From Regulation of Secondary Metabolites to Increased Virulence under Sublethal Dosage of Antibiotics: An Unprecedented Role of Global Regulator, MftR, in Burkholderia thailandensis

Sudarshan Singh Thapa

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FROM REGULATION OF SECONDARY METABOLITES TO INCREASED VIRULENCE UNDER SUBLETHAL DOSAGE OF ANTIBIOTICS: AN UNPRECEDENTED ROLE OF GLOBAL REGULATOR, MFTR, IN BURKHOLDERIA THAILANDENSIS

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Biological Sciences

by

Sudarshan Singh Thapa
B.Sc., Louisiana State University, 2013
December 2020
My journey of PhD, as for most, has been a roller coaster ride. In bacterial terms, I had a long lag phase before I could actually sense some growth in myself. The biggest thing I learnt in my journey was acorn does not turn into oak in a day, so one needs to be patient. I am thankful to my family and friends who supported me throughout my journey where I did not always make right choices. I would like to take this opportunity to dedicate my dissertation to some of the most important people in my life.

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# TABLE OF CONTENTS

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

LIST OF TABLES ....................................................................................................................... vii

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

ABSTRACT ............................................................................................................................... x

CHAPTER 1. DO GLOBAL REGULATORS HOLD THE KEY TO PRODUCTION OF BACTERIAL SECONDARY METABOLITES? ................................................................. 1
   Introduction .......................................................................................................................... 1
   Bioactive secondary metabolites ....................................................................................... 3
   Global Regulators of biosynthetic gene clusters ............................................................... 18
   Trimethoprim as an inducer of cryptic biosynthetic gene clusters ................................. 25
   Conclusion and future outlook ......................................................................................... 28
   References ......................................................................................................................... 30

CHAPTER 2. IMPAIRED PURINE HOMEOSTASIS PLAYS A PRIMARY ROLE IN TRIMETHOPRIM-MEDIATED INDUCTION OF VIRULENCE GENES IN BURKHOLDERIA THAILANDENSIS ......................................................................................... 44
   Introduction ....................................................................................................................... 44
   Experimental Procedures ................................................................................................. 47
   Results ............................................................................................................................... 53
   Discussion ......................................................................................................................... 65
   References ......................................................................................................................... 71

CHAPTER 3. GLOBAL REGULATOR MFTR-MEDIATED VIRULENCE AND ANTIBIOTIC RESISTANCE IN BURKHOLDERIA THAILANDENSIS ................................................. 75
   Introduction ....................................................................................................................... 75
   Experimental procedures ................................................................................................. 78
   Results ............................................................................................................................... 85
   Discussion ......................................................................................................................... 92
   Conclusion ......................................................................................................................... 95
   References ......................................................................................................................... 96

CHAPTER 4. CONCLUSION/FUTURE OUTLOOK ................................................................ 101
   Conclusion ....................................................................................................................... 101
   Future outlook ............................................................................................................... 103
   References ....................................................................................................................... 106

APPENDIX: COPYRIGHT PERMISSION ........................................................................ 107
   Copyright permission chapter1 ...................................................................................... 107
   Copyright permission chapter2 ...................................................................................... 108
LIST OF TABLES

1.1. Local regulators of biosynthetic gene clusters ......................................................... 7

2.1. Primers used for qRT-PCR .................................................................................... 50

2.2. Primers used for ChIP and FLAG-tagging ............................................................... 52

3.1. Primers used for qRT-PCR ..................................................................................... 81

3.2. Primers used for ChIP ............................................................................................ 82

3.3. Primers used for verification of Δs ........................................................................ 84
LIST OF FIGURES

1.1. Chemical structures of selected secondary metabolites ..................................................5
1.2. Organization of biosynthetic gene clusters .................................................................8
1.3. Regulation of biosynthetic gene clusters by Major Facilitator Transport Regulator (MftR) and Secondary Metabolite Regulator (ScmR) ..................................................................................20
2.1. Effect of trimethoprim on growth of *B. thailandensis* ..................................................54
2.2. Trimethoprim alters cellular levels of intermediates in purine metabolism ....................55
2.3. Modulation of purine metabolism is linked to changes in *mal* gene expression ............56
2.4. No change in mRNA level on addition of NaOH ........................................................58
2.5. Alkaline pH increases trimethoprim sensitivity ..........................................................59
2.6. Trimethoprim and allopurinol affect both *xdh* and *mal* gene expression ...................61
2.7. MftR binds *malR* and affects its expression ..............................................................63
2.8. Survival of *C. elegans* ..............................................................................................65
2.9. Regulation of the *mal* gene cluster .............................................................................67
3.1. Deletion of *mftR* leads to increased expression of ADI pathway genes, biofilm formation, and motility ..................................................................................................................87
3.2. Variable malleobactin production in *B. thailandensis* Δ strains ....................................88
3.3. MftR binds *ecf* directly ............................................................................................90
3.4. Δ*mftR* is more virulent than wild-type cells .............................................................91
3.5. *mftR* deletion alters resistance to antibiotics ..........................................................92
ABSTRACT

The rise of multi-drug resistant bacteria combined with a decreasing pool of effective antibiotics has placed an increasing need for the development of novel antibiotics. Bacterial natural products or secondary metabolites have been the greatest source for development of novel antibiotics. The genus *Burkholderia* has recently emerged as a source of promising compounds with antibacterial, antifungal, and anti-cancer activities. Bacterial secondary metabolites provide added advantage to bacteria under stressful environments such as during host infection, evading predators or nutrient deficient conditions. However, genes involved in synthesis of these novel compounds remain silent under normal laboratory growth, creating a hurdle in isolation and characterization of these compounds. Understanding the underlying mechanism of how these genes are regulated could hold the key to unlocking the production of the secondary metabolites.

A large number of biosynthetic gene clusters in *Burkholderia thailandensis* are known to be under direct or indirect control of the global regulator, MftR (Major facilitator transport regulator). MftR, a MarR (Multiple antibiotics resistance regulator) homolog, is conserved even in the pathogenic strains. My work focuses specifically on the role of MftR in regulation of genes encoding proteins required for production two secondary metabolites involved in increasing fitness of the bacteria through increased virulence a) malleilactone, a cytotoxic compound, and b) malleobactin, the major siderophore. The data presented here indicate that under normal growth conditions MftR directly represses the expression of genes encoding local activators, MalR and ECF (extra-cytoplasmic sigma factor), which are essential for production of malleilactone and malleobactin, respectively. Further, my work shows disruption of the purine metabolic pathway induced by sub-lethal dosage of trimethoprim in *B. thailandensis*, and that such disruption leads
to increased virulence as indicated by increased killing of *Caenorhabditis elegans* by bacterial cells grown with trimethoprim. Moreover, disruption of *mftR* makes *B. thailandensis* more virulent as indicated by increased motility, biofilm production, siderophore production, killing of *C. elegans*, and rot on onion bulb.

Overall, the work conducted so far not only addresses the role of secondary metabolites, malleilactone and malleobactin, in virulence of bacterial physiology, but also that targeting a global regulator could be an effective way of eliciting production of secondary metabolites. Moreover, the role of a global regulator in increasing bacterial fitness under antibiotic stress through production of secondary metabolites is highlighted.
CHAPTER 1
DO GLOBAL REGULATORS HOLD THE KEY TO PRODUCTION OF BACTERIAL SECONDARY METABOLITES?

INTRODUCTION

A large number of antibiotics are in clinical use, and they target a variety of pathways that range from cell wall, protein, and DNA synthesis to folate and nucleotide metabolism [1]. Over the last decade, occurrences of multiple drug resistant bacteria have been on the rise, and the development of new effective methods or drugs to combat such strains has become critical [2]. Studies focused on the identification and isolation of novel natural products from microbes that are effective against pathogenic bacteria or possess other clinical significance, e.g., anti-fungal, anti-cancer, and immunosuppressant activities, have become a priority [3, 4]. A considerable number of current pharmaceutical drugs have been directly derived from, or inspired by, bacterial natural products or secondary metabolites. These secondary metabolites, which are not essential for growth under normal conditions, are produced by bacteria in response to environmental stress or host interaction and provide them with a competitive advantage. Many bacterial species produce bioactive secondary metabolites, of which members of the genus *Streptomyces* and lately the genus *Burkholderia* are prominent sources. *Streptomyces* is the source of about 80% of the antibiotics that are produced today, which includes neomycin, kanamycin, vancomycin, streptomycin, tetracycline, and chloramphenicol [5, 6]. Proteins that are essential for the production of these bioactive compounds are generally encoded by large cryptic gene clusters, which remain silent under normal laboratory conditions, a circumstance that creates hurdle in discovery of novel

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compounds [7, 8]. This review article focuses on such cryptic gene clusters in the genus *Burkholderia* and on potential mechanisms for eliciting their expression.

When originally coined in 1992, the genus *Burkholderia* comprised seven species [9]. At present, nearly a hundred validly named *Burkholderia* species exist [10]. These *Burkholderia* species occupy diverse ecological niches (free living, saprophytic, obligate endosymbionts, phytopathogens, opportunistic pathogens, or obligate parasites) and they include species that can serve as biocontrol and bioremediation agents as well as pathogens. Some *Burkholderia* species, mainly *B. pseudomallei*, *B. mallei*, and members of the *B. cepacia* complex (Bcc), have caught attention because of their pathogenicity [11]. Genome sequencing of *Burkholderia* spp., driven largely by a desire to understand virulence mechanisms, has led to the discovery of a large number of cryptic natural product biosynthetic gene clusters [12]. The regulatory mechanism of some of these cryptic gene clusters has been studied in detail, primarily in *B. thailandensis*. *B. thailandensis*, which is a relatively non-pathogenic strain, shares a large number of genes with other members of the *B. pseudomallei* complex (Bpc), some of which are involved in synthesis of bioactive compounds.

The *B. thailandensis* genome features ~22 natural product biosynthetic gene clusters [12, 13]. Understanding how these gene clusters are regulated by local or global regulators (repressors or activators) and how different stress conditions (such as oxidative stress, osmolarity stress, phosphate starvation, and amino acid starvation) or inducing ligands can elicit gene expression is key to unlocking the potential of these cryptic gene clusters. Here, we focus on how the gene clusters that are responsible for encoding proteins involved in the synthesis of well-characterized bioactive compounds are regulated by local transcription factors dedicated to a specific gene cluster and how global regulators exert control over multiple gene clusters. Based on recent reports
that a multitude of compounds are concurrently produced under conditions such as antibiotic stress and that the corresponding gene clusters are induced by inactivating a single transcription factor, we propose that such global regulators hold the key to the discovery of novel compounds.

**BIOACTIVE SECONDARY METABOLITES**

**Malleilactone**

Bacterially produced cytotoxic products are an important contributor to pathogenesis during host infection. To conserve energy, such compounds are only produced when needed and in response to inducing signals; they are, therefore, generally not synthesized under normal laboratory conditions, and this renders their identification and characterization particularly challenging. Malleilactone (also identified as Burkholderic acid [14]), is a polyketide synthase (PKS)-derived cytotoxic product, produced by species in the *B. pseudomallei* complex (Bpc), and it has received much attention, as it has been shown to be essential for *B. pseudomallei* and *B. thailandensis* to cause infection in *Caenorhabditis elegans* (Figure 1.1). Malleilactone can also inhibit growth of Gram-positive bacteria and it is cytotoxic to cultured mammalian cells [15-17]. Proteins that are encoded by the *mal* gene cluster produce it (Table 1.1).

In *B. thailandensis*, the *mal* cluster is an ~35 kb cryptic gene cluster with 13 open reading frames (BTH_II2088 to BTH_II2099). The *mal* cluster is highly conserved in *B. pseudomallei* and *B. mallei* with about 80–90% amino acid identity across these three species [14, 15] (Figure 2A). The *mal* cluster has the same gene content in *B. pseudomallei*, *B. mallei*, and *B. thailandensis*, except that two annotated hypothetical genes that are upstream of *malA* and upstream of *malC* are absent in *B. thailandensis*. The *mal* cluster is divergent from the gene encoding the transcription factor MalR.
MalR has been shown to be essential for the expression of the *mal* cluster, and in both *B. thailandensis* and *B. pseudomallei*, MalR was shown to be required for the bacteria to infect *C. elegans* [18-21]. MalR is an orphan LuxR, which means that no cognate LuxI acyl homoserine lactone (AHL) synthase has been identified. The intergenic region between *malR* and *malA* contains a lux box-like region, to which MalR likely binds, and an intact lux box is required for the expression of *malA*, which indicates that MalR functions as an activator [13, 15, 18, 19]. Generally, LuxRs respond to either endogenously and/or exogenously produced AHLs, but MalR does not respond to AHLs. However, the deletion of all three *luxI* genes in *B. pseudomallei* Bp82 led to ~10-fold greater production of Malleilactone, and expression was restored to wild type levels upon the addition of all three AHLs exogenously to the Δ culture. This led to the inference that AHL levels indirectly regulate the production of Malleilactone [19]. As outlined below, the AHL-mediated repression is likely achieved via the global regulator ScmR (Secondary Metabolite Regulator).

**Bactobolin**

Bactobolin, which is a polyketide-peptide (a C₆-polyketide fused to a chlorinated hydroxy-valine residue), is produced by *B. thailandensis* (Figure 1). It was initially identified and characterized in *Pseudomonas* BMG13-A7 (1979 AD), where the compound was shown to have antibacterial as well as anti-tumor effects [22]. A number of studies have been conducted in *Burkholderia* spp. to understand the effect of the compound, its mechanism of action, and the genes that are involved in its synthesis. Approximately eight structurally different Bactobolin compounds have been identified in *B. thailandensis* (Bactobolin A-H) [23]. Bactobolins A-D have been tested for cytotoxicity to mammalian cells and antibacterial activity, with compounds A and C exhibiting more antibacterial activity than B and D. Further, there is a direct correlation between cytotoxicity
Figure 1.1. Chemical structures of selected secondary metabolites. Two-dimensional structures were obtained from PubChem (Malleilactone under the name Burkholderic Acid), except for Burkholdac B, which was obtained from ChEMBL (under the name Thailandepsin A) and Capistruin, for which the three-dimensional structure represents its conformation in complex with RNA polymerase (PDB ID 6N61). The complete amino acid sequence of Capistruin is GTPGFQTPDARVISRGFN, where bold letters denote residues linked by a backbone-sidechain lactam linkage to form the cyclic structure through which the C-terminal residues are threaded. Images were rendered with PyMol. C, yellow; O, red; N, blue; H, grey; S, orange; Cl, green.

and antibacterial activity [24]. The compounds seem to be particularly effective against Gram-positive bacteria. A mutational study in *B. subtilis* confirmed that Bactobolin targets the ribosome, but a different site than other known ribosome inhibitors [24]. Specifically, Bactobolin A’s biological activity derives from inhibiting protein synthesis by binding to a site in the 50S ribosomal subunit, which displaces tRNA from the P site of the ribosome [25].
Genes that are involved in synthesis of Bactobolin have been identified and characterized for *B. thailandensis* [24, 26]. It is one of the few gene clusters that include both polyketide synthase (PKS)-and nonribosomal peptide synthetase (NRPS)-encoding genes (Figure 2B). An ~37 kb gene cluster, btaA to btaU (BTH_II1222-BTH_II1242), which includes the LuxI-LuxR system btai2 (BTH_II1227) and btaR2 (BTH_II1231), is involved in the synthesis and regulation of Bactobolin (Table 1.1) [24, 26]. The gene cluster is relatively conserved in *B. pseudomallei*, but not in *B. mallei*. The btai2-btaR2 quorum sensing pair is absent in *B. mallei* (most likely lost due to deletion of genes not required for virulence) [24, 27]. The presence of the btai2-btaR2 system in the cluster suggests that the production of Bactobolin would be regulated in a quorum sensing-dependent manner, an inference that was confirmed by the generation of a quorum sensing-defective Δ [28]. That Btal2 and BtaR2 control the production of Bactobolin has been established while using the btaR2 Δ, which showed no antibiotic activity against Gram-positive bacteria. It is interesting to note that btaR2 could also be regulated by the product of BtaI3 [26]. As discussed below, the expression of genes encoding BtaR2 and Btal2, and therefore the bta gene cluster, is under the control of the global regulators ScmR and MftR (Major Facilitator Transport Regulator). Moreover, the production of Bactobolin has been suggested to be influenced by temperature, as its production was higher when *B. thailandensis* was grown at 30 °C as compared to 37 °C [24].

Capistruin

Capistruin belongs to the family of lasso peptides. Lasso peptides are bioactive peptides, which are ribosomally synthesized and post-translationally modified. They are characterized by an N-terminal macrolactam ring through which a C-terminal peptide tail is threaded [29-31]. The first lasso peptide, Anantin, was discovered in 1991 from a strain of *Streptomyces coerulescens* [32]. Capistruin from *B. thailandensis* was the first lasso peptide to be identified based on a genome
mining approach. This approach has since led to the more efficient identification of lasso peptides, and about 50 lasso peptides have been discovered so far. They have been classified into class I (which contains two disulfide bonds), class II (the most abundant type, which has no disulfide bonds, but the topology is held by steric hindrance), and classes III and IV (which both contain one disulfide bond that connects the ring to the tail or is within the tail region, respectively) [33].

Table 1.1 Local regulators of biosynthetic gene clusters.

<table>
<thead>
<tr>
<th>Bioactive Compound</th>
<th>Gene Cluster</th>
<th>Local Regulator</th>
<th>Cellular Target/Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malleilactone</td>
<td>mal</td>
<td>MalR (Orphan LuxR)</td>
<td>Unknown</td>
</tr>
<tr>
<td>Bactobolin</td>
<td>bta</td>
<td>BtaR2 (LuxR)</td>
<td>50S Ribosomal Subunit</td>
</tr>
<tr>
<td>Capistruin</td>
<td>cap</td>
<td>Unknown</td>
<td>RNA Polymerase</td>
</tr>
<tr>
<td>Thailandamide</td>
<td>tha</td>
<td>ThaA (Orphan LuxR)</td>
<td>Acetyl-CoA Carboxylase</td>
</tr>
<tr>
<td>Burkholdacs</td>
<td>bhc</td>
<td>BhcM (AraC)</td>
<td>Histone Deacetylase</td>
</tr>
<tr>
<td>Pyoverdine</td>
<td>pvc</td>
<td>BTH_II2035(LTTR)?</td>
<td>Siderophore</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Ornibactin</td>
<td>orb</td>
<td>OrbS (ECF)</td>
<td>Siderophore</td>
</tr>
<tr>
<td>Thailanstatin</td>
<td>tst</td>
<td>TstA (Orphan LuxR)</td>
<td>Spliceosome</td>
</tr>
</tbody>
</table>
Capistruin belongs to class II and it is similar to *E. coli* MccJ25 (Microcin J25; Figure 1). The mature Capistruin is a 19 amino acid peptide (excised from a 47 amino acid precursor), in which the N-terminal 9 residues form the macrolactam ring through which the 10-residues long C-terminal tail is threaded [29, 34, 35]. Unlike other antibacterial compounds, Capistruin from *B. thailandensis* is effective against closely related Burkholderia and Pseudomonas strains [35].

