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IDENTIFICATION AND CHARACTERIZATION OF GATase1-LIKE AraC-FAMILY TRANSCRIPTIONAL REGULATORS IN BURKHOLDERIA THAILANDENSIS.

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IDENTIFICATION AND CHARACTERIZATION OF GATASE1-LIKE ARAC-FAMILY TRANSCRIPTIONAL REGULATORS IN *BURKHOLDERIA THAILANDENSIS*.

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ABSTRACT

The ability of bacteria to detect their surroundings and enact an appropriate response is critical for survival. Translation of external signals into a coherent response requires specific control over the transcription of DNA into RNA. Much of the regulation at this step is accomplished by transcriptional regulators, proteins that bind to DNA and alter gene expression. A wide-spread variety of regulators in bacteria is the AraC-family. These regulators are divided into two conserved domains and respond to a variety of compounds owing to different N-terminal domains. A subfamily of these regulators, GATase1-like AraC-family transcriptional regulators (GATRs), is described. These proteins contain an N-terminal domain with structural characteristics similar to enzymes that synthesize amine-containing compounds. Members of this subfamily of transcriptional regulators are found in a wide range of bacteria, however, few are characterized. A relatively high number of GATRs are encoded in the *Burkholderia thailandensis* genome. Therefore, we utilized this bacterium as a model to explore the function and diversity of these regulators.

GATRs in *B. thailandensis* divided into two groups based on bioinformatics analysis. The first group includes three members which we identified that contribute to the positive regulation of glycine betaine (GB) catabolism. GB can be utilized as a nutrient source or as a potent osmoprotectant. The regulation of this pathway in *B. thailandensis* differs from previously established models due to the interplay of these regulators. Homologs of two other GATRs in this group were identified that regulate carnitine and arginine catabolism. The second group of GATRs contains uncharacterized members with no known functions. A genetic strategy for engineering constitutive GATRs was developed and employed to investigate the transcriptional regulons of these GATRs. This approach yielded the identification of a novel GATR that represses expression of an operon producing a formaldehyde detoxification system, and is the first example of a GATR that functions as a repressor.

CITATIONS

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CHAPTER 1:

INTRODUCTION

1.1 Sensing and responding to the environment.

Microbes must be able to survey their molecular surroundings and respond accordingly in order to survive and compete in a changing environment. A desiccating or nutrient poor environment may prompt a bacterium to undergo a metabolic slowdown to conserve resources, or even build a resilient spore (1). Perhaps a bacterium has been fortunate enough to colonize a favorable environment and metaphorically stakes its claim by building a robust biofilm (2). But how exactly is this achieved? Some signals are detected through the membrane by two-component regulatory systems that consist of a histidine kinase and a response regulator, which transmit information through phosphorylation relays (3). Other molecules may diffuse or be actively transported into the cytosol, where they impact the physiology of the cell directly or be detected by one-component regulatory systems more commonly referred to as transcriptional regulators (4). The response to these signals often relies on altering transcription or gene expression in the bacterium.

Transcription, the conversion of DNA into RNA by protein machinery, is a key to the central dogma of biology. DNA provides a master set of plans for the cell and a protected library of molecular information. The reading of the DNA and the production of RNA transcripts provide a translation of this information into functional directives for the cell to follow thus enabling it to persist and multiply. Control over transcription is a near universal point of regulation for the activities of all cells. A large portion of this

regulation is focused on the activities of the RNA polymerase holoenzyme, which is the protein machinery specifically responsible for the mechanistic process of transcription. The RNA polymerase must be recruited to the DNA at specific sites to produce functionally useful RNA. Transcription regulators bind to DNA elements to specifically recruit to or obscure RNA polymerase binding sites. There are a wide range of transcriptional regulators, which are classified by the architecture and function of the constituent protein domains.

1.2 Glycine betaine as a metabolite and signal.

Glycine betaine (GB) is found in abundance in the natural environment. Part of the reason for this abundance of availability is the widespread utilization of precursor molecules that can be catabolized into GB. Choline, for example, is an important moiety for both phosphatidylcholine and sphingomyelin. These phospholipids are major components of the plasma membrane, particularly in some eukaryotes (5). Another precursor molecule for GB is carnitine. Carnitine is used by eukaryotes in the process of transporting lipids across the mitochondrial membrane (6). For these two reasons, GB is thought to be particularly abundant in the environmental context of eukaryotes. GB is also significant because it is utilized as an osmoprotectant, a compatible solute that protects the cell against salt stress, by many prokaryotes, eukaryotes, and archaea (7-11). Many organisms can also metabolize GB for growth, using it as either a source of carbon or nitrogen (11). The abundance of GB and its metabolites in the environment may function as a signal to which bacteria can respond, especially in the context of association

with eukaryotes (11).

Pseudomonas aeruginosa is both able to utilize GB as an osmoprotectant, and contains a catabolic pathway that allows the bacterium to use GB as a nutrient source (11). GB and related metabolites in *P*. *aeruginosa* also stimulate production of the extracellular phospholipase PlcH, a prominent virulence factor (12). GbdR (*g*lycine *b*etaine and *d*imethylglycine response *r*egulator) is responsible for regulating the genes that catabolize choline into GB to dimethylglycine, and genes encoding proteins involved in the transport of these compounds (13). Two other closely related regulators also impinge on the regulation of catabolism related to GB in *P*. *aeruginosa*. SouR regulates the catabolic genes responsible for converting dimethylglycine to sarcosine and subsequently to glycine and serine (14). Additionally, the regulator for the carnitine catabolic and transport genes is CdhR (15). Carnitine provides an alternative metabolic route to GB. These three regulators share homology and are suggested to bind to the same or overlapping sequences in the promoter (14, 16). GbdR, SouR, and CdhR all belong to the AraC/XylS family of transcriptional regulators.

1.3 The AraC family of transcriptional regulators.

The AraC/XylS-family of transcriptional regulators (ATRs) is one of the most common types of prokaryotic transcriptional regulators. The namesake of this family, AraC, is responsible for the regulation of the *araBAD* operon that contains genes for the catabolism of arabinose (17-19). ATRs are defined by the homologous ninety-nine amino acid residues in the C-terminus of the ATR (20). The C-terminus is characterized by a helix-turn-helix DNA-binding domain, followed by a linker region joined to the N-

terminal region of the protein, typically containing ligand binding and dimerization capabilities (21-23) (Figure 1.1). ATRs regulate multiple or single operons, and are often themselves encoded divergently from genes they regulate. ATRs target genes that encode catabolic enzymes, virulence factors, secondary metabolism, transport, and others (24). AraC itself exists as a dimer at normal concentrations, whether or not it is bound to DNA and or arabinose (25). AraC regulates the L-arabinose operon by the 'light switch mechanism' (Figure 1.2). The operon is activated when each monomer binds an arabinose molecule, which increases affinity for the two proximal half sites, I_1 and I_2, activating transcription. In the absence of arabinose, one monomer binds I_1 while the other binds to an alternate half site (O_2) which is further upstream, resulting in repression of transcription by DNA looping (26). The I_1 and I_2 half site sequences are composed of direct repeat symmetry, as opposed to other transcription factors which have inverted repeats (27). This well-studied example has contributed to our general understanding of ATRs. However, not all of these insights are broadly applicable to the rest of this family of regulators.

The other canonical example of ATRs is XylS, which regulates alkyl benzoate catabolism and was discovered on the TOL plasmid of *Pseudomonas putida* (28). Unlike AraC, which makes some contacts with the σ^{70} subunit of RNA polymerase, XylS may utilize either σ^{70} or σ^{54} at its promoters (28, 29). Another difference is that while it also binds to similarly structured half sites and activates as a dimer, XylS typically exists in solution as a monomer (30). XylS and other dimerizing ATRs (e.g. RegA) only bind target promoters in the presence of the appropriate ligand rather than utilizing the 'light-switch mechanism' (31) (Figure 1.3). Still other ATRs like MarA, SoxS, and Rob, which

are involved in *E. coli* stress response and drug resistance, are examples of ATRs that bind promoters in respective regulons as monomers (32).

Many of the differences in ATRs stem from differences in the N-terminal domain of the protein that imparts ligand specificity and dimerization capacity. Notably, MarA and SoxS lack the ligand binding domain characteristic of many other ATRs altogether (33). The N-terminal domains of ATRs are derived from the binding domains of other protein families. An ATR in *Sinorhizobium meliloti* contains a cupin-like domain that enables a direct interaction with cyclic-di-GMP, quite different from binding sugars as a ligand (34). Still others utilize ligands such as urea and bicarbonate (35, 36).

The expression of genes coding for ATRs is often tightly regulated in order to exert fine-tuned control over the respective regulons. As regulators, the number of molecules per cell is often not significantly more than the number of target promoters, as is the case for AraC (27, 37). SoxS occurs at approximately 100 molecules per cell while impacting the regulation of about one hundred genes (38). XylS concentration per cell has also been experimentally determined and is in the range of 200 molecules per cell, while its target genes may vary in number due to variable copy number of the TOL plasmid (30). Many ATRs, including AraC, participate in some variety of auto-regulatory scheme. MarA, an ATR involved in multidrug resistance in pathogenic *Escherichia coli*, has also been demonstrated to bind to its own promoter (39). The turnover rate of MarA is only several minutes, allowing the TetR-family repressor MarR, which is located in the same operon, to bind in the absence of MarA ligand (33). SoxS and Rob, are ATRs that regulate genes in overlapping pathways with MarA and compete for similar promoters thus impacting the regulation of MarA induced genes including MarA itself (32).

The transcriptional control of ATR regulons is often also modulated by other types of transcriptional regulators, either directly or indirectly. The cyclic-AMP receptor protein (CRP), which is involved in the regulation of many genes in *E. coli*, is required for AraC-mediated induction of the L-arabinose catabolic operon (40). CRP is suggested to contribute to the relief of the repressive DNA looping caused by AraC as well as assist in the recruitment of RNA polymerase through direct contacts (27). ATRs are subjected to anti-activation from a family of proteins designated AraC negative regulators (ANRs) (41). These proteins interact with ATRs directly to prevent DNA binding. ANRs also interact with HN-S family proteins, which are responsible for DNA condensation and the silencing of AT-rich sequences (42). HN-S family proteins may also directly impact promoter regions of ATR targets, or the promoters of the ATRs themselves (43).

1.4 Glutamine amidotransferase 1 (GATase 1)-containing AraC family transcriptional regulators (GATRs).

We identified a subset of ATRs with common homology that we refer to as GATRs. GATRs contain two C-terminal helix-turn-helix motifs like other ATRs, but the N-terminal domain bears homology to Class I GATases (Figure 1.4), a family of enzymes that catalyzes the transfer of amide groups to various substrates. The distribution of these regulators is quite broad, including both Gram-positive and Gram-negative bacteria (Figure 1.5), however there are few characterized representatives of this type of ATR. These GATRs are particularly common amongst the Proteobacteria. The genomes of many members of the Proteobacteria contain multiple GATRs, rather than just one or two. Interestingly, GATRs are also found in eukaryotes including the pharaoh ant, pea

aphid, and *Entamoeba histolytica* (44). Many GATRs are labeled as FtrA or FtrA-like, owing to a described GATR in *Caulobacter crescentus*, which is suggested to be a putative regulator under limited oxygen conditions of an alternative branch of the thiamine synthesis pathway, myo-inositol degradation, and two aminotransferases (45). Analysis of amino acid sequence homology and synteny of adjacent genes reveals GATRs designated FtrA are unlikely to be orthologous and likely to have differential functions. Some GATRs contain a conserved cysteine residue within the N-terminal domain, which is a characteristic of the GAT superfamily of which GATases are part.

Members of the GAT superfamily of proteins are typified by a variable strand number Rossmann fold (46), which is series of parallel β-sheets suggested to play a role in nucleotide binding (47). This superfamily includes a wide variety of proteins with well characterized functions aside from the GATases. These include proteases like Pfp1 from the Archean *Pyrococcus furiosus* (48), and the chaperone Hsp31 in *E. coli* (49). The yeast homolog of Hsp31 in addition to chaperone activity, has a methylglyoxal detoxification activity under conditions of oxidative stress (50). Many members of the GAT superfamily appear to be evolutionary derivatives of ThiJ, a bacterial protein involved in the synthesis of thiamine (51). This includes the human protein DJ-1, which is protective against oxidative stress in the brain and associated with Parkinson's disease (52, 53). The GAT superfamily also shares evolutionary features with catalases, and typical sub-families may differ by the size of a variable region between strands of the Rossmann fold and occasional lack of the typical catalytic triad, cysteine-histidine-aspartate/glutamate (46). The superfamily is rounded out by particular members of the AraC-family (GATRs) and the GATases.

GATases are known to be involved in the biosynthesis of a wide variety of aminated molecules including amino acids, aminated sugars, nucleotides, and more (54). GATases typically utilize glutamine as the nitrogen donor, but some utilize primarily exogenous ammonia instead of or in addition to glutamine. The only noted exception is Glu-tRNA^{Gln} amidotransferase, which also utilizes asparagine at a low rate (55). GATases are divided both functionally and topologically into two domains of a monomer or as heterodimers of translationally separate subunits (56, 57).

The first domain, referred to as the GAT (glutamine amidotransferase) domain, consists of both the amine binding and amindotransferase activity. The second domain contains the synthase activity and is generally quite divergent owing to the chemically diverse amide receptors participating in the reaction to generate a particular product. The liberated ammonia is shuttled through a molecular channel between the amidotransferase and synthase domains (58-60).

The GAT domain is divided into two distinct classes, as defined by sequence homology (61). Class 1 has a conserved catalytic triad consisting of a cysteine, histidine, and glutamine. Class 2 members possess a single highly conserved cysteine in the amino terminus. The remaining amino acid residues of the catalytic triad are less well conserved.

The first characterized GATR was ArgR in *P. aeruginosa*. ArgR regulates the arginine succinyltransferase pathway of arginine catabolism (62). Although *P. aeruginosa* has three additional pathways for arginine utilization, it is unable to use arginine as a sole carbon source in the absence of *argR* (63). In *P. aeruginosa*, ArgR also plays a critical role in the catabolism of lysine. This is due to the requirement of ArgR for

expression of the *ldcA* gene which codes for a decarboxylase critical for the catabolism of lysine and its use as a sole carbon source (64). ArgR is unrelated to the similarly named, hexameric regulation protein found in *E. coli* and other enterics despite regulating similar components of the metabolic pathway (65, 66). The remaining recently characterized GATRs are implicated in different branches of GB catabolism in *P. aeruginosa*.

1.5 *Burkholderia thailandensis* as a model organism.

GATRs are especially prevalent in *Burkholderia*. *B. thailandensis* in particular possesses thirteen bioinformatically predicted GATRs (Figure 1.7). Because many examples of GATR-containing microorganisms only have one or two putative GATRs, a sample size of thirteen represents a significant survey of GATRs with respect to their different functions. For this reason, and the fact that *B. thailandensis* is a genetically tractable model organism for which there are preexisting genetic tools (67-69), we have concentrated our efforts on exploring this subset of GATRs in order to achieve a greater understanding of GATRs as a family.

Burkholderia thailandensis is a saprophytic, β-proteobacteria associated with the rhizosphere. The genome is arranged as two circular chromosomes possessing 5712 genes over 6.7 megabases (strain E264) (70). This genome is larger compared to familiar bacteria such as *E. coli* K-12 at 4.6 megabases, which indicates an increased number of genes based on the relatively linear correlation between genome size and the number of genes in bacterial species (71). A larger number of genes suggest an enhanced ability to take advantage of a larger diversity of compounds found in the environment, and is typical of free-living bacteria isolated from the environment (72). Initially, *Burkholderia*

species fell under the genus *Pseudomonas* due in part to metabolizing a similar array of compounds and filling a similar environmental niche. *Burkholderia* are now recognized as an independent genus (73), and have undergone several reclassifications in the scientific literature (Figure 1.6). More recently, a large number of primarily environmental *Burkholderia* have been reclassified and placed in the new genus, *Paraburkholderia* (74).

Burkholderia thailandensis was originally isolated from rice paddy soil samples in Thailand and recognized as distinct from the pathogenic *Burkholderia pseudomallei* due to its ability to catabolize arabinose and an apparent lack of clinical cases caused by any isolates with this ability (75-77). It is considered avirulent, and classified as a biosafety level 1 organism by many research institutions. *Burkholderia* species also tend to be naturally resistant to many antibiotics due to their contingent and variety of antimicrobial pumps and capsules (78). The metabolic flexibility of many Burkholderiales has made them a focus for studies in bioremediation (79). *Burkholderia* species are also notable for their capacity to produce secondary metabolites, including antifungal compounds (80, 81).

Utilizing *B. thailandensis* as a model organism has the advantage of serving as a substitute for its more pathogenic relatives due to its close genetic similarity (70). The *Burkholderia* genus can be roughly subdivided into two further distinct clades. The first group includes *Burkholderia pseudomallei* and *Burkholderia mallei*, potent pathogens and select agents. It is to this group that *B. thailandensis* is most closely related and the three organisms are thought to share a core metabolic genome (70). In addition, *B. thailandensis* also possesses many genes associated with virulence including fimbriae,

several capsule operons, several type III secretion systems, several type-VI secretion systems, and flagella suited to both extracellular and intracellular lifestyles (82). The presence of these virulence-associated genes in *B. thailandensis* may be related to the antagonistic and symbiotic interactions of these and other Burkholderiales have with different amoeba species (83-85). Members of this clade are also able to stimulate the fusion of different phagocytic cells into multinucleated giant cells in both humans and arthropods (86-88). The second group includes Burkholderiales that are often members of the rhizosphere or plant pathogens. This second group is also notable for including the namesake organism of the genus, *Burkholderia cepacia*, a pathogen of the onion plant that infiltrates the onion causing a tannish to yellow ooze of bacteria and rendering it inedible (89) (Figure 1.8A). However, these organisms may cause opportunistic infections in humans, particularly in immune compromised individuals. The best example of this opportunistic pathogenicity is in patients with cystic fibrosis (CF) where many of these Burkholderiales make up what is known as the *Burkholderia cepacia* complex (Bcc) and greatly increase morbidity (90). Similarly to the Pseudomallei group, *B. thailandensis* shares a large portion of its genes with members of the Bcc, but has the advantage of genetic tractability as the genomes of Bcc members are notoriously genetically malleable due to recombination between insertion elements, and are consequently less facile for genetic experimentation (91).

As a model for *B. pseudomallei* is particularly advantageous because *B. thailandensis* is significantly less virulent in mammals, and therefore less hazardous for laboratory work (92, 93). Although *B. thailandensis* retains the ability to live intracellularly in eukaryotic cell culture, the lack of a major capsule representing a

primary virulence factor in *B. pseudomallei*, allows the bacterium to be rapidly cleared by the innate immune system (94). Ultimately, the only incidents of disease attributed to *B. thailandensis* are a car accident were an infant was ejected from the vehicle into a drainage ditch (95), a traumatic leg amputation due to a motorcycle incident (96), and an unusual case of fever in an elderly man in Chongqing, China (97). In all of these cases, infection caused by *B. thailandensis* does not seem to be the primary health concern and the ability to identify the causative agent in a clinical setting can be limited by available resources and ability to isolate the correct bacterium.

B. pseudomallei is the causative agent of melioidosis, a disease that is rapidly gaining recognition in developing countries. Melioidosis, also known as Whitmore's disease after it's discoverer Alfred Whitmore (98) (Figure 1.8B), is difficult to diagnose in part because it has similar symptoms to other common illnesses and many countries where it is endemic lack the clinical facilities needed to accurately identify it (99). The infection has often progressed to an advanced stage by the time it is identified, often when abscesses are detectable on the spleen and other internal organs when the patient already may be septic. In addition, *B. pseudomallei* infection may be latent for an indeterminate amount of time before becoming active. The exact means by which this occurs is unknown, but may have to do with the ability of *B. pseudomallei* to infiltrate immune cells and survive intracellularly. Due to this ability, the illness was previously known as the "Vietnam Time Bomb", as American veterans of the Vietnam War might not become ill until years after returning home with one documented case occurring after sixty years. This phenomenon was particularly prevalent among helicopter crews, perhaps due to the generation of dust particles upon takeoff and landing (100). There are

a predicted 165000 cases of melioidosis per year with an approximately fifty percent mortality rate based on computer models (101). Infection may manifest a number of symptoms and result in abscesses and septic shock (102). Cases of melioidosis correspond to seasonal monsoons, possibly due to the resulting rising water table or the disturbance of the soil (103, 104). *B. thailandensis* and *B. pseudomallei* may directly compete for the same environmental niche, as isolation of these organisms in Northeastern compared to other regions of Thailand favors *B. thailandensis* and correspond with a lower level of melioidosis cases (105) (Figure 1.9). Additionally, *B. pseudomallei* and B. thailandensis both can compete experimentally with their multiple contact dependent inhibition systems, type-IV secretion systems, in addition to other systems (106-108).

The select agent status of *B. pseudomallei* and *B. mallei* also contribute to the difficulty of working with these pathogens. *B. mallei* is an extremely close relative of *B. pseudomallei* that has been genetically reduced putatively due to the high presence of transposons in its genome. As a consequence, *B. mallei* is an obligate intracellular pathogen and has never been isolated from soil. It is the causative agent of glanders in equines (horses, donkeys, mules) (Figure 1.8C), although it can also infect humans who have been in contact with infected equines (109). Due to efforts to eradicate infected animals, the last natural case of glanders in the United States occurred in 1937. However, it is still endemic in other countries where the use of equines as beasts of burden is common (109). *B. mallei* was actively used as an agent of biological warfare during World War I by Germany in an effort to sabotage the supply of horses to Europe from the United States (110). *B. pseudomallei* was also researched biological weapon by the

infamous Japanese unit 731 during World War II were the notable ability of *B. pseudomallei* to persist in pure water for long periods of time was exploited to contaminate well water in China (111). The potential of *B. pseudomallei* for use as a biological weapon was also researched by the Soviet Union as part of their notorious Biopreparat biological weapons program (112). Due to this colorful history, the politics and legal restrictions on studying these pathogens impose great monetary costs and hurdles to research. This makes the avirulent *B. thailandensis* an attractive, cost-effective alternative model organism.

1.6 Focus of study and approach.

The work in this thesis focuses on understanding the role of GATRs in the context of *B. thailandensis*. Because of the importance of GB catabolism, we first sought to identify an ortholog for GbdR (113). In Chapter 2 we identify multiple orthologs of GbdR and describe an alternative model for regulation of the GB catabolic pathway. Regulons for the identified GATRs are determined through RNA-Seq, reporter assays, and electro mobility shift assays. Chapter 3 describes our efforts to identify the function of additional GATRs in *B. thailandensis*. Strains with constitutive GATRs were generated by combining structural data with information about known constitutive mutations in a characterized GATR. This allowed for the determination of the regulon of an unknown GATR, I0685. I0685 is the first GATR found to act as a repressor, and prevents expression of a formaldehyde detoxification system. Two other GATRs were characterized by screening *B. thailandensis* GATR deletion strains against a small library

of compounds used in growth assays as either a sole carbon or sole nitrogen source. Finally, Chapter 4 explores the significance of these findings, what unanswered questions remain, and suggests future experiments to expand on these studies.

1.7 Chapter 1 Figures

Figure 1.1 – General Representation of ATRs.

AraC-family transcriptional regulators are separated into an N-terminal and C-terminal domain, joined by a short linker. The N-terminal domain includes both effector binding and dimerization capacity. A short N-terminal arm, which affects the nature of the dimerization interaction, is also present. The C-terminal domain imparts DNA binding ability and consists of two helix-turn-helix (HTH) motifs.

Figure 1.2 – The light-switch mechanism of AraC.

Figure adapted from Yang et al. (31). (A) The schematic of AraC and the 'light-switch mechanism' are a model for the understanding of ATRs. (B) The AraC dimer binds to the upstream element O_2 and I_1 of the *araBAD* promoter in the absence of arabinose ligand. (C) In the presence of ligand, the N-terminal arm changes arrangement, affecting the dimerization interaction and preference for half sites to the I_1 and I_2 sites, making the complex capable of activating transcription.

Figure 1.3 – Mechanism of RegA.

Figure adapted from Yang et al. (31). (A) In the absence of ligand, RegA remains unbound. (B) In the presence of its ligand, HCO_3^- (bicarbonate), RegA binds to and activates its target promoters.

Figure 1.4 – General Representation of GATRs.

GATRs are markedly similar to other ATRs, consisting of similar functional domains. However, the N-terminal domain is homologous to glutamine amidotransferase class 1 enzymes (GATase1).

Figure 1.5 – Distribution of GATRs.

(A) Protein architecture used for Conserved Domain Architecture Retrieval Tool (CDART) query to find GATRs in the NCBI non-redundant protein sequence database (44). (B) Result of query showing the broad presence of GATRs in bacteria, particularly proteobacteria, and several examples in eukaryotes.

Figure 1.6 – Phylogeny of *Burkholderia*.

The *Burkholderia* genus may be broken up into a group containing pathogens similar to *B*. *pseudomallei*, a group encompassing opportunistic pathogens that include members of the BCC, and phytopathogens. Many classical members of the genus can now be separated into the new, but closely related genus, *Paraburkholderia*. This phylogenetic tree was generated using PATRIC (114).

Figure 1.7 – GATRs in *B*. *thailandensis*.

GATRs, and rough schematics of their genomic context bioinformatically, predicted in *B*. *thailandensis* by BLAST homology to GbdR from *P*. *aeruginosa*. GATRs are depicted in magenta, while other predicted genes are depicted in yellow.

Figure 1.8 – Beware of *Burkholderia*.

Members of the *Burkholderia* genus may cause a variety of diseases in plants and animals. (A) Example of sour skin of onion caused by *B. cepacia* (115). (B) Medical examples of cutaneous melioidosis in patients (116). (C) A horse afflicted with Glanders, caused by *B. mallei*. Source: The Brooke Hospitals for Animals (https://www.thebrooke.org/our-work/india/glanders-impact-horse-owners)

Figure 1.9 – Global endemicity of melioidosis.

Figure adapted from Center for Disease Control website. Cases of melioidosis are generally concentrated near the tropical regions, particularly in South East Asia.

