are produced recombinantly using genetically engineered Chinese hamster ovary cells as bio-factories.[62] Many of the reagents that have been developed by industry are becoming valuable tools in academia. Underpinning synthetic biology are well-characterized transcriptional regulators required to engineer the genetic circuits and tightly control bio-production. The TetR inducible expression system is an example that is widely employed due to its high specificity toward its recognition system and the high affinity to tetracycline, a well characterized antibiotic.[63] There is now a need fill our discovery toolbox with a greater variety of well-characterized transcriptional regulators with utility in systems biology. In this review, the mechanisms of BirA, BioQ and BioR in regulating their target genes are discussed. The high specificity of BirA to its target operator, as well its ability to regulate gene expression in response to external biotin, provides an attractive approach to developing novel ligand-regulated gene expression systems for use in bacteria, yeast plants and animal systems. To date this approach has not yet been exploited. Biotin provides a highly attractive ligand to regulate transcription due to its low cost, solubility in aqueous solutions, low toxicity to many cell types and has no regulatory issues. While the well-studied Class II BirA enzyme/repressor from *E. coli* provides one useful example for generating a controllable genetic switch, the emergence of other biotin responsive transcription factors extends our repertoire of potential systems. Other Class II BPLs may be more responsive to environmental biotin levels, such as that from *S. aureus*. The BioR and BioQ proteins provide alternative repressors with distinctive mechanisms of action to BirA/BPL. These may potentially be advantageous when generating new genetic circuits that are highly responsive to

external stimuli, such as the addition of a vitamin. Further research on biotin regulated transcriptional factors promise to replenish our toolkit with greater variety of new agents for systems biology.

Acknowledgements

This work was supported by the National Health and Medical Research Council of Australia (application APP1068885) and the Centre for Molecular Pathology, University of Adelaide. We are grateful to the Carthew and Wallace families for their financial support of this work.

References

1. Polyak SW, Chapman-Smith A. Biotin. In: Lennarz WJ, Lane MD, editors. Encyclopeaedia of biological chemistry. San Diego (CA): Academic Press; 2013. p. 221–5.
2. Cronan JE Jr, Waldrop GL. Multi-subunit acetyl-CoA carboxylases. *Prog Lipid Res* 2002;**41**:407–35.
3. Polyak SW, Abell AD, Wilce MC, Zhang L, Booker GW. Structure, function and selective inhibition of bacterial acetyl-coa carboxylase. *Appl Microbiol Biotechnol* 2012;**93**:983–92.
4. Jitrapakdee S, St Maurice M, Rayment I, Cleland WW, Wallace JC, Attwood PV. Structure, mechanism and regulation of pyruvate carboxylase. *Biochem J* 2008;**413**:369–87.
5. Deodato F, Boenzi S, Santorelli FM, Dionisi-Vici C. Methylmalonic and propionic aciduria. *Am J Med Genet C Semin Med Genet* 2006;**142C**:104–12.
6. Polyak SW, Bailey LM, Azhar A, Booker GW. Biotin (Vitamin H or B7). In: Betancourt AI, Gaitan HF, editors. Micronutrients: sources, properties and health benefits. New York: Nova Publishers; 2012. p. 65–93.
7. Salaemae W, Azhar A, Booker GW, Polyak SW. Biotin biosynthesis in Mycobacterium tuberculosis: physiology, biochemistry and molecular intervention. *Protein Cell* 2011;**2**:691–5.
8. Lin S, Cronan JE. Closing in on complete pathways of biotin biosynthesis. *Mol Biosyst* 2011;**7**:1811–21.
9. Rodionov DA, Mironov AA, Gelfand MS. Conservation of the biotin regulon and the BirA regulatory signal in Eubacteria and Archaea. *Genome Res* 2002;**12**:1507–16.
10. Feng Y, Zhang H, Cronan JE. Profligate biotin synthesis in alpha-proteobacteria – a developing or degenerating regulatory system? *Mol Microbiol* 2013;**88**:77–92.
11. Chapman-Smith A, Morris TW, Wallace JC, Cronan JE Jr. Molecular recognition in a post-translational modification of exceptional specificity. Mutants of the biotinylated domain of acetyl-CoA carboxylase defective in recognition by biotin protein ligase. *J Biol Chem* 1999;**274**:1449–57.
12. Chapman-Smith A, Cronan JE Jr. The enzymatic biotinylation of proteins: a post-translational modification of exceptional specificity. *Trends Biochem Sci* 1999;**24**:359–63.
13. Cronan JE Jr, Wallace JC. The gene encoding the biotin-apoprotein ligase of Saccharomyces cerevisiae. *FEMS Microbiol Lett* 1995;**130**:221–9.
14. Leon-Del-Rio A, Leclerc D, Akerman B, Wakamatsu N, Gravel RA. Isolation of a cDNA encoding human holocarboxylase synthetase by functional complementation of a biotin auxotroph of Escherichia coli. *Proc Natl Acad Sci U S A* 1995;**92**:4626–30.
15. Polyak SW, Chapman-Smith A, Mulhern TD, Cronan JE Jr, Wallace JC. Mutational analysis of protein substrate presentation in the post-translational attachment of biotin to biotin domains. *J Biol Chem* 2001;**276**:3037–45.
16. Pendini NR, Bailey LM, Booker GW, Wilce MC, Wallace JC, Polyak SW. Microbial biotin protein ligases aid in understanding holocarboxylase synthetase deficiency. *Biochim Biophys Acta* 2008;**1784**:973–82.
17. Mayende L, Swift RD, Bailey LM, Soares da Costa TP, Wallace JC, Booker GW, et al. A novel molecular mechanism to explain biotin-unresponsive holocarboxylase synthetase deficiency. *J Mol Med* 2012;**90**:81–8.
18. Campeau E, Gravel RA. Expression in Escherichia coli of N- and C-terminally deleted human holocarboxylase synthetase. Influence of the N-terminus on biotinylation and identification of a minimum functional protein. *J Biol Chem* 2001;**276**:12310–6.
19. Duckworth BP, Geders TW, Tiwari D, Boshoff HI, Sibbald PA, Barry CE 3rd, et al. Bisubstrate adenylation inhibitors of biotin protein ligase from Mycobacterium tuberculosis. *Chem Biol* 2011;**18**:1432–41.
20. A. Wood Z, Weaver LH, Brown PH, Beckett D, Matthews BW. Co-repressor induced order and biotin repressor dimerization: a case for divergent followed by convergent evolution. *J Mol Biol* 2006;**357**:509–23.
21. Bagautdinov B, Kuroishi C, Sugahara M, Kunishima N. Crystal structures of biotin protein ligase from Pyrococcus horikoshii OT3 and its complexes: structural basis of biotin activation. *J Mol Biol* 2005;**353**:322–33.
22. Beckett D. Biotin sensing at the molecular level. *J Nutr* 2009;**139**:167–70.
23. Beckett D. Regulating transcription regulators via allostery and flexibility. *Proc Natl Acad Sci U S A* 2009;**106**:22035–6.
24. Chapman-Smith A, Turner DL, Cronan JE Jr, Morris TW, Wallace JC. Expression, biotinylation and purification of a biotin-domain peptide from the biotin carboxy carrier protein of Escherichia coli acetyl-CoA carboxylase. *Biochem J* 1994;**302**(Pt 3):881–7.
25. Forsyth RA, Haselbeck RJ, Ohlsen KL, Yamamoto RT, Xu H, Trawick JD, et al. A genome-wide strategy for the identification of essential genes in Staphylococcus aureus. *Mol Microbiol* 2002;**43**:1387–400.
26. Payne DJ, Gwynn MN, Holmes DJ, Pompliano DL. Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nat Rev Drug Discov* 2007;**6**:29–40.
27. Paparella AS, Soares da Costa TP, Yap MY, Tieu W, Wilce MC, Booker GW, et al. Structure guided design of biotin protein ligase inhibitors for antibiotic discovery. *Curr Top Med Chem* 2014;**14**:4–20.
28. Soares da Costa TP, Tieu W, Yap MY, Pendini NR, Polyak SW, Sejer Pedersen D, et al. Selective inhibition of biotin protein ligase from Staphylococcus aureus. *J Biol Chem* 2012;**287**:17823–32.
29. Tieu W, Soares da Costa TP, Yap MY, Keeling KL, Wilce MCJ, Wallace JC, et al. Optimising in situ click chemistry: the screening and identification of biotin protein ligase inhibitors. *Chem Sci* 2013;**4**:3533–7.
30. Feng Y, Chin CY, Chakravartty V, Gao R, Crispell EK, Weiss DS, et al. The atypical occurrence of two biotin protein ligases in Francisella novicida is due to distinct roles in virulence and biotin metabolism. *MBio* 2015;**6**.
31. Wilson KP, Shewchuk LM, Brennan RG, Otsuka AJ, Matthews BW. Escherichia coli biotin holoenzyme synthetase/bio repressor crystal structure delineates the biotin- and DNA-binding domains. *Proc Natl Acad Sci U S A* 1992;**89**:9257–61.
32. Bagautdinov B, Matsuura Y, Bagautdinova S, Kunishima N. Protein biotinylation visualized by a complex structure of biotin protein ligase with a substrate. *J Biol Chem* 2008;**283**:14739–50.
33. Eisenstein E, Beckett D. Dimerization of the Escherichia coli biotin repressor: corepressor function in protein assembly. *Biochemistry* 1999;**38**:13077–84.
34. Xu Y, Beckett D. Evidence for interdomain interaction in the Escherichia coli repressor of biotin biosynthesis from studies of an N-terminal domain deletion mutant. *Biochemistry* 1996;**35**:1783–92.
35. Chakravartty V, Cronan JE. The wing of a winged helix-turn-helix transcription factor organizes the active site of BirA, a bifunctional repressor/ligase. *J Biol Chem* 2013;**288**:36029–39.
36. Henke SK, Cronan JE. Successful conversion of the Bacillus subtilis BirA Group II biotin protein ligase into a Group I ligase. *PLoS ONE* 2014;**9**:e96757.
37. Abbott J, Beckett D. Cooperative binding of the Escherichia coli repressor of biotin biosynthesis to the biotin operator sequence. *Biochemistry* 1993;**32**:9649–56.
38. Streaker ED, Beckett D. Coupling of site-specific DNA binding to protein dimerization in assembly of the biotin repressor-biotin operator complex. *Biochemistry* 1998;**37**:3210–9.
39. Streaker ED, Gupta A, Beckett D. The biotin repressor: thermodynamic coupling of corepressor binding, protein assembly, and sequence-specific DNA binding. *Biochemistry* 2002;**41**:14263–71.
40. Chakravartty V, Cronan JE. Altered regulation of Escherichia coli biotin biosynthesis in BirA superrepressor mutant strains. *J Bacteriol* 2012;**194**:1113–26.
41. Kwon K, Streaker ED, Ruparelia S, Beckett D. Multiple disordered loops function in corepressor-induced dimerization of the biotin repressor. *J Mol Biol* 2000;**304**:821–33.
42. Taniguchi Y, Choi PJ, Li GW, Chen H, Babu M, Hearn J, et al. Quantifying E. coli proteome and transcriptome with single-molecule sensitivity in single cells. *Science* 2010;**329**:533–8.
43. Zhao H, Beckett D. Kinetic partitioning between alternative protein-protein interactions controls a transcriptional switch. *J Mol Biol* 2008;**380**:223–36.
44. Zhao H, Naganathan S, Beckett D. Thermodynamic and structural investigation of bispecificity in protein-protein interactions. *J Mol Biol* 2009;**389**:336–48.
45. Kwon K, Beckett D. Function of a conserved sequence motif in biotin holoenzyme synthetases. *Protein Sci* 2000;**9**:1530–9.
46. Pendini NR, Yap MY, Polyak SW, Cowieson NP, Abell A, Booker GW, et al. Structural characterization of Staphylococcus aureus biotin protein ligase and interaction partners: an antibiotic target. *Protein Sci* 2013;**22**:762–73.
47. Streaker ED, Beckett D. A map of the biotin repressor-biotin operator interface: binding of a winged helix-turn-helix protein dimer to a forty base-pair site. *J Mol Biol* 1998;**278**:787–800.
48. Buoncristiani MR, Howard PK, Otsuka AJ. DNA-binding and enzymatic domains of the bifunctional biotin operon repressor (BirA) of Escherichia coli. *Gene* 1986;**44**:255–61.
49. Weaver LH, Kwon K, Beckett D, Matthews BW. Corepressor-induced organization and assembly of the biotin repressor: a model for allosteric activation of a transcriptional regulator. *Proc Natl Acad Sci U S A* 2001;**98**:6045–50.
50. Eginton C, Naganathan S, Beckett D. Sequence-function relationships in folding upon binding. *Protein Sci* 2015;**24**:200–11.
51. Eginton C, Cressman WJ, Bachas S, Wade H, Beckett D. Allosteric coupling via distant disorder-to-order transitions. *J Mol Biol* 2015;**427**:1695–704.
52. Soares da Costa TP, Yap MY, Perugini MA, Wallace JC, Abell AD, Wilce MC, et al. Dual roles of F123 in protein homodimerization and inhibitor binding to biotin protein ligase from Staphylococcus aureus. *Mol Microbiol* 2014;**91**:110–20.
53. Weaver LH, Kwon K, Beckett D, Matthews BW. Competing protein:protein interactions are proposed to control the biological switch of the E coli biotin repressor. *Protein Sci* 2001;**10**:2618–22.
54. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. UCSF Chimera – a visualization system for exploratory research and analysis. *J Comput Chem* 2004;**25**:1605–12.
55. Adikaram PR, Beckett D. Protein:protein interactions in control of a transcriptional switch. *J Mol Biol* 2013;**425**:4584–94.

56. Solbiati J, Cronan JE. The switch regulating transcription of the Escherichia coli biotin operon does not require extensive protein-protein interactions. *Chem Biol* 2010;**17**:11–7.
57. Rodionov DA, Gelfand MS. Computational identification of BioR, a transcriptional regulator of biotin metabolism in Alphaproteobacteria, and of its binding signal. *FEMS Microbiol Lett* 2006;**255**:102–7.
58. Tang Q, Li X, Zou T, Zhang H, Wang Y, Gao R, et al. Mycobacterium smegmatis BioQ defines a new regulatory network for biotin metabolism. *Mol Microbiol* 2014;doi:10.1111/mmi.12817.
59. Brune I, Gotker S, Schneider J, Rodionov DA, Tauch A. Negative transcriptional control of biotin metabolism genes by the TetR-type regulator BioQ in biotin-auxotrophic Corynebacterium glutamicum ATCC 13032. *J Biotechnol* 2012;**159**:225–34.
60. Ramos JL, Martinez-Bueno M, Molina-Henares AJ, Teran W, Watanabe K, Zhang X, et al. The TetR family of transcriptional repressors. *Microbiol Mol Biol Rev* 2005;**69**:326–56.
61. Dobrin A, Saxena P, Fussenegger M. Synthetic biology: applying biological circuits beyond novel therapies. *Integr Biol (Camb)* 2016;doi:10.1039/C5IB00263J.
62. Brown AJ, James DC. Precision control of recombinant gene transcription for CHO cell synthetic biology. *Biotech Adv* 2015;doi:10.1016/j.biotechadv.2015.12.012.
63. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A* 1992;**89**:5547–51.

Chapter 2:

General materials and methods

2.1 Materials

2.1.1 Chemical reagents

All chemicals and reagents were purchased as analytical grade or higher.

Table 2.1: general reagents

Reagents	**Supplier**	**Catalogue number**
2log DNA ladder	New England Biolabs, MA, USA	B7025
Bradford protein reagent concentrate	Bio-rad Laboratories Inc., CA, USA	500205
T4 DNA ligase	New England Biolabs, MA, USA	M0202
Phusion®DNA polymerase	New England Biolabs, MA, USA	M0530S
deoxynucleotide (dNTP) mix	New England Biolabs, MA, USA	N0447
Phenylmethanesulfonyl fluoride (PMSF)	Sigma-Aldrich, MO, USA	329-98-6
Dithiorheitol (DTT)	Sigma-Aldrich, MO, USA	3483-12-4
Isopropyl-β-D-1-thiogalactopyranoside (IPTG)	Bio vectra, PE, USA	1882
5-Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside (X-Gal)	Bio vectra, PE, USA	1161
2-Nitrophenyl β-D-galactopyranoside	Sigma-Aldrich, MO, USA	369-07-3
Polymyxin B sulfate salt	Sigma-Aldrich, MO, USA	1405-20-5
MES SDS running buffer	Life technologies Inc, NY, USA	NP0002
Precision plus™Kaleidoscope™ prestained protein marker	Bio-rad Laboratories Inc., CA, USA	1610375
Gel red™ Nucleic acid gel stain	Bioitum Inc, CA, USA	7857
Big Dye terminator mix	Perkin Elmer, CA, USA	4303152
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, MO, USA	60-00-04
RNAprotect Bacteria reagent	Qiagen, GmbH, Germany	76506
DNAseI (RNAse free)	Life technology, Carlsbad, USA	AM2222
RNAse free water	Life technology, Carlsbad, USA	10977-015(500mL)
Lysostaphin	Sigma-Aldrich, MO, USA	7386-1mL

2.1.2 General materials

Table 2.2: general materials

Materials	Supplier	Catalogue number
amicon® centrifugal filter devices	Millipore, MA, USA	UFC900308
Ministart syringe filter 0.2uM	Sartorius, Goettingen, Germany	886-10276
Ministart syringe filter 0.4uM	Sartorius, Goettingen, Germany	886-10282
Ministart syringe filter 0.8uM	Sartorius, Goettingen, Germany	886-10291
Hybond P PVDF membrane	GE healthcare, Buckinghamshire, England	RPN303F
CEllusepT1 MWCO 3500 dialysis tube	Adelab scientific, Australia	5015-55
Nupage®4-12% Bis-Tris polyacrylamide gels	Life technologies Inc, NY, USA	NP0323BOX
Novex®4-20% TBE polyacrylamide gels	Life technologies Inc, NY, USA	EC62252BOX
Streptavidin sepharose™ High performance beads	GE healthcare, Uppsala, Sweden	71-5004-40

2.1.3 Antibodies

Antibodies were reconstituted and stored according to manufacturer's instructions. Working dilutions were made according to manufacturer's recommendations.

Table 2.3: List of antibodies

Antibodies	Supplier	Catalogue number
anti-His tag polyclonal antibody	Cell Signaling Technology, Danvers, MA, USA	2365S
anti-BirA monoclonal antibody	ABCAM, Cambridge, MA, USA	106159
Cy™5 conjugated Donkey anti rabbit IgG	Jackson Immuno research, PA, USA	711-175-152
Donkey anti chicken IgY-CY5 secondary antibody	Jackson Immuno research, PA, USA	703-175-155

2.1.4 Commercial kits

Table 2.4: Commercial kits

Kit	Supplier	Catalogue number
Qiaprep plasmid mini kit	QIAGEN, GmbH, Germany	12123
Qiaquick gel extraction kit	QIAGEN, GmbH, Germany	28704
Qiaquick PCR purification kit	QIAGEN, GmbH, Germany	28104
Rneasy mini kit	QIAGEN, GmbH, Germany	74104
Wizard™ genomic purification kit	Promega, Madison, WI, USA	A1120
Superscript II platinum sybr RT-PCR kit	Life technology, NY, USA	11736-051-100

2.1.5 General sequencing primers

Oligonucleotides were purchased from Geneworks Pty Ltd., Hindmarsh, South Australia. All primers used were of sequencing grade.

Table 2.5: General primers

Primer name	Sequence (5'-3')
M13 Forward	GTAAAACGACGGCCAGT
M13 Reversed	CAGGAAACAGCTATGAC
T7 Forward	CGAAATTAATACGACTCACTATAGGG
HK022-P1	GGAATCAATGCCTGAGTG
HK022-P2	ACTTAACGGCTGACATGG
HK022-P3	ACGAGTATCGAGATGGCA
HK022-P4	GGCATCAACAGCACATTC
Lambda-P1	GGCATCACGGC AATATAC
Lambda-P2	ACTTAACGGCTGACATGG
Lambda-P3	GGGAATTAATTCTTGAAGACG
Lambda-P4	TCTGGTCTGGTAGCAATG

2.1.6 Bacterial strains

For general cloning:

***E. coli* DH5α:** *sup*E44Δ*lac*U169(p80*lac*ZΔM15) *hsd*R17 *rec*A1 *end*AA1 *gyr*A96 *thi*-1 *rel*A1 (New England, Biolabs, CA, USA).

For protein expression:

***E. coli* BL21(DE3):** *fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5* (Stratagene, La Jolla, CA, USA).

2.1.7 Bacterial media

Luria Broth (LB): 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% NaCL, adjusted to pH 7.0 with 5M NaOH.

Mueller-Hinton media (Difco): 0.2% (w/v) beef extract, 1.75% (w/v) acid hydrolysate of casein, 0.15% (w/v) starch.

LB agar: LB supplemented with 1.5% (w/v) bacto-agar.

Super Optimal Media (SOC media): 2% (w/v) tryptone, 0.5% (w/v) yeast extact, 10mM NaCL, 2.5mM KCL, 10mM $MgCl_2$, 10mM MgSO4, 20mM Glucose.

The following antibiotics were added to the liquid or solid media as required:

Table 2.6: List of antibiotic and concentration

Antibiotic	**Concentration (μg/mL)**
Ampicillin	100
Chloramphenicol	50
Spectinomycin	30
Tetracycline	4

2.1.8 Plasmids

pET16b (Novagen): 5711 bp bacterial expression vector, used to generate fusions of a known protein with N-terminal- 6xHis-tag.

pGEM®-T vector (Addgene): 3000 bp cloning vector, used to clone particular gene or construct required. This vector had been modified previously in our laboratory; modifications include the presence of 6xHis-tag and additional restriction sites at its multiple cloning sites.

2.1.9 Buffers and solutions

Blocking solution (for western blotting): 1% non-fat dairy milk (w/v) in PBS containing 0.01% (v/v) Tween20.

SDS-PAGE Coomassie Brilliant Blue staining solution: 0.2% (w/v) Coomassie brilliant blue, 10% (v/v) Methanol, 10% (v/v) Acetic acid.

SDS-PAGE distaining solution: 10% (v/v) Methanol, 10% (v/v) Acetic acid.

Protein loading buffer (2X): 100mM Tris-HCL pH 6.8, 4% (v/v) SDS, 20% (v/v) glycerol, 25% (v/v) β mercaptoethanol, 0.05% (v/v) bromophenol blue.

Loading buffer (for DNA) 6x: 0.5xTBE, 40% (v/v) glycerol, 1mg/mL bromophenol blue.

Loading buffer (for protein) 2x: 100mM Tris-HCl pH6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% β –mercaptoethanol, 0.05% (w/v) Bromophenol blue.

PBS: 0.137 M NaCL, 2.7mM KCL, 1.46mM KH_2PO_4, 8.1mM Na_2HPO_4 (pH 7.4).

PBS-Tween: PBS, 0.01% (v/v) Tween20.

SDS-PAGE sample buffer (for proteins) 5X: 0.25 M Tris (pH 6.8), 10% (w/v) SDS, 0.5% (w/v) bromophenol blue, 50% (w/v) glycerol.

TBE: 216 g Trizma base, 100 g boric acid, 18.6 g EDTA to 1 L with MilliQ water.

TAE buffer: 40mM Tris (pH 7.6), 20mM Acetic acid, 1mM EDTA.

TBS: 25mM Tris pH 7.5, 150mM NaCL.

TE: 10mM Tris pH 7.5, 1mM EDTA.

10X annealing buffer: 200mM Tris pH 7.5, 100mM $MgCL_2$, 50mM NaCL.

western transfer buffer: 20mM Tris-HCL pH 7.5, 1.15 M Glycine, 20% Methanol, 0.1% (w/v) SDS.

Storage buffer (for purified soluble proteins) 1x: 25mM Tris buffer (pH 7.5), 30mM NaCL, 1mM EDTA, 1mM dithiothreitol (DTT) and 5% (v/v) glycerol.

Bacteria lysis buffer: 2%SDS, 10%(v/v) Beta- mercaptoethanol.

2.10 Computer software

Data were analyzed using Graphpad Prism 6 (Graphpad Software, Inc., CA, USA). ApE-A plasmid Editor version 1.17 (by M. Wayne Davis) and SnapGene viewer (GSL Biotech, www.snapgene.com) was used to generate constructs and plasmid maps as well as sequencing chromatogram viewings. USCF Chimera version 1.8.1 (UCSF, CA, USA) was used for viewing and analysis of PDB files. Imaging of agarose gel and western blotting membrane were done using Chemidoc™ -MP system imager and analysed using Image lab software (Bio-rad laboratory, California, USA)

2.11 Web resources

Sequence alignments were done using Clustal Omega (EMBL-EBI, www.ebi.ac.uk/Tools/msa/**clustal**o/). NCBI (http://www.ncbi.nlm.nih.gov/) was used to access protein and nucleotide reference sequences as well as Pubmed databases and Basic Local Alignment Tool (BLAST). Protein translation and ORF prediction was obtained from Https://www.expasy.org. Softberry-BProm bacteria promoter prediction tool : (http://www.softberry.com/cgi-bin/programs/gfindb/bprom.pl). USCF Chimera version 1.8.1 (UCSF, CA, USA) was used for viewing and analysis of protein structures and PDB files.

2.2 General methods

2.2.1 Plasmid DNA extraction

For purification of small (<10 μg) amounts of plasmid DNA, Pellet from 5mL of overnight bacterial culture harboring the desired plasmid was used. QIAGEN QIAprep Miniprep kit was employed following the manufacturer's instructions, with the exception of elution volume. The elution volume used for each purification was 35μL to yield higher concentration of DNA. The resulting purified DNA was quantified by measuring absorbance at 260 nm using NANODrop 2000 Spectrophotometer (ThermoFisher Scientific Pty Ltd, Australia).

2.2.2 Genomic DNA extraction

Two genomic DNA methods were employed, for colony screening, the genomic DNA was extraction by boiling method. For PCR template and sequencing, Promega Wizard™ Genomic DNA extraction kit was used.

2.2.2.1 Genomic DNA extraction by boiling

Bacterial pellet from 1mL of overnight *E. coli* culture was used. The pellet was then resuspend in 100μL TE Buffer in 1. 5mL Eppendorf tube and incubated in a heating block at temperature set to 100°C for 5 minutes and immediately cooled on ice for 10 minutes. The lysed mixture was then centrifuged at 17500 x g for 5 min at 4°C. 0.5μL of this lysate was then used in PCR screen, in a total PCR reaction volume of 20μL.

2.2.2.2 Genomic DNA extraction by Wizard ™ kit (Promega)

Genomic DNA extraction was performed according to the manufacturer's instructions, with the exception of the overnight culture volume. The overnight cultures used for extraction was 5mL instead of 1mL as recommended by manufacturer. The resulting purified DNA was quantified by measuring absorbance at 260nm using NANODrop 2000 Spectrophotometer (ThermoFisher Scientific Pty Ltd, Australia).

2.2.3 Total RNA extraction

2x volumes of RNAprotect bacteria reagent (Qiagen) was used to mix with 1x volume of bacteria resuspension and incubated at room temperature for 5 minutes, followed by centrifugation at 17500 x g for 5 minutes. The supernatant was then discarded and the pellet was resuspended in 100µl TE buffer containing 1µl of 0.1 mg/mL Lysostaphin and 1mg/mL Lysozyme. The suspension was then incubated at 37°C for 15 minutes with frequent mixing by inversion, every 5 minutes. 350µl of RLT buffer solution from RNeasy RNA extraction mini kit containing 1% Beta-mercaptoethanol was then added to the lysed solution and vortexed for 20 seconds. 200µL of 100% ethanol solution was added tubes were inverted 5 times. The lysate was then transferred to mini spin column from the RNeasy mini kit and centrifuged at full speed for 15 seconds. The column was then washed with 700µl of RW1 buffer followed by 2x500µl washes with RPE buffer, as per manufacturer instruction. The purified RNA was then eluted in 40µl of RNase free water and quantified using NANODrop 2000 Spectrophotometer (ThermoFisher Scientific Pty Ltd, Australia).

2.2.4 PCR amplification using Phusion® DNA polymerase (NEB)

For isolation of DNA template to be used in cloning, 0.5µl Phusion® DNA polymerase was mixed with 0.5µl of forward primer (100ng/µL), 0.5µl reversed primer (100ng/µL), 10µl of 5x HF buffer (NEB), 1µl of 10mM dNTP mix and 200ng of template DNA, in a total reaction volume of 50µl.

The following PCR program was then used to amplify the target:

Step 1: 98°C, 0:30

Step 2: 98°C, 0:10
55°C, 0:30
72°C, 1:00 (0.5Kb / min extension)] Repeat cycle 28x

Step 3: 72°C, 5:00

Step 4: hold at 4°C

The reaction was carried out on a MJ Research PTC 2000 Thermal Cycler (GMI Inc., MN, USA) The resulting PCR product was then purified using Qiaquick PCR purification kit (Qiagen).

2.2.5 Agarose gel electrophoresis

Analysis of DNA and separation of DNA fragments was performed using agarose gel electrophoresis. Gel slabs were prepared by melting 1% - 2%(w/v) agarose (depending on the size of fragment to be purified) in 1x TAE buffer. Prior to loading into wells, DNA samples were mixed with an appropriate volume of 6x DNA loading buffer. Samples were electrophoresed in 1X TAE buffer at 100 - 150 V and then stained in 1x GelRed™ Nucleic acid gel stain (Biotium Inc., CA, USA) for 10 minutes followed by briefly rinsing in distilled water to eliminate excess stain. DNA was visualized on a Chemidoc-MP system imager (Bio-rad laboratories,

California, USA).

2.2.6 Purification of PCR product

20µL -50µL of PCR reaction was added to 5X PB buffer provided in the Qiaquick PCR Clean up kit (Qiagen) and transferred into the purification column followed by centrifugation at 17500 x g as per manufacturer's instruction. The column was then washed with 650µL of PE Buffer and centrifuged for 1 minute to remove excess ethanol, at 17500 x g. The purified PCR product was then eluted in 35µL elution buffer.

2.2.7 DNA extraction from agarose gel

DNA was excised from the gel using a clean blade and weighed in 1.5 mL Eppendorf tube. The remaining procedures were performed according to the manufacturer's instructions.

2.2.8 Restriction digest of DNA

1-5µg of DNA was digested with 10 units of restriction enzyme in the appropriate NEB buffer for 2 hours at 37 °C. For cloning, DNA fragments were separated by agarose gel electrophoresis before purification form the excised gel slice using a QIAquick Gel Extraction kit.

2.2.9 Ligation of DNA fragments

Ligation reactions were carried out in a 20µl reaction volume, using 50ng of vector and an insert:vector molar ratio of 3:1 in 1x ligase buffer and 2 units of T4 DNA ligase overnight at 4°C .

2.2.10 Site-directed mutagenesis

The QuickChangeTM mutagenesis kit (Stratagene) was employed for site-directed mutagenesis according to the manufacturer's recommendation. Complementary strands of synthetic oligonucleotide primers containing the desired mutation were extended by the activity of Phusion® DNA polymerase during thermo cycling. The 50µL reaction mixture was comprised of 1x reaction buffer, 50ng DNA template, 1mM dNTPs, 2.5ng/µl each primer and 5 units of Pfu DNA polymerase. The reaction was carried out on a MJ Research PTC 2000 Thermal Cycler (GMI Inc., MN, USA) with the initial denaturation (94 °C) for 5 min followed by 16-20 cycles of denaturation (94 °C) for 1 min, annealing (50-60 °C) for 1 min, and extension (68 °C) for 9 min. Following the amplification, products were then treated with *D*pnI (target sequence: 5'-G^{m6}ATC-3') to digest the methylated parental DNA template. The digested vectors incorporating the desired mutation were then transformed into *E. coli* strain DH5α cells for amplification.

2.2.11 Heat shock bacteria transformation

2.2.11a Chemically competent cells preparation:

E. coli cells were grown in the appropriate media with antibiotic selection overnight and sub-cultured into 1L of media the next day, with starting OD_{600} = 0.05. Once the cells have reached mid-log phase (OD_{600} = 0.4-0.6) cells were cooled on ice for 30 minutes followed by centrifugation at 3200 x g , 4°C for 5 minutes. Cell pellet was then resuspended in 10 mL of transformation buffer I (15% glycerol (v/v), 10mM Potassium Acetate (CH_3CO_2K), 100mM RbCL, 10mM $CaCL_2$, 50mM $MnCL_2$) and cooled on ice for 10 minutes. The cells were then centrifuged at 3200 x g, 4°C for 5 minutes. The cell pellet was then resuspended in 1mL of

transformation buffer II (10mM MOPS, 10mM RbCL, 15% (v/v) glycerol, 75mM $CaCL_2$) and aliquoted into 10x 100µL before storage at -80°C.

2.2.11b Heat shock method:

An aliquot of 50µL *E. coli* competent cells was gently mixed with 50ng of DNA (or 10µL of ligation mixture). Cells were placed on ice for 30 min, incubated at 42 °C for 90 sec then placed on ice for an additional 5 min. The transformed cells were mixed with 450µL of SOC media, incubated at 37 °C for 1 hour with rotation and then centrifuged at 3200 x g for 1 min. The pellet was resuspend in 250µL SOC media and spread on LB agar plates containing the appropriate antibiotic selection and incubated overnight at 37 °C.

2.2.12 Bacteria transformation by electroporation.

Electro-competent cells preparation:

E. coli cells were grown in the appropriate media with antibiotic selection overnight and sub-cultured into 1L of media the next day, with starting OD_{600} = 0.05. Once the cells have reached mid-log phase (OD_{600} = 0.4-0.6) cells were cooled on ice for 30 minutes followed by centrifugation at 3200 x g, 4°C for 5 minutes. The supernatant was then discarded and the pellet was washed three times with 10 mL of ice cold sterile H_20. Cell pellet was then resuspended in 10mL of ice-cold 10% (v/v) glycerol, followed by centrifugation at 3200 x g, 4°C for 5 minutes. Supernatant was then discarded and the pellet was resuspended in 500µL of 10%(v/v) glycerol and divided into 100µl aliquot before storage at -80 °C.

Electroporation method:

Pre-cooled DNA (200ng - 1µg) was mixed with 20µL of cells thawed on ice, then transferred the mixture to pre-cooled 1mm electroporation cuvettes. Cells were transformed using a Bio-Rad MicroPulser™, following the manufacturer's recommended setting for *E. coli,* pre-set Ec1 program (1.8 kV). SOC medium was added immediately after electroporation, and cells were incubated at 37°C with shaking at 200 x g for 1 hour to recover and centrifuged at 3200 x g for 1 min. The pellet was resuspend in 250µL SOC media and spread on LB agar plates containing the appropriate antibiotic selection and incubated overnight at 37 °C.

2.2.13 Colony screening by PCR

Colonies resulting from transformation were picked using sterile toothpick and re-plated onto fresh agar plate containing the appropriate media and antibiotic selection, by gently touching the tip of the toothpick onto the fresh agar plate. The toothpick was then dipped into PCR mixture containing 0.25µL of 100ng/µL forward and reverse primer, 0.25µL of 10mM dNTP mix, 1X taq polymerase buffer (NEB), 0.2µL of taq polymerase (NEB) in a total of 20µL reaction. The following program was then used to amplify the specific region within the clones:

Step 1: 98°C, 3:00

Step 2: 98°C, 0:10
55°C, 0:30
72°C, 1:00 (1Kb / min extension)] Repeat cycle 28x
Step 3: 72°C, 5:00

Step 4: Hold at 4°C

The reaction was carried out on a MJ Research PTC 2000 Thermal Cycler (GMI Inc., MN, USA). The amplification product was then run on 1%-2% (w/v) agarose gel.

2.2.14 Glycerol stock

For long-term storage of plasmids, an overnight culture of the *E. coli* harboring the recombinant plasmid was prepared at 37 °C. An equal volumes of the overnight culture and 80% glycerol were mixed and stored at -80 °C.

2.2.15 Sequencing

500ng of purified plasmid or template DNA was used per sequencing reaction. Template was mixed with 1µL of Big Dye Terminator mix (Perkin Elmer), 1x Big dye terminator buffer, 1µL of primer (100ng /µL), in a total reaction of 20µL. The following Sequencing program was used:

Step 1: 96°C, 2:00

Step 2: 96°C, 0:10
50°C, 0:05
60°C, 4:00
] Repeat cycle 30x

Step 3: Hold at 4°C

The reaction was carried out on a MJ Research PTC 2000 Thermal Cycler (GMI Inc., MN, USA). The sequencing product was then mixed with 80µL of 75% (v/v) isopropanol, incubated at room temperature for 15 minutes followed by centrifugation at 17500 x g for 30 min and then washed once in 250µL of 75% (v/v) isopropanol. The pellet was then dried by placing the tube in 42°C heating block for 10 minutes. The cleaned up reaction was then sent to AGRF for Capillary separation. Sequencing results and chromatograms were analysed using Snapgene

Viewer (GSL Biotech).

2.2.16 Lysate preparation for 6xHis-tag purification

Pellet from 2L culture was resuspended in 30mL protein purification wash buffer (300mM KCL, 50mM KPO_4 pH 8.0, 5mM imidazole) and added 300µL of 300mM PMSF. The cells were then passed through cell disruptor (M110L homogenizer, Microfluidics, USA) set at 18000 psi, for 5-6 times. The homogenized solution was then centrifuged at 20000 x g for 10 minutes at 4°C using Avanti J26-XPI centrifuge (Beckman-coulter). Supernatant was then passed through 0.45µM filter disc (Sartorius) before loaded into the purification column.

2.2.17 6xHis-tag purification

His-tagged proteins were purified by immobilized nickel affinity chromatography using a 5mL Bio-ScaleTM Mini Profinity™ IMAC cartridge, in an FPLC-based purification using AKTA FPLC (Amersham Pharmacia Biotech). All buffers and solution used in this purification were filtered through 0.45µM filter disc (Sartorius). The flow was set at 5mL/min, making sure no air bubbles were introduced to the column at any stage of purification. For each wash, samples of the flow through were collected and ran on SDS-PAGE for analysis. The column was washed first with 5x column volume of 20% (v/v) ethanol, followed by 5x column wash with H_20. 6x column volume of wash buffer I (300mM KCL, 50mM KPO_4 pH 8.0, 5mM imidazole) was then loaded to equilibrate the column. Bacteria lysate was then loaded into the column and the flow through of the unbound materials were collected and sample was taken for SDS-PAGE analysis. The column was then washed with 6x column volume with wash buffer II (300mM KCL, 50mM

KPO_4 pH 8.0, 10mM imidazole). The purified protein was then eluted in 3x column volume of elution buffer (300mM KCL, 50mM KPO_4 pH 8.0, 250mM imidazole). The purified protein was then dialysed overnight in 4L of storage buffer (50mM Tris pH 8.0, 100mM KCL, 1mM EDTA pH 8.0, 5% (v/v) glycerol, 1mM DTT) at 4 °C.

2.2.18 Cartridge clean up and maintenance.

At the end of each purification, the column was cleaned by running 10x column volumes of cleaning solution I (500mM NaCL, 50mM Tris pH8.0), followed by 10x column volumes of cleaning solution II (500mM NaCL, 100mM sodium acetate). 10x column volumes of filtered distilled H_20 was then run through the column and finally, 10x column volume of 20% (v/v) ethanol was run through the column and stored at 4°C.

A thorough clean up and recharging procedure for the column was also conducted for maintenance, by running 10x column volume of 50mM EDTA, 10x column volume of H_20, 10x column volume of Guanidine Hydrochloride, 10x column volume of H_20, 10x column volume of $NiSO_4$, 10x column volume of H_20 and 10x column volume of 20% (v/v) ethanol.

2.2.19 Concentration of purified proteins

Concentration of protein solutions was performed using Amicon® Ultra-50 or -15 centrifugal filter devices (10000 MWCO) (Millipore, MA, USA) following manufacturer's instruction manual. The columns were rinsed with MilliQ water and then equilibrated in storage buffer by centrifugation at 5000 x g at 4 °C for 30 min or until reaching a required retentate volume. The storage buffer was discarded prior adding protein sample into the spin- column. Likewise, protein

was concentrated by centrifugation at 5000 x g at 4 °C until reaching a required retentate volume. For retentate recovery, the concentrate was collected using a pipette with 200µL tip to new pre-cold microcentrifuge tube. The protein was kept at -80 °C until needed. For storage of the Amicon® Ultra-50 or -15 centrifugal filter devices, the centrifuge tube was washed with distilled water to remove residual buffer components and kept in MilliQ water at 4 °C.

2.2.20 Bradford assay for protein concentration determination

Protein concentration was assayed using the Bradford Reagent (Bio-Rad Laboratories Inc., CA, USA) assay method. A standard curve of bovine serum albumin (BSA) was generated from 0 to 1 mg/ml. A 10µL of sample was mixed with 200µL of Bradford reagent in a 96 well plate (Falcon). Absorbance at 620 nm wavelength was measured on a microplate reader (Molecular Devices, CA, USA). Standard curves were generated and used to calculate protein concentration using Microsoft ™ Excell spread sheet.

2.2.21 SDS-PAGE electrophoresis and gel staining

Protein samples were diluted in Protein sample buffer to a final concentration of 1x. Samples were then boiled for 5 min, and centrifuged briefly to collect condensation from the top of the tube. The protein samples were fractioned on NuPage® 4-12% Bis-Tris polyacrylamide gels (Invitrogen) using 1x NuPAGE® MES running Buffer (Invitrogen) at 200V for approximately 40 min or until the dye front reached the bottom of the gel. The protein bands were visualized using SDS-PAGE Coomassie Blue staining solution. The gel was first soaked in staining solution (0.2% Coomassie Blue, 50% ethanol and 10% acetic acid) at room

temperature for 1 hour, before soaking in SDS-PAGE destaining solution (10% acetic acid and 5% methanol) overnight.

2.2.22 *E. coli* Lysate preparation for biotin assay and western blot

Pellet from the overnight culture was resuspended in 2 mL of 1 x PBS containing 100μM PMSF. Cells were then sonicated (6 x 30 seconds with incubation on ice in between sonication) and centrifuged at 17500 x g using microcentrifuge, at 4 °C. For ^{3H}-biotin incorporation assay, lysate was collected and total protein concentration was then determined using Bradford assay. For blotting, following bradford assay, samples were diluted to obtain uniform concentration for all the samples and was then mixed 1:1 with 2x protein loading buffer. Samples were heated at 98°C for 5 minutes following centrifugation at full speed for 5 minute prior to gel loading.

2.2.23 Western blotting

Proteins fractioned by PAGE were transferred onto a PVDF membrane using a semi- dry transfer unit (Hoefer SemiPhor, Amersham Pharmacia Biotech, CA, USA). Six sheets of Whatman filter paper and the PVDF membrane were pre-soaked in western transfer buffer prior to assembly of the 3 : 1 : 1: 3 ratio of paper : gel : membrane : paper sandwich. Proteins were transferred for 1 hour at 80 mA per gel. The membrane was then blocked in 1% (w/v) skim milk blocking buffer for 1 hour at room temperature or at 4 °C overnight. The membrane was then washed three times with PBS-Tween before being probed with a 6xHis Monoclonal Antibody (diluted in 1:10000) for 1 hour at room temperature. The membrane was then washed three times in PBS-Tween before addition of a CyTM 3-conjugated

AffiniPure Donkey Anti-mouse IgG (diluted in 1:5000) for 1 hour at room temperature. Finally the membrane was washed three times with PBS-Tween before being visualized using Chemidoc-MP system imager (Bio-rad laboratories, California, USA).

2.2.24 Chromosomal integration of vector containing promoter construct

Bacteria was made chemically competent as described in section 2.2.11a. Heat shock method was used to transform the competent cells with a temperature-sensitive helper plasmid pAH69. This plasmid contained gene required to express the integrase for attP-HK022 phage attachment site integration. The transformed cells were recovered for one hour at 30°C and then plated onto LB agar containing 100µg/mL ampicillin followed by overnight incubation at 30°C. The resulting colonies were then made chemically competent and transformed with the integration plasmid containing promoter::lacZ construct. Transformation was recovered for 1 hour at 37°C and plated onto LB agar containing 30µg/mL spectinomycin followed by overnight incubation at 37°C. The resulting colonies were screened using HK022 screening primers.

2.2.25 Chromosomal integration of vector containing repressor construct

Bacteria was made chemically competent as described in section 2.2.11a. Heat shock method was used to transform the competent cells with a temperature-sensitive helper plasmid pINTSα . This plasmid contained gene required to express the integrase for attP-λ phage attachment site integration. The transformed cells were recovered for one hour at 30°C and then plated onto LB agar containing 100µg/mL ampicillin followed by overnight incubation at 30°C. The resulting

colonies were then made chemically competent and transformed with the integration plasmid containing the promoter construct (pIT4_plac_repressor) . Transformation was recovered for 1 hour at 37°C and plated onto LB agar containing 4µg/mL tetracyline followed by overnight incubation at 37°C. The resulting colonies were screened by PCR using Lambda screening primers.

2.2.26 β- galactosidase assay

Table 2.7: Buffer and media used in β –galactosidase assay

Media / buffer	Composition	Source
Minimal media (liquid)	1x M9 salts, 0.1% casamino acid, 1mM $MgSO_4$, 1.0 µg/mL Thiamine, 0.4 % glucose	This study
Minimal media (solid)	1x M9 salts, 0.1% casamino acid, 1mM $MgSO_4$, 1.0 µg/mL Thiamine, 0.4 % glucose, 1.5% bacto agar	This study
TZ8 buffer	100mM Tris.HCl pH8.0, 10 mM KCL, 1mM MgSO4	St. Pierre *et al*, (2013){St-Pierre, 2013 #27}
Assay lysis buffer (Formula per well of lysis)	150 µL TZ8 buffer, 40 µ L of ONPG (o-nitrophenyl-β -D-galactoside , 4 mg/mL), 1.9 µL of 2-mercapto-ethanol, 0.95 µL of polymyxin B (20mg/mL), 30 µL of minimal media containing 0.4% glycerol.	St. Pierre *et al*, (2013)

E. coli reporter strains were streaked onto minimal media agar containing 100nM biotin and 30 µg/mL spectinomycin and grown overnight at 37°C. The next day, a single colony was used to inoculate 2 mL of the minimal media containing 100nM biotin and grown overnight at 37°C. The next day, the overnight culture was centrifuged and washed 3x times in 5 mL minimal media without any biotin to remove excess biotin. The culture was then resuspended in 1 mL of minimal media (without biotin) and OD_{600} was measured. For a standard assay, the concentrations of biotin tested were 0.5nM, 1nM, 3nM, 5nM, 10nM, 50nM, 100nM and 500nM and therefore these amount of biotin were added to the minimal media. Growth plate was set up by inoculating 100 µL of each concentration of biotin-supplemented media with the

overnight culture in a 96-well plate, and OD_{600} was diluted to 0.05. The plate was then incubated at 37°C with shaking at 200 x g until OD_{600} = 0.5-0.6 was achieved. To lyse the culture, 20 µL of the culture was mixed with 220uL of the lysis buffer. The increase in absorbance at A_{414} nM was then measured at 28 °C using a multiskan ascent microtitre plate reader. Reading was obtained every 2 minutes for 1 hour and 10 seconds shaking 960 rpm in between readings. As soon as each reading was obtained, the change in A414 per minute was determined and *β-galactosidase* units were calculated using the following formula:

$$\beta\text{-galactosidase units} = 200.000 \times (A_{414} / \text{min}) / (OD_{600} \times \text{culture volume in } \mu L)$$

Results from β-galactosidase assay were then graphed using GraphPad Prism 6 (Graphpad Software, Inc., CA, USA).

Chapter 3:

In vivo β-galactosidase reporter gene assay development

3.1 Introduction

In order to characterize the transcription repressor function of *Sa*BirA, it is important to be able to analyze the interaction of *Sa*BirA with its target promoters in a cellular environment, as it will provide further understanding of how *Sa*BirA regulates gene expression under physiological conditions. Therefore, having the appropriate *in vivo* tools to enable these analyses is crucial. Ideally, an *in vivo* assay should be carried out in *S. aureus*, however, genetic manipulation of *S. aureus* is challenging, due to the genetic make up of the bacteria. It is known that *S. aureus* possess a complex restriction modification barrier, which consists of type I, type II and type IV modification systems, that reduce the natural competency of this species [1]. Type I encodes for host-specificity proteins [2] and type II encodes for restriction endonucleases . Type IV is believed to be the dominant barrier in preventing DNA uptake, and encodes for proteins that detect methylation of DNA and cleaves foreign DNA [3, 4]. These barriers result in the genetic manipulation of *S. aureus* being very difficult and time-consuming to perform [5]. On the other hand, in the prototypical bacteria *E. coli*, genetic manipulations and *in vivo* experiments are relatively easy to perform due to advanced knowledge of the bacteria and resources available to genetically manipulate this species [6-11].

One way to avoid the need to manipulate *S. aureus*, is to re-construct functional genetic circuits in bacteria without such strong restriction barriers, such as *E. coli*. Studying the design and construction of such circuits has led to a more robust analysis of protein:protein and protein:DNA interactions [12-15], as illustrated by studies on zinc finger proteins and phage repressors (reviewed in [12]).

In this chapter, a bacterial reporter system was generated in a biotin auxotroph *E. coli* strain by engineering constructs into the bacterial chromosome. The assembly of the reporter strain involved an established method of site specific chromosomal integration [16]. First, the *birA* gene of interest under the control of a *lac* promoter was integrated into the attB- λ phage attachment site of the *E. coli* chromosome. A second construct containing the test promoter sequence fused to the lacZ gene was subsequently integrated into the attB-HK022 phage attachment site. The interaction between repressor and promoter was then analyzed by quantifying the amount of *LacZ* expression using a β-galactosidase assay. As the overall aim of this study was to compare the mechanism of action of *Sa*BirA with the well-studied *Ec*BirA transcriptional repressor, both repressors were investigated. In addition, mutants of these repressors that were shown to be monomeric in solution were also investigated, namely *Sa*BirA F123G [17] and *Ec*BirA R119W [18-20]. To facilitate this *in vivo* analysis, any repression generated by the endogenous *Ec*BirA has to be prevented, in order to ensure that any repression observed in the assay was due to the integrated repressor. Therefore, the endogenous *E. coli birA* gene was modified such that the protein retained its catalytic function to maintain viability, but the DNA binding activity was abolished. As biotin is an allosteric activator of DNA binding [21], hence the ability to control the level of biotin in the β – galactosidase assay was crucial. Therefore, in addition to using a biotin auxotroph strain, 0.1% casamino acid minimal media was chosen as the growth media in this assay in allow the concentration of biotin in the media to be tightly controlled.

3.1.1 Overview of stages involved in reporter strain assembly and *in vivo* assay development

In this chapter, the construction of an *E. coli* reporter strain and the development of *in vivo* β-galactosidase assays to measure biotin-inducible repression will be described. An overview of the steps involved in strain construction and *in vivo* assay is presented in **Figure 3.1**. *E. coli* JD26186 was used as the parent strain in this study, as this strain is unable to synthesize its own biotin due to the disruption of the biotin biosynthesis gene, *bioC*. The first stage of the strain construction involved the disruption of the N-terminal DNA binding domain of the endogenous *Ec*BirA of the parental strain, by insertion of a chloramphenicol cassette (CAT) into the 5′ region of the *birA* gene. This resulted in a new strain expressing *Ec*BirA lacking DNA binding activity, JD26186_*birA*::CAT. The precise disruption of the endogenous *birA* gene was confirmed by DNA sequencing and β–galactosidase assay. This strain was then used for the assembly of a series of reporter strains for use in this study.