**Figure 1.2.** Organization of biosynthetic gene clusters. (A) Malleilactone. (B) Bactobolin. (C) Capistruin. (D) Thilandamide. (E) Burkholdacs. (F) Pyoverdine. (G) Ornibactin. (H) Thailanstatin. Genomic loci and individual gene annotations correspond to *B. thailandensis* E264 and are obtained from the Burkholderia Genome Database ([https://www.burkholderia.com](https://www.burkholderia.com)), except for the Ornibactin biosynthetic gene cluster (*B. cenocepacia* J2315; Burkholderia Genome Database [36]) and the Thailanstatin gene cluster (*B. thailandensis* MSMB43; NCBI GenBank [JX307851.1]).

This leads to the suggestion that Capistruin-producing *Burkholderia* species either encode an immunity protein or feature some modification of the target. Capistruin and Microcin J25 biological activity is due to their ability to inhibit RNA polymerase (RNAP), although for Microcin J25, the over-production of reactive oxygen species (ROS) through a possible secondary target has
also been suggested [37-39]. Microcin J25 and Capistruin share the RNAP secondary channel as their binding site. Although Capistruin binds to *E. coli* RNAP as effectively as Microcin J25 in vitro, a nearly 10-fold higher concentration of Capistruin is required for inhibiting *E. coli* growth. Similarly, Microcin J25 can bind *Pseudomonas* RNAP as effectively as Capistruin, which inhibits *Pseudomonas* and *Burkholderia* growth, but no such inhibitory effect was seen with Microcin J25 [34, 35]. A recently discovered lasso peptide, named Citrocin, which was isolated from *Citrobacter pasteurii* and *Citrobacter braakii*, showed similar effect; the lasso peptide was nearly 100-fold more potent as an RNAP inhibitor compared to Microcin J25, but a higher concentration of Citrocin was required to inhibit *E. coli* growth when compared to Microcin J25 [40]. Despite having the same functional target, the effect of the compounds seems to be species-specific. The most likely reason for this species specificity is a variation in cellular uptake/export [34]. Another plausible cause could be a variation in how the compounds inhibit RNAP. The crystal structure of Microcin J25 and Capistruin in complex with RNAP has revealed that Microcin J25 binds within the active site of RNAP, which limits or prevents access to NTPs, and its inhibition of RNAP is partially competitive with respect to NTP binding. This is not the case for Capistruin, which binds further away from the active site, its inhibition is partially non-competitive with NTP binding, and it lowers the rate of phosphodiester bond formation by eight-fold. This suggests that the Capistruin-mediated inhibition of RNAP catalysis is primarily due to interference with the proper folding of the trigger loop, a mobile element within the RNAP catalytic subunit [39].

By comparison to the *E. coli* MccJ25 gene cluster (*mcjABCD*), a similar cluster was found in *B. thailandensis* and was determined to be the Capistruin biosynthetic gene cluster (*capABCD*; Figure 2C). *capABCD* encodes (i) the capistruin precursor protein CapA (BTH_I2437a), (ii) two modifying enzymes for converting the precursor to mature lasso peptide, a putative protease CapB
(BTH_I2438) and asparagine synthase CapC (BTH_I2439), and (iii) an ABC transporter CapD involved in export/immunity (BTH_I2440). CapD is most likely involved in the export of Capistruin from the cell, thereby mediating both transport and detoxification. Overproduction of the CapD homolog McjD in *E. coli* was sufficient to establish resistance against Capistruin [41]. The 4.5 kb lasso peptide gene cluster is conserved in *B. pseudomallei*.

The mechanism of expression of lasso peptide biosynthetic genes is poorly understood (Table 1.1). The *E. coli* *mcjABCD* was shown to be up-regulated under iron deficient conditions [42]. Similarly, Capistruin production in *Burkholderia* was upregulated when cells were grown in minimal media under heat stress (42 °C). Both cases can be related in terms of nutrient deficient conditions, but if nutrient deficiency were the main trigger for expression, one would expect Capistruin production to be higher in the stationary phase as compared to the exponential phase. However, unlike many other secondary metabolites with antibacterial activity, Capistruin can be detected in exponential phase, and its synthesis is arrested while transiting from late exponential to early stationary phase [35], which suggests a different mode of regulation as compared to other biosynthetic gene clusters. While no local regulator has been reported, Capistruin biosynthesis genes are under the control of ScmR and MftR, as discussed below.

**Thailandamide**

Thailandamide, which is a linear, long-chain, unstable polyene natural product, is a fatty acid synthesis blocker (Figure 1.1). It is a broad-spectrum antibiotic that has effect against both Gram-positive and Gram-negative bacteria. The structure of three distinctive forms of Thailandamide have been elucidated (Thailandamide A, Thailandamide B, and Thailandamide lactone). Thailandamide A is effective against Gram-positives, but it is less effective against Gram-negative bacteria (except *Neisseria gonorrhoeae*). It is interesting to note that a change in the *E.*
coli cell wall structure, which increased uptake, led to an increased efficiency of Thailandamide-A. This prompted the suggestion that the otherwise broad-spectrum activity of Thailandamide is limited by poor uptake in Gram-negative bacteria [43]. A study involving insertional mutation for characterization of new antibiotics revealed Thailandamide B to be the major product formed by B. thailandensis [44]; this is contrary to other analyses, where Thailandamide A was shown to be the major product [43, 45-47]. Thailandamide B was revealed to have bactericidal activity and it was shown to be toxic to human cells as well. Since Thailandamide is unstable, these differences might derive from variations in the extraction techniques; alternatively, mutations in genes encoding regulatory factors could lead to variations in the types of Thailandamide produced. For example, Thailandamide lactone, which was only detected in cells in which the tha gene cluster was highly active, displayed moderate anti-proliferative activity against tumor cell lines [46].

B. subtilis is sensitive to Thailandamide, and it was shown that a mutation in the accA gene, which encodes acetyl-CoA carboxylase, conferred resistance against Thailandamide A. Similarly, a single base mutation in accA was found in Thailandamide B-resistant Salmonella Δs. Further, in B. subtilis, over-expression of Δ AccA was sufficient to relieve the Thailandamide-induced inhibition. Thus, the cellular target for Thailandamide was suggested to be AccA protein or the AccA/AccD complex, which is involved in the first committed step of fatty-acid biosynthesis [43, 44].

The B. thailandensis gene locus BTH_II1662-1681 encodes proteins that are involved in the synthesis of Thailandamide (BTH_II1662-1676), resistance (BTH_II1679), and regulation of its production (BTH_II1681/ThaA; Figure 2D). thaC or accA-2 is responsible for resistance against Thailandamide (B. thailandensis and closely related species are resistant to Thailandamide). The presence of a second copy of accA (thaC) likely affords the resistance to
Thailandamide, which further strengthens the inference that AccA is the target for Thailandamide [43, 44], as cells that express thaC are less susceptible to Thailandamide activity. It is interesting to note that several Burkholderia spp., which lack the tha gene cluster still encode a thaC homolog [43]. This could explain the Thailandamide resistance characteristic of these Burkholderia spp.

The mode of regulation of the tha biosynthetic gene cluster has not been elucidated or agreed upon completely (Table 1.1). Thailandamide production seems to be regulated by more than one gene product, and environmental cues appear to be responsible for its regulation. Under normal growth conditions, only vanishingly small amounts of Thailandamide are produced and exclusively in the early growth phase. The disruption of thaA (luxR5) abolished production of Thailandamide A, whereas the disruption of the thaA promoter resulted in increased production, likely on account of increased thaA expression. ThaA (LuxR5) has an AHL motif and is similar to LuxR regulators, but it has no known cognate LuxI [46]. In a study that was designed to elucidate a quorum sensing-controlled regulon in B. thailandensis, it was shown that ThaA is autoregulatory and represses thaA. Interestingly, thaA was activated upon addition of exogenous AHLs [48]. Whether ThaA directly responds to AHL levels or if thaA is activated by another transcription factor remains to be determined. Most likely, ThaA activates the expression of genes that are involved in Thailandamide synthesis. As cells progress from early growth phase, levels of AHLs increase, which should lead to an increased production of ThaA; therefore, the observed decrease in Thailandamide production speaks to the repression of the tha cluster by a different mechanism. As noted below, increased AHL-dependent production of ScmR might explain such repression. Recently, it has been shown that transposon-mediated disruption of momS (BTH_I0633) led to increased production of Thailandamide B. MomS has 66% sequence identity to AtsR (Adhesion
and Type Six Secretion System Regulator), which has been shown to be a global regulator in *B. cenocepacia* [44].

Burkholdacs

Burkholdacs belong to the class of histone deacetylase (HDAC) inhibitors, which includes drugs, such as vorinostat, romidepsin, belinostat, and panobinostat (Figure 1.1). HDACs are a relatively new class of anti-cancer agents that induce death, apoptosis, and cell-cycle arrest. In eukaryotic cells, expression of genes is regulated through chromatin remodeling. One of the mechanisms by which such remodeling can be brought about is through either acetylation or deacetylation of lysine residues in core histones. While HATs (histone acetyl transferases), as the name suggests, acetylate the core histones, leading to uncoiled or less compact DNA, providing access to the transcription machinery, HDACs remove acetyl groups leading to the condensation of DNA around histone and repression of transcription. Thus, HDAC inhibitors prevent deacetylation, leading to an accumulation of hyperacetylated nucleosomes and differential gene expression [49].

Burkholdacs A and B from *B. thailandensis* were first isolated based on the overexpression of transcription factors linked to genes encoding secondary metabolite biosynthetic enzymes. In *B. thailandensis*, the *bhc* gene cluster includes two adjacent operons BTH_I2357-2358 and BTH-2359-2367, a hybrid-NRPS/PKS biosynthetic gene cluster (Figure 2E). The gene cluster has been shown to be under control of BTH_I2369 (encoded by *bhcM*), an AraC family transcription factor (Table 1.1) [50]. Members of the AraC/XylS family have three common regulatory functions: carbon metabolism, stress response, and pathogenesis [51]. Most members function as transcriptional activators, but some act as repressors or both, depending upon promoter architecture or the presence or absence of effectors [52, 53].
The above-mentioned study involving an overexpression of transcription factors within or adjacent to NRPS/PKS gene clusters identified BhcM as an activator of the bhc gene cluster, but it did not reveal how bhcM expression is controlled. [50]. Members of AraC/Xyls can be classified into two groups: Either the signal receptor and regulatory function resides in same polypeptide or transcription of the regulatory protein is controlled by another regulator, be it repressor or activator [51]. BhcM seems to fall in the latter group, as discussed below.

Pyoverdines

Pyoverdines, fluorescent yellowish-green pigments, are the primary siderophores in \textit{P. aeruginosa} (Figure 1.1). Siderophores are small, metal-chelating molecules with high affinity for Fe (III), which are produced by almost all bacteria and generally under iron limiting conditions. The role of siderophores becomes even more critical for pathogens that face a challenging low iron environment inside a host [54, 55]. Besides acting as iron chelators, siderophores (catecholate types) can serve an anti-oxidant role during host-pathogen interactions [56]. Siderophores can also bind other essential metals, such as Mn, Mo, Co, and Ni, and deliver them to the microbe [57]. Various strains of \textit{Pseudomonas} secrete different Pyoverdines, but commonalities in their structure include: (i) a fluorescent chromophore that is quenched upon binding of Fe$^{3+}$, (ii) a strain-specific peptide that interacts with Fe$^{3+}$ by chelating it, and (iii) an acyl side-chain bound to the chromophore whose functionality has not been completely understood [58]. Pyoverdines have been shown to be involved in both acquisition of iron and as signaling molecules for production of virulence factors [59-62]. No systematic analyses of Pyoverdine function have been reported in \textit{B. thailandensis}. However, a study was carried out to determine the interactions between different cystic fibrosis pathogens discovered that Pyoverdine produced by \textit{P. aeruginosa} inhibited the growth of \textit{B. cenocepa}cia J2315 [63].
The *B. thailandensis* chromosome harbors a predicted Pyoverdine gene cluster (BTH_I10229-0234) that is similar to *P. aeruginosa* pvcABCD (PyoVerdine Chromophore) cluster, which encodes proteins needed to synthesize Pyoverdine [17, 64]. In *B. thailandensis*, a convergently oriented LysR-type transcriptional regulator (LTTR; BTH_I10235) is encoded immediately downstream of the cluster (Figure 2F). Similarly, *P. aeruginosa* pvcABCD has a convergently oriented ptxR that encodes an activator PtxR, also an LTTR. ptxR has two promoter sites, of which one is regulated in an iron dependent manner, while the other promoter is iron-independent [65].

*pvcABCD* expression has been shown to be repressed by presence of iron and to be positively regulated by the alternate sigma factor PvdS and by the activator PtxR [65, 66]. A *ptxR* mutant did not produce detectable Pyoverdine, even under iron deficient conditions, which suggests that PtxR is an essential activator for expression of *pvcABCD* in *P. aeruginosa* [66]. PvdS synthesis is repressed by another transcription factor, called Fur (Ferric uptake regulator), which utilizes Fe^{2+} as a corepressor [67]. A low level of iron causes the dissociation of ferrous ion from Fur, leading to the derepression of *pvdS*; in turn, PvdS can possibly facilitate the expression of *ptxR*. *B. thailandensis* encodes PtxR and several extracytoplasmic function (ECF) sigma factors, whose roles have not been determined. A Fur homolog, BTH_I1206, is also present in *B. thailandensis*. Thus, the Pyoverdine gene cluster may be similarly regulated in *B. thailandensis* as in *P. aeruginosa* (Table 1.1).

**Ornibactin**

Ornibactin, which is a tetrapeptide siderophore with an L-ornithine-D-hydroxyaspartate-L-serine-L-ornithine backbone (Figure 1.1), was first identified in *B. cepacia* [68]. Various strains of *Burkholderia* produce Ornibactin, but it has been primarily characterized in members of the
Bcc. Ornibactin is produced under iron deficient conditions and its expression is completely inhibited by the presence of more than 15 µM of ferric iron in the media [69]. The function of Ornibactin, besides being an iron-acquiring molecule, has been established by studies of its function in different strains of *Burkholderia*. In *B. cepacia*, Ornibactin was shown to be critical for establishing infection in a murine chronic respiratory infection model. Moreover, it was noted that Ornibactin was critical in adherence and colonization [55, 70]. Evolution of the role of Ornibactin was highlighted by a study that was conducted in *B. contaminans* MS14, where the production of Ornibactin was shown to be critical for the production of an antibacterial compound, which is effective against a wide range of plant-pathogenic bacteria [71].

The Ornibactin gene cluster, *orbA* through *orbS*, has been described in *B. cenocepacia* J2315 (BCAL 1688-1702). The gene cluster includes a gene encoding an ECF sigma factor, *orbS* (*BCAL 1688*), whose product OrbS has a high degree of similarity to PvdS (Figure 2G). The Ornibactin gene cluster contains a promoter region, to which OrbS could possibly bind and thus activate the expression of the gene cluster. Further, no production of Ornibactin was detected in an *orbS* mutant, suggesting that OrbS is an essential activator (Table 1.1). The *orbS* promoter has a region to which Fur protein may bind and repress its expression under iron sufficiency [69]. Thus, regulation of Ornibactin synthesis by OrbS and Fur could work in similar fashion as described for Pyoverdine.

An interesting thing to note in the case of the Ornibactin gene cluster, which is present in a large number of *Burkholderia* species, is that it has conserved NRPS genes (*orbI* and *orbJ*), but contains diversity within the genes that are involved in initiation, transport, regulation, and modification, suggesting the possibility for differential roles and regulation across species, as exemplified by the antibacterial activity of the *B. contaminans* MS14-derived Ornibactin [71].
While Ornibactin has been characterized from members of the Bcc, members of the Bpc produce the related siderophore malleobactin, with the biosynthetic genes being organized and regulated in a similar fashion [72].

**Thailanstatin**

Thailanstatin belongs to the spliceostatin class of natural products, which inhibit the spliceosome. Four forms of the compound (Thailanstatin A-D; Figure 1.1) have been isolated and characterized from *B. thailandensis* MSMB43 [73-75]. Alternative splicing, which is carried out by spliceosomes, generates an abundance of protein variants, however, cancer cells exhibit increased splicing levels, mutations in the splicing machinery, and aberrant alternative splicing. Thus, compounds that belong to the spliceostatin class can serve as potent anti-cancer agents [73, 76].

FR901464, the first natural product of the spliceostatin family, was identified in 1996 from *Pseudomonas* sp. No. 2663 (subsequently, 16S rRNA sequence analysis showed the correct phylogenetic classification to be *Burkholderia* sp. FERM BP-342117 [77, 78]), and it was shown to have marked anti-tumor activity [79]. As the compound is chemically unstable, a more stable methylated derivative, Spliceostatin A, was produced. In vitro assays revealed that Thailanstatin A, which was shown to be the most potent, could inhibit pre-mRNA splicing as efficiently as FR901464; moreover, it possesses anti-proliferative activity and it is more chemically stable than FR901464 [73, 74]. Overall, Thailanstatin A has been shown to be less toxic to normal human cells and effective against human cancer cells lines. Spliceostatin A and FR901464 target the splicing factor 3b (SF3b) subcomplex of the U2 small nuclear ribonucleoprotein particle of the spliceosome, leading to the inhibition of pre-mRNA splicing and causing pre-mRNA leakage to the cytoplasm [80].
A gene cluster with homology to the fr9 gene cluster, which encodes proteins that are required for production of FR901464, was discovered in B. thailandensis MSMB43 and it was named the tst gene cluster (Figure 2H); this gene cluster is not conserved in closely related species such as B. thailandensis E264. The tst gene cluster is a 78.1 kb DNA region comprising 15 ORFs (tstA through tstR). tstA, which is divergently oriented to the rest of the gene cluster, encodes a LuxR type transcriptional factor with no cognate LuxI within the gene cluster (Table 1.1). TstA has been suggested to be involved in the regulation of the gene cluster [73]. This genomic locus and its arrangement is very similar to the mal gene cluster arrangement in B. thailandensis E264 suggesting that TstA could possibly serve as an activator of the gene cluster similar to MalR. Whether TstA responds to AHL levels or any other ligand has not been reported.