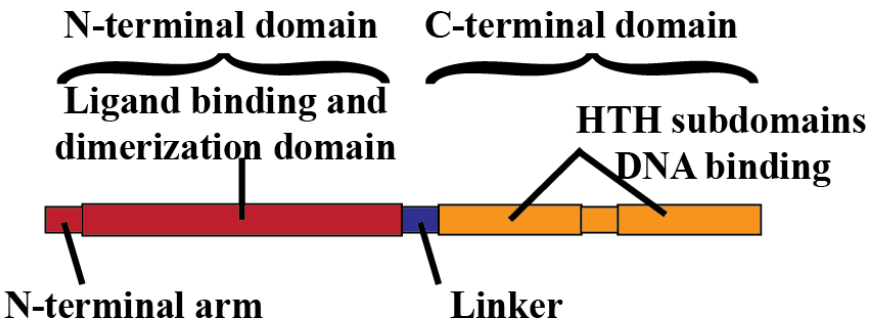


Figure 1.1 - General Representation of ATRs

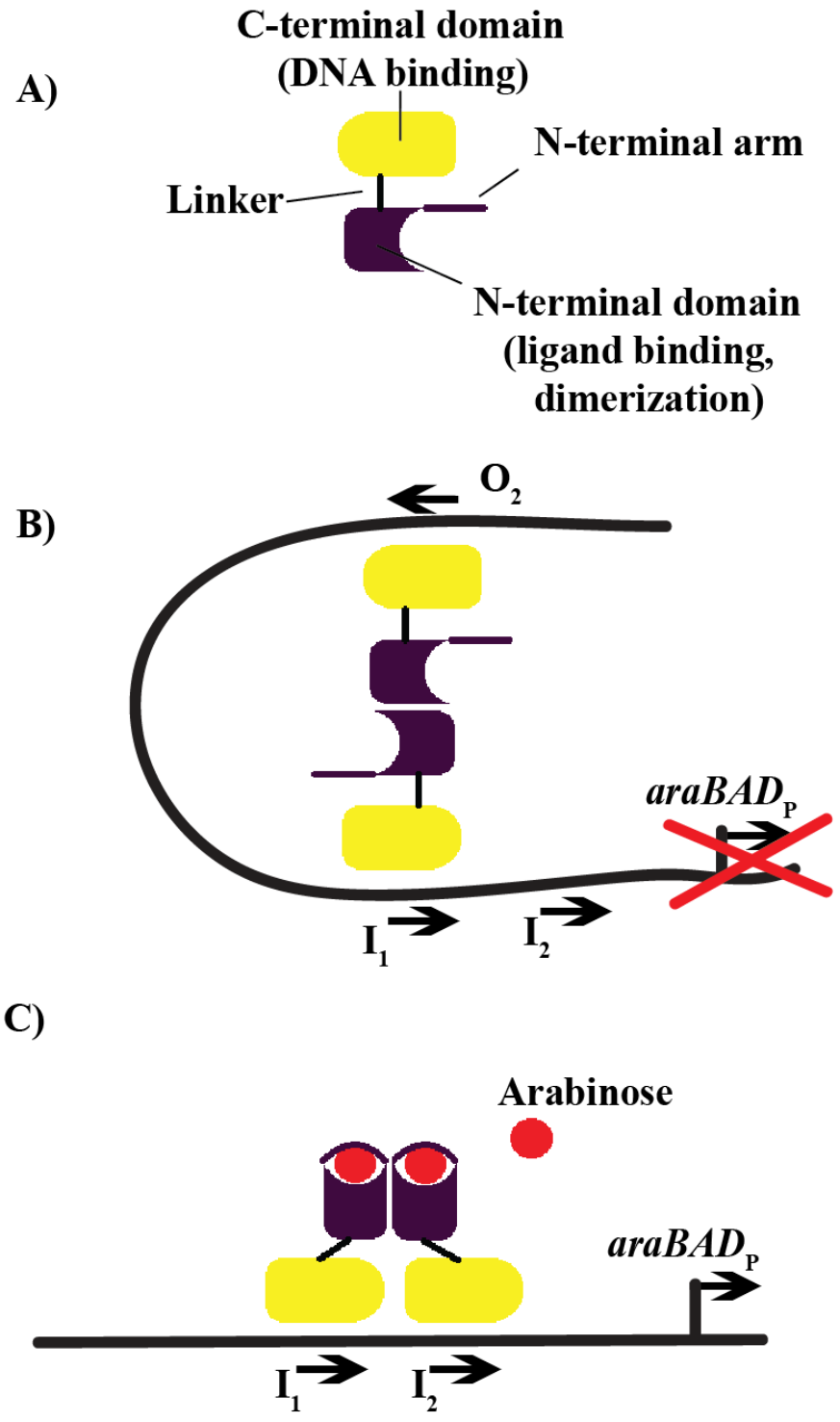


Figure 1.2 - The light-switch mechanism of AraC

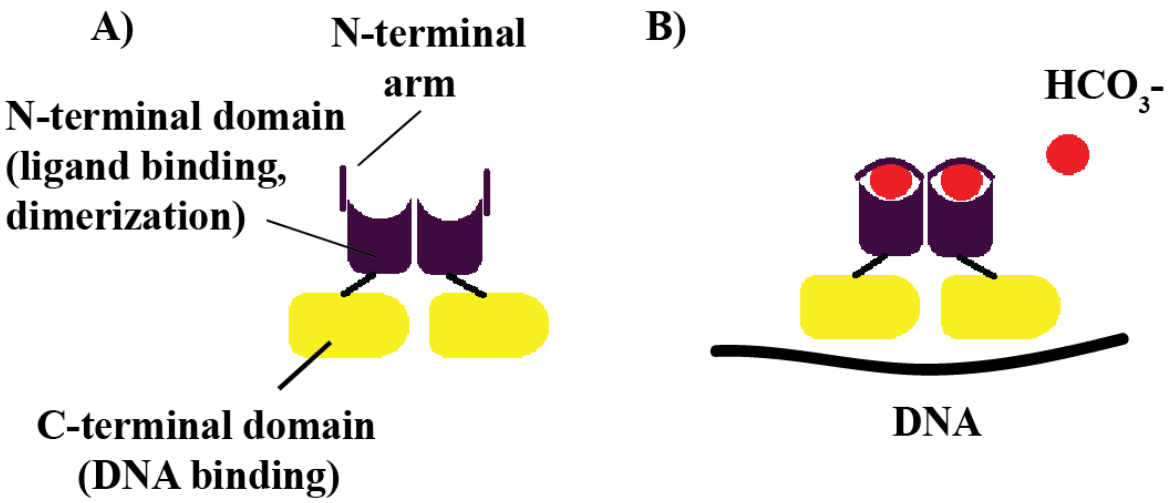


Figure 1.3 - Mechanism of RegA

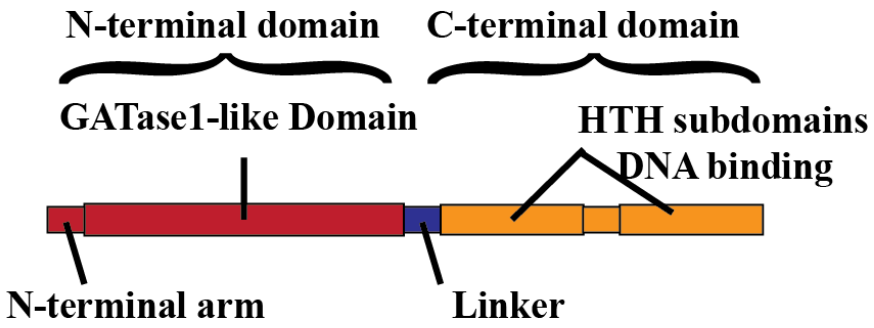


Figure 1.4 - General Representation of GATRs

A)

B)

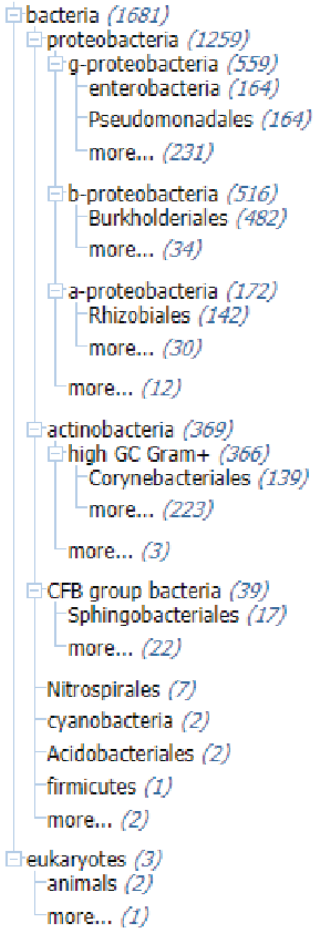


Figure 1.5 - Distribution of GATRs

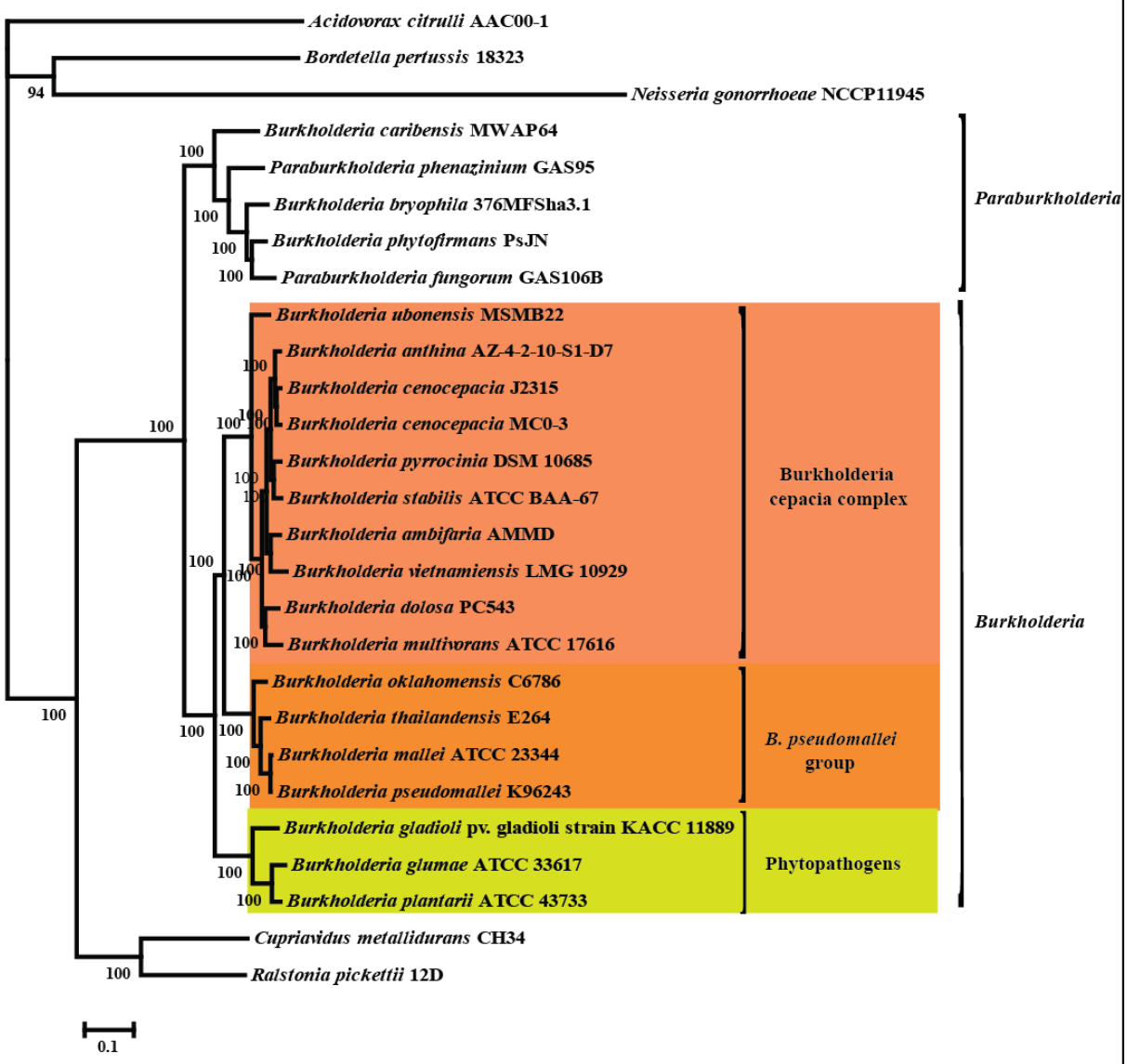


Figure 1.6 - Phylogeny of *Burkholderia*

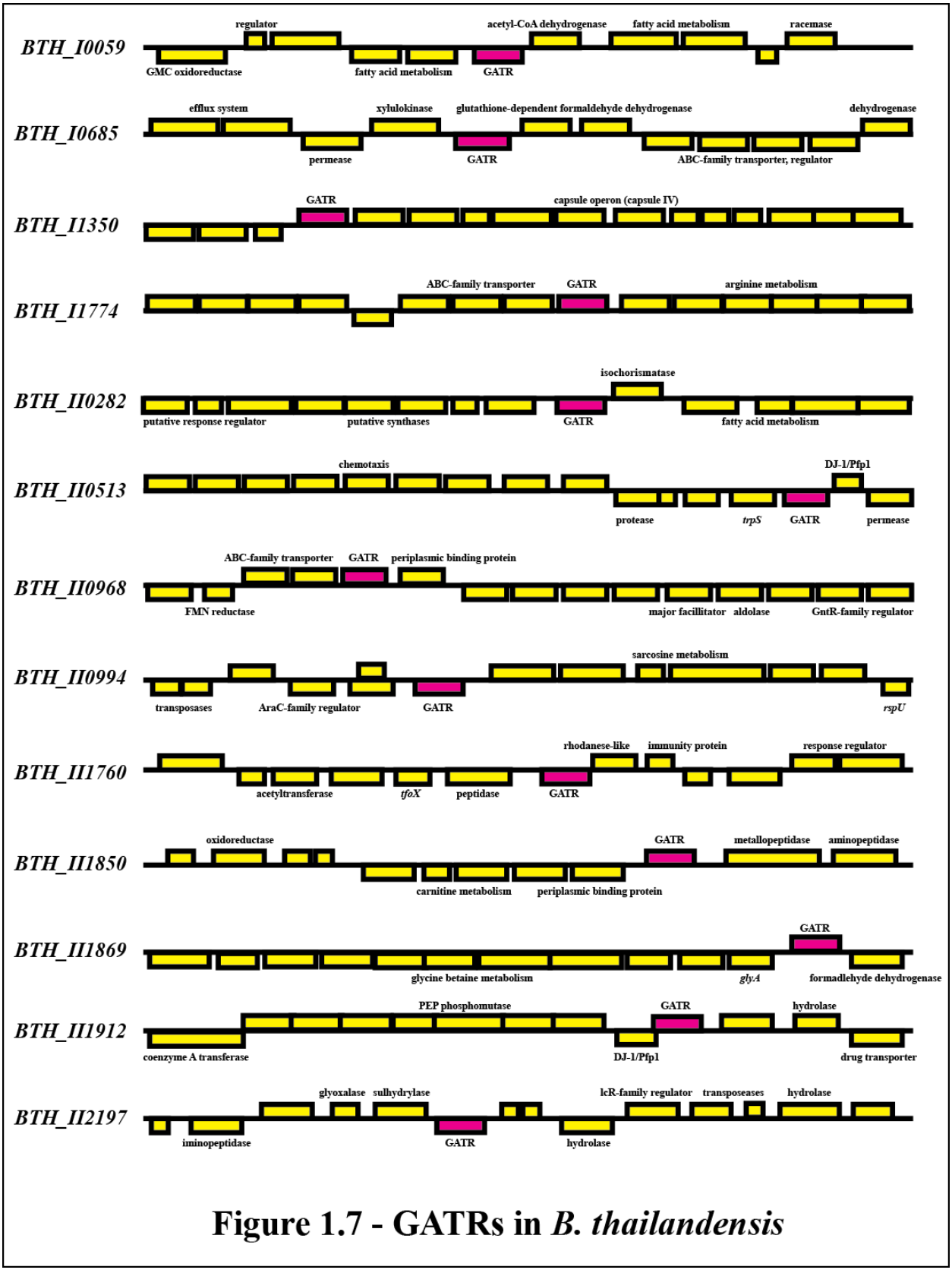
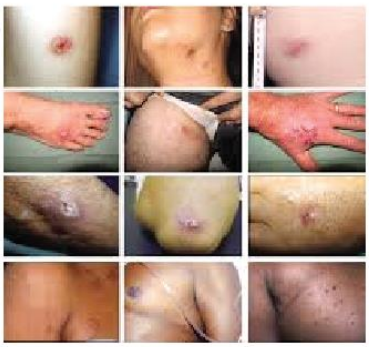


Figure 1.7 - GATRs in *B. thailandensis*

A)

B)



C)

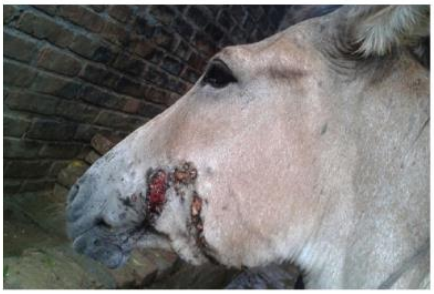


Figure 1.8 - Beware of *Burkholderia*

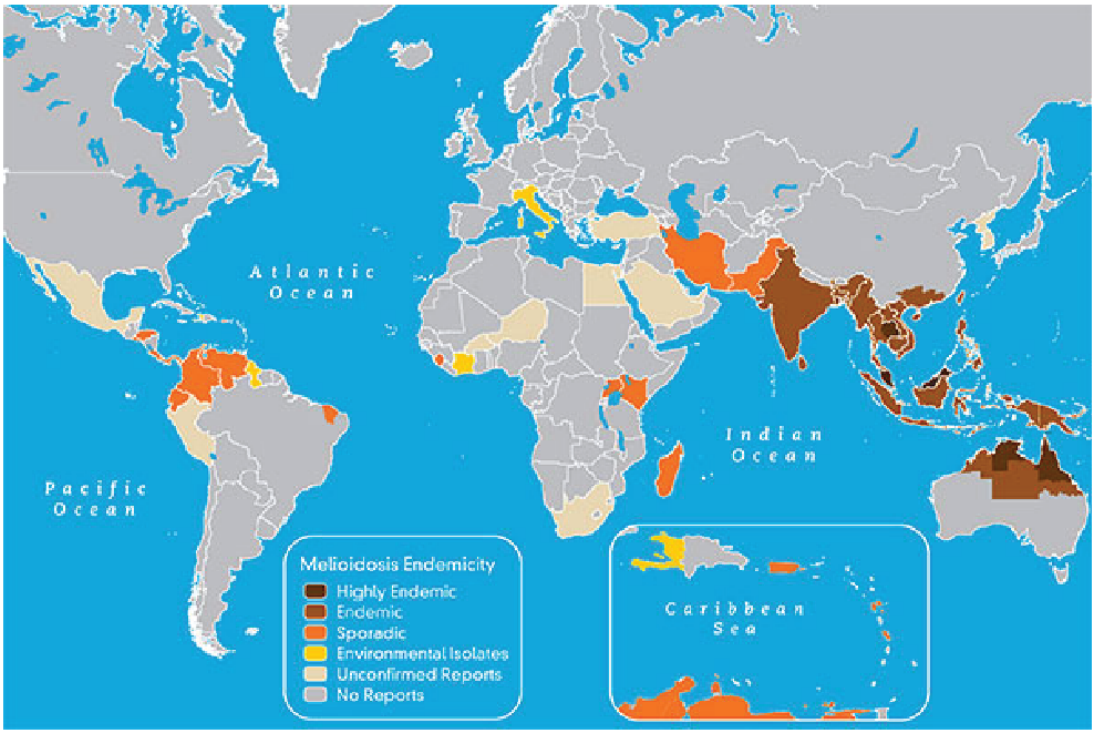


Figure 1.9 - Global endemicity of melioidosis.

1.8 Chapter 1 References

1. **Tocheva EI, Ortega DR, Jensen GJ.** 2016. Sporulation, bacterial cell envelopes and the origin of life. Nat Rev Microbiol **14:**535-542.
2. **Flemming HC, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S.** 2016. Biofilms: an emergent form of bacterial life. Nat Rev Microbiol **14:**563-575.
3. **Mitrophanov AY, Groisman EA.** 2008. Signal integration in bacterial two-component regulatory systems. Genes Dev **22:**2601-2611.
4. **Ulrich LE, Koonin EV, Zhulin IB.** 2005. One-component systems dominate signal transduction in prokaryotes. Trends Microbiol **13:**52-56.
5. **van Meer G, Voelker DR, Feigenson GW.** 2008. Membrane lipids: where they are and how they behave. Nature Reviews Molecular Cell Biology **9:**112-124.
6. **Bourdin B, Adenier H, Perrin Y.** 2007. Carnitine is associated with fatty acid metabolism in plants. Plant Physiol Biochem **45:**926-931.
7. **Lai MC, Gunsalus RP.** 1992. Glycine Betaine and Potassium-Ion Are the Major Compatible Solutes in the Extremely Halophilic Methanogen Methanohalophilus Strain Z7302. Journal of Bacteriology **174:**7474-7477.
8. **Lai MC, Sowers KR, Robertson DE, Roberts MF, Gunsalus RP.** 1991. Distribution of Compatible Solutes in the Halophilic Methanogenic Archaebacteria. Journal of Bacteriology **173:**5352-5358.
9. **Nicolaus B, Lanzotti V, Trincone A, Derosa M, Grant WD, Gambacorta A.** 1989. Glycine Betaine and Polar Lipid-Composition in Halophilic Archaebacteria in Response to Growth in Different Salt Concentrations. Fems Microbiology Letters **59:**157-160.
10. **Ashraf M, Foolad MR.** 2007. Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environmental and Experimental Botany **59:**206-216.
11. **Wargo MJ.** 2013. Homeostasis and catabolism of choline and glycine betaine: lessons from Pseudomonas aeruginosa. Appl Environ Microbiol **79:**2112-2120.
12. **Wargo MJ, Ho TC, Gross MJ, Whittaker LA, Hogan DA.** 2009. GbdR regulates Pseudomonas aeruginosa plcH and pchP transcription in response to choline catabolites. Infect Immun **77:**1103-1111.
13. **Wargo MJ, Szwergold BS, Hogan DA.** 2008. Identification of two gene clusters and a transcriptional regulator required for Pseudomonas aeruginosa glycine betaine catabolism. J Bacteriol **190:**2690-2699.
14. **Willsey GG, Wargo MJ.** 2015. Sarcosine catabolism in Pseudomonas aeruginosa is transcriptionally regulated by SouR. J Bacteriol doi:10.1128/JB.00739-15.
15. **Wargo MJ, Hogan DA.** 2009. Identification of genes required for Pseudomonas aeruginosa carnitine catabolism. Microbiology **155:**2411-2419.
16. **Meadows JA, Wargo MJ.** 2018. Transcriptional Regulation of Carnitine Catabolism in Pseudomonas aeruginosa by CdhR. mSphere **3**.
17. **Lee N, Francklyn C, Hamilton EP.** 1987. Arabinose-induced binding of AraC protein to araI2 activates the araBAD operon promoter. Proc Natl Acad Sci U S A **84:**8814-8818.

18. **Lee N, Gielow W, Martin R, Hamilton E, Fowler A.** 1986. The organization of the araBAD operon of Escherichia coli. Gene **47:**231-244.
19. **Englesberg E, Irr J, Power J, Lee N.** 1965. Positive control of enzyme synthesis by gene C in the L-arabinose system. J Bacteriol **90:**946-957.
20. **Gallegos MT, Michan C, Ramos JL.** 1993. The XylS/AraC family of regulators. Nucleic Acids Res **21:**807-810.
21. **Cass LG, Wilcox G.** 1986. Mutations in the araC regulatory gene of Escherichia coli B/r that affect repressor and activator functions of AraC protein. J Bacteriol **166:**892-900.
22. **Bustos SA, Schleif RF.** 1993. Functional domains of the AraC protein. Proc Natl Acad Sci U S A **90:**5638-5642.
23. **Eustance RJ, Bustos SA, Schleif RF.** 1994. Reaching out. Locating and lengthening the interdomain linker in AraC protein. J Mol Biol **242:**330-338.
24. **Martin RG, Rosner JL.** 2001. The AraC transcriptional activators. Curr Opin Microbiol **4:**132-137.
25. **Wilcox G, Meuris P.** 1976. Stabilization and size of araC protein. Mol Gen Genet **145:**97-100.
26. **Saviola B, Seabold R, Schleif RF.** 1998. Arm-domain interactions in AraC. J Mol Biol **278:**539-548.
27. **Schleif R.** 2010. AraC protein, regulation of the l-arabinose operon in Escherichia coli, and the light switch mechanism of AraC action. FEMS Microbiol Rev **34:**779-796.
28. **Du Y, Holtel A, Reizer J, Saier MH, Jr.** 1996. sigma54-dependent transcription of the Pseudomonas putida xylS operon is influenced by the IIANtr protein of the phosphotransferase system in Escherichia coli. Res Microbiol **147:**129-132.
29. **Dhiman A, Schleif R.** 2000. Recognition of overlapping nucleotides by AraC and the sigma subunit of RNA polymerase. J Bacteriol **182:**5076-5081.
30. **Dominguez-Cuevas P, Marin P, Busby S, Ramos JL, Marques S.** 2008. Roles of effectors in XylS-dependent transcription activation: intramolecular domain derepression and DNA binding. J Bacteriol **190:**3118-3128.
31. **Yang J, Tauschek M, Robins-Browne RM.** 2011. Control of bacterial virulence by AraC-like regulators that respond to chemical signals. Trends Microbiol **19:**128-135.
32. **Duval V, Lister IM.** 2013. MarA, SoxS and Rob of Escherichia coli - Global regulators of multidrug resistance, virulence and stress response. Int J Biotechnol Wellness Ind **2:**101-124.
33. **Griffith KL, Shah IM, Wolf RE, Jr.** 2004. Proteolytic degradation of Escherichia coli transcription activators SoxS and MarA as the mechanism for reversing the induction of the superoxide (SoxRS) and multiple antibiotic resistance (Mar) regulons. Mol Microbiol **51:**1801-1816.
34. **Schaper S, Steinchen W, Krol E, Altegoer F, Skotnicka D, Sogaard-Andersen L, Bange G, Becker A.** 2017. AraC-like transcriptional activator CuxR binds c-di-GMP by a PilZ-like mechanism to regulate extracellular polysaccharide production. Proc Natl Acad Sci U S A **114:**E4822-E4831.