The second stage of the reporter strain construction involved cloning a series of integration vectors suitable for chromosomal integration into the *E. coli* genome at specific phage attachment sites. The final step of the strain construction involved integrating these vectors chromosomally into JD26186-*birA*::CAT using methods previously described [16]. The resulting reporter strains were then used in β-galactosidase assays to assess repression of target promoters, following changes in extracellular biotin concentrations. The outcome of each stage of the strain assembly and assay development is outlined and discussed in detail in the following sub-sections.

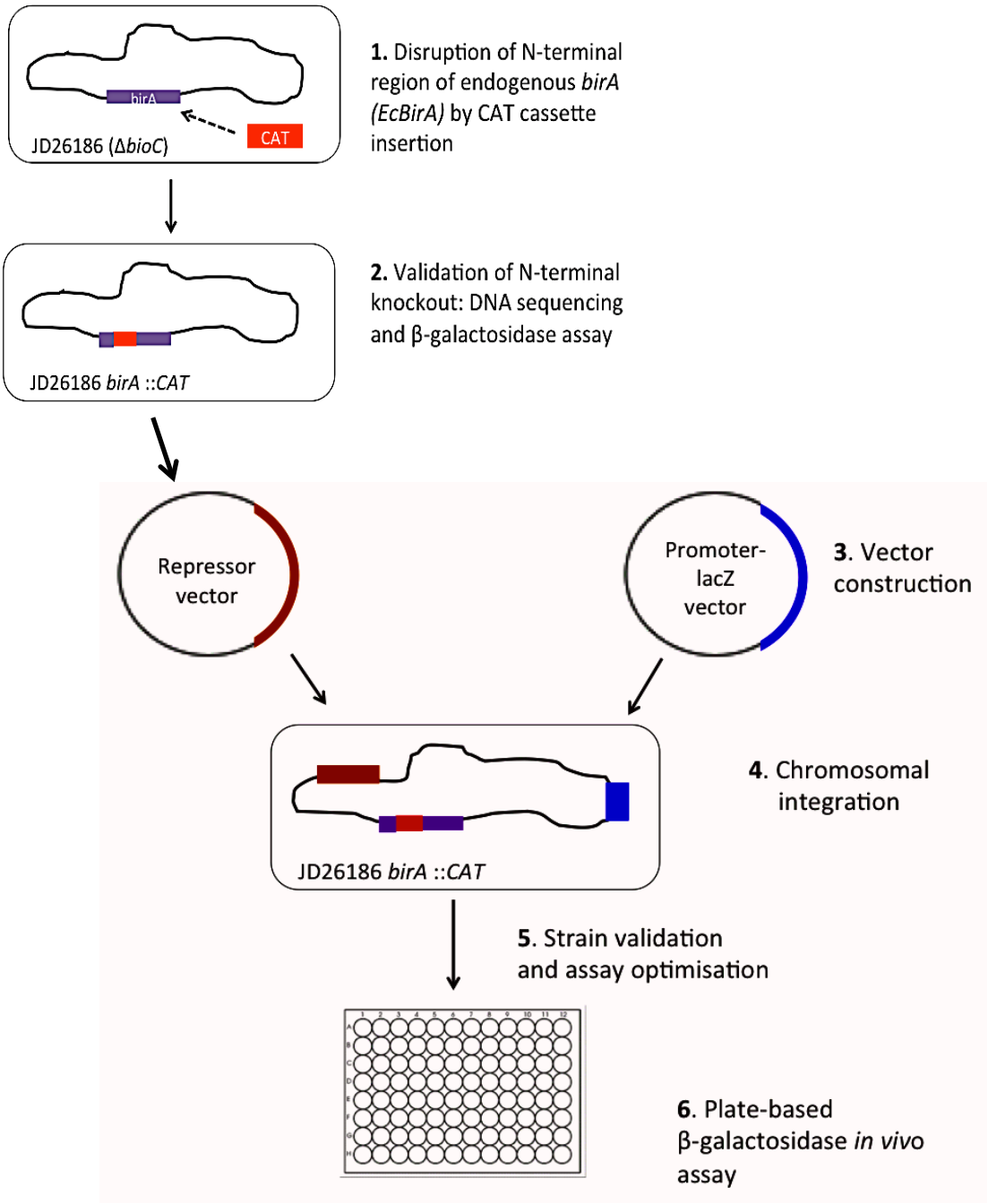


Figure 3.1: Summary of procedures involved in constructing reporter strain and *In vivo* assay establishment. *E. coli* JD26186 with disrupted *bioC* was chosen as the parent strain to generate the reporter strain. N-terminal domain of the endogenous *Ec*BirA was disrupted by the insertion of chloramphenicol cassette. A series of integration vectors containing the promoter and repressor genes of interest were constructed and chromosomally integrated into JD26186_ *birA*::CAT strain. The resulting strains were used in β-galactosidase assays to analyze the DNA binding mechanism of each repressor *in vivo*.

3.2 Materials and methods

3.2.1 Materials

All customized oligonucleotides used in this study are listed in **Table 3.1**. All DNA constructs and plasmids used in this study are shown in **Table 3.2** and the strains generated are shown in **Table 3.3**. The parental strain for the *in vivo* reporter strain, JD26186, was purchased from National Bioresource Project (NIG, Japan). This strain is not able to synthesize biotin, due to the disruption of the *bioC* gene by a kanamycin resistance gene (Kan^R) [22]. Consequently, the strain was maintained on either minimal media containing 100nM biotin or Luria Bertani medium without any additional biotin

3.2.2 Methods

Chromosomal integration, western blot and β-galactosidase assay protocol are presented in **chapter 2**. Specific methods involved in the assembly of the specific reporter strain are presented in each section, combined with the experimental result.

Table 3.1: List of oligonucleotides used in reporter strain construction

Oligo name	Sequence 5'-3'	Description
B391	CGTCAGGCATCGATCGGAAGCTGTGGTATGGCTGTGC	Amplify ptac-hBPL-H_6 (For), linker on ClaI site, HsBPL cloning into pKD46
B392	CAATATTATTGAAGCATCGATCAGGGTTATTGTCTCATGAGCG	Amplify ptac-hBPL-H_6 (Rev), linker on ClaI site, HsBPL cloning into pKD46
B106	GCTCTCTCTTAGATTTGTTTCATCC	forward primer to screen *Hs*BPL gene
B107	GAGATAATCGGCTCTTAAGG	reversed primer to screen *Hs*BPL gene
B393	GGAATAAGGGCGACACGGAAATGTTG	sequencing primer1 for pKD46-*Hs*BPL (Sequence from 5' end)
B394	CGGTCGCAATGTTGGTTTTGACG	sequencing primer1 for pKD46-*Hs*BPL (Sequence from 3' end)
B395	GAAGGATAACACCGTGCCACTGAAATTGATTGCCCTGTTAGCGAACGGTGAATTTCACGGCAGCATCACCCGACGCAC	Forward primer with birA homologous sequence for *CAT- Cm^R* isolation from pCY216 *birA*
B396	GGATAGGCTCAGGCAGGCTGTATCCTTTACCCGGAACGGTAAAGACATCAACGCCCCAGTTACGCCCCGCCCTGCCACTC	Reversed primer with birA homologous sequence for *CAT- Cm^R* isolation from pCY216 *birA*
B398	CGATTTAAGCTCTCCGATACGATCAAG	reversed sequencing primer for N-terminal birA::CAT
B399	GAAGGATAACACCGTGCCACTGAAATTG	Forward primer for *JD26186 birA::CAT* PCR screening
B133	CCATTTCCGACCCCGACCACCACGGCCAGC	Reversed primer for *JD26186 birA::CAT* PCR screening
B409	GGTGTGTCATATGCGCACCACCAAACTCC	Forward primer for N-terminal birA::CAT sequencing template
B410	GGCCTTGTTCCAGACGCCAGAACATCGAC	Reversed primer for N-terminal birA::CAT sequencing template
B410	GGCCTTGTTCCAGACGCCAGAACATCGAC	sequencing primer to sequence *pTac-birA* in integration plasmid pIT4_TL152002
B411	GGCAGCAGGTTGGCTTATCGATCAGTGCC	Forward sequencing primer for N-terminal birA::CAT
M13_F	ACTGGCCGTCGTTTTAC	universal primer M13 (forward) used to sequence promoter sequence in integration plasmid pIT3_SH_lacZtrim
pTac oligo 1	CATGCGGTACCTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAA	Top strand of double stranded sequence of pTac and lac operator, cloned into pGEMT-*SaBPL*(6xHis) / pGEMT-*birA* (6xHis)
pTac oligo 2	CATGTTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACATTATACGAGCCGATGATTAATTGTCAAGGTACCG	Bottom strand of double stranded sequence of pTac and lac operator, cloned into pGEMT-*SaBPL*(6xHis) / pGEMT-*birA* (6xHis)
EcBioO oligo 1	CTTGTCATAATCGACTTGTAAACCAAATTGAAAAGATTTAGGTTTACAAGTCTACACCGAATTAACAACAAAAGCATG	Top strand of double stranded *EcBioO* operator sequence, cloned into pIT3_SH_lacZtrim
EcBioO oligo 2	CTTTTGTTGTTAATTCGGTGTAGACTTGTAAACCTAAATCTTTTCAATTTGGTTTACAAGTCGATTATGACAAGGTAC	Bottom strand of double stranded *EcBioO* operator sequence, cloned into pIT3_SH_lacZtrim
SaBioO oligo 1	CGAAAAACATGCGCCTTAAATGTAAACTTATTAATTATAAAAGTTTACATTCGGATTGAGGTGCTTATTTTTTGCATG	Top strand of double stranded *SaBioO* operator sequence, cloned into pIT3_SH_lacZtrim
SaBioO oligo 2	CAAAAAATAAGCACCTCAATCCGAATGTAAACTTTTATAATTAATAAGTTTACATTTAAGGCGCATGTTTTTCGGTAC	Bottom strand of double stranded *SaBioO* operator sequence, cloned into pIT3_SH_lacZtrim
yHFS-T oligo 1	CAAAAATACTTATTCATTATATAATGTTAACAAGATGTATTTTAAAGTTTACATTGAGTGAGGGATATTGGCATG	Top strand of double stranded yHFS-T operator sequence, cloned into pIT3_SH_lacZtrim
yHFS-T oligo 2	CCAATATCCCTCACTCAATGTAAACTTTAAAATACATCTTGTTAACATTATATAATGAATAAGTATTTTTGGTAC	Bottom strand of double stranded yHFS-T operator sequence, cloned into pIT3_SH_lacZtrim
B386	GACTAAAATGTTGAATCGCATTCTTATCCCTAAATCAATAAATAAATTAAATTTAGATATCATTGAGAATGC	sequencing primer to sequence *pTac-SaBPL* in integration plasmid pIT4_TL152002
HK022-P1	GGAATCAATGCCTGAGTG	attp-HK022 PCR screening primer (St. Pierre *et a*l, 2013)
HK022-P2	ACTTAACGGCTGACATGG	attp-HK022 PCR screening primer (St. Pierre *et a*l, 2013)
HK022-P3	ACGAGTATCGAGATGGCA	attp-HK022 PCR screening primer (St. Pierre *et a*l, 2013)
HK022-P4	GGCATCAACAGCACATTC	attp-HK022 PCR screening primer (St. Pierre *et a*l, 2013)
Lambda P1	GGCATCACGGC AATATAC	attp-λ PCR screening primer (St. Pierre *et a*l, 2013)
Lambda P2	ACTTAACGGCTGACATGG	attp-λ PCR screening primer (St. Pierre *et a*l, 2013)
Lambda P3	GGGAATTAATTCTTGAAGACG	attp-λ PCR screening primer (St. Pierre *et a*l, 2013)
Lambda P4	TCTGGTCTGGTAG CAATG	attp-λ PCR screening primer (St. Pierre *et a*l, 2013)
B460_R119W_F	GGCCGTGGTCGCTGGGGTCGGAAATGG	Forward mutagenesis primer for birA R119W
B461_R119W_R	CCATTTCCGACCCCAGCGACCACGGCC	reverse mutagenesis primer for birA R119W
B479	GACTCATCATGAAGGATAACACCGTGCCAC	Forward primer to clone birA R119W into integration plasmid pIT4_TL 152002
B320	ACTAGTGATAAGCTTAATGATGATGATGATGATGTCC	reverse primer to clone birA R119W into integration plasmid pIT4_TL_152002

Table 3.2 List of plasmid used and generated in this study

Plasmid	Description	Source
pKD46	Amp^R, *oriR101*, λ-Red recombinase expression plasmid	Datsenko & Warner (2000)
pK(*HsBPL*)	template for HsBPL to clone into pKD46 (Amp^R, pBR322/pUC ori, *Hs*BPL-6xHis, pTac-uV5)	Mayende, *et al* (2012)
pKD46(*HsBPL*)	pKD46 containing *HsBPL* gene under the control of arabinose promoter	this study
pCY216(*birA*)	p15A ori, medium copy plasmid containing Cm^R and CAT promoter	Cronan & Wallace (1995)
pGEMT-SaBirA(6xHis)	pGEMT plasmid containing SaBirA with 6x his-tag	Pendini *et al*, (2008)
pGEMT-*birA*(6xHis)	pGEMT plasmid containing *birA* with 6x his-tag	Soares Da costa, *et al* (2014)
pIT3_CLlacZ_Trim	Chromosomal integration plasmid (λ-attP, Cm^R, R6Kγ ori, *lacZ*)	Cui *et a*l PNAS (2013)
pIT3-SH-152002	Chromosomal integration plasmid (HK022-attP, $Spec^R$, R6Kγ ori, *ccdB,* pUC ori)	Shearwin lab, Adelaide university
placZ_SH_Trim	Chromosomal integration plasmid (HK022-attP, $Spec^R$, R6Kγ ori, *lacZ*)	this study
pIT4_TL_152002	Chromosomal integration plasmid (λ-attP, Tc^R, R6Kγ ori, *ccdB,* pUC ori)	St. Pierre, *et a*l (2013)
pGEMT-*birA R119W* (6xHis)	pGEMT plasmid containing *birA R119W* (6x his-tag)	this study
pGEMT-SaBirA F123G (6xHis)	pGEMT plasmid containing SaBirA F123G (6xhis-tag)	Soares Da costa, *et al* (2014)
peT16b-*birA R119W* (6xHis)	pET16b expression vector containing *birA R119W*(6x his-tag)	this study
pET16b-SaBirA F123G (6xHis)	pET16b expression vector containing SaBirA F123G with (6xhis-tag)	Soares Da costa, *et al* (2014)
placZ_SH_*SabioO*_LacZ	SabioO promoter sequence cloned into pIT3_SH_LacZTrim, upstream of the lacZ gene	this study
placZ_SH_*SabioY*_LacZ	SabioY promoter sequence cloned into pIT3_SH_LacZTrim, upstream of the lacZ gene	this study
placZ_SH_yhfST_LacZ	yHFS-T promoter sequence cloned into pIT3_SH_LacZTrim, upstream of the lacZ gene	this study
pIT4_TL_SaBirA (WT)	plac-UV5 fused with SaBirA (wildtype) sequence cloned into pIT4_TL_152002	this study
pIT4_TL_SaBirA (F123G)	plac-UV5 fused with SaBirA (F123G) sequence cloned into pIT4_TL_152002	this study
pIT4_TL_*birA* (R119W)	plac-UV5 fused with birA (R119W) sequence cloned into pIT4_TL_152002	this study
pIT4_TL_*Empty*	Chromosomal integration plasmid (λ-attP, Tc^R, R6Kγ ori, Δ*ccdB,* pUC ori)	this study

Table 3.3 List of strain generated in this study

Strain name	Genotype	Description	Source	parent strain	plasmid/ PCR product used to transformed the strain
JD26186	bioC::KanR	E. coli BW25113 derivative with disrupted bioC gene	NBRP, Japan	JD26186	none
JD26186 birA::CAT	bioC::KanR birA::CAT	JD28186 strain with N-terminal CAT cassette insertion (knockout)of its	this study	JD26186	recombineering using CAT Casette
JD26186 birA::CAT-SabioO-SaBirA	bioC::KanR birA::CAT (SabioO-lacZ)HK(placUV5-SaBirA)I	JD26186 birA::CAT strain with SaBioO lacZ reporter chromosomally integrated	this study	JD26186-birA::CAT	pLacZ_SH_SabioO and pIT4_TL_SaBirA (WT)
JD26186 birA::CAT-SabioY-SaBirA	bioC::KanR birA::CAT (SabioY-lacZ)HK (placUV5-SaBirA)I	JD26186 birA::CAT strain with SaBioY-lacZ reporter chromosomally integrated	this study	JD26186-birA::CAT	pLacZ_SH_SabioY and pIT4_TL_SaBirA (WT)
JD26186 birA::CAT-yhfST-SaBirA	bioC::KanR birA::CAT (yHFT-lacZ)HK (placUV5-SaBirA)I	JD26186 birA::CAT strain with yHFT-lacZ reporter chromosomally integrated at	this study	JD26186-birA::CAT	pLacZ_SH_yhfST and pIT4_TL_SaBirA (WT)
JD26186 birA::CAT-SabioO-SaBirA F123G	bioC::KanR birA::CAT (SabioO-lacZ)HK (placUV5-SaBirA F123G)I	JD26186 birA::CAT strain with SaBioO-lacZ reporter chromosomally integrated	this study	JD26186-birA::CAT	placZ_SH_SabioO and pIT4_TL_SaBirA (F123G)
JD26186 birA::CAT-SabioY-SaBirA F123G	bioC::KanR birA::CAT (SabioY-lacZ)HK (placUV5-SaBirA F123G)I	JD26186 birA::CAT strain with SaBioY-lacZ reporter chromosomally integrated	this study	JD26186-birA::CAT	placZ_SH_SabioY and pIT4_TL_SaBirA (F123G)
JD26186 birA::CAT-yhfST-SaBirA F123G	bioC::KanR birA::CAT (yHFT-lacZ)HK (placUV5-SaBirA F123G)I	JD26186 birA::CAT strain with yHFT-lacZ reporter chromosomally integrated at	this study	JD26186-birA::CAT	placZ_SH_yhFS and pIT4_TL_SaBirA (F123G)
JD26186-EcBioO-EcbirA	bioC::KanR (EcBioO-lacZ)HK	JD26186 strain with EcBioO-lacZ reporter chromosomally integrated at	this study	JD26186	placz_SH_EcbioO
JD26186 birA::CAT-EcBioO-EcBirA R119W	bioC::KanR birA::CAT (EcBioO-lacZ)HK (placUV5-birA R119W)I	JD26186 birA::CAT strain with EcBioO promoter and placUV5-birA R119W	this study	JD26186-birA::CAT	placZ_SH_EcbioO and pIT4_TL_EcBirA (R119W)
JD26186 birA::CAT-SaBioO-empty	bioC::KanR birA::CAT (SabioO-lacZ)HK(placUV5-no repressor)I	JD26186 birA::CAT strain with SaBioO lacZ reporter chromosomally integrated	this study	JD26186-birA::CAT	placZ_SH_SabioO and pIT4_TL_Empty
JD26186 birA::CAT-SaBioY-empty	bioC::KanR birA::CAT (SabioY-lacZ)HK(placUV5-no repressor)I	JD26186 birA::CAT strain with SaBioY lacZ reporter chromosomally integrated	this study	JD26186-birA::CAT	placZ_SH_SabioY and pIT4_TL_Empty
JD26186 birA::CAT-yhfST-empty	bioC::KanR birA::CAT (yhfST-lacZ)HK(placUV5-no repressor)I	JD26186 birA::CAT strain with yhfS lacZ reporter chromosomally integrated at	this study	JD26186-birA::CAT	placZ_SH_yhfS and pIT4_TL_Empty
JD26186 birA::CAT-EcBioO-empty	bioC::KanR birA::CAT (EcbioO-lacZ)HK(placUV5-no repressor)I	JD26186 birA::CAT strain with EcBioO promoter and placUV5-pIT4-empty	this study	JD26186-birA::CAT	placZ_SH_EcbioO and pIT4_TL_Empty
JD26186 birA::CAT-Trim-SaBirA	bioC::KanR birA::CAT (no promoter-lacZ)HK(placUV5-SaBirA)I	JD26186 birA::CAT strain with no promoter construct integrated at HK022	this study	JD26186-birA::CAT	placZSH_Trim and pIT4_TL_SaBirA (WT)
JD26186 birA::CAT-Trim-EcBirA R119W	bioC::KanR birA::CAT (no promoter-lacZ)HK (placUV5-birA R119W)I	JD26186 birA::CAT strain with no promoter construct integrated at HK022	this study	JD26186-birA::CAT	placZSH_Trimand pIT4_TL_EcBirA(R119W)

3.3 Specific methods and results.

3.3.1 N-terminal disruption of endogenous *Ec*BirA of *E. coli* JD26186

3.3.1.1 Cloning of *Hsbpl* gene into pKD46 plasmid to complement the catalytic activity of the endogenous EcBirA

As mentioned in the introduction, for the assay to function, any background repression caused by endogenous *Ec*BirA should be avoided. This was done by insertion of CAT cassette in a specific region of the N-terminal domain of *Ec*BirA. Previous studies have indicated that *EcBirA* is an essential gene in *E. coli* and deletion of the N-terminal region of *EcBirA* resulted in reduced affinity to biotin [23, 24]. Based on these reports, it was possible that any modification performed on the N-terminal domain of the endogenous *Ec*BirA could result in less efficient biotinylation activity, which could be detrimental to the bacteria's growth. To prevent any viability issue, another functional BPL gene (Human BPL or *Hsbpl*) was transformed into the parent strain, in order to complement the compromised endogenous *Ec*BirA catalytic activity.

The *Hsbpl* gene was isolated from pK(*Hsbp*l) [25] as a *C*laI fragment, using B391-B392 primers and cloned into the same helper plasmid (pKD46) that encodes the *λ-red* genes (*β, exo and gam*), essential for recombineering [26, 27](**Figure 3.2**). Following ligation, heat shock transformation was performed and transformed cells were plated onto LB agar containing 100 µg/mL ampicillin and incubated overnight at 30 °C. Plasmid from positive colonies were extracted and confirmed by sequencing.

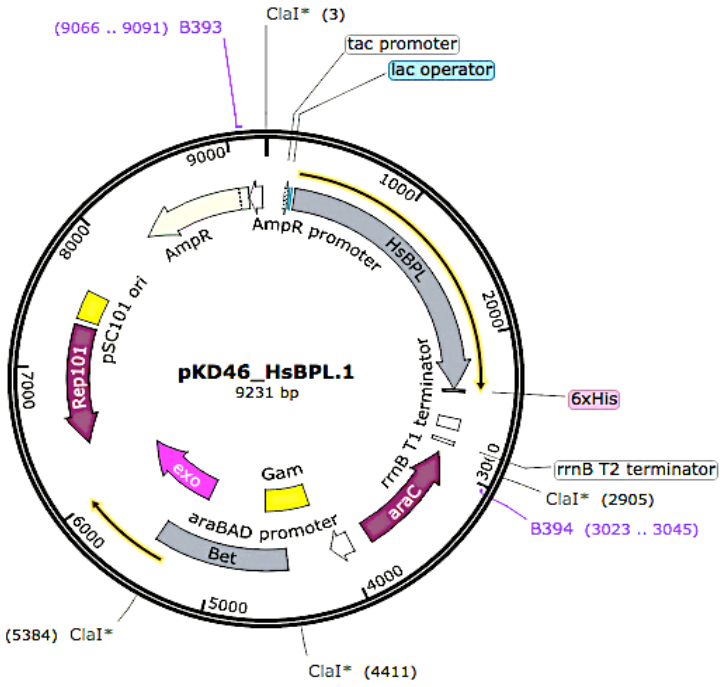


Figure 3.2: Helper plasmid PKD46-*Hsbpl*. *Hsbpl* gene encoding for human BPL, along with lac promoter was isolated from pK(*Hsbpl*) and cloned into the parent helper plasmid pKD46. This plasmid also encodes for genes required in recombineering process (*β, exo and gam*) and a temperature sensitive replication origin. Removal of this helper plasmid was done by incubation at temperatures ≥37°C.

3.3.1.2. Construction of Chloramphenicol cassette

The chloramphenicol resistance gene (*Cam*^{R}) and its promoter (CAT promoter) was isolated from pCY216 plasmid [28] using a set of oligonucleotides (B395 and B396) that contained a flanking sequence homologous to that of the *EcBirA* N-terminal region. The specific sequence of the CAT cassette insertion point within the *EcBirA* gene and the flanking homologous sequence incorporated into the oligonucleotides is outlined in **Figure 3.3**. Deep vent polymerase (NEB) was used to amplify the cassette, according to the manufacturer's instructions. The expected CAT cassete size of 1012 bp was confirmed on 1% agarose gel, followed by gel purification.

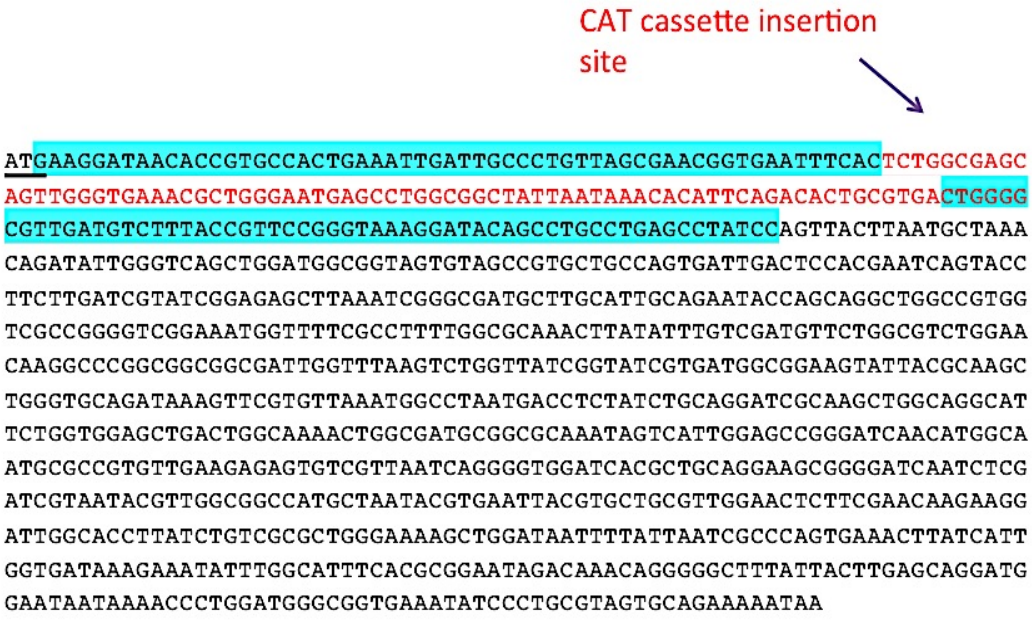


Figure 3.3: Diagram showing the target site for CAT cassette insertion in the endogenous *EcBirA* gene. The gene encoding for endogenous *EcBirA* of the parent strain *E. coli* JD26186 is outlined. The translation initiation codon (Met) is underlined. Blue shading indicates the homologous sequence that was engineered in the B395-B396 oligonucleotides to target *EcBirA* gene. Red letters indicate the sequence targeted for replacement by CAT cassette during the recombineering process.

3.3.1.3 Insertion of CAT cassette into *E. coli* JD26186 endogenous *Ec*BirA N-terminal domain.

Electrocompetent JD26186 cells carrying the temperature sensitive pKD46-*Hs*bpl plasmid were prepared at 30°C in the presence of 100 µg/mL ampicillin to maintain the plasmid and 10mM arabinose to induce the expression of the *λ-red* genes. The PCR product containing a CAT cassette flanked by homologous DNA sequence was then transformed into the cells, using recombineering method previously described by Sharan *et al* [29]. Transformed cells were recovered at 30°C in 500 µL SOC media for 1 hour and plated onto LB agar containing 100nM biotin, 0.2mM IPTG and 10 µg/mL chloramphenicol. The plate was then incubated overnight at 37°C. PCR screening was then performed

to isolate positive transformants, using B409 and B410 oligonucleotides. Positive clones should exhibit a PCR fragment of ~1.6Kb whereas wild-type colonies should produce a fragment of ~826bp. These fragments produced by B409-410 oligonucleotides were also used as a template in sequencing reaction to confirm the insertion.

A low concentration of chloramphenicol (10 μg/ mL) was chosen in this selection step to allow cells to grow in the minimal concentration necessary for antibiotic selection. However, it is known that at low concentrations, chloramphenicol is bacteriostatic against *Enterobacteriaceae* family of bacteria [30] and it has been shown that chloramphenicol concentrations as high as 80 μg/mL was needed for a bactericidal effect against *E. coli* [31]. As a result, PCR screening showed that the first generation of colonies grown on 10 μg/mL chloramphenicol plate exhibited a mixed population of wild-type and N-terminally disrupted colonies, as detected by PCR (**Figure 3.4a**). Therefore, more stringent enrichment of the mutant strain was needed. This was achieved by plating the resulting colonies onto increasingly higher chloramphenicol concentrations, up to 100 μg/mL. PCR screening of colonies grown at 100 μg/mL chloramphenicol concentration confirmed that wild-type contamination had been eliminated (**Figure 3.4b**).

To remove the helper plasmid, pKD46-*Hsbpl*, bacteria was plated onto LB agar and incubated overnight at 40 °C. A growth assay was performed to confirm the removal of this helper plasmid, by plating the colonies onto LB agar without antibiotic selection and LB agar containing ampicillin, followed by overnight incubation at 40 °C. Incubation at this high temperature should eliminate the temperature-sensitive pKD46-*Hsbpl* plasmid and as this plasmid conferred

ampicillin resistance, only colonies that retained this plasmid should grow in the presence of ampicillin. The result confirmed that the isolated colonies were able to grow on the media without ampicillin but failed to grow on media with ampicillin, which indicated that these colonies no longer retained the helper plasmid (**Figure 3.4c - 3.4d.)**

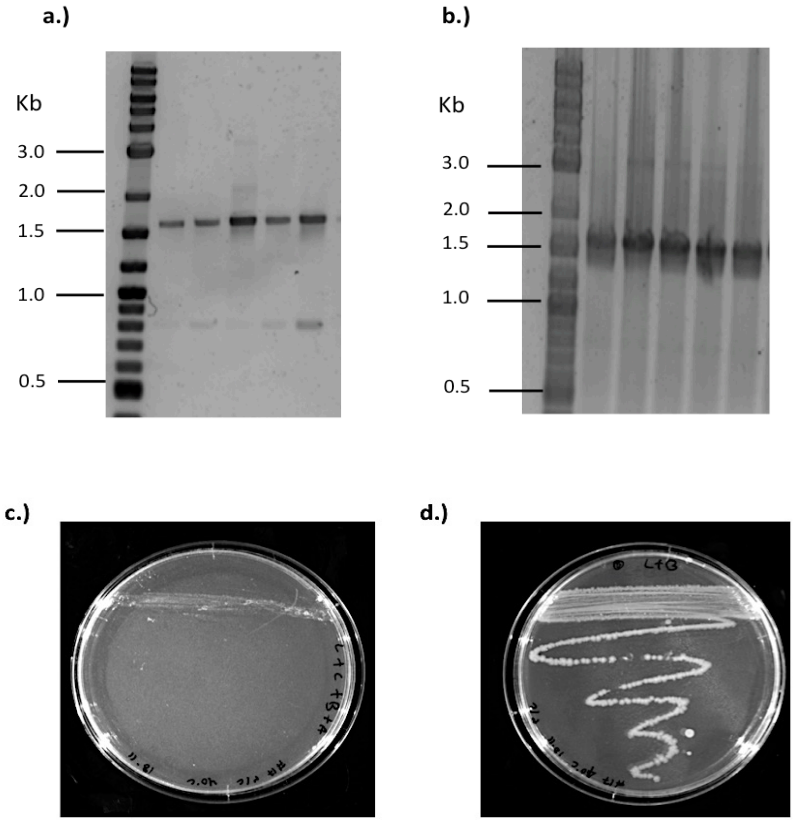


Figure 3.4: JD26186 *birA*::*CAT* mutant PCR screening and growth test. (a.) Low concentrations of chloramphenicol result in mixed population of wild-type and N-terminal disruption mutants as shown by amplified fragments of ~ 826 bp and ~1.6 Kb fragment, respectively. (b.) After re-plating onto LB media containing higher chloramphenicol concentration, single colony of the mutant, JD26186 *birA* :: CAT, was obtained. (c.) Growth tests confirmed the loss of pKD46-*Hsbpl*, as bacteria were unable to grow in media containing ampicillin but still viable in media without ampicillin as shown in (d.).

3.3.2 Bioinformatics prediction of frame shift created by the CAT cassette insertion into the specific target site of *birA* N-terminal domain.

In the genome, *EcBirA* is located in the same operon as another gene, *murB* that encodes for UDP-N-acetylenolpyruvoyl glucosamine reductase (**Figure 3.5a**). This enzyme is essential for the bacterial cell wall synthesis [32] and, consequently, cell growth. The promoter that drives the expression of both *murB* and *EcBirA* is not known. The open reading frame (ORF) of these genes and the ORF resulting from the disrupted N-terminal domain of *EcBirA* was predicted using bioinformatics analysis; ORF prediction tool (Https://www. expasy.org). The result showed that in the genome, the coding sequence of *murB* and *EcBirA* constituted two different ORFs. The first ORF encodes for *murB* and the second ORF encodes for *EcBirA* (**Figure 3.5a**). These ORFs overlap each other, where the end of *murB* coincides with the start of *EcBirA*.

The insertion of CAT cassette in the specific site of *EcBirA* (nucleotide 59-134) resulted in a shift within these two ORFs as shown in (**Figure 3.5b**). The first ORF encodes for *murB* and chloramphenicol resistance gene, respectively, whereas the second ORF encodes for *EcBirA* with the disrupted N-terminal domain but an intact C-terminal (amino acid 274-317) and central domains (amino acid 68-269) [33]. This result suggests that the insertion of the CAT cassette within the specific targeted site, should result in an endogenous *Ec*BirA with abolished DNA binding capability, but the protein should remain functionally active as a catalyst. The transformed colonies resulting from the N-terminal domain disruption were indeed viable , which suggests that the CAT cassette insertion did not interfere with the expression of *murB* and that these

mutants should have abolished DNA binding properties and a compromised BirA catalytic activity, as confirmed subsequently by *in vivo* and *in vitro* assay.

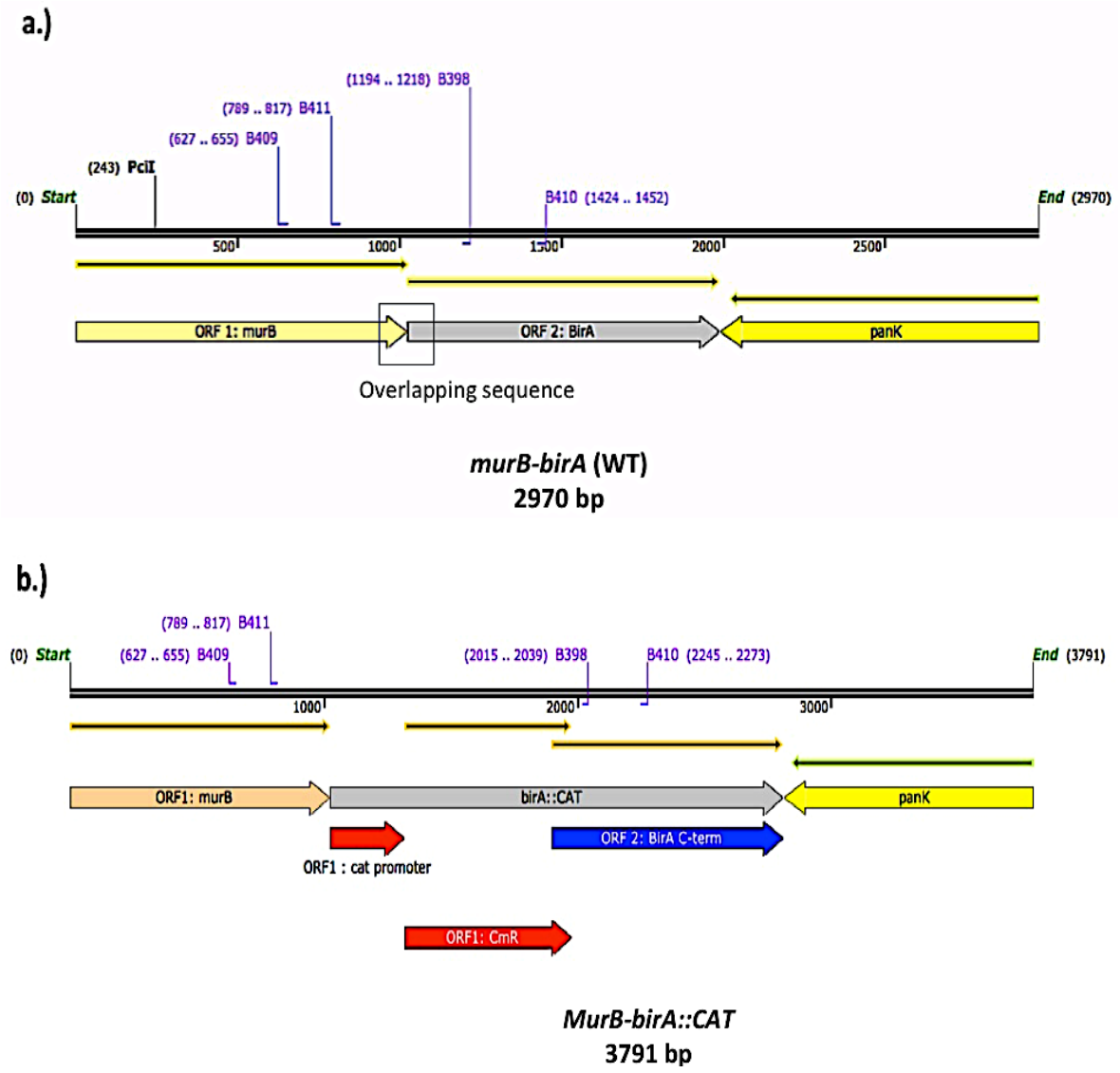


Figure 3.5: Open reading frames within the *E. coli* genome of the disrupted N-terminal domain of *EcBirA,* and wild-type *EcBirA*, as predicted using ORF prediction tool. (a) In the genome, *EcBirA* is located in the same operon as another essential gene, *murB*. One ORF represent the translation of *murB* and the second ORF encodes for *EcBirA*(denote as *birA* in the diagram) **(b)** Insertion of CAT cassette result in a shift within these two ORFs. The expression of *murB* and Chloramphenicol resistance gene (Cm^R) corresponds to ORF1 whereas the disrupted N-terminal domain of *EcBirA* with conserved C-terminal domain corresponds to ORF2. Oligonucleotides B409-B411 used in the template isolation for sequencing and oligonucleotides B411 and B398 used in a sequencing reaction to confirm the N-terminal region disruption are shown in purple text.

3.3.3 Validation of CAT cassette insertion into the *birA* N-terminal domain

The insertion of the CAT cassette in the genome was confirmed by DNA sequencing from the genomic template. A set of oligonucleotides (B409-B410) were designed and utilized to amplify the disrupted *EcBirA* sequence by PCR from the genomic DNA preparations and then sequenced using oligonucleotide B411 and B398 (**Figure 3.5b**, purple text). The PCR analysis with B409 and B410 yielded a 1.6 Kb fragment, as expected.

Furthermore, DNA sequencing results confirmed the insertion of the CAT cassette on the N-terminal domain of *EcBirA*, as predicted **(Figure 3.6)**. Alignment of the amino acid sequence resulting from translation of these sequences against the wild-type *EcBirA* amino acid sequence showed a mismatched in alignment of amino acids 1-45, which corresponds to the N-terminal domain, but perfect alignment from amino acid 45-321, which corresponds to central domain and C-terminal domain, as predicted in bioinformatics analysis **(Figure 3.7a)**. The mismatched alignment between amino acid 1-45 validated the disruption of the helix-turn-helix DNA binding domain of *EcBirA*, as illustrated in **(Figure 3.7b,** red shading**)** and therefore, it is expected that the endogenous *EcBirA* of JD26186_*birA::*CAT would no longer be able to bind DNA.

Overall, DNA sequencing has confirmed the insertion of CAT cassette. However, the loss of DNA binding ability due to the insertion of this CAT cassette, still needed to be tested *in vivo*. In order to test this, a reporter strain containing integrated construct of the promoter (*EcbioO*) fused to a *lacZ* gene (*EcbioO::lacZ*) has to be created using this mutant as the background strain, as well as the wild-type strain JD26186, to act as a control. The construction the pIT3_*EcbioO*::LAcZ integration vector and other integration vectors are described in section **3.3.6**.

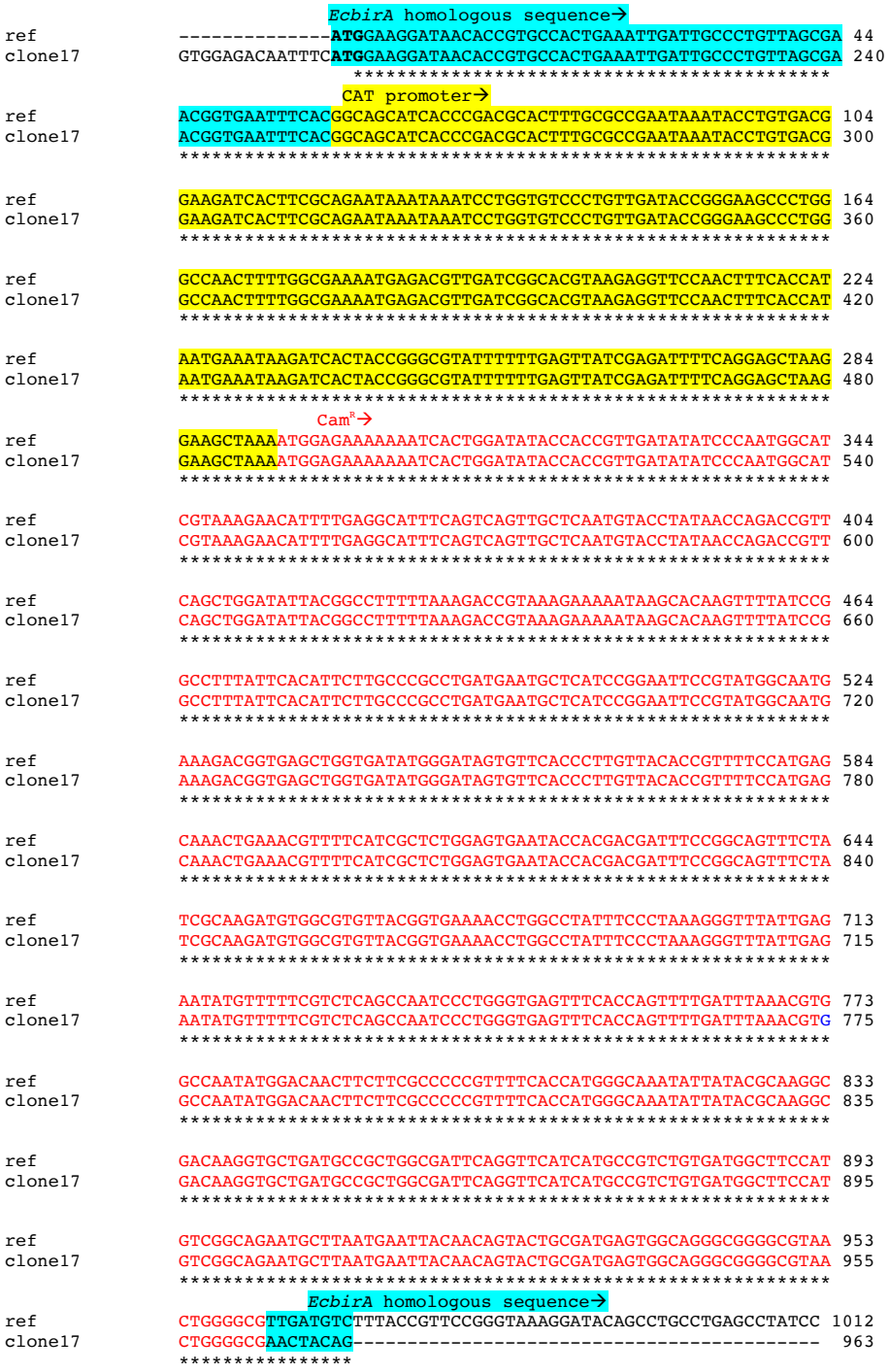


Figure 3.6: Sequence alignment to confirm the disrupted N-terminal domain of *EcBirA*. A reference sequence of *EcBirA* containing N-terminal CAT cassette insertion (*birA*::CAT) was aligned with the sequencing result obtained from the isolated clone containing the disrupted N-terminal domain. *EcBirA* homologous sequence is highlighted in blue, yellow highlight represents CAT promoter sequence and red text denotes the chloramphenicol resistance gene insertion (*Cam*^{R}).

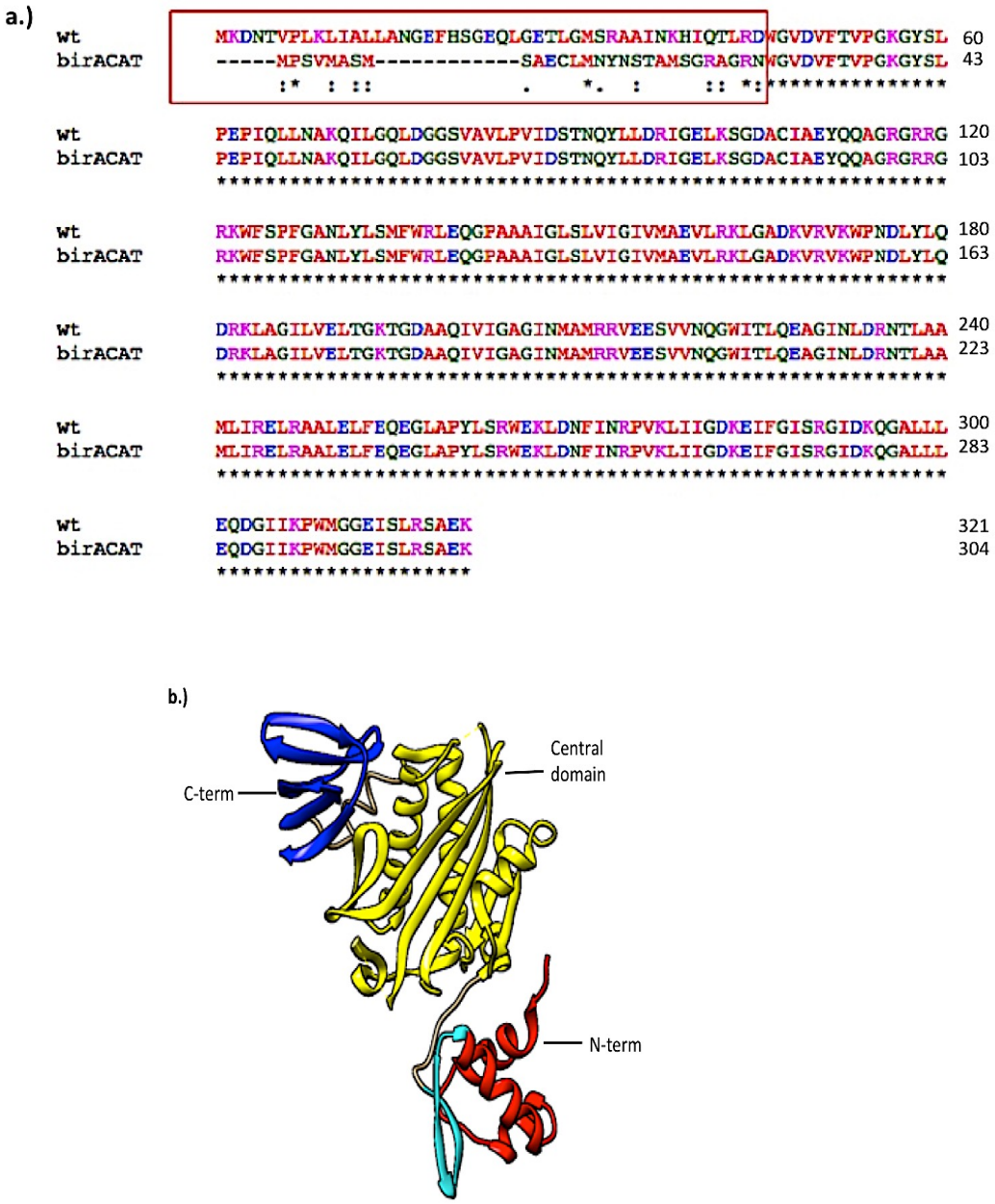


Figure 3.7: Amino acid sequence alignment of JD26186_*birA::CAT* vs. Wild-type. (**a.**) Altered sequence of amino acid 1-45 are shown in red box. The remaining sequence (amino acid 46-321) aligned perfectly with the wild-type sequence (**b.**) The out of frame translation corresponds to the disruption within the Helix-turn-Helix domain of *Ec*birA, as highlighted in red.

3.3.4 *In vivo* validation of the loss of *Ec*BirA repressor activity in *E. coli* JD26186 *birA*::CAT

In vivo analysis to validate the loss of DNA binding activity of the JD26186_*birA*::CAT, was performed by conducting β-galactosidase assays on JD26186_*birA*::CAT strain harboring a *EcbioO* promoter. *In vivo* assay using the parent strain, JD26186, containing the same promoter was also performed alongside the mutant strain, to serve as a control. Reduced affinity for biotin as a result of N-terminal disruption of endogenous *EcBirA*, in combination with the biotin auxotroph background of the parental strain, caused the mutant strain to require higher biotin concentrations to grow.

The use of rich media such as LB should provide a high biotin environment that is sufficient to support the growth of JD26186_*birA*::CAT and any repression capability of this mutant strain should be apparent under this high biotin condition. However, the concentration of biotin in LB media will vary, according to the specific batch and source of raw materials [35]. Therefore, to ensure a high biotin concentration was achieved and able to support the growth of the mutant strain, 500 nM of biotin was added to the LB media used in the *in vivo* assays, in parallel to LB media without additional biotin. Both wild-type and mutant strains were tested for *lacZ* repression in these media.

The result showed that the mutant strain was able to grow in both LB media with and without additional biotin, indicating there was sufficient biotin in the LB media to support growth, without the need to supplement biotin. β-galactosidase assay showed that in LB with additional biotin, the *EcbioO* promoter in JD26186_birA::CAT strain was not repressed, with activity measured at 150 ± 3.6 units, whereas the parental strain with an unmodified N-terminal

domain showed 11-fold lower β-galactosidase expression (13.7 ± 3.7 unit, $p < 0.0001$), suggesting repression of *EcbioO*. Interestingly, similar results were observed in LB media without additional biotin, where JD26186_*birA*::CAT strain also exhibited high β-galactosidase expression (140 ± 5.9 unit), whereas JD26186 exhibited 15.7-fold lower β-galactosidase expression (8.3 ± 1.8 unit13, $p < 0.0001$). These results confirmed that the ability of the modified *Ec*BirA in the JD26186_*birA*::CAT strain to repress *EcbioO* promoter has been successfully abolished, as indicated by the high expression of β-galactosidase in both of the LB media [**Figure 3.8a**].