GLOBAL REGULATORS OF BIOSYNTHETIC GENE CLUSTERS

ScmR (Secondary Metabolite Regulator)

As discussed above, dedicated pathway-specific regulators control the expression of many biosynthetic gene clusters. However, efforts to elucidate mechanisms by which cryptic biosynthetic gene clusters might be activated have recently led to the discovery of global regulators with a role in controlling an array of biosynthetic gene clusters. One of these regulators is ScmR, an LTTR, which has been shown to be involved in the production of various secondary metabolites in B. thailandensis E264. ScmR is conserved in B. thailandensis (BTH_I1403) with orthologs in B. pseudomallei and B. mallei. The scmR promoter contains a lux box, and gene expression was shown to be about two-fold greater when the cells were grown to higher cell density [16, 48, 81]. Moreover, no activation of scmR was seen in a mutant strain that was deleted for all three btaI genes, and therefore deficient in AHL synthesis. Similarly, a B. pseudomallei Bp82 triple btaI mutant exhibited ~3-fold lower scmR transcript levels when compared to wild type. Interestingly,
the *B. thailandensis* triple *btaI* mutant featured an increased production of cryptic secondary metabolites, as also seen in the Δ*scmR* strain, which suggests that ScmR—and therefore production of some secondary metabolites—is regulated by quorum sensing. Further, production of AHLs is drastically reduced in the Δ*scmR* strain, indicating reciprocal regulation of AHL synthesis by ScmR. In the *B. thailandensis ΔscmR* strain, 13 of the >20 predicted biosynthetic gene clusters in *B. thailandensis* were differentially regulated [16]. We will focus on the role of ScmR in regulation of some of the compounds that are discussed above.

The inactivation of *scmR* leads to a 7- to 13-fold upregulation of genes in the *bta* cluster, indicating that ScmR represses the production of Bactobolin [16]. The *bta* gene cluster is locally activated by BtaR2, which is induced by AHLs. AHLs are produced at a higher cell density and they serve to activate both BtaR2 and ScmR. The repression of genes in the *bta* cluster by ScmR could therefore be achieved by ScmR binding directly to the *btaI2* promoter and repressing transcription, consistent with the ~5-fold increased expression of *btaI2* in Δ*scmR*, forming a negative feedback loop that is designed to limit bactobolin production by attenuating activation by the AHL-dependent BtaR2 (Figure 1.3). Recently, a proteomic profiling of *B. thailandensis* during host infection revealed that ScmR is overexpressed in host-associated bacteria; while nine of 11 proteins encoded by genes that are repressed by ScmR were detected at lower levels, as expected, a Bactobolin biosynthetic enzyme was an exception. BtaC, encoded by *BTH_I11224*, was overproduced during infection. This suggests that, at least under infection conditions, Bactobolin synthesis is not solely determined through ScmR-mediated transcriptional repression [82].

The deletion of *B. thailandensis scmR* also revealed upregulation of genes involved in Capistruin biosynthesis, implicating ScmR as a repressor. During the stationary phase, AHL levels increase and *scmR* expression has been shown to be higher in presence of AHLs. Thus, the decrease
in Capistruin production that was observed as cells enter stationary phase might be due to more efficient repression of Capistruin biosynthetic genes by ScmR [16]. Whether ScmR directly controls the Capistruin gene cluster or whether it controls the expression of a cluster-specific regulator remains to be determined.

![Figure 1.3. Regulation of biosynthetic gene clusters by Major Facilitator Transport Regulator (MftR) and Secondary Metabolite Regulator (ScmR). Cluster-specific regulators, where known, are identified in green. Repression is shown as lines, and activation as arrows. Deletion of mftR or scmR results in the greatest upregulation of Bactobolin and Burkholdac, respectively, as indicated by heavier lines. ScmR-mediated activation of Pyoverdine biosynthetic genes is marginal (dotted line). For ScmR-mediated regulation of the Bactobolin biosynthetic gene cluster, direct repression of btaI2 was inferred, with BtaR2 reciprocally activating scmR.](image)

The metabolomics analysis showed 61-fold increased production of Burkholdac A in ΔscmR cells, and the corresponding transcriptome analysis revealed 50- to 135-fold upregulation of the various genes in the bhc cluster [16]. Further, the complementation of the ΔscmR strain resulted in reduced production of Burkholdac, as seen in wild type cells, which indicated that ScmR represses the bhc gene cluster. Among the genes that were upregulated in ΔscmR cells is BTH_I2369 (bhcM), encoding the AraC-family activator of the bhc gene cluster, BhcM. The repression of the bhc cluster by ScmR can therefore be explained by its repression of bhcM,
possibly by direct binding to its promoter, resulting in failure to produce an essential activator. As AHLs are produced at higher cell density even under normal laboratory settings, which leads to increased production of ScmR and repression of bhcM, this would result in little to no production of Burkholdac under these conditions.

The regulation of Malleilactone production by ScmR seems to be more complex. The metabolomics analysis of ΔscmR cells showed 210-fold overproduction of Malleilactone A, previously shown to be essential for the bacteria to cause infection in C. elegans. This overproduction was supported by the 8- to 18-fold upregulation of the different genes in the mal gene cluster and by the ~90% worm killing in a span of just 20 minutes when only ~5% of the worms were killed by wild type B. thailandensis [16]. Complementing the ΔscmR strain with plasmid-encoded scmR resulted in diminished production of Malleilactone, which verifies that ScmR acts as a repressor of Malleilactone synthesis. MalR has been established as an essential activator of the mal gene cluster, yet no change in expression of malR was noticed in the ΔscmR cells. This led the authors to the suggestion that ScmR possibly competes with MalR for binding to the mal promoter or that the accumulation of an unknown molecule only occurs in ΔscmR cells and functions as a MalR coinducer to activate the mal gene cluster further; we favor the first interpretation as no coinducers for MalR have been suggested in other studies. As discussed below, the global regulator MftR represses the mal gene cluster by also repressing the malR gene, which suggests an even more complex regulatory network. In a B. pseudomallei Bp82 ΔscmR strain, only ~4-fold increased production of Malleilactone was reported as compared to wild type cells, and the production was reduced to wild type levels upon complementing the mutant. In B. pseudomallei Bp82, malR expression was reported to modestly increase in a mutant deficient in AHL synthesis and malR transcript levels were two-fold higher in a ΔscmR strain, but only when
cells were grown to late stationary phase and not in exponential or early stationary phase [16, 19]. While the change in malR expression in the AHL mutant would be consistent with repression of malR by the quorum-sensing activated ScmR, the growth phase-dependent malR expression in ΔscmR cells points to regulation by a different mechanism.

Major Facilitator Transport Regulator (MftR)

*B. thailandensis* encodes 12 annotated MarR (Multiple Antibiotic Resistance Regulator) homologs, all of which are conserved in *B. mallei* and *B. pseudomallei* [83]. Members of the MarR family are transcription factors that are ubiquitous in the domains bacteria and archaea, and they have been shown to regulate various biological functions, such as response to environmental stress (for instance, antibiotic and oxidative stress or a change in pH), regulation of genes that are involved in virulence, and catabolism of aromatic compounds [84-86]. The genomic locus in which mftR, *BTH_I2391* in *B. thailandensis*, is divergently oriented to the mftP-fenI operon (*BTH_I2392* and *BTH_2393*) is conserved across members of the Bpc. MftR is a MarR homolog, while MftP (Major Facilitator Transport Protein) encodes an efflux pump and FenI is a predicted glycosyl hydrolase. MftR binds to the intergenic region between these divergently oriented genes, thereby repressing the expression of both mftR as well as the mftP-fenI operon [87].

MftR has been previously classified as a member of the MarR subfamily UrtR (Urate Responsive Transcriptional Regulator) [87, 88]. Urate and xanthine, which are products of purine metabolism [8, 89], bind MftR and attenuate its binding to DNA. Urate was predicted to bind MftR in a pocket that spans the DNA-binding and dimerization regions of the protein, a prediction that was based on the structure of the homologous urate-binding MarR protein HucR [87, 90]. By binding in this pocket, the ligand is predicted to reconfigure the disposition of DNA recognition helices to create a conformation that is unfavorable for DNA binding. Urate is produced by host
xanthine oxidase in response to bacterial infection at levels that can exceed 200 μM [91], which suggests that MftR might regulate genes upon host colonization. Indeed, determination of the MftR regulon by using an mftR knockout mutant revealed differential expression of ~400 genes, such as genes that are involved in biosynthetic processes, metabolism, and pathogenesis [17]. A total of 331 genes were upregulated, while 70 genes were down-regulated in the ΔmftR strain, which suggests that MftR is a negative regulator of most genes directly or indirectly under its control. Notably, a number of large biosynthetic gene clusters encoding various secondary metabolites, which are not expressed under normal laboratory settings were upregulated in the ΔmftR strain as well as when B. thailandensis was grown in media containing urate. As urate attenuates MftR binding to DNA, this observation is consistent with MftR functioning as a global repressor of these gene clusters, and it identifies urate as a common signal for the production of secondary metabolites. A proteomics analysis of differential protein accumulation in host-associated B. thailandensis showed a correlation between overproduced proteins and genes that are upregulated on the addition of urate, in accord with this interpretation [82]. The gene encoding ScmR is among those upregulated (2–3-fold) in the ΔmftR strain, whereas the expression of mftR is unaltered on the deletion of scmR, which indicates that MftR acts upstream of ScmR [16, 17].

As discussed above, secondary metabolite production might be controlled by quorum sensing via AHL-activated expression of scmR. By contrast, MftR may be important for regulation of gene expression under conditions of host infection that involve the activation of host xanthine oxidase and therefore increased urate production. However, gene expression analyses indicate that these regulatory networks are intricately intertwined. The expression of genes that are involved in quorum sensing (btaI2-btaR2, btaI3-btaR3) as well as AHL levels are elevated in the ΔmftR strain, which directly implicates MftR in the quorum sensing circuit [17]. Thus, scmR expression might
be repressed either by direct binding of MftR to the *scmR* promoter or—more likely—indirectly via the MftR-mediated regulation of AHL synthesis (Figure 1.3). Genes encoding Bactobolin, including *btaR2/btaI2*, are upregulated ~15-fold on the deletion of MftR or on the addition of urate, and *scmR* expression is modestly increased. This suggests that the ScmR-mediated repression of *btaI2* (and in turn the repression of the *bta* gene cluster) inferred to occur during balanced growth is bypassed when MftR is absent or the inducing ligand for MftR (urate) is present, and it is consistent with the overproduction of a Bactobolin biosynthetic enzyme in host-associated *B. thailandensis*, even under conditions of increased ScmR levels [82]. As *scmR* expression is increased in Δ*mftR* cells, a viable interpretation is that an activator of *btaI2* becomes abundant in the absence of MftR and competes with ScmR for binding; this activator could potentially be BtaR2, the expression of which is markedly increased in Δ*mftR* cells [17].

ScmR represses the expression of the local activator BhcM that is required for expression of the *bhc* gene cluster. Both *scmR, bhcM* and the Burkholdac biosynthetic gene cluster are upregulated ~two-fold in the Δ*mftR* strain, an expression pattern that suggests a more complex mechanism for the control of this gene cluster. The modestly increased expression of the Burkholdac biosynthetic genes in Δ*mftR* cells may therefore also derive from the accumulation of an activator of *bhcM* that can compete with ScmR for binding.

Expression of the *mal* and *tha* gene clusters that are involved in production of Malleilactone and Thailandamide, respectively, are upregulated two- to four-fold in *B. thailandensis* deleted for *mftR*. Genes encoding the local regulators MalR and ThaA are similarly upregulated in the Δ*mftR* strain, which indicates the possibility of direct regulation of these regulators by MftR. Both of these secondary metabolites and their respective gene clusters are upregulated in a Δ*scmR* strain, the *mal* gene cluster in particular, but no upregulation of the respective local regulators was seen.
Increased expression of the *mal* gene cluster in Δ*mftR* cells could be explained by derepression of *malR*, which encodes an activator, while the regulation by ScmR might entail a competition between ScmR and MalR for the *mal* promoter, as noted above. A similar mode of regulation may pertain in the case of *thaA*, with ScmR more effectively competing with ThaA for the regulation of the *tha* gene cluster when *thaA* is repressed by MftR.

Several genes that were upregulated in *B. thailandensis ΔmftR* cells encode proteins that are involved in the production and transport of siderophores (Figure 1.3). This includes the gene clusters linked to production of Pyoverdine (BTH_II0229-0234) and the Ornibactin-like siderophore Malleobactin (BTH_I2414-2427). The mechanism of regulation of the Pyoverdine gene cluster remains unknown; specifically, we note that the convergent gene encoding a predicted LTTR activator of this gene cluster is not differentially expressed on the deletion of *mftR* [17]. The expression of genes in this gene cluster is modestly (<two-fold) lower in Δ*scmR* cells, which suggests moderate activation by ScmR. By contrast, the ECF sigma factor encoded as part of the Malleobactin gene cluster (BTH_I2427), which is predicted to activate expression based on comparison to the *P. aeruginosa* homolog PvdS, is upregulated in Δ*mftR* cells as well as in Δ*scmR* cells. Whether the gene encoding this sigma factor is directly or indirectly regulated by either MftR and/or ScmR has not been reported. This ECF is unlikely to participate in the activation of the Pyoverdine biosynthetic genes, as its upregulation in Δ*scmR* cells would have been expected to result in an increased expression of this gene cluster.

**TRIMETHOPRIM AS AN INDUCER OF CRYPTIC BIOSYNTHETIC GENE CLUSTERS**

Genome mining has advanced the discovery of cryptic biosynthetic gene clusters and prompted efforts to optimize production of bioactive secondary metabolites. A variety of approaches, such as changes in fermentation conditions (media composition, temperature, and pH),
co-cultivation, deletion of local regulators, and random insertional mutagenesis, have been successfully employed for the isolation and characterization of specific compounds in *Burkholderia* spp. For instance, production of Capistruin was greatly enhanced by growing *B. thailandensis* in M20 medium at 42 °C, while the isolation of Thailanstatin was optimized by growing cells in defined fermentation medium [34, 35, 73, 74]. The production of siderophores was favored by growing the bacteria in iron deficient media or by cocultivation, and Malleilactone and Thailandamide were isolated as a result of an inducible promoter exchange strategy [15, 44, 71, 92]. Such approaches have been inspired by the successful production of bioactive compounds from streptomycetes, which are well known as sources of clinically relevant compounds. In these species, strain development and metabolic engineering approaches have also been implemented for the improved production of select compounds [93]. While promising, metabolic engineering remains challenging, due to unintended consequences of competing pathways or the accumulation of toxic pathway intermediates.

In general, engineering approaches, including expression in heterologous hosts, have focused on the production of specific compounds. In contrast, chemical elicitors that create a stressful environment for the bacteria have the potential to induce the expression of multiple biosynthetic gene clusters. The activation of cryptic gene clusters by small molecules or ligands may not only increase the yield of individual compounds, but it is also a time and cost-saving technique by comparison to the more laborious culture optimization or systems metabolic engineering approaches [94, 95]. Notably, a recent study that was focused on discovering novel elicitors of cryptic biosynthetic gene clusters in *B. thailandensis* found that, among the 640 candidates tested, a sub-lethal dosage of the antibiotic trimethoprim was the most potent elicitor [13]. It should be noted that a cocktail of trimethoprim and sulfamethoxazole (Co-trimoxazole) is
a therapeutic drug prescribed for the treatment of *Burkholderia* infections [96, 97]. Metabolomic profiling of cells that were treated with trimethoprim showed an upregulation of over 100 compounds, including molecules related to Capistruin, Malleilactone, Burkholdac, Thailandamide, and Bactobolin. Trimethoprim is a dihydrofolate reductase inhibitor, which prevents the conversion of dihydrofolate to tetrahydrofolate, a one-carbon donor that is essential in a variety of biosynthetic reactions, including the production of glycine, methionine, thymidine, and purines [98]. For trimethoprim to bind all of the structurally different, pathway-specific regulators of these cryptic biosynthetic gene clusters to induce gene expression would be unlikely. A more plausible scenario would be for trimethoprim or a metabolite that accumulates in cells treated with this antibiotic to modulate the function of a global regulator. Indeed, a correlation exists between the compounds that were detected on the addition of trimethoprim and the gene clusters upregulated when *B. thailandensis* is grown in media with urate added. However, for MftR, it has already been shown that trimethoprim does not directly bind to modulate DNA binding, and neither does 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), an intermediate in *de novo* purine biosynthesis shown to accumulate in the presence of trimethoprim [13, 17]. Thus, the inducing ligand, which accumulates in cells grown with trimethoprim, has yet to be discovered.

A recent article has reported that homoserine, a precursor in methionine biosynthesis, is accumulated under the effect of trimethoprim and that it is responsible for the over-production of malleilactone. The mechanism by which homoserine could lead to over-production of not just malleilactone, but other secondary metabolites as well was not considered. The experimental approach used to test if homoserine could bring about a similar effect as trimethoprim relied on the usage of a very high concentration of homoserine (5 to 10 mM) [99]. Homoserine is first
converted to homocysteine whose accumulation has been shown to be detrimental in bacteria, yeast as well as humans [100-102]. An excessive amount of homoserine could thus syphon away THF necessary for the de novo purine bio-synthesis and other pathways in an effort to convert homocysteine to methionine, a reaction that requires 5-methyl THF, to prevent accumulation of the toxic homocysteine. That trimethoprim-mediated inhibition of folate synthesis negatively impacts de novo purine biosynthesis is well-established. In fact, in yeast excessive accumulation of homocysteine has been shown to cause a defect in purine biosynthesis [102]. Thus, it remains a possibility that an excessive amount of homoserine in the media could impact the THF level similar to trimethoprim and mimic the effect of trimethoprim rather than being the metabolite that is directly responsible for the accumulation of malleilactone.