35. **D'Orazio SE, Thomas V, Collins CM.** 1996. Activation of transcription at divergent urea-dependent promoters by the urease gene regulator UreR. Mol Microbiol **21:**643-655.
36. **Hart E, Yang J, Tauschek M, Kelly M, Wakefield MJ, Frankel G, Hartland EL, Robins-Browne RM.** 2008. RegA, an AraC-like protein, is a global transcriptional regulator that controls virulence gene expression in Citrobacter rodentium. Infect Immun **76:**5247-5256.
37. **Kolodrubetz D, Schleif R.** 1981. Identification of araC protein and two-dimensional gels, its in vivo instability and normal level. J Mol Biol **149:**133-139.
38. **Griffith KL, Shah IM, Myers TE, O'Neill MC, Wolf RE, Jr.** 2002. Evidence for "pre-recruitment" as a new mechanism of transcription activation in Escherichia coli: the large excess of SoxS binding sites per cell relative to the number of SoxS molecules per cell. Biochem Biophys Res Commun **291:**979-986.
39. **Martin RG, Jair KW, Wolf RE, Jr., Rosner JL.** 1996. Autoactivation of the marRAB multiple antibiotic resistance operon by the MarA transcriptional activator in Escherichia coli. J Bacteriol **178:**2216-2223.
40. **Lobell RB, Schleif RF.** 1991. AraC-DNA looping: orientation and distance-dependent loop breaking by the cyclic AMP receptor protein. J Mol Biol **218:**45-54.
41. **Santiago AE, Yan MB, Tran M, Wright N, Luzader DH, Kendall MM, Ruiz-Perez F, Nataro JP.** 2016. A large family of anti-activators accompanying XylS/AraC family regulatory proteins. Mol Microbiol **101:**314-332.
42. **Santiago AE, Yan MB, Hazen TH, Sauder B, Meza-Segura M, Rasko DA, Kendall MM, Ruiz-Perez F, Nataro JP.** 2017. The AraC Negative Regulator family modulates the activity of histone-like proteins in pathogenic bacteria. PLoS Pathog **13:**e1006545.
43. **Giangrossi M, Zattoni S, Tramonti A, De Biase D, Falconi M.** 2005. Antagonistic role of H-NS and GadX in the regulation of the glutamate decarboxylase-dependent acid resistance system in Escherichia coli. J Biol Chem **280:**21498-21505.
44. **Geer LY, Domrachev M, Lipman DJ, Bryant SH.** 2002. CDART: protein homology by domain architecture. Genome Res **12:**1619-1623.
45. **Crosson S, McGrath PT, Stephens C, McAdams HH, Shapiro L.** 2005. Conserved modular design of an oxygen sensory/signaling network with species-specific output. Proc Natl Acad Sci U S A **102:**8018-8023.
46. **Horvath MM, Grishin NV.** 2001. The C-terminal domain of HPII catalase is a member of the type I glutamine amidotransferase superfamily. Proteins-Structure Function and Genetics **42:**230-236.
47. **Hanukoglu I.** 2015. Proteopedia: Rossmann fold: A beta-alpha-beta fold at dinucleotide binding sites. Biochem Mol Biol Educ **43:**206-209.
48. **Halio SB, Blumentals, II, Short SA, Merrill BM, Kelly RM.** 1996. Sequence, expression in Escherichia coli, and analysis of the gene encoding a novel intracellular protease (PfpI) from the hyperthermophilic archaeon Pyrococcus furiosus. J Bacteriol **178:**2605-2612.

49. **Mujacic M, Baneyx F.** 2007. Chaperone Hsp31 contributes to acid resistance in stationary-phase Escherichia coli. Appl Environ Microbiol **73:**1014-1018.
50. **Bankapalli K, Saladi S, Awadia SS, Goswami AV, Samaddar M, D'Silva P.** 2015. Robust glyoxalase activity of Hsp31, a ThiJ/DJ-1/PfpI family member protein, is critical for oxidative stress resistance in Saccharomyces cerevisiae. J Biol Chem **290:**26491-26507.
51. **Bandyopadhyay S, Cookson MR.** 2004. Evolutionary and functional relationships within the DJ1 superfamily. BMC Evol Biol **4:**6.
52. **Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, Dekker MC, Squitieri F, Ibanez P, Joosse M, van Dongen JW, Vanacore N, van Swieten JC, Brice A, Meco G, van Duijn CM, Oostra BA, Heutink P.** 2003. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science **299:**256-259.
53. **Ariga H, Takahashi-Niki K, Kato I, Maita H, Niki T, Iguchi-Ariga SM.** 2013. Neuroprotective function of DJ-1 in Parkinson's disease. Oxid Med Cell Longev **2013:**683920.
54. **Massiere F, Badet-Denisot MA.** 1998. The mechanism of glutamine-dependent amidotransferases. Cell Mol Life Sci **54:**205-222.
55. **Jahn D, Kim YC, Ishino Y, Chen MW, Soll D.** 1990. Purification and functional characterization of the Glu-tRNA(Gln) amidotransferase from Chlamydomonas reinhardtii. J Biol Chem **265:**8059-8064.
56. **Gerhart JC, Schachman HK.** 1965. Distinct subunits for the regulation and catalytic activity of aspartate transcarbamylase. Biochemistry **4:**1054-1062.
57. **Henderson EJ, Zalkin H, Hwang LH.** 1970. The anthranilate synthetase-anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase aggregate. Catalytic and regulatory properties of aggregated and unaggregated forms of anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase. J Biol Chem **245:**1424-1431.
58. **Krahn JM, Kim JH, Burns MR, Parry RJ, Zalkin H, Smith JL.** 1997. Coupled formation of an amidotransferase interdomain ammonia channel and a phosphoribosyltransferase active site. Biochemistry **36:**11061-11068.
59. **Thoden JB, Raushel FM, Benning MM, Rayment I, Holden HM.** 1999. The structure of carbamoyl phosphate synthetase determined to 2.1 A resolution. Acta Crystallogr D Biol Crystallogr **55:**8-24.
60. **Raushel FM, Thoden JB, Holden HM.** 2003. Enzymes with molecular tunnels. Acc Chem Res **36:**539-548.
61. **Zalkin H.** 1993. The amidotransferases. Adv Enzymol Relat Areas Mol Biol **66:**203-309.
62. **Park SM, Lu CD, Abdelal AT.** 1997. Cloning and characterization of argR, a gene that participates in regulation of arginine biosynthesis and catabolism in Pseudomonas aeruginosa PAO1. J Bacteriol **179:**5300-5308.
63. **Silver S, American Society for Microbiology.** 1990. Pseudomonas : biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.

64. **Chou HT, Hegazy M, Lu CD.** 2010. L-lysine catabolism is controlled by L-arginine and ArgR in Pseudomonas aeruginosa PAO1. J Bacteriol **192:**5874-5880.
65. **Maas WK.** 1994. The arginine repressor of Escherichia coli. Microbiol Rev **58:**631-640.
66. **Lu CD, Houghton JE, Abdelal AT.** 1992. Characterization of the arginine repressor from Salmonella typhimurium and its interactions with the carAB operator. J Mol Biol **225:**11-24.
67. **Thongdee M, Gallagher LA, Schell M, Dharakul T, Songsivilai S, Manoil C.** 2008. Targeted mutagenesis of Burkholderia thailandensis and Burkholderia pseudomallei through natural transformation of PCR fragments. Appl Environ Microbiol **74:**2985-2989.
68. **Choi KH, DeShazer D, Schweizer HP.** 2006. mini-Tn7 insertion in bacteria with multiple glmS-linked attTn7 sites: example Burkholderia mallei ATCC 23344. Nat Protoc **1:**162-169.
69. **Choi KH, Mima T, Casart Y, Rholl D, Kumar A, Beacham IR, Schweizer HP.** 2008. Genetic tools for select-agent-compliant manipulation of Burkholderia pseudomallei. Appl Environ Microbiol **74:**1064-1075.
70. **Kim HS, Schell MA, Yu Y, Ulrich RL, Sarria SH, Nierman WC, DeShazer D.** 2005. Bacterial genome adaptation to niches: divergence of the potential virulence genes in three Burkholderia species of different survival strategies. BMC Genomics **6:**174.
71. **Hou YB, Lin SJ.** 2009. Distinct Gene Number-Genome Size Relationships for Eukaryotes and Non-Eukaryotes: Gene Content Estimation for Dinoflagellate Genomes. Plos One **4**.
72. **Land M, Hauser L, Jun SR, Nookaew I, Leuze MR, Ahn TH, Karpinets T, Lund O, Kora G, Wassenaar T, Poudel S, Ussery DW.** 2015. Insights from 20 years of bacterial genome sequencing. Funct Integr Genomics **15:**141-161.
73. **Yabuuchi E, Kosako Y, Oyaizu H, Yano I, Hotta H, Hashimoto Y, Ezaki T, Arakawa M.** 1992. Proposal of Burkholderia gen. nov. and transfer of seven species of the genus Pseudomonas homology group II to the new genus, with the type species Burkholderia cepacia (Palleroni and Holmes 1981) comb. nov. Microbiol Immunol **36:**1251-1275.
74. **Sawana A, Adeolu M, Gupta RS.** 2014. Molecular signatures and phylogenomic analysis of the genus Burkholderia: proposal for division of this genus into the emended genus Burkholderia containing pathogenic organisms and a new genus Paraburkholderia gen. nov. harboring environmental species. Front Genet **5:**429.
75. **Wuthiekanun V, Smith MD, Dance DA, Walsh AL, Pitt TL, White NJ.** 1996. Biochemical characteristics of clinical and environmental isolates of Burkholderia pseudomallei. J Med Microbiol **45:**408-412.
76. **Smith MD, Angus BJ, Wuthiekanun V, White NJ.** 1997. Arabinose assimilation defines a nonvirulent biotype of Burkholderia pseudomallei. Infect Immun **65:**4319-4321.
77. **Brett PJ, DeShazer D, Woods DE.** 1998. Burkholderia thailandensis sp. nov., a Burkholderia pseudomallei-like species. Int J Syst Bacteriol **48 Pt 1:**317-320.

78. **Rhodes KA, Schweizer HP.** 2016. Antibiotic resistance in Burkholderia species. Drug Resist Updat **28:**82-90.
79. **O'Sullivan LA, Mahenthiralingam E.** 2005. Biotechnological potential within the genus Burkholderia. Letters in Applied Microbiology **41:**8-11.
80. **Vial L, Lepine F, Milot S, Groleau MC, Dekimpe V, Woods DE, Deziel E.** 2008. Burkholderia pseudomallei, B. thailandensis, and B. ambifaria produce 4-hydroxy-2-alkylquinoline analogues with a methyl group at the 3 position that is required for quorum-sensing regulation. Journal of Bacteriology **190:**5339-5352.
81. **Roitman JN, Mahoney NE, Janisiewicz WJ, Benson M.** 1990. A New Chlorinated Phenylpyrrole Antibiotic Produced by the Antifungal Bacterium Pseudomonas-Cepacia. Journal of Agricultural and Food Chemistry **38:**538-541.
82. **Angus AA, Agapakis CM, Fong S, Yerrapragada S, Estrada-de los Santos P, Yang P, Song N, Kano S, Caballero-Mellado J, de Faria SM, Dakora FD, Weinstock G, Hirsch AM.** 2014. Plant-associated symbiotic Burkholderia species lack hallmark strategies required in mammalian pathogenesis. PLoS One **9:**e83779.
83. **Noinarin P, Chareonsudjai P, Wangsomnuk P, Wongratanacheewin S, Chareonsudjai S.** 2016. Environmental Free-Living Amoebae Isolated from Soil in Khon Kaen, Thailand, Antagonize Burkholderia pseudomallei. PLoS One **11:**e0167355.
84. **DiSalvo S, Haselkorn TS, Bashir U, Jimenez D, Brock DA, Queller DC, Strassmann JE.** 2015. Burkholderia bacteria infectiously induce the proto-farming symbiosis of Dictyostelium amoebae and food bacteria. Proc Natl Acad Sci U S A **112:**E5029-5037.
85. **Inglis TJJ, Rigby P, Robertson TA, Dutton NS, Henderson M, Chang BJ.** 2000. Interaction between Burkholderia pseudomallei and Acanthamoeba species results in coiling phagocytosis, endamebic bacterial survival, and escape. Infection and Immunity **68:**1681-1686.
86. **Whiteley L, Meffert T, Haug M, Weidenmaier C, Hopf V, Bitschar K, Schittek B, Kohler C, Steinmetz I, West TE, Schwarz S.** 2017. Entry, intracellular survival and multinucleated giant cell-forming activity of Burkholderia pseudomallei in human primary phagocytic and non-phagocytic cells. Infect Immun doi:10.1128/IAI.00468-17.
87. **Fisher NA, Ribot WJ, Applefeld W, DeShazer D.** 2012. The Madagascar hissing cockroach as a novel surrogate host for Burkholderia pseudomallei, B. mallei and B. thailandensis. BMC Microbiol **12:**117.
88. **Galyov EE, Brett PJ, DeShazer D.** 2010. Molecular insights into Burkholderia pseudomallei and Burkholderia mallei pathogenesis. Annu Rev Microbiol **64:**495-517.
89. **Burkholder WH.** 1950. Sour skin, a bacterial rot of onion bulbs. Phytopathology **40:**115-117.
90. **Mahenthiralingam E, Urban TA, Goldberg JB.** 2005. The multifarious, multireplicon Burkholderia cepacia complex. Nat Rev Microbiol **3:**144-156.
91. **Anonymous.** 2007. Burkholderia: Molecular Microbiology and Genomics. Horizon Scientific Press.

92. **Kespichayawattana W, Intachote P, Utaisincharoen P, Sirisinha S.** 2004. Virulent Burkholderia pseudomallei is more efficient than avirulent Burkholderia thailandensis in invasion of and adherence to cultured human epithelial cells. Microbial Pathogenesis **36:**287-292.
93. **Yu YT, Kim HS, Chua HH, Lin CH, Sim SH, Lin DX, Derr A, Engels R, DeShazer D, Birren B, Nierman WC, Tan P.** 2006. Genomic patterns of pathogen evolution revealed by comparison of Burkholderia pseudomallei, the causative agent of melioidosis, to avirulent Burkholderia thailandensis. Bmc Microbiology **6**.
94. **Warawa JM, Long D, Rosenke R, Gardner D, Gherardini FC.** 2009. Role for the Burkholderia pseudomallei capsular polysaccharide encoded by the wcb operon in acute disseminated melioidosis. Infect Immun **77:**5252-5261.
95. **Glass MB, Gee JE, Steigerwalt AG, Cavuoti D, Barton T, Hardy RD, Godoy D, Spratt BG, Clark TA, Wilkins PP.** 2006. Pneumonia and septicemia caused by Burkholderia thailandensis in the United States. J Clin Microbiol **44:**4601-4604.
96. **Lertpatanasuwan N, Sermsri K, Petkaseam A, Trakulsomboon S, Thamlikitkul V, Suputtamongkol Y.** 1999. Arabinose-positive Burkholderia pseudomallei infection in humans: case report. Clin Infect Dis **28:**927-928.
97. **Chang K, Luo J, Xu H, Li M, Zhang F, Li J, Gu D, Deng S, Chen M, Lu W.** 2017. Human Infection with Burkholderia thailandensis, China, 2013. Emerg Infect Dis **23:**1416-1418.
98. **Whitmore AK, CS.** 1912. An account of the discovery of a hitherto undescribed infectious disease among the population of Rangoon. Ind Med Gaz **46:**262–267.
99. **Lau SK, Sridhar S, Ho CC, Chow WN, Lee KC, Lam CW, Yuen KY, Woo PC.** 2015. Laboratory diagnosis of melioidosis: past, present and future. Exp Biol Med (Maywood) **240:**742-751.
100. **Howe C, Sampath A, Spotnitz M.** 1971. The pseudomallei group: a review. J Infect Dis **124:**598-606.
101. **Limmathurotsakul D, Golding N, Dance DA, Messina JP, Pigott DM, Moyes CL, Rolim DB, Bertherat E, Day NP, Peacock SJ, Hay SI.** 2016. Predicted global distribution of Burkholderia pseudomallei and burden of melioidosis. Nat Microbiol **1:**15008.
102. **Perumal Samy R, Stiles BG, Sethi G, Lim LHK.** 2017. Melioidosis: Clinical impact and public health threat in the tropics. PLoS Negl Trop Dis **11:**e0004738.
103. **Currie BJ, Jacups SP.** 2003. Intensity of rainfall and severity of melioidosis, Australia. Emerg Infect Dis **9:**1538-1542.
104. **Thomas AD, Forbesfaulkner J, Parker M.** 1979. Isolation of Pseudomonas-Pseudomallei from Clay Layers at Defined Depths. American Journal of Epidemiology **110:**515-521.
105. **Trakulsomboon S, Vuddhakul V, Tharavichitkul P, Na-Gnam N, Suputtamongkol Y, Thamlikitkul V.** 1999. Epidemiology of arabinose assimilation in burkholderia pseudomallei isolated from patients and soil in Thailand. Southeast Asian J Trop Med Public Health **30:**756-759.

106. **Ngamdee W, Tandhavanant S, Wikraiphat C, Reamtong O, Wuthiekanun V, Salje J, Low DA, Peacock SJ, Chantratita N.** 2015. Competition between Burkholderia pseudomallei and B. thailandensis. Bmc Microbiology **15**.
107. **Nikolakakis K, Amber S, Wilbur JS, Diner EJ, Aoki SK, Poole SJ, Tuanyok A, Keim PS, Peacock S, Hayes CS, Low DA.** 2012. The toxin/immunity network of Burkholderia pseudomallei contact-dependent growth inhibition (CDI) systems. Mol Microbiol **84:**516-529.
108. **Schwarz S, West TE, Boyer F, Chiang WC, Carl MA, Hood RD, Rohmer L, Tolker-Nielsen T, Skerrett SJ, Mougous JD.** 2010. Burkholderia Type VI Secretion Systems Have Distinct Roles in Eukaryotic and Bacterial Cell Interactions. Plos Pathogens **6**.
109. **Van Zandt KE, Greer MT, Gelhaus HC.** 2013. Glanders: an overview of infection in humans. Orphanet J Rare Dis **8:**131.
110. **Wheelis M.** 1998. First shots fired in biological warfare. Nature **395:**213.
111. **Wheelis M, Rózsa L, Dando M.** 2006. Deadly cultures : biological weapons since 1945. Harvard University Press, Cambridge, Mass.
112. **Leitenberg M, Zilinskas RA, Kuhn JH.** 2012. The Soviet biological weapons program : a history. Harvard University Press, Cambridge, Massachusetts.
113. **Nock AM, Wargo MJ.** 2016. Choline Catabolism in Burkholderia thailandensis Is Regulated by Multiple Glutamine Amidotransferase 1-Containing AraC Family Transcriptional Regulators. J Bacteriol **198:**2503-2514.
114. **Wattam AR, Abraham D, Dalay O, Disz TL, Driscoll T, Gabbard JL, Gillespie JJ, Gough R, Hix D, Kenyon R, Machi D, Mao C, Nordberg EK, Olson R, Overbeek R, Pusch GD, Shukla M, Schulman J, Stevens RL, Sullivan DE, Vonstein V, Warren A, Will R, Wilson MJ, Yoo HS, Zhang C, Zhang Y, Sobral BW.** 2014. PATRIC, the bacterial bioinformatics database and analysis resource. Nucleic Acids Res **42:**D581-591.
115. **Schwartz HF, Mohan SK.** 2008. Compendium of onion and garlic diseases and pests, 2nd ed. American Phytopathological Society, St. Paul, MN.
116. **Gibney KB, Cheng AC, Currie BJ.** 2008. Cutaneous melioidosis in the tropical top end of Australia: a prospective study and review of the literature. Clin Infect Dis **47:**603-609.

CHAPTER 2:

Choline catabolism in *Burkholderia thailandensis* is regulated by multiple glutamine amidotransferase1-containing AraC-family transcriptional regulators

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2.1.1 Abstract

Burkholderia thailandensis is a soil-dwelling bacterium that shares many metabolic pathways with the ecologically similar, but evolutionarily distant, *Pseudomonas aeruginosa*. Among the diverse nutrients it can utilize is choline, metabolizable to the osmoprotectant glycine betaine and subseqeuntly catabolized as a source of carbon and nitrogen, similar to *P*. *aeruginosa*. Orthologs of genes in the choline catabolic pathway in these two bacteria showed distinct differences in gene arrangement as well as an additional orthologous transcriptional regulator in *B*. *thailandensis*. In this study, we showed that multiple glutamine amidotransferase1 (GATase1)-containing AraC-family transcription regulators (GATRs) are involved in regulation of the *B*. *thailandensis* choline catabolic pathway (*gbdR1*, *gbdR2*, *souR*). Using genetic analyses and sequencing the transcriptome in the presence and absence of choline, we identified the likely regulons of *gbdR1* (*BTH_II1869*) and *gbdR2* (*BTH_II0968*). We also identified a functional ortholog for *P*. *aeruginosa souR*, a GATR that regulates the metabolism of sarcosine to glycine. GbdR1 is absolutely required for expression of the choline catabolic locus, similar to *P*. *aeruginosa* GbdR, while GbdR2 is important to increase expression of the catabolic locus. Additionally, the *B*. *thailandensis* SouR ortholog (*BTH_II0994*) is required for catabolism of choline and its metabolites as carbon sources, whereas in *P*. *aeruginosa*, SouR function can by bypassed by GbdR. The strategy employed by *B*. *thailandensis* represents a distinct regulatory solution to control choline catabolism and thus provides both an evolutionary counterpoint and an experimental system to compare the acquisition and regulation of this pathway during environmental growth and infection.

2.1.2 Importance

Many proteobacteria that occupy similar environmental niches have horizontally acquired orthologous genes for metabolism of compounds useful in their shared environment. The arrangement and differential regulation of these components can help us understand both the evolution of these systems and the potential roles these pathways have in the biology of each bacterium. Here we describe the transcriptome response of *Burkholderia thailandensis* to the eukaryote-enriched molecule choline, identify the regulatory pathway governing choline catabolism, and compare the pathway to that previously described for *Pseudomonas aeruginosa*. These data support a multi-tiered regulatory network in *B. thailandensis*, with conserved orthologs in the select agents *Burkholderia pseudomallei* and *Burkholderia mallei*, as well as the opportunistic lung pathogens in the *Burkholderia cepacia* clade.

2.2 Introduction

Burkholderia thailandensis is a saprophytic, soil dwelling bacterium common in tropical and subtropical regions, and is an opportunistic pathogen of insects, plants, nematodes, and amoeba (1-3). *B. thailandensis* is used as a less virulent model for the select agents *B. pseudomallei* and *B. mallei*, the causative agents of melioidosis and glanders, respectively. The reduced virulence of *B. thailandensis* is primarily due to the absence of the major capsule locus important for *B. pseudomallei* virulence in mammals (4), although it is used to study the effect of type III section systems on phagocytic escape and retains other genes associated with virulence (5, 6). Despite virulence

differences, a great deal of the core genome is shared between *B*. *thailandensis* and its relatives, including many pathways for accessory metabolism.

Genes predicted to be involved in choline catabolism are found throughout the *B*. *pseudomallei* group (BPG) and *Burkholderia cepacia* complex (BCC) (7, 8). Many soil bacteria can use choline as a sole carbon and nitrogen source, and this catabolic pathway may be particularly important for bacteria associated with eukaryotes (9). Choline is part of the polar head group of both phosphatidylcholine and sphingomyelin, which together constitute the majority of lipids on the outer leaflet of eukaryotic cell membranes (10), and are also abundant in pulmonary surfactant (11). In addition to its role as a nutrient source, choline metabolism can generate glycine betaine (GB), an important osmoprotectant (12) and inducer of virulence factor production (13). The conversion of choline to GB has been shown to be important for *Escherichia coli* survival in urine (14) and *P*. *aeruginosa* survival in the mammalian lung (15, 16). Many organisms, including Burkholderiales, maintain an intracellular GB pool, potentially as a hedge against future osmotic stress (17). Choline can also be found in the rhizosphere, exuded from plant roots, likely to influence populations of bacteria in this environment (18). In fact, many plants accumulate high levels of GB as an osmoprotectant, notably beets from whence the appellation 'betaine' was derived (19). In the environment, secreted or decaying organic matter provides a metabolic opportunity that holds much promise to microbial opportunists poised to exploit them.

Our lab has previously studied the choline catabolic pathway and its regulation in *Pseudomonas aeruginosa* (20-22), and examination of orthologous genes in *B*. *thailandensis* suggested an alternate, perhaps more complex, regulatory strategy. In *P*.

aeruginosa, the critical regulator of GB catabolism is GbdR, a type-I glutamine amidotransferase (GATase1)-like AraC-family transcription regulator (GATR) that regulates the genes required for sequential demethylation of GB to glycine in *P. aeruginosa* (Fig 2.1A, left side) (22). In contrast to *P. aeruginosa*, *B. thailandensis* and other Burkholderiales appeared to have two GbdR orthologs (Fig 2.1B, C), whose co-occurrence and conserved synteny suggested that they could play non-overlapping roles in *B. thailandensis* choline catabolism. In this study, we have investigated the contributions of the two *B. thailandensis* GbdR orthologs to choline and GB catabolism and to global gene expression in response to choline. These data and follow-up analyses reveal that the choline catabolism pathway in *B. thailandensis*, while bearing many similarities to *P. aeruginosa*, contains key differences in organization and regulation.

2.3 Methods

2.3.1 Culture conditions.

Burkholderia thailandensis E264 (ATCC® 700388™) (23) cultures were inoculated from -80 ^{o}C glycerol stocks onto LB (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) with 1.5% agar and incubated at 37 ^{o}C overnight. For protein expression and molecular cloning, *Escherichia coli* DH5α or *E. coli* T7 express cells (New England BioLabs (NEB)) were then grown in LB broth containing 100 μg/mL carbenicillin, kanamycin, or trimethoprim (Tp), as appropriate.

2.3.2 Strain Construction.

Deletion strains (Table S1) were generated as described in Thongdee et al. and the resistance markers were removed using the methods described in Choi et al. (24, 25). Briefly, a PCR product was amplified via splice overlap extension PCR that consisted of a trimethoprim resistance marker amplified from pUC18mini-TN7T-Tp, including the flanking Flp recombinase sites, and with regions of homology upstream and downstream of the gene of interest (See primers in Table S2). The resultant PCR product was then used to naturally transform *B. thailandensis* that had been grown in defined media (DM) as previously described [24]. To remove the resistance marker, pFLPe2 was electroporated into the Tp resistant strain and plated onto low-salt LB (LSLB: 10 g/L tryptone, 5 g/L yeast extract, 3 g/L NaCl, 1.5% agarose, pH 7.5 with 250 µg/mL zeocin and 0.2% L-rhamnose for Flp recombinase expression). Cells were cured of pFLPe2 by growth at 42 ^{o}C. Resultant colonies were streaked onto plates with zeocin, Tp, or without antibiotics to identify colonies where the trimethoprim marker and pFLPe2 had been lost. The Δ*gbdR1*, Δ*gbdR2,* and *ΔsouR* phenotypes were complemented by cloning each gene and ~250 bps of the upstream regulatory region into pUC18mini-TN7T-Zeo and integration into one of the two *att*Tn7 sites in *B. thailandensis* (26, 27) (Table 2.S1). Appropriate control strains were generated by *att*Tn7 integration with the empty pUC18mini-TN7T-Zeo vector (Table 2.S1).

2.3.3 Growth assays.

Growth assays were performed by starting overnight cultures in MOPS minimal media supplemented with 20 mM pyruvate and 5 mM glucose incubated at 37 ^{o}C (28). Cultures were diluted in MOPS media to an OD_{600} of 0.07 and 30 µL were used to

inoculate 470 µL of MOPS media supplemented with carbon sources as described in a flat bottom 48-well plate (Costar). These plates were incubated at 37 ^{o}C with agitation and OD_{600} measured using a Biotek Synergy 2 plate reader.