Moreover, the aim of the assay development is to create a system that would facilitate measurable repression of target promoters, in response to varying external biotin concentrations. Development of the β-galactosidase assay in LB media was therefore not feasible, as a substantial repression of *EcbioO* promoter was observed in LB media without any additional biotin, indicating that the level of biotin in LB media was too high to allow measurable biotin-responsive repression. As an alternative, minimal media supplemented with a range of biotin concentrations (0.5nM – 500nM) was used in the β–galactosidase assay. Both JD26186 and JD26186_birA::CAT were tested for repression activity in this media. Both strains were able to grow in this media and, as expected, the wild-type strain showed dose-responsive repression, as opposed to JD26186_birA::CAT, where β–galactosidase expression remained high at all biotin concentrations tested [**Figure 3.8b**].

Furthermore, the mutant strain was able to grow in minimal media, even when the media was supplemented with low concentrations of biotin. As the cells were grown overnight in 100nM biotin-supplemented minimal media (refer to

materials and methods, **Chapter 2**), it was predicted that during this time the cells were able to accumulate biotinylated proteins and were able to pass on these biotinylated proteins to the daughter cells during replication, which was enough to sustain growth over a short period of time, prior to lysis. Multiple passage of this overnight culture in low biotin media showed the inability of the cells to grow (data not shown).

Overall, it is clear that the insertion of the CAT cassette into the designated section of the N-terminal domain of the endogenous *birA* was successful in rendering the endogenous *Ec*BirA to a non-functional repressor, as the inability to repress the target promoter was evident in this assay. Therefore, any repression observed in the reporter strain generated using this background strain should be due to the particular integrated repressor. In addition, dose-responsive repression exhibit by the wild-type strain in the minimal media indicated the suitability of this media to be used in the assay development.

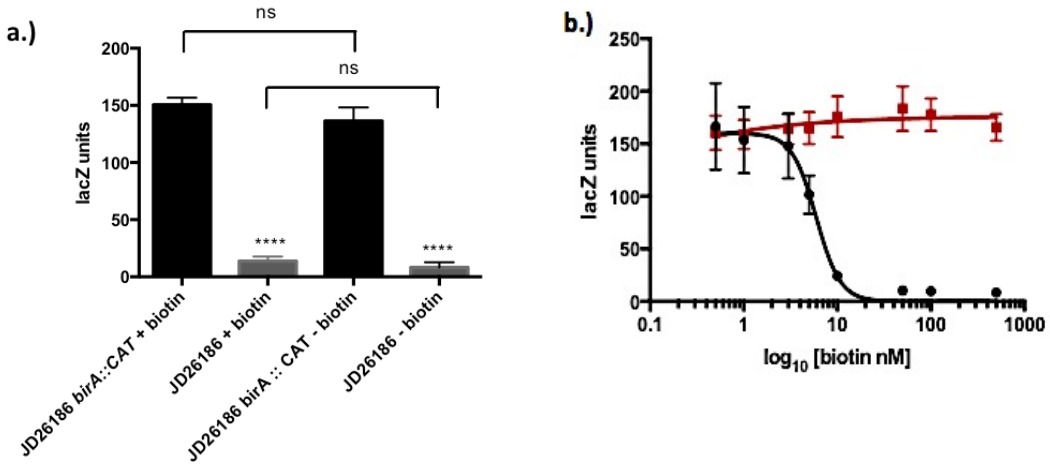


Figure 3.8: Validation of *E. coli* JD26186_*birA::CAT* DNA binding property impairment. *β-galactosidase* assay was employed to determine the DNA binding ability of the disrupted N-terminal mutant within the JD26186_*birA::CAT* strain compared to the wild-type *Ec*BirA within the JD26186 strain. (a.) Assay was performed initially in LB media (-/+) 500nM biotin, to ensure there was enough biotin to support the growth of the mutant strain. Error bars denote S.E.M, n = 3 (b.) Assay was repeated in minimal media with varying biotin concentrations. Both wild-type and mutant strains were able to grow in this media. Wild-type strain (black) was responsive to the increased biotin concentration, whereas repression in JD26186_*birA::CAT* strain (red) was not responsive to biotin concentration, indicating an un-repressed promoter. **** denote p <0.0001 as calculated by 2 tailed t-test. Error bars denote S.E.M, n =12

3.3.5. N-terminal disruption result in lower biotinylation efficiency of *Ec*BirA

The disruption of the N-terminal domain of *EcBirA* was predicted to affect its catalytic activity. Previous study performed by Xu and co-workers [23] had shown that *Ec*BirA with N-terminal deletion (*Ec*BirA 65-324) binds 100-fold weaker to biotin and 1000-fold weaker to the reaction intermediate, biotinyl-5′-AMP, compared to the wild-type. A BPL activity assay was then performed upon whole cell lysates from wild-type cells JD26186 and JD26186_*birA*::CAT, in order to compare their catalytic activities [**Figure 3.9**].

Whole cell lysate from JD26186 and JD26186 *birA*::*CAT* were prepared as described in **chapter 2**. An assay to measure the ligase activity of JD26186 *birA*::*CAT* was performed as described previously by Polyak *et al* [34]. Briefly, a biotinylation reaction was carried out by adding 4 µg of whole cell lysate into reaction buffer containing 50mM Tris.HCl pH 8.0, 3mM ATP, 5.5 mM $MgCl_2$, 5 µM biotin, 5 pmol ^{3}H-Biotin, 0.1 mM DTT, 0.1 mg/mL BSA and 10 µM of purified *S. aureus* biotin acceptor domain (*Sa*PC90). Reaction was performed at 37 °C for 7 hours. 20 µL of the reaction was then spotted onto filter papers that had been treated with biotin and trichloroacetic acid (TCA). Filter papers were then dried and washed 2x in 10% ice cold TCA and 1x in 100% ethanol. Filters were then dried and submerged in Optiphase supermix scintillation fluid (Perkin Elmer, USA) and radioactivity was measured using 1214 Racbetta liquid scintillation counter (LKB instrument, Australia).

The results clearly indicated that the biotinylation activity of JD26186 *birA*::*CAT* was compromised, as the amount of radiolabelled biotin incorporated into the acceptor protein was approximately 4-fold lower compared to the wild-type ($p < 0.05$). Thus, the disruption of the N-terminal domain by the insertion of a CAT cassette in this study is in agreement with the previous study described above, concluding that disruption of the N-terminal domain of *EcBirA* also affects catalytic function of this enzyme. However, this reduced enzyme activity due to N-terminal domain disruption was still sufficient to maintain cell growth.

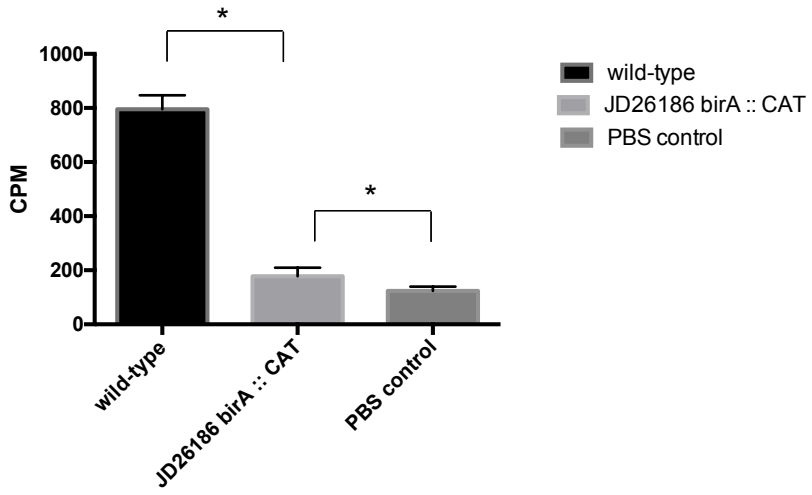


Figure 3.9: Activity assay of JD26186_*birA::CAT* vs. wild-type. ^{3}H-biotin was used in an *in vitro* activity assay of wild-type JD26186 and the N-terminal disrupted mutant, JD26186 *birA*::*CAT*. Approximately 4-fold less activity was observed in the mutant compared to wild-type. Assay was performed using 4 µg whole cell lysates as described in materials and methods, and no lysate sample (PBS only) served as control. The displayed asterisk symbolized statistical significance of $p < 0.05$.

3.3.6 Cloning of integration vectors and reporter strain assembly.

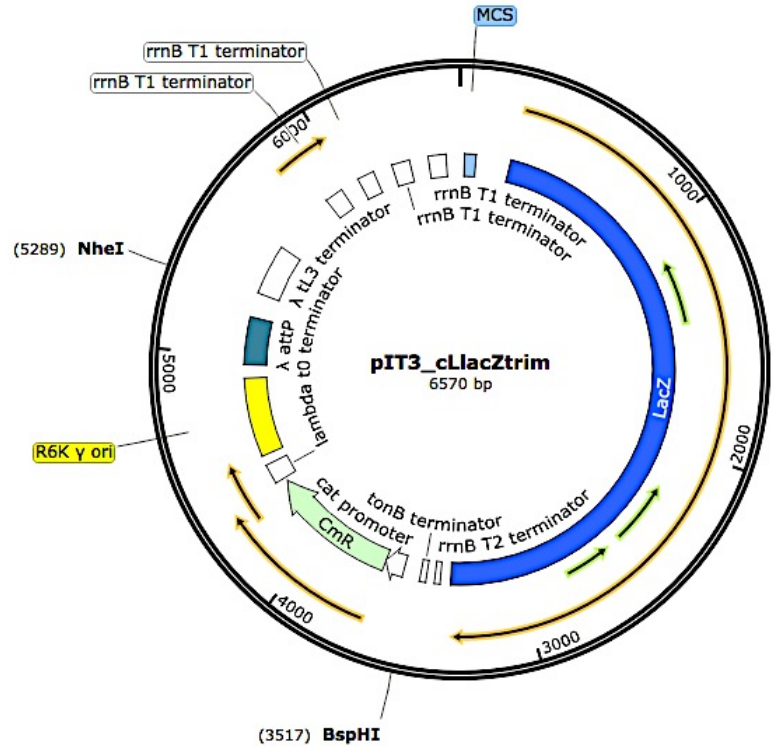
3.3.6.1 Construction of integration vectors containing fusion of target promoter and a *lacZ* gene

A series of vectors containing specific phage attachment sites for precise genome integration was designed. The placZSH_Trim integration vector carrying the attP-HK022 phage attachment site was engineered to contain the sequence of BirA binding site, in order to allow regulation by BirA. This integration vector was constructed by digesting pIT3_CLlacZ _Trim vector [36], **(Figure 3.10a)** with *B*spHI and *N*heI restriction enzyme to remove the Cam^R gene and replacing it with spectinomycin resistance gene (Sm^R) from pIT3-SH-152002 vector

containing attB-HK022 Phage attachment site [16] **(Figure 3.10b)**, to give rise to another vector, placZSH-Trim.

Double-stranded oligonucleotide containing promoter sequence (*EcbioO*, *SabioO, SabioY* and *SayhfS-SayhfT,* respectively) was ligated upstream of the *lacZ* gene in the placZSH-Trim vector, as *K*pnI and *S*phI fragments. These oligonucleotides were designed to include BirA binding sites. It is known that *Ec*BirA binds to the *bioO* operator site and occupies the -35 and -10 of the operator region [37, 38] **(Figure 3.11a)**. Therefore, oligonucleotides were designed to comprise the -50 bp to +20bp of the transcription start site. For *S. aureus*, it is not known if *Sa*BirA also occupies the -35 and -10 region of the operator region of all three target promoters. Therefore, the *S. aureus* promoters were designed to include -50 bp of the transcription start site, to the ATG translation start codon **(Figure 3.11b- 3.11d)**. Vector transformation was conducted in *E. coli* E2878 *Pir*$^{+}$ strain, to allow replication of these vectors. The resulting integration vector pLacZSH_Trim is outlined in **(Figure 3.12).**

a.)



b.)

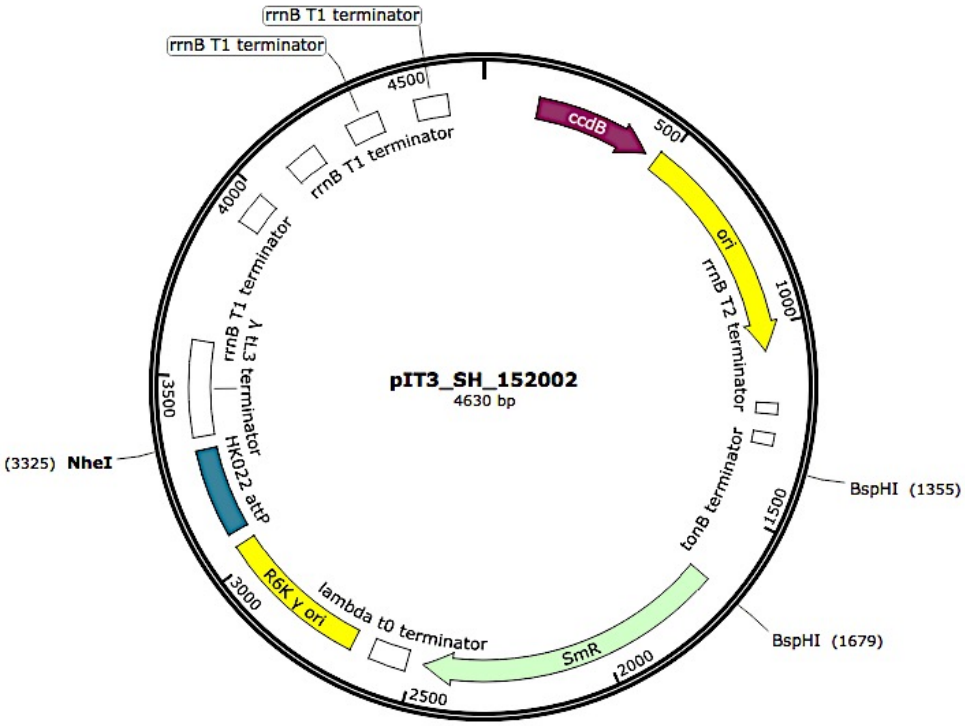


Figure 3.10. **Parent vectors of pLacZSH_Trim**. Integration vector pLacZSH_Trim was created using the parent vector (a.) pIT3_CLlacZtrim containing *lacZ* gene. (b.) attP-HK022 and Spectinomycin resistance gene was acquired from pIT3_SH_15002 vector.

a.) ***EcbioO* DS oligo**

5′ CTTGTCATAATCGACTTGTAAACCAAATTGAAAAGATTTAGGTTTACAAGTCTACACCGAATTAACAACAAAAGCATG
3′ CATGGAACAGTATTAGCTGAACATTTGGTTTAACTTTTCTAAATCCAAATGTTCAGATGTGGCTTAATTGTTGTTTTC

b.) ***SabioO* DS oligo**

5′ CGAAAAACATGCGCCTTAAATGTAAACTTATTAATTATAAAAGTTTACATTCGGATTGAGGTGCTTATTTTTTGCATG
3′ CATGGCTTTTTGTACGCGGAATTTACATTTGAATAATTAATATTTTCAAATGTAAGCCTAACTCCACGAATAAAAAAC

c.) ***SabioY* DS oligo**

5′ CTAATTTATATAGAACAACTTATTGTAAACTTTTCATTTCTTAAAGTTTACAATGGTGCTATAATAATGGTCATGAAATACGAAAGGAAGTAAAGCATG
3′ CATGGATTAAATATATCTTGTTGAATAACATTTGAAAAGTAAAGAATTTCAAATGTTACCACGATATTATTACCAGTACTTTATGCTTTCCTTCATTTC

d.) ***SayhfS-SayhfT* DS oligo**

5′ CAAAAATACTTATTCATTATATAATGTTAACAAGATGTATTTTAAAGTTTACATTGAGTGAGGGATATTGGCATG
3′ CATGGTTTTTATGAATAAGTAATATATTACAATTGTTCTACATAAAATTTCAAATGTAACTCACTCCCTATAACC

Figure 3.11: Double stranded oligonucleotide design for *S. aureus* promoter constructs Each DS-oligonucleotide contained the BirA binding sites (red letters) and bases upstream and down stream of this binding site, comprising the promoter. Blue highlighted sequences are the overhang of the restriction enzymes used in ligation. (a.) *EcbioO* promoter sequence is well characterized, the -35 and -10 region of the operator are underlined. (b.) *SabioO,* (c.) *SabioY* and (d.) *SayhfS-SayhfT* promoter sequence chosen for the DS-oligonucleotide.

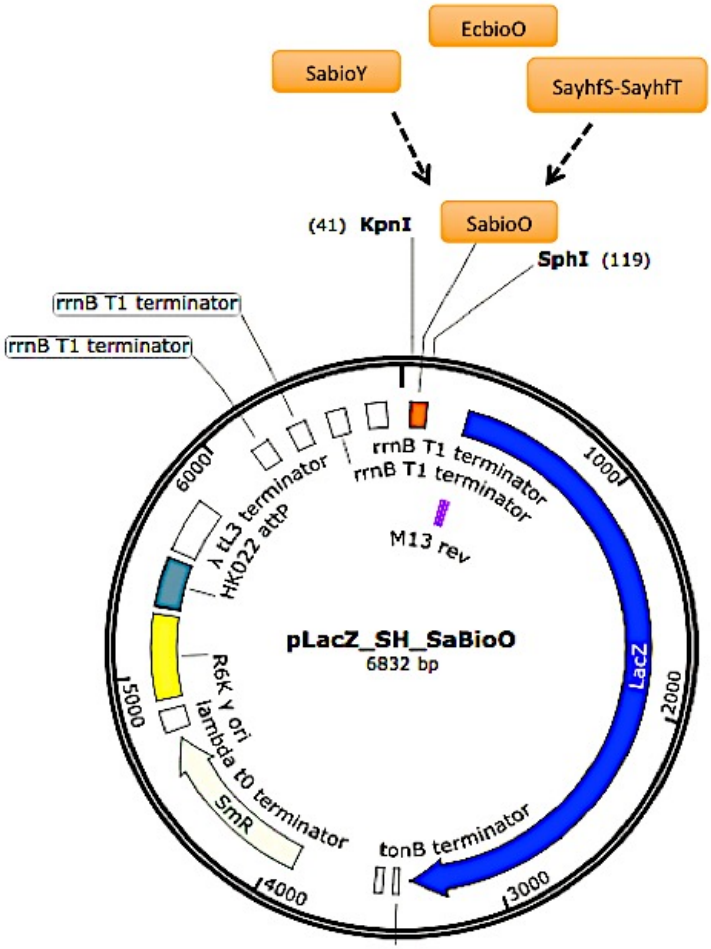


Figure 3.12: Integration vector pLacZSH_Trim. Promoter constructs (orange boxes, representing sequences outlined in Figure 3.11) were designed to be ligated upstream of LacZ gene as *K*pnI and *S*phI fragment. Chromosomal integration of construct assemble in this vector is targeted for HK022 phage attachment site (green box). Spectinomycin selection of positive integrant is made possible due to the spectinomycin resistance gene donated by the pIT3_SH_152002 parent vector.

3.3.6.2 Construction of integration vectors containing repressor gene

Integration vectors containing repressor genes (*Ec*BirA and *Sa*BirA) were engineered for IPTG-inducible protein expression. Double-stranded oligonucleotide containing *plac* promoter sequence flanked with *Bs*phI and *P*ciI flanking sequences on the 5′- and 3′- end of the oligonucleotide, respectively

(Figure 3.13a), was first ligated upstream of the *SaBirA* gene in the *P*ciI-treated pGEMT-*SaBirA*(6x his) vector [39] **(Figure 3.13b)**. The *B*spHI overhang is compatible with the *P*ciI overhang, but ligation of these overhangs will eliminate both restriction sites. The *plac-SaBirA* construct was then excised from the purified pGEMT_*plac*_SaBirA **(Figure 3.13c)** using *K*pnI and *S*phI restriction enzymes and sub-cloned into pIT4_TL_152002 vector carrying attP-λ phage attachment site [40], to generate a new integration vector pIT4_*pLac_SaBirA* (6xhis) **(Figure 3.14)**. Sequence manipulation in the pGEMT cloning vector was preferred, as this vector is a high copy vector that yielded suitable quantities of plasmid for cloning, as opposed to the low copy integration vector pIT4_TL_152002.

For the monomeric repressor (*SaBirA* F123G) construct, the same *pLac* oligonucleotide was ligated upstream of previously constructed vector, pGEMT-*SaBirA* F123G (6x his)[17]. The resulting fusion of *pLac- SaBirA (F123G)* construct was then sub-cloned into pIT4_TL152002 as a *K*pnI/*S*phI fragment, generating integration vector construct pIT4_*pLac_SaBirA F123G* (6xhis).

For the integration vector carrying the monomeric *Ec*BirA repressor (*EcBirA* R119W) construct, site-directed mutagenesis was performed on pGEMT-*EcBirA* (6xhis) plasmid using B460-B461 primers to generate pGEMT-*EcBirA* R119W (6xhis). Mutation was confirmed by sequencing. PCR was then performed to isolate the *EcBirA* R119W gene from this plasmid using primers B479-B480 to yield a fragment of *EcBirA* R119W with a *B*spHI restriction site on the 5′ end and *S*phI restriction site on the 3′ end. This fragment was then digested with the respective restriction enzymes and ligated to a vector backbone pIT4-plac, prepared by digesting pIT4-plac-*Sa*BirA vector with *P*ciI and *S*pHI restriction

enzyme. Manipulations of the pIT4- derived vectors were performed in *E. coli* E2878 *Pir*$^{+}$ strain, to allow replication of the integration vector and transformed colonies were plated onto LB agar containing 4 μg/mL tetracycline.

Furthermore, several attempts to clone the wild-type *Ec*BirA gene into the integration plasmid were not successful. Therefore, another strategy was employed in order to generate a reporter strain containing wild-type *Ec*BirA repressor, the details involved in generating this strain will be discussed in **section 3.3.6.5**.

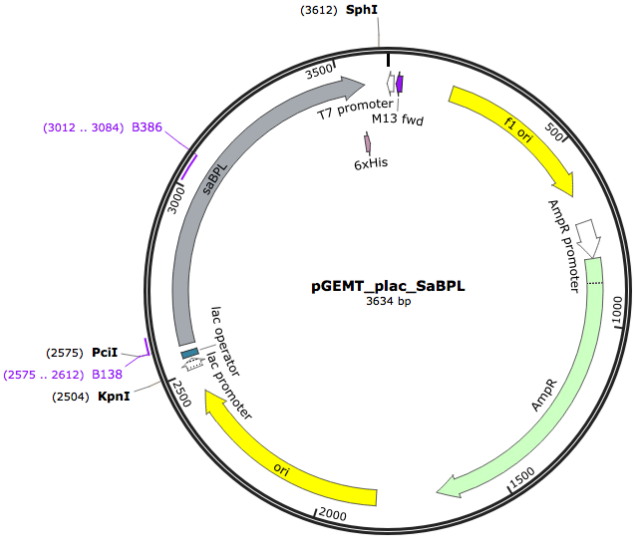
3.3.6.3. Construction of integration vectors to be used in control strains.

Strains containing pLacZSH-Trim (i.e. no-promoter construct) and pIT4-TL-empty (i.e. no-repressor construct) were required to act as controls in the *in vivo* β-galactosidase assay. As pIT4_TL_152002 vector contained the *ccdB* gene encoding a toxic protein [41-43], direct transformation of this plasmid would not result in viable colonies. Therefore, the *ccdB* gene was removed from this vector prior to transformation, by digesting the vector with *B*amHI / *B*glII restriction enzymes followed by self-ligation to generate a new vector, pIT4_TL_Empty. Diagrams of integration vectors used in the assembly of control strains are presented in **Figure 3.15-3.16**

a.)

BamHIKpnI pLac lacO RBS PciI
5′catgcggtaccTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAa
gccatggAACTGTTAATTAGTAGCCGAGCATATTACACACCTTAACACTCGCCTATTGTT AAAGTGTGTCCTTTGTTgtac

b.)



C.)

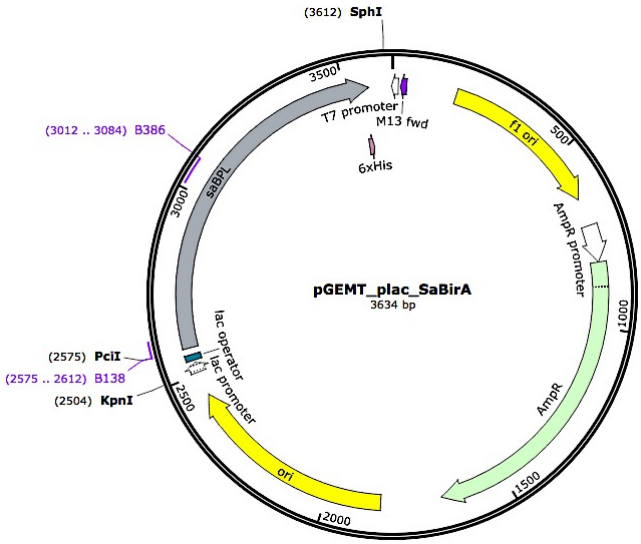


Figure 3.13: Construction of integration vector carrying the repressor genes. (a.) Double stranded oligonucleotide containing *plac* promoter sequence was ligated upstream of the repressor gene in the parent vector, **(b.)** pGEMT-*SaBirA*(6x his) vector. (c.) The resulting construct of pGEMT-plac-*SaBirA*(6x his) contained plac promoter and a *K*pnI restriction site, introduced in the double stranded oligo. *SaBirA* gene is indicated as *Sa*BPL in the diagram.

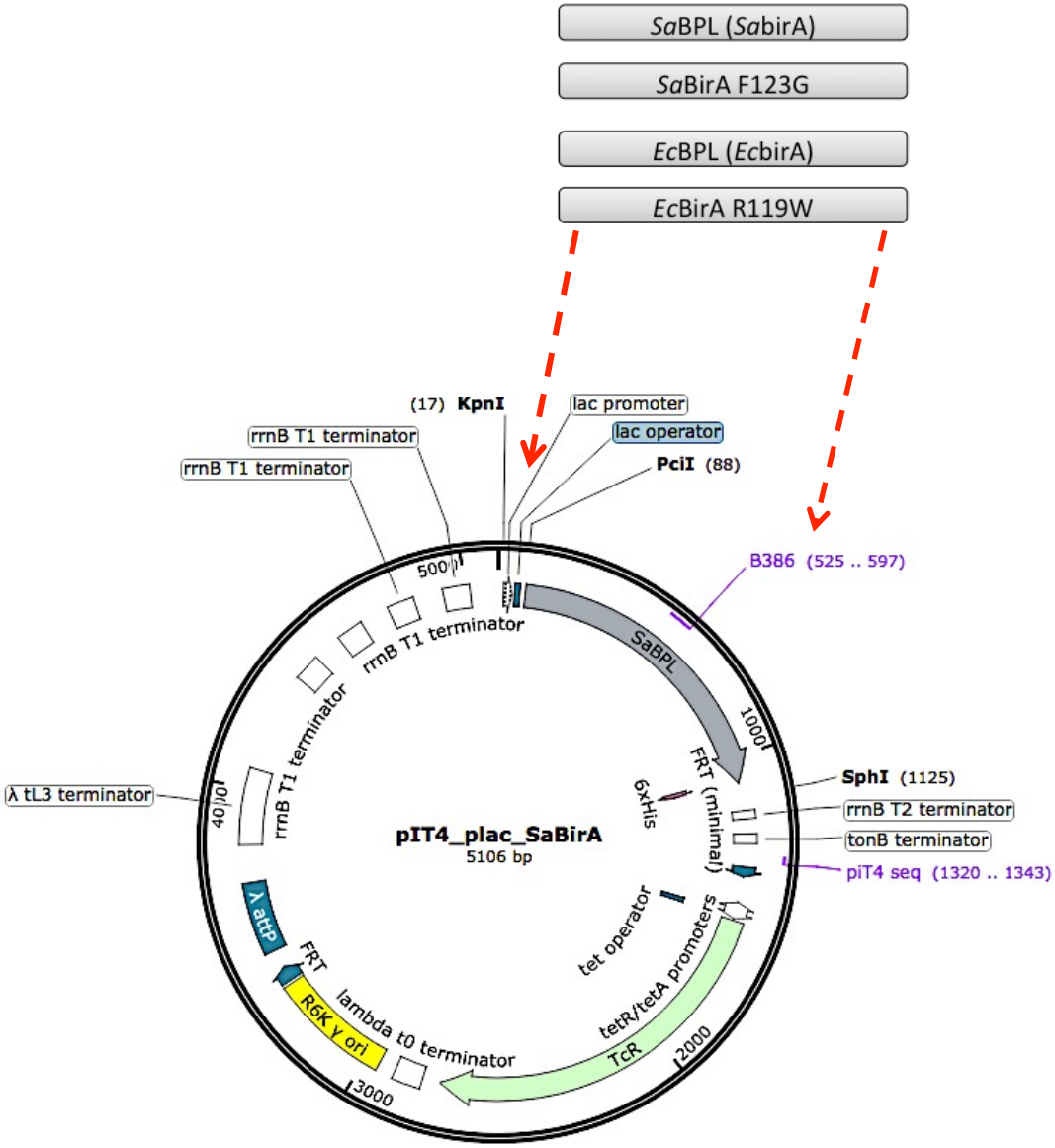


Figure 3.14: Vector map of integration construct carrying the repressor gene
Fusion of plac::repressor (plac::*Sa*BirA) extracted from pGEMT-plac-*SaBirA*(6x his) was ligated into the parent integration vector, pIT4_TL_152002, in order to generate the final construct, pIT4_plac_*Sa*BirA. Other genes encoding for different repressors were also generated using the same construction method. *SaBirA* gene is indicated as *Sa*BPL in the diagram.

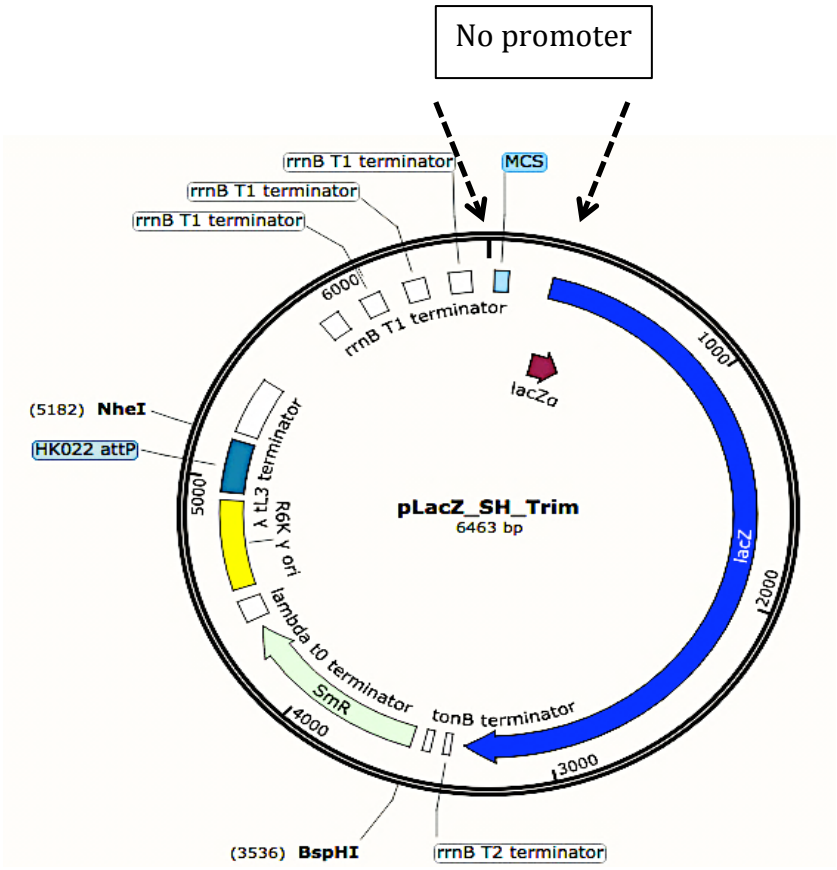


Figure 3.15: Integration vector to be used in assembly of control strain containing "no-promoter" construct. placZSH_Trim with no-promoter was used to transform the background strain in order to generate a "no-promoter " control strain thus l*acZ* gene with no promoter construct was integrated into attB-HK022 in this strain.

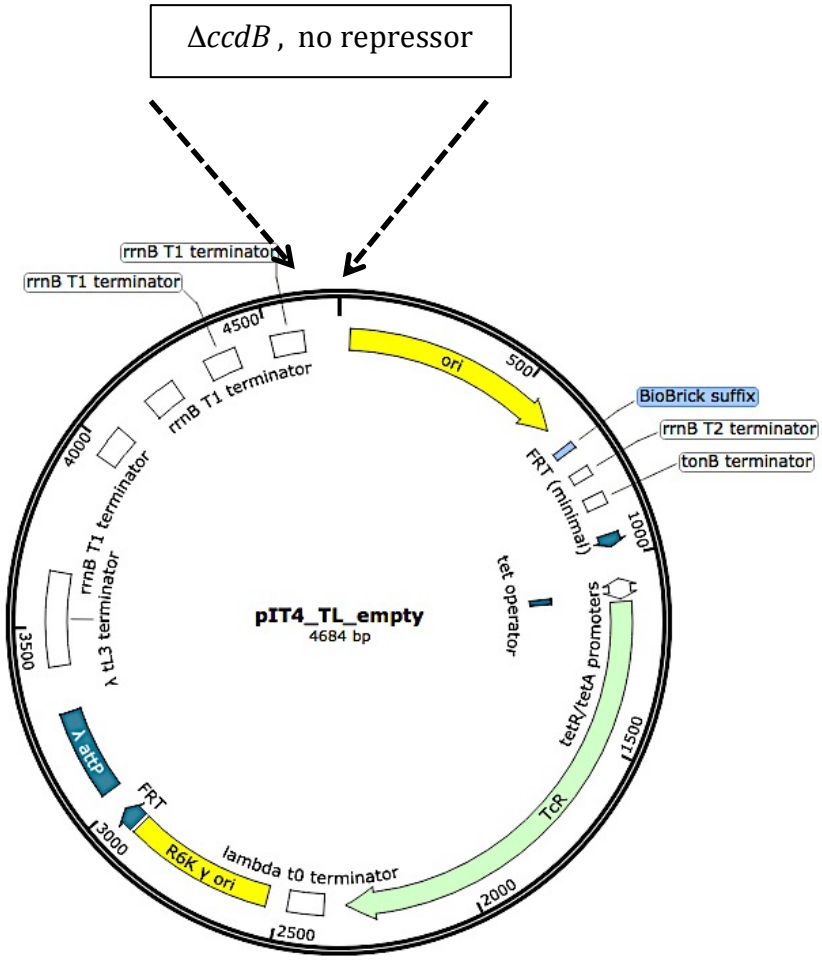


Figure 3.16: Integration vector to be used in assembly of control strain containing "no-repressor" construct. pIT4_TL_152002 was modified by removing the toxic *ccdB* gene followed by self-ligation, no repressor gene was cloned into this vector, this empty plasmid with no repressor construct was integrated into the attP- λ in the background strain to provide a "no-repressor " control strain

3.3.6.4 Integration vector transformation and reporter strain assembly

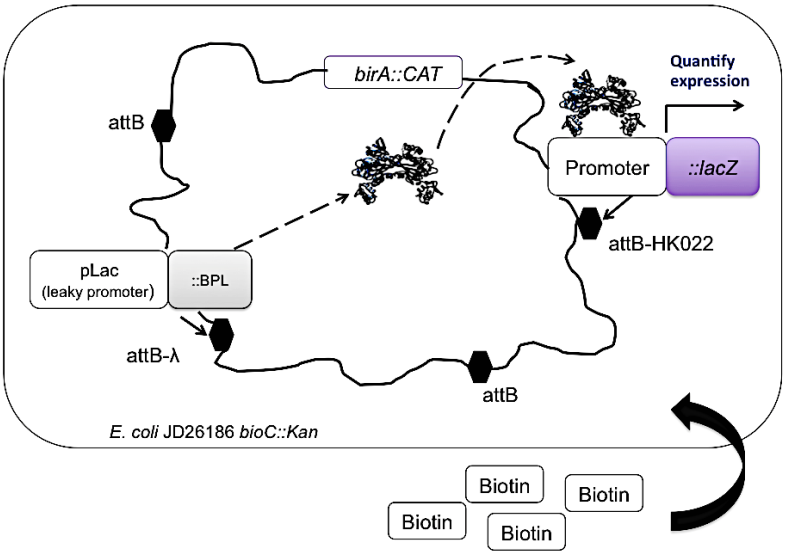
These integration vectors were subsequently employed to transform the background strain JD26186_ *birA*::CAT in order to integrate a single copy of each construct, site specifically into the *E. coli* chromosome using the designated phage attachment site. Firstly, the strain was transformed with the pLacZSH_Trim-

derived integration vectors containing the promoter construct. Transformants were screened using PCR and positive colonies were subsequently selected and transformed with the pIT4-TL-152002 -derived integration vectors containing the repressor construct. The protocol of the chromosomal integration and PCR screening is presented in **chapter 2**. Diagrams outlining these reporter strains are presented in **(Figure 3.17)**

3.3.6.5. Construction of reporter strain containing wild-type *Ec*BirA

Despite multiple attempts, cloning of wild-type *EcBirA* construct into the integration vector was not successful. Previous study revealed that the over-expression of *EcBirA* affects cell viability due to a toxicity effect [44, 45]. It is likely that in the host strain, *E. coli* E2878 (pir^+), the production of lac repressor was insufficient to create a complete suppression of the toxic gene product, which resulted in non-viable colonies during the cloning process. This hypothesis, however, was not investigated further and cloning of the wild-type *Ec*BirA into the integration vector was aborted. As an alternative, the parent strain JD26186 with non-disrupted *birA* was utilized to provide wild-type *EcBirA*. Therefore, a reporter strain containing wild-type *birA* was generated by transforming *pLacZ-SH-EcbioO*, into JD26186 **(Figure 3.18)**. This same reporter strain was also used as a control in *in vivo* assays to validate the loss of repressive ability of endgenous *Ec*BirA in the JD26186_birA::CAT strain, as described in **section 3.3.5**.

a.)



b.)

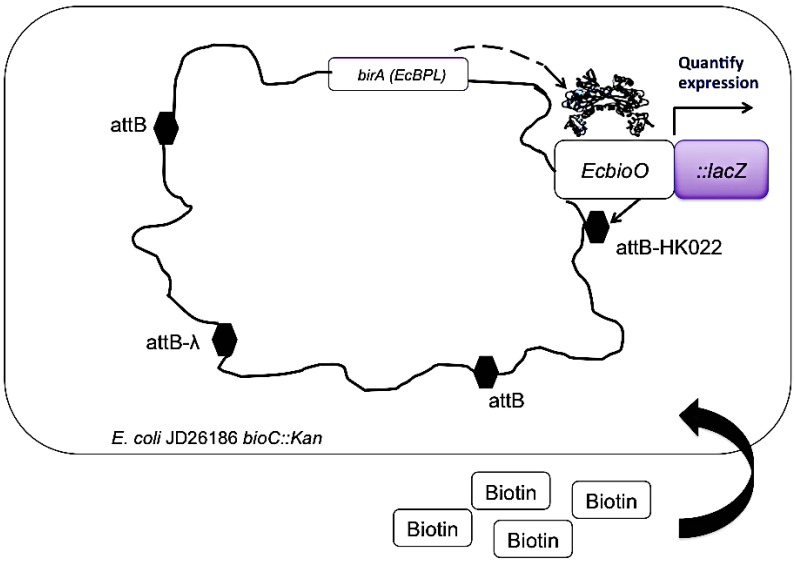
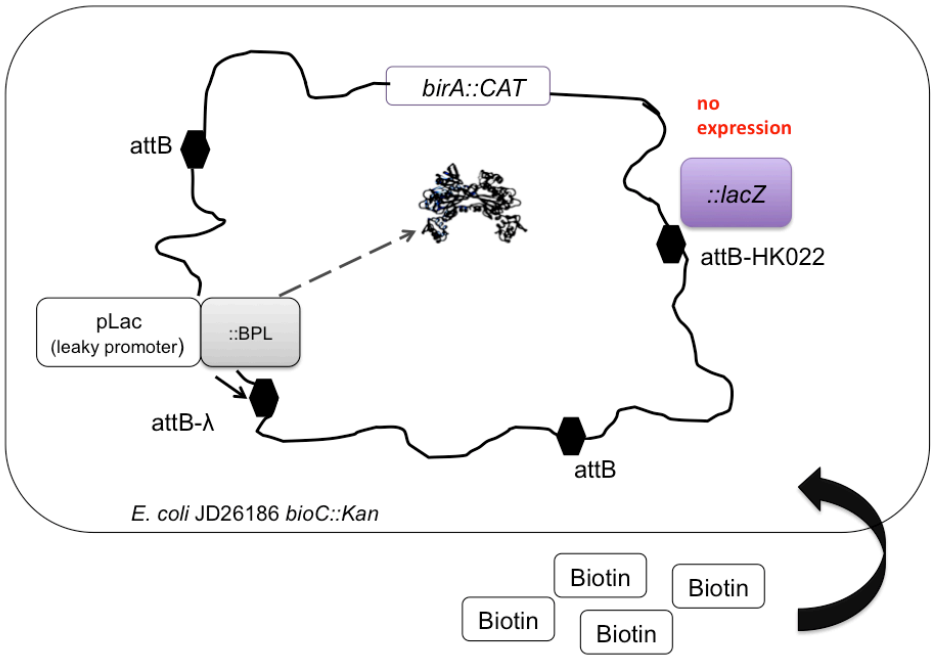


Figure 3.17: Diagram of reporter strains generated in this study. **(a.)** A modified version of the parent strain, JD26186_*birA::CAT* was used to generate reporter strain containing BirA constructs and the respective promoter. Expression of the repressor gene is driven by a leaky *pLac* promoter, as discussed in **section 3.3.8 (b.)** For wild-type *EcBirA* construct, parent strain JD26186 was used as the background strain and *EcBirA* production were sourced from the endogenous gene. Biotin levels could be controlled by adding them directly to the media, due to the biotin auxotroph nature of the strain. *In vivo* β-galactosidase assay to assess the repression of *lacZ* gene by each repressor in response to varying biotin concentration could then be performed.

a.)



b.)

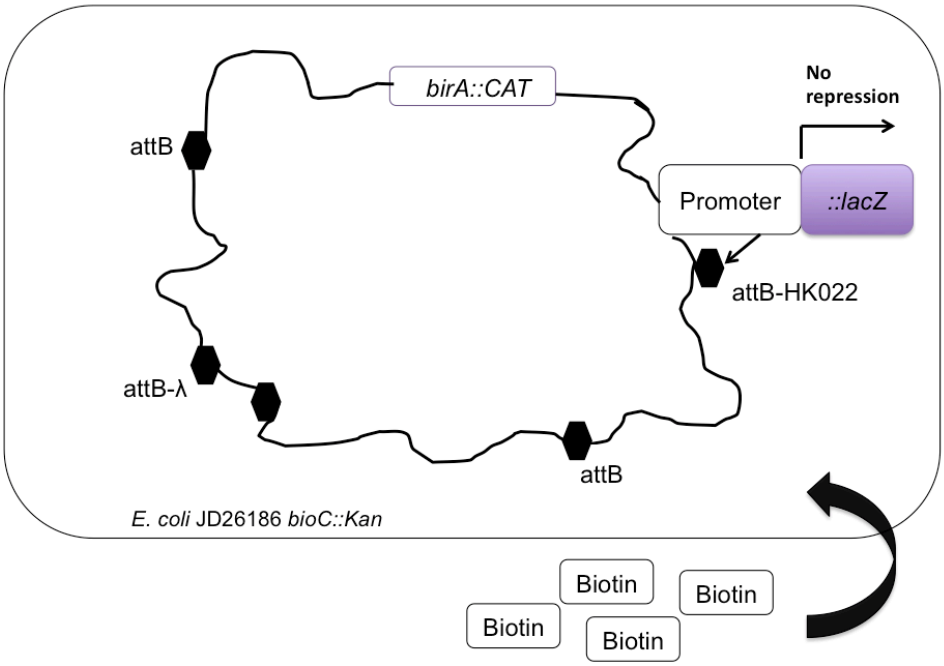


Figure 3.18: Control strains containing "no-promoter" and "no-repressor" constructs. (a.) no-promoter control strain and (b.) no-repressor control strain were also generated to validate that any repression observed was specific to integrated repressor and promoter.

3.3.7 β-galactosidase assay optimisation.

Following the completion of reporter strain construction, these strains were then tested in β-galactosidase assays for their ability to generate repression of target promoters *in vivo*. The expression of repressor genes in the reporter strain were driven by the lac promoter, therefore, the addition of IPTG should induce the production of these repressors which will then interact with their respective promoter, in the manner that is dictated by the introduced biotin level. Control strains provide evidence that the repression was specific, such that the control strain without integrated repressor should only show basal expression of β –galactosidase as produced by a promoter of interest and control strain without this promoter should not produce any β –galactosidase expression.

In order to determine the optimal IPTG concentration in this assay, a strain containing *Sa*BirA repressor and *SabioO* promoter was tested for repression in the presence of 0.01 mM IPTG and no IPTG. In addition, to ensure that *Sa*BirA F123G could be expressed in this system and that the mutation created does not interfere with the expression of this monomeric mutant, the reporter strain with integrated *Sa*BirA F123G construct was also included in the test.

It was expected that without IPTG, the β-galactosidase activity would remain high, as no repressor should be produced without induction. Unexpectedly, the result showed that in the absence of IPTG, biotin-responsive repression of *SabioO* promoter was still observed **(Figure 3.19a)**, which suggests the possibility that *Sa*BirA was still being produced. In addition, the result also showed that when the strains were induced with 0.01mM IPTG, no expression of β–galactosidase was observed at any biotin concentration **(Figure 3.19b)**. This

result indicated that the addition of IPTG at this concentration did not result in biotin-responsive repression, but instead, complete repression of the promoter was observed. This was probably due to the high level of *Sa*BirA expression, as a result of the 0.01 mM IPTG induction. Based on this data, it was concluded that the addition of IPTG was not necessary as biotin-responsive repression of the promoter was achieved without any addition of IPTG, most likely due to the leaky *pLac* promoter.

Additionally, in order to verify this leaky promoter, a western blot was performed to detect the production of *Sa*BirA in the absence and presence of 0.01 mM and 0.1mM IPTG. Each of the reporter constructs were engineered to express *Sa*BirA (6xhis) therefore expression can easily be detected using an anti-6xhis antibody in a western blot. The result confirmed "leaky" expression of *Sa*BirA, as expression of *Sa*BirA was detected in the whole cell lysate grown in the absence of IPTG. Addition of 0.01mM and 0.1 mM IPTG resulted in substantial increased in *Sa*BirA expression, as indicated in **(Figure 3.19c)**. The parent strain JD26186 was derived from *E. coli* BW25113, a strain known to possess $lacI^{+}$ $lacZ^{-}$ phenotype. Therefore, this strain should produce lac repressor. However, in this instance, it is likely that this strain might not produce enough lac repressor to fully repress the strong activity of the *lac* promoter, which resulted in basal expression of the repressor protein. The response generated from this "leaky" lac promoter appeared to be sufficient to generate a measureable response, as shown in **(Figure 3.19a)**. Thus, subsequent assays were performed without the addition of IPTG.

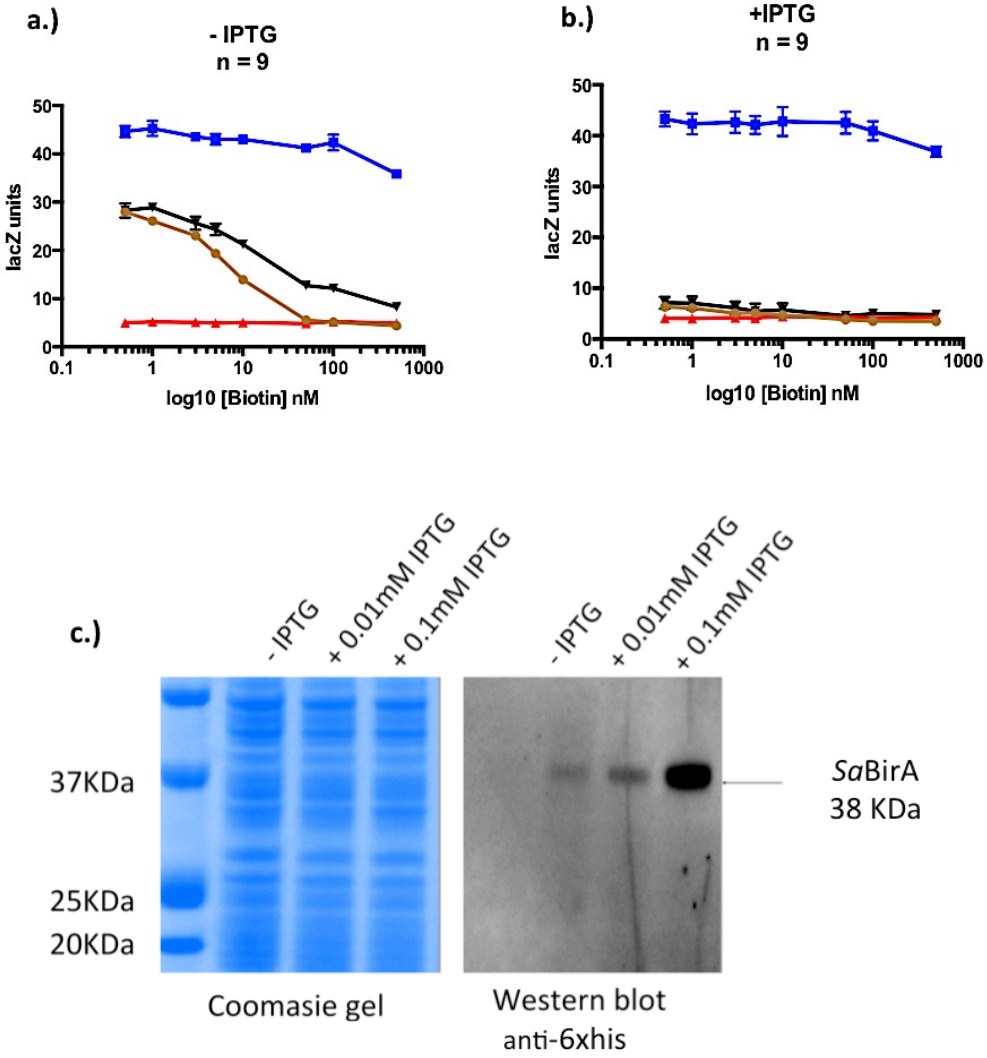


Figure 3.15: Repression profile in the presence and absence of IPTG.
(a.) Repression profile of the reporter strains in the absence of IPTG and (b) in the presence of 0.01mM IPTG; blue line represents no-repressor control, red line represents no-promoter control, black line represents repressor strain containing repressor monomeric mutant (*Sa*BirA F123G) and brown line represents strain with wild-type *Sa*BirA. β-galactosidase assay was performed to test the repression pattern in response to the increasing amount of biotin. Error bars denote S.E.M. of n = 9. (c.) Leaky expression of *Sa*BirA was detected in western blot experiment (right panel) and loading control of Coomassie gel was shown (left panel).