**CONCLUSIONS AND FUTURE OUTLOOK**

Secondary metabolites confer a competitive advantage to bacteria in a hostile environment, including the inhospitable environment that is created by host defenses, yet the mechanisms that regulate their production have not been completely deciphered. The gene clusters that encode enzymes responsible for the production of these secondary metabolites, whether being harmful virulence factors or novel compounds with potential clinical activity, such as antibiotics, generally remain silent or cryptic to conserve cellular resources. Understanding how the expression of these gene clusters is elicited could shed light on mechanisms of pathogenicity as well as advance the discovery of novel beneficial compounds. The identification of global regulators, which control cryptic biosynthetic gene clusters, has opened a possible path towards achieving these goals.

Global regulators have previously been shown to be involved in the regulation of specialized genes, such as those that are involved in pathogenesis, quorum sensing, and biofilm formation. Increased biofilm formation, which also aids in bacterial antibiotic resistance, is a
characteristic of the ΔscmR strain. In a similar vein, increased anaerobiosis has been reported for the ΔmftR strain, a metabolic state that is critical for the survival of bacterial species in an oxygen-deprived environment, such as the interior of a biofilm and abscesses that are caused by infection. A role for both ScmR and MftR in virulence is further supported by their regulation of genes encoding enzymes that are responsible for the production of siderophores, which become critical in the iron-deficient environment inside a host [16, 17, 103, 104]. These findings not only suggest that global regulators can control bacterial fitness in a host environment and increase their antibiotic resistance, but they also suggest global regulators as suitable targets for drugs. A recent study in P. aeruginosa showed that the global regulator MvfR (also known as PqsR), an LTTR, is involved in biofilm formation. A drug (M64) targeting MvfR interfered with biofilm formation of P. aeruginosa and increased the antibiofilm activity of other antibiotics when used in conjunction with M64 [105].

The secondary metabolites that are discussed above likely represent just the tip of the iceberg and additional, novel secondary metabolites isolated from Burkholderia spp. await characterization. Inactivation of the global regulators ScmR and MftR is associated with remarkable changes in secondary metabolite production and a corresponding induction of biosynthetic gene clusters. Based on these observations, we predict that identifying global regulators in other bacterial species and understanding their regulatory mechanisms through a combination of genome-wide transcriptomics, metabolomics, and ChIP-Seq may enhance our chances of discovering potentially bioactive compounds as well as novel drug targets for pathogenic strains.
REFERENCES


CHAPTER 2
IMPAIRED PURINE HOMEOSTASIS PLAYS A PRIMARY ROLE IN TRIMETHOPRIM-MEDIATED INDUCTION OF VIRULENCE GENES IN BURKHOLDERIA THAILANDENSIS

INTRODUCTION

The genus *Burkholderia* comprises about 100 species, which occupy diverse ecological niches (free living, saprophytic, obligate endosymbionts, phytopathogens, opportunistic pathogens, or obligate parasites)\(^2\). Some of these species have potential for use as bioremediation or biocontrol agents, but the genus is primarily known for the pathogenic strains, namely *B. pseudomallei*, *B. mallei* and the closely related *B. cepacia* complex (Bcc) species \(^1, 2\). *B. thailandensis* is a relatively nonpathogenic strain, which has been used as a model to study mechanisms of gene regulation and virulence in *B. pseudomallei* and *B. mallei*. Due to high genetic similarity between these species, they have been classified together as the *Burkholderia pseudomallei* complex (Bpc) \(^3, 4\). *B. pseudomallei* and *B. mallei* cause melioidosis and glanders, respectively, potentially fatal diseases that occur in animals as well as humans. No effective vaccination has been developed against these diseases, and only few antibiotics are effective for treatment \(^5\). One of the most commonly prescribed anti-bacterials for treatment of glanders and melioidosis as well as Bcc infections is co-trimoxazole, a cocktail of trimethoprim and sulfamethoxazole \(^6\)–\(^8\). Trimethoprim is an antifolate, which inhibits dihydrofolate reductase (DHFR), the most well-characterized consequence of which is inhibition of thymidine biosynthesis, leading to “thymine-less” death in bacteria \(^9, 10\). Folate metabolism is generally

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required for one-carbon (1C) metabolism, and its inhibition interferes with transfer of 1C units in biosynthetic processes, including purine and thymidine synthesis and amino acid homeostasis [11].

Recently, a metabolomic study in *B. thailandensis* revealed that a sub-lethal dose (30 µM) of trimethoprim causes accumulation of a large number of secondary metabolites that are not produced under normal laboratory growth conditions, including malleilactone [12]. Secondary metabolites are often synthesized by products of large biosynthetic gene clusters that remain silent until induced by elicitors generated under specific conditions. Secondary metabolites, which are generally produced in response to environmental stress or host interaction provide a competitive advantage to the bacteria [13, 14]. The trimethoprim-mediated increase in malleilactone production was ascribed to accumulation of homoserine, a precursor in the methionine biosynthetic pathway, but the mechanism by which homoserine in turn leads to malleilactone production remains unknown [15]. Notably, many gene clusters associated with the trimethoprim-induced metabolome are also repressed by MftR (Major Facilitator Transport Regulator), a MarR (Multiple Antibiotic Resistance Regulator) homolog [3, 13, 16]. This correlation raises the possibility that trimethoprim or a metabolite produced when cells are grown with this antibiotic functions as ligand for MftR; like many MarR family proteins, DNA binding by MftR is relieved on ligand binding, resulting in upregulation of target genes [3, 17]. However, while two intermediates of purine metabolism, urate and xanthine, are ligands for MftR, trimethoprim is not. Neither is 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), an intermediate in *de novo* purine biosynthesis, which accumulates on inhibition of DHFR [16, 18-20]. As noted above, purine homeostasis is expected to be impacted by antifolates such as trimethoprim, pointing to a link between trimethoprim and ligands for MftR.
MftR is a master regulator in \textit{B. thailandensis} and disruption of \textit{mftR} leads to differential expression of \textasciitilde400 genes. In general, MftR functions as a repressor as 331 of these genes are upregulated in an \textit{mftR} knockout mutant. These include genes involved in metabolic pathways, production of secondary metabolites, and virulence [16]. Interestingly, the secondary metabolites that accumulate in \textit{B. thailandensis} under the effect of trimethoprim correspond strikingly to genes that are upregulated when the MftR ligand urate is added to the growth media. Among such accumulated products and upregulated genes is malleilactone and the \textit{mal} gene cluster.

The \textit{mal} gene cluster, comprising \textit{malA} to \textit{malM}, encodes enzymes essential for production of the polyketide malleilactone. This 35 Kb region is largely conserved in Bpc members, which have diverse lifestyles (saprophyte, opportunistic pathogen or obligate parasite) suggesting that \textit{mal} gene products are useful in diverse environments. Further, a functional \textit{mal} gene cluster has been previously shown to be essential for \textit{B. thailandensis} to infect \textit{Caenorhabditis elegans} [21].

The \textit{mal} gene cluster is under the control of MalR, an orphan LuxR, which is an essential activator for malleilactone production as a \textit{malR} mutant showed no detectable production of malleilactone, even in the presence of trimethoprim or homoserine [15, 22]. \textit{B. thailandensis} MalR is not a typical LuxR in the sense that it is not responsive to AHLs (Acyl-Homoserine Lactones) [22, 23]. MalR and malleilactone are also important virulence factors in \textit{B. pseudomallei}, and trimethoprim-mediated induction of \textit{malR} expression is conserved [24].

We show here that trimethoprim induces accumulation of the MftR ligand xanthine, which leads to derepression of \textit{malR}, and that \textit{malR} is directly regulated by MftR. Increased expression of \textit{malR} in turn correlates with overexpression of the \textit{mal} gene cluster and with increased virulence in \textit{C. elegans}. Since upregulation of the \textit{mal} gene cluster is greater on addition of trimethoprim than in an \textit{mftR} mutant, we propose that \textit{malR} expression is repressed by both MftR and by a
separate transcription factor, which also responds to a metabolite that accumulates on exposure to trimethoprim.

**EXPERIMENTAL PROCEDURES**

**Growth Media and Stock Solutions**

Bacterial cells were grown in LB (Luria Broth) media. Trimethoprim (100 mM) was dissolved in dimethyl sulfoxide (DMSO). Colony morphology was assessed on LB agar containing Congo Red (40 mg/L). *C. elegans* assay was performed on 60 mm NGM (Nematode Growth Media) plates. *C. elegans* (Bristol strain N2) were maintained at room temperature on NGM media with *E. coli* OP50 as the food source. For qRT-PCR (quantitative Real Time PCR), xanthine and urate assays, and growth analysis, cells were grown in 2×YT media. An overnight culture was diluted 1:100 for subculture unless indicated otherwise. Allopurinol and xanthine were dissolved in 0.4 M NaOH and used at final concentrations of 5 mM and 2 mM, respectively. *

**Growth Analysis**

Cells grown overnight were diluted 1:100 in 50 ml 2×YT media and were grown at 37 °C with constant shaking until OD\textsubscript{600} reached ~0.2. At that point, trimethoprim, NaOH, or NaOH plus trimethoprim was added to the respective flasks and OD\textsubscript{600} was recorded every two hours for a total of 10 hours. Growth curves represent means of triplicate cultures.

**Colony Morphology**

Colony morphology was performed as described previously with some modifications [16, 25]. An overnight culture of *B. thailandensis* was diluted 1:100 in 50 ml LB media and placed in a shaker at 37 °C until OD\textsubscript{600} reached approximately 2.0. One ml of the culture was added to a microcentrifuge tube and centrifuged at 13,000 RPM for 1 min. The supernatant was discarded,
and the cell pellet was washed with 1 ml of LB. Cells were resuspended in 200 µl of LB and 10 µl of the suspension was spotted on Congo Red-LB agar plates with or without 25 µM trimethoprim. Plates were allowed to dry and placed at 37 °C for 4-5 days before taking pictures. The experiment was performed with three independent cultures.

**C. elegans Survival Assay**

Ten µl of *E. coli* OP50 or *B. thailandensis* grown overnight was spotted on NGM (nematode growth medium) plates containing DMSO or trimethoprim (25 µM). Plates were incubated at 37 °C for 24 hours and then allowed to equilibrate to room temperature for 12 h. At the end of the 12 h period, about 10-12 *C. elegans* (L4 larval stage) were added to the plates and left for 24 h at room temperature before determining survival. Worms were observed under a dissecting microscope (Olympus SZ-ST) for their ability to maintain body posture and sensitivity to touch. Worms that were completely insensitive to touch and had lost their ability to maintain normal body posture were declared as dead. The percentage of surviving worms was calculated as: Survival (%) = (live worms/total worms used) × 100. The experiment was performed with three separate biological samples, each in triplicate.

**Urate and Xanthine Assays**

Cellular concentrations of urate were determined using the Amplex Red Urate/Uricase Assay (ThermoFisher, Waltham, MA) as per the user manual. An overnight culture of *B. thailandensis* was diluted 1:100 in 50 ml 2×YT media and placed in a shaker at 37 °C. Once OD$_{600}$ reached 0.2, trimethoprim (25 µM) or allopurinol (5 mM) was added to the respective flasks and cultures were grown until OD$_{600}$ reached ~0.6. Forty ml of the culture was centrifuged at 3,500 RPM at 4 °C for 8 minutes and the supernatant was discarded. Cells were resuspended in 1 ml of 1× reaction buffer from the assay kit and centrifuged at 13,000 RPM at 4 °C for 1 minute and the
supernatant was removed. Cells pellets were washed again with the 1× reaction buffer and stored at -80 °C. Cells pellets were thawed on ice and resuspended in 1 ml of 1× reaction buffer and lysed by sonication (30% output, pulse mode, total time 10 seconds, 1 minute in ice between each cycle, 8 cycles in total). Sonicated samples were then centrifuged at 13,000 RPM at 4 °C for 10 minutes and the lysate was transferred to a clean tube. The lysate was centrifuged again for another 3 minutes to remove any transferred residual debris and this cell free lysate was utilized for the assay. Three hundred µl of the lysate was mixed with 300 µl of the reaction mix (prepared as per the kit protocol) in a microcentrifuge tube and placed at 37 °C in a well-covered box to avoid light interference. The absorbance at 560 nm was recorded after 1.5 h. Urate levels were normalized using the total protein concentration measured at 280 nm. The results obtained are the mean (±SD) from three independent experiments.

Determination of xanthine concentrations was performed utilizing the Amplex Red Hypoxanthine/Xanthine assay kit (ThermoFisher) as per the user manual. The assay was conducted, and data were analyzed as described above. Note that determination of xanthine concentrations requires addition of xanthine oxidase from the assay kit, and cells pellets from cultures grown with xanthine were therefore washed more extensively.

qRT-PCR

An overnight culture of *B. thailandensis* was sub-cultured in 50 ml 2×YT media and grown until OD<sub>600</sub> reached ~0.2. Trimethoprim (25 µM), allopurinol (5 mM), a cocktail of allopurinol and trimethoprim, and/or 0.4 M NaOH (800 ml) was added to the cultures and cells were grown until OD<sub>600</sub> reached ~0.6. Once cells reached this density, 1 ml of the cell culture was pelleted and washed twice with autoclaved diethyl pyrocarbonate (DEPC)-treated water and stored at -80 °C.
RNA was extracted utilizing illustra RNAspin mini kit (GE Healthcare, Chicago, IL) according to the manufacturer’s protocol. RNA was electrophoresed on agarose gels to ascertain integrity and PCR was performed to verify absence of genomic DNA contamination. Five hundred ng of RNA was used for preparation of cDNA using AMV RT (Reverse Transcriptase; New England Biolabs, Ipswich, MA) and gene-specific primers (Supplemental Table S1). Quantitative PCR was performed on Applied Biosystem QuantStudio 6 Flex System using SYBR Green I dye and Luna qPCR master mix (New England Biolabs). Expression of the HGPRT gene was utilized as reference. For measuring expression of XDH, One-Step qPCR was done using Luna One step universal master mix (New England Biolabs). Data represent means (± SD) from biological triplicates (each determined from technical triplicates) using the comparative C_T method (2^{-ΔΔC_T}).

Table 2.1 Primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>malA forward</td>
<td>GACGATCACCGGACCTT</td>
</tr>
<tr>
<td>malA reverse</td>
<td>TCGATCACGGACGATGAG</td>
</tr>
<tr>
<td>malR forward</td>
<td>GGTCTTCAGGGTCTTTCG</td>
</tr>
<tr>
<td>malR reverse</td>
<td>CGAAGGTCTTGAAGCCCAGT</td>
</tr>
<tr>
<td>xdh forward</td>
<td>GATCTGGGCGACGTGCTGTA</td>
</tr>
<tr>
<td>xdh reverse</td>
<td>ATCAGGCCGCGCATCGAATC</td>
</tr>
<tr>
<td>mftP forward</td>
<td>TGCCCATGCTTAATTCTCCT</td>
</tr>
<tr>
<td>mftP reverse</td>
<td>GATCGGCAAGGTCGTCAG</td>
</tr>
<tr>
<td>hgprt forward</td>
<td>CGAGAAGAGGCTCCACAT</td>
</tr>
<tr>
<td>hgprt reverse</td>
<td>TCGAACTCGAGCGGAAATC</td>
</tr>
</tbody>
</table>
ChIP Assay

A synthetic DNA construct was used to create a C-terminally FLAG-tagged MftR strain; a 379 bp fragment comprising 292 bp of MftR coding sequence immediately followed by sequence encoding the 3×FLAG tag and flanked by XbaI and XhoI restriction sites was obtained (Synbio Technologies, Monmouth Junction, NJ) in the pUC57-Amp vector. The fragment was subcloned into the mobilizable suicide vector pKNOCK-Tc[26] and transformed into E. coli SM10 λ pir. B. thailandensis E264 encoding genomic MftR-FLAG was created through biparental mating and transformants selected as described[16]. Presence of the correct sequence was verified by PCR (for primers, see Table 2.2) and sequencing.

For ChiP, an overnight culture of B. thailandensis MftR-FLAG was diluted 1:100 in 50 ml LB. Once the cells reached OD600~0.6, 1% formaldehyde was added to the flask, which was placed on a shaker for 16 minutes to allow crosslinking to occur. The cell culture was then centrifuged at 3,500 RPM at 4 °C for 8 minutes and the supernatant was discarded. Cell pellets were washed twice by adding 20 ml phosphate buffered saline (PBS) followed by another wash with 5 ml of PBS and centrifuging at 3,500 RPM for 5 minutes. Finally, the pellets were resuspended in 1 ml of PBS and the supernatant was removed after centrifuging at 13,000 RPM for one minute. Cell pellets were stored at -80 °C. ChIP was performed as previously described with some modifications [27]. Briefly, cells were suspended in 1 ml of lysis buffer with protease inhibitor cocktail and placed on ice for 1 hour. Genomic DNA was sheared by sonicating 5 times at 35% output at 10 secs pulse with 1-minute gap in between. Cell/cell debris-free lysate was collected by centrifugation at 13,000 RPM at 4 °C for 12 min and transferred to a clean tube. The lysate was centrifuged for another 3 min and the final lysate was collected. The lysate was precleared using protein G-Sepharose beads (GE Healthcare) to reduce non-specific binding to the beads. For
immunoprecipitation, 5 μl of anti-FLAG (M2; MilliporeSigma, Burlington, MA) antibodies was used. Eluted DNA from ChIP samples or input DNA was analyzed by PCR (for primer sequences, see Table 2.2). PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide. Signal intensities from PCR data was quantified from the TIFF images by using ImageJ software. Experiments were performed in triplicate.