2.3.4 Alignments and phylogenetic tree construction.

Sequence alignments and phylogenetic analysis were preformed using MEGA version 6 (29). Amino acid sequences of the relevant GATRs were aligned using MUSCLE (30). The *ftrA* sequence, a GATR homolog from *Escherichia coli*, was included as an outgroup to root the phylogenetic tree.

2.3.5 RNA-Seq.

For RNA-Seq, wild type, Δ*gbdR1*, Δ*gbdR2*, and Δ*gbdR1*Δ*gbdr2* strains were streaked from frozen stocks onto LB agar plates and incubated overnight at 37 ^{o}C. These plates were used to inoculate 3 mL MOPS with 20 mM pyruvate and 5 mM glucose cultures which were incubated for 18 hours at 37 ^{o}C on a rotary wheel. To initiate the experiment, 1 mL of these cultures was added to 2 mL of pre-warmed MOPS with 30 mM pyruvate or 2 mL MOPS with 30 mM pyruvate and 1.5 mM choline; resulting in final concentrations of 20 mM pyruvate and 1 mM choline. These cultures were incubated for four hours at 37 ^{o}C. Cells from 1.5 mL of culture were collected by centrifugation and the resultant pellets were resuspended in RNA Protect Bacteria Reagent (Qiagen) and incubated at room temperature for 10 minutes. Cells were collected by centrifugation and resuspended in 50 µL 10 mM Tris, 1 mM EDTA (TE) buffer with 3 mg/mL lysozyme and incubated at room temperature for 5 minutes. To each of these

resuspensions, 1 µL 20 mg/mL proteinase K, 0.5 µL of 10% SDS, 1 µL of DNase I (RNase-free, Ambion), and 2 µL of 50 mM $MgCl_2$ were added and samples were incubated for 5 minutes at room temperature. RNA was then prepared using the Qiagen RNeasy kit according to the manufacturer's instructions. Eluate from this purification was further treated with 11 µL 10x DNase I buffer and 2 µL DNase I (RNase-free, Ambion) and incubated at 37 ^{o}C for 20 minutes to remove contaminating DNA and re-purified using the Qiagen RNeasy protocol with the optional on-column DNase I step. The resulting purified RNA was assessed for purity by PCR and quantified by bioanalyzer chip, depleted of 16S and 23S rRNA by using MICROBExpress™ Bacterial mRNA Enrichment (Ambion), and subsequently reassessed by Bioanalyzer (Agilent Technologies). RNA-Seq DNA libraries were prepared by the Vermont Cancer Center – College of Medicine Massively Parallel Sequencing Facility using an Illumina TruSeq Stranded Total RNA Library Prep Kit and were run on an Illumina Hi-Seq 1500 to generate single end reads. Read quality was checked with FastQC (31). Samples were aligned to the *B. thailandensis* E264 chromosomes I (NC_007651) and II (NC_007650) using BWA (32), duplicate reads were marked using Picard (version 1.110; Broad Institute of MIT and Harvard, [http://broadinstitute.github.io/picard/]) , and gene counts were calculated using summarizeOverlaps from the GenomicRanges package (33) before differential expression was called using the DESeq package (34) in R (version 3.0.1; R Development Core Team, [https://www.r-project.org/]) with assistance from the University of Vermont College of Medicine Bioinformatics Shared Resource group. RNA-Seq data has been deposited in NCBI GEO under record GSE81652.

2.3.6 qRT-PCR to confirm RNA-Seq findings.

Wild-type, Δ*gbdR1*, Δ*gbdR2*, and Δ*gbdR1*Δ*gbdr2* strains were grown and induced as described for the RNA-Seq experiment. RNA was extracted similarly to the RNA-Seq, except that a third DNase I treatment was performed using the Ambion DNA-free kit (AM1906) as per the manufacturer's protocol. cDNA was generated using Superscript IV First-Strand Synthesis System (Invitrogen) using 24 ng of RNA combined with the 5'-NSNSNSNSNS-3' primer previously described (35). qPCR was performed as described in Willsey et al. (36). Briefly, 0.2x SYBR Green I nucleic acid gel stain (Thermo Fisher Scientific (TFS)) was used with Q5 High-Fidelity 2x master mix (NEB) and amplified using cycle conditions of 98 ^{o}C for two minutes, 98 ^{o}C for 20 seconds, annealing at 60 ^{o}C for 30 seconds, and extension at 72 ^{o}C for 20 seconds repeated by going back to the second step 39 times. Reactions were performed in technical duplicate and biological triplicate with RT primers designed for *BTH_I1140* (*rplU*), *BTH_II1869* (*gbdR1*), *BTH_II1861* (*gbcA*), *BTH_II1853* (putative porin), *BTH_II0968* (*gbdR2*), *BTH_II0966* (*cbcV*), *BTH_II0994* (*souR*), and *BTH_II0996* (*soxB*). A dilution series was used to generate a standard curve for each primer set. Each reaction was normalized to its respective *rplU* value, and then induced values were divided by uninduced values to derive a fold effect value.

2.3.7 Purification of GbdR1, GbdR2, and SouR.

gbdR1, *gbdR2*, and *souR* were each cloned into the pMAL-C2X expression vector generating an N-terminal maltose binding protein tag. The constructs were transformed

into *E. coli* T7 Express, transformants selected on LB agar + 100 μg/mL carbenicillin, and positive colonies used to inoculate 20 mL cultures of LB + 100 μg/mL carbenicillin. After 18 hours, these cultures were used to inoculate 500 mL flasks of LB + 100 μg/mL LB carbenicillin, grown to an OD_{600} of 0.3, and induced for 4 hours at 30 ^{o}C with 500 μM IPTG. Cells were collected by centrifugation. Cells were then washed, collected by centrifugation, and the resulting cell pellets were flash frozen in liquid nitrogen. Cell pellets were resuspended on ice in 10 mL of chilled chromatography and lysis buffer (20 mM Tris pH 7.4, 500 mM NaCl, 20 mM imidazole, 1x HALT protease inhibitor (TFS)). 10 μL DNase I was added to the resuspended cell pellet, and the mixture was incubated for 10 minutes on ice. The resuspended cells were lysed with a French cell press using three 1100 PSI passages and NP-40 was then added to a final concentration of 0.05%. The lysate was clarified by centrifugation for 15 minutes at 15,000 rpm at 4 ^{o}C. The supernatant was passed over a column containing 1 mL bed volume of amylose resin beads (NEB). The column was then washed with 10 mL of column buffer 300 (20 mM Tris pH 7.4, 300 mM KCl, 1 mM EDTA, 0.05% NP-40, 0.7 μL/mL β-mercaptoethanol), then 10 mL column buffer 150 (20 mM Tris pH 7.4, 150 mM KCl, 1 mM EDTA, 0.05% NP-40, 0.7 μL/mL β-mercaptoethanol). Protein was eluted in column buffer 150 containing 10 mM maltose and twelve 300 μL fractions were collected. Fractions were analyzed by SDS-PAGE acrylamide gels stained with Coomassie. Since GATR proteins tend to precipitate at higher concentrations, fractions of lower concentration were pooled and dialyzed in Slide-A-Lyzer® Dialysis Cassette G2 (TFS) against 2 L column buffer 150. Aliquots were flash frozen in liquid nitrogen and stored at -80 ^{o}C.

2.3.8 Electrophoretic mobility shift assays (EMSA).

Biotinylated or unlabeled primers were used to generate DNA probes from appropriate templates containing the putative promoter regions using Q5® High Fidelity DNA polymerase (NEB), then subjected to QIAquick PCR Purification Kit (Qiagen) and quantified using a Nanodrop 1000 (TFS). 20 µL reactions were assembled using 2x binding buffer (10 mM Tris pH 7.4, 200 mM KCl, 2 mM EDTA, 2 mM DTT, 10% glycerol, and 200 µg/µL bovine serum albumin (37)), 5 ng/µL poly (dI-dC), 25-75 nM purified MBP-tagged protein, 1 nM biotinylated DNA probe, and optionally 20 nM unlabeled competitor DNA probe. Reactions were incubated in a 30 ^{o}C waterbath for 20 minutes, then 5 µL 5x EMSA loading buffer (1x TBE, 20% glycerol, 0.01% xylene cyanol, 0.01% bromophenol blue) was added and samples were loaded onto a 5% acrylamide 0.5x TBE gel. Gels were run for 1 hour at 100 volts then transferred to a BioDyne-B nylon membrane (Pierce) for 1 hour at 80 volts in 0.5x TBE at 4 ^{o}C. DNA was crosslinked to the membrane using a UV Stratalinker 2400 (Stratagene) on the auto crosslink setting, and the biotin-labeled probe visualized using the Pierce LightShift Chemiluminescent EMSA Kit (TFS) and imaged on a ChemiDoc XRS+ molecular imager (Bio-Rad). Density of resulting bands was quantified using Quantity One software from Bio-Rad.

2.3.9 Generation of reporter constructs and β-galactosidase assays.

Yeast homologous recombination was used to replace the kanamycin resistance gene in the shuttle vector pMQ131 (38) (*apaHA3*) with the Tp resistance gene (*dhrFII*)

resulting in pAN1. Yeast recombination was also used to replace the *lacZ*α fragment with the full *lacZYA* operon resulting in pAN7. The intergenic region between *gbdR1* and *glyA*, as well as truncations of this region, were ligated into the SphI site proximal to the *lacZYA* ribosomal binding site, and transformants selected on LB agar + 100 μg/mL Tp at 37 ^{o}C. Tp resistant colonies were used to inoculate 3 mL MOPS media with 20 mM pyruvate, 5 mM glucose, and 100 μg/mL Tp, which were incubated for 48 hours at 37 ^{o}C. Cells from these cultures were collected by centrifugation at 13000 rpm for 2 min at room temperature, washed with 1 mL MOPS media, collected again by centrifugation and the pellets resuspended in MOPS media. 30 μL of this resuspension was used to inoculate each well in a 48-well plate that contained 470 μL MOPS media with 20 mM pyruvate and with or without 1 mM choline. These cultures were incubated for 48 hours at 37 ^{o}C on an orbital shaker at 170 rpm in a humidified chamber. The extended induction time was shown to be necessary for detectable β-galactosidase activity empirically in our hands, and as indicated by Kang et al. (39). β-galactosidase activity was quantified as previously described (21).

2.4 Results

2.4.1 Organization of predicted *B. thailandensis* orthologs of *P. aeruginosa* choline catabolic genes.

The choline catabolic pathway and its regulation in *P. aeruginosa* have been described previously (20, 40). Briefly, in *P. aeruginosa* the TetR family transcription factor BetI represses expression of *betIBA* and is derepressed by choline, allowing production of the BetA and BetB enzymes that to convert choline into GB (41-43). The

GATase1-like AraC-family transcription regulator (GATR) GbdR responds to GB and dimethylglycine (DMG) by inducing expression of catabolic genes including *gbcA-B*, the *dgc* operon (*PA5376*, *PA5377*, *dgcA*, *dgcB*, *PA5400*, *PA5401*), and the sarcosine oxidase genes, which together contribute to the sequential demethylation of GB to DMG, sarcosine, and finally glycine (Fig 2.1A,C). SouR, also a GATR, controls transcriptional induction of the sarcosine oxidase genes in response to sarcosine (36). Homologs of *betI* and *betBA* in *B*. *thailandensis* have been described previously (39).

Sequence analysis revealed two likely GbdR orthologs in *B*. *thailandensis*, BTH_*II1869* (*gbdR1*) and BTH_*II0968* (*gbdR2*), with strong amino acid sequence homology to *P*. *aeruginosa* GbdR (48.2% and 47.0% identity and 75.5% and 71.3% similarity, respectively, using BLOSUM50 along the entire protein length). Alignments of the characterized *P*. *aeruginosa* GATRs (ArgR, CdhR, SouR), and their putative *B*. *thailandensis* orthologs, revealed that the *B*. *thailandensis* GbdR orthologs were more similar to each other than to *P*. *aeruginosa* GbdR or any other GATRs (Fig 2.1B). This suggested that they might also have similar functions or play separate roles within the same catabolic pathway. The presence of a more distantly related GATR divergently transcribed from the sarcosine oxidase operon (Fig 2.1B, C), led us to predict that *BTH_II0994* was a functional ortholog of *P*. *aeruginosa* SouR, though the phylogenetic tree suggests that they share no recent paralogy.

On the *P*. *aeruginosa* chromosome, *gbdR* is located one gene away from the *cbcXWV* transport genes (Fig 2.1C), previously implicated in the transport of choline and its immediate metabolites into the cytosol from the periplasm (44), and adjacent to the *sdaB* gene known to participate in the pathway (45). In *B*. *thailandensis*, *gbdR1* is located

adjacent to a putative operon containing the bulk of predicted orthologous genes encoding enzymes needed for GB catabolism to sarcosine, while *gbdR2* is present within a locus containing the putative *cbcXWV* orthologs (Fig 2.1C). The homology of GbdR1 and GbdR2 with *P. aeruginosa* GbdR, combined with their chromosomal locations adjacent to genes involved in choline metabolism, strongly suggested that both transcription factors were involved in the regulation of this pathway. The synteny of the *gbdR1* and *gbdR2* loci in *B. thailandensis* E264 is conserved throughout the various clades of Burkholderiales, including species such as *B. pseudomallei*, *B. mallei*, *B. cenocepacia*, *B. ambifaria*, and *B. multivorans*. This conservation in synteny suggests that both GbdR1 and GbdR2 play roles in the regulation of choline metabolism in *Burkholderia*.

2.4.2 *gbdR1* and *gbdR2* contribute differentially to choline catabolism.

B. thailandensis, like *P. aeruginosa* (46), can use choline as a sole source of carbon and/or nitrogen (Fig 2.2A). Based on our predictions of *gbdR1* and *gbdR2* involvement in choline metabolism we tested the ability of each single deletion strain and the double deletion strain to grow using choline as a sole carbon source. Wild type *B. thailandensis* grows on choline as a sole carbon source, while a Δ*gbdR1* or Δ*gbdR1*Δ*gbdr2* strain cannot (Fig 2.2A, right side). Deletion of *gbdR2* alone resulted in reduced growth on choline as a sole carbon source (Fig 2.2A, right side). On plates, none of the manipulations appeared to alter growth on pyruvate as a sole carbon source (Fig 2.2A, left side). The impaired but visible growth of the Δ*gbdR2* strain led us to examine the growth kinetics of these strains on choline and its downstream metabolites GB and

DMG. Deletion of *gbdR2* results in slower growth than wild type on choline, GB, and DMG, comprised of both an extended lag phase and a lower growth rate during exponential phase (Fig 2.2B). Deletion of these transcription factors did not substantially alter growth kinetics using pyruvate as a sole carbon source, but did alter maximal yield in liquid culture in the double deletion strain (Fig 2.2B), although this difference was not apparent on solid media (Fig 2.2A, left side). The Δ*gbdR1* and Δ*gbdR1*Δ*gbdr2* strains showed no measurable growth on any of the choline-related metabolites tested within 100 hours.

Integration of *gbdR1* at the *att*Tn7 site restored growth of a Δ*gbdR1* strain on choline, GB, and DMG (Fig 2.3A). Growth in GB and DMG was very similar to wild type, while growth of the complement strain on choline exceeded that of wild type on choline (Fig 2.3A). Similarly, integration of *gbdR2* at the *att*Tn7 site restored growth of a Δ*gbdR2* strain on choline-related carbon sources and resulted in more rapid growth than in wild type for choline, GB, and DMG (Fig 2.3B). We also tested complementation of Δ*gbdR1*Δ*gbdR2* with either *gbdR1* or *gbdR2*. The Δ*gbdR1*Δ*gbdr2 att*Tn7::*gbdR1* strain was able to grow on choline as a sole carbon source in MOPS minimal media, though not at wild type levels, whereas Δ*gbdR1*Δ*gbdr2 att*Tn7::*gbdR2* was not capable of growth on choline (Fig 2.S1). Taken together, these data suggest that *gbdR1* and *gbdR2* regulate the choline catabolic pathway, with *gbdR1* absolutely required and *gbdR2* playing an accessory role.

2.4.3 GbdR1 and GbdR2 regulate transcription of genes involved in the choline catabolic pathway.

To identify the GbdR1 and GbdR2 regulons, we exposed our deletion strains to minimal media with pyruvate as a primary carbon source and with or without 1 mM choline, and analyzed the transciptomes by RNA-seq. Exposure of wild-type *B. thailandensis* to choline resulted in 57 transcripts being induced or repressed more than 2.3 fold, many predicted to be involved in quaternary amine catabolism and transport (Table 2.1). A prominent cluster of induced genes from *BTH_II1868* to *BTH_II1853* includes orthologs to the P. *aeruginosa glyA* (*BTH_II1868*), the *dgc* operon (*BTH_II1867*, *BTH_II1866*, *BTH_II1865*, *BTH_II1864*, *BTH_II1863*, and *BTH_II1862*), *gbcA* (*BTH_II1861*), *gbcB* (*BTH_II1860*), and betX (*BTH_II1859*). Based on these predictions, the putative operon consisting of *glyA* to *BTH_II1858* (Fig 2.1C) encodes the genes likely to be responsible for the breakdown of GB to sarcosine. Also induced was a nearby predicted operon consisting of *BTH_II1855- BTH_II1853*, encoding likely orthologs of *P. aeruginosa betC*, *cosX*, and a predicted porin. Another GATR, *BTH_II0994*, and the divergently transcribed operon consisting of *BTH_II0995* (*sdaA-2*), *BTH_II0996* (*soxB*), *BTH_II0997* (*soxD*), *BTH_II0998* (*soxA*), *BTH_II0999* (*soxG*), and *BTH_II1000* were also induced and we hypothesized that these are the *B. thailandensis souR* and the sarcosine oxidase genes responsible for the breakdown of sarcosine to glycine (36).

Homologs of *cbcXWV* in *P .aeruginosa*, an operon important for the transport of choline, are present in *B. thailandensis* and are also induced by choline. The *gbdR2* gene is situated in this operon, between *BTH_II0967* (*cbcW*) and *BTH_II0969* (*cbcX*). The aforementioned genes are induced by choline in the wild type strain, but are not in either Δ*gbdR1* or Δ*gbdR1*Δ*gbdr2*, suggesting an absolute requirement of *gbdR1* in the

regulation of these genes. Cells lacking *gbdR2* express all of the genes in the major catabolic operon, *glyA-BTH_II1858*, in response to choline but to a lower level than the wild type. Interestingly, the relative induction of the transcripts in the transport operon containing *gbdR2* are unaffected by the absence of *gbdR2*, suggesting that there is no autoregulation of the *gbdR2* operon.

To confirm our RNA-Seq results, we preformed qRT-PCR on a subset of induced genes and the transcription factors of interest, normalized to *rplU* transcript levels (Fig S2). The wild type *B*. *thailandensis* response to choline in our qRT-PCR experiment reflected our RNA-Seq results. *gbcA*, *cbcV*, and *soxB* are all induced in response to choline, suggesting overall induction of their putative operons. The qRT-PCR data also shows that the GATRs *gbdR1* and *souR* transcripts were slightly induced in response to choline while *gbdR2* was induced at a higher level. This slight increase in *gbdR1* expression observed may be negligible and therefore it is unclear if induction of *gbdR1* is a mechanism by which GbdR2 regulates choline catabolism. Overall, we interpret the matching trends in expression between the RNA-Seq and qRT-PCR experiments as a validation of our findings.

2.4.4 The *glyA* promoter is induced by choline under the control of GbdR1 and GbdR2.

pAN27 is a reporter plasmid containing the full 408 bp intergenic region between *gbdR1* and *glyA*, such that the *glyA* promoter drives *lacZYA* expression. This reporter was used to assess the transcriptional control of the *glyA* promoter. This putative promoter likely controls the expression of *BTH_II1868* to *BTH_II1857* and thus governs the bulk

of genes needed to convert GB to sarcosine. In a *B*. *thailandensis* wild-type background, pAN27 *lacZ* expression is induced by choline, GB, and DMG, but not ethylcholine (Fig 2.4A). Ethylcholine is a non-metabolizable inducer of GbdR-dependent transcription in *P*. *aeruginosa* (40). The inability of this choline analog to elicit similar effects in a GbdR/GbdR2 dependent promoter suggests specificity differences between GbdR and GbdR1 or GbdR2, a metabolite transport difference, or a difference in the specificity of choline oxidase. No β-galactosidase activity was detected when pAN27 in *B*. *thailandensis* was induced using 1 mM sarcosine, similar to findings in *P*. *aeruginosa* for *gbdR*-dependent induction of *choE*, a choline esterase (40), or for the phospholipase *plcH* (47, 48) (data not shown).

Δ*gbdR1*, Δ*gbdR2*, and Δ*gbdR1*Δ*gbdr2* deletion strains carrying pAN27 failed to show significant choline-induced β-galactosidase induction when compared to the wild type control (Fig 2.4B), and basal expression was also low. This suggests that both *gbdR1* and *gbdR2* are required for robust induction of the *glyA* promoter. We would have predicted that Δ*gbdR2* would produce some β-galactosidase activity in response to choline, as Δ*gbdR2* can use choline as a sole carbon source (Fig 2.2). The incongruity may be due to the unusually long time it takes to observe β-galactosidase activity in *B*. *thailandensis*, combined with the long delay in growth of Δ*gbdR2* on choline (Fig 2.2). As a follow-up experiment, we transformed pAN27 into a Δ*souR* to determine if SouR is required for *glyA* promoter induction in *B*. *thailandensis* (Fig 2.S3). β-galactosidase activity in Δ*souR* in response to choline is robust, higher than in wild type, potentially due to blockage of the catabolic pathway and subsequent buildup of intermediate metabolites. This indicates that SouR is not required for induction of *glyA*, but could also

point to positive feedback control between sarcosine metabolism and regulation of upstream steps.

Reporter plasmids derived from pAN27 with progressively smaller portions of the intergenic region between *gbdR1* and *glyA* were produced (-340, -251, -147, -85 with respect to the *glyA* putative translational start site) in order to map the choline-dependent portion of the *glyA* regulatory region. Exposure of cells containing these constructs to choline resulted in choline-dependent induction of β-galactosidase activity for -340 and -251, but not -147 or -85 (Fig 2.4C). This suggests that the region from -251 bp to the translation start site of *glyA* is necessary for a response to choline.

2.4.5 GbdR1 and GbdR2 bind directly to the *glyA* promoter.

N-terminally tagged MBP-GbdR1 and MBP-GbdR2 were produced and assayed by EMSA using biotinylated DNA probes representing sections of the intergenic region between *gbdR1* and *glyA*. Probe 1 is 216 bp covering the -320 to -104 bp region with respect to the *glyA* translational start site, while probe 2 is 178 bp covering the -160 to +18 region. MBP-GbdR1 could bind probe 1 in a concentration-dependent manner, and binding to biotinylated probe 1 could be significantly competed with unlabeled probe 1 (Fig 2.5A). MBP-GbdR1 did not interact with probe 2 (right-most lane, Fig 2.5A), and unlabeled probe 2 was markedly less able to compete with MBP-GbdR1 binding to labelled probe 1. These data support a GbdR1 binding site within probe 1. Similar results were obtained using MBP-GbdR2, which was also able to bind to probe 1, not probe 2, and could be similarly competed from labeled probe 1 using unlabeled probe 1 (Fig 2.5B), and again much less so with the unlabeled probe 2. The shift produced by MBP-

GbdR2 consists of multiple bands that are likely the result of oligomerization. Results using 6xHis-N-terminally tagged versions of GbdR1 and GbdR2 produced the same results and banding patterns, although the solubility of the 6-His version was much lower than the MBP fusions (data not shown). These results provide evidence that both GbdR1 and GbdR2 can both bind to the *glyA* promoter. When combined with results from our reporter assays (Fig 2.4), we infer that the binding site for GbdR1 and GbdR2 is likely to be contained within the -251 to -160 bp region with respect to the *glyA* translational start site. As we have previously shown for *P*. *aeruginosa* GbdR and SouR, addition of their presumptive ligand did not impact binding to DNA (data not shown).

2.4.6 *B*. *thailandensis* SouR regulates sarcosine metabolism.

Sarcosine is a downstream metabolite of choline and orthologs of *P*. *aeruginosa* sarcosine oxidase genes were identified in our RNA-Seq results (Table 2.1, Fig 2.1C). *P*. *aeruginosa* uses sarcosine as a sole carbon and nitrogen source and this is regulated by the transcription factor, SouR (36). While *B*. *thailandensis* can use choline as a sole carbon or sole carbon and nitrogen source, *B*. *thailandensis* is unable to grow on sarcosine as a sole carbon source, or as a sole carbon and nitrogen source (Fig 2.S4). We hypothesize that the ability of *B*. *thailandensis* to transport extracellular sarcosine may be limited in comparison with *P*. *aeruginosa*, and the rate of import is insufficient to support growth as a sole carbon source. It is, however, able to utilize sarcosine as a sole nitrogen source when pyruvate is available as a primary carbon source, and does so in a SouR dependent manner (Fig 2.6A). Deletion of *gbdR1* or *gbdR2* has no major effect on the ability of *B*. *thailandensis* to utilize sarcosine as a nitrogen source, but Δ*souR* does show

a reduced level of growth.

BTH_II0994 is a GATR divergently transcribed from the sarcosine oxidase operon and we hypothesized that it might be functionally orthologous to *souR* in *P*. *aeruginosa* (Fig 2.1C), despite BTH_II0994 and SouR not sharing a high degree of similarity when compared to the other GATR orthologous pairs (Fig 2.1B). To test if *BTH_II0994* was a functional *souR*, we generated a *B*. *thailandensis* Δ*souR* strain and used it to determine if *souR* is required for growth on choline. Δ*souR* fails to grow on choline, GB, or DMG as a sole carbon source (Fig 6B). This is contrary to regulation in *P*. *aeruginosa*, where choline, GB, and DMG can still be used as sole carbon sources for growth in the absence of *souR*, although at a diminished growth rate (36). This result also suggests that GbdR1 and GbdR2 are insufficient to cause induction of the sarcosine oxidase genes by themselves or together, contrary to the regulation scheme in *P*. *aeruginosa* (Fig 2.1A). This phenotype can be complemented by chromosomal integration of *souR* at an *att*Tn7 site under the control of its native promoter (Fig 2.6B).

MBP-SouR was produced and its DNA binding assessed using EMSA with a biotinylated 230 bp oligo designated probe 3, covering the region -208 to +22 base pairs relative to the putative translation start site of *sdaA-2*, the first gene in the likely sarcosine oxidase operon of *B*. *thailandensis*. MBP-SouR was able to bind the biotinylated probe 3, but not the probe 2 negative control. The binding of MBP-SouR to Biotinylated probe 3 could be competed off with unlabeled probe 3, but not unlabeled probe 2, suggesting that this interaction is specific (Fig 2.6C). The binding of MBP-SouR to this putative promoter region coupled to the phenotype of the Δ*souR* strain suggests that SouR is responsible for the regulation of the sarcosine oxidase genes in *B*. *thailandensis*.