3.3.9 Discussion

In this study, a series of *E. coli* reporter strains were successfully generated. Firstly, the repressor ability of the endogenous *Ec*BirA in the parental strain, *E. coli* JD26186, was abolished by insertion of CAT cassette into a specific site in the N-terminal domain, as confirmed in β-galactosidase assay. This modified strain was then used as the background strain in generating various reporter strains. Each strain contained a particular repressor of interest (*Sa*BirA wild-type, *Sa*BirA F123G, or *Ec*BirA R119W) and their respective promoter. Due to unsuccessful attempts to clone the wild-type *Ec*BirA into the integration vector, endogenous *Ec*BirA of the parental strain was used in the reporter strain containing wild-type *Ec*BirA.

The use of β-galactosidase reporter gene assay to study the repressor function of BirA has been demonstrated in previous studies. Chakravartty and co-workers [46] developed a similar assay to screen for super-repressor mutants of *Ec*BirA. The *in vivo* system created by these researchers involved deletion of the endogenous *Ec*BirA and a disruption of biotin biosynthesis operon by insertion of *LacZY* gene into the *bioF* coding region of *E. coli* MG1655 derivative, using phage P1 bacteria transduction. Although this system allowed measurable repression of *lacZ* by the *Ec*BirA repressor, the generation of this strain involved many experimental stages, and would have taken a long time to complete.

The general protocol for P1 phage transduction required up to five days to complete [47, 48] as opposed to two days required to generate the reporter strain described in this chapter. In addition, the fact that the endogenous *Ec*BirA was deleted, the bacteria needed to be transformed with another plasmid expressing another BirA protein to complement the loss of *Ec*BirA, as the absence

expressing another BirA protein to complement the loss of *Ec*BirA, as the absence of this enzyme would lead to cell death. In the assay developed in this study, the need to complement the loss of catalytic ability of *Ec*BirA is avoided, as the endogenous *Ec*BirA of the reporter strain still retained its catalytic activity but at the same time, would not create any repression background, as the DNA binding domain is disrupted. Moreover, the use of the i*n vivo* system described by these authors was limited to analysis involving *EcbioO* promoter and *Ec*BirA, as opposed to the assay developed in this study where the system can be applied to analyze the interactions of other transcription factors with their respective promoters.

Additionally, another reporter assay was developed in *Bacillus subtillis* by Henke *et al* [49] to assess the ability of wild-type and mutants with deleted DNA binding domain of *B. subtillis* BPL to regulate biotin biosynthesis. These authors created a reporter assay whereby the gene encoding one of the biotin biosynthesis enzymes, *bioW*, under the control of *B. Subtillis bioO* promoter (*Bs-bioO*) was fused to *LacZY* gene using single cross over integration. Although the *B. subtillis birA* in this modified strain could be replaced by other *BirA* protein, such as *Sa*BirA, using gene replacement method as demonstrated in the subsequent work from these same authors [50], this system only allowed analysis involving *Bs-bioO*. Modification of the promoter in this system would be difficult and has not been demonstrated. In contrast, the *in vivo* system described in this study allowed analysis of more than just one type of promoter to be performed.

The reporter strains generated in this study were then used to analyze the binding of *Sa*BirA to the three different *S. aureus* promoters *in vivo,* and comparing the interaction to the well established binding mechanism of *Ec*BirA to

the *EcbioO* promoter, as discussed in **chapter 4**. In addition, this platform was also used to analyze the interaction of a *Sa*BirA mutant (*SaBirA* D200E), isolated from advanced resistance studies, with *SabioO* and *SabioY* promoters, as detailed in **chapter 5**. Finally, the integration vectors generated in this study have been designed in such a way that cloning of other promoter or repressor constructs should be relatively straight forward, therefore allowing this reporter strain to be applied to other transcription factors involved in biotin synthesis in organism with class I BPL, such as BioQ [51, 52] and BioR [53], as will be discussed in **chapter 6**.

Reference:

1. Monk, I.R. and T.J. Foster, *Genetic manipulation of Staphylococci-breaking through the barrier.* Front Cell Infect Microbiol, 2012. **2**: p. 49.
2. Loenen, W.A., et al., *Type I restriction enzymes and their relatives.* Nucleic Acids Res, 2014. **42**(1): p. 20-44.
3. Xu, S.Y., et al., *A type IV modification-dependent restriction enzyme SauUSI from Staphylococcus aureus subsp. aureus USA300.* Nucleic Acids Res, 2011. **39**(13): p. 5597-610.
4. Corvaglia, A.R., et al., *A type III-like restriction endonuclease functions as a major barrier to horizontal gene transfer in clinical Staphylococcus aureus strains.* Proc Natl Acad Sci U S A, 2010. **107**(26): p. 11954-8.
5. Monk, I.R., et al., *Complete Bypass of Restriction Systems for Major Staphylococcus aureus Lineages.* MBio, 2015. **6**(3): p. e00308-15.
6. Arber, W. and S. Linn, *DNA modification and restriction.* Annu Rev Biochem, 1969. **38**: p. 467-500.
7. Datta, N., *Escherichia coli as a genetic tool.* J Hyg (Lond), 1985. **95**(3): p. 611-8.
8. Davison, J., *Genetic tools for pseudomonads, rhizobia, and other gram-negative bacteria.* Biotechniques, 2002. **32**(2): p. 386-8, 390, 392-4, passim.
9. Feng, Y., et al., *A Francisella virulence factor catalyses an essential reaction of biotin synthesis.* Mol Microbiol, 2014. **91**(2): p. 300-14.
10. Dussoix, D. and W. Arber, *Host Specificity of DNA Produced by Escherichia Coli. Iv. Host Specificity of Infectious DNA from Bacteriophage Lambda.* J Mol Biol, 1965. **11**: p. 238-46.
11. Whipple, F.W., *Genetic analysis of prokaryotic and eukaryotic DNA-binding proteins in Escherichia coli.* Nucleic Acids Res, 1998. **26**(16): p. 3700-6.
12. Brophy, J.A. and C.A. Voigt, *Principles of genetic circuit design.* Nat Methods, 2014. **11**(5): p. 508-20.
13. Bertram, R. and W. Hillen, *The application of Tet repressor in prokaryotic gene regulation and expression.* Microb Biotechnol, 2008. **1**(1): p. 2-16.
14. Prindle, A., et al., *Genetic Circuits in Salmonella typhimurium.* ACS Synth Biol, 2012. **1**(10): p. 458-464.
15. Sleight, S.C., et al., *Designing and engineering evolutionary robust genetic circuits.* J Biol Eng, 2010. **4**: p. 12.
16. St-Pierre, F., et al., *One-Step Cloning and Chromosomal Integration of DNA.* Acs Synthetic Biology, 2013. **2**(9): p. 537-541.
17. Soares da Costa, T.P., et al., *Dual roles of F123 in protein homodimerization and inhibitor binding to biotin protein ligase from Staphylococcus aureus.* Mol Microbiol, 2014. **91**(1): p. 110-20.
18. Barker, D.F. and A.M. Campbell, *Genetic and biochemical characterization of the birA gene and its product: evidence for a direct role of biotin holoenzyme synthetase in repression of the biotin operon in Escherichia coli.* J Mol Biol, 1981. **146**(4): p. 469-92.
19. Barker, D.F. and A.M. Campbell, *The birA gene of Escherichia coli encodes a biotin holoenzyme synthetase.* J Mol Biol, 1981. **146**(4): p. 451-67.
20. Kwon, K. and D. Beckett, *Function of a conserved sequence motif in biotin holoenzyme synthetases.* Protein Sci, 2000. **9**(8): p. 1530-9.

21. Weaver, L.H., et al., *Corepressor-induced organization and assembly of the biotin repressor: a model for allosteric activation of a transcriptional regulator.* Proc Natl Acad Sci U S A, 2001. **98**(11): p. 6045-50.
22. Miki, T., Yamamoto, Y., Matsuda, H, *A novel, simple, high-throughput method for isolation of genome-wide transposon insertion mutants of Escherichia coli K-12*. Methods in Molecular Biology. Vol. 416. 2008. 195-204.
23. Xu, Y. and D. Beckett, *Evidence for interdomain interaction in the Escherichia coli repressor of biotin biosynthesis from studies of an N-terminal domain deletion mutant.* Biochemistry, 1996. **35**(6): p. 1783-92.
24. Chakravartty, V. and J.E. Cronan, *The wing of a winged helix-turn-helix transcription factor organizes the active site of BirA, a bifunctional repressor/ligase.* J Biol Chem, 2013. **288**(50): p. 36029-39.
25. Mayende, L., Swift, R. D., Bailey, L. M., Soares da Costa, T. P., Wallace, J. C., Booker, G. W., Polyak, S. W., *A novel molecular mechanism to explain biotin-unresponsive holocarboxylase synthetase deficiency.* J Mol Med (Berl), 2012. **90**(1): p. 81-8.
26. Datta, S., N. Costantino, and D.L. Court, *A set of recombineering plasmids for gram-negative bacteria.* Gene, 2006. **379**: p. 109-15.
27. Datsenko, K.A. and B.L. Wanner, *One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products.* Proc Natl Acad Sci U S A, 2000. **97**(12): p. 6640-5.
28. Cronan, J.E., Jr. and J.C. Wallace, *The gene encoding the biotin-apoprotein ligase of Saccharomyces cerevisiae.* FEMS Microbiol Lett, 1995. **130**(2-3): p. 221-9.
29. Sharan, S.K., et al., *Recombineering: a homologous recombination-based method of genetic engineering.* Nat Protoc, 2009. **4**(2): p. 206-23.
30. Rahal, J.J., Jr. and M.S. Simberkoff, *Bactericidal and bacteriostatic action of chloramphenicol against memingeal pathogens.* Antimicrob Agents Chemother, 1979. **16**(1): p. 13-18.
31. Feldman, W.E. and N.S. Manning, *Effect of growth phase on the bactericidal action of chloramphenicol against Haemophilus influenzae type b and Escherichia coli K-1.* Antimicrob Agents Chemother, 1983. **23**(4): p. 551-4.
32. Gilbert, A.M., et al., *Pyrazolidine-3,5-diones and 5-hydroxy-1H-pyrazol-3(2H)-ones, inhibitors of UDP-N-acetylenolpyruvyl glucosamine reductase.* J Med Chem, 2006. **49**(20): p. 6027-36.
33. Wilson, K.P., et al., *Escherichia coli biotin holoenzyme synthetase/bio repressor crystal structure delineates the biotin- and DNA-binding domains.* Proc Natl Acad Sci U S A, 1992. **89**(19): p. 9257-61.
34. Polyak, S.W., et al., *Biotin protein ligase from Saccharomyces cerevisiae. The N-terminal domain is required for complete activity.* J Biol Chem, 1999. **274**(46): p. 32847-54.
35. Corrine, J., *Nutritive medium for the culture of microorganism*, U.S. patendt, Editor. 1996, Bio Merieux, France: USA.
36. Cui, L., et al., *Enhancer-like long-range transcriptional activation by lambda CI-mediated DNA looping.* Proc Natl Acad Sci U S A, 2013. **110**(8): p. 2922-7.

37. Streaker, E.D. and D. Beckett, *Coupling of site-specific DNA binding to protein dimerization in assembly of the biotin repressor-biotin operator complex.* Biochemistry, 1998. **37**(9): p. 3210-9.
38. Otsuka, A. and J. Abelson, *The regulatory region of the biotin operon in Escherichia coli.* Nature, 1978. **276**(5689): p. 689-94.
39. Pendini, N.R., Bailey, L. M., Booker, G. W., Wilce, M. C., Wallace, J. C. and Polyak, S. W., *Microbial biotin protein ligases aid in understanding holocarboxylase synthetase deficiency.* Biochim Biophys Acta, 2008. **1784**(7-8): p. 973-82.
40. St-Pierre, F., et al., *One-step cloning and chromosomal integration of DNA.* ACS Synth Biol, 2013. **2**(9): p. 537-41.
41. Bernard, P., *Positive selection of recombinant DNA by CcdB.* Biotechniques, 1996. **21**(2): p. 320-3.
42. Afif, H., et al., *The ratio between CcdA and CcdB modulates the transcriptional repression of the ccd poison-antidote system.* Mol Microbiol, 2001. **41**(1): p. 73-82.
43. Dao-Thi, M.H., et al., *Crystallization of CcdB in complex with a GyrA fragment.* Acta Crystallogr D Biol Crystallogr, 2004. **60**(Pt 6): p. 1132-4.
44. Buoncristiani, M.R. and A.J. Otsuka, *Overproduction and rapid purification of the biotin operon repressor from Escherichia coli.* J Biol Chem, 1988. **263**(2): p. 1013-6.
45. Chapman-Smith, A., et al., *The C-terminal domain of biotin protein ligase from E. coli is required for catalytic activity.* Protein Sci, 2001. **10**(12): p. 2608-17.
46. Chakravartty, V. and J.E. Cronan, *Altered regulation of Escherichia coli biotin biosynthesis in BirA superrepressor mutant strains.* J Bacteriol, 2012. **194**(5): p. 1113-26.
47. HimediaLabs, *Hiper Transduction kit (HTM 005) instruction manual*. 2012, Himedia laboratory.
48. http://ecoliwiki.net/colipedia/index.php/Methods:P1_transduction. *Methods P1 transduction* 2009.
49. Henke, S.K. and J.E. Cronan, *Successful conversion of the Bacillus subtilis BirA Group II biotin protein ligase into a Group I ligase.* PLoS One, 2014. **9**(5): p. e96757.
50. Henke, S.K. and J.E. Cronan, *The Staphylococcus auresu group II biotin protein ligase birA is an effective regulator of biotin operon transcription and requires DNA binding domain for full enzymatic activity.* Molecular microbiology, 2016. **doi:10.1111/mm1.13470**.
51. Tang, Q., et al., *Mycobacterium smegmatis BioQ defines a new regulatory network for biotin metabolism.* Mol Microbiol, 2014.
52. Brune, I., Gotker, S., Schneider, J., Rodionov, D. A. and Tauch, A., *Negative transcriptional control of biotin metabolism genes by the TetR-type regulator BioQ in biotin-auxotrophic Corynebacterium glutamicum ATCC 13032.* J Biotechnol, 2012. **159**(3): p. 225-34.
53. Rodionov, D.A. and M.S. Gelfand, *Computational identification of BioR, a transcriptional regulator of biotin metabolism in Alphaproteobacteria, and of its binding signal.* FEMS Microbiol Lett, 2006. **255**(1): p. 102-7.

Chapter 4:

Characterization of *Sa*BirA transcriptional repressor function and investigation into the effect of biotin on *S. aureus* growth

Statement of Authorship

Title of Paper	Biotin-regulated gene expression in *Staphylococcus aureus* is more responsive to environmental biotin than *Escherichia coli*.
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Satiaputra, J., Sternicki, L.M., Eijkelkamp, B., McDevitt, C.A., Pukala, T.L., Booker, G.W., Shearwin, K.E. Polyak, S.W. (2016), Biotin-regulated gene expression in *Staphylococcus aureus* is more responsive to environmental biotin than *Escherichia coli*.

Principal Author

Name of Principal Author (Candidate)	Jiulia Satiaputra		
Contribution to the Paper	Performed most of the experiments and performed data analysis, wrote the manuscript, prepared the figures.		
Overall percentage (%)	90		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	08/12/16

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Louise Sternicki		
Contribution to the Paper	Performed the cross-linking and nESI-MS experiments. Contributed data and development of the "materials and method" section of the manuscript.		
Signature		Date	8/12/16

Name of Co-Author	Bart Eijkelkamp
Contribution to the Paper	Contribute to qRT-PCR and *S. aureus* growth experiments and data analysis. Helped evaluate and review the manuscript
Signature	Date 8/12/16

Name of Co-Author	Christopher McDevitt
Contribution to the Paper	Provided valuable input for the development of this work and review the manuscript.
Signature	Date 8/12/16

Name of Co-Author	Tara Pukala
Contribution to the Paper	Supervised the cross-linking and nESI-MS experiment. Contributed to "materials and method" section and nESI-MS figure of the manuscript
Signature	Date 9/12/16

Name of Co-Author	Grant Booker
Contribution to the Paper	Provided overall direction of the research
Signature	Date 12/12/2016

Name of Co-Author	Keith Shearwin
Contribution to the Paper	Supervised the *in vivo* reporter assay experiments and provided valuable input and resources. Helped evaluate and review the manuscript.
Signature	Date 8/12/2016

Name of Co-Author	Steven Polyak		
Contribution to the Paper	Supervised the project, provided valuable input and contributed to manuscript preparation. The corresponding author for the paper.		
Signature		Date	8/12/2016.

Suggested Title:

Biotin-regulated gene expression in *Staphylococcus aureus* is more responsive to environmental biotin than *Escherichia coli*.

Satiaputra, J.[1], Sternicki, L.M.[1], Eijkelkamp, B.A.[1], McDevitt, C.A.[1], Pukala, T.L.[2], Shearwin, K.E.[1], Booker, G.W.[1], Polyak, S.W[1].

[1] School of Biological Sciences, University of Adelaide, South Australia 5005, Australia
[2] School of Physical Sciences, University of Adelaide, South Australia 5005, Australia

Abstract:

For *Staphylococcus aureus*, an adequate supply of biotin is vital for the bacteria's survival and pathogenesis. The key protein responsible for maintaining biotin homeostasis in bacteria is the biotin retention protein A (BirA, also known as biotin protein ligase). BirA is a bi-functional protein that serves both as a ligase to catalyze the biotinylation of important metabolic enzymes, as well as a transcriptional repressor that regulates biotin biosynthesis, biotin transport and fatty acid elongation. The mechanism of BirA regulated transcription has been extensively characterized in *Escherichia coli,* but not other bacteria. In *E. coli*, biotin is the co-repressor that induces homodimerization of *E. coli* BirA (*Ec*BirA), which is a prerequisite for DNA binding. *Ec*BirA is unable to dimerize, or bind DNA, in its non-liganded form. In contrast, *S. aureus* BirA (*Sa*BirA) can dimerize in the absence of biotin and bind DNA. We propose that this activity permits *Sa*BirA greater control of gene expression than *Ec*BirA. In this study we show that *Sa*BirA senses varying biotin levels in the environment, and displays a more rapid repression of biotin biosynthesis than *Ec*BirA. Our data also show that a mutant *Sa*BirA with impaired dimerization ability was able to bind DNA, in contrast to the analogous *Ec*BirA mutant. Based on these data, we proposed an alternative DNA binding mechanism for *Sa*BirA that is not employed by *Ec*BirA. This alternative mechanism would potentially allow *Sa*BirA more intricate gene regulation in response to environmental biotin, compared to *Ec*BirA. This mechanism may contribute to survival of *S. aureus* during pathogenesis.

Introduction

Staphylococcus aureus is a highly adaptable bacterial pathogen with an extraordinary ability to colonize a wide range of niche microenvironments. *S. aureus* infection is responsible for various disorders affecting the skin, respiratory organs, soft tissues, bones, joints and endovascular system. The bacterium can also rapidly evolve resistance to host immune responses and antibiotic therapies. To establish a successful infection, *S. aureus* must co-ordinate its metabolism in response to its immediate environment. If the bacterium is unable to fulfill its requirements for certain micronutrients through scavenging exogenous material then it must commit to the synthesis of these factors. One example of this is biotin (also known as vitamin H or B7). Biotin serves as a cofactor for two important biotin-dependent enzymes in *S. aureus*, namely acetyl CoA carboxylase (ACC) and pyruvate carboxylase (PC) [1]. These enzymes play key roles in important metabolic pathways required for growth and virulence, respectively [1-4]. ACC catalyzes the first committed step in the fatty acid synthesis pathway necessary for biogenesis and maintenance of cell membranes [5]. PC catalyzes the production of oxaloacetate that is fed into metabolic pathways required for virulence, such as the TCA cycle and aspartate biosynthesis [6, 7]. As ACC and PC share the same requirement for protein biotinylation, balancing the cellular demand for biotin with its supply is critical.

Certain bacteria, such as *Lactococcus lactis* [8] and *Streptococcus suis* [9], rely only on import from the environment to fulfill their biotin demand, whereas bacteria such as *Mycobacterium tuberculosis* rely solely on *de novo* synthesis [10]. On the other hand, bacteria such as *E. coli* and *S. aureus* can both scavenge biotin from the environment or synthesize it *de novo*. In *E. coli,* the YigM protein has been

identified as the protein responsible for biotin import [11]. However, it is believed that this protein is not the only means of biotin acquisition from the environment for *E. coli,* as a mutant deficient for the YigM transporter and biotin synthesis was still viable [12]. *S. aureus* transports biotin from the external environment through the activity of a high-affinity transporter, BioY [13]. The synthesis of biotin is metabolically costly requiring at least four gene products and 20 ATP equivalents for each molecule of biotin [14]. Therefore, uptake of the micronutrient from the immediate environment is likely to be preferred as it will allow resources to be diverted to alternative fates. However, the concentration of biotin in the niche microenvironments that the bacteria colonize is likely to be limiting, given that mammalian cells have no equivalent biosynthetic pathway for *de novo* synthesis of the vitamin. The concentration of biotin in human serum has been measured at between 0.5 – 3.0 nM [15, 16].

In both *E. coli* and *S. aureus,* biotin retention A protein (BirA, also known as Biotin Protein Ligase or BPL) is the protein responsible for maintaining biotin homeostasis. BirA is a bi-functional protein capable of both enzymatic biotinylation of ACC and PC, as well as transcriptional repression of biotin biosynthesis. By combining both activities in a single protein, BirA is uniquely placed as the key regulator of biotin metabolism. BirA from the prototypical bacteria *Escherichia coli* (*Ec*BirA), has been well studied through genetic, biochemical and structural biology studies [17-23], and provides insights into the maintenance of biotin homeostasis. *Ec*BirA binds to its ligands, biotin and ATP, in an ordered manner [20, 24, 25]. Conformational changes induced by biotin create the binding pocket necessary for ATP binding. This is followed by the synthesis of biotinyl-5′-AMP that serves as both a reaction intermediate for biotin ligation as

well as a co-repressor. BirA:biotinyl-AMP complex, known as the holo enzyme complex, then has one of two fates: to function as a biotin protein ligase or homodimerize and serve as a transcriptional repressor.

The transcriptional repressor function of *Ec*BirA involves a co-operative interaction between two *Ec*BirA subunits and an inverted palindromic repeat sequence present in the promoter of the biotin biosynthetic operon (*bioO*). Homodimerization of the holo enzyme complex ($K_D^{2\text{-}1}$ = 1.5 x 10^{-6} M) is a pre-requisite for DNA binding [26, 27]. The unliganded enzyme (i.e. apo-*Ec*BirA) does not dimerize at physiological concentrations ($K_D^{2\text{-}1}$ = 1.4 x 10^{-3} M) [28] and is unable to bind DNA [23]. Amino acid substitutions in the interface between the two *Ec*BirA subunits that disrupt dimerization, such as R119W, are devoid of DNA-binding activity [26, 29]. Therefore, *Ec*BirA R119W mimics the monomeric state of apo *Ec*BirA.

S. aureus BirA (*Sa*BirA) shares many features with its *E. coli* equivalent. X-ray crystallography has revealed the two proteins share homologous structures, and both undergo the analogous conformational changes that define the ordered ligand binding mechanism [30, 31]. *Sa*BirA binding to the *bioO* target sequence within the promoter of the biotin biosynthetic operon has been validated experimentally by us and others [30-32]. Bioinformatic studies have predicted the presence of two additional BirA binding sites in the *S. aureus* genome [33], suggesting biotin-regulated expression of the BioY biotin transporter (encoded by *SabioY*), as well as an operon containing *SayhfS* and *SayhfT* genes that encodes for homologs of acetyl-CoA acetyl transferase and long-chain fatty acid-CoA ligase, respectively. However, transcriptional regulation of these genes by BirA has not

yet been validated experimentally. It is also not known if *Sa*BirA discriminates between the three promoters, thereby producing a hierarchy of regulation. Another distinction between *S. aureus* and *E. coli* is the ability of apo-*Sa*BirA to dimerize and bind DNA. Analytical ultracentrifugation studies have revealed that *Sa*BirA can dimerize at significantly lower protein concentrations ($K_D^{2\text{-}1}$ = 29 x 10^{-6} M) than *Ec*BirA, *in vitro* [25]. Furthermore, we and others have shown that apo *Sa*BirA is competent to bind DNA, as evidenced by small angle X-ray scattering analysis and *in vitro* DNA binding experiments [25, 31, 32]. Again, it is not clear whether the findings from these biophysical and structural studies are physiologically important.

In this study, we analyze the effect of biotin on *S. aureus* growth and *Sa*BirA-regulated transcription. We probe the repressor activity of *Sa*BirA and *Ec*BirA both *in vitro* and *in vivo* using an *E. coli* reporter strain. Wild-type and dimerization-defective mutants (*Ec*BirA R119W and the *Sa*BirA equivalent F123G) were also employed to understand the monomer:dimer state and its requirement for repression. EMSA analysis, in solution crosslinking and native mass spectrometry techniques were also adopted to further delineate differences between the two BirA proteins.

Materials and methods

General bacteria culture and molecular biology reagents.

S. aureus and *E. coli* strains used in this study were purchased from the American Tissue Culture Collection except the biotin auxotroph *E. coli* strain JD26186 (bioC::Kan) that was obtained from National BioResource Project (Japan). Unless otherwise stated, all bacteria were cultured at 37°C with vigorous shaking in cation-adjusted Mueller-Hinton II broth (Becton Dickinson Company) media containing the appropriate antibiotic. Plasmid extractions were performed using the Plasmid Mini Kit (Qiagen) and genomic extractions were performed using the Wizard® Genomic DNA purification kit (Promega). All molecular biology enzymes (DNA polymerase and restriction enzymes) and buffers were supplied by New England Biolabs. Chemically competent *E. coli* DH5α was prepared in-house and used as general cloning strain, unless otherwise specified. Oligonucleotides, purchased from Geneworks Ptd Ltd, are shown in Supplementary Table 4 and 5. Site directed mutants were generated using the Quikchange Site Directed Mutagenesis kit.

Preparation of biotin depleted media.

Biotin depletion of bacterial growth media was performed following methods established previously [34]. Briefly, slurry of streptavidin-agarose resin (GE healthcare) was prepared by resuspending the packed resin in an equal volume of sterile water. For biotin depletion, 1.2 mL of the 50% slurry was used to treat 200mL Mueller-Hinton media overnight at 4°C with continuous mixing, then filtered through 0.2 μM steritop filter (Millipore).

Comparison of growth curve in biotin depleted- biotin supplemented media.

S. aureus NCTC 8325 and *E. coli* strain ATCC 25922 were prepared in triplicate and grown overnight at 37°C in 5 mL Mueller-Hinton media with medium agitation. Each culture was washed three times in 5 mL of sterile PBS and re-suspended in 2 mL of sterile PBS. The absorbance of each culture OD_{600} was measured and the cell suspension diluted to a final OD_{600} of 0.04 in fresh with culture media containing varying concentrations of biotin, from 0 to 100 nM. A final volume of 200 µl per well was plated into 96 well flat-bottom plates (Costar) in triplicate and the sealed using Breathe-easy® gas permeable membrane (Diversified biotech, BEM-1). Cultures were grown for 24 hours with OD_{600} measured every 30 minutes using a Fluostar Omega plate reader (BMG labtech). Growth was normalized against the control (i.e. non-inoculated wells) and the average of each of the triplicate wells were calculated as a function of time, allowing the time required to reach half-maximum OD_{600} to be determined.

Biotin uptake assay

Overnight cultures of *S. aureus* NCTC 8325 or *E. coli* ATCC 25922 were grown in Mueller-Hinton media. Each culture was then centrifuged at 3200 x g for 10 minutes (4°C) and the pellet was washed three times in 5 mL of sterile PBS before resuspension in 2 mL of sterile PBS. This cell suspension was used to inoculate 10 mL of biotin-depleted media to give a final OD_{600} = 0.10 for *S. aureus* and OD_{600} = 0.05 for *E. coli*. Biotin treatment was performed at mid-log phase by addition of 10 nM biotin solution containing a mixture of 80% (v/v) non-labeled biotin and 20% (v/v) ^{3}H-biotin (*d*-8,9-^{3}H-biotin, Perkin Elmer). Samples of each culture, 200 µL, were taken at various time up to 90 minutes post-biotin treatment.

Samples were mixed with 800 µL of ice cold PBS followed by centrifugation at 3200 x g for 2 minutes. Pellets were resuspend in 1 mL ice cold PBS and washed twice before resuspension in 110 µL PBS. 100 µL of each sample was then mixed with an equal volume of Optiphase Supermix scintillation solution (Perkin Elmer). ^{3}H-Biotin uptake was measured using a MicroBeta^{2}® microplate counter (Perkin Elmer). OD_{600} at each time point was also measured and the rate of uptake was expressed as a measure of counts per minute/ OD_{600}. As a control, bacteria were subcultured into 10 nM ^{3}H-biotin supplemented media and grown continuously for the same duration as the biotin-treated cultures. Samples were taken at the same time as each time point as the biotin-treated samples. For *S. aureus*, viable cell counts were determined on samples collected at time 0, 15, 30 and 90 minutes post-biotin treatment, as detailed in the supporting experimental procedure.

Quantification of gene expression using QRT-PCR

Biotin-treatment was performed using the same method as described in biotin uptake assay except 10 nM of non-labeled biotin was used. Cells were harvested at relevant time points and treated with RNAprotect bacteria reagent (Qiagen), following the manufacturer's protocol. The bacterial cell pellet was resuspended in 100 µL of TE buffer containing 1 mg/mL lysozyme. For *S. aureus*, 1 µL of 10 mg/mL of lysostaphin (Sigma Aldrich) was added to the solution. Lysis was performed by incubating the solution at 37°C for 30 minutes with frequent mixing by inverting the tubes, until the solution became clear. RNA extractions were performed using RNAeasy mini kit (Qiagen), following the manufacturer's instructions. Total RNA was measured using a Nanodrop spectrophotometer (Thermo fisher scientific). Two micrograms of total RNA was treated with 20 units

of DNAse1 at 37°C for 1 hour using a DNAseI kit (Life technology), followed by inactivation using 2 µL RNase-free EDTA reagent (Sigma Aldrich) at 65°C for 10 minutes. A total of 10 ng of RNA was used as a template in the qRT-PCR reaction and a total of 0.1 ng of RNA was used as a template in 16s rRNA amplification. The qRT-PCR reactions were performed using the Superscript® III platinum ® SYBR® 1-step QRT-PCR kit (Life technologies) with 0.2 nM of oligonucleotides. The reaction and data analysis were performed using the Quantstudio™-Dx real-time PCR instrument and software (Thermofisher). Relative expression was normalized against 16s rRNA and corrected to t = 0. For each experiment, at least 3 independent biological replicates were obtained and each PCR reaction was performed in triplicates.

Construction of in vivo reporter strains

Reporter strains were constructed in the biotin auxotroph *E. coli* JD26186 (*bioC::Kan*^{R}). The DNA binding activity of the endogenous *Ec*BirA was eliminated by inserting a chloramphenicol cassette (CAT cassette) into the N-terminal region of the *EcBirA* gene, as described in supplementary material section SE.2. The resulting *E. coli* strain, JD26186 *birA*::CAT was used as the parent for all reporter strains constructed in this study.

Chromosomal integration vectors containing either the *birA* gene or the target promoter fused to a *lacZ* gene were constructed and site specifically introduced into the chromosome of *E. coli* JD26186 *birA*::CAT. Coding regions for the repressors, *SaBirA* and *EcbirA*, as well as mutants *SabirA* F123G and *EcbirA* R119W, were fused to the plac-UV5 promoter and ligated into the integration vector pIT4_TL_15002. Sequences of the target promoters (*SabioO, SabioY* and

SayhfS-SayhfT and *EcbioO*) were ligated upstream of a *lacZ* reporter gene in the integration vector pIT3_SH_LacZTrim. Chromosomal integration of repressor gene and target promoter sequence of interest was performed using the clonetegration method [35]. Positive integrants were screened by PCR, as previously described [35]. Detailed cloning procedures for all the constructs can be found in the supporting experimental procedures. The plac-UV5::*Sa*BirA construct integrated into the reporter strain indicated leaky expression as *Sa*BirA was obtained without the addition of IPTG, as shown by western blot, **sup. Fig. S7.** The expression of *Sa*BirA generated by this leaky pLac-UV5 promoter was sufficiently strong that β-galactosidase assays in this study were performed without the addition of IPTG.

β-Galactosidase assay

β -Galactosidase assays were performed on the reporter strains as described [35] Briefly, bacteria were grown overnight in minimal media (0.1% casamino acid , M9 salts, 1 mM $MgSO_4$, 1 μg/mL thiamine, 0.4% glucose) containing 1.5% bacto agar and 100 nM biotin. The overnight culture was pelleted and washed three times with minimal media containing no biotin and used to inoculate each well of the 96-well growth plate containing minimal media supplemented with 0.5 nM - 500 nM biotin. Cells were grown at 37°C with gentle agitation until the optical density reached OD_{600} = 0.50-0.60, at which point the OD_{600} was recorded, cells lysed and β-galactosidase activity measured as described previously [35]. The β-galactosidase activity from each strain was subtracted from the readings of the corresponding no-promoter control strain. Results were analyzed using Graphpad Prism.

Protein methods

Expression and purification of apo- *Sa*BirA and apo-*Ec*BirA, as well as the Western blot procedure to detect the purified apo-protein, were performed as described in [25]. Purified proteins were dialyzed overnight in 4 L of storage buffer (50mM Tris pH 8.0, 100 mM KCl, 1 mM DTT, 5% glycerol) and stored at -80°C until required. Protein SDS-PAGE was performed using Nupage™ Bis-Tris 4-12% gel (Invitrogen).

Native nESI-MS

Purified apo-*Sa*BirA was buffer exchanged into 200 mM ammonium acetate using Vivaspin 500 MWCO 10,000 spin columns. Holo-*Sa*BirA samples were prepared by pre-incubating apo-*Sa*BirA with 500 µM biotin, 1 mM ATP and 1 mM $MgCl_2$ prior to buffer exchange. Proteins were diluted to 10 µM in 200 mM ammonium acetate for analysis by nano-electrospray ionization-mass spectrometry (nESI-MS). MS measurements were performed on a Synapt HDMS system (Waters, UK) with the sample introduced by nano-electrospray ionisation in positive ion MS mode from platinum-coated borosilicate capillaries prepared in-house. Instrument parameters were optimized to remove adducts while preserving non-covalent interactions, and were as follows; capillary voltage, 1.5 kV; cone voltage, 60 V; trap collision energy, 20 V; transfer collision energy, 15 V; source temperature, 50°C; backing pressure, 3.95 mbar.

Electrophoretic Mobility Shift Assay (EMSA).

HPLC-purified double stranded oligonucleotides containing the operator sequence of interest were purchased from Integrated DNA Technology (USA). The binding reactions were performed at room temperature for 30 minutes using EMSA buffer (50 mM Tris pH 8.0, 50 mM NaCl, 1 mM ATP, 1 mM $MgCl_2$ and 10% (v/v) glycerol) together with 10 nM of the double stranded oligonucleotide and varying concentrations of BirA. For analysis of holo-enzyme binding reaction 0.1 mM biotin was added to the EMSA. Gel retardation was performed using 4-12% TBE polyacrylamide gels (Life technology) run at 100 volts (constant) for 45 minutes and stained in GelRed (Biotium) solution for 5 minutes. After washing five times in distilled water, the gels were imaged using ChemiDoc imaging (Bio-Rad). Each EMSA experiment was performed in triplicate. A list of oligonucleotides used in EMSA experiments are presented in **Supplementary Table 6**.

Protein Cross-Linking.

*Sa*BirA (13.5 μM) was pre-incubated with 26 μM double stranded oligonucleotide, 0.1 mM biotin, 10 mM ATP and 10 mM $MgCl_2$ in PBS, 100 mM KCl, 5% glycerol and 0.1 mM EDTA pH 8.0 at room temperature for 15 min. A 30-fold molar excess of dithiobis succinimidyl propionate (DSP) cross linker (ThermoFisher) solubilised in PBS containing 10% DMSO was added to the reaction, such that the final DMSO content was 1(v/v)%. The reaction was incubated at 37 °C for 15 min. The amine modifying cross-linking reaction was terminated by the addition of 50 mM Tris pH 7.5 at room temperature for 10 min. Samples were analysed by SDS-PAGE in the absence of reducing agents for cross-linked species.

Results

Analysis of the *bioO, SabioY* and *SayhfS-SayhfT* promoters

*Ec*BirA binding to its target sequence *bioO* has been the subject of numerous studies [23, 36-38], and the architecture of its binding site in the promoter of the biotin biosynthetic operon is well characterized (**Fig.1a)**. The *Ec*BirA binding sites are composed of two inverted palindromic sequences separated by 16 bp of A and T rich nucleotides. Each half site accommodates a single *Ec*BirA monomer, the transcriptional repressor functioning as a homodimer. *Ec*BirA regulates two face-to-face promoters located between two coding regions required for biotin biosynthesis, namely a single gene for *bioA* and the biotin biosynthesis operon containing *bioB, bioF, bioC* and *bioD.* The BirA binding site directly overlaps with -35 and -10 sequences in the *bioB* promoter, thereby directly competing with RNA polymerase for DNA binding [36, 39]. Occupation of this binding site allows simultaneous repression of *bioA* and the biotin operon. *Sa*BirA target promoters have not been the subjects of such detailed studies.

Figure 1b-1d shows the proposed biotin-regulated promoter sequences from the prototypical *S. aureus* strain NCTC 8325. Highlighted are the *Sa*BirA binding sites, predicted from previous bioinformatics studies [33]. We, and others, have confirmed that *Sa*BirA binds to the *bioO* promoter sequence through gel-shift and solution based DNA binding assays, as well as small angle X-ray scattering experiments [25, 32]. *Sa*BirA binding to the *SabioY* and *SayhfS-SayhfT* was verified for the first time in this study (vide infra). All three *Sa*BirA binding sites contain two conserved palindromic sequences separated by 15 base pairs. Like the *Ec*BirA binding element, this spacer is rich in A and T nucleotides with the *SabioO*

sequence being devoid of any G or C nucleotides and the two other sequences containing only two G-C base pairs (**Fig. 1d**). All *Sa*BirA binding sites are composed of perfect inverted repeat sequences, except for the *SayhfS-SayhfT* promoter where a single base pair change gives an imperfect sequence in one half site (**Fig. 1d**, grey highlight).

S. aureus growth is affected by biotin and biotin uptake rate is increased following changes in biotin levels in the environment.

To mimic the low biotin conditions experienced *in vivo*, *E. coli* and *S. aureus* were grown in Mueller-Hinton broth pre-treated with streptavidin-agarose resin to remove biotin. Using radio-labeled biotin, we have demonstrated this approach is highly effective in removing biotin from the growth media [34]. Defined concentrations of biotin were then added back to the depleted media. The optical density of each culture was measured every 30 minutes for 24 hours (**Supp Fig. 1**) and the time required to reach half-maximum OD_{600} was determined (**Fig. 2 and Supp Table 1**). The data revealed that the growth of *E. coli* was unchanged under the 5 different conditions investigated (**Fig. 2a**). In contrast, the time for *S. aureus* to reach half-maximum OD_{600} in depleted media was longer (5.5 ± 0.3 hours) compared to biotin-supplemented media. The addition of 10 – 100 nM biotin improved the growth of *S. aureus* (**Fig. 2b**), with 10 nM being sufficient to accelerate the time to reach half-maximum OD_{600} (4.9 ± 0.3 hours, $p = 0.0058$, 10 nM *vs* depleted). This biotin concentration was subsequently used in all further experiments. These data are in agreement with previous studies that demonstrated biotin is a growth promoting factor for *S. aureus* [40, 41].

Biotin transport activity in response to external biotin concentrations was next analyzed. Both *E. coli* and *S. aureus* were sub-cultured into biotin-depleted media such that the cells were starved of exogenous biotin and became dependent upon *de novo* biotin synthesis. At mid-log phase, 10 nM of ^{3}H-biotin was introduced into the media and biotin uptake quantitated by liquid scintillation. Cell growth was monitored at this stage by both spectroscopy and viable cell counts. Parallel cultures were prepared as controls, where bacteria were subjected to an environment where biotin was readily available, by growing continuously in 10 nM ^{3}H-biotin supplemented media. The amount of radioactivity imported into the cells under the two conditions was compared to assess how biotin transport was influenced by the availability of environmental biotin.

The amount of ^{3}H-biotin imported into *E. coli* did not change throughout the time course, regardless of the media used (**Fig. 3a**). In sharp contrast, intracellular ^{3}H-biotin accumulation increased in *S. aureus* for the first 30 minutes post treatment. This data was consistent with *S. aureus* possessing a high-affinity, active transport system that is responsive to environmental biotin [42-44]. After 30 minutes, the amount of tracer accumulated in the cells decreased through mechanisms that are not understood (**Fig. 3b,** solid black line). Viable cell counts throughout the time course demonstrated that this was not due to cell lysis. Hence, the observed decrease is most likely the result of inhibited import or increased efflux once sufficient intracellular concentrations of biotin are obtained. In contrast to biotin-treated *S. aureus*, the control *S. aureus* grown continuously with 10 nM ^{3}H-biotin showed a lower and constant uptake rate compared to the biotin-treated *S. aureus* (**Fig 3b**, grey line). The data suggests that *S. aureus* was able to finely tune biotin import according to cellular demand and the availability of biotin

from the external environment, as opposed to *E. coli* where the activity of biotin transporters remained unchanged.

Expression of biotin-regulated genes is elevated in a low biotin environment, but not the expression of *Sa*BirA.

Expression of biotin–regulated genes was next investigated using qRT-PCR. *S. aureus* NCTC 8325 was grown until mid-log phase in either non-depleted Mueller Hinton broth, biotin-depleted media or biotin-depleted media supplemented with 10 nM biotin. Total RNA was harvested and used to quantify the expression of *SabioD, SabioY, SayhfS* and *SabirA* transcripts relative to the *16s rRNA* housekeeping gene **(Fig. 4).** The result showed that the expression of *SabioD, SabioY* and *SayhfS* were all up regulated in biotin-depleted media compared to non-depleted media ($p \leq 0.001$). Consistent with its co-repressor function, the addition of 10 nM biotin in the growth media repressed expression of all three target-genes to the same low levels observed in the non-depleted conditions. In contrast, the *SaBirA* transcript showed no change in any of the conditions analyzed (**Fig. 4d**). This lack of biotin-controlled regulation was expected, as the *birA* promoter is devoid of any potential binding sites for *Sa*BirA [33].

Biotin-regulated gene expression in *S. aureus* and *E. coli* in response to changing environmental biotin levels.

To further investigate the kinetics of biotin-regulated repression, transcription of *SabioD* (the first gene in *S. aureus* biotin biosynthesis operon), *SabioY* and *SayhfS* were quantitated alongside *E. coli bioA* (*EcbioA*) and *bioB (EcbioB)*. Here cells were again grown to mid-log phase in biotin-depleted media

then treated with 10 nM biotin. Total RNA was harvested from cells at 0, 15, 30 and 90 minutes post-biotin treatment and analyzed by qRT-PCR. The abundance of both the *E. coli* transcripts was reduced by approximately half within the first 15 mins (**Fig. 5a** and **5b**). The greatest response in *E. coli* was observed for *EcbioB* at 30 minutes post-treatment, when the transcript level was reduced by 5.5-fold (p = 0.0019, t = 0 *vs* 30 mins) (**Fig. 5b**). In stark contrast, *S. aureus bioD (SabioD)* was highly responsive to exogenous biotin with 111-fold repression observed at 15 minute post-treatment (p = 0.0001, t = 0 *vs* 15 mins). This strong repression was maintained throughput the time course (**Fig. 5c**). Repression of *SabioY* and *SayhfS* was not as pronounced as for *SabioD*, and similar to the kinetics observed for *EcbioA* and *EcbioB.* Abundance of the *SabioY* transcript was reduced by 2.5-fold at 15 minutes (p = 0.0003, t=0 *vs* 15 min), 4.2-fold at 30 minutes (p = 0.0001, t=0 vs 30 mins) and 6.0-fold at 90 minutes (p = 0.0001, t = 0 vs 90 mins) **(Fig. 5d)**. Similarly, *SayhfS* repression was reduced by 1.8-fold at 15 minutes (p = 0.0001, t = 0 *vs* 15 mins), 4.9-fold at 30 minutes (p = 0.0001, t = 0 *vs* 30 mins) and 9.0-fold at time 90 minutes (p = 0.0001, t = 0 vs 90 mins) (**Fig. 5e**). As controls, *S. aureus* grown in parallel in depleted media showed no change in transcript levels between the 0 and 90 minute time points (**Sup. Fig. S3**), implying that the repression observed was specifically due to biotin. Together these data highlight a key difference between *E. coli* and *S. aureus*, namely that the latter is highly effective at repressing the biotin biosynthetic operon when exposed to sufficient concentrations of environmental biotin. While some of the differences observed here might be, in part, due to the differences in RNA stability, the data clearly suggest a hierarchy of control between the three target genes of *Sa*BirA, with the

biotin biosynthetic operon being the most responsive to the changing environment.

_Ec_BirA and _Sa_BirA _in vivo_ DNA binding assay.

To specifically define the role of BirA in biotin-regulated gene expression, a bacterial reporter system was constructed in the genome of a biotin auxotroph strain of *E. coli*. These experiments permitted the study of both *Ec*BirA and *Sa*BirA in a homogenous genetic background, thereby eliminating species-specific differences that complicate a direct comparison of the two bacteria in the initial series of cellular assays already described. Here, a *birA* gene (either *Sa*BirA or *Ec*BirA) under the control of pLac-UV5 promoter was site specifically integrated into the Lambda attB phage attachment site present in the *E. coli* chromosome. Likewise, a second construct containing a BirA-target promoter fused to a *lacZ* reporter gene was integrated into the HK022 attB phage attachment site (**Fig. 6a**). This approach facilitated *in vivo* studies of biotin-mediated gene control using β-galactosidase activity as a convenient readout for gene expression. Importantly, the effect of exogenous biotin on these engineered genetic circuits could be measured by simply adding appropriate concentrations of the micronutrient into the growth media. To eliminate the DNA-binding activity of endogenous BirA in the host *E. coli* strain JD26186, a chloramphenicol resistance gene was inserted into the 5′ region of the *birA* gene encoding the DNA binding-domain. The loss of DNA-binding activity in the resulting strain, JD26186 *birA::CAT*, was confirmed following integration of an *EcbioO*-lacZ reporter. Whilst increasing concentrations of biotin in the growth media repressed β-galactosidase expression in JD26186,

repression was abolished in JD26186 *birA::CAT* (**Fig. 6b**). JD26186 *birA::CAT*, served as the parent strain for all reporter constructs in this study.

For construction of the *E. coli bioO* promoter, the DNA sequence encompassing -50 to +20 of the promoter region was cloned upstream of the *lacZ* reporter gene (**Fig. 1a**). For the three *S. aureus* promoters, the region spanning from -50 of the estimated transcription start site all the way to the start of the translation was cloned. The DNA sequences of all the promoters employed in the study are shown in **Fig 1**. These sequences were all functional promoters in *E. coli* capable of driving expression of β-galactosidase. As expected for a biotin-inducible repressor, both wild-type *Ec*BirA and *Sa*BirA inhibited β-galactosidase expression when increasing levels of biotin were included in the growth media (black curves, **Fig. 6c-f**). The concentrations of biotin required to achieve half-maximum repression at equilibrium (K_R) were calculated for all the constructs tested (**table 1**). For *Ec*BirA, half maximal repression was achieved with 4.7 nM biotin (**Fig. 6c**). Similar low values were observed for *Sa*BirA with all three of its targets (**Fig. 6d-f**) implying that both BPLs have similar repressor activities *in vivo*. In all cases 500nM biotin was sufficient to completely repress β-galactosidase expression.

A previous study revealed that un-liganded (apo) *Sa*BirA was able to dimerize and bind DNA, with a dimerization constant of 30 μM [25]. Considering this high dimerization constant, it is therefore expected that under physiological condition, apo-*Sa*BirA is primarily monomeric. As it is not possible to investigate the apo-form of BirA in these *in vivo* assays, mutant proteins with properties that mimic the apo state were employed, namely *Ec*BirA R119W [26] and its counterpart *Sa*BirA F123G [25]. Both mutants abolished homodimerization in solution, and *Ec*BirA R119W has been shown to be devoid of DNA binding activity

[26, 45]. As expected, *Ec*BirA R119W was devoid of repressor activity *in vivo* (**Fig. 6c,** grey) yielding similar levels of β-galactosidase activity as the no-repressor control (**Fig. 6c**, blue). Unexpectedly, *Sa*BirA F123G was a functional, biotin-regulated repressor that reduced β-galactosidase activity for all three target promoters (**Fig 6d –6f**, grey). For *SayhfS-SayhfT*, the K_R values were 2-fold higher for *Sa*BirA F123G, relative to the wild-type protein (p = 0.0009, WT vs mutant) whereas for *SabioO* the K_R values were 3-fold higher for *Sa*BirA F123G relative to the wild-type protein (p = 0.0016 WT vs mutant). This decrease in activity is consistent with a 3-fold higher K_M for biotin that has been reported for F123G relative to the wild-type enzyme [25]. For *SabioY*, an accurate estimate of the K_R was not possible as 500 nM was insufficient to completely inhibit expression down to the background necessary to generate a concentration-dependent repression curve. At the lowest biotin concentration tested (1 nM), β-galactosidase expression from the *bioO* promoter was significantly lower than the corresponding no-repressor control implying both the wild-type and F123 mutant proteins partly occupied the DNA. This was also observed for *Sa*BirA with *SayhfS-SayhfT*, but was less pronounced for the mutant protein. Together these data suggest that an interaction exists between DNA and dimerization-impaired *Sa*BirA that is not evident with the *E. coli* system.

Validation of the oligomeric state of *Sa*BirA (wild-type) and *Sa*BirA F123G in apo and holo-form by nESI-MS.

For further biochemical analysis, *Sa*BirA wild-type and *Sa*BirA F123G were purified in apo-form. Purification of apo material was achieved by incubating the cell lysates containing over-expressed proteins with ATP and a biotin-accepting

substrate protein to facilitate protein biotinylation and the concomitant loss of the biotinyl-5′-AMP co-repressor from the protein's active site prior to the IMAC purification step. Confirmation of the apo state was confirmed through two alternative methods; a streptavidin-blot method we have previously described [25] (**Sup Fig S4**) and nano-electrospray ionization mass-spectroscopy (nESI-MS, **Fig 7**). Prior to the mass spectroscopy analysis, samples were exchanged into 200 mM ammonium acetate, the optimal buffer for electrospray ionization that allows measurement of protein mass. The native nESI-MS allowed characterization of both apo and the biotinyl-5′-AMP-bound proteins (i.e. holo proteins) and their multimeric states. A table summarizing the molecular weight detected in nESI-MS is presented in **Table 2**. Mass spectrometry analysis confirmed that at 10 μM >95% of apo-*Sa*BirA (wild-type) was monomeric, with a molecular mass of 37892 Da (theoretical molecular mass 37892 Da). The addition of biotin and MgATP to the apo-*Sa*BirA (wild-type) resulted in two species, both consistent with that of monomeric wild-type *Sa*BirA bound to the reaction intermediate biotinyl-5′-AMP (measured 38470 Da, theoretical molecular mass of 38465 Da) and the dimeric form of this *Sa*BirA complex (measured 76930 Da, theoretical molecular mass 76925 Da). This data was in excellent agreement with the wildtype protein existing in a monomer-dimer equilibrium in solution.