**Table 2.2** Primers used for ChIP and FLAG-tagging.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>mftP-mftR</em> intergenic region chip forward</td>
<td>GAGCTGCGCGATCCATTAAC</td>
</tr>
<tr>
<td><em>mftP-mftR</em> intergenic region chip reverse</td>
<td>TGCAGGAGAATTAAGCATGCGG</td>
</tr>
<tr>
<td><em>malR</em> upstream forward</td>
<td>GGTCTTCAGCGGTCTTTTC</td>
</tr>
<tr>
<td><em>malR</em> upstream reverse</td>
<td>CGAAGTTCTTGAAGCCCAGT</td>
</tr>
<tr>
<td><em>ohrR</em> upstream forward</td>
<td>GGTCTTCATGGCGAGATTTTC</td>
</tr>
<tr>
<td><em>ohrR</em> upstream reverse</td>
<td>GTCGTTCAATGGCGAGATTTTC</td>
</tr>
<tr>
<td>Flag-tag cloning verification forward</td>
<td>AACGCACTGAGAAGCCCTTA</td>
</tr>
<tr>
<td>Flag-tag cloning verification reverse</td>
<td>TTGCAGGAGAAGCCCTTA</td>
</tr>
<tr>
<td>Flag-tag chromosomal integration verification forward</td>
<td>GTCGTCGATGATCGTGCTC</td>
</tr>
<tr>
<td>Flag-tag chromosomal integration verification reverse</td>
<td>TTGCAGGAGAAGCCCTTA</td>
</tr>
</tbody>
</table>
Statistical analyses

For determination of statistically significant differences (gene expression, cellular urate/xanthine levels, and worm survival), an unpaired, Student’s t-test was used.

RESULTS

Sub-lethal dosage of trimethoprim leads to upregulation of virulence genes

The trimethoprim-induced accumulation of secondary metabolites such as malleilactone points to a modulation of numerous metabolic pathways and consequently an altered physiological state. While wild type *B. thailandensis* grown on LB-Congo Red plates have a rugose morphology, cells grown on plates with 25 µM trimethoprim gave rise to a smooth colony morphology (Fig. 2.1A-B). A rugose colony morphology is associated with increased surface area, which enhances access to oxygen and serves to balance cellular redox homeostasis [28]. The 25 µM concentration of trimethoprim used for our experimental purpose did not arrest cell growth but led to a reduced growth rate compared to cells grown in LB without the antibiotic (Fig. 2.1C).

MalR is an essential activator of the *mal* gene cluster, binding to a Lux-box immediately upstream of *malA* (Fig. 2.1D), and it is required for trimethoprim-based activation of the *mal* genes, a requirement that was inferred to reflect trimethoprim-mediated upregulation of *malR* [15, 21, 22, 24]. To verify that the sub-lethal (25 µM) dose of trimethoprim caused upregulation of *malR* and *malA*, (encoding the first ORF in the *mal* gene cluster) relative transcript levels of *malR* and *malA* were measured in wildtype cells grown in liquid culture with trimethoprim. Consistent with expectations, *malR* was upregulated ~11-fold in cells grown with trimethoprim compared to cells grown without trimethoprim, and *malA* expression increased ~50-fold when cells were grown with trimethoprim (Fig. 2.1E). Trimethoprim-based upregulation of *malR* and the *mal* gene cluster has been reported in *B. pseudomallei* as well [24].
Figure 2.1. Effect of trimethoprim on growth of *B. thailandensis*. (A-B) Colony morphology of cells grown on LB-Congo Red plates without (A) or with trimethoprim (B). (C) Growth of cells in 2×YT without (blue line) or with trimethoprim (25 µM; orange line). (D) Intergenic region between the divergently encoded *malR* and the *mal* gene cluster; only part of *malA* is shown. The *Lux*-box to which MalR binds is identified (green line). (E) Increase in expression of *malA* and *malR* induced by addition of 25 µM trimethoprim to liquid cultures. The transcript levels were calculated using $2^{-\Delta\Delta C_T}$ relative to the reference gene and reported relative to expression in the corresponding unsupplemented (2×YT) cultures. Asterisks indicate statistically significant differences (**, p<0.01) based on a Student’s t-test.

**Xanthine accumulates in cells grown with trimethoprim**

We have previously reported that urate elicits an upregulation of *malR* and the *mal* gene cluster, suggesting the possibility that the changes in purine homeostasis expected on treatment with trimethoprim include elevated urate levels [16]. While xanthine is an intermediate in purine salvage, its conversion to urate diverts purines away from salvage pathways for complete degradation (Fig. 2.2A) [18, 19]. A coupled enzymatic assay was performed to determine the cellular urate level in cells grown with or without trimethoprim, and we found that urate levels decreased by ~0.6-fold in cells grown with trimethoprim compared to cells grown without trimethoprim (Fig. 2.2B). This outcome not only suggests that purine degradation may be disfavored under conditions of trimethoprim-mediated inhibition of DHFR, and hence inhibition of *de novo* purine biosynthesis (Fig. 2.2A), but it also indicates that urate is unlikely to be involved in differential gene expression on exposure to trimethoprim. We also determined the intracellular
level of xanthine in cells grown with or without trimethoprim by utilizing a similar coupled enzymatic assay and found it to be increased ~2-fold in cells grown with trimethoprim compared to cells grown without trimethoprim (Fig. 2.2C). These results make intuitive sense as degradation of xanthine to urate would prevent recovery of purines through purine salvage (Fig. 2.2A), a pathway that would likely be favored in response to trimethoprim-mediated inhibition of de novo synthesis.

**Figure 2.2.** Trimethoprim alters cellular levels of intermediates in purine metabolism. (A) Outline of purine salvage pathway in *B. thailandensis* (several intermediate steps have been omitted for simplicity). Pathway is represented based on the KEGG pathway database ([https://www.genome.jp/keggbin/show_pathway?org_name=bte&mapno=00230&mapscale=&show_description=show](https://www.genome.jp/keggbin/show_pathway?org_name=bte&mapno=00230&mapscale=&show_description=show)). Trimethoprim interferes with de novo synthesis (red arrow), and purine salvage is expected to be favored on inhibition of de novo synthesis; red arrows next to urate and xanthine reflect observed changes in cellular level of the indicated metabolite on addition of trimethoprim. (B-C) Relative cellular urate (B) and xanthine levels (C) in *B. thailandensis* grown with trimethoprim compared to cells grown without trimethoprim, reported as mean ± SD from three independent experiments. Asterisks indicate statistically significant differences (**, p<0.01; *, p<0.05) for cells grown in media with trimethoprim compared to cells grown without trimethoprim.

To address the potential role of xanthine in gene regulation, relative expression levels of *malA* and *malR* were measured in cells grown with or without xanthine. An ~4-fold upregulation of *malA* and ~2-fold increase in *malR* expression was seen upon addition of xanthine to the media (2 mM) (Fig. 2.3A), suggesting that accumulation of xanthine leads to upregulation of *malA* and *malR*. The relatively modest increase in expression of *malA* and *malR* seen upon addition of
xanthine could be the result of a) xanthine (which is dissolved in 0.4 M NaOH) being poorly soluble and precipitating due to the decrease in pH upon addition to the growth media, b) uptake of xanthine possibly being inefficient, and c) excess xanthine being easily metabolized in an unstressed cell. Combined, these factors would work to reduce the xanthine concentration within the cell. We also note that *malR* and *malA* expression is increased 2.5-fold and 5-fold, respectively, based on RNA-seq data on exposure to 5 mM of the also poorly soluble and readily metabolizable urate, levels of induction that are comparable to those seen here with xanthine, and that equivalent urate-mediated increases in gene expression are not observed in Δ*mftR* cells [16]. Taken together, our results indicate that xanthine accumulation may contribute to the increased expression of *malR* and *malA* on exposure to trimethoprim and that it may do so, at least in part, by acting as a ligand for MftR.

**Figure 2.3.** Modulation of purine metabolism is linked to changes in *mal* gene expression. (A) Increase in *malA* and *malR* expression induced by addition of 2 mM xanthine. (B) Relative cellular urate content in *B. thailandensis* grown with trimethoprim (Tri; 25 µM) or allopurinol (Allo; 5 mM). (C) Relative cellular xanthine content in *B. thailandensis* grown with trimethoprim (Tri), allopurinol (Allo), xanthine (Xan) or Trimethoprim + Allopurinol (Tri+Allo). (D) Increase in *malA* and *malR* expression induced by addition of allopurinol. All data are reported as mean ± SD from three independent experiments. The transcript levels were calculated using 2^ΔΔC_T relative to the reference gene and reported relative to expression in the corresponding unsupplemented (2×YT) cultures. Asterisks indicate statistically significant differences relative to unsupplemented cultures, except where bracket indicates otherwise (**, p<0.01; *, p<0.05).
Xdh inhibition leads to derepression of malR and malA

The enzyme that converts xanthine to urate is xanthine dehydrogenase (Xdh), whereas xanthine can be produced by the actions of either Xdh or guanine deaminase (Fig. 2.2A). Allopurinol, a competitive inhibitor of Xdh, is clinically utilized for treatment of gout, which is caused by accumulation of uric acid crystals [29], and it functions in several bacterial species to block Xdh activity [30-32]. Allopurinol not only inhibits conversion of xanthine to urate, but an adverse effect of allopurinol in humans is the accumulation of xanthine [33, 34]. Thus, allopurinol could possibly mimic the effect of trimethoprim by promoting an accumulation of xanthine. To evaluate this possibility, 5 mM allopurinol was added to the growing culture when the cell density reached OD$_{600}$~ 0.2, and the cellular urate level was measured. We found that allopurinol mediated a decrease in urate level by ~0.6-fold compared to unsupplemented cultures (Fig. 2.3B). This decrease in urate level is consistent with reduced Xdh activity and it mimics that induced in cells grown with trimethoprim. In accord with the second expectation that inhibition of Xdh would result in xanthine accumulation, cellular levels of xanthine were found to increase ~2-fold, equivalent to the increase observed on addition of trimethoprim. By comparison, addition of 2 mM xanthine to the media resulted in a <1.5-fold increase in cellular xanthine (Fig. 2.3C).

The relative transcript levels of malR and malA were measured in cells grown with or without allopurinol. The expression of malA and malR increased by ~43-fold and 11-fold, respectively (Fig. 2.3D), similar to cells grown with trimethoprim (Fig. 2.1E). Addition of allopurinol changed the pH of the media from 6.89±0.03 to 7.54±0.03 (Fig. 2.4A). In order to ensure that the change in transcript level was not due to a change in pH (allopurinol was dissolved in 0.4 M NaOH), an equivalent volume of 0.4 M NaOH was added to the control culture. When only NaOH was added to the media, the pH of the media also increased (to 7.53±0.04; Fig. 4A).
and no significant change in expression of *malA* or *malR* was seen (Fig. 2.4). This outcome indicates that the change in *malA* and *malR* expression is due to allopurinol and not to changes in pH, and that inhibiting Xdh activity leads to overexpression of both genes.

![Graph showing mRNA levels](image)

**Figure 2.4.** No change in mRNA level on addition of NaOH. The transcript levels were calculated using $2^{-\Delta\Delta CT}$ relative to the reference gene and reported relative to expression in the corresponding unsupplemented (2xYT) cultures.

**Increase in pH promotes trimethoprim sensitivity**

Adding NaOH or allopurinol to the media modestly increased the pH, whereas trimethoprim addition did not alter the external pH (Fig. 2.5A). In *E. coli*, an increase in pH leads to more efficient uptake of trimethoprim as evidenced by a slower growth rate and an increased effect of trimethoprim [35]. If such were the case in *B. thailandensis*, then adding trimethoprim along with NaOH should result in slower growth rate as well as increased expression of *malA* and *malR*. To examine if the slight change in pH was sufficient to increase uptake of trimethoprim in *B. thailandensis*, cells were grown at low (15 µM) and high (30 µM) concentration of trimethoprim with or without NaOH added. We found that growth of the bacteria at low concentration of trimethoprim with NaOH added to the media is similar to its growth at the higher concentration of trimethoprim, consistent with increased uptake of trimethoprim at elevated pH (Fig. 2.5B).
Moreover, expression of \textit{malR} and \textit{malA} increased \(\sim 159\) and 21-fold, respectively, when trimethoprim (25 \textmu M) was added along with NaOH to the growth media (Fig. 2.5C). This change in expression of \textit{malA} and \textit{malR} seen upon addition of trimethoprim with NaOH was significantly greater compared to that observed upon addition of trimethoprim only. We also note that adding trimethoprim with NaOH imposed a further slight decrease in the cellular urate concentration (\(\sim 0.5\)-fold; Fig. 2.5D) compared to trimethoprim only (\(\sim 0.6\)-fold). We infer that uptake of trimethoprim by \textit{B. thailandensis} is more efficient at higher pH and that the expression of \textit{malA} and \textit{malR} is affected by trimethoprim in a concentration dependent manner.

\textbf{Figure 2.5.} Alkaline pH increases trimethoprim sensitivity. (A) Measurement of pH upon addition of allopurinol (Allo; dissolved in NaOH), NaOH, or trimethoprim (Tri) to the growth media (2xYT). (B) Increase in pH leads to slower growth rate in cultures supplemented with trimethoprim. Growth was measured in 2xYT without or with NaOH added (light and dark blue), in cultures containing 15 \textmu M trimethoprim without or with NaOH added (Tri15; light and dark orange) or 30 \textmu M trimethoprim without or with NaOH added (Tri30; light and dark grey). (C) Cells grown with trimethoprim and NaOH show increased upregulation of \textit{malA} and \textit{malR} compared to cultures grown with trimethoprim only. The transcript levels were calculated using \(2^{-\Delta\Delta C_T}\) relative to the reference gene and reported relative to expression in the corresponding un-supplemented (2xYT) cultures. (D) Relative cellular urate content in cells grown with trimethoprim (Tri) without or with NaOH added. Data represent mean ± SD from three independent experiments. Asterisks indicate statistically significant differences relative to un-supplemented cultures, except where bracket indicates otherwise (**, \(p<0.01\); *, \(p<0.05\)).
Xanthine is involved in trimethoprim-mediated gene regulation

The Xdh inhibitor allopurinol elicited a similar effect as trimethoprim in terms of upregulation of malR and malA (Figs. 2.1E and 2.3D) with corresponding increases in intracellular xanthine concentrations (Fig. 2.3B). In some bacterial species, xanthine has been reported to induce an increase in expression of the genes encoding Xdh [36, 37], so we wanted to ascertain if growth with allopurinol or trimethoprim led to changes in xdh expression. Both allopurinol and trimethoprim caused modestly (<2-fold) increased expression of xdh a change in expression also seen on addition of xanthine (Fig. 2.6A), further indicating that trimethoprim and allopurinol elicit similar changes in purine metabolism. When trimethoprim was added along with NaOH, an ~4-fold upregulation in xdh expression was observed, which speaks to a concentration-dependent effect considering the facilitated uptake of trimethoprim at the slightly more alkaline pH. By comparison, a mixture of allopurinol and trimethoprim (5 mM and 30 µM, respectively) led to an ~5-fold increased expression of xdh. Notably, this elevated level of xdh expression corresponded to a 2.5-fold higher cellular level of xanthine compared to an ~2-fold increase with either trimethoprim or allopurinol alone (Fig. 2.3C) and to a high level of expression of both malA (~162-fold) and malR (~21-fold; Fig. 2.6B) that is comparable to that seen with trimethoprim and NaOH (Fig. 2.5C).

The transcription factor MftR controls expression of ~400 genes in B. thailandensis, and its DNA binding is attenuated on binding of the ligands urate or xanthine [16, 38]. This raises the possibility that MftR participates in controlling gene expression in response to accumulation of intracellular xanthine. The gene encoding MftR is divergent to mftP, which encodes the efflux pump MftP; MftR binds the mftR-mftP intergenic region, causing repression of both genes, and urate induces expression of mftP in a concentration-dependent manner, with increased expression.
observed when ≥5 mM urate was added to the media [16]. Accordingly, we would expect upregulation of mftP only if intracellular xanthine levels reach a certain threshold. Consistent with this expectation, upregulation of mftP is seen when cells are grown with trimethoprim and either NaOH or allopurinol, conditions under which cellular xanthine levels are the highest (Fig. 2.6C). Evidently, the efficiency of trimethoprim uptake correlates with cellular levels of xanthine, which in turn elicit an upregulation of xdh and mftP, and with malA and malR expression.

Figure 2.6. Trimethoprim and allopurinol affect both xdh and mal gene expression. (A) Relative xdh expression in cells grown with NaOH, allopurinol (Allo), trimethoprim (Tri), trimethoprim+NaOH, trimethoprim+allopurinol, or xanthine (Xan) compared to cells grown in 2×YT. (B) Change in expression of malA and malR upon addition of trimethoprim and allopurinol together. (C) Relative change in mftP transcript levels compared to cells grown in 2×YT. The transcript levels were calculated using $2^{-\Delta\Delta C_T}$ relative to the reference gene and reported relative to expression in the corresponding unsupplemented (2×YT) cultures. Asterisks indicate statistically significant differences relative to unsupplemented cultures, except where brackets indicate otherwise (**, p<0.01; *, p<0.05).

MftR participates in controlling malR expression

In order to determine if MftR directly participates in controlling malR expression in vivo we performed chromatin immunoprecipitation (ChIP). A B. thailandensis strain harboring C-
terminally FLAG-tagged MftR was created and expression of the tagged protein was verified by Western blotting (data not shown). For ChIP, we used as a positive control the intergenic region between mftR and the divergently oriented gene mftP to which MftR binds in vitro [20, 38], and we verified MftR binding to this region in vivo (Fig. 2.7A). A negative control using the ohrR upstream region to which the unrelated transcription factor OhrR binds [39] was used to ensure the immunoprecipitation was specific, and no indication of MftR binding was detected (Fig. 6B).

The ability of MftR to bind upstream of malR was tested by amplifying a fragment comprising 74 bp upstream of malR and part of the coding region, and MftR was found to bind this region, consistent with the notion that it directly controls malR expression (Fig. 2.7C).

We previously characterized a B. thailandensis ΔmftR strain and documented a markedly increased expression of mftP, which was completely reversed on complementation with mftR [16]. As shown in Fig. 2.7D, mftP expression was >100-fold higher in the ΔmftR strain compared to wild-type cells, and addition of trimethoprim had no further effect on mftP expression. By contrast, expression of malA and malR were increased ~14-fold and 4-fold, respectively, in the ΔmftR strain compared to wild-type cells, pointing to a role for MftR in controlling gene expression. A further increase in expression of malA and malR was observed on addition of trimethoprim (~135-fold and 16-fold, respectively, relative to the expression in wild-type cells without any supplements; Fig. 2.7E-F). This translates into 10-fold and 4-fold higher expression of malA and malR in ΔmftR cells on addition of trimethoprim (Fig. 2.6G). These trimethoprim-induced changes in gene expression in ΔmftR cells were lower than those seen in wild-type cells grown with trimethoprim (50-fold and 11-fold, respectively; Fig. 2.1E; note that measurements of mRNA levels in wild-type and ΔmftR cells were performed side-by-side in the same experiment). These observations are consistent with MftR playing a role in trimethoprim-mediated induction of gene expression.
Taken together, we infer that MftR participates in controlling \textit{malR} (and in turn \textit{malA}) expression along with a second transcription factor, and that both repressors respond to metabolites produced when cells are grown with trimethoprim.