2.5 Discussion

The ability to utilize GB as a compatible solute, providing protection against osmotic and other stresses without creating significant disruption of normal cell processes, is nearly ubiquitous among bacteria, however the ability to catabolize GB is not as widespread (49). *B*. *thailandensis* and other species in this genus possess the enzymes for utilizing choline and its metabolites as carbon and nitrogen sources, taking advantage of this widely available biomolecule as befits their description as metabolically adaptable bacteria. *P*. *aeruginosa*, another soil-dwelling and metabolically adaptable microorganism, shares this ability and was our original model for the study of the GB catabolic pathway and its regulation (20). *B*. *thailandensis* and *P*. *aeruginosa* inhabit similar environments, possess similarly sized genomes, encode diverse metabolic pathways, and are thus considered to have similar generalist strategies, despite *B*. *thailandensis* and *P*. *aeruginosa* belonging to the β-proteobacteria and γ-proteobacteria classes, respectively. The metabolic enzymes in the choline catabolic pathway are well conserved and the orthologous genes in different species are readily identifiable. However, the identification of *gbdR2* and the alternate gene organization in *B*. *thailandensis* prompted us to ask if the choline regulatory network was as well conserved. We show here that *B*. *thailandensis* uses an alternate regulatory solution to control this pathway compared to *P*. *aeruginosa*. Both *B*. *thailandensis* and *P*. *aeruginosa* can store pools of GB (17) and metabolize it as a carbon and nitrogen source, therefore the different regulatory networks suggest evolution of alternate strategies to control the decision to store or catabolize.

Although the data presented here indicates that choline catabolism is regulated differently in *B*. *thailandensis* than in *P*. *aeruginosa* (Fig 1A), the basic components of the pathway are conserved and thus the overall scheme of the pathway is the same. In both organisms, choline catabolism begins with choline-dependent de-repression of the catabolic genes responsible for the conversion of choline to GB, *betBA*, mediated by the choline-sensing TetR-family transcription factor BetI. These genes have been described previously (41), and evidence suggests they function in *B*. *thailandensis* as they do in *P .aeruginosa* (39). In *P*. *aeruginosa* GB is subsequently metabolized to DMG by GbcA and GbcB heterodimer (50, 51), and from DMG to sarcosine by enzymes in the *dgc* operon (52), all under the regulation of GbdR (20). In *B*. *thailandensis* the same enzymatic steps are under the control of GbdR1, modulated by GbdR2. Sarcosine is then demethylated to glycine by components of the sarcosine oxidase operon and *glyA1*, controlled in tandem by GbdR and SouR in *P*. *aeruginosa*, and controlled separately by SouR, and GbdR1 and GbdR2 respectively in *B*. *thailandensis* as described below.

In this study we determined that both GbdR1 and GbdR2 participate in the regulation of GB catabolism, and characterized their regulons. Both regulators are GATRs with strong amino acid sequence similarity to GbdR in *P*. *aeruginosa*, and deletion strains confirmed that both play roles in the regulation of choline catabolism and catabolism of its downstream metabolites (Fig 2, 3). Combining the transcriptional data with the growth phenotypes of the deletion strains supports *gbdR1* as required for expression of the major catabolic cluster of genes and the transport operon that includes *gbdR2*. *gbdR2*, on the other hand, is important for a robust response to choline, enhancing expression of many genes required to import and catabolize choline and its metabolites,

particularly the major catabolic cluster (Fig 2.1C, Table 2.1). The operon containing *gbdR2* and the putative *cbcWVX* orthologs, is likely regulated solely by GbdR1. Although the presence of *gbdR1* is sufficient for growth (Fig 2.2A-B), reporter assays and growth studies showed that both *gbdR1* and *gbdR2* are required for a robust transcriptional response (Fig 2.4A) and growth on choline (Fig 2.2). In agreement with promoter mapping results (Fig 2.4C), EMSA data suggests that GbdR1 and GbdR2 likely share a similar binding site, raising the question of whether or not they compete for the same binding site or act synergistically. A possibility is that GbdR1 and GbdR2 possess differential DNA binding K_d values that result in differential response to choline and its metabolites under different physiological conditions.

In addition to *gbdR1* and *gbdR2*, our transcriptome analysis revealed a third GATR induced in response to choline, *BTH_II0994*, which we confirmed as a functional *souR* ortholog in *B*. *thailandensis*. In *P*. *aeruginosa*, SouR regulates the expression of the sarcosine oxidase catabolic operon, the components of which are responsible for converting sarcosine to glycine (36). SouR in *B*. *thailandensis* is less similar to GbdR, GbdR1, and GbdR2 than is the SouR in *P*. *aeruginosa* (Fig 1B). *B*. *thailandensis souR* (*BTH_II0994*) is divergently transcribed from the sarcosine oxidase operon, whereas *P*. *aeruginosa souR* (*PA4184*) is distantly located and part of a two gene operon. These factors suggest a divergence in how *souR* was acquired or evolved in both organisms. The inability of *B*. *thailandensis* to grow on sarcosine as a sole carbon source was surprising given that choline is readily catabolized and the enzymes for sarcosine catabolism are expressed (Fig 2.6A). We found that *souR* in *B*. *thailandensis* is required for sarcosine catabolism, unlike in *P*. *aeruginosa* where GbdR can induce the sarcosine

oxidase operon in the absence of *souR* (36). The complementation and EMSA for the sarcosine oxidase operon putative promoter provides further evidence that *BTH_II0994* is indeed the functional *souR* ortholog in *Burkholderia* (Fig 2.6B, C). It is not clear if SouR is capable of autoregulation of *souR* or if GbdR1 controls its expression directly.

GbdR1, GbdR2, and SouR are all GATRs implicated in the regulation of genes involved in the catabolism of quaternary amines or their metabolites. GATRs are members of the AraC transcription factor family, grouped by their canonical C-terminal DNA binding domain, but unlike classic AraC proteins they contain a GATase1-like N-terminal domain. The GATase1-like domain is characterized by its homology to class-I glutamine amidotransferases, which bind glutamine or ammonia and participate in the amidation/deamidation reaction (53, 54). Many of the GATR N-termini, notably excluding SouR in *P. aeruginosa*, also retain a bioinformatically identified cysteine residue that would be part of the functional catalytic triad of GATase1 family enzymes. The N-terminal domain of AraC proteins is typically involved in ligand binding and dimerization, which is a characteristic important for their functionality, and dimerization is often affected by the binding of ligand (55). *P. aeruginosa* possesses other GATRs implicated in the catabolism of N-substituted amines including *argR*, the regulator of arginine catabolism (56), and *cdhR*, the regulator of carnitine catabolism (20). Putative *argR* and *cdhR* orthologs are also present in *B. thailandensis* (unpublished data). We hypothesize that the GATase1-like domain has been combined with the AraC-style DNA binding domain to enable detection of charged amine-containing compounds and that GATRs as a whole may represent a sub-family of transcription factors that regulate metabolism of accessory nitrogen sources, including choline and its metabolites.

The transport of choline and derivative molecules also appears to be handled differentially between *B*. *thailandensis* and *P*. *aeruginosa* based on genomic information. *P*. *aeruginosa* possesses an array of BCCT and ABC-family transporters that have been implicated in choline and GB transport that are effective under different osmotic conditions or primarily utilized when choline is in such abundance that it can be used as a carbon or nitrogen source (44). *B*. *thailandensis* possesses fewer transporters, with only one putative homolog of the BCCT-family transporter (*BTH_II1109*), compared to the three in *P*. *aeruginosa* (*betT1*, *betT2*, *betT3*). *B*. *thailandensis* possess a putative amino acid permease, *BTH_II1858*, as part of the major GB catabolic operon with no obvious ortholog in *P*. *aeruginosa*. The placement of *BTH_II1858* and conserved synteny among *Burkholderia* suggests a role as a transporter for choline or its derivatives, but there is no functional evidence as of yet. Some Pseudomonads have a putative ortholog of *BTH_II1858* identified as an ethanolamine transporter, ethanolamine being similar to choline in its role as a head group for phospholipids, suggesting the possibility of a link to general fatty acid metabolism. Both *B*. *thailandensis* and *P*. *aeruginosa* also possess orthologs of the choline/GB transporter, *opuC* that was described in *P*. *syringae* and found to function under hyperosmolar conditions (57). *B*. *thailandensis* orthologs to many of the *P*. *aeruginosa* periplasmic binding proteins that mediate ABC-transporter dependent import of quaternary amines (*cbcX* (*BTH_II0969*), *caiX* (*BTH_II1849*), *betX* (*BTH_II1859*), *cosX* (*BTH_II1853*)), but these are apparently not sufficient for efficient sarcosine transport (Fig 2.S4). This result is perhaps not surprising as limited current data suggest that sarcosine does not compete well with choline for these transporters in *P*. *aeruginosa* (58). To date the sarcosine transporter has not been identified, and the

functional differences between *P*. *aeruginosa* and *B*. *thailandensis* for sarcosine utilization in the background of a similar metabolic pathway may provide a platform for identification of this transporter.

In this study we have identified an alternative model for the regulation of choline catabolism that incorporates multiple GATRs and examined their respective regulons. The conservation of *gbdR1* and *gbdR2* throughout the *Burkholderia* genus suggests that differences in regulation of choline catabolism outlined here represent a model that can be extrapolated to the more pathogenic strains, as well those strains associated with the rhizosphere. Contrasting the models of choline catabolism in *B*. *thailandensis* and *P*. *aeruginosa* will serve as a useful tool to probe the remaining unanswered questions concerning the pathway.

2.6 Acknowledgements

We would like to thank Jeff Bond, Julie Dragon, Robert Devins, and David J Shirley for bioinformatics assistance. We also owe thanks to Graham Willsey and Lauren Hinkel for critical reading of the manuscript.

2.7 Chapter 2 Figures

Figure 2.1 - Comparison of *B*. *thailandensis* and *P*. *aeruginosa* choline metabolism and associated regulators.

(**A**) Model of choline catabolic regulation in *P*. *aeruginosa* (left) and *B*. *thailandensis* (right). (**B**) Phylogenetic tree of relevant GATRs in *B*. *thailandensis* and *P*. *aeruginosa* rooted to FtrA, a GATR present in *E*. *coli*. (C) Diagram of the chromosomal context of *gbdR*, *gbdR1*, *gbdR2*, *souR* and major catabolic genes for the catabolism of GB and subsequent metabolites for *B*. *thailandensis* and *P*. *aeruginosa*.

Figure 2.2 - Deletion of *gbdR1* and *gbdR2* result in altered growth on choline and its metabolites as sole carbon sources.

(**A**) Growth of *B*. *thailandensis* deletions strains on MOPS minimal media agar plates supplemented with either 20 mM pyruvate or 20 mM choline and incubated at 37 ^{o}C for 72 hours. Δ*gbdr1* and Δ*gbdR1*Δ*gbdR2* fail to grow on choline while Δ*gbdR2* exhibits less growth. (**B**) Growth curves reveal the inability of Δ*gbdr1* and Δ*gbdR1*Δ*gbdR2* to grow on choline or its metabolites while Δ*gbdR2* exhibits a slow-growth phenotype on choline, glycine betaine, and dimethylglycine. Growth curves are representative of at least three experiments per condition at each time point, each with biological triplicates, and the error bars represent standard deviation.

Figure 2.3 - The growth phenotypes of Δ*gbdR1* and Δ*gbdR2* can be complimented.

(**A**) Complementation of Δ*gbdR1* at *att*Tn7 with *gbdR1* and its putative promoter results in restoration of ability to grow on choline and its metabolites. (**B**) Complementation of Δ*gbdR2* at *att*Tn7 with *gbdR2* results in an increased growth rate on choline and its metabolites, surpassing the growth rate of wild type. Growth curves are representative of at least three experiments per condition at each time point, each with biological triplicates, and the error bars represent standard deviation.

Figure 2.4 - The *glyA* promoter is induced by choline and its metabolites under the combined control of *gbdR1* and *gbdR2*.

(**A**) β-galactosidase activity from pAN27 is induced in the presence of choline and its metabolites, but not pyruvate. (**B**) *gbdR1* and *gbdR2* are both required for significant β-galactosidase induction in the presence of choline. (**C**) Promoter truncations of the *glyA* promoter reveal that the critical region is located between -147 and -340 with respect to the predicted translational start site of *glyA*. In each case, data presented represents the standard error of the mean determined from three independent experiments, each with three technical replicates. Statistical notation in panel (A) based on one-way ANOVA with Dunnett's post-test using the pyruvate group as the comparitor, while (B) & (C) were analyzed by two-way ANOVA with Sidak's post-test comparing with vs. without choline. Abbreviations and symbols: pyruvate (Pyr), choline (Cho), glycine betaine (GB), dimethylglycine (DMG), ethylcholine (EtCho), not significant (n.s.), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

Figure 2.5 - MBP-GbdR1 and MBP-GbdR2 bind to the regulatory region of the major GB catabolic operon (*BTH_II1868-BTH_II1858*).

(**A**) MBP-GbdR1 binds to biotin-probe 1 in a dose dependent manner (lanes 3-5). The interaction is specific as it does not bind to biotin-probe 2 (lane 8) and can be competed off biotin-probe 1 with unlabeled probe 1 (lane 6 compared to lane 4, 17.2% shift versus 75.2% shift respectively), but less so with unlabeled probe 2 (lane 7 compared to lane 4, 51.5% shift versus 75.2% shift respectively). (**B**) MBP-GbdR2 also binds specifically to probe 1 in a dose dependent manner (lanes 3-5) while failing to bind to biotin-probe 2 (lane 8). The binding between MBP-GbdR2 and biotin-probe 1 is also specific, as it can be competed by unlabeled probe 1 (lane 6 compared to lane 4, 4.3% shift versus 16% respectively) but less so by unlabeled probe 2 (lane 7 compared to lane 4, 7.7% shift versus 16% respectively).

Figure 2.6 - SouR is a critical regulator of the choline catabolic pathway.

(**A**) *B*. *thailandensis* can utilize choline as a nitrogen source. Strains were grown on 20 mM Pyruvate with either 5 mM or choline (C) or sarcosine (S) for 72 hours at 37 ^{o}C. Δ*gbdR1*, Δ*souR* and Δ*gbdR1*Δ*gbdR2*Δ*souR* were significantly diminished in their ability to utilize choline as a nitrogen source. Δ*gbdR2* utilized both choline and sarcosine as a nitrogen source at similar levels to wild type. Δ*souR* used sarcosine as a nitrogen source at reduced rates compared to the wild type. OD_{600} measurements were normalized to basal growth without a nitrogen source and then normalized to wild type and represented as percent growth. (**B**) Deletion of *souR* prevents *B*. *thailandensis* from using choline as a carbon source. Complementation of *souR* at an *att*Tn7 site with *souR*, under the control of

its putative native promoter, restores the ability of *B*. *thailandensis* to utilize choline and its metabolites as carbon sources. Strains were grown with either 40 mM pyruvate (P), choline (C), GB (G), or DMG (D) as a sole carbon source in MOPS minimal media for 72 hours at 37 ^{o}C. (**C**) MBP-SouR binds to the putative promoter of the sarcosine oxidase operon represented by biotin-probe 3 in a dose dependent manner (lanes 3-5). The interaction is specific, as MBP-Sour does not bind to biotin-probe 2 (lane 8) and can be competed off by unlabeled probe 3 (lane 6 compared to lane 4, 22% shift versus 37.1% respectively), but not by unlabeled probe 2 (lane 7 compared to lane 4, 38.2% shift versus 37.1% respectively). Data in panels (A) & (B) are averaged from three experiments each with three biological replicates and error bars represent SEM. These data were both analyzed by two-way ANOVA with Tukey's post-test testing for strain effect for each carbon source separately, not comparing the carbon sources. For (A), except for Δ*gbdR2*, all choline changes are different from WT and are not noted. Only the sarcosine comparisons different from WT are noted. For (B), pyruvates were not significantly different, but all other comparisons were except WT vs. the mutant in dimethylglycine. $p < 0.01$ (**), $p < 0.001$ (***).

Figure 2.S1 – Complementation of Δ*gbdR1*Δ*gbdR2* with *gbdR1* or *gbdR2*. Complementation of Δ*gbdR1*Δ*gbdR2* with *gbdR1* alone is able to restore the ability of *B*. *thailandensis* to utilize choline as a carbon source, but *gbdR2* alone is unable to complement the metabolic deficiency. Data shown are averaged from three experiments each with three biological replicates and error bars represent SEM. Data analyzed by two-

way ANOVA with Dunnett's post-test using the double deletion mutant as the comparator making comparisons within each time point. The asterisks indicate $p < 0.01$.

Figure 2.S2 – Validation of RNA-Seq experiments.

Validation of RNA-Seq experiments by qRT-PCR supports the findings of the RNA-Seq experiment. RNA-Seq values were normalized within DEseq based on library size while the RT-qPCR values were normalized to the *rplU* transcript levels. Both data sets are expressed here as fold change in expression scaled to their own respective y-axes.

Figure 2.S3 – pAN27 is still responsive to choline in Δ*souR*.

pAN27 is still responsive to choline in Δ*souR*, and β-galactosidase activity seems to be induced to even higher levels as compared to wild type. This suggests that SouR is not required for the induction of *glyA* promoter. Data shown are averaged from three experiments each with three biological replicates and error bars represent SEM.

Figure 2.S4 – Usage of choline and sarcosine as sole carbon or nitrogen sources.

B. thailandensis can utilize choline as either a carbon or carbon and nitrogen source. *B. thailandensis* is unable to utilize sarcosine as a carbon or carbon and nitrogen source, unlike P. aeruginosa. Strains were grown in MOPS minimal media or MOPS minimal media without NH_4, with either 40 mM or choline (C) or sarcosine (S) for 72 hours at 37 ^{o}C. Data shown are averaged from three experiments each with three biological replicates and error bars represent SEM.

2.8 Chapter 2 Tables

Table 2.1 – Transcript changes of *B*. *thailandensis* in response to choline.

Table 2.S1 – Strains and plasmids.

Table 2.S2 – Primers used in study.

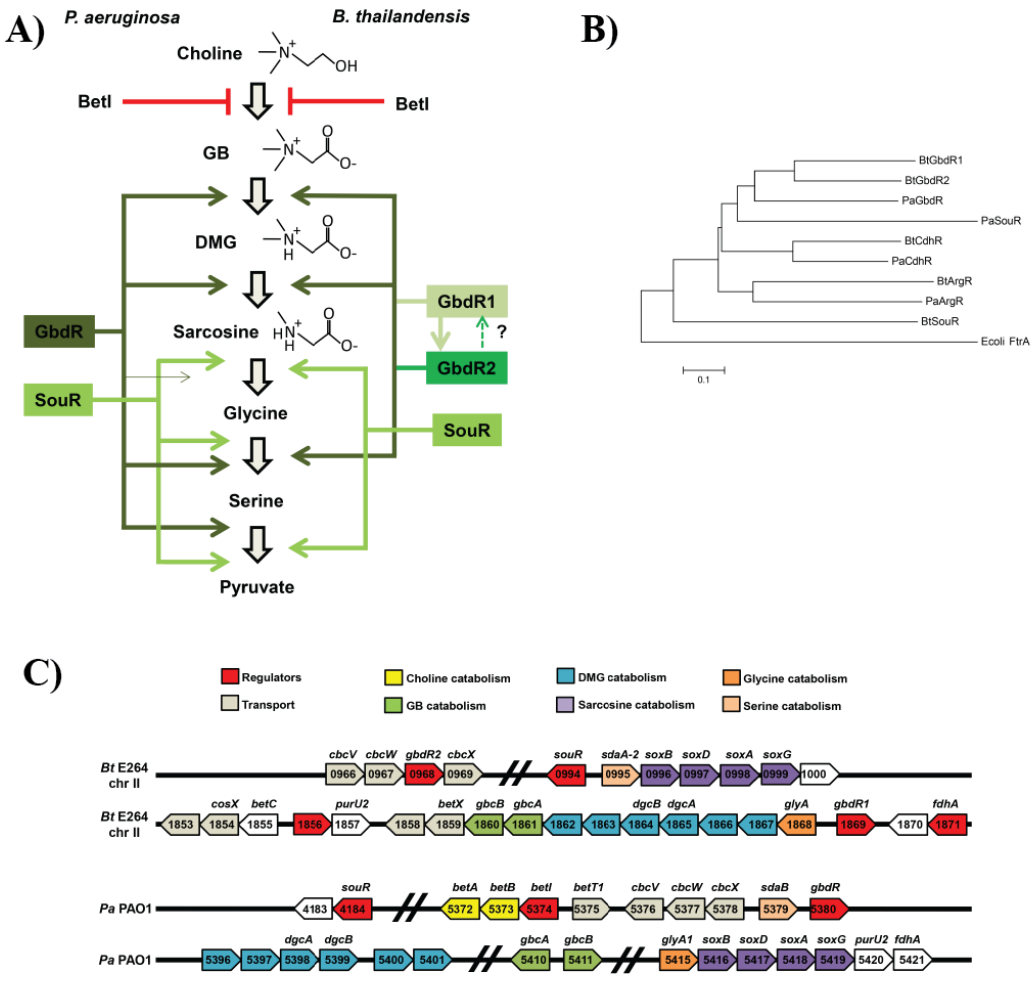


Figure 2.1 - Comparison of *B. thailandensis* and *P. aeruginosa* choline metabolism and associated regulators.

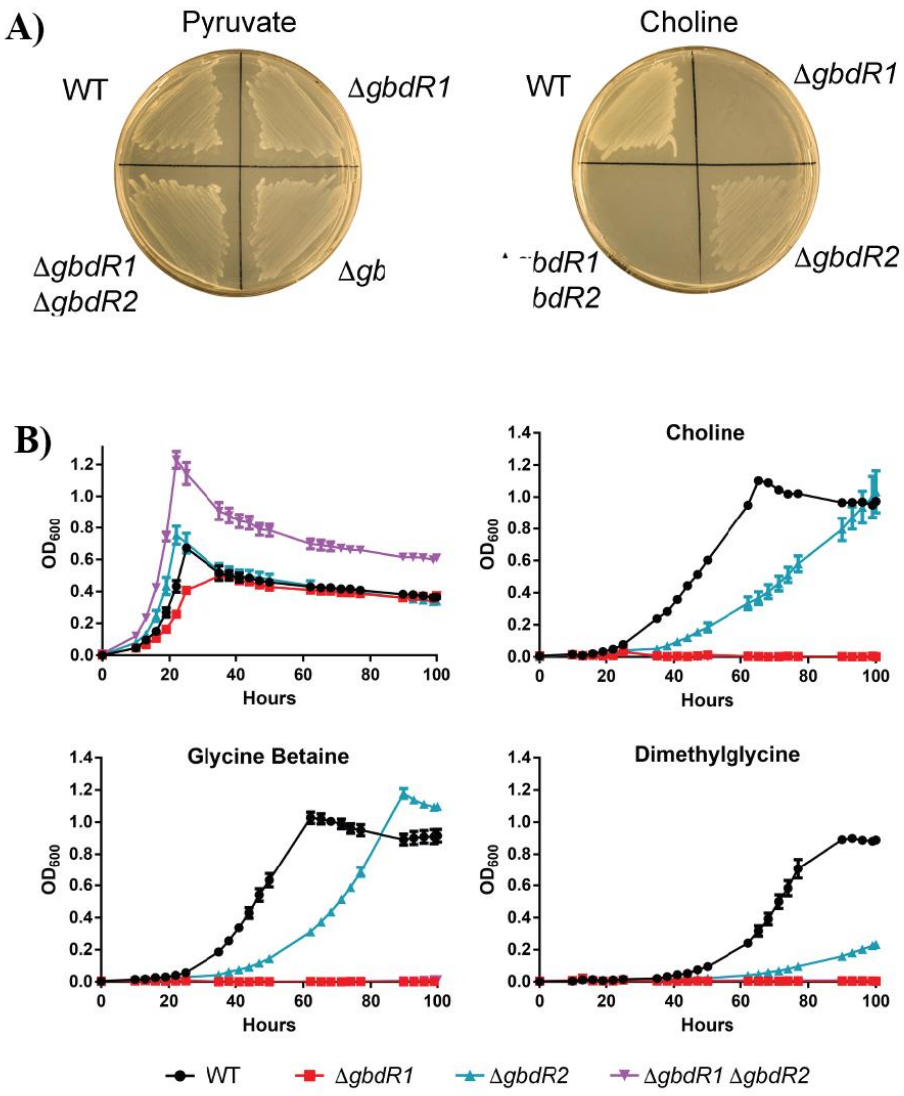


Figure 2.2 - Deletion of *gbdR1* and *gbdR2* result in altered growth on choline and its metabolites as sole carbon sources.

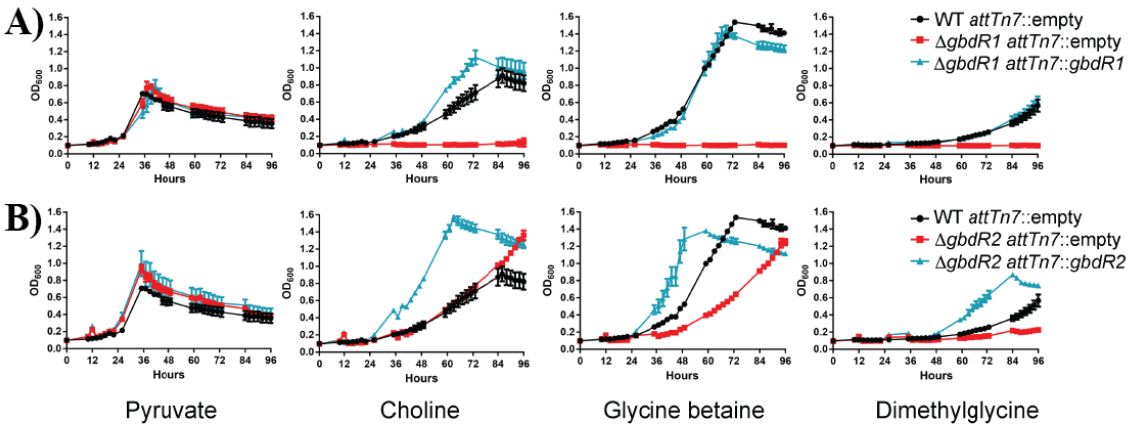


Figure 2.3 - The growth phenotypes of Δ*gbdR1* and Δ*gbdR2* can be complimented.