At the same protein concentration, *Sa*BirA F123G was monomeric in the apo form (apo monomer measured 37802 Da, theoretical molecular mass of 37800 Da). The addition of biotin and ATP yielded a species with a mass consistent with a monomer in complex with biotinyl-5′-AMP (holo measured 38381 Da, theoretical molecular mass of 38373 Da). As no evidence of dimer was seen, these data suggest that the F123G mutation abolished dimerization in solution, but that

*Sa*BirA F123G was active and compotent to catalyze synthesis of the reaction intermediate. The data also showed that the enzyme:biotinyl-5′-AMP complex was stable in the volatile buffer used in the mass spectrometry.

DNA enhances *Sa*BirA dimerization

To address the role of DNA on the monomer-dimer equilibrium, in solution cross-linking studies were performed. Both apo and holo *Sa*BirA were treated with dithiobis succinimidyl propionate (DSP) in the presence and absence of the *SabioO* probe. This approach allowed the covalent capture of transient protein dimers that could be visualized after fractionation by reducing SDS-PAGE. The inclusion of DNA in the reaction enhanced protein dimerization. A 77 KDa band corresponding to the *Sa*BirA dimer was observed in the presence and absence of DNA for the wild-type protein in both apo and holo forms (**Figure 8**). For *Sa*BirA F123G, both apo and holo produced weakly staining bands of this size suggestive of a possible transient interaction in solution. However, the addition of *SabioO* in the reaction yielded a more intense product at 77 kDa, implying that dimerization is intensified by the presence of DNA. The DNA probes containing *SabioY* and *SayhfS-SayhfT* promoter sequence were also tested and were able to induce dimerization, as seen in *SabioO* probe (**Sup. Fig. S5**). These data were consistent with DNA-enhanced dimerization of wild-type *Sa*BirA and the *Sa*BirA F123G mutant.

***In vitro* DNA binding assay of *Sa*BirA and *Ec*BirA**

To further compare *in vitro* binding properties of *E. coli* and *S. aureus* BirAs, a series of electrophoretic mobility gel shift assays (EMSA) were performed. Ideally, an in-solution DNA binding assay was preferred to the gel-based EMSA that

has well documented limitations [46-50]. However, our attempts at developing an in-solution assay using FITC-labeled oligonucleotides and fluorescence polarization were unsuccessful due to poor sensitivity at low protein concentrations and non-specific binding at higher concentrations. For example, we observed non-specific binding of *Sa*BirA to a labeled probe with both binding sequences mutated (data not shown). Subsequently, we turned to EMSA to assess the binding of *Sa*BirA to its target promoters and comparing them with the binding of *Ec*BirA to *EcbioO* promoter. 10 nM of double stranded oligo and the purified apo proteins described previously were used in the EMSA. To generate holo samples, biotin was added to the EMSA binding buffer that contained MgATP. As expected, holo-*Ec*BirA was able to bind to *EcbioO* (**Fig. 9a**) and holo-*Sa*BirA was also able to bind to all three target promoters. A complete shift was observed at 50 nM holo-*Sa*BirA concentrations for all promoters (**Fig 9b-d**). The result also suggests that the interaction between holo-*Sa*BirA and its target promoters are stronger compared to the interaction between holo-*Ec*BirA and *EcbioO* promoter, as the last apparent band shift in holo-*Ec*BirA EMSA was observed at an *Ec*BirA concentration of 15.6 nM as opposed to 1.56 nM observed for holo-*Sa*BirA. A control reaction using oligonucleotides containing mutated *Sa*BirA binding sites resulted in no protein binding (**Fig.9e.**), suggesting that binding events are sequence specific.

In agreement with the *in vivo* assay data, *Ec*BirA R119W EMSA did not bind to the *EcbioO* promoter sequence (**Fig. 10a.**), whereas *Sa*BirA F123G did indeed bind DNA, albeit through a weaker interaction compared to wild-type *Sa*BirA (**Fig. 10b-d**). Interestingly, the result clearly indicated that the binding of the *Sa*BirA F123G to *SabioO* exhibits similar affinity to *SabioY*. However, binding to the *SayhfS-SayhfT* probe was much weaker (**Fig. 10d.**). This result might be a function of the

imperfect inverted palindromic sequence of the binding sites in the *SayhfS-SayhfT* promoter (**Fig. 1d**). Based on this result, we also tested the binding of holo-*Sa*BirA to a probe containing only one functional half-site and found no binding was observed in this oligonucleotide (**sup Fig. S6**). This suggests that both binding sites need to be present in order for *Sa*BirA to bind and that binding to DNA requires two *Sa*BirA monomers to occupy each binding site.

Discussion

In this study, direct comparison of the role of biotin upon the growth of prototypical bacteria *E. coli* and clinically important pathogen *S. aureus* was performed. We have shown that biotin is a growth-promoting factor in biotin-starved *S. aureus* but not for *E. coli.* Biotin-starved *S. aureus* also exhibited a higher uptake rates of ^{3}H-biotin compared to *E. coli.* Additionally, transcriptomic data revealed that following the addition of 10 nM biotin to the growth media, the transcript level of *S. aureus* biotin biosynthesis gene, *SabioD*, was more reduced compared to other biotin-regulated genes, *SabioY* and *SayhfS*, as well as compared with *E. coli* biotin biosynthesis genes. This data is in agreement with previous studies where 10-45 nM of biotin was found to be insufficient to repress *EcbioO* operator *in vivo* [51-53] and to down regulate biotin biosynthesis in enterohaemorragic *E. coli* isolated from the mouse colon [54]. These findings suggest that the down regulation of *S. aureus* biotin biosynthesis requires less exogenous biotin compared to *E. coli* and other *S. aureus* biotin-regulated genes.

Furthermore, we performed *in vivo* and *in vitro* analyses to obtain new insights into *Sa*BirA, the protein that is responsible for regulating biotin homeostasis in *S. aureus.* We performed these analyses alongside the well-studied *E. coli* counterpart protein, *Ec*BirA. We also assessed the ability of the dimerization compromised mutants (*Sa*BirA F123G and *Ec*BirA R119W) to bind DNA and to regulate gene expression. Interestingly, our data clearly showed that *Sa*BirA F123G was able to interact with DNA and exhibit repression of all target promoters, albeit weaker than the wild-type. These results suggest that *Sa*BirA's DNA-binding ability is very distinct to *Ec*BirA, since *Ec*BirA R119W was unable to bind DNA as demonstrated in this study and past studies [26, 45]. Crosslinking revealed

showed that dimerization of the *Sa*BirA F123G was enhanced in the presence of DNA. This data suggests a DNA-mediated dimerization mechanism that is not apparent in *E. coli*. Furthermore, a high dimerization constant reported for apo-*Sa*BirA ($K_D^{2\text{-}1}$ = 29 ± 0.2 μM) [25] suggests that apo-*Sa*BirA is likely to be monomeric within the intracellular environment. Therefore, it is reasonable to suggest that *Sa*BirA F123G mimics the *in vivo* oligomeric state of apo-*Sa*BirA. This argument was supported by the nESI-MS results, where both *Sa*BirA F123G and apo-*Sa*BirA were found to be monomeric in solution. The fact that *Sa*BirA F123G binds weakly to DNA is also in agreement with previous studies on apo-*Sa*BirA where binding to DNA exhibited a K_D value that was 6-fold lower compared to the wild-type [25], as measured in an EMSA and 60-fold lower as measured by fluorescence anisotropy [32].

By combining our *in vitro* and *in vivo* data, we propose two possible binding pathways of *Sa*BirA to DNA, as illustrated in **Fig. 11.** We propose that in high biotin conditions, dimerization is favoured and thus a pre-formed dimer of holo-*Sa*BirA binds DNA, similar to that observed for *Ec*BirA [19, 21] (**Fig. 11a**). This pre-formed dimer provides an optimal structure to promote strong binding to DNA and simultaneous occupation of both binding sites on the target operator. In a low biotin environment, where *Sa*BirA is predominantly monomeric, binding to DNA may proceed by the interaction of monomers with the operator half sites, which are then stabilized by dimerization on the DNA (**Fig. 11b**). We suggest that this proposed mechanism contributes to the lower biotin threshold required to initiate gene repression by *Sa*BirA, compared to higher biotin concentrations required to repress *EcbioO*. In addition, the arrangement of target promoters, including variation between the operator sequence and the position of the -35 and -10

elements relative to *Sa*BirA binding sites, also contributes to the different magnitude of repression and the different level of expression between *Sa*BirA-regulated genes. Future studies are still required to precisely define the position of the -35 and -10 elements of the *S. aureus* promoters and how binding of *Sa*BirA affects the activity of RNA polymerase.

This study reinforces the link between biotin, fatty acid synthesis and cell proliferation. It is known that biotin-dependent ACC catalyzes the first committed step in fatty acid synthesis [55] and its expression is controlled by the bacteria growth rate [56, 57]. ACC also plays an important role in the regulatory switch between catalytic and repressor function of *Sa*BirA [21]. In dividing cells, high intracellular ACC concentration leads to de-repression of *Sa*BirA-regulated genes. This default co-ordination allows biotin to be obtained through *de novo* synthesis as well as biotin import. In addition, it is known acetyl CoA acetyltransferase encoded by *SayhfS* is involved in mevalonate pathway, which leads to the synthesis of important membrane biomolecules such as cholesterol [58], while long-chain fatty acid ligases encoded by *Sayhf*T plays an important role in fatty acid elongation [59, 60]. The transcriptional control of the *SayhfS-SayhfT* operon by *Sa*BirA provides a molecular basis to coordinate biotin-mediated membrane synthesis during active growth stage.

Overall, in this study we have validated the role of *Sa*BirA as a functional repressor for biotin biosynthesis. We have also presented the first *in vivo* evidence of *Sa*BirA as a functional regulator for *SabioY* and *SayhfS-SayhfT* and provided the first direct comparison of biotin-controlled gene transcription in *S. aureus* and *E. coli*. The findings presented in this study supported the hypothesis that *Sa*BirA regulation of biotin-related gene is different to *Ec*BirA and involves more than one

pathway of DNA binding. Physiologically, this complex process of gene regulation displayed by *Sa*BirA makes sense, considering biotin concentrations may vary markedly in the niche environments occupied by *S. aureus*. Thus efficient switching between biotin transport and *de novo* synthesis must be carefully orchestrated to support survival.

Reference:

1. Paparella, A.S., et al., *Structure guided design of biotin protein ligase inhibitors for antibiotic discovery.* Curr Top Med Chem, 2014. **14**(1): p. 4-20.
2. Forsyth, R.A., et al., *A genome-wide strategy for the identification of essential genes in Staphylococcus aureus.* Mol Microbiol, 2002. **43**(6): p. 1387-400.
3. Benton, B.M., et al., *Large-scale identification of genes required for full virulence of Staphylococcus aureus.* J Bacteriol, 2004. **186**(24): p. 8478-89.
4. Malachowa, N., et al., *Global changes in Staphylococcus aureus gene expression in human blood.* PLoS One, 2011. **6**(4): p. e18617.
5. Polyak, S.W., et al., *Structure, function and selective inhibition of bacterial acetyl-coa carboxylase.* Appl Microbiol Biotechnol, 2012. **93**(3): p. 983-92.
6. Jitrapakdee, S., Booker, G. W., Cassady, A. I. and Wallace, J. C., *Cloning, sequencing and expression of rat liver pyruvate carboxylase.* Biochem J, 1996. **316 (Pt 2)**: p. 631-7.
7. Jitrapakdee, S., et al., *Structure, mechanism and regulation of pyruvate carboxylase.* Biochem J, 2008. **413**(3): p. 369-87.
8. Zhang, H., et al., *Deciphering a unique biotin scavenging pathway with redundant genes in the probiotic bacterium Lactococcus lactis.* Sci Rep, 2016. **6**: p. 25680.
9. Ye, H., et al., *Functional definition of BirA suggests a biotin utilization pathway in the zoonotic pathogen Streptococcus suis.* Sci Rep, 2016. **6**: p. 26479.
10. Salaemae, W., Azhar, A., Booker, G. W., Polyak, S. W., *Biotin biosynthesis in Mycobacterium tuberculosis: physiology, biochemistry and molecular intervention.* Protein Cell, 2011. **2**(9): p. 691-5.
11. Ringlstetter, S.L., *identification of the biotin transporter in Escherichia coli, biotinylation of histones in saccharomyces cerevisiae and analysis of biotin sensin th saccharomyces cerevisiae*. 2010, University of regensburg: Germany.
12. Weiner, D. and B. Wolf, *Biotin uptake, utilization, and efflux in normal and biotin-deficient rat hepatocytes.* Biochem Med Metab Biol, 1991. **46**(3): p. 344-63.
13. azhar, A., Booker, G.W, Polyak, S.W., , *Mechanisms of biotin transport.* Biochemistry & Analytical Biochemistry, 2015. **4**(4).
14. Feng, Y., et al., *A Francisella virulence factor catalyses an essential reaction of biotin synthesis.* Mol Microbiol, 2014. **91**(2): p. 300-14.
15. Sakamoto, O., et al., *Relationship between kinetic properties of mutant enzyme and biochemical and clinical responsiveness to biotin in holocarboxylase synthetase deficiency.* Pediatr Res, 1999. **46**(6): p. 671-6.
16. Livaniou, E., et al., *Serum biotin levels in patients undergoing chronic hemodialysis.* Nephron, 1987. **46**(3): p. 331-2.
17. Zhao, H., et al., *Protein-protein interactions dominate the assembly thermodynamics of a transcription repression complex.* Biochemistry, 2007. **46**(47): p. 13667-76.
18. Streaker, E.D. and D. Beckett, *Coupling of site-specific DNA binding to protein dimerization in assembly of the biotin repressor-biotin operator complex.* Biochemistry, 1998. **37**(9): p. 3210-9.

19. Streaker, E.D. and D. Beckett, *Coupling of protein assembly and DNA binding: biotin repressor dimerization precedes biotin operator binding.* J Mol Biol, 2003. **325**(5): p. 937-48.
20. Wood, Z.A., et al., *Co-repressor induced order and biotin repressor dimerization: a case for divergent followed by convergent evolution.* J Mol Biol, 2006. **357**(2): p. 509-23.
21. Adikaram, P.R. and D. Beckett, *Protein:protein interactions in control of a transcriptional switch.* J Mol Biol, 2013. **425**(22): p. 4584-94.
22. Adikaram, P.R. and D. Beckett, *Functional versatility of a single protein surface in two protein:protein interactions.* J Mol Biol, 2012. **419**(3-4): p. 223-33.
23. Xu, Y. and D. Beckett, *Evidence for interdomain interaction in the Escherichia coli repressor of biotin biosynthesis from studies of an N-terminal domain deletion mutant.* Biochemistry, 1996. **35**(6): p. 1783-92.
24. Streaker, E.D. and D. Beckett, *Ligand-linked structural changes in the Escherichia coli biotin repressor: the significance of surface loops for binding and allostery.* J Mol Biol, 1999. **292**(3): p. 619-32.
25. Soares da Costa, T.P., et al., *Dual roles of F123 in protein homodimerization and inhibitor binding to biotin protein ligase from Staphylococcus aureus.* Mol Microbiol, 2014. **91**(1): p. 110-20.
26. Kwon, K. and D. Beckett, *Function of a conserved sequence motif in biotin holoenzyme synthetases.* Protein Sci, 2000. **9**(8): p. 1530-9.
27. Kwon, K., et al., *Multiple disordered loops function in corepressor-induced dimerization of the biotin repressor.* J Mol Biol, 2000. **304**(5): p. 821-33.
28. Streaker, E.D., A. Gupta, and D. Beckett, *The biotin repressor: thermodynamic coupling of corepressor binding, protein assembly, and sequence-specific DNA binding.* Biochemistry, 2002. **41**(48): p. 14263-71.
29. Buoncristiani, M.R., P.K. Howard, and A.J. Otsuka, *DNA-binding and enzymatic domains of the bifunctional biotin operon repressor (BirA) of Escherichia coli.* Gene, 1986. **44**(2-3): p. 255-61.
30. Soares da Costa, T.P., et al., *Selective inhibition of biotin protein ligase from Staphylococcus aureus.* J Biol Chem, 2012. **287**(21): p. 17823-32.
31. Pendini, N.R., et al., *Structural characterization of Staphylococcus aureus biotin protein ligase and interaction partners: an antibiotic target.* Protein Sci, 2013. **22**(6): p. 762-73.
32. Henke, S.K. and J.E. Cronan, *The Staphylococcus auresu group II biotin protein ligase birA is an effective regulator of biotin operon transcription and requires DNA binding domain for full enzymatic activity.* Molecular microbiology, 2016. **doi:10.1111/mm1.13470**.
33. Rodionov, D.A., Mironov, A. A. and Gelfand, M. S., *Conservation of the biotin regulon and the BirA regulatory signal in Eubacteria and Archaea.* Genome Res, 2002. **12**(10): p. 1507-16.
34. Bailey, L.M., et al., *Artifactual detection of biotin on histones by streptavidin.* Anal Biochem, 2008. **373**(1): p. 71-7.
35. St-Pierre, F., et al., *One-Step Cloning and Chromosomal Integration of DNA.* Acs Synthetic Biology, 2013. **2**(9): p. 537-541.
36. Streaker, E.D. and D. Beckett, *A map of the biotin repressor-biotin operator interface: binding of a winged helix-turn-helix protein dimer to a forty base-pair site.* J Mol Biol, 1998. **278**(4): p. 787-800.

37. Barker, D.F. and A.M. Campbell, *Genetic and biochemical characterization of the birA gene and its product: evidence for a direct role of biotin holoenzyme synthetase in repression of the biotin operon in Escherichia coli.* J Mol Biol, 1981. **146**(4): p. 469-92.
38. Weaver, L.H., et al., *Corepressor-induced organization and assembly of the biotin repressor: a model for allosteric activation of a transcriptional regulator.* Proc Natl Acad Sci U S A, 2001. **98**(11): p. 6045-50.
39. Otsuka, A. and J. Abelson, *The regulatory region of the biotin operon in Escherichia coli.* Nature, 1978. **276**(5689): p. 689-94.
40. Porter, J.R., Pelczar, M.J., *The nutrition of Staphylococcus aureus the influence of biotin, BioS IIB and vitamin H on the growth of several strains.* Journal of bacteriology, 1941. **41**(2): p. 173-92.
41. Gretler, A.C., Mucciolo,P., Evans, J.B., Niven, JR., C.F., *Vitamin nutrition of staphylococci with special reference to their biotin requirements.* Journal of bacteriology, 1955. **70**(1): p. 44-49.
42. Finkenwirth, F., F. Kirsch, and T. Eitinger, *Solitary BioY proteins mediate biotin transport into recombinant Escherichia coli.* J Bacteriol, 2013. **195**(18): p. 4105-11.
43. Finkenwirth, F., F. Kirsch, and T. Eitinger, *A versatile Escherichia coli strain for identification of biotin transporters and for biotin quantification.* Bioengineered, 2014. **5**(2): p. 129-32.
44. Hebbeln, P., et al., *Biotin uptake in prokaryotes by solute transporters with an optional ATP-binding cassette-containing module.* Proc Natl Acad Sci U S A, 2007. **104**(8): p. 2909-14.
45. Barker, D.F. and A.M. Campbell, *Use of bio-lac fusion strains to study regulation of biotin biosynthesis in Escherichia coli.* J Bacteriol, 1980. **143**(2): p. 789-800.
46. Pagano, J.M., C.C. Clingman, and S.P. Ryder, *Quantitative approaches to monitor protein-nucleic acid interactions using fluorescent probes.* RNA, 2011. **17**(1): p. 14-20.
47. Alves, C., Cunha, C., *Gel electrophoresis-Advanced techniques*, in *Electrophoretic Mobility Shift Assay: Analyzing protein-Nucleic acid interactions*, D.S. Magdeldin, Editor. 2012, InTech.
48. Cann, J.R., *Phenomenological theory of gel electrophoresis of protein-nucleic acid complexes.* J Biol Chem, 1989. **264**(29): p. 17032-40.
49. Fried, M.G. and G. Liu, *Molecular sequestration stabilizes CAP-DNA complexes during polyacrylamide gel electrophoresis.* Nucleic Acids Res, 1994. **22**(23): p. 5054-9.
50. Fried, M. and D.M. Crothers, *Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis.* Nucleic Acids Res, 1981. **9**(23): p. 6505-25.
51. Cronan, J.E., Jr., *Expression of the biotin biosynthetic operon of Escherichia coli is regulated by the rate of protein biotination.* J Biol Chem, 1988. **263**(21): p. 10332-6.
52. Chakravartty, V. and J.E. Cronan, *Altered regulation of Escherichia coli biotin biosynthesis in BirA superrepressor mutant strains.* J Bacteriol, 2012. **194**(5): p. 1113-26.

53. Chakravartty, V. and J.E. Cronan, *The wing of a winged helix-turn-helix transcription factor organizes the active site of BirA, a bifunctional repressor/ligase.* J Biol Chem, 2013. **288**(50): p. 36029-39.
54. Yang, B., et al., *Enterohemorrhagic Escherichia coli senses low biotin status in the large intestine for colonization and infection.* Nat Commun, 2015. **6**: p. 6592.
55. Beckett, D., *Biotin sensing: universal influence of biotin status on transcription.* Annu Rev Genet, 2007. **41**: p. 443-64.
56. Cronan, J.E., *Biotin and Lipoic Acid: Synthesis, Attachment, and Regulation.* EcoSal Plus, 2014. **6**(1).
57. Li, S.J. and J.E. Cronan, Jr., *Growth rate regulation of Escherichia coli acetyl coenzyme A carboxylase, which catalyzes the first committed step of lipid biosynthesis.* J Bacteriol, 1993. **175**(2): p. 332-40.
58. Dibrova, D.V., M.Y. Galperin, and A.Y. Mulkidjanian, *Phylogenomic reconstruction of archaeal fatty acid metabolism.* Environ Microbiol, 2014. **16**(4): p. 907-18.
59. Fujita, Y., H. Matsuoka, and K. Hirooka, *Regulation of fatty acid metabolism in bacteria.* Mol Microbiol, 2007. **66**(4): p. 829-39.
60. Dong, Y., et al., *Characterization of two long-chain fatty acid CoA ligases in the Gram-positive bacterium Geobacillus thermodenitrificans NG80-2.* Microbiol Res, 2012. **167**(10): p. 602-7.

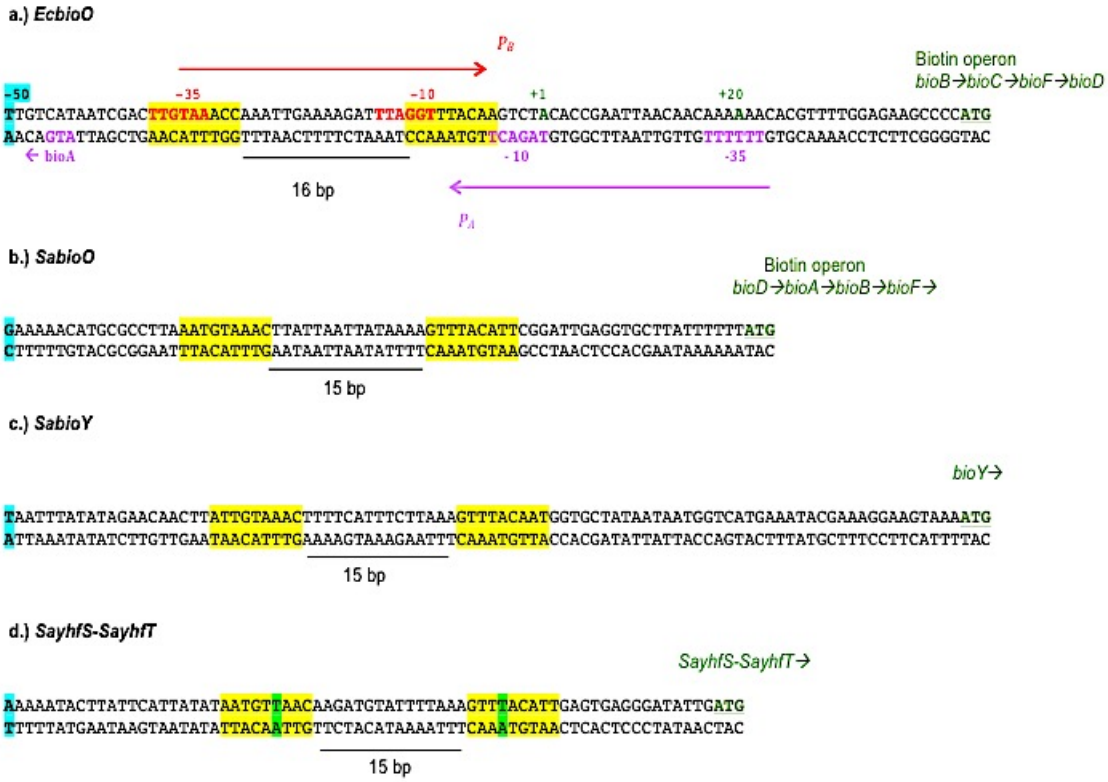


Figure 1: Outline of *E. coli* and *S. aureus* promoter region. BirA binding sequence within: a.) *EcBioO,* b.) *SabioO*, c.) *SabioY* and d.) *SayhfS-SayhfT* promoters are shown. The BirA binding sites are highlighted in yellow. The *Ec*BirA binding site is located between two face-to-face promoters; P_B (red arrow) that regulates the expression of *bioB, bioC, bioF, bioD* and P_A (purple arrow) for *bioA* expression. The *Ec*BirA binding site overlaps with the -35 and -10 element of P_B (bold, red). All BirA binding sites are composed of perfect inverted repeats, except for the *SayhfS-SayhfT* promoter, which is one base pair away from forming perfect inverted repeat (green highlight). The spacing between the inverted repeats is also shown. Blue highlighted letters indicate the starting oligonucleotide of each promoter construct that was cloned into the integration vector, to generate the reporter strain, utilized in β-galactosidase *in vivo* assay.

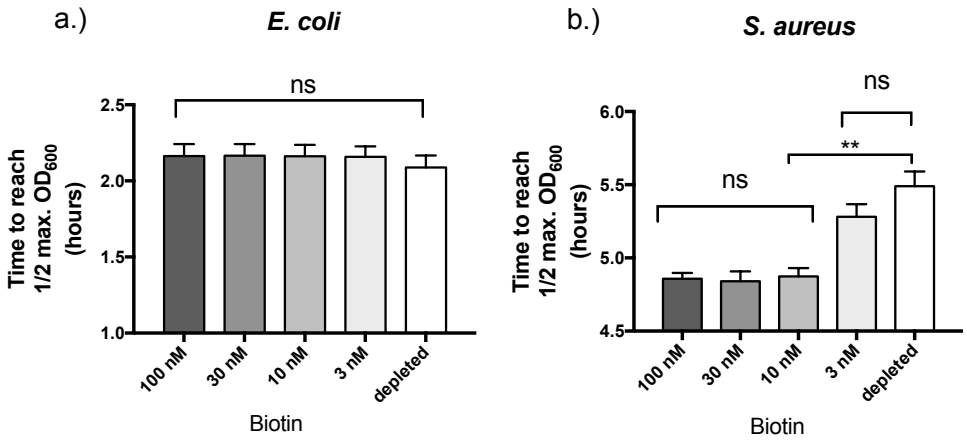


Figure 2: Growth curve of *S. aureus* and *E. coli* in biotin depleted and biotin supplemented media. *E. coli* (ATTC 25922) and *S. aureus* (NCTC 8325) were grown in biotin-depleted media and biotin-supplemented media. Time to reach half maximum growth (half maximum OD_{600}) was calculated for both (a.) *E. coli* and (b). *S. aureus* and graphed. Error bars indicate S.E.M from independent biological replicates (n =3). ** = p <0.01. Statistical calculation was performed using two-tailed t-test.

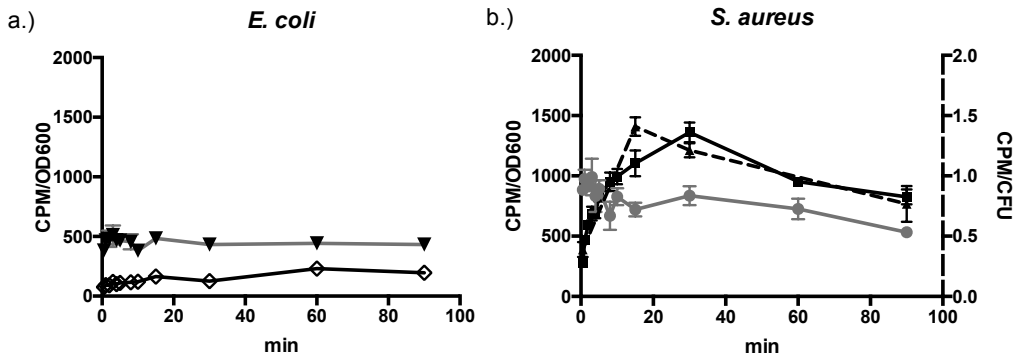


Figure 3: Biotin uptake assay of *S. aureus* and *E. coli*. (a.) *E. coli* Intracellular ^{3}H-biotin count in: (◇)^{3}H-biotin -treated *E. coli* and (▼) *E. coli* grown continuously in the presence of 10 nM ^{3}H-biotin (b.) *S. aureus* intracellular ^{3}H-biotin count in: (■)^{3}H-biotin–treated *S. aureus*. (●) *S. aureus* grown continuously in 10 nM ^{3}H-biotin. (▲) represent CPM/ CFU of ^{3}H-biotin–treated *S. aureus*. Intracellular ^{3}H-biotin was expressed CPM/OD_{600}, X-axis represents minutes post- mid-log phase (or post biotin addition in biotin-treated cultures). Error bars represent S.E.M from independent biological replicates (n = 6).

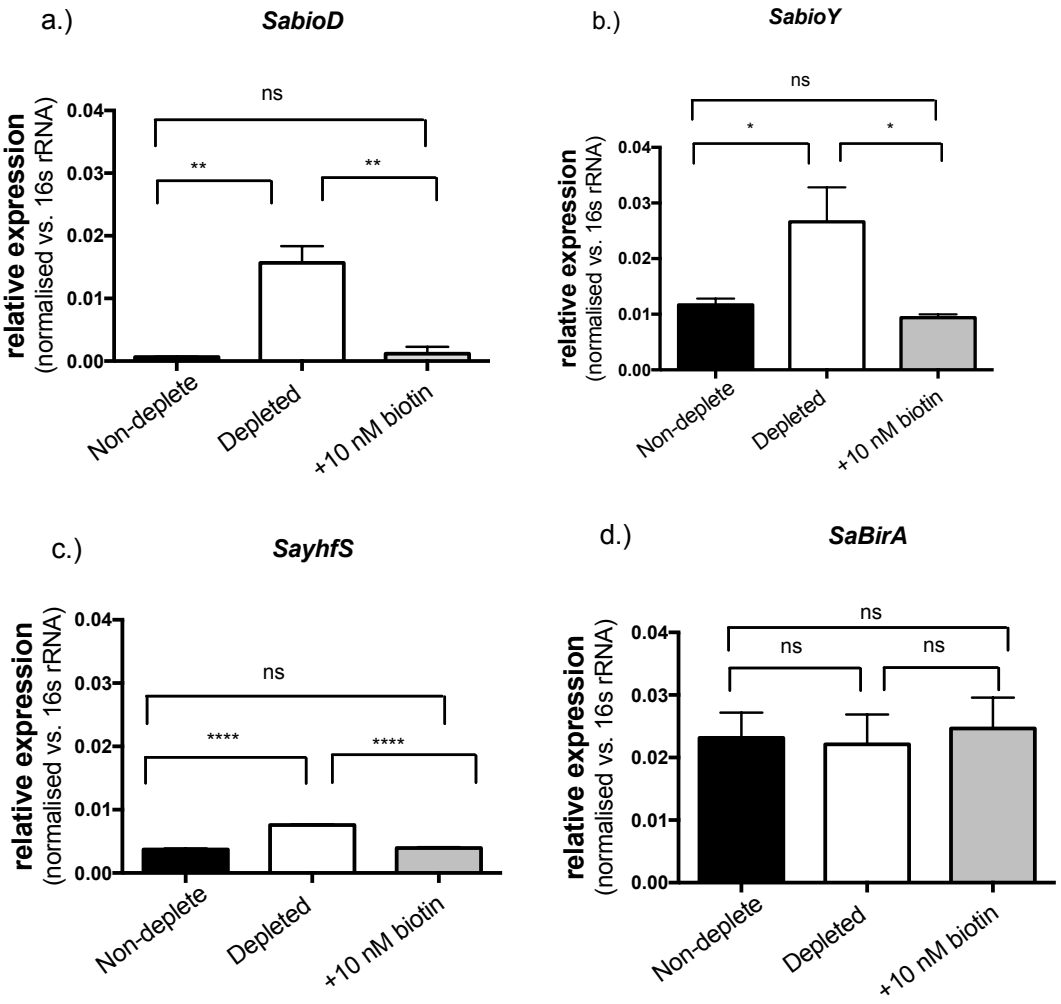


Figure 4: Up-regulation of *Sa*BirA regulated genes in biotin-depleted media. Total RNA was harvested from *S. aureus* during mid-log phase. *S. aureus* culture was grown in Mueller-Hinton media (black), biotin-depleted Mueller-Hinton media (white) and depleted media supplemented with 10 nM biotin (grey). qRT-PCR was performed to detect the expression of (a.) S*abioD,* (b.) *SabioY*, (c.) *SayhfS* and (d.) *SaBirA*. Relative expression was normalized against *16s rRNA*, error bars represent S.E.M from independent biological replicate of (n = 3). Statistical significance was calculated using one-way ANOVA (* = p <0.05, ** = p <0.01, *** = p <0.001, **** = p <0.0001, ns = non-significant).

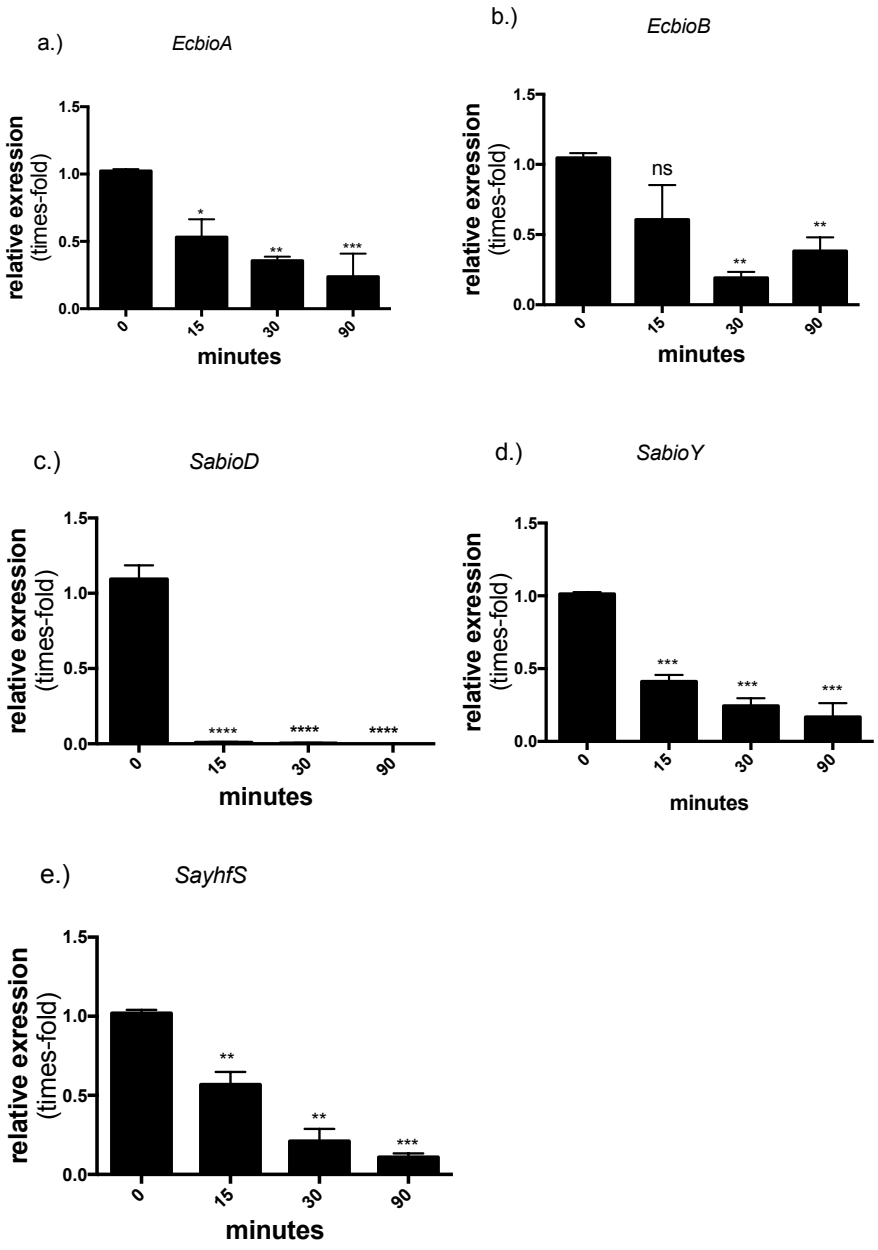


Figure 5: qRT-PCR analysis of time course biotin-treated samples of BirA-regulated genes. *S. aureus* and *E. coli* was grown in biotin-depleted media until mid-log phase was reached and treated with 10 nM biotin. RNA samples were collected at 0,15,30 and 90 minutes post biotin addition. The relative expression of biotin biosynthesis genes (a.) *E. coli bioA* and (b.) *E. coli bioB* were quantified and compared with (c.) *S. aureus bioD.* The expression of two other *Sa*BirA-regulated genes, (d.) *SabioY* and (e.) *SayhfS* were also quantified. Transcription levels were corrected to t = 0, after internal normalization against 16s rRNA. Error bars represent S.E.M from independent biological replicate of at least (n = 3). Statistical significance was calculated using one-way ANOVA (* = p <0.05, ** = p <0.01, *** = p >0.001, **** = p <0.0001, ns = non-significant).

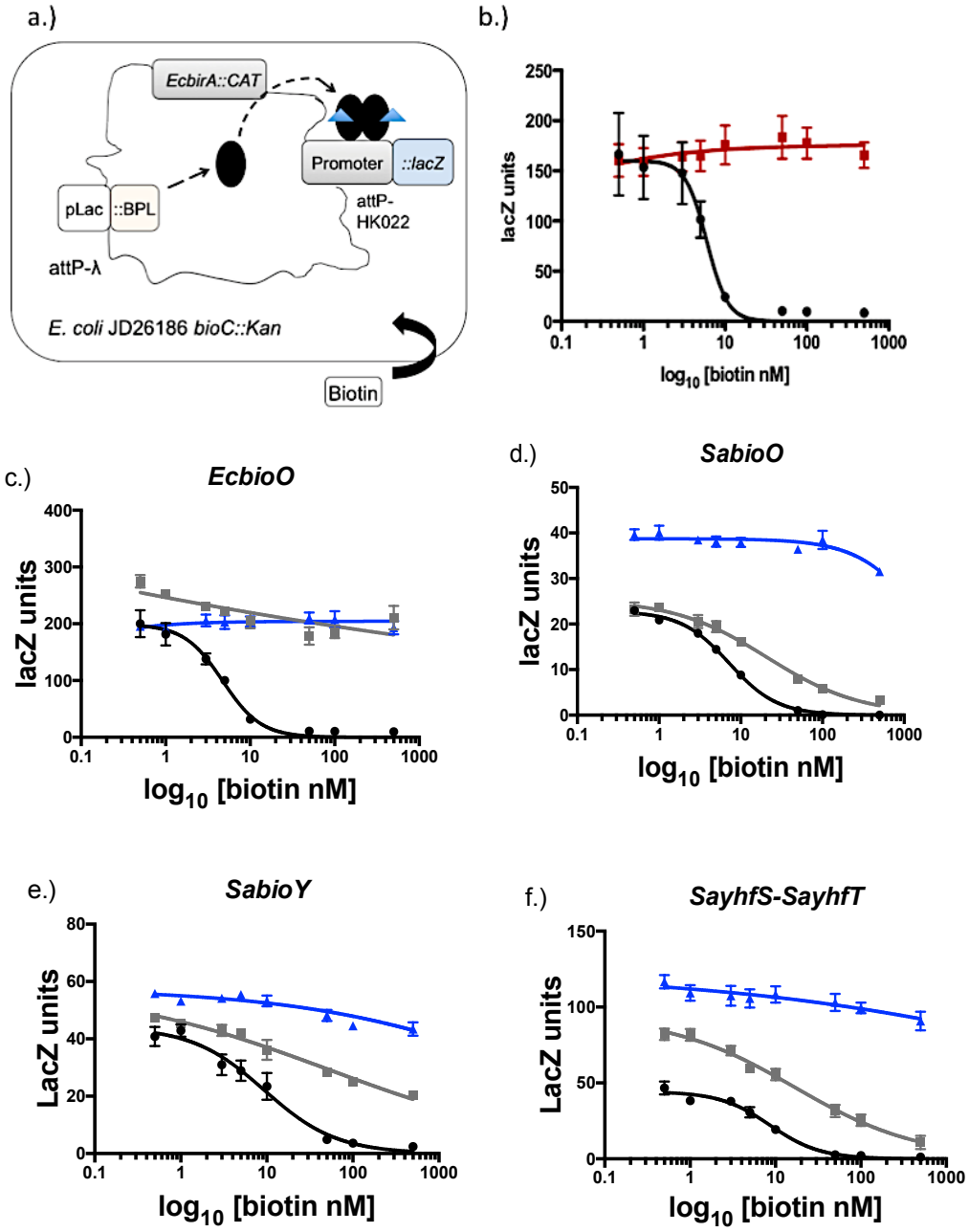


Figure 6: *In vivo* β-galactosidase assay and *E. coli* reporter strain outline. (a.) Overview of *E. coli* reporter strain containing integrated constructs. β-galactosidase assay validation showing: (b.) the loss of repressor function of the JD26186 birA::CAT strain (red) compared to the wild-type JD26186 with un-modified *Ec*BirA (black), (c.) *Ec*BirA interaction with *EcbioO.* The interaction of *Sa*BirA with (d.) *SabioO*, (e.) *SabioY*, (f.) *SayhfS-SayhfT* promoter is shown. Wild type proteins are shown in (black) and dimerization mutants are shown in (grey). Strain with no integrated BirA was also included to serve as control (blue). A control strain without integrated promoter was used to measure the background *lacZ* activity at each biotin concentration (≤ 10 units), which has been subtracted to give values shown in the graphs. Error bars denote S.E.M from independent biological replicate of at least (n = 6).

	BirA	
	Wild-type $K_{R\ biotin}$ (nM)	Dimerization mutant $K_{R\ biotin}$ (nM)
SabioO	7.2 ± 0.3	20.9 ± 5.0
SabioY	9.4 ± 2.4	≥ 500
SayhfS-SayhfT	8.6 ± 1.3	16.9 ± 6.4
EcBioO	4.7 ± 0.5	≥ 500

Table 1: *In vivo* binding assay equilibrium binding constant for biotin (K_R). The amount of biotin required to achieve half-maximum repression at equilibrium was calculated from the graph using GraphPad Prism one-site specific binding equation: Y= Bmax*X^h /(K_R^h + X^h). Where Y = specific binding (*LacZ* unit), X = Biotin concentration (nM), Bmax = maximum binding (*LacZ* unit) and h = Hill-slope value (binding as a dimer is represented as h = 2).

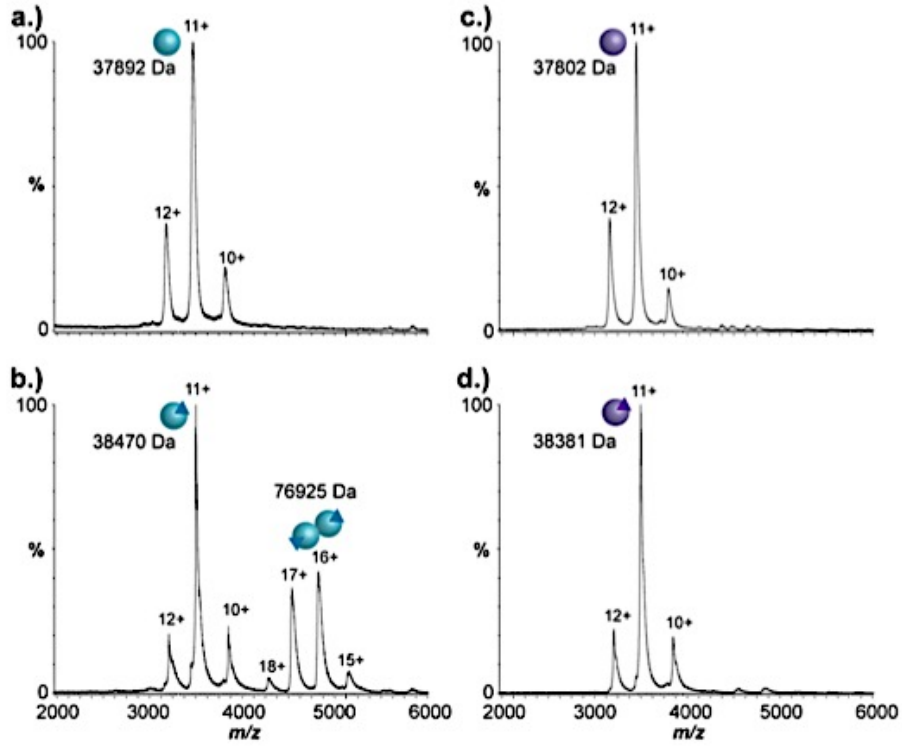


Figure 7: nESI-MS of wild-type *Sa*BirA and *Sa*BirA F123G. Nano-electrospray ionization-mass spectrometry of (a.) apo-*Sa*BirA (wild-type), (b.) holo-*Sa*BirA (wild-type), (c.) apo-*Sa*BirA F123G and (d.) holo-*Sa*BirA F123G is shown. Peaks representing the oligomeric state of the protein are marked by the sphere symbols. Monomeric apo protein is presented by a single solid blue sphere (wild-type *Sa*BirA) and purple sphere (*Sa*BirA F123G). The presence of ligand is presented by a triangle. Dimeric protein is denoted by two joined-spheres. Molecular weight of the major species detected are presented in the figures.

***Sa*BirA**	**Expected Oligomeric state**	**Expected MW (Da)**	**MW detected (Da)**	**Confirmed oligomeric state**
Apo- wild type	Monomer	37892 (-Met)	37892	Monomer, no ligand
Holo-wild type	Monomer Dimer	38465 76930	38470 76925	Monomer, biotinyl-5′-AMP bound Dimer, biotinyl-5′-AMP bound
Apo-F123G	Monomer	37800 (-Met)	37802	Monomer, no ligand
Holo-F123G	Monomer	38373	38381	Monomer, biotinyl-5-AMP bound

Table 2: Native nano-electrospray ionisation mass-spectroscopy (nESI-MS) result. Apo-purified *Sa*BirA was analysed using ESI-MS. Holo-*Sa*BirA was prepared by incubating the apo-purified *Sa*BirA with 500 µM biotin, 1mM ATP and 1 mM $MgCl_2$, prior to analysis. Predicted molecular mass and detected mass is outlined. Predicted oligomeric state and detected oligomeric state is also outlined. Relative error mass of the detected MW is within the expected range of approximately 1% in 1 MDa.

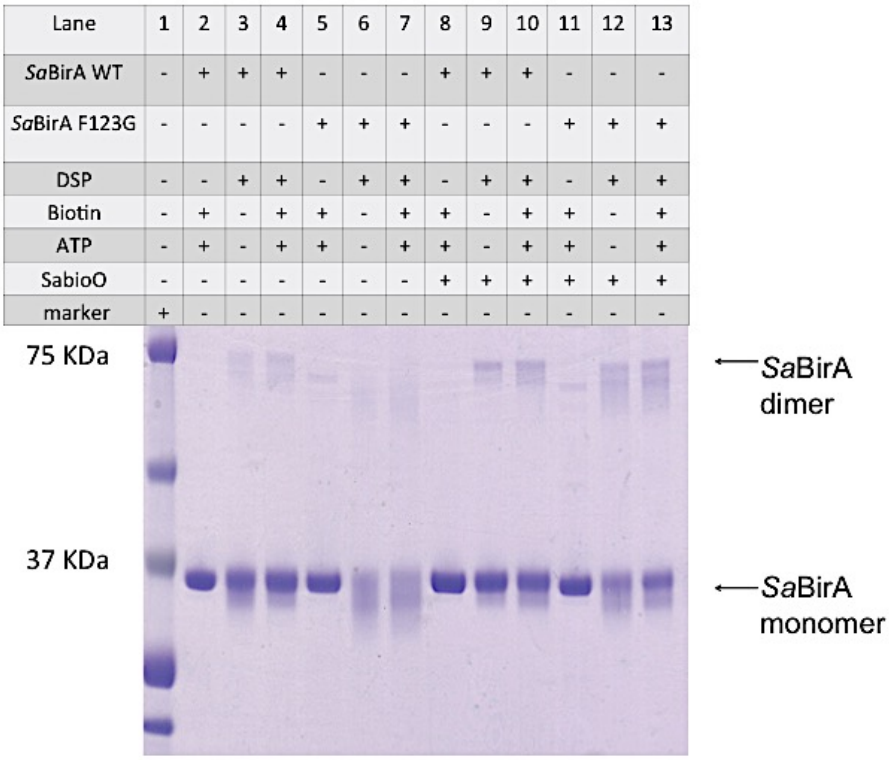


Figure 8: DSP cross-linked *Sa*BirA SDS-PAGE. Cross-linking reactions were performed using DSP cross linker as described in materials and method. *Sa*BirA wildtype and *Sa*BirA F123G were tested for dimerization in the absence or presence of 44 bp double-stranded oligo containing sequence from *SabioO* promoter. Binding was also tested in the absence and presence of biotin and ATP as indicated in each lane.