\textbf{Figure 2.7.} MftR binds \textit{malR} and affects its expression. (A-C) \textit{In vivo} binding of MftR determined by chromatin immunoprecipitation. PCR products electrophoresed on 1.5\% agarose gels and visualized with ethidium bromide for \textit{mftR-mp} intergenic region to which MftR binds \textit{in vitro} (A), \textit{ohrR} promoter, which is not regulated by MftR (B), and \textit{malR} (C). Results are representative of three biological replicates. L, 100 bp ladder (at the left, in bp); IC, input control; NC, negative control without antibody; IP, immunoprecipitated with anti-FLAG antibody. (D-F) Relative changes in \textit{mftP}, \textit{malA}, and \textit{malR} transcript levels, respectively, in \textit{ΔmftR} cells grown without or with trimethoprim (Tri) compared to cells grown in 2×YT. The transcript levels were calculated using $2^{-ΔΔC_T}$ relative to the reference gene and reported relative to expression in wild type cells grown in 2×YT. Asterisks indicate statistically significant differences relative to unsupplemented wild-type cultures, except where brackets indicate otherwise. (G) Increase in expression of \textit{malA} and \textit{malR} induced by addition of 25 µM trimethoprim to \textit{ΔmftR} cells in liquid culture. Asterisks indicate statistically significant differences relative to unsupplemented \textit{ΔmftR} cultures (**, p<0.01).
Trimethoprim increases virulence of *B. thailandensis*

It has been previously reported that expression of the mal gene cluster is critical for both *B. pseudomallei* and *B. thailandensis* to infect *C. elegans* [22, 24]. It has also been demonstrated that trimethoprim elicits production of malleilactone [12]. One would therefore expect that trimethoprim-mediated over-production of malleilactone would affect the ability of *B. thailandensis* to infect *C. elegans*. To determine if the upregulation of malA is physiologically relevant, a survival assay was conducted using *C. elegans* as the model organism. It is to be noted that *C. elegans* has been shown to be unaffected by 1 mM trimethoprim [40], which is a much higher concentration compared to what we utilized for our experimental purpose. We observed a difference between *B. thailandensis* grown on plates containing a sub-lethal dosage of trimethoprim (25 μM) and those grown on regular NGM plates; approximately 80% of the *C. elegans* died when co-incubated with *B. thailandensis* on plates containing trimethoprim compared to only 20% death of *C. elegans* when co-incubated with cells on NGM plates containing DMSO after a 24 hour incubation (Fig. 2.8A). While *C. elegans* incubated with *B. thailandensis* looked sicker and exhibited reduced mobility compared to those incubated with *E. coli* OP50, only worms that were completely insensitive to touch and unable to maintain their normal body posture were considered as dead (Fig. 2.8 B-C). After prolonged incubation, all *C. elegans* died on incubation with *B. thailandensis*, regardless of the presence of trimethoprim (data not shown) [41]. By contrast, worms deposited on plates seeded with *E. coli* XL1-Blue transformed with plasmid pSCRhaB2, which confers resistance to trimethoprim, remained healthy whether or not trimethoprim was added to the media.
Figure 2.8. Survival of C. elegans. (A) Survival of C. elegans co-incubated with E. coli OP50 (gray bar), B. thailandensis (blue bar), B. thailandensis grown with 25 μm trimethoprim (Tri; orange bar), E. coli XL1-Blue(pSCrhaB2) (XL-B; gray striped bar), or E. coli XL1-Blue(pSCrhaB2) grown with 25 μm trimethoprim (XL-B/Tri; gray/orange striped bar) on NGM plates at room temperature for 24 hours (mean ± SD). Asterisks indicate statistically significant differences (**, p<0.01) (B) Live C. elegans being able to maintain its body posture on plate containing B. thailandensis with DMSO. (C) Dead C. elegans insensitive to touch and unable to maintain its regular body posture on plate containing B. thailandensis with trimethoprim.

DISCUSSION

MftR represses malR expression

MalR is an essential activator of the mal gene cluster [22]. It also appears evident that the increased malleilactone production characteristic of cells grown with trimethoprim is due to an effect on malR expression, with MalR in turn activating the mal genes [15]. In addition to MalR, the LysR-type transcriptional regulator ScmR also participates in controlling mal genes by repressing gene expression in an acyl-homoserine lactone-dependent fashion [42, 43]. While deletion of the ∆scmR gene leads to increased production of malleilactone, no effect on malR expression was seen in ∆scmR cells [42]. An explanation that would be consistent with these observations is that the activator MalR and the repressor ScmR compete for binding to the mal gene promoter (Fig. 2.9A). In support of these interpretations, the already high levels of
malleilactone produced in ∆scmR cells are even greater after addition of trimethoprim, suggesting that the trimethoprim-mediated induction of malleilactone production is independent of ScmR [15].

Upregulation of numerous biosynthetic gene clusters, including malR and mal, was previously shown by RNA-seq in B. thailandensis deleted for mftR and in wild-type cells grown with the MftR ligand urate [16]. We show here that MftR binds the malR promoter in vivo (Fig. 2.7C), and that malR expression is increased in ∆mftR cells and in cells grown in presence of the MftR ligand xanthine (Figs. 2.3A and 2.7F). Consistent with the increased malR expression, malA is upregulated accordingly. This indicates that MftR contributes to repression of malR expression.

Addition of trimethoprim elicits an increase in malR expression, and this increase is even greater in ∆mftR cells (Figs. 2.1E and 2.7E-F). This observation also points to repression of malR by MftR, and it indicates that the malR gene is repressed by both MftR and by a second repressor (Fig. 2.9B). Trimethoprim does not bind MftR [16], and it has also been demonstrated that accumulation of secondary metabolites requires inhibition of DHFR and not trimethoprim per se [15], indicating that it is metabolites accumulating as a consequence of trimethoprim-mediated inhibition of DHFR that induce malR. Since deletion of scmR has no effect on malR expression, the second repressor is unlikely to be ScmR. The possibility that malR is autoregulated by MalR has not been explicitly reported, however, the identified Lux-box to which MalR binds is far from the malR gene and therefore less likely to be part of the malR regulatory sequence. The identity of the second repressor notwithstanding, both MftR and the second repressor apparently respond to metabolites that accumulate when DHFR is inhibited by trimethoprim. The genomic locus comprising malR and the mal gene cluster as well as mftR are conserved in the pathogenic strains, suggesting a similar mode of MftR-regulated control of malR expression in these strains.
**Fig. 2.9.** Regulation of the *mal* gene cluster. Intergenic region between the divergently encoded *malR* (blue) and *malA* (green) is shown. The Lux-box to which MalR binds is identified as a green line. (A) A competition between the activator MalR (blue) and the repressor ScmR (yellow) for binding to the *malA* promoter is illustrated; deletion of *scmR* results in increased *mal* expression. (B) Repression of *malR* by MftR (grey) and a second repressor (brown); in absence of MalR production, ScmR represses *mal* expression efficiently (top panel). Addition of trimethoprim results in inhibition of DHFR and the attendant accumulation of specific metabolites such as xanthine (red oval). These metabolites act to induce expression of *malR*, presumably by binding directly to both MftR and the second repressor and attenuating their DNA binding. As a consequence, MalR activates *mal* expression and outcompetes binding and repression by ScmR (bottom panel).

**Modulation of purine homeostasis by trimethoprim is linked to changes in gene expression**

Trimethoprim blocks DHFR activity, thereby preventing the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate (THF), which supplies 1C units for various reactions, such as the synthesis of glycine, methionine, thymidine triphosphate (dTTP), and purines [19, 35, 44]. Such inhibition leads to accumulation of pathway intermediates; *de novo* purine biosynthesis generates inosine monophosphate (IMP; Fig. 2.2A), which can then be converted to either ATP or GTP, but inhibition of DHFR leads to accumulation of the intermediate AICAR instead of production of IMP [19]. Under such conditions, purine salvage may be favored, including reducing
the conversion of xanthine to urate, as urate formation siphons purines away from the salvage pathway and precludes recycling. Increased purine salvage has also been reported during the starvation-induced stringent response, another condition that disfavors de novo purine biosynthesis, where upregulation of genes encoding Xdh is linked to production of the signaling molecules guanosine 5′-triphosphate-3′-diphosphate (pppGpp) and guanosine 5′-diphosphate-3′-diphosphate (ppGpp) [14, 30, 31]. Stringent response amplifies the effect of trimethoprim in B. thailandensis, as evidenced by reduced malleilactone accumulation in cells deleted for enzymes responsible for synthesis of (p)ppGpp [15].

Our data are consistent with increased purine salvage in response to administration of trimethoprim, as urate levels decreased significantly whereas xanthine accumulated (Fig. 2.5B-C). The remarkable similarity in terms of both cellular urate and xanthine levels and malR/malA expression in response to either trimethoprim or the Xdh inhibitor allopurinol identifies impaired purine homeostasis as a key factor in the response to trimethoprim. Xanthine can be converted to xanthine monophosphate (XMP) by hypoxanthine guanosine ribosyl transferase (HGPRT; Fig. 2.2A) and XMP can be aminated to generate GMP, which can then be converted to other phosphorylated guanosine derivatives. GMP reductase catalyzes the reductive deamination of GMP to IMP, which allows the synthesis of ATP. Since impaired dNTP homeostasis can affect genome integrity and have genotoxic consequences [45], an imbalance in the dNTP levels could occur if purine salvage were not tightly regulated and excess xanthine were readily converted to XMP and in turn to GTP. Xdh is considered the rate-limiting enzyme in purine salvage where it favors production of guanosine derivatives [46], and upregulation of genes encoding Xdh in response to xanthine accumulation (Fig. 2.6A) would serve to avert such imbalance.
Expression of malR/malA correlates with cellular xanthine levels. Allopurinol inhibits Xdh and it causes both xanthine accumulation and an increased malR/malA expression, which directly implicates impaired purine homeostasis in eliciting changes in gene expression. Since trimethoprim is structurally unrelated to allopurinol, we consider it unlikely that it induces xanthine accumulation by directly inhibiting Xdh activity. However, trimethoprim results in a buildup of the purine precursor AICAR [19], and it is conceivable that this compound might interfere with Xdh activity.

It was reported that growth of B. thailandensis with homoserine mimics the effect of trimethoprim in terms of malleilactone production; using a malA promoter-lacZ reporter, this effect was concentration-dependent, requiring 10 mM homoserine to elicit a similar increase in β-galactosidase activity as 30 µM trimethoprim [15]. Homoserine is a precursor in the biosynthesis of methionine and other amino acids, and it accumulates ~2-fold on addition of trimethoprim to B. thailandensis since the final step in the methionine biosynthetic pathway, the S-methylation of homocysteine, is THF-dependent [15]. In E. coli, homocysteine, which is derived from homoserine, is toxic and it perturbs branched-chain amino acid biosynthesis [47]. We must therefore also consider the possibility that a vast excess of homoserine forces a conversion of homocysteine to methionine to alleviate potential toxicity, and that this reaction reduces the available pool of THF, adversely affecting other THF-dependent enzymes. Regardless of mechanism, the observation that allopurinol elicits a similar increase in malR/malA expression as trimethoprim leads us to favor the interpretation that impaired purine homeostasis plays a primary role in trimethoprim-mediated induction of malR and in turn malA.

Our data show that a sub-lethal dosage of trimethoprim, which is in clinical use for the treatment of Burkholderia infections, causes increased worm death after a 12 h incubation period
(Fig. 2.8). Since trimethoprim causes production of malleilactone, and since malleilactone is critical for virulence, we surmise that the observed increase in worm death is largely contributed by the elevated production of malleilactone; however, we cannot rule out a contribution from other gene products that accumulate due to the presence of trimethoprim. This scenario illustrates the importance of proper administration of trimethoprim and adherence to treatment protocols. Trimethoprim promotes accumulation of xanthine, which is a ligand for MftR. Since MftR participates in expression of numerous genes, including genes involved in virulence, other genes may be differentially expressed on account of trimethoprim-mediated changes in purine homeostasis.
REFERENCES


CHAPTER 3
GLOBAL REGULATOR MFTR-MEDIATED VIRULENCE AND ANTIBIOTIC RESISTANCE IN *BURKHOLDERIA THAILANDENSIS*

INTRODUCTION

*Burkholderia thailandensis*, a relatively non-pathogenic strain, has been used as a model organism for studying virulence mechanisms of the pathogenic strains, *B. pseudomallei* and *B. mallei*, due to its high genetic similarity with these strains. In addition, it has also recently emerged as a source of novel secondary metabolites with antifungal, anti-tumor and antibacterial activities [1-3]. *B. thailandensis* encodes 12 annotated Multiple Antibiotic Resistance Regulator (MarR) homologs, which are conserved in the pathogenic strains as well, out of which MftR is one [4]. In general, MarRs are transcription factors, which bind to the intergenic region between two divergently oriented genes, with their binding sites often overlapping with -10 and-35 promoter elements. Thus, binding of a MarR prevents access of RNA polymerase to the promoter region and prevents transcription [5]. MarRs frequently serve as environmental sensors; upon change in environmental conditions such as antibiotic stress, oxidative stress and host-pathogen interaction, the binding of MarR to the DNA can be altered and derepression occurs [6].

MarR homologs have been characterized and studied in various bacteria such as *Deinococcus radiodurans* (HucR), *Dickeya dadantii* (PecS), *Pseudomonas aeruginosa* (MexR), and *Xanthomonas campestris* (OhrR) [7-11]. Further, some of the MarRs such as PecS (*D. dadantii*), MgrA (*Staphylococcus aureus*), and MvfR (*P. aeruginosa*) have been reported to function as master regulators [7, 12, 13]. MftR (Major facilitator transport regulator) has been reported to serve as a global regulator in *B. thailandensis*. MftR belongs to the UrtR (Urate responsive transcriptional Regulator) sub-family of wHTH (winged helix-turn-helix) family of DNA binding protein. MftR has been reported to generally function as a repressor of various genes,
as 331 genes are upregulated in a ΔmftR strain, including those involved in regulation of genes involved in metabolic pathways, secondary metabolite production, and virulence [14]. Urate and xanthine, products of purine metabolism, have been reported to serve as ligands for MftR [15, 16]. Binding of these ligands to the protein brings a conformational change in the protein and attenuates its DNA binding ability [8, 16]. Urate is a potent anti-oxidant produced by a host’s xanthine oxidase in the process of making ROS (reactive oxygen species) [17]. ROS are generated as a primary line of defense by host cells in response to bacterial infection [18]. Thus, MftR could serve as sensor of host-pathogen interaction using urate as an effector molecule. Upon considering the aforementioned premise it can be presumed that MftR could play a vital role in regulating genes essential for successful host colonization ultimately leading to bacterial infection. Based on the above hypothesis I predict that a ΔmftR strain could be more virulent. Further, increased biofilm formation observed in the ΔmftR strain along with an ability to survive better under anaerobic conditions [14] could result in increased resistance to antibiotics in the ΔmftR strain.

Despite *B. thailandensis* being relatively non-pathogenic it is to be noted that on comparison to pathogenic strains a number of genes encoding virulence factors such as type III secretion system, quorum sensing components, cytotoxic secondary metabolites, and siderophores are conserved [14]. In chapter 2, I have reported the role of MftR in regulation of genes encoding proteins involved in production of the cytotoxic product malleilactone. Here I focus on the role of MftR in siderophore production. Iron (Fe) is essential for various biological processes such as respiration, photosynthesis, enzymatic processes and so on. Although Fe is abundant in nature, its bioavailability is markedly reduced because its most common form Fe$^{3+}$ is insoluble at physiological pH. Moreover, in a host, Fe is bound to other proteins, which makes iron acquisition systems a crucial requirement for bacterial growth [19, 20]. To overcome iron deficiency, most
bacteria utilize the mechanism of producing iron chelating molecules, siderophores. Siderophores are small metal-chelating molecules with high affinity for Fe(III), which are produced by almost all bacteria and generally under iron limiting conditions [21]. The role of siderophores becomes even more critical for pathogens who face a challenging low iron environment inside a host [22, 23]. In general, bacteria have multiple types of siderophore receptors and can produce more than one type of siderophore. A large number of studies in various bacteria have revealed that siderophores are essential for causing virulence [24]. Besides acting as an iron chelating molecule, siderophores can serve an anti-oxidant role during host-pathogen interaction [25]. Siderophores can also bind other essential metals such as Mn, Mo, Co and Ni and deliver it to the microbe [26]. Thus, understanding the mechanism of regulation of siderophore production could help us develop tactics to lower the pathogenicity as well as viability of bacteria.

_Burkholderia_ spp. encode more than one type of siderophore namely: ornibactin, malleobactin, cepaciachelin, cepabactin, pyoverdine, and pyochelin. Ornibactin has been characterized among members of the _Burkholderia cepacia_ complex (Bcc), while the structurally related malleobactin is produced by members of the _Burkholderia pseudomallei_ complex (Bpc) [27]. Production of both of these siderophores has been reported to be under the control of a local regulator belonging to the family of ECF (extra cytoplasmic sigma factor) [28, 29]. Malleobactin has been reported to be the major siderophore in _B. pseudomallei_ whose production is under the control of an activator named Mbas, an ECF [29]. Malleobactin is the only conserved siderophore in _B. mallei_ indicating its importance in a virulent strain. Further, malleobactin has been shown to be able to remove iron from both transferrin and lactoferrin [30]. A genetic locus similar to the one found in _B. pseudomallei_, comprising the genes encoding enzymes essential for production and uptake of siderophore along with _ecf_, is conserved in _B. thailandensis_. Interestingly,
upregulation of the genes encoding enzymes essential for production of malleobactin as well as increased siderophore production has been reported in ΔmftR. Here I report that ECF, encoded by ecf (BTH_I2427), is an essential activator for regulating the genes involved in encoding enzymes essential for production of major siderophore, malleobactin, in *B. thailandensis*. My results show that ecf is directly repressed by MftR and that ΔmftR is more virulent as indicated by decreased survival of *Caenorhabditis elegans* and increased maceration of onion tissues.