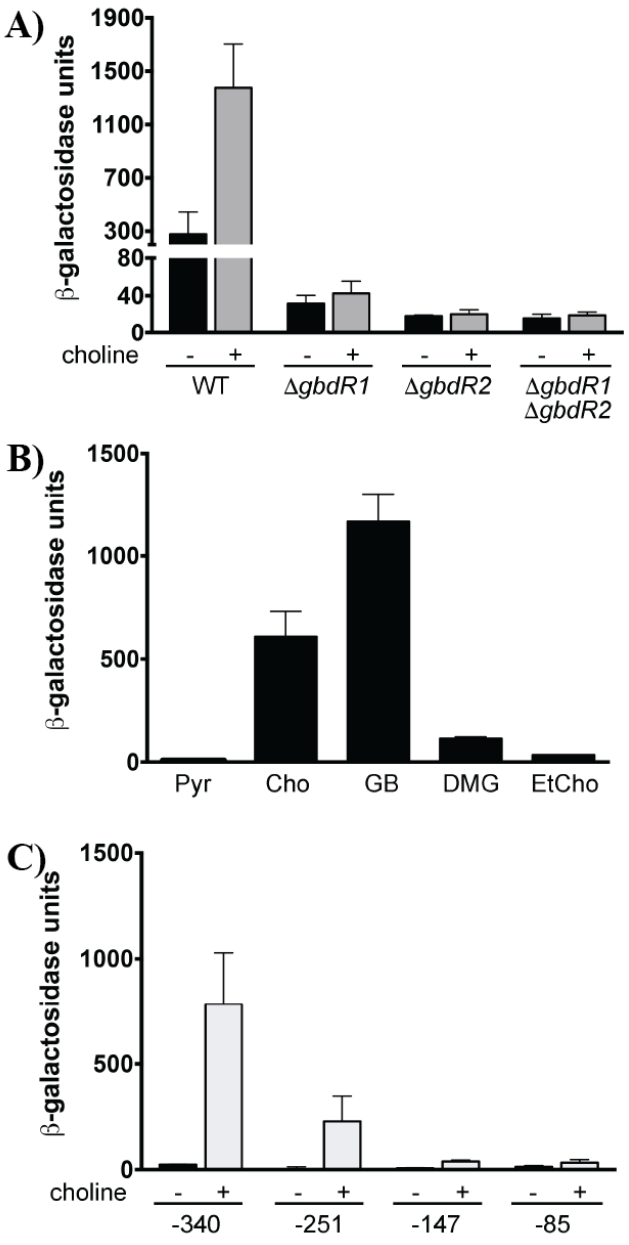


Figure 2.4 - The *glyA* promoter is induced by choline and its metabolites under the combined control of *gbdR1* and *gbdR2*.

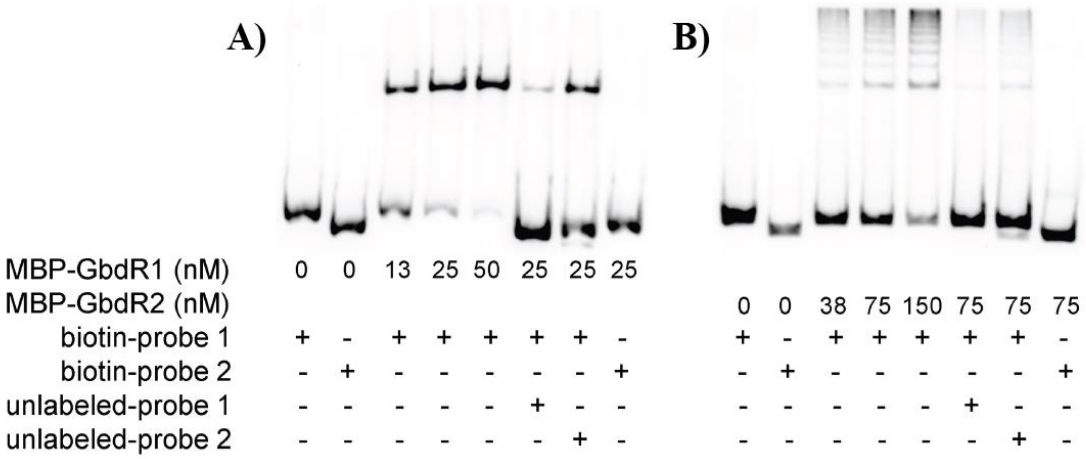


Figure 2.5 - MBP-GbdR1 and MBP-GbdR2 bind to the regulatory region of the major GB catabolic operon (*BTH_II1868-BTH_II1858*).

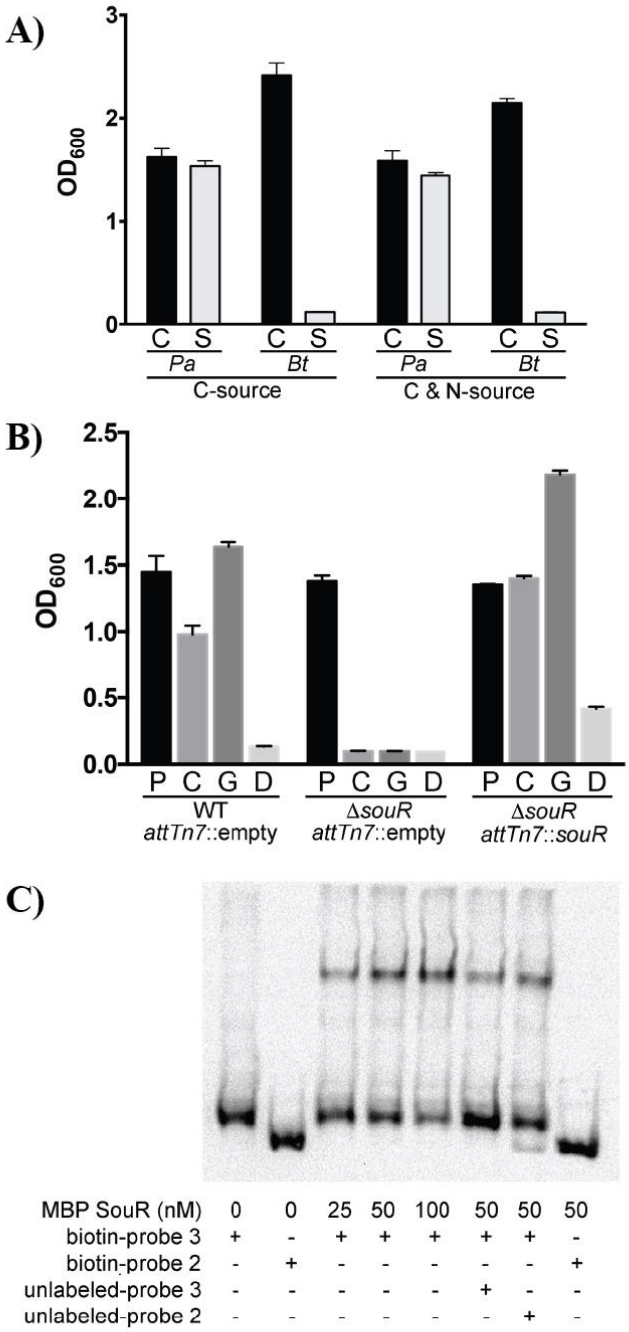


Figure 2.6 - SouR is a critical regulator of the choline catabolic pathway.

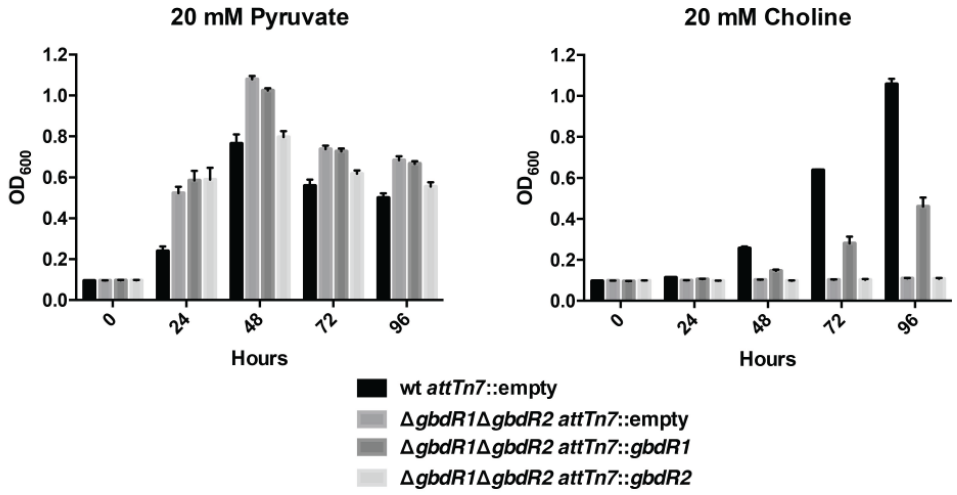


Figure 2.S1 - Complementation of ΔgbdR1ΔgbdR2 with *gbdR1* or *gbdR2.*

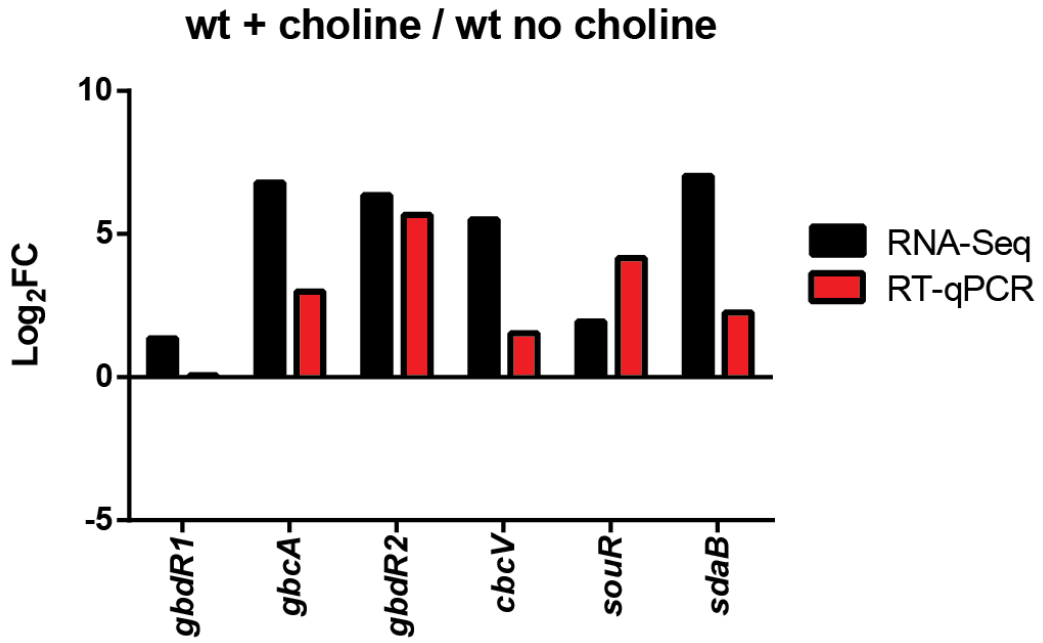


Figure 2.S2 - Validation of RNA-Seq experiments.

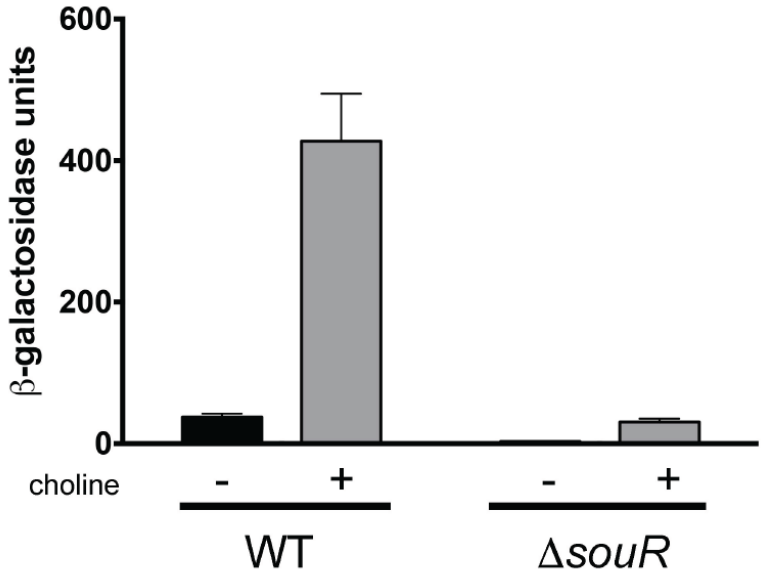


Figure 2.S3 - pAN27 is still responsive to choline in Δ*souR*.

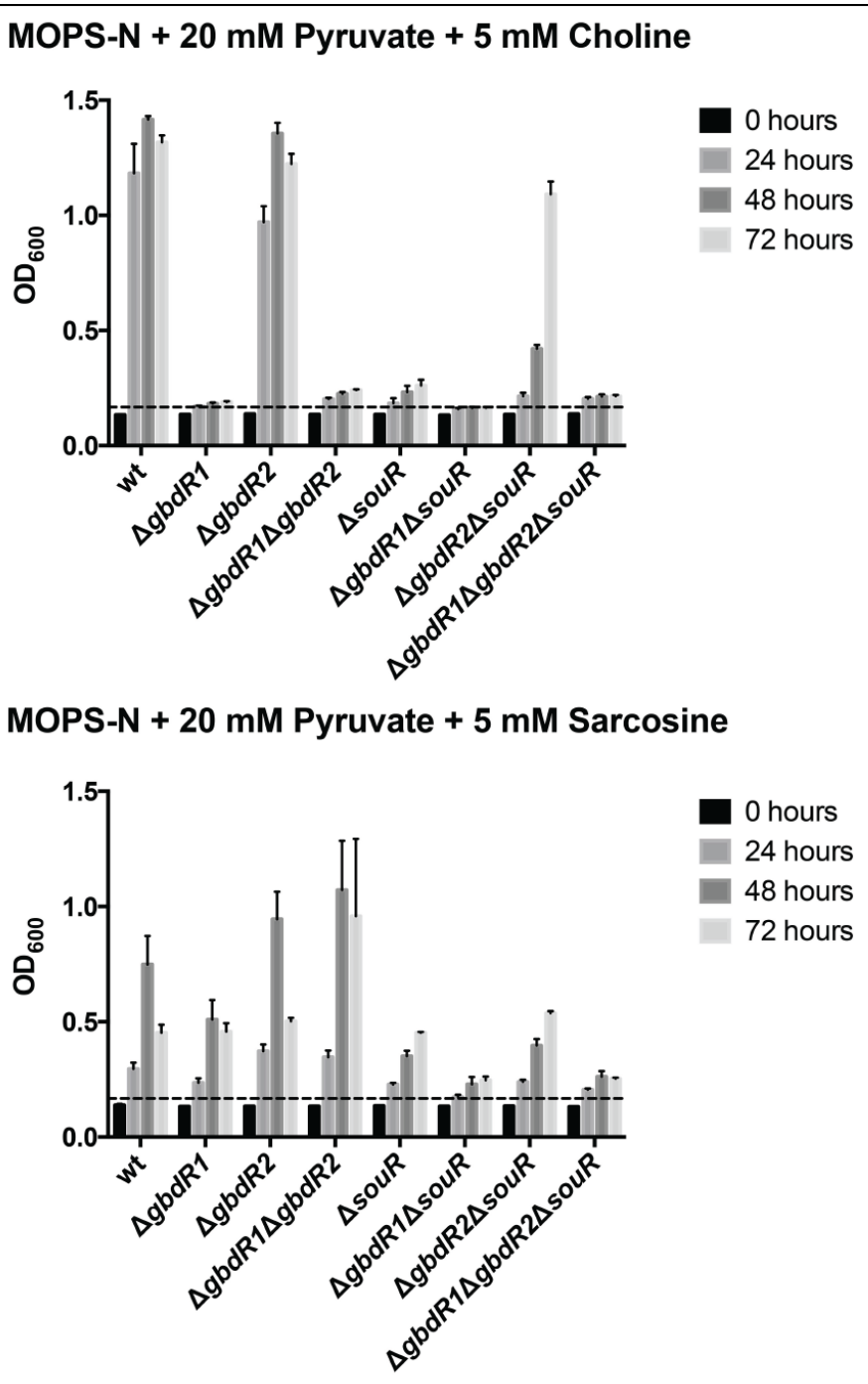


Figure 2.S4 - Usage of choline and sarcosine as sole carbon or nitrogen sources.

Table 2.1 Transcript changes of *B*. *thailandensis* in response to choline.

Locus ID	Gene[b]	Linear fold change in transcript over pyruvate alone[a]:			
		wt	Δ*gbdR1*	Δ*gbdR2*	Δ*gbdR1* Δ*gbdR2*
BTH_I0192		-2.6	NC[c]	NC	NC
BTH_I0393		-4.8	NC	NC	NC
BTH_I0394		-5.1	NC	NC	NC
BTH_I0395		-8.3	NC	NC	NC
BTH_I0396		-4.3	NC	NC	NC
BTH_I0398		-3.9	NC	NC	NC
BTH_I0687		23.8	NC	10.5	NC
BTH_I0688		19.3	NC	NC	NC
BTH_I0698		11.3	NC	NC	NC
BTH_I0700		12.0	NC	NC	NC
BTH_I0866		5.8	5.3	NC	NC
BTH_I0959		-2.6	NC	-5.3	-5.0
BTH_I1406		-2.3	NC	-4.4	NC
BTH_I1620		5.7	NC	4.4	4.4
BTH_I1621		61.2	NC	18.3	NC
BTH_I1622		52.7	NC	9.7	NC
BTH_I1623		40.0	NC	9.3	NC
BTH_I1624		43.7	NC	5.0	NC
BTH_I1625		5.5	NC	NC	NC
BTH_I3016		-2.8	NC	-3.6	-3.4

BTH_I3017		-3.1	NC	-4.0	-3.9
BTH_II0001		5.4	NC	9.0	6.5
BTH_II0643		2.4	NC	NC	NC
BTH_II0694		-3.4	NC	-2.4	NC
BTH_II0695		-2.6	NC	NC	NC
BTH_II0964		2.9	NC	NC	NC
BTH_II0966	*cbcV*	45.4	NC	NC	NC
BTH_II0967	*cbcW*	94.0	NC	NC	NC
BTH_II0968	*gbdR2*	81.7	NC	NC	NC
BTH_II0969	*cbcX*	136.6	NC	NC	NC
BTH_II0970		37.7	NC	NC	NC
BTH_II0971		8.8	NC	NC	NC
BTH_II0994	*souR*	3.9	NC	4.7	NC
BTH_II0995	*sdaA-2*	90.8	NC	32.3	NC
BTH_II0996	*soxB*	130.6	NC	39.3	NC
BTH_II0997	*soxD*	172.0	NC	102.3	NC
BTH_II0998	*soxA*	122.7	NC	58.6	NC
BTH_II0999	*soxG*	24.9	NC	11.7	NC
BTH_II1000		7.6	-3.2	8.7	-3.5
BTH_II1546		4.3	NC	3.7	NC
BTH_II1853		5.4	NC	NC	NC
BTH_II1854	*cosX*	7.1	NC	NC	NC
BTH_II1855	*betC*	3.8	NC	NC	NC

BTH_II1856		11.5	NC	NC	NC
BTH_II1857	*purU2*	8.4	NC	NC	NC
BTH_II1858		6.5	NC	NC	NC
BTH_II1859	*betX*	175.6	NC	38.6	NC
BTH_II1860	*gbcB*	115.8	NC	28.9	NC
BTH_II1861	*gbcA*	110.8	NC	23.7	NC
BTH_II1862		218.7	NC	44.4	NC
BTH_II1863		163.4	NC	97.2	NC
BTH_II1864	*dgcB*	154.1	NC	28.9	NC
BTH_II1865	*dgcA*	199.7	NC	23.4	NC
BTH_II1866		223.9	NC	29.8	NC
BTH_II1867		135.3	NC	47.4	NC
BTH_II1868	*glyA*	162.9	NC	20.7	NC
BTH_II1869	*gbdR1*	2.6	NC	NC	NC

[a] Constraints on inclusion in table were +/- 2.3 fold change and $p \leq 0.005$ based on the wild type.

[b] Gene names are putative based on homology to *P*. *aeruginosa*.

[c] NC, no change due to significance, lower fold change, or experimental deletion of the gene.

Table 2.S1 – Strains and plasmids.

Designation	Genotype or Description	Reference or Source
Burkholderia thailandensis strains		
MJ358	E264 wild type	(23)
AN3	Δ*gbdR1*	This study
AN19	Δ*gbdR2*	This study
AN16	Δ*gbdR1* Δ*gbdR2*	This study
AN126	Δ*souR*	This study
AN202	Δ*gbdR1* Δ*souR*	This study
AN205	Δ*gbdR2* Δ*souR*	This study
AN207	Δ*gbdR1* Δ*gbdR2* Δ*souR*	This study
AN136	*att*Tn7 vector, Zeo^r	This study
AN153	*att*Tn7 vector, Zeo^r	This study
AN128	*att*Tn7 vector, Zeo^r	This study
AN150	*att*Tn7 vector, Zeo^r	This study
AN158	Δ*gbdR1 att*::*gbdR1* comp, Zeo^r	This study
AN173	Δ*gbdR2 att*::*gbdR2* comp, Zeo^r	This study
AN162	Δ*gbdR1* Δ*gbdR2 att*::*gbdR1*, Zeo^r	This study
AN164	Δ*gbdR1* Δ*gbdR2 att*::*gbdR2*, Zeo^r	This study
AN151	*att*::*souR* comp, Zeo^r	This study
AN191	pAN27	This study
AN192	Δ*gbdR1* + pAN27	This study
AN193	Δ*gbdR2* + pAN27	This study
AN194	Δ*gbdR1* Δ*gbdR2* + pAN27	This study
AN192	Δ*souR* + pAN27	This study
AN226	pAN28	This study
AN227	pAN29	This study
AN228	pAN30	This study
AN229	pAN31	This study
Escherichia coli strains		
DH5α		
T7 Express	See manufacturer	NEB
AN133	pAN13 in T7 Express	
AN134	pAN14 in T7 Express	

AN188	pAN19 in T7 Express	
Plasmids		
pMQ132	Yeast recombineering vector, Gm^r	(38)
pAN1	pMQ132 derivative, Tp^r	This study
pAN7	pAN1 derivative, *lacZYA* reporter plasmid, Tp^r	This study
pAN27	pAN7-P_{glyA} -408	This study
pAN28	pAN7-P_{glyA} -340	This study
pAN29	pAN7-P_{glyA} -251	This study
pAN30	pAN7-P_{glyA} -147	This study
pAN31	pAN7-P_{glyA} -85	This study
pMAL-C2X	N-terminal MBP affinity tagging, Amp^r	NEB
pAN13	pMAL-C2X-*gbdR1*	This study
pAN14	pMAL-C2X-*gbdR2*	This study
pAN19	pMAL-C2X-*souR*	This study
pUC18-mini-TN7T-Zeo	*att*Tn7 integration vector, Zeo^r	(26)
pTNS2	carrying the *att*Tn7 transposase, Amp^r	(59)
pAN23	pUC18-mini-TN7T-Zeo-*gbdR1* comp	This study
pAN10	pUC18mini-TN7T-Zeo-*gbdR2* comp	This study
pAN22	pUC18-mini-TN7T-Zeo-*souR* comp	This study

Table 2.S2 – Primers used in study.

Bt-gbdR1-TpFLP-1F	gcgccccggccgcatcaagatcagatccaaggaacgcttcGAGCTCGAATTAGCTTCAAA	*gbdR1* (*BTH_II1869*) deletion
Bt-gbdR1-TpFLP-1R	ggtgtgcgatttcgggggccggtttcgagttccggtttccGAGCTCGAATTGGGGATCTT	*gbdR1* (*BTH_II1869*) deletion
Bt-gbdR1-2F	CGTAATCGATCAGCATCGTG	*gbdR1* (*BTH_II1869*) deletion
Bt-gbdR1-2R	GAAGCGTTCCTTGGATCTGA	*gbdR1* (*BTH_II1869*) deletion
Bt-gbdR1-3F	GGAAACCGGAACTCGAAAC	*gbdR1* (*BTH_II1869*) deletion
Bt-gbdR1-3R	ACGTCGAGACGCTGAGGAT	*gbdR1* (*BTH_II1869*) deletion

BtgbdR2 proximal F	AAG CCG TGC CGA TGT GGC TG	*gbdR2* (*BTH_II0968*) deletion
BtgbdR2 proximal R	AAG ATC GAC CAG CGG TAA TG	*gbdR2* (*BTH_II0968*) deletion
BtgbdR2 tp F REVISED	ACTACGTCGCGCGCGCGGACCATTACCG CTGGTCGATCTTgagctcgaattagcttcaaa	*gbdR2* (*BTH_II0968*) deletion
BtgbdR2 tp R REVISED	TACGCCTTGCTGAAATGGCACGGCGAGT GAAAGCCGCAGAgagctcgaattggggatctt	*gbdR2* (*BTH_II0968*) deletion
BtgbdR2 post F REVISED	TCTGCGGCTTTCACTCGCCG	*gbdR2* (*BTH_II0968*) deletion
BtgbdR2 post R REVISED	CGCCGCCGCTCAGATAGTCG	*gbdR2* (*BTH_II0968*) deletion
BtII0994proxF	CGGCAATCTACGATGAGGCT	*souR* (*BTH_II0994*) deletion
BtII0994proxR	CGATAGGGGGAAGAACACCG	*souR* (*BTH_II0994*) deletion
BtII0994postF	GGTCAGCGTGAAGTTAGGCA	*souR* (*BTH_II0994*) deletion
BtII0994postR	CCGATGTCGTCCTTCTCGTC	*souR* (*BTH_II0994*) deletion
BtII0994tpF	GCCGCCGGCCGCATCGGCGTCGGTGTTC TTCCCCCTATCGgagctcgaattagcttcaaa	*souR* (*BTH_II0994*) deletion
BtII0994tpR	GCGCTTCGGGATCGTGCTGCTGCCTAACT TCACGCTGACCgagctcgaattggggatctt	*souR* (*BTH_II0994*) deletion
gbdR1compFHindIII	CCTAAGCTTGAGCGACATCCCGAGCACC G	*gbdR1* complement
gbdR1compRKpnI	CTTGGTACCCGGGGGCCGGTTTCGAGTT C	*gbdR1* complement
KpnIgbdR2compF	CTT GGTACC CTGACGTTTTCCGCCTTCG	*gbdR2* complement
EcoRIgbdR2compR	CTT GAATTC GGGATCGGTTCGTGGCTG	*gbdR2* complement
KpnI II0994compF	CTT GGTACC CCTTGCGTAACGATGCGTGGT	*souR* complement (use MBP-SouR reverse primer)
Tp pMQ132F	TACGCCGTGGGTCGATGTTTGATGTTATG GAGCAGCAACGgatcccctgattccctttgt	Generation of pAN1
Tp pMQ132R	ACTCCGCGGCCGGGAAGCCGATCTCGGC TTGAACGAATTGagcgcttttgaagctgatgt	Generation of pAN1
BtPFusion YHRp1F	CTACTGCCGCCAGGCAAATTCTGTTTTAT CAGACCGCTTC GAATTC CTGCCCG	Generation of pAN7 step 1
BtPFusion	GCAGCTGGCACGACAGGTTTCCCGACTG	Generation of pAN7