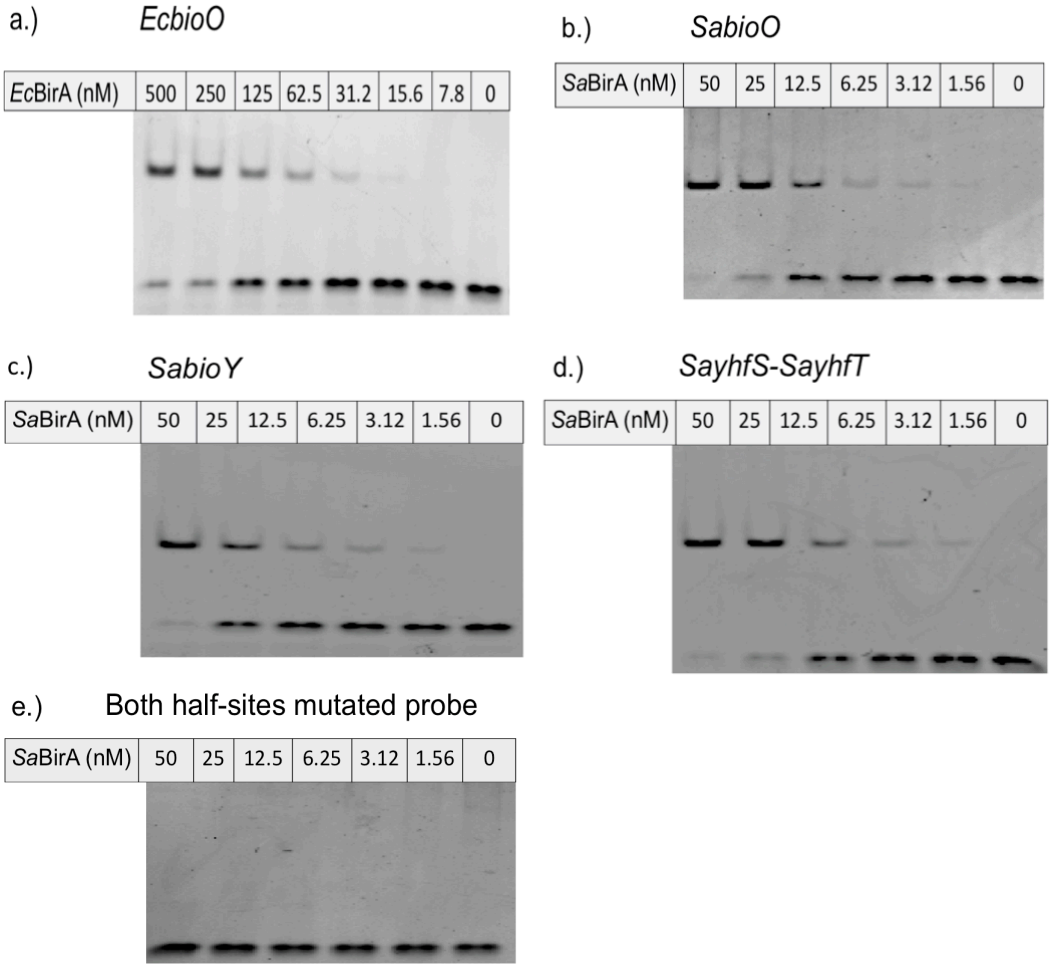


Figure 9: Electrophoretic mobility shift assay (EMSA) of holo-*Sa*BirA and holo-*Ec*BirA. EMSA of the holo-BirA were performed in standard binding buffer containing 1mM ATP and 0.1 mM biotin. 10 nM of DNA probe was incubated with protein for 0.5 hour at room temperature prior to electrophoresis. Gel was stained with GelRed, as outlined in materials and methods. The results are shown in panel; (a.) Holo-*Ec*BirA binding to *EcbioO.* Holo-*Sa*BirA binding to (b.) *SabioO* (c.) *SabioY* (d.) *SayhfS-SayhfT* and (e.) control probe containing mutated sequences of *Sa*BirA binding sites.

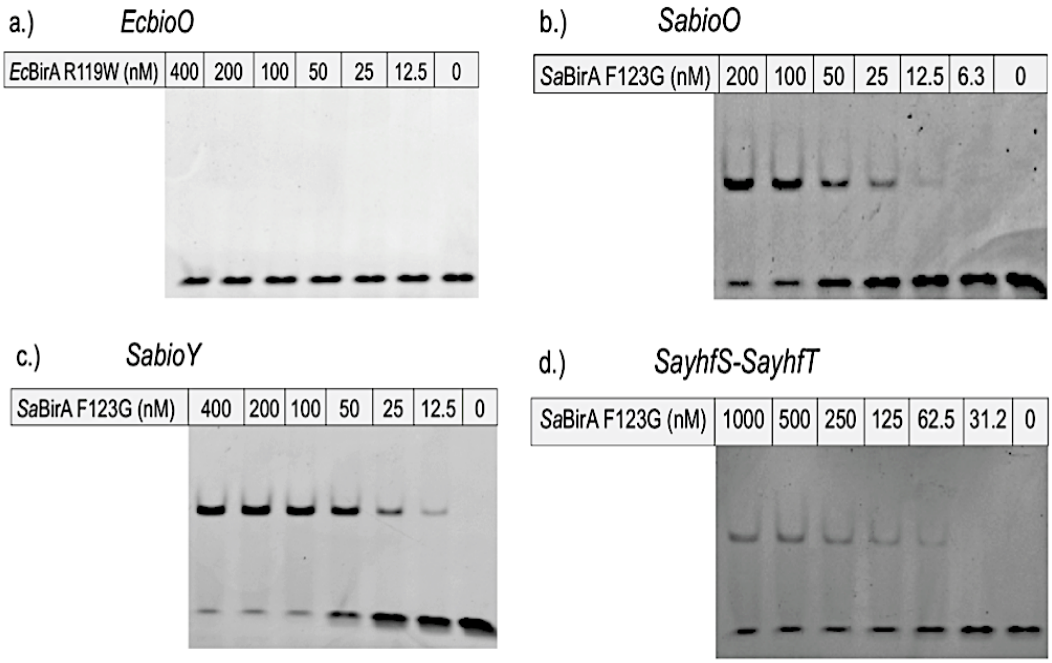


Figure 10: EMSA analysis of dimerization mutant *Sa*BirA F123G and *Ec*BirA R119W. EMSA of the dimerization mutants were performed in standard binding buffer containing 1mM ATP and 0.1 mM biotin. 10 nM of DNA probe was incubated with protein for 0.5 hour at room temperature prior to electrophoresis. Gel was stained with GelRed, as outlined in materials and methods. The results are shown in panel; (a.) *Ec*BirA R119W binding to *EcbioO. Sa*BirA F123G binding to (b.) *SabioO* (c.) *SabioY* (d.) *SayhfS-SayhfT*

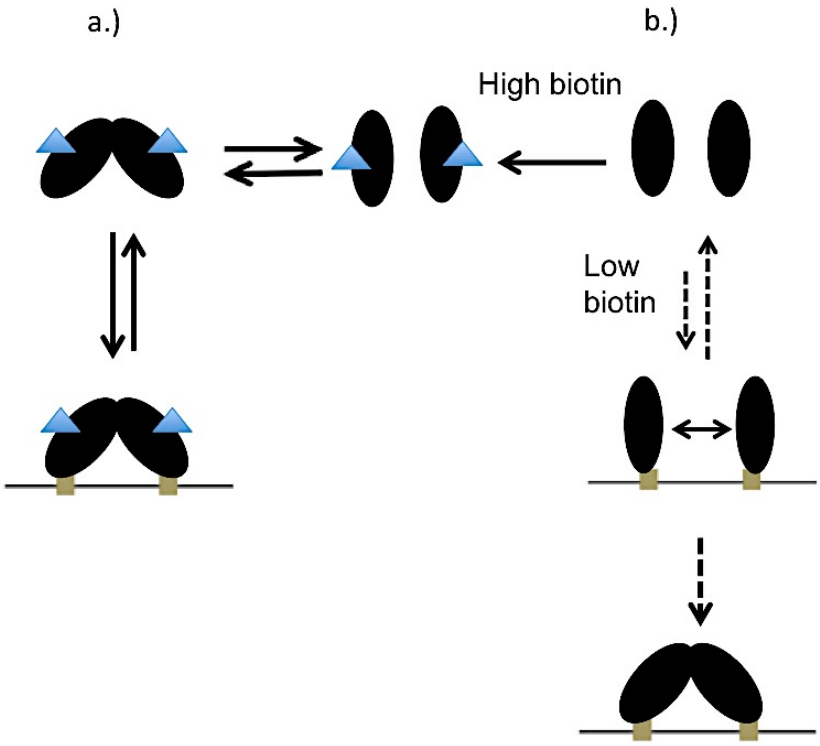
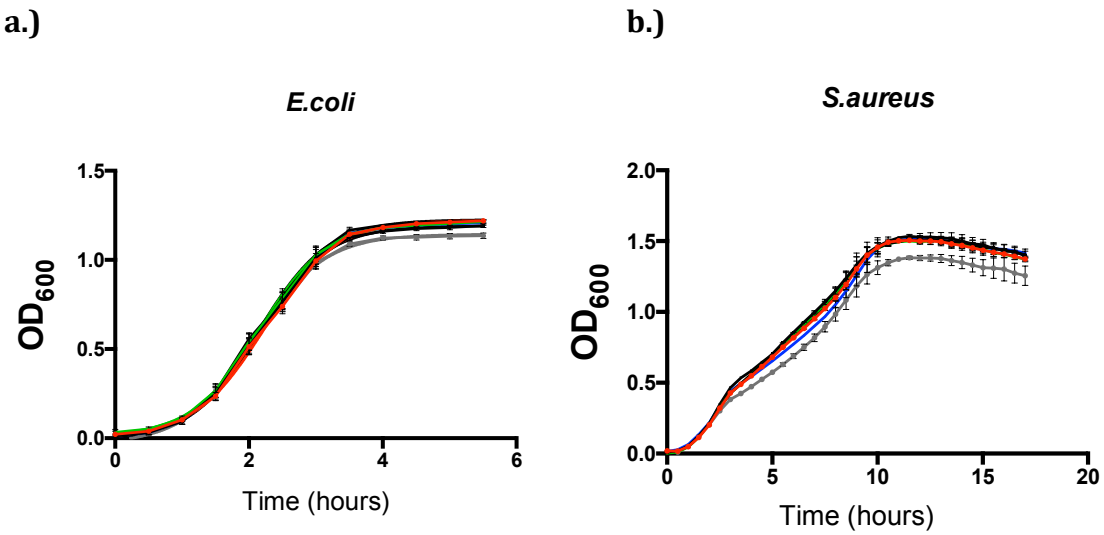


Figure 11: Proposed binding mechanism of *Sa*BirA. (a.) Under high biotin conditions, *Sa*BirA (black) binds biotin (blue triangles). Under these conditions, holo-*Sa*BirA follows the same binding mechanism as holo-*Ec*BirA where pre-formed dimer binds to DNA (brown squares represent operator half-sites). (b.) Under low biotin conditions, *Sa*BirA monomers can bind to DNA, followed by dimerization on the DNA, as indicated by the dashed arrows. Under the same conditions, *Ec*BirA cannot bind DNA.

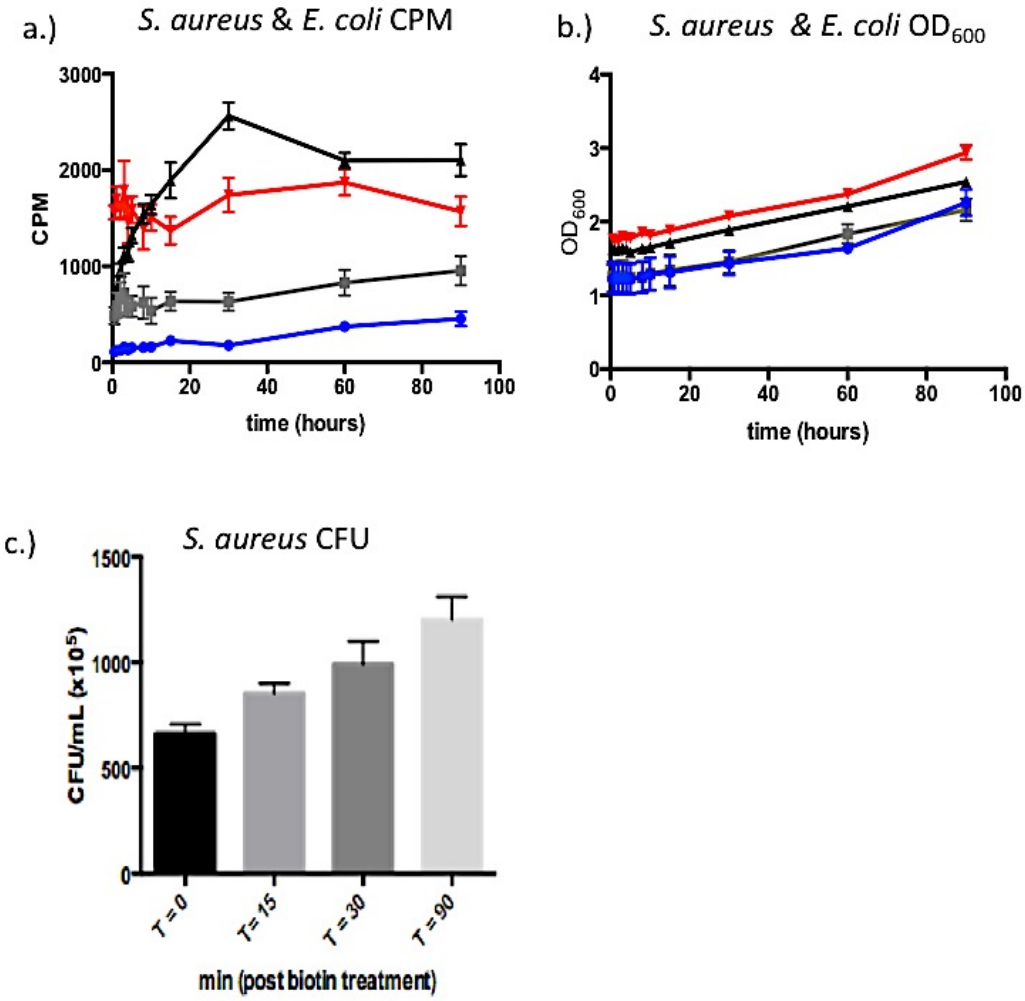
Supplementary figures and tables:



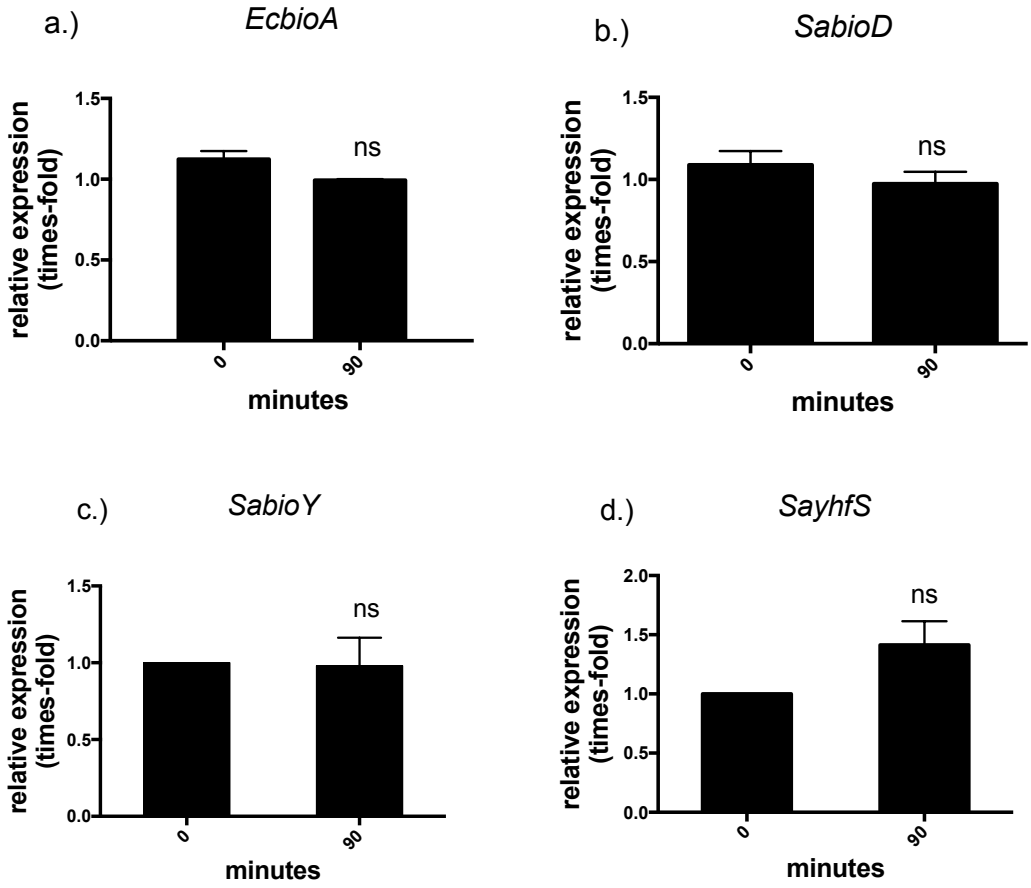
Supplementary Fig 1 S1: Growth curve of *E. coli* and *S. aureus* in biotin depleted media and biotin supplemented media. Growth curve of (a.) *E. coli* and (b.) *S. aureus* were obtained by growing bacteria in 100nM biotin supplemented (black), 30nM biotin supplemented (green), 10nM biotin supplemented (red), 3nM biotin supplemented (blue) and depleted media is shown in (grey). Cultures were grown for 24 hours and OD_{600} were measured every 30 minutes.

	S. aureus		*E. coli*	
Media	**Time to reach 1/2 Max. OD600**	**Max. OD600**	**Time to reach 1/2 Max. OD600**	**Max. OD600**
+ 100 nM biotin	4.8 ± 0.2	1.5 ± 0.0	2.2 ± 0.0	1.2 ± 0.0
+ 30 nM biotin	4.8 ± 0.3	1.5 ± 0.0	2.2 ± 0.0	1.2 ± 0.0
+ 10 nM biotin	4.9 ± 0.3	1.5 ± 0.0	2.2 ± 0.0	1.2 ± 0.0
+ 3 nM biotin	5.4 ± 0.3	1.5 ± 0.0	2.2 ± 0.0	1.2 ± 0.0
Biotin-depleted	5.5 ± 0.3	1.4 ± 0.0	2.2 ± 0.0	1.2 ± 0.0

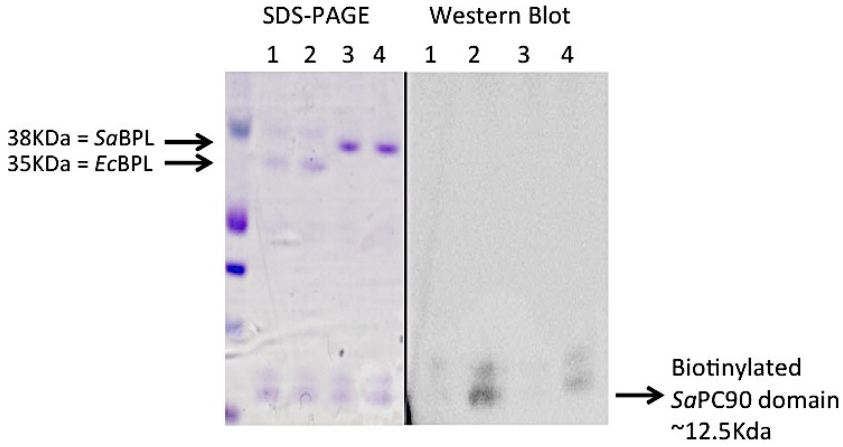
Supplementary Table 1: Summary of time required to reach half maximum OD_{600} (half maximum growth) of *E. coli* and *S. aureus* grown in different media. Values were obtained from a triplicate samples and represent mean ± S.E.M.



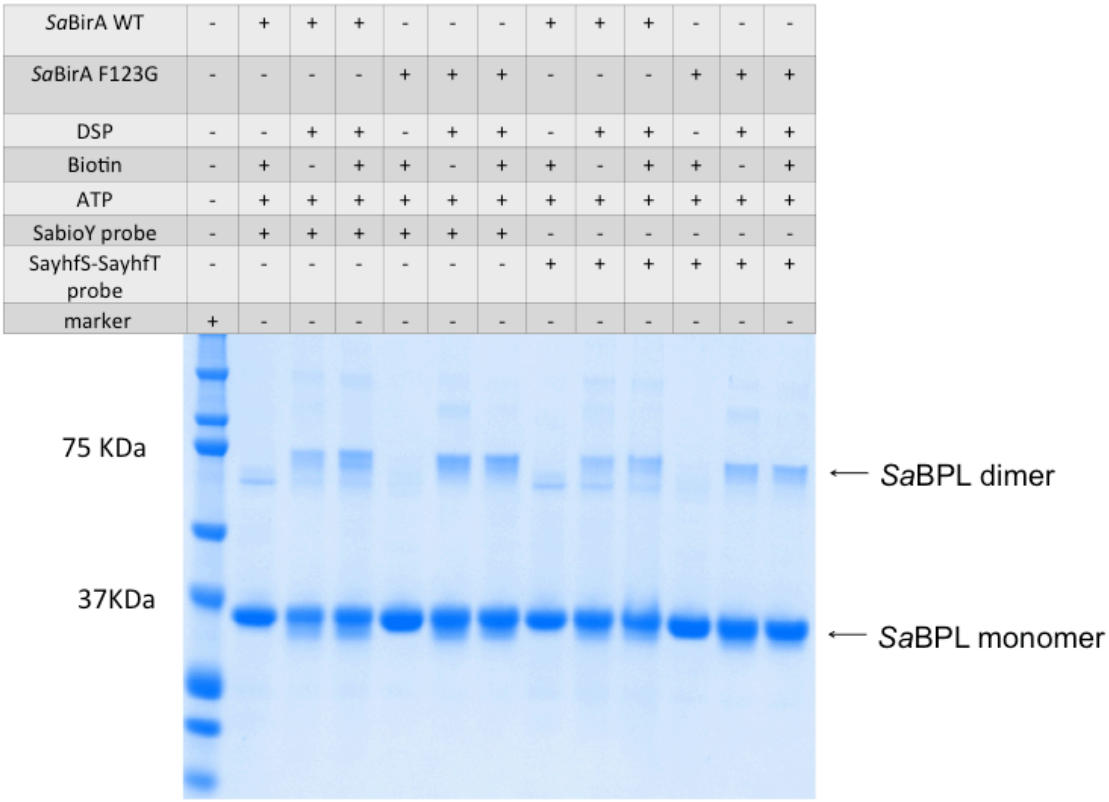
Supplementary Fig 2 S2: Biotin uptake assay results showing count per minute of ^{3}H-biotin uptake, cell density and viability assay. (a.) ^{3}H-biotin uptake vs. time. In *E. coli* biotin-treated samples (blue line), *E. coli* continuous growth in ^{3}H-biotin (grey line), *S. aureus* biotin-treated sample (black line) and *S. aureus* continous growth in 10nM ^{3}H-biotin (red line) at 60 minutes. (b.) Density of each culture taken per time point. (c.) CFU count of *S. aureus* taken at 0, 15, 30 and 90 minutes post biotin treatment.



Supplementary Fig 3 S3: QRT-PCR control for *S. aureus* and *E. coli*. Total RNA was harvested from *S. aureus* and *E. coli* grown in biotin-depleted media at time 0 and 90 minutes. The relative expression of (a.) *EcBioA*, (b.) *SabioD*, (c.) *SabioY* and (d) *SayhfS* were corrected to t = 0, after internal normalization against 16s rRNA. Error bars represent S.E.M from independent biological replicate of n = 3. Statistical significance was calculated using t test (ns = non-significant).



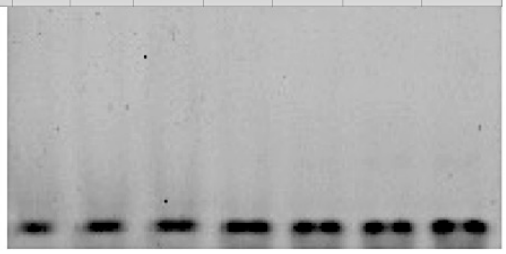
Supplementary Figure 4 S4: Western blot confirmation of purified apo-protein. Western blot was performed to confirm biotinyl-5′-AMP did not co-purify with apo-*Sa*BPL or apo-*Ec*BPL as described in supporting experimental procedures. Reactions containing (1) apo-*Ec*BPL incubated with *Sa*PC90, (2) holo-*Ec*BPL incubated with *Sa*PC90, (3) apo-*Sa*BPL incubated with *Sa*PC90 and (4) holo-*Sa*BPL incubated with *Sa*PC90 were probed using Alexa-Fluor488 conjugated streptavidin, to detect biotinylated product. The corresponding SDS-page gel stained with Coomassie blue is presented as a loading control.



Supplementary Figure 5 S5: SDS-PAGE of *Sa*BPL crosslinking in the presence and absence of *SabioY* and *SayhfS-SayhfT* probes. Cross-linking reactions were performed using DSP cross linker as described in materials and method. *Sa*BirA wildtype and *Sa*BirA F123G were tested for dimerization in the absence or presence of 44 bp double stranded oligo containing sequence from *SabioY* and *SayhfS-SayhfT* promoter.

a.)

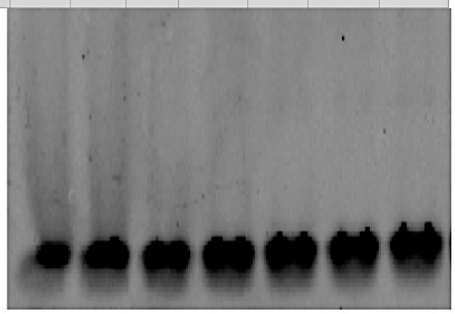
SaBirA (μM)	2.0	1.0	0.5	0.3	0.1	0.05	0
First-half mutated probe (nM)	10	10	10	10	10	10	10
0.1mM biotin	+	+	+	+	+	+	+
1mM ATP	+	+	+	+	+	+	+



AATGTAAAC
5′-GCCTTACACAGACGATTATTAATTATAAAAGTTTACATTCGGAT-3′

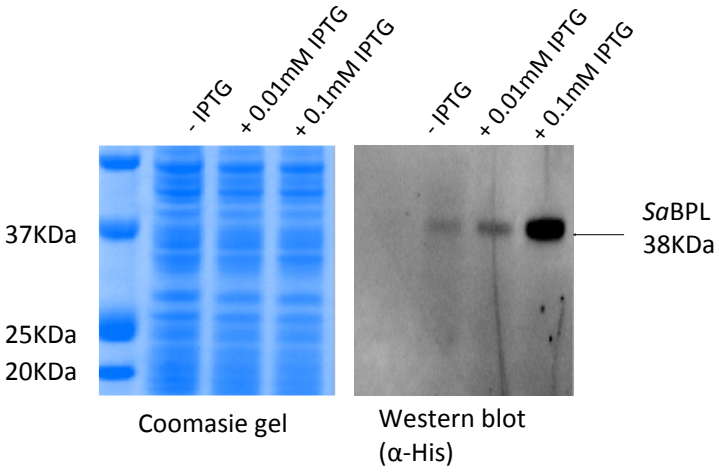
b.)

SaBirA (μM)	2.0	1.0	0.5	0.3	0.1	0.05	0
Second-half mutated probe (nM)	10	10	10	10	10	10	10
0.1mM biotin	+	+	+	+	+	+	+
1mM ATP	+	+	+	+	+	+	+



GTTTACATT
5′-GCCTTAAATGTAAACTTATTAATTATAAAAAGCAGACACCGGAT-3′

Supplementary Figure 6 S6: Gel shift assay of half-site probes. Gel shift was performed on double stranded DNA containing mutated sequence of (a.) the first half site (left half) of the *Sa*BirA binding site and (b.) the second half-site (right half) of the *Sa*BirA binding site. *Sa*BirA binding sites are highlighted in yellow, mutated sequence is highlighted in red with wild-type sequence displayed above (underlined).



Supplementary Figure 7 S7: Validation of *Sa*BPL(6xHis) expression in *E. coli* reporter strain. α-His antibody was used to detect the presence of *Sa*BPL (6xHis) in the *E. coli* reporter strain treated with no IPTG, 0.01mM and 0.1mM IPTG (right panel). The coomassie gel loading control is shown (left panel).

Strain name	Genotype	Description	Source
JD26186	*bioC::Kan*	E. coli MG1655 derivative with disrupted bioC gene	NBRP, Japan
JD26186 *birA::CAT*	*bioC::Kan birA::CAT*	JD28186 strain with N-terminal CAT cassette insertion (knockout)of its endogenous *birA*	this study
JD26186 *birA::CAT-SaBioO-SaBPL*	*bioC::Kan birA::CAT* $(SabioO\text{-}lacZ)_{HK}(placUV5\text{-}SaBPL)_{I}$	JD26186 birA::CAT strain with SaBioO lacZ reporter chromosomally integrated at HK022 att site, and placUV5-SaBPL(wildtype) cassette chromosomally integrated at lambda att site.	this study
JD26186 *birA::CAT-SaBioY-SaBPL*	*bioC::Kan birA::CAT* $(SabioY\text{-}lacZ)_{HK}$ $(placUV5\text{-}SaBPL)_{I}$	JD26186 birA::CAT strain with SaBioY-lacZ reporter chromosomally integrated at HK022 att site and placUV5-SaBPL(wildtype) cassette chromosomally integrated at lambda att site.	this study
JD26186 *birA::CAT-yHFS-T-SaBPL*	*bioC::Kan birA::CAT* $(yHFS\text{-}T\text{-}lacZ)_{HK}$ $(placUV5\text{-}SaBPL)_{I}$	JD26186 birA::CAT strain with yHFS-T-lacZ reporter chromosomally integrated at HK022 att site, and placUV5-SaBPL(wildtype) cassette chromosomally integrated at lambda att site.	this study
JD26186 *birA::CAT-SaBioO-SaBPL F123G*	*bioC::Kan birA::CAT* $(SabioO\text{-}lacZ)_{HK}$ $(placUV5\text{-}SaBPL\ F123G)_{I}$	JD26186 birA::CAT strain with SaBioO-lacZ reporter chromosomally integrated at HK022 att site, and plac-UV5-SaBPL (monomeric mutant) cassette chromosomally integrated at lambda att site.	this study
JD26186 *birA::CAT-SaBioY-SaBPL F123G*	*bioC::Kan birA::CAT* $(SabioY\text{-}lacZ)_{HK}$ $(placUV5\text{-}SaBPL\ F123G)_{I}$	JD26186 birA::CAT strain with SaBioY-lacZ reporter chromosomally integrated at HK022 att site, and plac-UV5-SaBPL (monomeric mutant) cassette chromosomally integrated at lambda att	this study
JD26186 *birA::CAT-yHFS-T-SaBPL F123G*	*bioC::Kan birA::CAT* $(yHFS\text{-}T\text{-}lacZ)_{HK}$ $(placUV5\text{-}SaBPL\ F123G)_{I}$	JD26186 birA::CAT strain with yHFS-T-lacZ reporter chromosomally integrated at HK022 att site and plac-UV5-SaBPL (monomeric mutant) cassette chromosomally integrated at lambda att	this study
JD26186-*EcBioO-birA*	*bioC::Kan* $(EcBioO\text{-}lacZ)_{HK}$	JD26186 strain with EcBioO-lacZ reporter chromosomally integrated at HK022 att site	this study
JD26186 *birA::CAT-EcBioO-birA R119W*	*bioC::Kan birA::CAT* $(EcBioO\text{-}lacZ)_{HK}$ $(placUV5\text{-}birA\ R119W)_{I}$	JD26186 birA::CAT strain with EcBioO promoter and placUV5-birA R119W (monomeric mutant) chromosomally integrated at lambda att	this study
BL21 *(λ DE3)*	*E. coli B ompT Dhsd*S $(rB^{-}mB^{-})$ *dcm gal* λ(DE3)	Protein expression strain	lab collection
BL21-CodonPlus(DE3)-RIPL	*E. coli B* F^{-} *ompT DhsdS* $(rB^{-}mB^{-})$ $dcm^{+}Tet^{R}$ *gal λ(DE3) endA Hte [argU proL* Cm^{R}*] [argU ileY leuW* $Strep/Spec^{R}$*]*	Protein expression strain	Agilent technology (USA)

Supplementary Table 2: list of bacterial strains used in this study.

Plasmid	description	Source
pKD46	Amp^R, *oriR101*, λ-Red recombinase expression plasmid	Datsenko & Warner (2000)
pK(*HsBPL*)	template for HsBPL to clone into pKD46 (Amp^R, pBR322/pUC ori, *Hs*BPL-6xHis, pTac-uV5)	Mayende, *et al* (2012)
pKD46(*HsBPL*)	pKD46 containing *HsBPL* gene under the control of arabinose promoter	this study
pCY216(*birA*)	p15A ori, medium copy plasmid containing Cm^R and CAT promoter	Cronan & Wallace (1995)
pGEMT-*Sa*BPL(6xHis)	pGEMT plasmid containing *saBPL* with 6x his-tag	Pendini *et al*, (2008)
pGEMT-*birA*(6xHis)	pGEMT plasmid containing *birA* with 6x his-tag	Soares Da costa, *et al* (2014)
pIT3_CLlacZ_Trim	Chromosomal integration plasmid (λ-attP, Cm^R, R6Kγ ori, *lacZ*)	Cui *et a*l PNAS (2013)
pIT3-SH-152002	Chromosomal integration plasmid (HK022-attP, $Spec^R$, R6Kγ ori, *ccdB,* pUC ori)	Shearwin lab, Adelaide university
pIT3_SH_LacZTrim	Chromosomal integration plasmid (HK022-attP, $Spec^R$, R6Kγ ori, *lacZ*)	this study
pIT4_TL_152002	Chromosomal integration plasmid (λ-attP, Tc^R, R6Kγ ori, *ccdB,* pUC ori)	St. Pierre, *et a*l (2013)
pGEMT-*birA R119W* (6xHis)	pGEMT plasmid containing *birA R119W* (6x his-tag)	this study
pGEMT-*Sa*BPL F123G (6xHis)	pGEMT plasmid containing *saBPL F123G* (6xhis-tag)	Soares Da costa, *et al* (2014)
peT16b-*birA R119W* (6xHis)	pET16b expression vector containing *birA R119W*(6x his-tag)	this study
pET16b-*SaBPL F123G* (6xHis)	pET16b expression vector containing *saBPL F123G* with (6xhis-tag)	Soares Da costa, *et al* (2014)
pIT3_SH_*SabioO*_LacZ	SabioO promoter sequence cloned into pIT3_SH_LacZTrim, upstream of the lacZ gene	this study
pIT3_SH_*SabioY*_LacZ	SabioY promoter sequence cloned into pIT3_SH_LacZTrim, upstream of the lacZ gene	this study
pIT3_SH_*yHFT*_LacZ	yHFT promoter sequence cloned into pIT3_SH_LacZTrim, upstream of the lacZ gene	this study
pIT4_TL_*Sa*BPL (WT)	plac-UV5 fused with SaBPL (wildtype) sequence cloned into pIT4_TL_152002	this study
pIT4_TL_*Sa*BPL (F123G)	plac-UV5 fused with SaBPL (F123G) sequence cloned into pIT4_TL_152002	this study
pIT4_TL_*birA* (R119W)	plac-UV5 fused with birA (R119W) sequence cloned into pIT4_TL_152002	this study

Supplementary Table 3: list of plasmid used in this study.

Oligo name	Sequence 5'-3'	Description
B391	CGTCAGGCATCGATCGGAAGCTGTGGTATGGCTGTGC	Amplify ptac-hBPL-H_6 (For), linker on ClaI site, HsBPL cloning into pKD46
B392	CAATATTATTGAAGCATCGATCAGGGTTATTGTCTCATGAGCG	Amplify ptac-hBPL-H_6 (Rev), linker on ClaI site, HsBPL cloning into pKD46
B106	GCTCTCTCTTAGATTTGTTTCATCC	forward primer to screen *Hs*BPL gene
B107	GAGATAATCGGCTCTTAAGG	reversed primer to screen *Hs*BPL gene
B393	GGAATAAGGGCGACACGGAAATGTTG	sequencing primer1 for pKD46-*Hs*BPL (Sequence from 5' end)
B394	CGGTCGCAATGTTGGTTTTGACG	sequencing primer1 for pKD46-*Hs*BPL (Sequence from 3' end)
B395	GAAGGATAACACCGTGCCACTGAAATTGATTGCCCTGTTAGCGAACGGTGAATTTCACGGCAGCATCACCCGACGCAC	Forward primer with birA homologous sequence for *CAT- Cm^R* isolation from pCY216 *birA*
B396	GGATAGGCTCAGGCAGGCTGTATCCTTTACCCGGAACGGTAAAGACATCAACGCCCCAGTTACGCCCCGCCCTGCCACTC	Reversed primer with birA homologous sequence for *CAT- Cm^R* isolation from pCY216 *birA*
B398	CGATTTAAGCTCTCCGATACGATCAAG	reversed sequencing primer for N-terminal birA::CAT
B399	GAAGGATAACACCGTGCCACTGAAATTG	Forward primer for *JD26186 birA::CAT* PCR screening
B133	CCATTTCCGACCCCGACCACCACGGCCAGC	Reversed primer for *JD26186 birA::CAT* PCR screening
B409	GGTGTGTCATATGCGCACCACCAAACTCC	Forward primer for N-terminal birA::CAT sequencing template
B410	GGCCTTGTTCCAGACGCCAGAACATCGAC	Reversed primer for N-terminal birA::CAT sequencing template
B410	GGCCTTGTTCCAGACGCCAGAACATCGAC	sequencing primer to sequence *pTac-birA* in integration plasmid pIT4_TL152002
B411	GGCAGCAGGTTGGCTTATCGATCAGTGCC	Forward sequencing primer for N-terminal birA::CAT
M13_F	ACTGGCCGTCGTTTTAC	universal primer M13 (forward) used to sequence promoter sequence in integration plasmid pIT3_SH_lacZtrim
pTac oligo 1	CATGCGGTACCTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAA	Top strand of double stranded sequence of pTac and lac operator, cloned into pGEMT-*SaBPL*(6xHis) / pGEMT-*birA* (6xHis)
pTac oligo 2	CATGTTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACATTATACGAGCCGATGATTAATTGTCAAGGTACCG	Bottom strand of double stranded sequence of pTac and lac operator, cloned into pGEMT-*SaBPL*(6xHis) / pGEMT-*birA* (6xHis)
EcBioO oligo 1	CTTGTCATAATCGACTTGTAAACCAAATTGAAAAGATTTAGGTTTACAAGTCTACACCGAATTAACAACAAAAGCATG	Top strand of double stranded *EcBioO* operator sequence, cloned into pIT3_SH_lacZtrim
EcBioO oligo 2	CTTTTGTTGTTAATTCGGTGTAGACTTGTAAACCTAAATCTTTTCAATTTGGTTTACAAGTCGATTATGACAAGGTAC	Bottom strand of double stranded *EcBioO* operator sequence, cloned into pIT3_SH_lacZtrim
SaBioO oligo 1	CGAAAAACATGCGCCTTAAATGTAAACTTATTAATTATAAAAGTTTACATTCGGATTGAGGTGCTTATTTTTTGCATG	Top strand of double stranded *SaBioO* operator sequence, cloned into pIT3_SH_lacZtrim
SaBioO oligo 2	CAAAAAATAAGCACCTCAATCCGAATGTAAACTTTTATAATTAATAAGTTTACATTTAAGGCGCATGTTTTTCGGTAC	Bottom strand of double stranded *SaBioO* operator sequence, cloned into pIT3_SH_lacZtrim
yHFS-T oligo 1	CAAAAATACTTATTCATTATATAATGTTAACAAGATGTATTTTAAAGTTTACATTGAGTGAGGGATATTGGCATG	Top strand of double stranded yHFS-T operator sequence, cloned into pIT3_SH_lacZtrim
yHFS-T oligo 2	CCAATATCCCTCACTCAATGTAAACTTTAAAATACATCTTGTTAACATTATATAATGAATAAGTATTTTTGGTAC	Bottom strand of double stranded yHFS-T operator sequence, cloned into pIT3_SH_lacZtrim
B386	GACTAAAATGTTGAATCGCATTCTTATCCCTAAATCAATAAATAAATTAAATTTAGATATCATTGAGAATGC	sequencing primer to sequence *pTac-SaBPL* in integration plasmid pIT4_TL152002
HK022-P1	GGAATCAATGCCTGAGTG	attp-HK022 PCR screening primer (St. Pierre *et al*, 2013)
HK022-P2	ACTTAACGGCTGACATGG	attp-HK022 PCR screening primer (St. Pierre *et al*, 2013)
HK022-P3	ACGAGTATCGAGATGGCA	attp-HK022 PCR screening primer (St. Pierre *et al*, 2013)
HK022-P4	GGCATCAACAGCACATTC	attp-HK022 PCR screening primer (St. Pierre *et al*, 2013)
Lambda P1	GGCATCACGGC AATATAC	attp-λ PCR screening primer (St. Pierre *et al*, 2013)
Lambda P2	ACTTAACGGCTGACATGG	attp-λ PCR screening primer (St. Pierre *et al*, 2013)
Lambda P3	GGGAATTAATTCTTGAAGACG	attp-λ PCR screening primer (St. Pierre *et al*, 2013)
Lambda P4	TCTGGTCTGGTAG CAATG	attp-λ PCR screening primer (St. Pierre *et al*, 2013)
B460_R119W_F	GGCCGTGGTCGCTGGGGTCGGAAATGG	Forward mutagenesis primer for birA R119W
B461_R119W_R	CCATTTCCGACCCCAGCGACCACGGCC	reverse mutagenesis primer for birA R119W
B479	GACTCATCATGAAGGATAACACCGTGCCAC	Forward primer to clone birA R119W into integration plasmid pIT4_TL 152002
B320	ACTAGTGATAAGCTTAATGATGATGATGATGATGTCC	reverse primer to clone birA R119W into integration plasmid pIT4_TL_152002

Supplementary Table 4: list of general oligos used in this study.

Target gene	Primer name	Primer sequence 5'-3'
S. aureus bioD	qSA2716_F	GCAAGGTGTGGTGATACAGG
	qSA2716_R	ACACGTGGTCATCGAGTTTG
S. aureus bioY	qSA2552_F	AATGGCAAGCCAGCAACTAC
	qSA2552_R	GGATTGGTACCGGTAATTCCA
S. aureus BPL	qSA1473_F	TCATTCGGCCATTTCACTTT
	qSA1473_R	AAAGGGCAAGGACTTTGGAT
S. aureus yHFT	qSA0557_F	AACTAAATGCCCGCATCACT
	qSA0557_R	TGCCATTTACCTTCCATCATC
S. aureus 16s rRNA	qSA0002_F	GAACCGCATGGTTCAAAAGT
	qSA0002_R	CGTAGGAGTCTGGACCGTGT
E. coli 16s rRNA	Ec_16s_F	GTTAATACCTTTGCTCATTGA
	EC_16s__R	ACCAGGGTATCTAATCCTGTT
E. coli bioB	Ec_BioB_F	ATTACTACAACCACAACCTGGACAC
	Ec_BioB_R	AATAAAATCAAAGGCATCGACATC
E. coli bioA	Ec_BioA_F	AGGTGGCGGATATTGAAGTACAG
	Ec_BioA_R	CAACTGTTGCGGGAGAATAATATAG
E. coli birA	Ec_BirA_F	TGTTGAAGAGAGTGTCGTTAATCAG
	Ec_BirA_R	AATGCCAAATATTTCTTTATCACCA

Supplementary Table 5: list of oligos used in quantitative-PCR

Oligo name	Sequence 5'-3'	Description
DS-SaBioO oligo 1	CCTTAAATGTAAACTTTTATAATTAATAAGTTTACATTTAAG	Top strand oligo containing *sabioO* wildtype sequence
DS-SaBioO oligo 2	CCTTAAATGTAAACTTATTAATTATAAAAGTTTACATTTAAGG	Bottom strand oligo containing *sabioO* wildtype sequence
DS-SabioY oligo 1	AACTTATTGTAAACTTTTCATTTCTTAAAGTTTACAATGGTGCT	Top strand oligo containing *sabioY* wildtype sequence
DS-SabioY oligo 2	AGCACCATTGTAAACTTTAAGAAATGAAAAGTTTACAATAAGTT	Bottom strand oligo containing *sabioY* wildtype sequence
DS-yHFS-T oligo 1	TTATATAATGTTAACAAGATGTATTTTAAAGTTTACATTGAGTGA	Top strand oligo containing yHFS-T wildtype sequence
DS-yHFS-T oligo 2	TCACTCAATGTAAACTTTAAAATACATCTTGTTAACATTATATAA	Bottom strand oligo containing yHFS-T wildtype
DS-HS1m oligo 1	GCCTTACACAGACGATTATTAATTATAAAAGTTTACATTCGGAT	Top strand oligo containing mutated sequence of the first half-site of *Sa*BPL recognition sequence for *sabioO*
DS-HS1m oligo 2	ATCCGAATGTAAACTTTTATAATTAATAATCGTCTGTGTAAGGC	Bottom strand oligo containing mutated sequence of the first half-site of *Sa*BPL recognition sequence for *sabioO*
DS-HS2m oligo 1	GCCTTAAATGTAAACTTATTAATTATAAAAAGCAGACACCGGAT	Top strand oligo containing mutated sequence of the second half-site of *Sa*BPL recognition sequence for *sabioO*
DS-HS2m oligo 2	ATCCGGTGTCTGCTTTTTATAATTAATAAGTTTACATTTAAGGC	Bottom strand oligo containing mutated sequence of the second half-site of *Sa*BPL recognition sequence for s*abioO*
DS-HSm oligo 1	GCCTTACACAGACGATTATTAATTATAAAAAGCAGACACCGGAT	Top strand oligo containing mutated sequence of both half-site of *Sa*BPL recognition sequence for *sabioO*
DS-HSm oligo 2	ATCCGGTGTCTGCTTTTTATAATTAATAATCGTCTGTGTAAGGC	Bottom strand oligo containing mutated sequence of both half-site of *Sa*BPL recognition sequence for *sabioO*

Supplementary Table 6: list of oligos used in gel shift assays. All oligos were purchased from Integrated DNA Technology Inc, (USA) as a double stranded, HPLC purified fragments.

Supporting experimental procedure

S.E.1: CFU count for samples taken post-biotin treatment.

Colony forming unit (CFU) assays were performed on *S. aureus* samples collected at time 0, 15, 30 and 90 minutes post-biotin treatment. 50µL of each sample were collected and serial dilutions were performed to obtain final dilution factor of $1X10^4$ and $1x\ 10^5$, respectively. 100µl of each diluted culture was then plated onto LB agar. Plates were incubated at 37°C overnight. The next day, colonies were counted from the overnight plates and the initial CFU/mL was calculated using the formula CFU/mL = (numbers of colonies/volume spread on the plate (mL) x dilution factor).

S.E. 2: N-terminal knockout of E. coli JD26186

In order to prevent possible cell death due to the reduced affinity to biotin and biotinyl-5'-AMP[1], as a result of the endogenous BirA N-terminal disruption of the parent strain, *E. coli* JD26186 strain was temporarily complemented with human BPL. The human BPL gene (*HsBPL*) was isolated from pK(*Hs*BPL) plasmid [2] and cloned into *C*laI restriction site of pKD46 [3]. The resulting clone was then transformed into *E. coli* JD26816.

The chloramphenicol resistance gene (Cm^R) under the control of CAT promoter was isolated from the pCY216(*birA)* expression vector [4] by PCR, using primers which contained sequences homologous to those of the N-terminal sequence of *birA*.

using primers which contained sequences homologous to those of the N-terminal sequence of *birA*.

The N-terminal knockout was performed using a homologous recombination based method previously described [3] Briefly, electro-competent cells of the parent strain, JD26186 containing pKD46(*HsBPL*), were transformed with 1 µg of *CAT-Cm*^{R} PCR product flanked with *birA* N-terminal homologous sequences. The transformation mixture was plated onto 1.5% LB agar containing 10 µM biotin + 0.2mM IPTG + 100µg/mL chloramphenicol and grown overnight at 30°C. The resulting colonies (JD26186 *birA*::*CAT*) were screened using PCR and the N-terminal knockout confirmed by sequencing. The abolition of DNA binding was also validated in β-galactosidase assay (**Fig. 6b**). The strain was then incubated at 42°C to eliminate the pKD46(*HsBPL)* helper plasmid, which carries a temperature sensitive origin of replication.

S.E.3 : Cloning of repressor gene and operator sequence into integration plasmids.

The chromosomal integration plasmid pIT3_SH_LacZTrim was created by excising the spectinomycin resistance gene (*Sm*^{R}) from pIT3-SH-152002 and ligating the *Sm*^{R} fragment into the NheI / BstBI backbone of pIT3_CLlacZ_Trim [5], to replace the *Cm*^{R} with *Sm*^{R}. The resulting plasmid piT3_SH_lacZtrim was digested with KpnI / SpHI and used to ligate the promoter sequence of interest, upstream of the *lacZ* gene.

Construction of integration plasmid containing BirA gene was done in two steps. Firstly, a double stranded oligo containing the pLac-UV5 promoter sequence and lac operator sequence upstream of the repressor gene (*SabirA* /

EcbirA) was inserted in the *K*pnI / *P*ciI fragment of pGEMT-*Sa*BirA (6x his) and pGEMT-*birA*(6x his) vector, respectively. The resulting pLac-UV5-repressor fragment was then excised from the pGEMT vector and ligated into the integration vector pIT4_TL152002 as KpnI/SphI fragment.

S.E.4 : Construction of JD26186_HK022::EcbioO

The integration of pLac-UV5-*EcbirA* (WT) into JD26186_*birA::CAT* strain did not yield any positive clones, despite multiple attempts. As an alternative, parental strain *E. coli* JD26186 containing endogenous *EcbirA* with non-modified N-terminal domain was used instead of JD26186_*birA::CAT*. The promoter construct *EcbioO*::*lacZ* was then integrated into this wild-type strain to give JD26186_HK022::*EcbioO* strain. This strain was then used to analyze the repression of *EcbioO* promoter by wild-type *Ec*BirA in the β-galactosidase assay.

S.E.5 : Validation of pLac-UV5 promoter basal activity

A preliminary assay was conducted to test the activity of pLac-UV5::*Sa*BirA(6x his) in the presence and absence of IPTG (data not shown). The result indicated that in the absence of IPTG the promoter was able to produce sufficient amount of repressor to give a quantifiable response in the assay. This suggests the possibility of residual expression being produced as a result of leaky promoter activity. A western blot detection using rabbit-anti-His antibody (Cell signaling technology) was done to confirm the presence of *Sa*BirA-(6Xhis) in the absence of IPTG, which validated this hypothesis (**Sup. Fig. S7)**. All subsequent assays were therefore conducted without IPTG addition. All integration vectors were confirmed by sequencing prior to integration.

Protein samples were run on SDS-PAGE and transferred onto Hybond-PVDF membrane (Amersham) using Hoefer semiphor TE70 apparatus (Pharmacia). Transfer were done at 80mA for 1 hour, all blocking was done for 1 hour at room temperature for 1 hour in 5% skim milk PBS/0.1% tween, followed by washing in PBS/0.1% tween. For detection of 6xHis recombinant protein, rabbit-anti-6xHis antibody (Cell Signaling Technology) was used as primary antibody and detected using donkey anti rabbit-CY5 antibody (Jackson immunoResearch). All antibodies were diluted in blocking buffer and used according to manufacturer's instruction. A chemiDoc imager (Bio-Rad) was used to image the resulting blot.

S.E.7: Biotinyl transferase assay

Biotinyl-transferase assay was performed as described in [6]. Briefly, *Sa*BirA was incubated in a reaction containing 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM ATP and 10 % glycerol and a 5x molar excess of *Sa*PC90. A control containing reaction was prepared with the addition of 5µM biotin. The reaction was incubated at 37°C for 1 hour, the products were run on SDS-PAGE before Western transfer onto PVDF membrane. The resulting blot was subsequently probed with Alexa-fluor 488 conjugated to streptavidin (Life technologies) to detect biotinylated protein.

Reference:

1. Xu, Y. and D. Beckett, *Evidence for interdomain interaction in the Escherichia coli repressor of biotin biosynthesis from studies of an N-terminal domain deletion mutant.* Biochemistry, 1996. **35**(6): p. 1783-92.
2. Mayende, L., Swift, R. D., Bailey, L. M., Soares da Costa, T. P., Wallace, J. C., Booker, G. W., Polyak, S. W., *A novel molecular mechanism to explain biotin-unresponsive holocarboxylase synthetase deficiency.* J Mol Med (Berl), 2012. **90**(1): p. 81-8.
3. Datsenko, K.A. and B.L. Wanner, *One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products.* Proc Natl Acad Sci U S A, 2000. **97**(12): p. 6640-5.
4. Cronan, J.E., Jr. and J.C. Wallace, *The gene encoding the biotin-apoprotein ligase of Saccharomyces cerevisiae.* FEMS Microbiol Lett, 1995. **130**(2-3): p. 221-9.
5. St-Pierre, F., et al., *One-Step Cloning and Chromosomal Integration of DNA.* Acs Synthetic Biology, 2013. **2**(9): p. 537-541.
6. Soares da Costa, T.P., et al., *Dual roles of F123 in protein homodimerization and inhibitor binding to biotin protein ligase from Staphylococcus aureus.* Mol Microbiol, 2014. **91**(1): p. 110-20.