**EXPERIMENTAL PROCEDURES**

**Growth Media and Stock Solutions**

Bacterial cells were grown in LB (Luria Broth) media. *C. elegans* assay was performed on 60 mm NGM (Nematode Growth Media) plates. *C. elegans* (Bristol strain N2) were maintained at room temperature on NGM media with *E. coli* OP50 as the food source. For qRT-PCR (quantitative Real Time PCR) cells were grown in LB media. An overnight culture was diluted 1:100 for subculture unless indicated otherwise. *B. thailandensis* E264 strain was obtained from American Type Culture Collection (ATCC). The ΔmftR strain in which the coding region is disrupted by a transposon, was obtained from the Manoil lab [31].

**Construction of Δecf**

Δecf was created as described previously [14]. Briefly, a 346 bp fragment of the ecf open reading frame was amplified using the primers Ecf_ko_Fwd and Ecf_ko_Rev. The PCR product was cloned into the suicide vector pKnockTc. The construct was transformed into *E. coli* sm10 λpir cells. For creating the Δecf strain, mating was performed by mixing the overnight culture of sm10λpir with *B. thailandensis* at a 2:1 ratio (donor:recipient). The mixture was washed twice with 1 ml of LB and resuspended in 60 μl of LB. The mixture was then spotted on an LB plate and incubated at 37°C for 12 hours. The mating mixture was scraped off and resuspended in 1 ml of
LB. Serial dilutions (up to $10^{-5}$) of the culture were made and transformants were selected on LB agar plates containing 80 µg/ml of tetracycline and 15 µg/ml of gentamicin. To verify the $\Delta ecf$, PCR was conducted using ECF_veri_Fwd and Pknoc_tc_rev primers.

**Pellicle and Biofilm Formation Assay**

Pellicle and biofilm formation assays were conducted as previously described [32]. Briefly, an overnight culture of wild type and $\Delta mftR$ strains were diluted to 1 ODU. 30 µl of the diluted culture was added to 3 ml of the LB media in a plastic tube and kept stationary at 37 degrees for 72 hrs. At the end of the incubation period, pellicle formation was visually observed. For measuring the biofilm formation, the media was gently removed, and the tubes were washed with water to remove planktonic cells. One ml of 0.1% crystal violet was added to the tube and gently rolled around to ensure that the crystal violet spread evenly around the tube. After 15 minutes of incubation, excess crystal violet was dumped off and the tubes were washed two times with water to remove excess crystal violet. Tubes were then allowed to air dry and 1 ml of DMSO was added to dissolve the visible crystal violet stain. The solution was then allowed to incubate at room temperature for 15 minutes and OD was measured at 560 nm using a spectrophotometer. For biofilm assays, the graph represents the mean value of two technical replicates of three independent biological samples.

**Swarming and Swimming Assay**

Swarming assay was conducted as previously described [33]. For swarming assay, agar plates (0.5% (wt/vol) of Difco Bacto-agar, 8 g/liter of Difco nutrient broth, 5 g/liter of glucose) were prepared and allowed to dry at room temperature for 8-10 hours. An overnight culture was diluted to OD$_{600}$~1 and 1 µl of the culture was spotted at the center of the plate. Plates were incubated at 37 degrees for 24 hours before taking the final image. Swimming assay was conducted
in similar way as swarming assay except the agar plates were composed of 0.3% (wt/volume) of Difco Bacto-agar, 10 g/liter of tryptone, and 5 g/liter of NaCl. For swimming assay, plates were incubated for 48 hours before taking the final image. Results are representative of three biological replicates.

**CAS (Chrome Azurol S) Assay**

Variation in amount of siderophores was detected by CAS assay, performed as previously described [34]. Briefly, cells grown over night were diluted to OD$_{600}^{-0.6}$ and pellets were collected by centrifuging at 13,000 RPM for 1.5 minutes. The supernatant was discarded, and cells were resuspended in 20 µl of LB and 10 µl of the cells were spotted at the center of the CAS plate. Plates were incubated at 37 degrees for 48 hours before taking the final image. Results are representative of three biological replicates.

**qRT-PCR**

An overnight culture of *B. thailandensis* was sub-cultured in 50 ml LB media and grown until OD$_{600}^{-0.6}$. Once cells reached the desired density, 1 ml of the cell culture was pelleted and washed twice with autoclaved diethyl pyrocarbonate (DEPC)-treated water and stored at -80 °C. RNA was extracted utilizing Monarch Total RNA Miniprep Kit (New England Biolabs, Ipswich, MA) according to the manufacturer’s protocol. RNA was electrophoresed on agarose gels to ascertain integrity and PCR was performed to verify absence of genomic DNA contamination. For measuring gene expression, One-Step qPCR was performed using Luna One step universal master mix (New England Biolabs). Data represent means (± SD) from biological duplicates (each determined from technical triplicates) using the comparative C_T method ($2^{-\Delta\Delta C_T}$).
Table 3.1. Primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ecf forward</td>
<td>TCGAGAACACCTACCACGC</td>
</tr>
<tr>
<td>ecf reverse</td>
<td>TCGATCAGCCAGTCGATGAG</td>
</tr>
<tr>
<td>arcA forward</td>
<td>TCCCAAGCCATCCCTGAAG</td>
</tr>
<tr>
<td>arcA reverse</td>
<td>CAGGTTGTGCATCTCGAGC</td>
</tr>
<tr>
<td>hgp rt forward</td>
<td>CGAGAAGAAGCCCTCCACAT</td>
</tr>
<tr>
<td>hgp rt reverse</td>
<td>TCGAACTCGAGC GGAAATC</td>
</tr>
</tbody>
</table>

Chromatin Immunoprecipitation (ChIP) Assay

For ChiP, an overnight culture of *B. thailandensis* MftR-FLAG was diluted 1:100 in 50 ml LB. Once the cells reached OD$_{600}$~0.6, 1% formaldehyde was added to the flask, which was placed on a shaker for 16 minutes to allow crosslinking to occur. The cell culture was then centrifuged at 3,500 RPM at 4 °C for 8 minutes and the supernatant was discarded. Cell pellets were washed twice by adding 20 ml phosphate buffered saline (PBS) followed by another wash with 5 ml of PBS and centrifuging at 3,500 RPM for 5 minutes. Finally, the pellets were resuspended in 1 ml of PBS and the supernatant was removed after centrifuging at 13,000 RPM for one minute. Cell pellets were stored at -80 °C. ChIP was performed as previously described with some modifications [35]. Briefly, cells were suspended in 1 ml of lysis buffer with protease inhibitor cocktail and placed on ice for 1 hour. Genomic DNA was sheared by sonicating 5 times at 35%
output at 10 secs pulse with 1-minute gap in between. Cell/cell debris-free lysate was collected by centrifugation at 13,000 RPM at 4 °C for 12 min and transferred to a clean tube. The lysate was centrifuged for another 3 min and the final lysate was collected. The lysate was precooled using protein G-Sepharose beads (GE Healthcare) to reduce non-specific binding to the beads. For immunoprecipitation, 5 µl of anti-FLAG (M2; MilliporeSigma, Burlington, MA) antibodies was used. Eluted DNA from ChIP samples or input DNA was analyzed by PCR (for primer sequences, see Table 3.2). PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide. Signal intensities from PCR data was quantified from the TIFF images by using ImageJ software. Experiments were performed in triplicate.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mftP-mftR intergenic region chip forward</td>
<td>GAGCTGCAGCGATCCATTAAC</td>
</tr>
<tr>
<td>mftP-mftR intergenic region chip reverse</td>
<td>TGCAGGAGAATTAAGCATGGG</td>
</tr>
<tr>
<td>ecf upstream forward</td>
<td>ATGATCGACGAGCGAAGTG</td>
</tr>
<tr>
<td>ecf upstream reverse</td>
<td>GAGGATCGGTAACGGTTTTC</td>
</tr>
<tr>
<td>ohrR upstream forward</td>
<td>GGTCGTCTCCCATCGTTTTC</td>
</tr>
<tr>
<td>ohrR upstream reverse</td>
<td>GTCGTTCATGGCGAGATTTTC</td>
</tr>
</tbody>
</table>
Removal of Antibiotic Resistance Cassette from ΔmftR

The ΔmftR strain in which transposon T23 (ISlacZ-PrhaBo-Tp/FRT) was inserted in *B. thailandensis mftR* (at genomic position 2719492, open reading frame location 4) was obtained from the Manoil laboratory and grown on LB-agar plates with 50 µg/ml trimethoprim. Single colonies were selected to verify correct insertion of the transposon. PCR was performed using primers Mftr_Veri_Fwd and LacZ. Removal of the antibiotic cassette was performed as previously described [36]. Briefly, Flp recombinase-encoding pFLPe4 plasmid was transformed into *E. coli* RHO3 cells. For mating, 6-8 single colonies of *E. coli* RHO3 and *B. thailandensis* were overlayed on LSLB (Low salt LB) plates containing 200 µg/ml DAP (2,6-diaminopimelic acid) and incubated at 30 °C for 6 hours. Selection was done on LSLB/kanamycin (250 µg/ml) plates at 30 °C. To induce *flp* expression, selected colonies were streaked on LSLB/kanamycin/rhamnose plates and incubated at 30 °C. Colonies sensitive to trimethoprim but resistant to kanamycin were streaked on LSLB plates and grown at 37 °C to induce loss of the temperature-sensitive pFlpe4. Removal of antibiotic cassettes was confirmed by PCR using primers Mftr_no cassette_Fwd and MftR_no cassette_Rev.

**Antibiotic Sensitivity Test**

To determine the variation of resistance between the ΔmftRX strain (ΔmftR with no antibiotic cassette) and wild type, cells grown overnight were diluted to 1:100 in 50 ml LB media and placed in a shaker at 37 °C till OD₆₀₀ reached 0.6. Once cells reached the desired OD₆₀₀ 1ml of the culture was collected in an Eppendorf tube. The cells were then serially diluted 10-fold till final dilution of 1000-fold and were spotted on LB plates containing ampicillin, trimethoprim, tetracycline, kanamycin. The plates were incubated at 37 °C for 24 hours before taking the final image.
Table 3.3. Primers used for verification of $\Delta$s

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ecf_ko_Fwd</td>
<td>GCTCTAGAGTAAGCTCGTCGATTTCCC</td>
</tr>
<tr>
<td>Ecf_ko_Rev</td>
<td>GCGGTACCCGTGCGGACCACGTAAT</td>
</tr>
<tr>
<td>ECF_veri_Fwd</td>
<td>AGACGCTCGTGCATTTCAT</td>
</tr>
<tr>
<td>ECF_veri_Rev</td>
<td>TTGCGGAGAACTGTGAATGC</td>
</tr>
<tr>
<td>Mftr_Veri_Fwd</td>
<td>GAGCACAGGACGCAGATTGA</td>
</tr>
<tr>
<td>LacZ</td>
<td>GGGTAACGCCAGGGTTTCT</td>
</tr>
<tr>
<td>MftR_no cassette_Fwd</td>
<td>GAGCACAGGACGCAGATTGA</td>
</tr>
<tr>
<td>Mftr_no cassette_Rev</td>
<td>ATGAGCTGCGGATCCATTA</td>
</tr>
</tbody>
</table>

**C. elegans Survival Assay**

For assessment of $\Delta$mftR strain virulence, 10 $\mu$l of *E. coli* OP50 (control), wild type *B. thailandensis*, and $\Delta$mftR strains grown over night were spotted on NGM plates. Plates were incubated at 37 °C for 24 hours and then allowed to equilibrate at room temperature for 12 hours. At the end of the 12 hours period about 10-12 *C. elegans* were added to the plates and left for 12 hours at room temperature before checking for survival. Worms were observed under light microscope (Olympus SZ-ST) for their ability to maintain body posture and sensitivity to touch. Worms that were completely insensitive to touch and had lost their ability to maintain normal body posture were declared as dead. The percentage of surviving worms was calculated as: Survival (%) = (live worms/total worms used) $\times$ 100. The experiment was performed with three separate biological samples, each in triplicate.
Onion tissue maceration

Onion tissue maceration was performed as previously described [37]. Yellow onions (Allium cepa) commercially available in grocery stores were utilized for the pathogenicity experiment. Scale from the outer bulb was removed completely before cutting the bulb vertically, to get long cylindrical scales, with a sterile razor. These scales were further cut to get uniform size. About 10-12 autoclaved napkins were placed in a plastic container and sprayed with autoclaved water to create a moist environment before introducing the onion slices. For this assay, wild type or ΔmftR was grown overnight (18-20 hrs) on LB plates. Cells were then resuspended in LB to get 10^10 CFU/ml of culture. The inner surface of individual scales was scraped gently with a sterile pipette tip (1- to 10-μl volume) to remove the outer epidermal layer and create a wound. Ten μl of bacterial culture (10^7 CFU/ml) was inoculated into the wound. The onion scales were incubated at 30°C for 72 hours. The degree of maceration was visually inspected, and images of individual slices were taken. Cell free media (LB only) was used as control. The experiment was performed with three separate biological samples of each strain.

RESULTS

Increased biofilm formation in ΔmftR

Biofilm has been reported as hallmark for chronic infection as bacteria growing in biofilm not only evade host immune responses but can be resistant to a large number of drugs, creating an obstacle in treatment of infection. Biofilm has been reported to be a major player in persistence of P. aeruginosa and members of Bcc, which infect lungs of people with cystic fibrosis, in the airway of CF patients [38, 39]. A common phenomenon associated to most biofilm is creation of a hypoxic environment. Thus, successful establishment of persistent biofilm requires not only surface attachment genes but also genes involved in anaerobiosis. Requirement of anaerobic fitness of
pathogens for virulence and pathogenesis has been reported in various bacteria [40]. The $\Delta mftR$ strain has been previously reported to grow better in anaerobic conditions compared to the wild type. One of the key pathways involved for improved survival under anaerobic condition is the arginine deiminase (ADI) pathway [41]. Genes of the ADI pathway, $arcDABC$, are found across a large number of bacteria. $arcD$, the first gene of the $arcDABC$ operon, encodes an arginine/ornithine antiporter (ArcD), and $arcABC$ encodes the enzymes involved in anaerobic degradation of arginine with concomitant production of ATP. Under anaerobic conditions, ArcD helps for continuous operation of the ADI pathway via energy-efficient exchange of exogenous arginine with endogenous ornithine [42]. To evaluate if the ADI pathway is upregulated in $B. thailandensis$, relative expression of $arcD$ was compared between wild type and $\Delta mftR$. $arcD$ expression was increased by ~3.5-fold in the $\Delta mftR$ strain compared to wild type (Fig 3.1A). Further, increased expression of arginine deiminase pathway has been reported in various bacteria under biofilm formation [43]. To determine if biofilm formation ability is enhanced in $\Delta mftR$, a crystal violet staining assay was performed. After incubation for 72 hours a higher level of pellicle biofilm (formed at air-liquid interference) was observed in the $\Delta mftR$ strain compared to the wild type (Fig. 3.1B). Upon quantification, nearly 2-fold higher production of biofilm was observed in the $\Delta mftR$ strain compared to wild type (Fig. 3.1C). Increased expression of $arcD$ along with the crystal violet staining assay suggests that $\Delta mftR$ strain can readily form more persistent biofilm compared to wild-type cells.

**Increased swimming and swarming motility in $\Delta mftR$**

Bacterial flagellar motility plays a key role in adhesion, biofilm formation, and translocating virulent proteins. Swarming motility is considered as a precursor for biofilm formation [44]. Since an increased biofilm formation was observed in the $\Delta mftR$ strain (Fig.3.1B)
I assumed a variation could be seen in terms of motility between the wild type and the \( \Delta mftR \) strain. In order to determine if \( \Delta mftR \) cells show variation in flagellar activity compared to wild type cells, swimming and swarming motility assays were performed. Increased swimming and swarming motility were observed in \( \Delta mftR \) compared to wild type as indicated by increased swarm and swimming diameter (Fig. 3.1 D and E). In case of \( B. \) pseudomallei it has been suggested that flagella are important for invasion of macrophage as well as non-phagocytic cell lines. Moreover, flagellum has been determined to be essential in \( B. \) pseudomallei during intranasal and intraperitoneal infection of mice [45]. Thus, increased flagellar activity seen in \( \Delta mftR \) along with ability to survive better in anaerobic conditions suggests \( \Delta mftR \) possesses increased virulence characteristics.

**Figure 3.1.** Deletion of \( mftR \) leads to increased expression of ADI pathway genes, biofilm formation, and motility. (A) Increased \( arcD \) expression in the \( \Delta mftR \) strain compared to wild type. (B) Pellicle formation in static culture. (C) Quantification of biofilm formation using crystal violet staining. (D) Swimming (top) and (E) Swarming (bottom) motility.
**ECF is an essential activator for malleobactin production**

Mbas, an ECF, has been previously reported to be an essential activator of malleobactin production in *B. pseudomallei* [29]. In *B. thailandensis* a locus similar to that of *B. pseudomallei*, encoding malleobactin along with an *ecf* is present. As stated earlier, the role of siderophore is critical specially in pathogenic bacteria that encounter an iron limitation condition in the host environment. To verify if *ecf* of *B. thailandensis* plays a similar role in malleobactin synthesis, a Δ*ecf* strain was created. To determine if ECF functions similar to MbaS or OrbS in regulating the production of siderophore, a CAS assay was performed. As seen (Fig 3.2A), the amount of siderophore produced in Δ*ecf* strain is reduced compared to wild type suggesting that ECF is an essential activator for production of the siderophore malleobactin in *B. thailandensis*.

**Figure 3.2.** Variable malleobactin production in *B. thailandensis* Δ strains. (A) Disruption of *ecf* leads to decreased production of siderophore while in Δ*mftR* an increased production of malleobactin is observed. The lower panel is the zoomed version of the plate to give a clear view of the halo ring formed. (B) *mftR* mutation leads to increased expression of *ecf*. 
Previously, an upregulation of ecf was reported in the ΔmftR strain based on genome-wide RNA-seq data [14]. Next, I compared the variation in siderophore production between the wild type and the ΔmftR strain. The ΔmftR strain produces a larger orange halo ring indicating production of more siderophore compared to wild type (Fig. 3.2A). Further, ecf is upregulated by ~ 4.5-fold in ΔmftR strain (Fig. 3.2B) The gene expression analysis along with CAS assay data not only shows increased production of siderophore in ΔmftR, but it also indicates that MftR regulates ecf expression.