YHRp1R	GAAAGCGGGCAG GAATTC GAAGCGG	step 1
BtPFusion YHRp2F	CTACTGCCGCCAGGCAAATTCTGTTTTAT CAGACCGCTTC ccaagcttgcatgcctgcag	Generation of pAN7 step 2
BtPFusion YHRp2R	GCAGCTGGCACGACAGGTTTCCCGACTG GAAAGCGGGCAG gaattCGCAGCGTATCAGGC	Generation of pAN7 step 2
glyAtogbd R1interF	CCTGCATGCAAGGCGTCTCGTCACTGGA T	Generation of pAN27
glyAtogbd R1interR	CTTGCATGCGGGGAAGCGTTCCTTGGAT CT	Generation of pAN27
SphI P.glyA1Lac Z1	CTT GCATGC CGCAACTAGCGTGCCATACG	Generation of pAN28, use with glyAtogbdR1interR
SphI P.glyA1Lac Z2	CTT GCATGC CTGCGCAGCGCGAGTG	Generation of pAN29, use with glyAtogbdR1interR
SphI P.glyA1Lac Z3	CTT GCATGC GAAAGCGTCTGAATTCATCAATCGG	Generation of pAN30, use with glyAtogbdR1interR
SphI P.glyA1Lac Z4	CTT GCATGC CCGCTGCGGCGGAAC	Generation of pAN31, use with glyAtogbdR1interR
gbdR1EMS A2F	GGGGTTGGCGTTCGACATAA	EMSA Probe 2
gbdR1EMS A2R	CGGAAAAGGGAAAGAAAGCGT	EMSA Probe 2
BIOgbdR1 EMSA2F	BIO-GGGGTTGGCGTTCGACATAA	EMSA Probe 2
gbdR1EMS A3R	GCTAAACCCTAGATCGGCGG	EMSA Probe 1
gbdR2EMS A1F	CAGCGTATGCAGGAACGGG	EMSA Probe 1
BIOgbdR1 EMSA3F	BIO-TGTCGTTCGGATTTTTAGCCG	EMSA Probe 1
SarOxiEM SA1F	CGGTCATGTCTGCAAAATCGT	EMSA Probe 3
SarOxiEM SA1R	GCCGATAGAACAAAAACGGCA	EMSA Probe 3
BioSarOxi EMSA1F	BIO-CGGTCATGTCTGCAAAATCGT	EMSA Probe 3
pMALC2X gbdR1F	CCTGGATCCTCGCCCGACCGCACCGCG	MBP-GbdR1
RSFgbdR1 R	CTT AAG CTT TCA GCG CGC CGA CAC GCG	MBP-GbdR1
MALgbdR 2F	CCG GGATCC GTG ACG TCC GCC GCC GCT	MBP-GbdR2

6hisgbdR2 R	CTTAAGCTTTCAGCGCGCCGACACGCG	MBP-GbdR2
BamHI II0994 pMAL F	CCG GGATCC GTGCGTTTCGGCGATGGTTC	MBP-SouR
HindIII II0994 pMAL R	CCT AAGCTT TCAGCGAGCGCCCCTC	MBP-SouR and *souR* complementation
BtRplURT F	CAGTACAAGGTTGCCGTTGG	*rplU* qRT-PCR set
BtRplURT R	CCGTGCTTTTGGTAGTGCTTC	*rplU* qRT-PCR set
II1861RTF	GCAGCAGATACGGCTCCATC	*gbcA* qRT-PCR set
II1861RTR	GCAACGAGGAAAAAGGGTCG	*gbcA* qRT-PCR set
II0996RTF	CTCGAACTACCTGTGGGACG	*soxB* qRT-PCR set
II0966RTR	AAGTTGATCGTCGGCTCGAT	*soxB* qRT-PCR set
II0994RTF	CGTTGCCTTTCTGCATCTCG	*souR* qRT-PCR set
II0994RTR	TGCGTGAGCTGGTTCCATTA	*souR* qRT-PCR set
BtgbdR1 RT F	AGTGCTCAGGATGGCAAACT	*gbdR1* qRT-PCR set
BtgbdR1 RT R	CAGATTTTCCCAATGGATCG	*gbdR1* qRT-PCR set
gbdR2 set3 RTF	CAGGTGTCCGAGCAGTTCAT	*gbdR2* qRT-PCR set
gbdR2 set3 RTR	CGGCGACACGTTCAGATAGA	*gbdR2* qRT-PCR set

2.9 Chapter 2 References

1. **Pilatova M, Dionne MS.** 2012. Burkholderia thailandensis Is Virulent in Drosophila melanogaster. Plos One **7**.
2. **Inglis TJJ, Rigby P, Robertson TA, Dutton NS, Henderson M, Chang BJ.** 2000. Interaction between Burkholderia pseudomallei and Acanthamoeba species results in coiling phagocytosis, endamebic bacterial survival, and escape. Infection and Immunity **68:**1681-1686.
3. **Lee YH, Chen YH, Ouyang XZ, Gan YH.** 2010. Identification of tomato plant as a novel host model for Burkholderia pseudomallei. Bmc Microbiology **10**.
4. **Reckseidler SL, DeShazer D, Sokol PA, Woods DE.** 2001. Detection of bacterial virulence genes by subtractive hybridization: identification of capsular polysaccharide of Burkholderia pseudomallei as a major virulence determinant. Infect Immun **69:**34-44.
5. **Angus AA, Agapakis CM, Fong S, Yerrapragada S, Estrada-de los Santos P, Yang P, Song N, Kano S, Caballero-Mellado J, de Faria SM, Dakora FD, Weinstock G, Hirsch AM.** 2014. Plant-associated symbiotic Burkholderia species lack hallmark strategies required in mammalian pathogenesis. PLoS One **9:**e83779.
6. **Haraga A, West TE, Brittnacher MJ, Skerrett SJ, Miller SI.** 2008. Burkholderia thailandensis as a Model System for the Study of the Virulence-Associated Type III Secretion System of Burkholderia pseudomallei. Infection and Immunity **76:**5402-5411.
7. **Yu YT, Kim HS, Chua HH, Lin CH, Sim SH, Lin DX, Derr A, Engels R, DeShazer D, Birren B, Nierman WC, Tan P.** 2006. Genomic patterns of pathogen evolution revealed by comparison of Burkholderia pseudomallei, the causative agent of melioidosis, to avirulent Burkholderia thailandensis. Bmc Microbiology **6**.
8. **Kim HS, Schell MA, Yu Y, Ulrich RL, Sarria SH, Nierman WC, DeShazer D.** 2005. Bacterial genome adaptation to niches: divergence of the potential virulence genes in three Burkholderia species of different survival strategies. BMC Genomics **6:**174.
9. **Wargo MJ.** 2013. Homeostasis and catabolism of choline and glycine betaine: lessons from Pseudomonas aeruginosa. Appl Environ Microbiol **79:**2112-2120.
10. **Zachowski A.** 1993. Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. Biochem J **294 (Pt 1):**1-14.
11. **Bernhard W, Hoffmann S, Dombrowsky H, Rau GA, Kamlage A, Kappler M, Haitsma JJ, Freihorst J, von der Hardt H, Poets CF.** 2001. Phosphatidylcholine molecular species in lung surfactant: composition in relation to respiratory rate and lung development. Am J Respir Cell Mol Biol **25:**725-731.
12. **Chambers S, Kunin CM.** 1985. The osmoprotective properties of urine for bacteria: the protective effect of betaine and human urine against low pH and high concentrations of electrolytes, sugars, and urea. J Infect Dis **152:**1308-1316.
13. **Shortridge VD, Lazdunski A, Vasil ML.** 1992. Osmoprotectants and phosphate regulate expression of phospholipase C in Pseudomonas aeruginosa. Mol Microbiol **6:**863-871.

14. **Kunin CM, Hua TH, White LV, Villarejo M.** 1992. Growth of Escherichia-Coli in Human Urine - Role of Salt Tolerance and Accumulation of Glycine Betaine. Journal of Infectious Diseases **166:**1311-1315.
15. **Wargo MJ.** 2013. Choline catabolism to glycine betaine contributes to Pseudomonas aeruginosa survival during murine lung infection. PLoS One **8:**e56850.
16. **Sun ZX, Kang Y, Norris MH, Troyer RM, Son MS, Schweizer HP, Dow SW, Hoang TT.** 2014. Blocking Phosphatidylcholine Utilization in Pseudomonas aeruginosa, via Mutagenesis of Fatty Acid, Glycerol and Choline Degradation Pathways, Confirms the Importance of This Nutrient Source In Vivo. Plos One **9**.
17. **Fitzsimmons LF, Hampel KJ, Wargo MJ.** 2012. Cellular choline and glycine betaine pools impact osmoprotection and phospholipase C production in Pseudomonas aeruginosa. J Bacteriol **194:**4718-4726.
18. **de Rudder KE, Sohlenkamp C, Geiger O.** 1999. Plant-exuded choline is used for rhizobial membrane lipid biosynthesis by phosphatidylcholine synthase. J Biol Chem **274:**20011-20016.
19. **Craig SA.** 2004. Betaine in human nutrition. Am J Clin Nutr **80:**539-549.
20. **Wargo MJ, Szwergold BS, Hogan DA.** 2008. Identification of two gene clusters and a transcriptional regulator required for Pseudomonas aeruginosa glycine betaine catabolism. J Bacteriol **190:**2690-2699.
21. **Fitzsimmons LF, Flemer S, Jr., Wurthmann AS, Deker PB, Sarkar IN, Wargo MJ.** 2011. Small-molecule inhibition of choline catabolism in Pseudomonas aeruginosa and other aerobic choline-catabolizing bacteria. Appl Environ Microbiol **77:**4383-4389.
22. **Hampel KJ, Labauve AE, Meadows JA, Fitzsimmons LF, Nock AM, Wargo MJ.** 2013. Characterization of the GbdR regulon in Pseudomonas aeruginosa. J Bacteriol doi:10.1128/JB.01055-13.
23. **Brett PJ, DeShazer D, Woods DE.** 1998. Burkholderia thailandensis sp. nov., a Burkholderia pseudomallei-like species. Int J Syst Bacteriol **48 Pt 1:**317-320.
24. **Thongdee M, Gallagher LA, Schell M, Dharakul T, Songsivilai S, Manoil C.** 2008. Targeted mutagenesis of Burkholderia thailandensis and Burkholderia pseudomallei through natural transformation of PCR fragments. Appl Environ Microbiol **74:**2985-2989.
25. **Choi KH, Mima T, Casart Y, Rholl D, Kumar A, Beacham IR, Schweizer HP.** 2008. Genetic tools for select-agent-compliant manipulation of Burkholderia pseudomallei. Appl Environ Microbiol **74:**1064-1075.
26. **Choi KH, Schweizer HP.** 2006. mini-Tn7 insertion in bacteria with single attTn7 sites: example Pseudomonas aeruginosa. Nat Protoc **1:**153-161.
27. **Choi KH, DeShazer D, Schweizer HP.** 2006. mini-Tn7 insertion in bacteria with multiple glmS-linked attTn7 sites: example Burkholderia mallei ATCC 23344. Nat Protoc **1:**162-169.
28. **LaBauve AE, Wargo MJ.** 2012. Growth and laboratory maintenance of Pseudomonas aeruginosa. Curr Protoc Microbiol **Chapter 6:**Unit 6E 1.
29. **Tamura K, Stecher G, Peterson D, Filipski A, Kumar S.** 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol **30:**2725-2729.

30. **Edgar RC.** 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res **32:**1792-1797.
31. **Andrews S.** 2009. FastQC: a quality control tool for high throughput sequence data., http://www.bioinformatics.babraham.ac.uk/projects/fastqc.
32. **Li H, Durbin R.** 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics **25:**1754-1760.
33. **Lawrence M, Huber W, Pages H, Aboyoun P, Carlson M, Gentleman R, Morgan MT, Carey VJ.** 2013. Software for computing and annotating genomic ranges. PLoS Comput Biol **9:**e1003118.
34. **Anders S, Huber W.** 2010. Differential expression analysis for sequence count data. Genome Biol **11:**R106.
35. **Wargo MJ, Gross MJ, Rajamani S, Allard JL, Lundblad LK, Allen GB, Vasil ML, Leclair LW, Hogan DA.** 2011. Hemolytic phospholipase C inhibition protects lung function during Pseudomonas aeruginosa infection. Am J Respir Crit Care Med **184:**345-354.
36. **Willsey GG, Wargo MJ.** 2015. Sarcosine catabolism in Pseudomonas aeruginosa is transcriptionally regulated by SouR. J Bacteriol doi:10.1128/JB.00739-15.
37. **Brutinel ED, Vakulskas CA, Brady KM, Yahr TL.** 2008. Characterization of ExsA and of ExsA-dependent promoters required for expression of the Pseudomonas aeruginosa type III secretion system. Mol Microbiol **68:**657-671.
38. **Shanks RM, Kadouri DE, MacEachran DP, O'Toole GA.** 2009. New yeast recombineering tools for bacteria. Plasmid **62:**88-97.
39. **Kang Y, Norris MH, Barrett AR, Wilcox BA, Hoang TT.** 2009. Engineering of tellurite-resistant genetic tools for single-copy chromosomal analysis of Burkholderia spp. and characterization of the Burkholderia thailandensis betBA operon. Appl Environ Microbiol **75:**4015-4027.
40. **Hampel KJ, LaBauve AE, Meadows JA, Fitzsimmons LF, Nock AM, Wargo MJ.** 2014. Characterization of the GbdR regulon in Pseudomonas aeruginosa. J Bacteriol **196:**7-15.
41. **Rkenes TP, Lamark T, Strom AR.** 1996. DNA-binding properties of the BetI repressor protein of Escherichia coli: the inducer choline stimulates BetI-DNA complex formation. J Bacteriol **178:**1663-1670.
42. **Velasco-Garcia R, Villalobos MA, Ramirez-Romero MA, Mujica-Jimenez C, Iturriaga G, Munoz-Clares RA.** 2006. Betaine aldehyde dehydrogenase from Pseudomonas aeruginosa: cloning, over-expression in Escherichia coli, and regulation by choline and salt. Arch Microbiol **185:**14-22.
43. **Andresen PA, Kaasen I, Styrvold OB, Boulnois G, Strom AR.** 1988. Molecular cloning, physical mapping and expression of the bet genes governing the osmoregulatory choline-glycine betaine pathway of Escherichia coli. J Gen Microbiol **134:**1737-1746.
44. **Malek AA, Chen C, Wargo MJ, Beattie GA, Hogan DA.** 2011. Roles of three transporters, CbcXWV, BetT1, and BetT3, in Pseudomonas aeruginosa choline uptake for catabolism. J Bacteriol **193:**3033-3041.
45. **Diab F, Bernard T, Bazire A, Haras D, Blanco C, Jebbar M.** 2006. Succinate-mediated catabolite repression control on the production of glycine betaine

catabolic enzymes in Pseudomonas aeruginosa PAO1 under low and elevated salinities. Microbiology **152:**1395-1406.
46. **Kortstee GJ.** 1970. The aerobic decomposition of choline by microorganisms. I. The ability of aerobic organisms, particularly coryneform bacteria, to utilize choline as the sole carbon and nitrogen source. Arch Mikrobiol **71:**235-244.
47. **Shortridge VD, Lazdunski A, Vasil ML.** 1992. Osmoprotectants and Phosphate Regulate Expression of Phospholipase-C in Pseudomonas-Aeruginosa. Molecular Microbiology **6:**863-871.
48. **Domenech CE, Garrido MN, Lisa TA.** 1991. Pseudomonas aeruginosa cholinesterase and phosphorylcholine phosphatase: two enzymes contributing to corneal infection. FEMS Microbiol Lett **66:**131-135.
49. **Collins RE, Deming JW.** 2013. An inter-order horizontal gene transfer event enables the catabolism of compatible solutes by Colwellia psychrerythraea 34H. Extremophiles **17:**601-610.
50. **Smith LT, Pocard JA, Bernard T, Le Rudulier D.** 1988. Osmotic control of glycine betaine biosynthesis and degradation in Rhizobium meliloti. J Bacteriol **170:**3142-3149.
51. **White RF, Kaplan L, Birnbaum J.** 1973. Betaine-homocysteine transmethylase in Pseudomonas denitrificans, a vitamin B 12 overproducer. J Bacteriol **113:**218-223.
52. **Meskys R, Harris RJ, Casaite V, Basran J, Scrutton NS.** 2001. Organization of the genes involved in dimethylglycine and sarcosine degradation in Arthrobacter spp.: implications for glycine betaine catabolism. Eur J Biochem **268:**3390-3398.
53. **Weng ML, Zalkin H.** 1987. Structural role for a conserved region in the CTP synthetase glutamine amide transfer domain. J Bacteriol **169:**3023-3028.
54. **Nyunoya H, Lusty CJ.** 1984. Sequence of the small subunit of yeast carbamyl phosphate synthetase and identification of its catalytic domain. J Biol Chem **259:**9790-9798.
55. **Schleif R.** 2010. AraC protein, regulation of the l-arabinose operon in Escherichia coli, and the light switch mechanism of AraC action. FEMS Microbiol Rev **34:**779-796.
56. **Hashim S, Kwon DH, Abdelal A, Lu CD.** 2004. The arginine regulatory protein mediates repression by arginine of the operons encoding glutamate synthase and anabolic glutamate dehydrogenase in Pseudomonas aeruginosa. J Bacteriol **186:**3848-3854.
57. **Chen C, Beattie GA.** 2007. Characterization of the osmoprotectant transporter OpuC from Pseudomonas syringae and demonstration that cystathionine-beta-synthase domains are required for its osmoregulatory function. J Bacteriol **189:**6901-6912.
58. **Chen C, Malek AA, Wargo MJ, Hogan DA, Beattie GA.** 2010. The ATP-binding cassette transporter Cbc (choline/betaine/carnitine) recruits multiple substrate-binding proteins with strong specificity for distinct quaternary ammonium compounds. Mol Microbiol **75:**29-45.
59. **Choi KH, Gaynor JB, White KG, Lopez C, Bosio CM, Karkhoff-Schweizer RR, Schweizer HP.** 2005. A Tn7-based broad-range bacterial cloning and expression system. Nat Methods **2:**443-448.

CHAPTER 3:

EXPLORATION OF GATRS IN *B. THAILANDENSIS*

3.1 Abstract

Glutamine amidotransferase1 (GATase1)-containing AraC-family transcriptional regulators (GATRs) represent a widely disseminated class of regulator about which little is known. Given the particularly high presence of GATRs in β-proteobacteria, we choose to use the genetically tractable *Burkholderia thailandensis* as a model organism to increase our knowledge of these regulators. Phylogenetic comparisons of GATRs between *Burkholderia* species were made to determine how representative *B. thailandensis* is of other Burkholderiales. A small library of molecules was assembled based on functional predictions and potential orthologs of unknown GATRs. GATR deletions strains were screened against the library for growth on these compounds as either a carbon or nitrogen source, resulting in the identification of two additional GATRs in *B. thailandensis*.

To assess the functions of the remaining unknown GATRs, we developed a strategy to generate constitutive mutants by perturbing the predicted dimerization domain based on known constitutive mutations and a known crystal structure. We constructed a constitutively active mutant of a known GATR, GbdR, in *P. aeruginosa*, which led to the induction of genes involved in choline and glycine betaine transport and catabolism. When this strategy was applied to a GATR of unknown function in *B. thailandensis*, I0685, we found that I0685 acts as a repressor of an operon encoding glutathione-

dependent formaldehyde dehydrogenase. This function was additionally confirmed by growth assays in the presence of formaldehyde.

3.2 Introduction

Glutamine amidotransferase1 (GATase1)-containing AraC-family transcriptional regulators (GATRs) are a broadly occurring, yet poorly understood subfamily of AraC-family transcriptional regulators (ATRs). Currently there are few well-characterized members of the GATR subfamily. In order to better understand this subfamily of regulators, we sought to identify and characterize additional GATRs.

The ATR family is a prevalent and widespread family of regulators in bacteria (1-3). ATRs are typically around 300 to 350 amino acids long. Structurally, ATRs may be divided into two domains separated by a short linker region (Figures 1.1). The C-terminal domain contains two helix-turn-helix motifs that are responsible for promoter recognition. The N-terminal domain is responsible for ligand binding and dimerization, and typically includes an unstructured N-terminal arm. ATRs typically function as dimers and bind two degenerate direct repeat half sites proximal to the transcriptional start site where they function as activators. The mechanism of activation for ATRs involves the perturbation of dimerization between monomers caused by ligand binding.

The namesake member of the family, AraC, activates transcription by the 'light-switch' mechanism whereupon transcription is repressed by DNA looping due to a preference for a distal upstream half site (Figures 1.2) (4). Binding of arabinose to the AraC dimer results in a shift in preference for binding sites to the two half sites proximal

to the transcriptional start site, thereby activating expression of the target operon. This shift in orientation of the dimer is caused by interaction between the N-terminal arm, and the ligand binding domain when arabinose is bound (5). Alternatively, dimers of other ATRs such as RegA remain in solution until their ligand is bound, which then causes the nature of the dimer to change and bind to its target promoter and activate transcription (Figures 1.3) (3, 6).

GATRs are distinguishable from other ATRs based on the homology of the N-terminal dimerization and ligand recognition domain to the GATase1 family of enzymes (7, 8). GATases catalyze the transfer of amide groups to various substrates (9). GATase1 domain-containing enzymes include anthranilate synthases, CTP synthases, GMP synthetases, and 2-amino-2-desoxyisochorismate synthases among many more, which are responsible for producing a wide range of both primary and secondary metabolites (10-12). GATase1 domains may be fused to a synthase domain or translated as an independent peptide, which forms a heterodimer with a synthase domain (9). GATase1 domain-containing proteins often function as dimers or sometimes higher order oligomers. In some cases, higher order oligomerization leads to the production of proteinaceous filaments, such as in CTP synthetase, where filament formation negatively regulates enzyme activity in the presence of excess product (13). Many GATRs also share a conserved cysteine, the remnant of a cysteine/glutamate/histidine catalytic triad stemming from the homology to GATase1 enzymes.

GATRs are found in both Gram-positive and Gram-negative bacteria, with a notably higher presence in proteobacteria based on currently available sequence data (14). While many GATR-possessing bacteria encode only one or two homologs, other

genomes may contain more. For example, *Pseudomonas aeruginosa* encodes seven (15) and *Burkholderia cenocepacia* encodes fifteen (16). To investigate the diversity and functionality of GATRs, *Burkholderia thailandensis* was chosen as a model organism based on the availability of genetic tools and its genetic tractability (17-20).

The genome of *B. thailandensis* contains thirteen bioinformatically predicted GATRs including putative orthologs of all GATRs present in the closely related select agents *Burkholderia pseudomallei* and *Burkholderia mallei*, the etiological agents of melioidosis and glanders (21, 22). *B. thailandensis* also contains putative orthologs of GATRs found elsewhere in the *Burkholderia* genus, making the model a respectable survey of the GATR family found here and in more distally related bacteria (Figures 3.1, 3.2, and Table 3.4).

Previously identified GATRs in *P. aeruginosa*, GbdR and ArgR, have been shown to function as transcriptional activators for genes involved in the transport and catabolism of glycine betaine (GB) and arginine respectively (23-26). To better understand the function of *B. thailandensis* GATRs, and what compounds GATRs might enable the bacterium to utilize, we generated deletion strains of each GATR. Synthesizing the functional predictions for adjacent and co-occurring genes with our knowledge of known GATRs, deletion strains were screened against a collection of potential metabolites as sole carbon and nitrogen sources. Using these screen we identified orthologs of the GATRs ArgR and CdhR.

In order to identify the regulons of additional unknown GATRs, we designed an approach based on perturbation of the dimerization of GATRs to achieve differential expression of their target operons independent of ligand by combining information about

known constitutive mutants in characterized GATRs with predicted protein structures. Utilizing this method, we found that the unknown GATR, I0685, is responsible for regulating glutathione-dependent formaldehyde detoxification. Interestingly, this regulation is due to repression by I0685 revealing that GATRs may act as repressors, contrary to previous data that suggested GATRs function solely as activators (26-29).

The regulation of formaldehyde metabolism is important for bacteria, as formaldehyde at high concentrations may damage and interfere with other biological processes (30). Simultaneously, lower amounts of formaldehyde may be useful as singly carbon building blocks for more complex molecules (31). Choline catabolism in *B. thailandensis* produces three molecules of formaldehyde per molecule of choline, and three different GATRs are already known to impact the regulation of this pathway (32). Formaldehyde can also be generated in bacteria as a result of methylglyoxal degradation, which can be generated by glyceraldehyde oxidation and the degradation of glyceraldehyde-3-phosphate or dihydroxyacetone-phosphate (33). The finding of an additional GATR that impacts formaldehyde catabolism is an interesting link to the GATR-regulated catabolism of choline.

3.3 Results

3.3.1 Phylogenetic comparison of GATRs in *Burkholderia*.

Alignment of the full length sequences of *Burkholderia* species GATRs resulted in a general grouping of the GATRs into three groups (Figure 3.1). The first group consists of orthologs of GbdR1, GbdR2, SouR, CdhR, and ArgR, all except ArgR

regulate different metabolic pathways related to GB catabolism. The second group includes I1350, I0685, II1912, II0513, and several *B*. *cenocepacia* GATRs that do not have apparent orthologs in the other Burkholderiales. The third group includes II2197, II0282, and II1760. All the members of this clade are bioinformatically labeled as FtrA or FtrA-like, a GATR accociated with accessory nitrogen metabolism present in *Caulobacter crescentus* (34). However, upon comparison of the clustering with FtrA from *Yersinia enterocolitica* and *C*. *crescentus*, and the conserved synteny of a putative rhodanese divergently transcribed from it, II1760 is likely the genuine ortholog of FtrA as identified from *E*. *coli* (Figures 3.1, 3.2). Because the DNA binding domain might drive alignments due to similarities in HTH motifs subjected to possible selective pressure based on similarities between target binding sites, we also generated a phylogenetic tree based on alignment of only the GATase1-like domain (Figure 3.2). The similar results suggest that the alignments produced were not strongly influenced by the C-terminal domains. The GATR I0059 does not fit into any one of the clade in either alignment.

To further investigate the groupings of putative orthologs derived from the alignments, gene synteny and co-occurrence of the nearby genes using the STRING database and the *Burkholderia* Genome Database were utilized (16, 35), generating Table 3.4. These comparisons suggest that the phylogenetic clustering is accurate. However, the association of BCAS0761 with a particular group based on Table 3.4 was not clear. The phylogenetic alignments group BCAS0761 with I0685 and it co-occurs with orthologs of genes encoding glutathione-dependent formaldehyde dehydrogenase and an associated esterase (*I0686* and *I0687*). Orthologs of *I0686* and *I0687* in *P*. *aeruginosa* are regulated by GfnR, a LysR-family regulator. GfnR is not present in *B*. *cenocepacia* suggesting an

alternative regulator such as an ortholog of I0685 may be required for regulation of these genes (28).