Chapter 5

Investigation into the effect of *Sa*BirA inhibitor on *Sa*BirA-regulated gene transcription

5.1 Introduction

Antibiotic resistance evolves due to bacteria's ability to adapt to the exposure of toxic agents [1]. It is evident that the increasing resistance to current antimicrobials is a serious problem and that there is an urgent need to replenish the current antibiotic discovery pipeline with new chemotherapeutics [2]. Increases in economic burden, health care costs and mortality rates are all attributed to antibiotic resistance [3]. Efforts to generate novel classes of antibiotics necessary to combat drug resistance have been disappointing, with only four new classes of antibiotic discovered in the past forty years [4]. Targeting essential metabolic enzymes, such as BPL, is an attractive approach for the discovery of new antibiotics [5]. A number of antiviral drugs have been discovered using similar approach whereby new drugs were designed as analogues of the enzyme's natural ligand [6].

In recent years, research targeting *S.aureus* BPL (*Sa*BPL, also known as *Sa*BirA) has generated multiple promising inhibitors with high selectivity towards *Sa*BirA over its human equivalent [7-10]. Most of the compounds generated are mimics of the reaction intermediate, biotinyl-5′-AMP (**Figure 1,** structure 1). The first generation inhibitor was obtained by replacing the phosphoanhydride linker of biotinyl-5′-AMP with the more stable, non-hydrolysable phosphodiester linker to give rise to biotinol-5′-AMP (**Figure 1,** structure 2). This compound lacks specificity as it also inhibits human BPL (*Hs*BPL)[11]. Improved selectivity was achieved by substituting the phosphoanhydride linker with a 1,2,3 triazole [**Figure 1**, structure 3]. Moreover, further studies performed on the biotin triazole series revealed that the ribose moiety shown in structure 3 was dispensable, as its removal improved the K_i by 3-fold [12] [**Figure 1,** structure 4]. Replacement of the

linker in structure (1) with acylsulfonamide resulted in structure 5; a potent BPL inhibitor with whole cell activity against *Mycobacterium tuberculosis* (IC_{50} = 140 nM)[13]. For reasons that are not understood**,** this compound does not have antimicrobial activity against *S. aureus*. We recently designed and characterized a potent *Sa*BirA inhibitor containing the acylsulfonamide linker but devoid of the ribose group that was previously shown to be dispensable. This compound, known as BPL199 [**Figure 1,** structure 6], was found to be highly potent against *Sa*BirA (*K*i = 2.4 nM), and demonstrated whole cell activity against *S. aureus* (Minimum Inhibitory Concentration (MIC) = 0.25-0.50 μg/mL)[12]. A crystal structure of *Sa*BirA in complex with BPL199 has now been determined (**Figure 2**). As expected, the compound occupies the biotin and ATP binding pockets in the same mode adopted by the reaction intermediate, biotinyl-5′-AMP.

This structural data reveals that BPL199 is an inhibitor due to competitive binding in the active site. However, none of the compounds have been tested for their ability to act as co-repressors for DNA binding. Compounds that inhibit both ligase activity as well as repress the expression of the biotin biosynthesis operon and biotin transporter (*SabioY*) are attractive, as interference with more than one metabolic pathway would possibly make target-based resistance more difficult [14]. In this chapter, the effect of the lead compound, BPL199, on DNA binding and gene expression was investigated using both *in vitro* and *in vivo* methods.

Furthermore, advanced resistance studies have been performed in our laboratory to investigate possible resistance mechanisms to BPL199. Here, *S. aureus* NCTC 8325 evolved resistance *in vitro* by passaging cultures continuously in sub-optimal concentrations of BPL199, as described in previous literature [15]. DNA sequencing of the *birA* gene revealed a missense mutation in one strain,

where aspartic acid 200 had been substituted with glutamic acid (D200E). D200 localizes in the dimerization interface, where its side chain interacts with the side chains of R122 and F123 of the neighboring subunit through hydrogen bond and hydrophobic interaction, respectively [16]. Mutation of F123 has been previously demonstrated to inhibit *Sa*BirA dimerization [16] resulting in altered DNA-binding activity and, as a consequence, altered repressor function as described in **chapter 4**. Therefore, it is proposed that the D200E substitution may likewise impact BirA dimerization and subsequently, DNA binding and transcriptional regulation activity. As aspartic acid is chemically similar to glutamic acid, with only a single carbon chain length difference distinguishing the two amino acids, which raised the hypothesis that the effect of the D200E mutation is likely to be subtle. The effect of this single-base substitution mutation on DNA-binding activity of *Sa*BirA and its comparison to both *Sa*BirA wild-type and the previously characterized F123G is also addressed in this chapter.

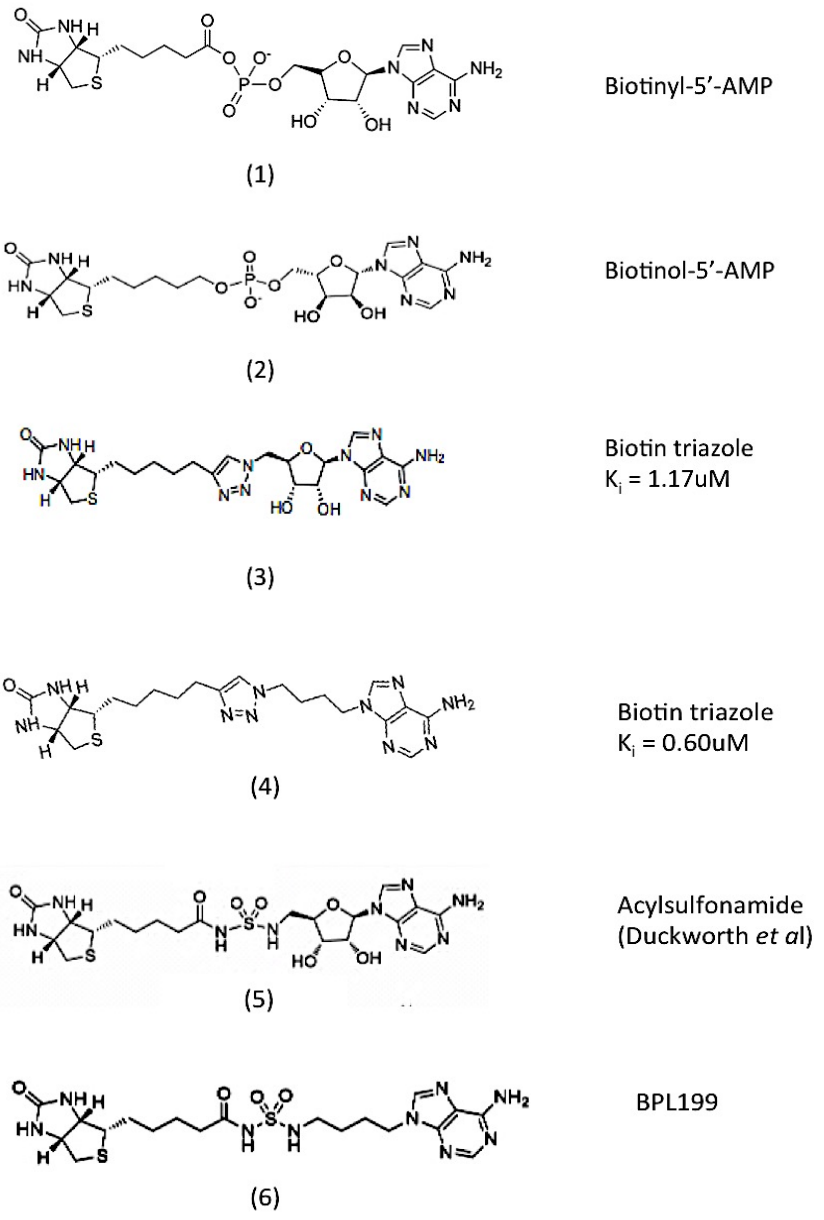


Figure 1: Structure of lead compounds against *Sa*BirA.(1.) Reaction intermediate biotinyl-5′-AMP, (2.) derivative of intermediate reaction with non-hydrolysable phosphodiester linker, biotinol-5′-AMP is shown in, (3.) next generation of inhibitor with higher potency containing 1,2,3 triazole linker (4.) improved stucture of 1,2,3 triazole with removed ribose moeity (5.) recent active compound against *Mt*BPL containing acylsulfanomide linker (6.) derivative of Acylsulfonamide, give rise to potend *Sa*BirA inhibitor, BPL 199.

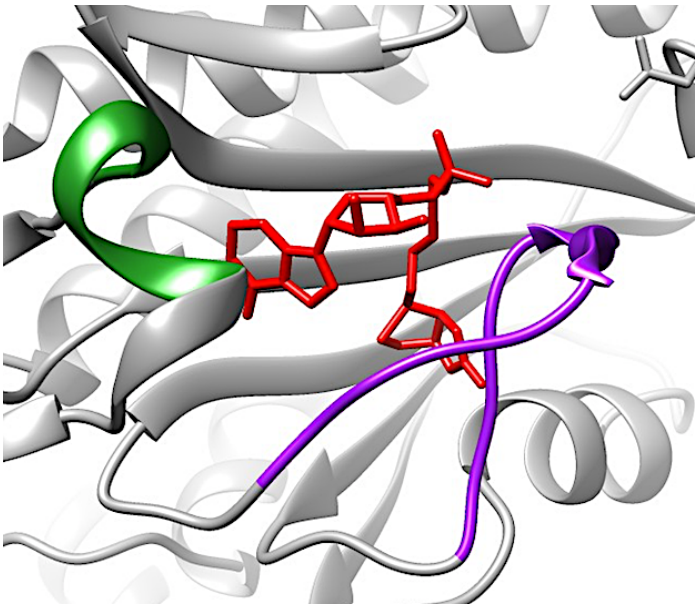


Figure 2: Crystal structure of BPL199 in complex with *Sa*BirA . BPL199 (red) occupies the catalytic site of the enzyme, which is located between the ATP Binding Loop (green) and biotin binding loop (purple). These loops were structured upon binding of BPL199, in the same manner as that observed for Biotinyl-5′-AMP.

5.2 Materials and methods

5.2.1 Structural comparison

Alignment of X-ray crystal structures was performed using the structural analysis program, UCSF Chimera. Superimposition of *Sa*BirA in complex with either BPL199 (Prof. Matthew Wilce, Monash University, unpublished data) or biotinyl-5′-AMP (PDB ID# 3RIR) was performed using this program. The result of this superimposition is presented in **Figure 3**.

5.2.2 Electrophoretic Mobility Shift Assay (EMSA)

An EMSA protocol was adapted from methods previously published in [17]. The EMSA binding buffer contained 50mM Tris pH 8.0, 50 mM NaCl, 1 mM ATP, 1 mM $MgCl_2$ and 5% (v/v) glycerol. Double stranded oligonucleotides (10 nM) containing the promoter sequence of *SabioD* and *SabioY*, were included in the reaction along with varying concentrations *Sa*BirA. Oligo sequences are presented in supplementary **Table 6 in Chapter 4**. To generate holo-*Sa*BirA, 100 µM biotin was also added to the EMSA binding buffer. Binding reactions were performed in a final volume of 10 µL and incubated for 30 minutes at room temperature. For analysis of BPL199, 100 µM of compound was added to the EMSA buffer. As BPL199 was reconstituted in 100% DMSO, the final DMSO concentration in the binding buffer was 2.5% (v/v). In order to directly compare *Sa*BirA-BPL119 bound and holo-*Sa*BirA (biotinyl-5′-AMP-bound *Sa*BirA), a control reaction was prepared by adding 100µM biotin and 2.5% (v/v) DMSO to the binding reaction. The reaction was run on 4-12% TBE gradient gel in 0.5x TBE buffer (Life technologies) at 100V for 45 minutes, at room temperature, followed by staining with staining solution containing 100mM NaCl, 1x Gel Red (Biotium).

5.2.3 *S. aureus* culture preparation for QRT-PCR analysis

S. aureus NCTC 8325 was grown overnight at 37 °C in biotin-depleted Mueller-Hinton media. The overnight culture was then used to inoculate 10 mL of fresh biotin-depleted Mueller-Hinton media and grown until mid-log phase (OD_{600} = 1.4-1.6). The culture was then treated by adding either 10 nM BPL 199 or 3.9 µM (i.e. equivalent of 4x MIC). 500µL of culture was withdrawn at time point 0, 15, 30 and 90 minutes post- BPL199 addition. The bacterial pellet from

each time point was then treated with 1 mL RNAprotect solution (Qiagen), according to manufacturer's instructions. Total RNA was extracted following method described in **Chapter 2 section 2.2.3.**

5.2.4 QRT-PCR analysis

Following RNA extraction, genomic DNA was further removed by digesting the extracted samples with DNaseI enzyme (Life technologies), according to manufacturer's instructions. A mixture of RNA template, PCR primers and Superscript® III platinum® SYBR® 1- step QRT-PCR kit (Life technologies) was prepared as outlined in **Chapter 2**. QRT-PCR was performed using Quantstudio™ instrument (ThermoFisher). Result was normalized against *16s rRNA* and relative expression was corrected against t = 0, according to the Livak method [18] and analyzed using Graphpad Prism.

5.2.5 Cloning of *Sa*BirA D200E into expression vector pET16b and integration vector pIT4-TL-152002

*Sa*BirA D200E was generated in the pGEMT-*Sa*BirA (6x his) plasmid using the Quickchange Site Directed Mutagenesis Kit® and Mutagenesis primers, B481 (5′-GGTTGCTAATAATGAAGGTATAGAAGCAATAATATGTGG-3′) and B482 (5′-CCACATATTATTGCTTCTATACCTTCATTATTAGCAACC -3′). The mutagenesis experiment was performed by Mr. Andrew Hayes. The resulting plasmid, pGEMT-*Sa*BirA-D200E (6xhis) was digested with *N*coI and *H*indIII fragment and the 1 Kb fragment cloned into similarly treated pET16b expression vector. To clone *Sa*BirA D200E into the integration vector, the pGEMT-*Sa*BirA-D200E (6xhis) was digested with *K*pnI and *S*pHI restriction enzymes and ligated into similarly treated

pIT4_TL_152002 integration vector, to give a final integration vector; pIT4-pLac-*Sa*BirA-D200E (6xhis). All constructs were verified by DNA sequencing.

5.2.6 Protein expression and apo-purification

pET16B-*Sa*BirA-D200E-(6xhis) and pET16b-*Sa*BirA-wildtype-(6xhis) plasmids were transformed into the expression strain *E. coli* BL21(DE3)-RIPL. Expression of this protein was performed following previous protocols [16, 19]. To generate apo-BirA, bacterial lysate was incubated at 30°C for 1 hour with purified biotin domain-GST fusion from *S. aureus* [20] (*Sa*PC90). Treated lysate was centrifuged at 17500 x g for 20 minutes and filtered through 0.45 μM ministart filter disc (Sartorius stedim, Germany) prior to loading into Nickel affinity purification column. Nickel affinity chromatography was performed, according to the methods outlined in **chapter 2, section 2.2.17.** Protein concentration was determined using Bradford assay. Western blot analysis was performed to confirm the purified apo-*Sa*BirA D200E and apo-*Sa*BirA (wild-type), as previously described [16]

5.2.7 Chromosomal integration of pIT4-pLac- *Sa*BirA D200E (6xhis).

Incorporation of *Sa*BirA (D200E) into attB-λ phage attachment site of the *E. coli* reporter strain containing *β-galactosidase* gene under the control of either *SabioO* or *SabioY* promoters, (JD26186_*birA*::*CAT_SabioO* and JD26186_*birA*::*CAT_SabioY*, respectively) was performed by previously established integration methods [21]. PCR screening to select the positive integrants was outlined in **Chapter 2, section 2.2.13**. PCR screening was performed using lambda P1-P4 primers **(Table 2.5, Chapter 2)**.

5.2.8 β-galactosidase assay of reporter strain containing *Sa*BirA D200E

β-galactosidase assay was performed on *E. coli* reporter strain containing *Sa*BirA D200E repressor in order to analyze its *in vivo* DNA binding activity. Activity was compared against reporter strains containing *Sa*BirA wild-type and the dimerization-impaired mutant *Sa*BirA F123G, respectively. β-galactosidase assay performed following methods described in **Chapter 2, section 2.2.26.**

5.2.9 Native nESI-MS

Apo-purified *Sa*BirA D200E were buffer exchanged into 200 mM ammonium acetate using 500 MWCO 10,000 Vivaspin® centrifugal concentrator (Vivaproducts, USA). Holo-*Sa*BirA D200E was prepared by incubating the apo protein with 500 μM biotin, 1 mM ATP and 1 mM $MgCl_2$ at 37 °C for 0.5 hour prior to buffer exchange. Protein was diluted to 10 μM prior to analysis. MS measurements were performed on a Synapt HDMS system (Waters, UK) with the sample introduced by nano-electrospray ionisation in positive ion MS mode from platinum-coated borosilicate capillaries prepared in-house. Instrument parameters were optimized to remove adducts while preserving non-covalent interactions, and were as follows; capillary voltage, 1.5 kV; cone voltage, 60 V; trap collision energy, 20 V; transfer collision energy, 15 V; source temperature, 50°C; backing pressure, 3.95 mbar.

5.3 Results and discussion

5.3.1 BPL199-bound *Sa*BirA displayed the same structural changes as biotinyl-5′-AMP-bound *Sa*BirA

A series of well-characterized ligand-induced conformational changes are required to facilitate *Sa*BirA binding to DNA. Upon biotin binding, residues 118–129 within the central domain of *Sa*BirA, which form the biotin binding loop (BBL), become ordered. The adenosine binding loop (ABL) formed by residues I224 and A228, also undergo structural changes, to accommodate the binding of adenylate moiety [22]. The protein then undergoes a transition from monomer to dimer, as discussed in the introduction. This homodimerization involves residues R122 and F123 that localize within the biotin binding loop from one subunit with the D200 residue that resides in the central domain of the neighboring subunit. Upon homodimer formation, the two N-terminal DNA binding domains are optimally positioned for an interaction with DNA [22]. In order to compare the structure of BPL199-bound *Sa*BirA with the structure of *Sa*BirA bound to biotinyl-5′-AMP, structural alignment between the two available x-ray crystal structures was performed. The superposition showed that both BPL199-bound *Sa*BirA and the holo-enzyme are perfectly aligned (RMSD = 1.2Å), as shown in **Figure3**. This indicated that the binding of BPL199 to *Sa*BirA initiates the same conformational changes that are required for dimerization as biotinyl-5′-AMP and resulted in N-terminal position that is receptive to DNA binding. Based on this structure alignment, it is reasonable to conclude that the *Sa*BirA-BPL199 complex is a functional co-repressor. In order to investigate this further, a series of *in vitro* and *in vivo* assays were performed.

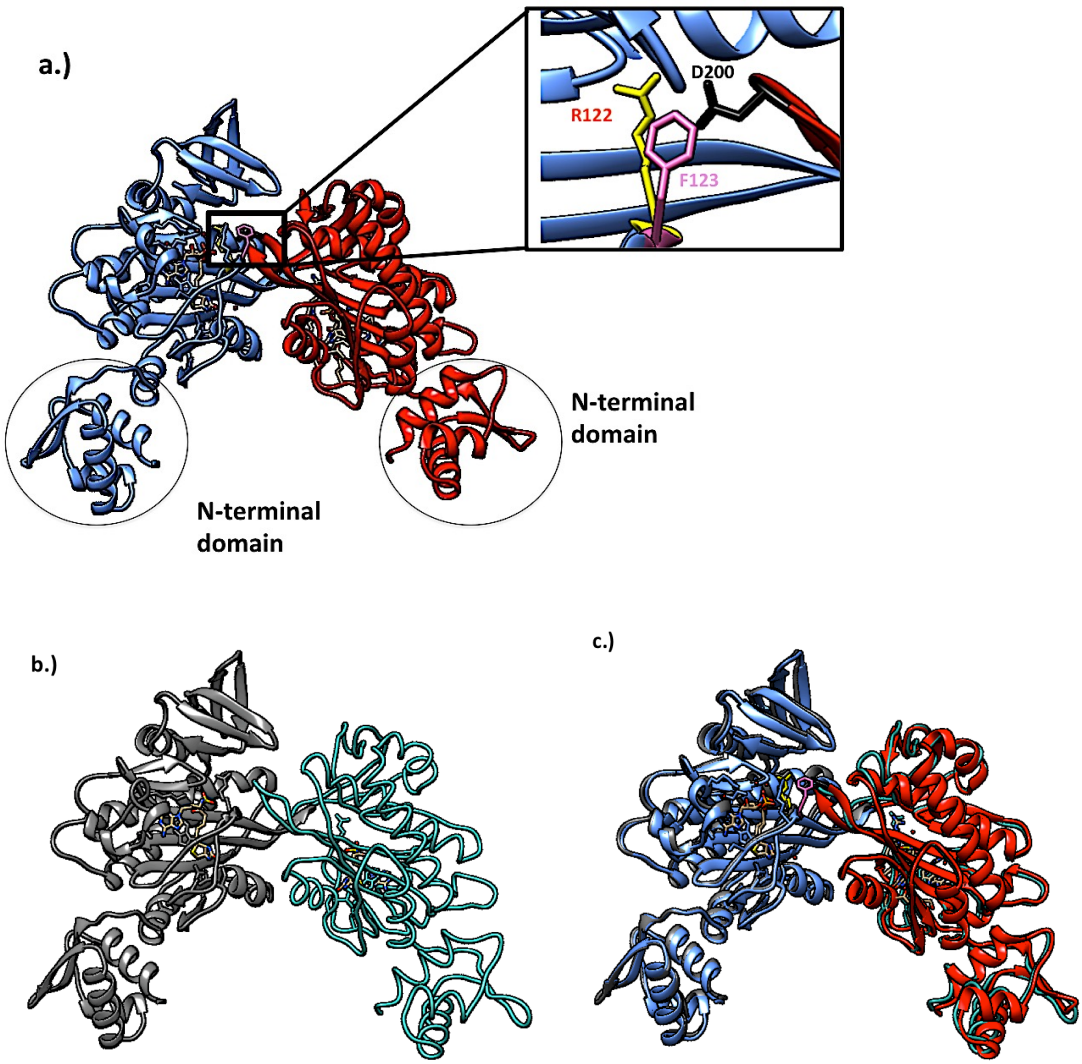


Figure 3: Structural alignment of BPL199-bound *Sa*BirA vs. biotinyl-5′-AMP-bound *Sa*Bir*A*. (a.) The structure of biotinyl-5′-AMP-bound *Sa*BirA. Each *Sa*BirA subunit is highlighted (blue and red). Residues located in the dimerization interface are shown (boxed diagram). N-terminal domain with helix-turn-helix motif for DNA binding of each subunit is highlighted in circle (b.) The structure of BPL199-bound *Sa*BirA. Each *Sa*BirA subunit is highlighted (gray and green) (c.) BPL199-bound *Sa*BirA was aligned with biotinyl-5′-AMP-bound *Sa*BirA (RMSD = 1.2Å).

5.3.2 Purification of *Sa*BirA wild-type and *Sa*BirA D200E.

Wild-type *Sa*BirA was purified alongside *Sa*BirA D200E identified from the advance resistance study. These purified proteins were then used in EMSA analysis. For this work, it was critical that both proteins were purified in their apo-form as any co-purified biotin or biotinyl-5′-AMP in the active site might influence DNA binding activity. Proteins were purified using a previously established method that removes biotinyl-5′-AMP from the samples. Briefly, cell lysates were incubated with ATP and substrate protein (*Sa*PC90) to encourage the removal of the biotin from the enzyme, as described in materials and method **section 5.2.6**. The apo material was tested using both native nano-electrospray ionisation-mass spectroscopy (nESI-MS) and Western blot probed with alexa488-conjugated streptavidin in order to assess the ability of the purified enzyme to biotinylate *Sa*PC90.

The result showed that both wild-type and *Sa*BirA D200E were purified in their apo-forms, as co-purified biotinyl-5′-AMP was not detected by nESI-MS (**Table 1**) and biotinylation activity failed to be detected by western blot (**Figure 4**). In addition, nESI-MS data indicated that both wild-type and D200E *Sa*BirA were catalytically active, as biotinyl-5′-AMP was detected when the apo-purified proteins were treated with biotin and ATP (Table 1, holo-*Sa*BirA and holo-*Sa*BirA D200E respectively). Furthermore, nESI-MS detected two different molecular mass for holo-*Sa*BirA, which correspond to a monomer and a dimer form of *Sa*BirA, suggesting that the wild-type protein was able to dimerize in solution. In contrast, a single molecular mass detected for holo-*Sa*BirA D200E, which correspond to that of monomeric protein, suggesting that the dimerization ability of this mutant protein might be compromised.

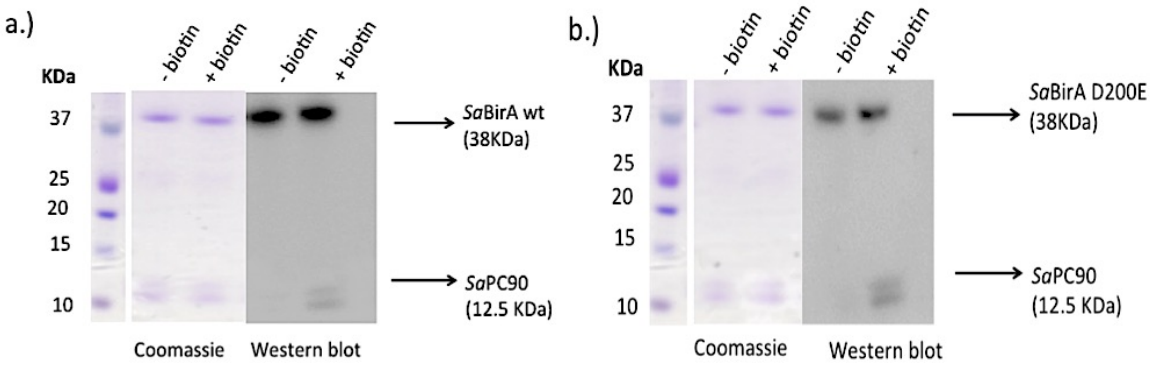


Figure 4: Confirmation of purified wild-type and *Sa*BirA D200E in their apo-forms. Western blot detection using Alexa 488 conjugated streptavidin was used to detect biotinylated *Sa*PC90 domain in the absence of biotin and in the presence of biotin as a control. The result for (a.) *Sa*BirA wild-type and (b.) *Sa*BirA D200E are shown. A small proportion of biotin might be covalently linked to a non-specific lysine residue within *Sa*BirA during purification, which result in detection of *Sa*BirA in Western blot. Coomassie stained gel was used as a loading control.

***Sa*BirA**	**Expected oligomeric state**	**Expected MW (Da)**	**Oligomeric state and ligand detected by ESI-MS**	**MW detected (Da)**
Apo-Wildtype	Monomeric	37892 (-Met)	Monomeric, no ligand	37892
Holo-Wildtype	Monomeric	38465	Monomeric, biotinyl-5′-AMP bound	38470
	Dimeric	76930	Dimeric, biotinyl-5′-AMP bound	76925
Apo-D200E	Monomeric	37905 (-met)	Monomeric, no ligand	37910
Holo-D200E	Monomeric	38478	Monomeric, biotinyl-5′-AMP bound	38491

Table 1: Native nESI-MS analysis of apo and Holo *Sa*BirA D200E and wild-type *Sa*BirA. Summary of molecular weight and protein species detected by nESI-MS for wild-type *Sa*BirA and *Sa*BirA D200E. The predicted and the detected molecular weight (MW) in the absence and presence of biotinyl-5′-AMP (MW = 573 Da), as well as the oligomeric state are presented.

5.3.3 The effect of BPL199 on DNA binding activity of *Sa*BirA

5.3.3.1 BPL199 promotes DNA-binding *in vitro*

EMSA analysis was performed to determine the co-repressor activity of BPL199. The EMSA reaction was performed by first incubating apo-*Sa*BirA with either BPL199 or biotin and ATP to allow the synthesis of biotinyl-5′-AMP (i.e. Holo-*Sa*BirA). Since BPL199 was reconstituted in 100% DMSO, dilution of BPL199 in the reaction buffer give a final DMSO concentration of 2.5% (v/v). A control reaction containing biotin, ATP and 2.5% (v/v) DMSO was performed in parallel to adjust for these binding conditions. DNA binding was tested against double stranded DNA probes containing either *SabioO* or *SabioY* promoter sequences, representing the promoters that regulate the biotin biosynthesis operon and biotin transporter, respectively. A control of the *Sa*BirA-*SabioO* and *Sa*BirA-*SabioY* interaction in the presence of biotinyl-5′-AMP performed in binding buffer lacking DMSO obtained from separate experiment (as described in chapter 4), was also included in the figure as a comparison (**Figure 5a-b**)

By comparing the *Sa*BirA-*SabioO* interaction in binding buffer lacking DMSO with the same interaction in binding buffer containing DMSO (**Figure 5c**), it is evident that the presence of DMSO reduces the binding affinity by approximately 3-fold. In DMSO-containing buffer, 100% binding was observed at 156 nM *Sa*BirA concentration compared to 50 nM in buffer without DMSO. To my knowledge, the exact mechanism of how DMSO affects *Sa*BirA-DNA interaction has not been investigated previously. However, the ability of DMSO to change the topological structure of DNA has been well documented [23-25]. Therefore, the discrepancy in binding affinity between the two buffers was not surprising, as the DNA confirmation might be different in the two different buffers.

Furthermore, the data also showed that BPL199-bound *Sa*BirA exhibited equipotent DNA binding to that of biotinyl-5′-AMP-bound *Sa*BirA (**Figure 5c-f**). *Sa*BirA binding to *SabioO* exhibit 100% binding at 156 nM *Sa*BirA concentration for both BPL199 and biotinyl-5′-AMP (**Figure 5c-d**). *Sa*BirA binding to *SabioY* probe was 2-fold weaker compared to *SabioO*, as the lowest concentration of protein required to produce maximum binding was 312 nM for both ligands (**Figure 5e-f**). This observation confirmed the hypothesis that BPL199 induced the same DNA binding activity as biotinyl-5′-AMP. In addition, this observation also suggests that in binding buffer containing DMSO, wild-type *Sa*BirA binds weaker to *SabioY* probe compared to *SabioO*. This observation is in contrast to the wild-type protein EMSA performed in binding buffer without DMSO, where binding to *SabioO* and *SabioY* exhibit similar affinity (**Figure 5a-b**). This result indicated that the change in DNA topology caused by the DMSO might be different between *SabioO* and *SabioY*, which resulted in weaker binding of SaBirA to *SabioY*.

Overall, the binding affinities displayed by BPL199-bound *Sa*BirA was similar to that of biotinyl-5′-AMP-bound *Sa*BirA under these conditions, which suggests that BPL199 is capable of producing similar DNA binding interaction as the natural substrate, *in vitro.* Based on these results, it was reasonable to predict that BPL199 should also able to promote DNA binding *in vivo* and exhibit similar ability to regulate gene expression as biotinyl-5′-AMP.

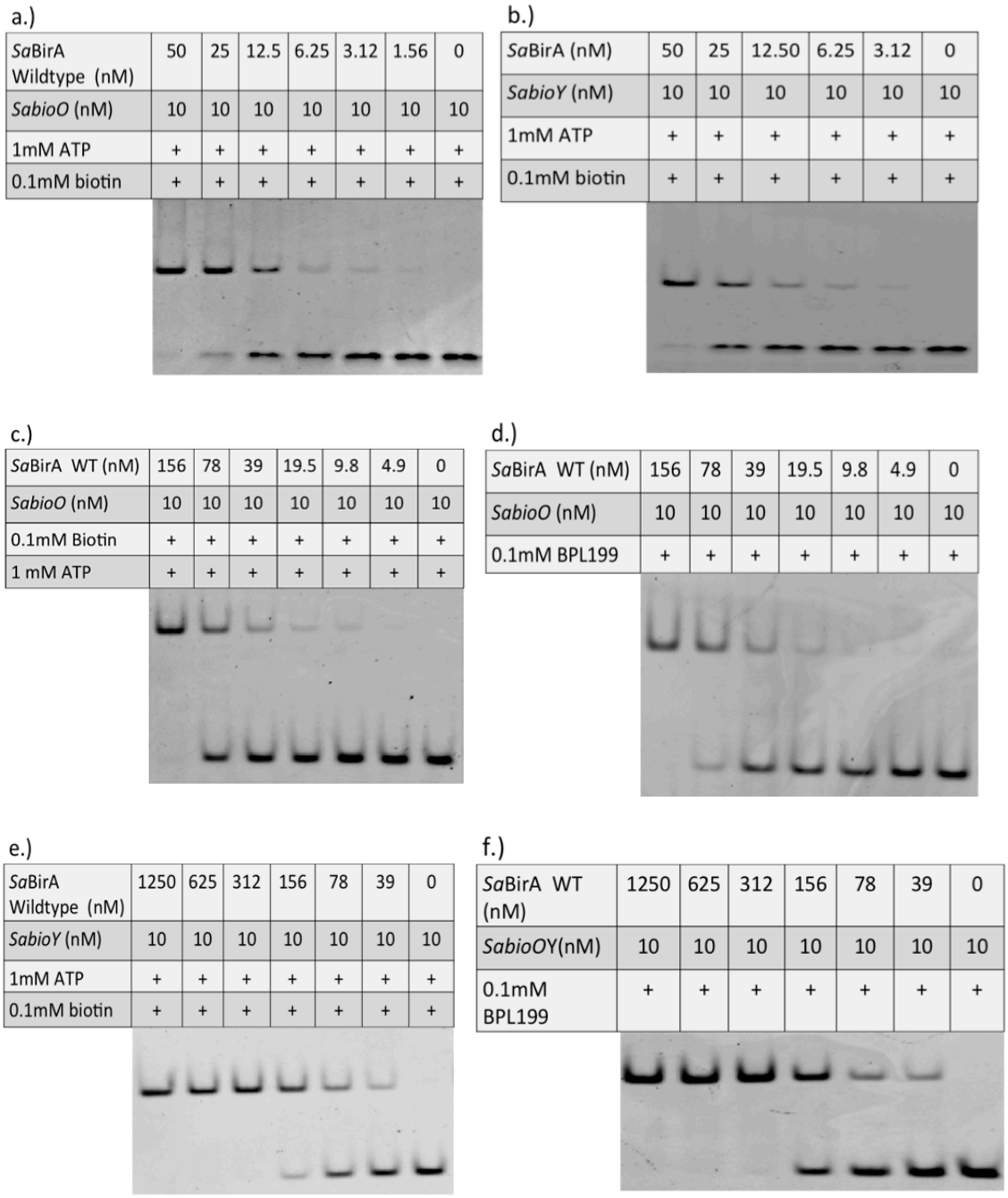


Figure 5: EMSA of BPL199-bound *Sa*BirA vs biotinyl-5′-AMP-bound *Sa*BirA. EMSA was performed using 10 nM of 44 bp double stranded oligo and titrated wild-type *Sa*BirA as indicated, in strandard binding buffer containing 50 mM Tris pH 8.0, 50 mM NaCl and 5% (v/v) glycerol. (a.) Binding reaction of *Sa*BirA–*SabioO* and (b.) *Sa*BirA-*SabioY* in the absence of DMSO were performed in separate experiment as comparisons to the binding reaction containing 2.5% (v/v) DMSO. Gel shift assay in standard buffer containing 2.5% DMSO is outlined in (c.) biotinyl-5′-AMP-bound *Sa*BirA with *SabioO* (d.) BPL199-bound *Sa*BirA with *SabioO* (e.) biotinyl-5′-AMP-bound *Sa*BirA with *SabioY* (f.) BPL199-bound *Sa*BirA with *SabioY.*

5.3.3.2 BPL199 is an active co-repressor *in vivo*.

In order to test the ability of BPL199 to repress gene expression in *S. aureus*, cultures of *S. aureus* NCTC 8325 were prepared in biotin depleted Mueller-Hinton media and grown until mid-log phase. At this point, the culture was treated with BPL199, either at 10 nM or 4x MIC (i.e. 3.9 μM). Cells were harvested at 0, 15, 30 and 90 minutes post treatment and total RNA was prepared to analyze the level of transcript of *SabioD* (the first gene in the biotin biosynthesis operon) and *SabioY* (biotin transporter gene) using qRT-PCR. All data was normalized against *16S rRNA*. Relative mRNA expression was quantified and corrected against t = 0, according to the Livak method [18]. The results were then compared with qRT-PCR results from *S. aureus* treated with 10 nM biotin, obtained from separate experiment, as presented in **Chapter 4**.

As discussed in **Chapter 4**, *S. aureus* treated with 10 nM biotin showed a strong repression, with 111-fold decrease in *SabioD* expression displayed at 15 minute post-biotin addition and this strong repression was maintained throughout the time course (**Figure 6a**). On the other hand, the results obtained from *S. aureus* treated with At 10 nM BPL199 indicated that *SabioD* expression was repressed by 14-fold at 15 minutes post- BPL199 addition relative to time = 0 ($p \leq 0.0001$). This level of repression was maintained throughout the time course. This results suggest that whilst 10 nM BPL199 was functional, the potency of repression was lower compared to the natural ligand, biotin (**Figure 6b**).

Increasing the dosage of BPL199 to 3.9 μM (i.e. 4x MIC) resulted in stronger repression of *SabioD*. The level of transcripts collected at 15 minutes showed a 52-fold decrease, 32-fold at 30 minutes and 71-fold at 90 minutes compared to time 0

($p \leq 0.0001$, **Figure 6c**). The inconsistent trend of repression level reflects the difficulty in accurately quantifying the levels of low quantity transcript by qRT-PCR [26-29]. Overall, it was clear that the level of repression generated by biotin was greater compared to BPL199. Nevertheless, it was evident that a significant down regulation of *SabioD* was observed following addition of BPL199 at both 10 nM and 4x MIC, with the *P* values indicating a significant difference ($p < 0.0001$). In addition, the results also indicated BPL199 dose-dependent repression, as increasing the concentration of BPL199 also resulted in increase in repression. This result confirmed that the mode of action of BPL199 was consistent with BPL199 binding to *Sa*BirA and that BPL199 is an active co-repressor *in vivo*. Moreover, the kinetics of ligand binding to the *Sa*BirA target was equivalent for BPL199 and the natural ligand, biotin.

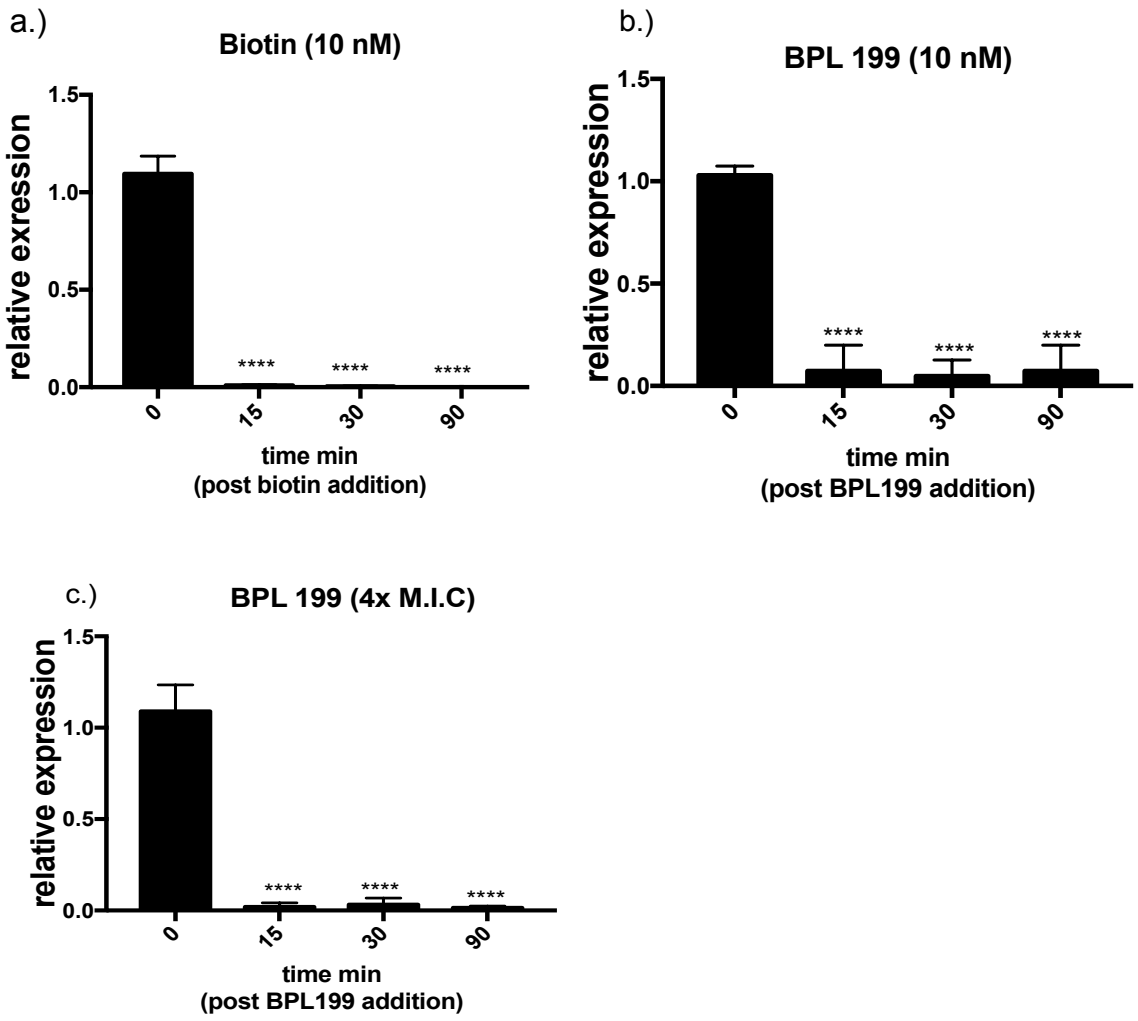


Figure 6: qRT-PCR analysis to determine the effect of BPL199 on *SabioD* gene expression. *S. aureus* treated with (a.) 10 nM biotin as previously presented in chapter 4, (b.) 10nM BPL199 and (c.) 4x MIC or 3.9 µM of BPL 199. Relative expression was calculated by using the Livak method [18]. Error bars represent S.E.M from independent biological replicate of (n = 3), experiment was performed in triplicate. **** indicated $p \leq 0.000.1$

In chapter 4, repression of *SabioY* induced by the addition of 10 nM biotin was presented. 10 nM biotin only yielded 2-fold repression at 15 minutes ($p < 0.01$), 5–fold at 30 minutes ($p < 0.01$) and 6-fold at 90 minutes ($p < 0.01$) (**Figure 7a**). The effect of BPL199 on *SabioY* gene expression was also tested at 10 nM and compared to the repression induced by biotin. The result showed that

BPL199 was also capable in promoting repression of *SabioY* expression, with similar potency as biotin (**Figure 7b)**. For BPL199, *SabioY* expression was reduced by1.5-fold at 15 minutes ($p < 0.01$), 2-fold at 30 minutes ($p < 0.01$) and 6-fold at 90 minutes ($p \leq 0.0001$). It is worthwhile to note that the mode of transport utilized by *S. aureus* to acquire BPL199 has not been determined. Thus, it is possible that the rate of uptake between BPL199 and biotin could be different, which could influence the level of repression observed. Nonetheless, these results further indicated that BPL199 was clearly able to act as a co-repressor and promote gene repression in *S. aureus*, similar to the gene repression induced by the *Sa*BirA natural substrate, resulting in lower transcription levels of biotin-related genes.

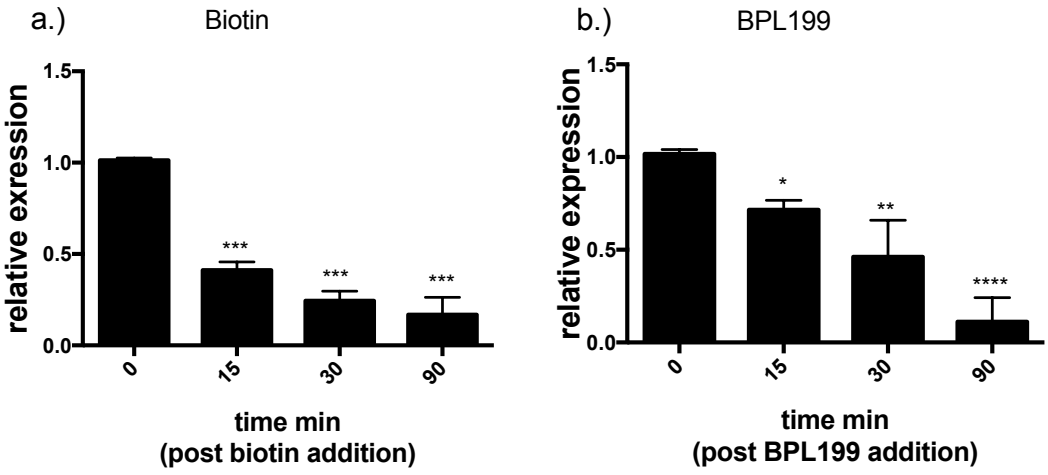


Figure 7: qPCR analysis to determine the effect of BPL199 on *SabioY* gene expression. *S. aureus* treated with ; (a.)10 nM biotin as previously presented in chapter 4 and (b.) 10 nM BPL199. Relative expression was calculated by using the Livak method [18]. Error bars represent S.E.M from independent biological replicate of (n = 3), experiment was performed in triplicate. * indicated $p < 0.05$, ** indicated $p < 0.01$, *** indicated $p < 0.001$ and **** indicated $p \leq 0.000.1$. Statiscal analysis was performed using one-way ANOVA.

5.3.4 Characterization of *Sa*BirA D200E DNA-binding properties

5.3.4.1 *Sa*BirA D200E binds to DNA in both holo and apo-form *in vitro*

Apo-purified *Sa*BirA D200E was then tested for DNA binding activity using EMSA. DNA-binding of holo-*Sa*BirA D200E was carried out in binding buffer containing 1mM ATP, 1mM $MgCl_2$ and 100 μM biotin. For analysis of the apo-enzyme biotin was omitted from the binding buffer. These EMSA results are presented in **Figure 8** and compared to the EMSA analysis of wild-type *Sa*BirA presented in **Figure 5a-b**. From the data, it was clear that apo-*Sa*BirA D200E binds weaker to both *SabioO* and *SabioY*, compared to the holo-protein. This result is in agreement with wild-type *Sa*BirA, where weaker interaction with DNA was also observed for apo-*Sa*BirA, compared to holo-*Sa*BirA [16, 30].

The results also indicated that, for both apo and holo *Sa*BirA D200E, complete binding to *SabioY* failed to be obtained at the highest protein concentration, as indicated by the appearance of the unbound DNA (**Figure 8b and 8d**). Conversely, 100% binding was observed to the *SabioO* probe at 625 nM and 78 nM for apo and holo-*Sa*BirA D200E, respectively. This result suggested that, unlike the wild-type protein that has equivalent affinity for *SabioO* and *SabioY* (**Chapter 4, Figure 9**), the *Sa*BirA D200E interaction with *SabioY* was compromised. The consequence of this is that bacteria harboring the D200E mutation would have greater expression of BioY, allowing the bacteria to accumulate biotin from the environment more readily compared to the wild-type strain. It is possible that this mechanism contributed to the bacteria's resistance to BPL199. Furthermore, by comparing the EMSA results of holo-*Sa*BirA D200E binding to *SabioO* and *SabioY* (Figure 8c-d), to the EMSA results of wild-type *Sa*BirA binding to the same probes (presented in **chapter 4, Figure 9b-c**,

respectively), it is clear that holo-*Sa*BirA D200E binds weaker to these probes compared to the wild-type *Sa*BirA. Maximum binding of *Sa*BirA D200E occurred at concentrations ≥ 100 nM, whereas the wild-type *Sa*BirA produced 100% binding at 50 nM protein concentration.

Overall, these results showed that the binding of *Sa*BirA D200E to both *SabioO* and *SabioY* generated a weaker interaction compared to the wild-type protein. This suggests that *S. aureus* containing the D200E mutation may have elevated rate of *de novo* biotin synthesis and transport. Analysis comparing *in vivo* repression of *SabioO* and *SabioY* by *Sa*BirA D200E is discussed in the next section. In addition, further studies comparing the rate of biotin uptake between *S. aureus* bearing this D200E mutation against a wild-type *S. aureus*, as well as transcriptomics studies of biotin biosynthesis genes from both strains, still need to be conducted in order to validate this hypothesis.

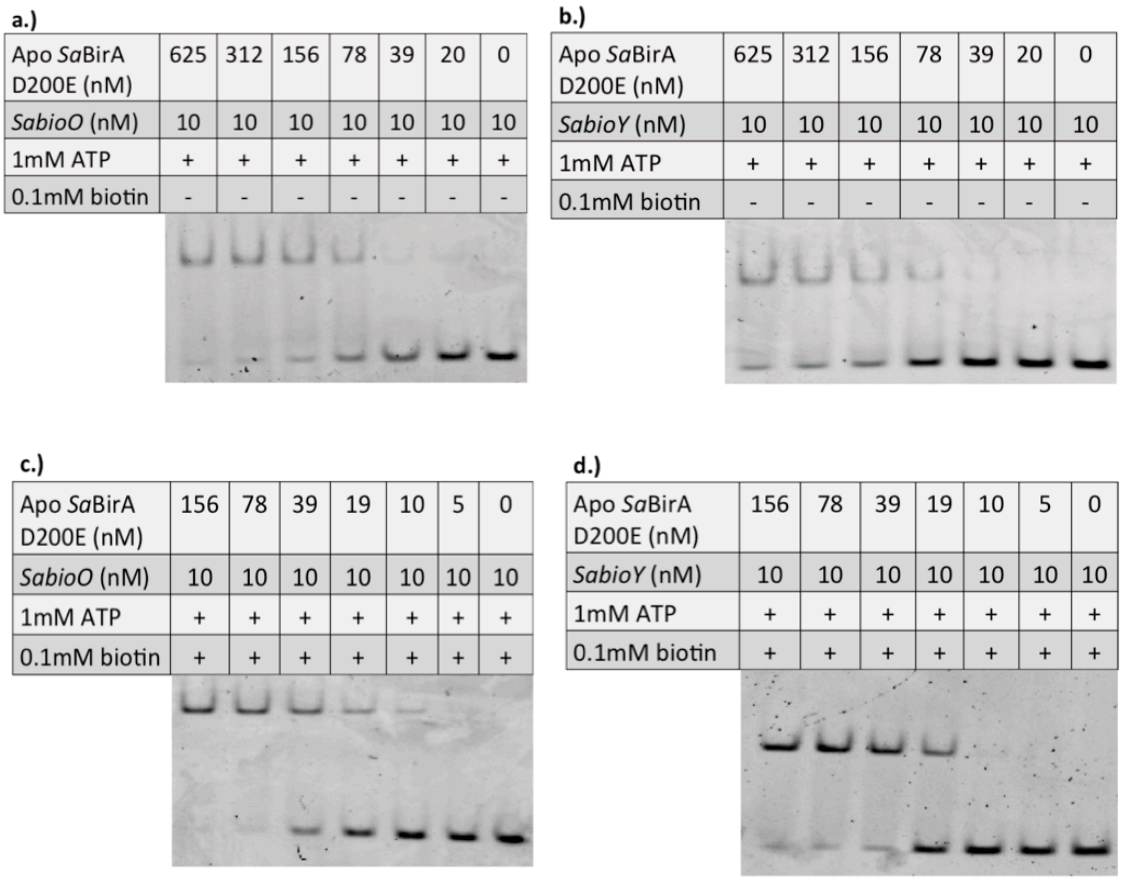


Figure 8: EMSA of holo-*Sa*BirA D200E. EMSA was performed in a standard binding buffer containing 50 mM Tris pH 8.0, 50 mM NaCl, 1 mM ATP, 5% (v/v) glycerol and 10 nM of 44 bp double stranded oligo. Holo reactions were performed by adding 0.1 mM biotin, as indicated. EMSA of apo-*Sa*BirA D200E binding to (a.) apo-*SabioO* probe, (b.) *SabioY* probe were performed, as well as EMSA of the holo-*Sa*BirA binding to (c.) *SabioO* probe and (d.) *SabioY* probe.