**MftR binds ecf regulating siderophore production**

To determine if ecf is directly regulated by MftR, a ChIP-assay was performed. For ChIP, the intergenic region between mftR and the divergently oriented gene mftP to which MftR binds in vitro [15, 16] was used as the positive control, and I verified MftR binding to this region in vivo (Fig. 3.3A). To ensure the obtained immunoprecipitation was specific, a negative control using the ohrR upstream region to which the unrelated transcription factor OhrR binds [46] was used, and no indication of MftR binding was detected (Fig. 3.3A). The ability of MftR to bind upstream of ecf was tested by amplifying a fragment comprising 128 bp upstream of ecf and part of the coding region, and MftR was found to bind this region (Fig 3.3B). Our ChIP data is consistent with the gene expression analysis and siderophore production assay supporting the notion that MftR binds to the upstream region of ecf directly regulating its expression. Thus, our data indicate that ECF is an activator of the genes involved in encoding enzymes essential for malleobactin production and transport and its expression is directly regulated by MftR.
Figure 3.3. MftR binds ecf directly. (A and B) In vivo binding of MftR determined by chromatin immunoprecipitation. PCR products electrophoresed on 1.5% agarose gels and visualized with ethidium bromide for mftR-mftp intergenic region to which MftR binds in vitro (A), ohrR promoter, which is not regulated by MftR (A), and ecf (B). Results are representative of three biological replicates. L, 100 bp ladder (at the left, in bp); IC, input control; NC, negative control without antibody; IP, immunoprecipitated with anti-FLAG antibody.

**ΔmftR is more virulent**

My data so far clearly indicates that the ΔmftR strain demonstrates various physiological characteristics generally associated with virulence. Moreover, in chapter 2 I have reported that MftR along with an unknown transcription factor directly regulates malR, encoding MalR. MalR is an essential activator of the mal gene cluster which encodes enzymes essential for production of the cytotoxic compound malleilactone. Production of malleilactone is essential for killing of *C. elegans* by *B. thailandensis*. Combining the characteristics described above with the production of malleilactone led us to hypothesize that disruption of mftR could make cells more virulent towards *C. elegans*. In order to test this hypothesis, a *C. elegans* survival assay was conducted. Approximately 60% of the *C. elegans* died when co-incubated with ΔmftR cells compared to only 20% death of *C. elegans* when co-incubated with wild type cells on NGM plate (Fig 3.4).

Several species in the genus *Burkholderia* have been known to cause rotting in onions but no such effect has been reported for *B. thailandensis* [37, 47]. Since genes involved in production
of various secondary metabolites are upregulated in ΔmftR cells and since pathogenic characteristics (increased biofilm formation, motility and siderophore production) is a hallmark of the ΔmftR strain I presumed a possibility that it could be successful in causing rot in onion. To test this hypothesis, onion scales were wounded and incubated with wildtype or ΔmftR cells. Upon infecting onion wounds with wild type cells and ΔmftR cells a significantly higher area of maceration for the ΔmftR strain compared to wild type was observed (Fig. 3.4 B and C). Thus, it can be concluded that the ΔmftR strain is virulent in both animal and plant models utilized.

**Figure 3.4.** ΔmftR is more virulent than wild-type cells. (A) Survival of *C. elegans* co-incubated with *E. coli* OP50 (gray bar), *B. thailandensis* (blue bar), or ΔmftR (red bar) on NGM plates at room temperature for 24 hours (mean ± SD from three independent experiments). Asterisks indicate statistically significant differences between wild-type and ΔmftR cells ((**, p<0.01). Increased maceration observed in onion scales when incubated with the ΔmftR strain (B and C). Maceration observed in the outermost scale underneath the tunic (B) and the second layer of scales (C) after 72 hours of coinoculation with LB (control), wildtype and ΔmftR.
**ΔmftR is more resistant to ampicillin and kanamycin**

The ability of ΔmftR to readily form biofilm and to survive better under anaerobic conditions, along with upregulation of various efflux pumps, could potentially make it more resistant to antibiotics compared to wild type. To test if MftR could play a direct or indirect role in antibiotic resistance, an antibiotic sensitivity test was conducted with different classes of antibiotics (ampicillin, kanamycin, trimethoprim and tetracycline). ΔmftRX (ΔmftR with no antibiotic cassette) showed increased resistance against kanamycin and ampicillin. MftR directly regulates mftP, encoding MftP, whose substrate is still unknown. Upregulation of mftP in ΔmftR has been previously reported. The increased resistance of ΔmftR to ampicillin or kanamycin raises the possibility of these antibiotics being substrates for MftP.

![Figure 3.5. mftR deletion alters resistance to antibiotics (A) LB, (B) Kanamycin (75 µg/ml), and (C) Ampicillin (20 µg/ml)]

**DISCUSSION**

For successful survival and multiplication in a hostile host environment, bacteria require mechanisms to sense the change in the environmental conditions and to counteract the host defense. The role of MarR family proteins in regulation of genes involved in cellular response, oxidative stress, antibiotic resistance, and virulence in response to change in environmental conditions has been characterized in various bacteria [4, 48]. One of the preliminary steps required for host-pathogen interaction is successful attachment of the bacterial cell to the host. Flagella not only help chemotaxis movement to avoid harsh conditions but also help in successful adhesion to
the host surface. Studies in various bacteria have revealed that defects in flagellar protein lead to bacteria becoming less virulent or unable to infect a host [45, 49]. In *B. cenocepacia*, flagella have been reported to play an important role in virulence in a murine agar bead model of infection [50]. The experimental data reported here shows that ∆*mftR* has increased flagellar motility (Fig 3.1 D and E).

Secondly, successful colonization of the host would require a mechanism to avoid host defense, oxidative stress, and antibiotic stress. One of the well-studied mechanisms that a large number of bacteria employ for successful colonization and survival in host is biofilm formation. Biofilm not only provides resistance against changes in pH, osmolarity, and nutrients scarcity but also against antibiotics and it helps the bacteria evade the host immune system [51]. Due to the anoxic environment created in a biofilm, genes involved in anaerobiosis play a critical role in establishment of persistent biofilm. ∆*mftR* survives better in an anaerobic environment compared to wild type [14] and *arcD* upregulation (Fig. 3.1 A), involved in anaerobiosis, would be expected to promote the ability of ∆*mftR* to form a persistent biofilm. Moreover, it has been reported that *Burkholderia* spp. grown under anaerobic conditions have increased resistance against antibiotics. The study also reported that drug-tolerant cells were characterized by an anaerobic metabolic signature [40, 52]. I observed that ∆*mftR* shows not only increased biofilm formation (Fig 3.1 B and C) but also increased antibiotic resistance against kanamycin and ampicillin (Fig 3.5 B and C). The antibiotic resistance observed in ∆*mftR* needs to be further analyzed. MftR directly regulates *mftP*, encoding MFTP, whose substrate has not been determined yet. Gene expression analysis of *mftP* in the presence and absence of these antibiotics along with susceptibility test of cells deleted for *mftP* would help to determine if MftR plays a direct role in resistance against these antibiotics via regulating *mftP* or is indirectly involved through upregulating other genes.
For bacteria, especially pathogenic ones, to survive in a host environment, an efficient iron acquisition mechanism is critical [53]. *Burkholderia* spp. encode more than one type of siderophore but in case of the pathogenic *B. mallei* the only known siderophore is malleobactin. Here, I have reported that an ECF (encoded by Bth_I2427), like Mbas, is an essential activator of the genes involved in malleobactin production and uptake. Ornibactin, structurally related to malleobactin, has been shown to be essential for *B. cenocepacia* to cause infection in *C. elegans* and *Galleria mellonella* [54]. Further, *ecf* is directly under the control of MftR as shown by ChiP (Fig 3.3 C) and further supported by the increased expression of *ecf* (Fig. 3.2 B) and the increased siderophore production (Fig. 3.2A) observed in ΔmftR. A *C. elegans* or *G. mellonella* survival study in Δecf could help shed light on the role of malleobactin in survival and pathogenicity in members of Bpc.

*B. thailandensis* possesses the ability to kill *C. elegans*. Although *B. thailandensis* encodes a large number of secondary metabolites, malleilactone has been reported to be an essential compound required to cause infection and death in *C. elegans* [55]. In chapter 2, MftR was shown to directly regulate *malR*, encoding MalR. Further, upregulation of *malA* and *malR* in ΔmftR has also been reported. Bactobolin, a cytotoxic polyketide-peptide, has also been reported as an important pathogenic toxin utilized by *B. pseudomallei* to cause infection in *C. elegans* [56]. Upregulation of genes involved in production of bactobolin (>20-fold) as well as increased antibacterial ability has been reported in ΔmftR [14]. Upregulation of cytotoxic compounds along with increased pathogenic traits is indicative of MftR-mediated regulation of virulence genes in *B. thailandensis*. The *C. elegans* survival assay showed increased killing of worms when co-incubated with ΔmftR indicating increased production of cytotoxic compounds. Further, the ΔmftR caused increased maceration of onion tissue, which could also be a result of accumulation or over
production of cytotoxic compounds in this strain. Thus, based on results of both the invertebrate and plant models utilized (Fig. 3.4 A, B, and C) it can be established that MftR plays a significant role in regulation of virulence in B. thailandensis.

**CONCLUSION**

MftR, a MarR homolog, has been reported to be a global regulator in *B. thailandensis*. Differential regulation of genes involved in metabolic pathways, secondary metabolite production and pathogenicity in Δ*mftR* was reported in a genome wide RNA-seq study. Further, MftR is conserved in the related pathogenic strain implicating a similar mode of regulation in the virulent strains. The data so far shows that MftR is involved in regulation of biofilm formation, motility, siderophore production as well as antibiotic resistance. Further, the role of MftR in production of bactobolin and the cytotoxic compound, malleilactone, has been discussed earlier in chapter 1 and 2 respectively. The data obtained so far indicate that Δ*mftR* not only displays virulent phenotypic characteristics but plays a critical role in pathogenesis of *B. thailandensis.*
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38. Murphy, M.P. and E. Caraher, Residence in biofilms allows Burkholderia cepacia complex (Bcc) bacteria to evade the antimicrobial activities of neutrophil-like dHL60 cells. Pathog Dis, 2015. 73(8): p. ftv069.


CHAPTER 4
CONCLUSION/FUTURE OUTLOOK

CONCLUSION

*Burkholderia thailandensis*, a relatively non-pathogenic strain, has served as a model organism for the study of pathogenic strains, namely *B. pseudomallei* and *B. mallei*, due to its high genetic similarity with these strains [1]. The recent discovery that the *B. thailandensis* genome features a large number of biosynthetic gene clusters, encoding enzymes essential for production of secondary metabolites, has added another dynamic of focus on *B. thailandensis* as a source for production of novel compounds. These biosynthetic gene clusters that encode enzymes responsible for production of secondary metabolites, whether virulence factors or novel compound with potential clinical activity (antifungal, anti-cancer, antibiotics), generally remain silent under normal growth conditions [2]. Bacterial secondary metabolites confer a competitive advantage to bacteria in a hostile environment, yet the mechanisms by which their production is regulated has not be been deciphered completely. Understanding how expression of these gene clusters could be elicited would help shed light on mechanisms of pathogenicity as well as aid in the discovery of novel beneficial compounds. Various strategies such as variation in growth media, alternation of pH, and antibiotic stress have been utilized to elicit the expression of these secondary metabolites. Unfortunately, local regulation of the biosynthetic gene clusters differs from one another creating a hurdle in discovery of overall secondary metabolites synthesized by the bacteria [3]. The recent discovery that global regulators, MftR and ScmR, play a critical role in regulation of a large number of biosynthetic gene clusters in *Burkholderia* has opened a new path for eliciting production of secondary metabolites through modulation of global regulators [4, 5]. Concurrently, in *B. thailandensis*, a sub-lethal dosage of trimethoprim has been identified as a potent elicitor of
these biosynthetic gene clusters, however, the molecular mechanism by which trimethoprim causes change in gene expression was not delineated [4].

The first chapter in this dissertation describes the importance of secondary metabolites and describes some of the well-known secondary metabolites produced by *B. thailandensis*. The *B. thailandensis* genome includes ~22 natural product biosynthetic gene clusters, potentially involved in production of novel compounds [6]. The chapter not only describes the function of the secondary metabolites and various local regulators involved in production of these secondary metabolites, but it also describes the role of global transcriptional regulators in regulating production of these secondary metabolites. Further, the observation that a sub-lethal dosage of trimethoprim is an elicitor of biosynthetic gene clusters has been briefly described. Compounds accumulated under the effect of trimethoprim and genes upregulated in ΔmftR strain has staggering similarity. The mechanism by which trimethoprim causes upregulation of various secondary metabolites similar to global regulator MftR is considered in the second chapter. Thus, the first chapter in general describes some of the important secondary metabolites present in *B. thailandensis* with emphasis on alternative paths for unlocking the production of secondary metabolites through targeting global regulators.

The second chapter provides a mechanism of trimethoprim-based upregulation of secondary metabolites in *B. thailandensis*. Although a striking similarity between the compounds accumulated under sub-lethal dosage of trimethoprim and genes upregulated in ΔmftR has been reported, MftR is not a direct target of trimethoprim [5]. In order to disentangle this convoluted situation, my work focused on regulation of the genes involved in regulation of genes comprising the *mal* gene cluster, encoding enzymes essential for production of malleilactone. Malleilactone, a cytotoxic product known to be an important virulence factor, is accumulated under sub-lethal
dosage of trimethoprim. My work shows that *malR*, encoding an essential activator (MalR) of the *mal* gene cluster is under direct control of MftR. Further, a previously unreported consequence of sub-lethal dosage of trimethoprim causing disturbance in the purine metabolism pathway has been demonstrated. The result of this phenomenon is accumulation of xanthine, a ligand for the global regulator MftR, causing increased expression of *malR* and ultimately the *mal* gene cluster. Allopurinol, a Xdh inhibitor, led to similar upregulation of *malR* and the *mal* gene cluster, which indicates that disturbance in the purine pathway indeed leads to increased expression of virulence factors. Lastly, an increased killing of *C. elegans* upon coinubcation with bacterial cells grown with trimethoprim puts forth a grave situation of increased virulence under sub-lethal dosage of antibiotics.

The third chapter describes the virulent phenotype observed upon disruption of the *mftR* gene. In Δ*mftR*, increased biofilm formation, motility, and siderophore production, which aid in increased virulence, was observed. Moreover, my work shows that *ecf*, encoding ECF – an essential activator for malleobactin synthesis – is directly under the control of MftR. Taken together all these features along with the increased production of malleilactone (described in Chapter 2) indicate Δ*mftR* cells to be more virulent. An increased killing of *C. elegans* and increased maceration observed in onion scales when co-incubated with the Δ*mftR* strain helps to confirm it to be more virulent than wildtype. Moreover, an increased resistance against ampicillin and kanamycin was observed in the Δ*mftR* strain. Thus, in overall this chapter emphasizes the role of global regulator, MftR, in not just pathogenicity but also in antibiotic resistance.

**FUTURE OUTLOOK**

The need for novel effective antibiotics is greater today than ever and bacterial secondary metabolites have for a long time been a source for their discovery. My dissertation has emphasized
that identifying global regulators in different bacterial species through a combination of genome-wide transcriptomics, metabolomics, and ChIP-Seq could increase our chances of discovering novel compounds with not just antibacterial, but various other beneficial effects. Moreover, these global regulators could be a novel drug target against pathogenic strains.

Co-trimoxazole, (a cocktail of trimethoprim and sulfamethoxazole) is often prescribed to treat glanders and melioidosis [7]. My work shows that a sub-lethal dosage of trimethoprim leads to increase in virulence via modulating purine metabolism pathway. That a sub-lethal dosage of antibiotic leading to increased virulence is a grave scenario as an incomplete regimen of prescribed medicine could lead to a harmful effect. This unintended consequence of increased virulence in bacteria upon treatment with sub-lethal dosage of an antibiotic needs to be further explored. Such studies will be helpful in determining an effective cocktail and dosage of drugs to be prescribed. Secondly, accumulation of xanthine seen under effect of trimethoprim can have a clinical significance as usage of trimethoprim could lead to xanthinuria. This could lead to a complicated situation in people suffering from misregulated purine metabolism [8]. Inhibition of Xdh by allopurinol led to accumulation of xanthine and similar changes in gene expression of malR and malA as trimethoprim. This scenario raises a possibility that metabolites accumulated under the effect of trimethoprim could causes inhibition of Xdh. Identification of this compound could help in discovery of novel metabolites that could be utilized in patient suffering from gout diseases [9]. Purification of Xdh and conducting enzymatic activity assay with compounds accumulated under the effect of trimethoprim could help achieve this goal. Further, computational modeling and docking could help to shortlist possible compounds that could be tested against Xdh activity. Lastly, a cocktail of trimethoprim and allopurinol was shown to further enhance the expression of virulence genes. This outcome highlights that using trimethoprim to treat *Burkholderia* infections
in persons prescribed allopurinol as gout medication, which inhibits Xdh, could have deleterious outcome.

MftR is conserved in pathogenic strains, and it could be a drug target for treatment of glanders and melioidosis. Based on the outcome of my third chapter, the increased virulence observed in a \( \Delta mftR \) strain helps to establish its roles in virulence. Ligands that target MftR, outcompeting xanthine and urate, without altering its DNA binding ability can be an effective drug target. Study focused on crystallization of MftR would be a leading step towards this approach. A crystal structure of the protein bound with DNA and ligands could help us get a better understanding of the important residues as well as the conformational changes the protein undergoes. Such study could be followed up with computational modeling to help shortlist possible compounds that can interact with the protein. Protein-DNA binding assay such as EMSA could help determine if the compound selected could actually bind the protein without interfering with its DNA binding ability.

The gene locus comprising \( mftR \) along with the \( mftp-fenI \) operon present is \( B. thailandensis \) is conserved in pathogenic strains as well indicating its importance under different environmental conditions. Thus, the role of \( mftP \), identifying its substrate/s and function, could be an interesting path to follow. That \( mftp \) is regulated by MftR [10] has been already demonstrated, and this study could be effectively utilized in two ways: (1) Identifying the substrate effluxed by MftP along with a combination of ligands that can bind MftR without altering its DNA binding ability could prove to be a successful cocktail for treatment of melioidosis and glanders (2) Could the ligand for MftR be a substrate for MftP or might a novel substrate for MftP, if identified, be a ligand for MftR.
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