5.3.4.2 *Sa*BirA D200E exhibited weak interaction with DNA *In vivo*, similar to the interaction displayed by *Sa*BirA F123G.

To determine the DNA binding activity of *Sa*BirA D200E *in vivo*, an integration vector containing the gene encoding *Sa*BirA D200E was constructed (**Figure 9a**) and integrated into the chromosome of *E. coli* reporter strains JD26186_*birA::CAT_SabioO* and JD26186_*birA::CAT_SabioY*, containing *SabioO* and

SabioY regulated *lacZ* reporter genes, respectively. β-galactosidase activity was then measured to assess *in vivo* DNA binding activity of *Sa*BirA D200E and compared to wild-type *Sa*BirA and *Sa*BirA F123G. The expression of β-galactosidase from each reporter strain was normalized by subtracting the background values generated by the control strain containing no promoter sequence. The results were then graphed (**Figure 9b-9c**) and the concentration of biotin required to generate half-maximum repression (K_R) was calculated and presented in **Table 2**. The result showed that wild-type *Sa*BirA interaction with *SabioO* promoter was the strongest (K_R = 4.3 ± 1.9 nM) and about 2-fold weaker interaction was observed for interaction with *SabioY* (K_R = 8.2 ± 0.7 nM). *Sa*BirA D200E exhibited a similar repression profile as *Sa*BirA F123G (**Figure 9b-9c**). *Sa*BirA F123G interaction with *SabioO* exhibited a 3.6-fold lower K_R value (15.3 ± 3.5 nM) compared to the wild-type ($p < 0.01$). This result was similar to *Sa*BirA D200E binding to *SabioO,* where the K_R value (13.9 ± 3.4 nM) was 3.2-fold lower compared to the wild-type ($p < 0.01$). In stark contrast, binding of *SabioY* to both *Sa*BirA F123G and *Sa*BirA D200E failed to give K_R values with the concentrations tested in this assay ($K_R \geq 500$nM). This suggested that the biotin requirement to generate full repression is higher for these mutants. Overall, these data indicated that both *Sa*BirA mutants required higher biotin concentration to generate full repression of *SabioO* and *Sabio*Y and that the biotin level required to repress *SabioY* is significantly higher compared to *SabioO*.

Based on the *in vivo* data obtained, I proposed that the weaker DNA interaction displayed by both *Sa*BirA mutants is an indication of higher biotin requirement of these mutants to form the reaction intermediate. It is known that biotinyl-5′-AMP is a positive allosteric effector of DNA binding by promoting

dimerization, which gives the protein the optimal conformation to bind DNA [31-33]. Therefore, it is possible that weaker interaction with DNA was due to the fact that the given biotin concentrations utilized in this assay were not high enough to induce optimal structural change of the dimerization-impaired mutants, in order to promote strong binding to DNA. It is possible that repression of *SabioY* by *Sa*BirA D200E would be apparent at higher biotin concentrations, higher than 500 nM tested in this assay. This hypothesis is supported by the EMSA result, where the concentration of biotin in the binding buffer was fixed at 100 μM, the binding of *Sa*BirA D200E to DNA was evident as indicated by the band shifts on the gel.

Furthermore, EMSA analysis of the apo-*Sa*BirA D200E indicated that DNA-binding also occurred in the absence of biotin. However, in the *in vivo* assay, no repression was observed at low biotin concentrations (≤1 nM). It is possible, that the protein concentration *in vivo* was lower than those tested in the EMSA. Although the concentration of intracellular *Sa*BirA has not been reported, previous studies estimated that the concentration of intracellular *E. coli* BirA was between 2-100 nM [31, 34, 35]. Assuming that the integrated *lac* promoter that drives the expression of SaBirA in the *in vivo* system produces similar concentration of protein as the predicted BirA concentrations it was not surprising that no repression at lower biotin concentration (≤1 nM) was observed. Therefore, it is reasonable to conclude the repression generated by this mutant is a function of repressor concentration and biotin concentration. Experiments determining the dimerization constant of *Sa*BirA D200E and the K_m for biotin, would need to be performed in order to verify this hypothesis.

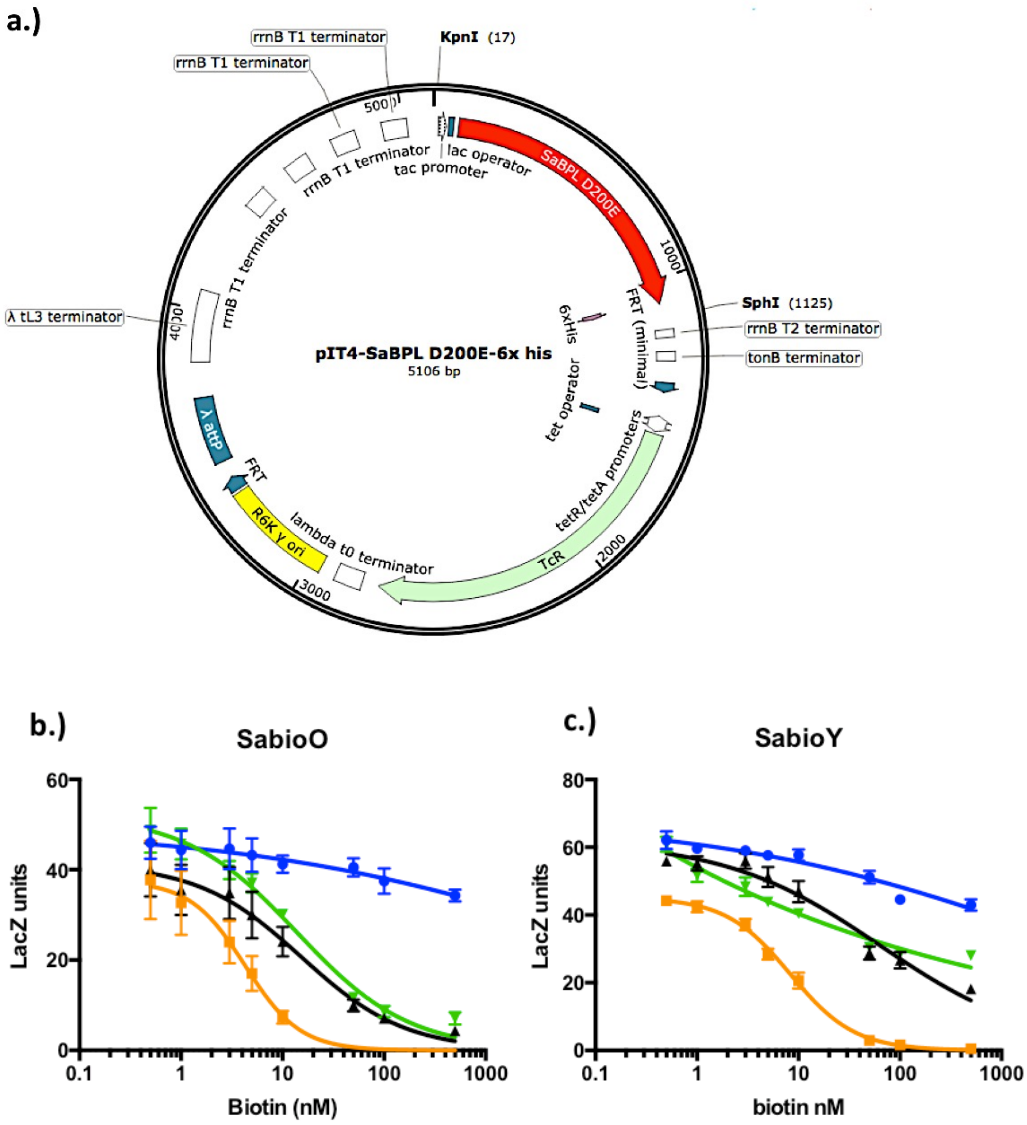


Figure 9: *In vivo* β-galactosidase assay of *Sa*BirA D200E compared to Wild-type *Sa*BirA and *Sa*BirA F123G. *In vivo* assay was carried out in *E. coli* reporter strain as described in chapter 3. (a.) Integration vector containing *Sa*BirA D200E. β -galactosidase assay showing the repression of (b.) *SabioO* and (c.) *SabioY* by *Sa*BirA D200E (green), *Sa*BirA F123G (black) and *Sa*BirA wild-type *Sa*BirA (orange). No-repressor control represented in (blue). Error bars represent S.E.M from independent biological replicate (n = 6).

*Sa*BirA	Half-maximum repression K_R biotin (nM) *SabioO*	Half-maximum repression K_R biotin (nM) *SabioY*
Wild-type	4.3 ± 1.9	8.2 ± 0.7
F123G	15.3 ± 3.5	≥ 500
D200E	13.9 ± 3.4	≥ 500

Table 2: Summary of $K_{R\ biotin}$ obtained from *in vivo* assay. The amount of biotin to reach half-maximum repression (K_R) ± S.E.M was calculated from the results generated in **Figure 9,** using Graphpad Prism. Data was collected from at least n = 6 biological replicates.

5.4 Conclusion

In this study, the effect of *Sa*BirA inhibitor, BPL199, on the transcriptional repressor function of *Sa*BirA was investigated. EMSA analysis indicated that both biotinyl-5'-AMP and BPL199 was able to induce DNA-binding and generated similar affinity, as seen in **Figure 5**. In addition, qRT-PCR analysis indicated that *S. aureus* treated with biotin exhibited stronger *SabioD* and *Sabio*Y repression compared to *S. aureus* treated with BPL199, suggesting that although BPL199 was an active co-repressor *in vivo*, the level of repression generated by this inhibitor was still less compared to biotin. Overall, in this chapter, the co-repressor ability of BPL199 has been validated both *in vitro* and *in vivo*.

Furthermore, in this chapter, the transcriptional repressor activity of *Sa*BirA D200E mutant generated from the advance resistance study was also investigated and compared to the wild-type protein. The EMSA result indicated that while wild-type *Sa*BirA binds to *SabioO* and S*abioY* with similar strength, *Sa*BirA D200E binding to *SabioY* was weaker compared to *SabioO*. This suggested that the strain bearing this D200E mutation would be more readily to de-repress

SabioY compared to the biotin biosynthesis operon. Moreover, *in vivo* β-galactosidase assay results also showed that while the wild-type *Sa*BirA was able to exhibit a complete repression of both *SabioO* and *SabioY*, *Sa*BirA D200E failed to give a complete repression of *SabioY* under the given biotin concentrations (≤ 500 nM). Overall, these results suggest that repression of *SabioO* might be preferred by *Sa*BirA D200E over *SabioY*.

Furthermore, the K_R value generated by *Sa*BirA D200E binding to *SabioO* was lower by 3.2-fold compared to the K_R value generated by wild-type protein binding to the same probe ($p \leq 0.01$). This suggests that binding of *Sa*BirA D200E to *SabioO* is weaker compared to the wild-type. Based on this data and the data described above, it was reasonable to conclude that *S. aureus* BPL199-resistant mutant bearing the *Sa*BirA D200E mutation would have a higher rate of transcription of both biotin biosynthesis and biotin transporter, compared to the wild-type strain. Moreover, although the intracellular biotin concentration in *S. aureus* has not been reported, in *E. coli*, the intracellular biotin concentration was between 10-100 nM [36, 37]. Considering the high K_R value exhibited by *Sa*BirA D200E for binding to *SabioY* ($K_R \geq 500$ nM), if the intracellular biotin concentration in *S. aureus* is similar to *E. coli*, the mutant strain containing the *Sa*BirA D200E would have to accumulate biotin at ≥ 5-fold higher concentration than the intracellular biotin concentration. Therefore, it would not be surprising if the *SabioY* is being expressed constitutively *in vivo* in the mutant strain, based on these results.

Overall, these data suggest that the single mutation within the dimerization interface of *Sa*BirA, (D200E) weakens the repressor-DNA interaction, possibly to facilitate the *S. aureus* resistant mutant to acquire more biotin through both *de*

novo synthesis and biotin transport, compared to the wild-type strain. The data also suggested that biotin transport was the preferred mechanism to obtain biotin over *de novo* synthesis. Further studies are still required in order to validate this hypothesis further, for example, by comparing the level of intracellular biotin between wild-type *Sa*BirA and D200E mutant, as well as biotin uptake studies and transcriptomics studies comparing the level of biotin biosynthesis transcripts between the two strains. The accumulation of intracellular biotin may be necessary to outcompete the toxic compound (in this case, BPL199) in occupying the active site of *Sa*BirA. This competitive binding mechanism was common in resistance development involving vitamin biosynthesis, as suggested by previous studies in antimicrobial resistance [38-40]. A detail discussion of this resistance mechanism, and future development of antibacterial compounds targeting *Sa*BirA, is presented in **chapter 6**.

Reference:

1. Desai, K.K. and B.G. Miller, *Recruitment of genes and enzymes conferring resistance to the nonnatural toxin bromoacetate.* Proc Natl Acad Sci U S A, 2010. **107**(42): p. 17968-73.
2. Cooper, A., Shlaes, D, *Fix the antibiotics pipeline.* Nature, 2011. **472**: p. 32.
3. Rai, J., Randhawa, G.K., Kaur, M., *recent advances in antibacterial drugs.* International Journal of Applied and Basic Medical Research, 2013. **3**(1): p. 3-9.
4. Payne, D.J., Gwynn, M.N., Holmes, D.J., and Pompliano, D.I., *Drugs for bad bugs: confronting the challenges of antibacterial discovery.* Nature reviews drug discovery, 2007. **287**(21): p. 21-40.
5. Hughes, D. and A. Karlen, *Discovery and preclinical development of new antibiotics.* Ups J Med Sci, 2014. **119**(2): p. 162-9.
6. Colman, P.M., *New antivirals and drug resistance.* Annu Rev Biochem, 2009. **78**: p. 95-118.
7. Paparella, A.S., et al., *Structure guided design of biotin protein ligase inhibitors for antibiotic discovery.* Curr Top Med Chem, 2014. **14**(1): p. 4-20.
8. Soares da Costa, T.P., et al., *Biotin analogues with antibacterial activity are potent inhibitors of biotin protein ligase.* ACS Med Chem Lett, 2012. **3**(6): p. 509-14.
9. Tieu, W., et al., *Heterocyclic acyl-phosphate bioisostere-based inhibitors of Staphylococcus aureus biotin protein ligase.* Bioorg Med Chem Lett, 2014. **24**(19): p. 4689-93.
10. Soares da Costa, T.P., et al., *Selective inhibition of biotin protein ligase from Staphylococcus aureus.* J Biol Chem, 2012. **287**(21): p. 17823-32.
11. Tieu, W., et al., *Improved Synthesis of Biotinol-5'-AMP: Implications for Antibacterial Discovery.* ACS Med Chem Lett, 2015. **6**(2): p. 216-20.
12. Feng, J., *Development of Biotin Protein Ligase Inhibitors from Staphylococcus aureus as new antibiotics*, in *chemistry*. 2016, University of Adelaide. p. 90-114.
13. Duckworth, B.P., et al., *Bisubstrate adenylation inhibitors of biotin protein ligase from Mycobacterium tuberculosis.* Chem Biol, 2011. **18**(11): p. 1432-41.
14. Silver, L.L., *Challenges of antibacterial discovery.* Clin Microbiol Rev, 2011. **24**(1): p. 71-109.
15. Friedman, L., J.D. Alder, and J.A. Silverman, *Genetic changes that correlate with reduced susceptibility to daptomycin in Staphylococcus aureus.* Antimicrob Agents Chemother, 2006. **50**(6): p. 2137-45.
16. Soares da Costa, T.P., et al., *Dual roles of F123 in protein homodimerization and inhibitor binding to biotin protein ligase from Staphylococcus aureus.* Mol Microbiol, 2014. **91**(1): p. 110-20.
17. Hellman, L.M. and M.G. Fried, *Electrophoretic mobility shift assay (EMSA) for detecting protein-nucleic acid interactions.* Nat Protoc, 2007. **2**(8): p. 1849-61.
18. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.

19. Pendini, N.R., Bailey, L. M., Booker, G. W., Wilce, M. C., Wallace, J. C. and Polyak, S. W., *Microbial biotin protein ligases aid in understanding holocarboxylase synthetase deficiency.* Biochim Biophys Acta, 2008. **1784**(7-8): p. 973-82.
20. Chapman-Smith, A., et al., *Expression, biotinylation and purification of a biotin-domain peptide from the biotin carboxy carrier protein of Escherichia coli acetyl-CoA carboxylase.* Biochem J, 1994. **302 (Pt 3)**: p. 881-7.
21. St-Pierre, F., et al., *One-Step Cloning and Chromosomal Integration of DNA.* Acs Synthetic Biology, 2013. **2**(9): p. 537-541.
22. Pendini, N.R., et al., *Structural characterization of Staphylococcus aureus biotin protein ligase and interaction partners: an antibiotic target.* Protein Sci, 2013. **22**(6): p. 762-73.
23. Juang, J.K. and H.J. Liu, *The effect of DMSO on natural DNA conformation in enhancing transcription.* Biochem Biophys Res Commun, 1987. **146**(3): p. 1458-64.
24. Helmling, C., et al., *Rapid NMR screening of RNA secondary structure and binding.* J Biomol NMR, 2015. **63**(1): p. 67-76.
25. Chakrabarti, R. and C.E. Schutt, *The enhancement of PCR amplification by low molecular-weight sulfones.* Gene, 2001. **274**(1-2): p. 293-8.
26. Lekane Deprez, R.H., Fijnvandraat, A.C., Ruijter, J.M, Moorman, A.F.M., *Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depends on cDNA synthesis conditions.* Analytical Biochemistry, 2002. **307**: p. 63-69.
27. Suzuki, M.T. and S.J. Giovannoni, *Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR.* Appl Environ Microbiol, 1996. **62**(2): p. 625-30.
28. Polz, M.F. and C.M. Cavanaugh, *Bias in template-to-product ratios in multitemplate PCR.* Appl Environ Microbiol, 1998. **64**(10): p. 3724-30.
29. Smith, C.J., Osborn, M.A., *Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology.* FEMS Micriobiol Ecol, 2009. **67**: p. 6-20.
30. Henke, S.K. and J.E. Cronan, *The Staphylococcus auresu group II biotin protein ligase birA is an effective regulator of biotin operon transcription and requires DNA binding domain for full enzymatic activity.* Molecular microbiology, 2016. **doi:10.1111/mm1.13470**.
31. Eisenberg, M.A., O. Prakash, and S.C. Hsiung, *Purification and properties of the biotin repressor. A bifunctional protein.* J Biol Chem, 1982. **257**(24): p. 15167-73.
32. Xu, Y. and D. Beckett, *Evidence for interdomain interaction in the Escherichia coli repressor of biotin biosynthesis from studies of an N-terminal domain deletion mutant.* Biochemistry, 1996. **35**(6): p. 1783-92.
33. Eisenstein, E. and D. Beckett, *Dimerization of the Escherichia coli biotin repressor: corepressor function in protein assembly.* Biochemistry, 1999. **38**(40): p. 13077-84.
34. Tungtur, S., et al., *In vivo tests of thermodynamic models of transcription repressor function.* Biophys Chem, 2011. **159**(1): p. 142-51.
35. Streaker, E.D. and D. Beckett, *The biotin regulatory system: kinetic control of a transcriptional switch.* Biochemistry, 2006. **45**(20): p. 6417-25.

36. Xu, Y. and D. Beckett, *Kinetics of biotinyl-5'-adenylate synthesis catalyzed by the Escherichia coli repressor of biotin biosynthesis and the stability of the enzyme-product complex.* Biochemistry, 1994. **33**(23): p. 7354-60.
37. Beckett, D., *Biotin sensing: universal influence of biotin status on transcription.* Annu Rev Genet, 2007. **41**: p. 443-64.
38. Blount, K.F.a.B., R.R, *Riboswitches as antibacterial drug targets.* nature biotechnology, 2006. **24**(12): p. 1558-1564.
39. Du, Q., H. Wang, and J. Xie, *Thiamin (vitamin B1) biosynthesis and regulation: a rich source of antimicrobial drug targets?* Int J Biol Sci, 2011. **7**(1): p. 41-52.
40. Pedrolli, D.B., et al., *A highly specialized flavin mononucleotide riboswitch responds differently to similar ligands and confers roseoflavin resistance to Streptomyces davawensis.* Nucleic Acids Res, 2012. **40**(17): p. 8662-73.

Chapter 6

Final discussion and future directions

6.1. *Sa*BirA –regulated gene expression

6.1.1 Validation of *Sa*BirA as transcriptional regulator in *S. aureus*.

The role of *Ec*BirA as a transcriptional repressor has been well characterized (reviewed in [1, 2]). In contrast, the transcriptional repressor function of *Sa*BirA has not been explored as extensively as *Ec*BirA. Previously, a computational study performed by Rodionov *et al* [3] predicted that as opposed to *Ec*BirA, which only regulates biotin biosynthesis operon, *Sa*BirA regulates multiple genes including the biotin biosynthesis operon (*SabioO*), biotin transporter (*SabioY*) and homologs of long-chain fatty acid CoA ligase and acetyl CoA acetyltransferase (*SayhfS-SayhfT*). Since then, few studies have investigated the interaction between *Sa*BirA with *SabioO* [4-6]. However, the interaction of *Sa*BirA with *SabioY* and *SayhfS-SayhfT* has not been elucidated experimentally. In this study, I have confirmed the role of *Sa*BirA as a biotin-responsive transcriptional repressor for *SabioO*, *SabioY* and *SayhfS-SayhfT*, using both *in vitro* and *in vivo* analyses. The results obtained from this study suggest a hierarchy of *Sa*BirA-regulated genes, with biotin biosynthesis as the most stringently controlled over the other targets, possibly to conserve energy as biotin synthesis is metabolically costly [7].

The molecular interaction of *Sa*BirA with DNA was also investigated and compared to that of *Ec*BirA. Extensive studies on *Ec*BirA revealed that homodimerization is a pre-requisite for DNA binding (reviewed in [1] and [8]). The resolved structure of both apo and holo-*Sa*BirA revealed structural similarity between the *Ec*BirA and *Sa*BirA [4], which suggests that the repression mechanism of these two proteins could be similar. However, Soares Da Costa *et al* [5] discovered that *Sa*BirA was able to dimerize and bind DNA in its apo-form.

These authors also showed that the F123G mutation within the dimerization interface abolished dimerization of *Sa*BirA and resulted in a 3-fold lower K_m for biotin compared to the wild-type.

In vivo and *in vitro* data obtained from this present study revealed that *Sa*BirA F123G was able to bind DNA and dimerize (chapter 4, Figure 6, 8 and 10). Interestingly, native nESI-MS data confirmed that this mutant is primarily monomeric in solution, hence it was predicted that the dimerization of this monomeric mutant was induced by contact with DNA. In addition, similar results were also obtained for apo-*Sa*BirA, where native nESI-MS data showed that this apo-protein was essentially monomeric in solution, but was able to dimerize and bind DNA, as shown in cross-linking experiments. Given the high dimerization constant of apo-*Sa*BirA ($K_D^{2\text{-}1}$ = 29.0 μM), it was predicted that in a cellular context, apo-*Sa*BirA is largely monomeric. Therefore, *Sa*BirA F123G could also represent the apo-protein in the *in vivo* environment. Therefore, it was not surprising that both apo and *Sa*BirA F123G exhibited the same outcome in the native nESI-MS and cross-linking experiments. Although apo-*Sa*BirA could bind DNA *in vitro*, the physiological relevance of this feature still needs further clarification. Overall, these data provided evidence that *Sa*BirA exhibits an extra layer of complexity in its interaction with DNA compared to *Ec*BirA.

6.1.2 *Sa*BirA ability to bind as apo and holo-form is similar to other transcription factors.

A study by Balderaz-Martinez and colleagues recently analyzed 982 known transcription factor-promoter interactions in the prototypical bacteria *E. coli* [9]. The study revealed that the majority of transcription factors are active in

their holo forms (37% holo-repressor, 42% by holo-activators vs. 15% apo-repressor and 3.25% by apo-activators). Similar work on *S. aureus* transcription factors has not been reported. However, it is possible that apo-regulators might be common in *S. aureus*. It is also possible that transcription factors can act in both apo and holo-form such as *Sa*BirA. Moreover, certain transcription factors, such as the acyl-CoA–responsive activator, FadR, displays the ability to act as apo-repressor. FadR interaction with ligand acyl-CoA disrupted the apo-dimer formation and DNA binding, thereby activating gene expression [10]. As there was evidence of the ability of apo-*Sa*BirA to dimerize and bind DNA, it is possible that apo-*Sa*BirA may exhibit the same repression mechanism as FadR.

Another transcription factor that shares similarities to *Sa*BirA, is the CodY transcription factor. CodY regulates its own gene expression, as well as regulates the expression of the virulence gene *prfA*. Like *Sa*BirA, CodY regulates the synthesis of its own co-repressor, by regulating the *ilvD* operon that encodes for isoleucine synthesis [11]. Lobel *et al* showed that *Listeria monocytogenes* CodY was able to bind a DNA probe containing the promoter sequence as both apo and holo-forms with different affinity. These authors revealed that when isoleucine is abundant, holo-CodY binds to the *ilvD* operon and *codY*, to act as a repressor. However, when isoleucine is scarce, apo-CodY binds to the *prfA* promoter to act as an activator. These authors proposed that during host invasion, the level of branch amino acids, such as isoleucine is limited. This is the cue for the bacteria to activate its virulence gene, *prfA*. Although there was no evidence of apo-*Sa*BirA to act as a transcriptional activator, both CodY and *Sa*BirA show the ability to bind DNA in both apo and holo-form. In addition, the limited environmental level of biotin [12, 13] could be the cue for *S. aureus* to de-repress

*Sa*BirA-regulated genes and initiate transcription, just like the lack of isoleucine activates the *pfrA* virulence genes for CodY.

Furthermore, recent work on MerR family of transcription factors revealed a novel mechanism involved in apo and holo protein interaction with DNA. A study conducted by Philips *et al* on CueR protein, a member of MerR family, showed that in a repressor conformation, apo-CueR binds DNA and causes DNA bending that prevents contact between the -10 elements and the σ2 domain of RNA polymerase (RNAP) by forcing the -10 elements away from the σ2 domain by 40Å [14]. Upon ligand binding, the protein undergoes further conformational change and introduces major kinks to the DNA, bringing the -10 elements closer to the σ2 subunit of RNAP, completing the transformation of CueR from transcriptional repressor to an activator [14]. Counago *et al* provided similar findings from their work on another MerR protein, HiNmlR. These authors revealed that apo-HiNmlR binds to sequence between the -10 and -35 sites of the operator region to act as a repressor. Ligand binding to the protein induced DNA twisting leading to re-arrangement of the -10 and -35 orientation, allowing access for RNA polymerase to bind on the opposite face of the DNA helix to HiNmlR and form a complex to activate transcription [15].

Based on these examples of MerR transcription factors, it was clear that binding of transcription factors to DNA can result in a conformational change in DNA. In the case of *Ec*BirA, it has been predicted that binding to *EcbioO* operator caused DNA to bend by 40° from linearity [16]. In contrast, Pendini *et al* showed that probe containing *SabioO* operator sequence used in a SAXS experiment was bent in solution but became linearized upon *Sa*BirA binding [4]. However, this SAXS experiment was confined to *SabioO* and did not include the *SabioY* or

SayhfS-SayhfT DNA probes. Given the different binding hierarchy exhibited by *Sa*BirA towards these three target promoters, and that the sequence comprising these promoters are different, similar SAXS experiment should also be performed on *SabioY* and *SayhfS-SayhfT* probe. Future experiments involving SAXS analysis on these probes would determine whether the changes in DNA conformation exhibit by *sabioY* and *SayhfS-SayhfT* is the same as *SabioO*, or whether DNA conformation varies between these probes. The consequence of this change in DNA structure on gene regulation should also be investigated in the future.

6.1.3 The need for *Sa*BirA–DNA complex crystal structure and validation of -35 and -10 sites of the promoter region.

High-resolution molecular structure will provide crucial information on the *Sa*BirA-DNA interaction, including details on DNA bending. To date, a crystal structure of class II BPL in complex with DNA has not been reported. Our laboratory, in collaboration with Prof. Matthew Willce (Monash University) has attempted to grow crystals of *Sa*BirA in complex with *SabioO*, without success. Hundreds of crystallization conditions were trialed with only two yielding crystals. However, these crystals produced either weak or no diffraction data [17]. Increasing the stability of the protein-DNA complex may enhance the crystallization of the complex [18]. Future studies should focus on improving the stability of the protein-DNA complex such as shortening the length of the DNA probe and using alternative DNA sequence from *SabioY* and *SayhfS-SayhfT*.

In addition, the recent discovery of a *Ec*BirA super-repressor mutant (*Ec*BirA G154D) by Chakravarrty and Cronan suggested that this super-repressor

mutant could increase the chances of obtaining stable complexes for crystallization [19]. An equivalent mutation to generate super-repressor *Sa*BirA could also be used in future crystallization trials in order to improve the diffraction of the crystals. In this study, I have predicted the equivalent mutation in *Sa*BirA to be A158D, using structural alignment and sequence alignment. I have generated this mutant protein using site-directed mutagenesis and successfully purified this protein. However, the purified protein did not show any super-repressor activity, as tested in EMSA (data not shown). Future studies should focus on generating *Sa*BirA super-repressor mutants by using other techniques, such as random mutagenesis.

Furthermore, in addition to high-resolution molecular structure data, crucial information that is still lacking in the experimental validations is the identification of the -35 and -10 promoter site of *Sa*BirA target promoters. The location of *Ec*BirA binding sites within the promoter region has been well-defined. It is known that *Ec*BirA binding sites overlap with the -35 and -10 elements of two face-to-face promoters that regulate the biotin biosynthesis operon and *bioA* gene. This knowledge has been confirmed experimentally through DNAseI foot printing and hydroxy radical foot printing studies [20, 21]. However, similar experiments to determine the -35 and -10 sites relative to the *Sa*BirA binding sites in each promoter, has not been performed. A future study to delineate the -35 and -10 elements of the promoter site relative to *Sa*BirA binding sites would provide information in how *Sa*BirA interacts with RNA polymerase and, consequently, the effect on gene regulation.

6.1.4. Defining the relationship between biotin, fatty acid synthesis, cell growth and virulence.

It is known that the biotin-dependent enzyme, ACC, catalyzes the first limited step in fatty acid biosynthesis [2, 22]. At the same time, *Sa*BirA is also responsible for ensuring sufficient biotin is available, by acting as a transcriptional repressor of both *de novo* biotin synthesis and biotin transporter. As confirmed in this study, *Sa*BirA also regulates the transcription of long-chain fatty acid CoA ligase, an enzyme catalyzes the formation of acyl-CoA through an ATP-dependent process that requires fatty acids [23] (encoded by *SayhfS,* http://www.genome.jp/kegg-bin/show_pathway?ec00061+6.2.1.3). The *SayhfS* gene is located within the same operon as another enzyme, acetyl CoA acetyltransferase, an enzyme that is involved in many metabolic pathways, including fatty acid degradation and amino acid metabolism (encoded by *SayhfT*, http://www.genome.jp/dbget-bin/www_bget?2.3.1.9). Therefore, a certain dynamic has to exist between maintaining the level of biotin and fatty acid biogenesis, with *Sa*BirA being the central regulator of these events. Further studies are therefore required to elucidate the correlation between biotin and fatty acid metabolism.

Furthermore, the ability of bacteria to modify their membrane composition through the altered synthesis of various fatty acids can contribute to a successful infection [24, 25]. Considering the intimate role of biotin in fatty acid biosynthesis, future work should focus on metabolomics profiling of *S. aureus* fatty acid composition, from bacteria that are subjected to biotin-deprived and biotin replete environments. Such experiments would allow

identification of fatty acid modifications exhibited by this *S. aureus* strain under these conditions and how these modifications aid bacterial survival. Such a study was performed as part of this project. However, the results obtained from a sample size of n = 3 were inconclusive (data not shown). A larger sample size would be required to obtain more definitive data. In addition, as the composition of fatty acids will influence the fluidity of the bacterial membrane, experiments measuring membrane fluidity of *S. aureus* grown in these biotin-depleted and biotin-replete conditions should also be conducted to provide insights into how biotin affects viscosity of the membrane and, subsequently the function of the membrane, such as passive permeability, active transport and protein:protein interactions [24]. This knowledge would then aid in understanding the rapid adaptation ability of *S. aureus* during pathogenesis [26-28].

Evidence of the association between biotin and virulence in other bacteria have been well documented [29-32]. Therefore, future studies should address the relationship between biotin and virulence in *S. aureus*. The use of *S. aureus* mutants with a deleted *SabioY* gene (*ΔSabioY*) or a deleted biotin synthesis gene (for example, *ΔbioC*) will be useful to aid these future studies. In addition, *S. aureus* with deleted *SayhfS* and *SayhfT* genes should also be utilized in order to gain more understanding of the roles of these proteins in *S. aureus* metabolism and how they contribute to virulence. Comparing the ability of these mutants to establish an infection in animal models should be performed. Moreover, the use of biotin-deficient mice [33, 34] can also be considered in order to assess the efficiency of infection in low biotin environments *in vivo* and to determine the concentration of biotin in the niche environment inhabited by *S. aureus* and how this affects the virulence of the bacteria.

6.2 *In vivo* reporter assay

6.2.1 Application of the established *E. coli* reporter assay to study other DNA-binding protein.

Although transformation of *S. aureus* has been documented, the process is time consuming and generally yields a low transformation efficiency [35-38]. As an alternative to *S. aureus* transformation, *E. coli* reporter strains were generated in this study, as described in **chapter 3**. These reporter strains provided an efficient tool to study *Sa*BirA repressor function, *in vivo*. The integration vectors generated in this study were modified by introducing particular restriction sites to enable cloning of other target promoters and repressors. In addition, chromosomal integration procedures used to integrate these vectors into the genome has also been validated to be highly efficient [39]. Therefore, the assembly of reporter strain provided by this system is straightforward and offers high transformation efficiency of site-specific genomic integration. This system can therefore be applied to study other biotin-regulated transcription factors. For example, transcription factors from organisms with class I BPL such as BioR from *Brucella melitensis* [40], other *alphaproteobacteria* as described by Rodionov *et al* [41], and the BioQ protein from *Mycobacterium smegmatis* [42] and *Corynebacterium glutamicum* [43]

6.3 *Sa*BirA inhibitor and resistance mechanism

6.3.1 The effect of *Sa*BirA inhibitor (BPL199) on DNA binding

There has been much interest in understanding vitamin synthesis pathways in bacteria, especially as drug targets for new antibiotics. For example,

thiamine (vitamin B1), folic acid (vitamin B9) and nicotinic acid (vitamin B3) have all been the subject of antibiotic development [44]. Just like biotin, these vitamins are essential requirements to support bacterial growth and deprivation of these vitamins could be detrimental. Therefore, targeting the utilization of biotin through the inhibition of protein biotinylation is also an attractive target for new antibiotic development. One compound discovered in our laboratory, BPL199, is a promising pre-clinical candidate for antibiotic discovery against *S. aureus.* BPL199 binds *Sa*BirA with a low nM K_i value, inhibits the growth of *S. aureus* with an MIC of 0.5 μg/mL and exhibits low toxicity towards mammalian cells and a mouse model (unpublished data). However, whilst extensive biochemical and structural analysis of BPL199 as an enzyme inhibitor on the catalytic function has been performed in our laboratory (unpublished result), BPL199 function as a co-repressor has not been previously explored.

In this study, the effect of BPL199 on *Sa*BirA's DNA-binding ability and consequently, gene regulation, was investigated using *in vitro* and *in vivo* methods. Comparison of BPL199-bound *Sa*BirA and biotinyl-5′-AMP-bound *Sa*BirA revealed a high degree of structural homology (RMSD = 1.2Å), suggesting that the *Sa*BirA-BPL199 complex would be receptive to binding DNA. Gel-shift assays indeed confirmed the DNA-binding activity of BPL199-bound *Sa*BirA. In addition, qPCR analysis performed on *S. aureus* treated with BPL199, confirmed that BPL199 could down regulate the expression of biotin biosynthesis gene, *SabioD*, as well as the gene encoding biotin transporter protein, S*abioY*. Based on these findings, it is clear that BPL199 can function as a co-repressor and generated repression as effectively as the natural co-repressor, biotinyl-5′-AMP.

The fact that BPL199 interferes with both functions of *Sa*BirA by acting as enzyme inhibitor and a co-repressor, the development of resistance to this inhibitor potentially is more difficult compared to other inhibitors that only target one function of the protein. Future studies investigating the effect other compounds generated in our laboratory, such as the biotin-triazoles [45], should also be performed in order to assess their effect on gene transcription. Such data will provide crucial information to aid the development of potent antibiotics in the future with special care to ensure new compounds are both enzyme inhibitors and co-repressors.

6.3.2 *Sa*BirA D200E mutation contributes to resistance mechanism

In order to elucidate resistance mechanisms to BPL199, resistant mutants of *S.aureus* were generated by serial passage in sub-optimal concentrations of BPL199. The genome of one isolated mutant was sequenced and the result identified a single mutation within the *Sa*BirA dimerization interface, where aspartic acid 200 was replaced with glutamic acid (D200E). EMSA data indicated that the *Sa*BirA D200E binds weaker to *SabioY* compared to *SabioO* (Chapter 5, Figure 8). Further assessment of DNA binding ability of D200E *in vivo* was then performed by incorporating *Sa*BirA D200E into the *in vivo* reporter assay, alongside the wild-type *Sa*BirA and *Sa*BirA F123G.

The results from *β–galactosidase in vivo* assays clearly indicated that *Sa*BirA D200E was able to repress the *SabioO* and *SabioY* promoters *in vivo*, with similar potency to *Sa*BirA F123G. It is known that the side-chain of F123 forms a hydrophobic interaction with the D200 residue from the partner subunit in the *Sa*BirA dimer [5]. Therefore, it was proposed that these two mutants would

behave similarly in the *in vivo* reporter assay. Both *Sa*BirA D200E and *Sa*BirA F123G did indeed exhibit higher requirements for biotin compared to the wild-type. The $K_{R\ biotin}$ value for both mutants with *SabioY* promoter was strikingly higher ($K_R \geq 500$ nM) compared to the wild-type ($K_R = 8.2 \pm 0.7$ nM), suggesting a loss of biotin-regulated control over the expression of the biotin transporter had evolved during resistance.

Other studies have shown that one of the resistance mechanisms that can be employed by bacteria treated with antibiotics that target vitamin biosynthesis pathways is to increase the cellular concentration of the vitamin being targeted, to outcompete the binding of the toxic compound to the proteins [46-48]. For example, resistance to the thiamanine analogue, pyrithiamine, in *Bacillus subtillis*, *E. coli* and *Aspergillus oryzae* was shown to have occurred by de-repression of thiamine biosynthesis [46]. In another example, high intracellular concentration of thiamine accumulated through thiamin import and *de novo* synthesis reversed 4-amino-2-trifuloromethyl-5-hydroxymethylpyrimine (CF3-HMP) inhibition on thiamine biosynthesis enzyme, ThiE [47]. Likewise, resistance to roseoflavin, an inhibitor of riboflavin (vitamin B2) synthesis, was through the over-production of riboflavin [48]. Based on the previous studies outlined above, it is possible that high intracellular levels of biotin are required to reverse the effect of BPL199 by outcompeting the binding of this compound to the *Sa*BirA target. It is therefore logical that the D200E resulted in weaker interaction between the repressor and the promoters thereby increasing gene expression to subsequently allow biotin accumulation via *de novo* synthesis and biotin transport.

6.4 Conclusion

Overall, this study has confirmed the role of *Sa*BirA as a transcriptional repressor that regulates biotin-related genes, as well as the *SayhfS-SayhfT* operon involved in fatty acid metabolism. In this study, I have also developed an *in vivo* reporter assay that could be used as a tool to study other DNA-binding proteins. Future studies on solving the structure of *Sa*BirA-DNA complex still needs to be performed in order to provide detail understanding of the molecular interaction between this transcriptional repressor and DNA. This study also revealed that the resistance mechanism to BPL199 involves a mutation within the dimerization interface, of *Sa*BirA, which result in reduced affinity for DNA and higher requirement for biotin. Experiments to determine determining the K_m for biotin of the *Sa*BirA D200E, and experiments to identify other mutations within the BPL199 resistant *S. aureus* still need to be performed, in order to gain good understanding of the resistance mechanism so improvements can be made in the design of future inhibitors to prevent rapidly evolving resistance mutant.

The findings from this study highlighted the importance of biotin for the growth of *S. aureus* and provided new insight into *Sa*BirA-DNA interactions at molecular level and the down-regulation of *Sa*BirA-regulated genes in response to extracellular biotin concentrations. In addition, this study also provided more knowledge in the effect of *Sa*BirA inhibitor on transcriptional regulation function of this enzyme, as well as knowledge in resistance mechanism evolution.

Reference:

1. Beckett, D., *Biotin sensing: universal influence of biotin status on transcription.* Annu Rev Genet, 2007. **41**: p. 443-64.
2. Cronan, J.E., *Biotin and Lipoic Acid: Synthesis, Attachment, and Regulation.* EcoSal Plus, 2014. **6**(1).
3. Rodionov, D.A., Mironov, A. A. and Gelfand, M. S., *Conservation of the biotin regulon and the BirA regulatory signal in Eubacteria and Archaea.* Genome Res, 2002. **12**(10): p. 1507-16.
4. Pendini, N.R., et al., *Structural characterization of Staphylococcus aureus biotin protein ligase and interaction partners: an antibiotic target.* Protein Sci, 2013. **22**(6): p. 762-73.
5. Soares da Costa, T.P., et al., *Dual roles of F123 in protein homodimerization and inhibitor binding to biotin protein ligase from Staphylococcus aureus.* Mol Microbiol, 2014. **91**(1): p. 110-20.
6. Henke, S.K. and J.E. Cronan, *The Staphylococcus auresu group II biotin protein ligase birA is an effective regulator of biotin operon transcription and requires DNA binding domain for full enzymatic activity.* Molecular microbiology, 2016, Vol 102(3), pp. 417-429.
7. Solbiati, J. and J.E. Cronan, *The switch regulating transcription of the Escherichia coli biotin operon does not require extensive protein-protein interactions.* Chem Biol, 2010. **17**(1): p. 11-7.
8. Adikaram, P.R. and D. Beckett, *Protein:protein interactions in control of a transcriptional switch.* J Mol Biol, 2013. **425**(22): p. 4584-94.
9. Balderas-Martinez, Y.I., et al., *Transcription factors in Escherichia coli prefer the holo conformation.* PLoS One, 2013. **8**(6): p. e65723.
10. van Aalten, D.M., C.C. DiRusso, and J. Knudsen, *The structural basis of acyl coenzyme A-dependent regulation of the transcription factor FadR.* EMBO J, 2001. **20**(8): p. 2041-50.
11. Lobel, L., et al., *The metabolic regulator CodY links Listeria monocytogenes metabolism to virulence by directly activating the virulence regulatory gene prfA.* Mol Microbiol, 2015. **95**(4): p. 624-44.
12. Sakamoto, O., et al., *Relationship between kinetic properties of mutant enzyme and biochemical and clinical responsiveness to biotin in holocarboxylase synthetase deficiency.* Pediatr Res, 1999. **46**(6): p. 671-6.
13. Livaniou, E., et al., *Serum biotin levels in patients undergoing chronic hemodialysis.* Nephron, 1987. **46**(3): p. 331-2.
14. Philips, S.J., et al., *TRANSCRIPTION. Allosteric transcriptional regulation via changes in the overall topology of the core promoter.* Science, 2015. **349**(6250): p. 877-81.
15. Counago, R.M., et al., *Structural basis of thiol-based regulation of formaldehyde detoxification in H. influenzae by a MerR regulator with no sensor region.* Nucleic Acids Res, 2016. **44**(14): p. 6981-93.
16. Weaver, L.H., et al., *Corepressor-induced organization and assembly of the biotin repressor: a model for allosteric activation of a transcriptional regulator.* Proc Natl Acad Sci U S A, 2001. **98**(11): p. 6045-50.
17. Yap, M.Y., *Structural characterisation of biotin protein ligase and its interacting partners: a novel antibiotic*, in *Biochemistry and molecular*

biology. 2014, Monash University: Faculty of medicine, nursing and health sciences.
18. Hollis, T., *Crystallization of protein-DNA complexes*, in *Methods in molecular Biology*, S. Doublie, Editor. 2007, Humana Press Inc., Totowa, NJ. p. 225-237.
19. Chakravartty, V. and J.E. Cronan, *Altered regulation of Escherichia coli biotin biosynthesis in BirA superrepressor mutant strains.* J Bacteriol, 2012. **194**(5): p. 1113-26.
20. Otsuka, A. and J. Abelson, *The regulatory region of the biotin operon in Escherichia coli.* Nature, 1978. **276**(5689): p. 689-94.
21. Streaker, E.D. and D. Beckett, *Coupling of site-specific DNA binding to protein dimerization in assembly of the biotin repressor-biotin operator complex.* Biochemistry, 1998. **37**(9): p. 3210-9.
22. Polyak, S.W., et al., *Structure, function and selective inhibition of bacterial acetyl-coa carboxylase.* Appl Microbiol Biotechnol, 2012. **93**(3): p. 983-92.
23. Dong, Y., et al., *Characterization of two long-chain fatty acid CoA ligases in the Gram-positive bacterium Geobacillus thermodenitrificans NG80-2.* Microbiol Res, 2012. **167**(10): p. 602-7.
24. Zhang, Y.M. and C.O. Rock, *Membrane lipid homeostasis in bacteria.* Nat Rev Microbiol, 2008. **6**(3): p. 222-33.
25. Cronan, J.E., Jr. and E.P. Gelmann, *Physical properties of membrane lipids: biological relevance and regulation.* Bacteriol Rev, 1975. **39**(3): p. 232-56.
26. Alonzo, F., 3rd and V.J. Torres, *A lesson in survival: S. aureus versus the skin.* Cell Host Microbe, 2013. **13**(1): p. 3-5.
27. van der Mee-Marquet, N.L., et al., *Emergence of a novel subpopulation of CC398 Staphylococcus aureus infecting animals is a serious hazard for humans.* Front Microbiol, 2014. **5**: p. 652.
28. Pantosti, A., A. Sanchini, and M. Monaco, *Mechanisms of antibiotic resistance in Staphylococcus aureus.* Future Microbiol, 2007. **2**(3): p. 323-34.
29. Yang, B., et al., *Enterohemorrhagic Escherichia coli senses low biotin status in the large intestine for colonization and infection.* Nat Commun, 2015. **6**: p. 6592.
30. Feng, Y., et al., *A Francisella virulence factor catalyses an essential reaction of biotin synthesis.* Mol Microbiol, 2014. **91**(2): p. 300-14.
31. Malachowa, N., et al., *Global changes in Staphylococcus aureus gene expression in human blood.* PLoS One, 2011. **6**(4): p. e18617.
32. Salaemae, W., G.W. Booker, and S.W. Polyak, *The Role of Biotin in Bacterial Physiology and Virulence: a Novel Antibiotic Target for Mycobacterium tuberculosis.* Microbiol Spectr, 2016. **4**(2).
33. Tsuji, A., T. Nakamura, and K. Shibata, *Biotin-deficient diet induces chromosome misalignment and spindle defects in mouse oocytes.* Biosci Biotechnol Biochem, 2015. **79**(2): p. 292-9.
34. Watanabe, T., et al., *Biochemical alterations in the palatal processes in fetuses of biotin-deficient mice.* Congenit Anom (Kyoto), 2010. **50**(1): p. 21-8.
35. Monk, I.R. and T.J. Foster, *Genetic manipulation of Staphylococci-breaking through the barrier.* Front Cell Infect Microbiol, 2012. **2**: p. 49.

36. Monk, I.R., et al., *Transforming the untransformable: application of direct transformation to manipulate genetically Staphylococcus aureus and Staphylococcus epidermidis.* MBio, 2012. **3**(2).
37. Monk, I.R., et al., *Complete Bypass of Restriction Systems for Major Staphylococcus aureus Lineages.* MBio, 2015. **6**(3): p. e00308-15.
38. Jones, M.J., et al., *Improving transformation of Staphylococcus aureus belonging to the CC1, CC5 and CC8 clonal complexes.* PLoS One, 2015. **10**(3): p. e0119487.
39. St-Pierre, F., et al., *One-Step Cloning and Chromosomal Integration of DNA.* Acs Synthetic Biology, 2013. **2**(9): p. 537-541.
40. Feng, Y., et al., *Brucella BioR regulator defines a complex regulatory mechanism for bacterial biotin metabolism.* J Bacteriol, 2013. **195**(15): p. 3451-67.
41. Rodionov, D.A. and M.S. Gelfand, *Computational identification of BioR, a transcriptional regulator of biotin metabolism in Alphaproteobacteria, and of its binding signal.* FEMS Microbiol Lett, 2006. **255**(1): p. 102-7.
42. Tang, Q., et al., *Mycobacterium smegmatis BioQ defines a new regulatory network for biotin metabolism.* Mol Microbiol, 2014.
43. Brune, I., Gotker, S., Schneider, J., Rodionov, D. A. and Tauch, A., *Negative transcriptional control of biotin metabolism genes by the TetR-type regulator BioQ in biotin-auxotrophic Corynebacterium glutamicum ATCC 13032.* J Biotechnol, 2012. **159**(3): p. 225-34.
44. Mobegi, F.M., et al., *From microbial gene essentiality to novel antimicrobial drug targets.* BMC Genomics, 2014. **15**: p. 958.
45. Soares da Costa, T.P., et al., *Selective inhibition of biotin protein ligase from Staphylococcus aureus.* J Biol Chem, 2012. **287**(21): p. 17823-32.
46. Blount, K.F.a.B., R.R, *Riboswitches as antibacterial drug targets.* nature biotechnology, 2006. **24**(12): p. 1558-1564.
47. Du, Q., H. Wang, and J. Xie, *Thiamin (vitamin B1) biosynthesis and regulation: a rich source of antimicrobial drug targets?* Int J Biol Sci, 2011. **7**(1): p. 41-52.
48. Pedrolli, D.B., et al., *A highly specialized flavin mononucleotide riboswitch responds differently to similar ligands and confers roseoflavin resistance to Streptomyces davawensis.* Nucleic Acids Res, 2012. **40**(17): p. 8662-73.