B. *mallei* does not contain an ortholog for SouR, despite possessing orthologs for GbdR1, GbdR2, and CdhR.

3.3.2 GATR functionality by screening carbon and nitrogen utilization.

Our knowledge of the inducing ligands for GbdR and ArgR in *P*. *aeruginosa* (25, 36) and bioinformatic predictions for genes either adjacent or co-occurring with GATRs (Figure 1.7), allowed for the assembly of a collection of compounds to screen as a sole carbon or nitrogen sources to determine if the GATR deletions displayed phenotypes under any of these conditions. Deletion strains were generated for every GATR in *B*. *thailandensis*, except for *II1912*, which differs in length and orientation between differing strains of *B*. *thailandensis*, and may not conserved or functional in *B*. *thailandensis* E264.

Δ*gbdR1*, Δ*gbdR2*, and *ΔsouR* strains exhibited growth defects on choline, glycine betaine, dimethylglycine, and sarcosine as sole carbon sources (Figure 3.3). Δ*gbdR1* and Δ*gbdR2* grew on sarcosine as a sole nitrogen source. However, the *ΔsouR* strain demonstrated no growth on sarcosine (Figure 3.4). These results support our previous findings (32), thus serving as effective controls for the screen. Different strains appeared to exhibit large differences in growth rates on a large set of compounds, which may be partially explained by differences associated with the biofilm phenotype discussed in Appendix 1.

Δ*gbdR1*, Δ*gbdR2*, and Δ*souR* strains were unable to utilize choline-O-sulfate as

either a sole carbon or nitrogen source compared to wild type (Figures 3.3 and 3.4). Choline-O-sulfate is utilized in some plants, fungi, and bacteria as a compatible solute or as management for high levels of sulfate (37-39).

Two other deletion strains exhibited growth defects in the screen. Δ*II1850* was unable to grow on carnitine as either a sole carbon or nitrogen source (Figures 3.3 and 3.4). This suggests that II1850 is likely an ortholog for CdhR, a GATR in *P. aeruginosa* shown to regulate genes for carnitine transport and catabolism (40). The second strain, Δ*I1774*, was defective in growth on arginine as a sole carbon source (Figure 3.3) and appeared to utilize arginine as a sole nitrogen source at a diminished rate compared to wild type and other strains (Figure 3.4). This result suggested that I1774 may be an ortholog for ArgR, a GATR in *P. aeruginosa* responsible for regulating genes involved in arginine synthesis and catabolism (26).

3.3.3 Deletion of *II1850* results in a catabolic defect on carnitine as a sole carbon source.

The synteny of genes adjacent to *cdhR* in *P. aeruginosa* and *II1850* are identical and encode similar proteins, suggesting *II1850* is a *cdhR* ortholog (Figure 3.5A). The *cdhA*, *cdhB*, and *cdhC* genes encode proteins directly responsible for the catabolic degradation of carnitine into glycine betaine (40). *caiX*, a gene encoding a periplasmic binding protein, is responsible for the transport of carnitine from the periplasmic space through interaction with CbcWV, an ATP-binding cassette transporter (41). Finally, *hocS*

encodes hydrolase responsible for the degradation of short-chain O-acetyl carnitines that also feeds into this catabolic pathway (42).

Growth of Δ*II1850* on carnitine as a sole carbon source on MOPS minimal media plates confirms the catabolic defect previously observed in catabolic screens (Figure 3.5B). This defect was complemented by addition of *II1850* back onto the chromosome at the *attTn7* site, under the control of its native promoter (Figure 3.5B, right panel). The wild type, deletion, and complement strains are all capable of growing on pyruvate under these growth conditions (Figure 3.5B, left panel).

3.3.4 Deletion of *I1774* results in a catabolic defect on arginine as a carbon source.

Comparison of the genomic context of *I1774* in *B. thailandensis* with the genomic context of *argR* in *P. aeruginosa* reveals an adjacent operon containing predicted arginine succinyltransferase pathway (AST) genes (Figure 3.6A). These GATRs are both located at the end of a transport operon. This operon is described as being responsible for the transport of arginine and ornithine under the regulatory control of ArgR (25). The arrangement of these operons differs between these species, with the *B. thailandensis* operon containing only putative orthologs to *aotQ* (*I1771*), *aotM* (*I1772*), and *aotP* (*I1773*). A potential ortholog for *aotJ* (*I1783*), a periplasmic binding protein that primarily contributes to the uptake of arginine (25), is located proximally to AST genes. Additionally, an ortholog for *autO* (*II2133*) exists, but it is not strictly required for arginine catabolism (25).

Deletion of *I1774* results in a defect in growth on arginine as a sole carbon source (Figure 3.6B, right panel). This defect is complemented by addition of the *I1771-4* operon at the *attTn7* site under the control of its native promoter. All strains were able to grow on pyruvate as a sole carbon source under these conditions (Figure 3.6B, left panel). Efforts to complement the phenotype using just *I1774* and the immediate 200 bp upstream from it were unsuccessful, likely because *I1774* is part of an operon and the native promoter required for expression is located upstream from *I1771* (data not shown).

3.3.5 GbdR E36G constitutively upregulates transcription of members of the GbdR regulon.

Discovering the function of the remaining unknown GATRs presented a hurdle as neither their ligand nor the genes they regulate are known. The construction of constitutively active transcriptional regulators might be used to identify genes that are upregulated in a ligand independent manner. In order to determine whether constitutively active GATRs can be constructed based on examples of constitutive mutation in SouR from *P*. *aeruginosa* and the crystal structure 3GRA from *P*. *putida* (Figure 3.7), we focused on GbdR in *P*. *aeruginosa* as a model since its regulon and function is understood (23). A GbdR E36G strain was designed based on the predicted structural homology to the D48G allele constitutive mutant of SouR in *P*. *aeruginosa* PA14 (Table 3.5). This mutant allele was capable of rescuing the catabolic phenotype of *ΔgbdR* on choline as a sole carbon source (Figure 3.8), demonstrating that the protein is functional. When grown in the absence of choline, the inducing ligand, microarray analysis revealed

that transcripts in the GbdR regulon were upregulated (Table 3.6). These include genes responsible for the transport of choline and its metabolites such as *cbcW* and *betX*, as well as genes encoding enzymes that catabolize GB into glycine such as *gbcA*, *soxA*, *soxB*, and *glyA* among others. This result suggests successful construction of a constitutively active, ligand-independent version of GbdR. Similar mutations and analyses can be used to uncover the regulons of other GATRs.

3.3.6 I0685 regulates expression of a formaldehyde dehydrogenase.

Similarly to our approach with GbdR, we constructed a ligand independent, constitutive I0685 by incorporating an N32G mutation based on comparison to constitutive mutations in SouR of *P. aeruginosa* and the crystal structure of 3GRA in *P. putida* (Table 3.5). *I0685* is divergently transcribed from a highly conserved operon that includes a glutathione-dependent formaldehyde dehydrogenase and an associated esterase. Surprisingly, when the transcriptome of the N32G expressing strain was compared to the deletion strain, the adjacent dehydrogenase and esterase genes were significantly downregulated in the presence of the predicted "constitutive" mutant (Table 3.7). This was opposite of the result we expected to observe based on our knowledge that other GATRs function as activators (24, 26). The downregulation is dependent on the presence of the N32G mutant, and not simply due to accidental disruption of the promoter during deletion of *I0685*. It is possible that the mutation has resulted in a conformational change that causes insensitivity to de-repressing ligand that might normally be present.

In order to further assess the potential impact on formaldehyde detoxification, *I0685* deletions and complement strains were constructed in a Δ*II1870* (orthologs commonly named *fdhA*) background in order to separate the impact of the glutathione-dependent formaldehyde dehydrogenase from redundant detoxification by the glutathione-independent formaldehyde dehydrogenase. Previously, the multiple systems present in *Burkholderia* have been shown to successfully compensate for each other (43). Growth in the presence of formaldehyde revealed that the *I0685* deletion had a slight growth advantage compared to the wild type. Complementation of *I0685* back onto the chromosome was slightly deleterious, and complementation with the N32G mutant greatly inhibited growth in the presence of formaldehyde (Figure 3.9). This suggested that the *I0685* may function to repress the expression of *I0686* and *I0687*.

3.4 Discussion

3.4.1 Building constitutive regulators.

The construction of constitutively active transcriptional regulators is a useful tool both for uncovering details of mechanism and, as we have explored here, revealing target genes. Constitutive mutants in AraC have been previously published, with the majority being found to be localized in the N-terminal arm (44). Other constitutive mutations were found to occur in the dimerization domain itself and some in the DNA binding domain (45). Contrary to the experience of other groups, all constitutive mutations we have identified experimentally occur in the predicted dimerization face of the N-terminal domain (Figure 3.7). Although GATRs also appear to possess an unstructured N-terminal

arm, it is not well-conserved and it is unclear if the N-terminal arm plays an important mechanistic role in GATR function. However, GATase functionality is heavily dependent on ligand induced conformational changes, which are necessary for proper formation of ammonia channels to supply associated synthase domains (8). Because dimerization or higher oligomerization of GATase-containing enzymes is critical to proper formation of ammonia channels and orientation of active sites for functionality, it stands to reason that altering the ability to undergo conformational changes through mutations in the dimerization domain might greatly affect the functionality of the protein. Indeed, constitutive GATases have been constructed through mutation of residues critical for proper active conformation (46). It is possible that the homology of GATRs to GATase1 enzymes have contributed a mechanism for conformational change allowing GATRs to function through a conformational change-based mechanism similar to the mechanisms of other ATRs such as AraC and RegA.

In order to test our ability to generate constitutive GATRs, we utilized GbdR from *P. aeruginosa* as a model. GbdR is responsible for regulation of enzymes in the GB catabolic pathway, various transporters, and the extracellular phospholipase PlcH (23, 24, 27). This pathway converts choline into the ubiquitous osmoprotectant GB and can further catabolize it through a series of demethylation steps ultimately into glycine. *B. thailandensis* also shares this catabolic pathway, although the gene organization and regulation scheme differs from *P. aeruginosa* and includes two GbdR orthologs instead of just one (32). GbdR is thought to bind DNA as a dimer due not only to similarities to ATRs, but more specifically to ArgR which has been shown to dimerize and share similar structure of the orientation of the direct repeat binding sites of within target promoters

(23, 25, 47). Using the combined information from the crystal structure of the N-terminal domain of 3GRA from *P. putida* and the constitutive mutants of SouR from *P. aeruginosa*, we were able to successfully generate a constitutive GbdR that activates transcription of target genes by altering a residue in the predicted dimerization domain. The alteration to the dimerization domain may alter the conformational preference of the dimer, resulting in a bias toward the activating conformation even in the absence of ligand. Currently it is unclear whether the change in conformation of this constitutively activating GbdR is altering an interaction with the RNA polymerase holoenzyme or changing the way in which GbdR acts with its DNA binding half sites, as in the case for AraC.

3.4.2 Identification of a GATR acting as a repressor of formaldehyde detoxification.

Formaldehyde is a biologically significant molecule because it can be a useful one carbon building block, or a highly reactive molecule damaging proteins and DNA. Accordingly, the metabolism of formaldehyde must be tightly controlled (33). Consequently, bacteria utilize several pathways for formaldehyde detoxification. *B. thailandensis* is predicted to contain two formaldehyde detoxification pathways: a glutathione-independent formaldehyde dehydrogenase and a glutathione-dependent formaldehyde dehydrogenase. Other Burkholderiales have been shown to contain other, alternative pathways for formaldehyde detoxification. *Burkholderia cepecia* TM1 possesses genes encoding a ribulose monophosphate-dependent pathway shown to be important for the detoxification of formaldehyde generated by the degradation of lignin from plant matter (48, 49). Closely related and previously classified as a *Burkholderia*,

Paraburkholderia fungorum LB400 utilizes the pterin-dependent formaldehyde detoxification pathway which is dependent on either the pterin-containing tetrahydrofolate or tetrahydromethanopterin. This pathway is widespread amongst bacteria due to utilization in the one carbon transfer needed for the synthesis of purines and amino acids (33, 43).

The glutathione-independent formaldehyde dehydrogenase, II1870, is adjacent to gene required for GB catabolism. Transcription of *II1870* may be under the regulatory control of the GbdR orthologs GbdR1 and GbdR2 in addition to a conserved raffinose repressor in the same operon (32). This reaction relies on NAD^+ and results in the generation of formate (50). The close association between II1870 and GB catabolism is likely due to the three molecules of formaldehyde produced for every molecule of GB that is catabolized to pyruvate (36).

The glutathione-dependent pathway utilizes glutathione to react with formaldehyde resulting in S-hydroxymethylgluathione, which is then converted to S-formylglutathione by the formaldehyde dehydrogenase (I0686 in *B*. *thailandensis*) (51, 52). An associated esterase (I0687 in *B*. *thailandensis*) then converts this product into formate and regenerates glutathione (53). While the glutathione-dependent formaldehyde dehydrogenase and esterase are regulated by the LysR-family transcriptional regulator GfnR in *P*. *aeruginosa* and many other bacteria, *B*. *thailandensis* has no ortholog for GfnR. Instead an unknown GATR, I0685, is divergently transcribed from the operon containing these genes (28). Operons divergently transcribed from their regulator is a common gene arrangement (54).

We applied our strategy for constructing constitutive GATRs to I0685. We

expected a ligand-independent upregulation of the glutathione-dependent formaldehyde dehydrogenase and esterase genes. Surprisingly, the deletion strain expressing *I0685* N32G significantly downregulated expression of these genes when compared to an *I0685* deletion strain carrying the empty vector (Table 3.7). The change in expression of these genes stimulated the investigation of the effects of formaldehyde on the growth of various strains. Strains producing wild type, or particularly I0685 N32G, have reduced tolerance of formaldehyde compared to the deletion itself, likely due to direct repression of *I0686* and *I0687*.

The I0685 regulatory mechanism is novel in multiple ways. First, characterized ATRs are typically activators (3, 55) and this marks the first incidence of any GATR acting specifically as a repressor. Second, GATRs have been associated with smaller amine-containing compounds such as arginine, carnitine, GB, and sarcosine. However, *I0685-7* are not transcribed in a GB- or GbdR- dependent manner, and the upregulation of *I0686-7* has been observed in connection with the catabolism of GB, which produced formaldehyde (32). It is quite possible that formaldehyde itself is the ligand to which I0685 is responding, which suggests not all GATRs respond to amine-containing compounds as we had initially suspected.

3.4.3 II1850 regulates carnitine catabolism.

Carnitine is an abundant metabolite in many environments (56) and found in particularly high levels in muscle tissue (57). Carnitine is also found in plants, but in far lower concentration than in animals (58). Animals use carnitine to shuttle fatty acids by

using O-acylcarnitine shuttles, with the acylcarnitines being generated by the liver (59-61). Interest in carnitine has been piqued recently because gut microbiota have been shown to metabolize dietary carnitine into trimethylamine that is then converted by the host into trimethylamine-N-oxide, which has been linked to atherosclerosis (62, 63). In addition to utilizing carnitine as a carbon and/or nitrogen source, examples of both Gram-negative and -positive bacteria have been shown to use carnitine as an osmoprotectant directly, or by catabolism into GB (40, 64-67).

II1850 is clearly an ortholog of CdhR in *B*. *thailandensis* given the similarity in gene synteny and phenotype of the deletion strains when compared to *P*. *aeruginosa* (Figure 3.5). The presence of a carnitine catabolic pathway provides another route to acquire the osmolyte GB. In addition, the pathway provides yet another opportunity for taking advantage of available nutrients that may be present when the bacterium is in association with decaying plant or animal matter in the environment.

3.4.4 I1774 regulates arginine catabolism.

The metabolism of arginine in Gram-negative bacteria is well characterized and typically regulated in large part by one of two unrelated regulators named ArgR. ArgR in *E*. *coli* is a hexameric repressor regulating genes involved in arginine transport and metabolism (68, 69). In bacteria lacking the hexameric repressor, a GATR alternative (also called ArgR) is often found. The GATR ArgR has been shown to regulate genes involved in arginine biosynthesis, as well as genes in the AST pathway in *P*. *aeruginosa* (26). This pathway is one of four pathways responsible for arginine utilization and is

considered the primary pathway responsible for the aerobic catabolism of arginine (70).

ArgR in *P*. *aeruginosa* regulates *ldcA*, a gene responsible for lysine catabolism, in an arginine dependent manner. It is hypothesized that the lack of lysine-responsive control of these genes accounts for poor utilization of lysine as a sole carbon source, despite being able to utilize lysine when arginine is present (71). A similar regulation scheme may be present in *B*. *thailandensis*, either due to regulation of lysine decarboxylases or the specific regulation and specificity of the assorted substrate periplasmic binding proteins. The presence of such a regulation scheme might explain the lack of growth of *B*. *thailandensis* strains on lysine as a sole carbon source in our screen (Figure 3.3).

Based on the catabolic phenotype and high degree of homology between parts of the arginine transport and catabolic pathway components, I1774 is an ortholog of ArgR. Similarities between components of the *P*. *aeruginosa* AST pathway and observed phenotypes in *B*. *thailandensis* suggest a similar approach to regulation in both organisms.

3.4.5 II1760, a potential regulator of cyanide detoxification.

Many Proteobacteria possess a single GATR that has been bioinformatically classified as FtrA, while *B*. *thailandensis* contains three GATRs that are currently annotated as being FtrA-like: II0282, II1760, and II2197. In our phylogenetic analysis of the GATRs (Figure 3.11A), we included typical examples of FtrA from *E*. *coli*, *C*. *crescentus*, and *Y*. *enterocolitica*. The resulting phylogenetic trees definitively clustered these GATRs with II1760 and putative homologs from other *Burkholderia*. The synteny

of the divergently transcribed gene, *II1761*, versus its putative homologs is conserved in all of these examples (Figure 3.11B). This suggests that this adjacent gene is a likely regulatory target for II1760.

II1761 is predicted to encode rhodanese, an enzyme that detoxifies cyanide by converting it to thiocyanate (Figure 3.11C) (72). The toxicity of cyanide is often due to inhibition of cytochrome c or other electron receptors, consequently impairing aerobic respiration (73). Rhodanese-like proteins are widely distributed among eukaryotes, prokaryotes, and archaea, and may also contribute to sulfur and selenium metabolism in addition to their role in cyanide detoxification (74). Many bacteria, specifically those with rhodanese, are of interest as potential bioremediation agents (75, 76). *B. cepacia* has been previously shown to degrade a wide range of cyanide containing compounds (77). Bacteria and fungi often produce cyanide containing compounds to facilitate competition or avoid predation (78). An antagonistic relationship between *P. aeruginosa* and *B. cenocepacia* has also been described in the cystic fibrosis lung environment wherein cyanide containing compounds produced by *P. aeruginosa* inhibit the growth of a particular strain of *B. cenocepacia* (79).

This raises the possibility of a GATR responsible for cyanide detoxification and that potentially uses cyanide as a ligand. More direct evidence of regulation of rhodanese by II1760 in *B. thailandensis* is still needed, and the function of this putative rhodanese remains to be ascertained. It is likely that this information will reflect the function of FtrA, perhaps the most pervasive GATR in proteobacteria, and its relationship to rhodanese in other species.

3.4.6 Significance of choline-O-sulfate.

Some plants rely on osmoprotectant other than glycine betaine. In particular the Plumbaginaceae, a salt-tolerant group of plants, have evolved to accumulate choline-O-sulfate to high levels in contrast to osmoprotectants such as glycine betaine (80). Choline-O-sulfate has also been speculated to be involved in bacteria-plant communication (81). *B. thailandensis* possesses genes predicted to transport and catabolize choline-O-sulfate, which were found to be induced in the presence of choline in our previous studies (32), and could be expected to feed into the downstream choline and glycine betaine catabolic pathways. Because the precise environmental niche of many *Burkholderia* species is not yet fully appreciated, perhaps this metabolic capacity is suggestive of co-occurrence with organisms commonly capable of producing and exuding choline-O-sulfate.

3.4.7 **The absence of SouR in *B. mallei***.

Although it is tempting to speculate that one of the remaining GATRs may be responsible for regulation of the downstream portion of GB catabolism, further examination reveals that there are no putative orthologs of tetrameric sarcosine oxidase by BLAST (32). Sarcosine may also be catabolized by monomeric sarcosine oxidase (82, 83), but a putative ortholog is not obvious. It is possible that *B. mallei* either lacks the ability to utilize GB metabolites as carbon or nitrogen sources or that it possesses an alternative pathway not yet appreciated.

3.5 Methods

3.5.1 Alignment of GATRs and phylogenetic tree construction.

The full length sequence of all GATRs from *B. thailandensis* E264, *B. pseudomallei* K96243, *B. mallei* ATCC 23344, *B. cenocepacia* J2315, and several GATRs bioinformatically identified as orthologs of FtrA, in addition to AraC and PyrG from *Escherichia coli*, were aligned using the MUSCLE algorithm (84). In addition, the predicted GATase1-like domains for these GATRs and PyrG were also aligned and compared in order to eliminate similarity driven by HTH domains that bind similar DNA sequences. Maximum likelihood phylogenetic trees were generated using the MEGA6 software package (85). Each phylogenetic tree was bootstrapped using five hundred bootstrap repetitions.

3.5.2 Culture conditions for strains.

B. thailandensis, *P. aeruginosa*, and *E. coli* strains were typically plated on Lennox broth (LB) (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 1.5% agar) agar and grown at 37^{o}C prior to generating overnights for experiments. Modified MOPS minimal media (86, 87) with and without NH_4^+ was used for growth on particular carbon or nitrogen sources, as well as inductions.

3.5.3 Construction of deletion strains.

Deletion strains in *B. thailandensis* were generated by inducing natural competency in combination with the introduction of splice overlap extension PCR products as described in Thongdee et al. using primers in (Table 3.3) (17). Trimethoprim resistance markers in

the resulting insertion-deletion mutants were removed utilizing pFLPe4 as described by Choi et al. (18).

3.5.4 Carbon and nitrogen utilization screens.

A custom selection of compounds was derived from bioinformatics, functional predictions based on gene synteny, and co-occurrence of genes associated with unknown GATRs using the STRING database (35). Compounds for the carbon-source screen included pyruvate as a positive control, arginine, asparagine, β-alanine, N,N-dimethyl-β-alanine, 4-aminobenzoic acid (PABA), glutamate, glutamine, glycine, histidine, proline, hydroxyproline, serine, O-acetyl L-serine, ornithine, lysine, threonine, taurine, histamine, ethanolamine, agmatine, canavanine, citrulline, ecotine, theanine, xanthine, urea, uric acid, trigonelline, ɣ-amino butyric acid (GABA), ɣ-butyrobetaine (GBB), guanidine, aminoethylethanolamine (AEEA), carnitine, choline-O-sulfate, choline, glycine betaine (GB), dimethylglycine (DMG), sarcosine, cadaverine, spermidine, putricine, N-acetyl-D-glucose, N-acetyl-D-galactose, UDP-glucose, creatine, and creatinine. The nitrogen-source screen included arginine, asparagine, β-alanine, N,N-dimethyl-β-alanine, cysteine, glutamate, glutamine, glycine, histidine, hydroxyproline, hydroxyserine, lysine, O-acetyl-L-serine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, taurine, agmatine, canavanine, citruline, ectoine, thenine, xanthine, urea, uric acid, trigonelline, GBB, GABA, creatine, creatinine, carnitine, choline-O-sulfate, choline, GB, DMG, sarcosine, cadaverine, spermidine, putrescine, UDP-glucose, N-acetyl-glucosamine, N-acetyl-galactosamine, anthranilate, and PABA. Compounds were prepared in 48-well plates (Costar), one compound per well. For carbon-source screen plates, compounds

were added at 100 mM in 30 μL for a final concentration of 20 mM. For nitrogen-source screen plates, compounds were added at 25 mM in 30 μL for a final concentration of 5 mM. WT and deletion strains were struck onto LB agar plates and incubated overnight at 37^{o}C. These were used to inoculate 3 mL of MOPS with 20 mM pyruvate and 5 mM glucose, which were incubated at 37^{o}C overnight on a culture wheel. For carbon source screening, overnights were collected by centrifugation, washed with 1 mL MOPS, and resuspended in MOPS to an OD_{600} measurement of 0.05. For nitrogen source screening, overnights were spun down and washed with 1 mL MOPS minus NH_4^+ plus 20 mM pyruvate, then resuspended in MOPS minus NH_4^+ plus 20 mM pyruvate to an OD_{600} measurement of 0.05. In both cases, 120 μL of resuspended cells were added per well of the appropriate plates, which were sealed with sterile, gas-permeable plate film. The plates were incubated at 37^{o}C in humidity chambers on a shaker, and OD_{600} was measured at 24 and 48 hours.

3.5.5 Prediction of amino acid mutations resulting in constitutively active GATRs.

Understanding of the putative dimerization of GATRs is based off of the crystal structure of the N-terminal domain of the SouR ortholog, 3GRA, originating from *Pseudomonas putida* (88, 89). Unpublished data from Graham Willsey at the University of Vermont indicated mutations in particular residues of *P*. *aeruginosa* SouR that resulted in constitutive activation of SouR-regulated genes (Figure 3.7). The N-terminal domain of *P*. *aeruginosa* SouR was strung onto the 3GRA crystal structure using UCSF Chimera (90), which revealed that the constitutive mutations were localized in the putative

dimerization domain. Portions of the N-terminal domain from various GATRs were then modeled onto the structure of 3GRA to identify the structurally orthologous residues in each GATR, resulting in the predictions contained within Table 3.5.

3.5.6 Generation of complement and constitutive GATR strains.

Plasmid tools for *attTn7* integration produced by Choi and colleagues (19, 20, 91) were used for complementation in both *B*. *thailandensis* and *P*. *aeruginosa*. For *B*. *thailandensis*, complementing plasmids based on pUC18T-mini-TN7T-TP or pUC18-mini-TN7T-Zeo (Table 3.2) were co-transformed with pTNS2 using electroporation and subsequently selected on either LB agar plates containing 100 μg/mL trimethoprim or Low-salt LB plates (3 g/L NaCl, pH= 7.5 with NaOH) containing 250 μg/mL zeocin. For *P*. *aeruginosa*, a tri-parental mating strategy was utilized. Briefly, WT or Δ*gbdR P*. *aeruginosa* PA14 was combined with *E*. *coli* S17-1 containing pUC18-mini-TN7T-Gm based complement or empty vector and *E*. *coli* S17-1 containing pTNS2 in various proportions. Prior to conjugation, *P*. *aeruginosa* strains were heat-shocked for fifteen minutes at 42^{o}C. These mixtures were spotted onto LB plates without antibiotics and incubated overnight at 30^{o}C. Spots were scrapped off the plate and re-plated onto *Pseudomonas* isolation agar (PIA) (BD) plates containing 50 μg/mL gentamicin and incubated overnight at 37^{o}C. Resistant colonies were subcultured on PIA with 50 μg/mL gentamicin and selected clones were screened by PCR.

3.5.7 Growth of strains and RNA extractions.

Putative constitutive complement mutants were grown in tandem with their respective deletion strain as a control to avoid potential interference by wild type GATRs either through interaction between mutant and wild-type GATRs or competition for DNA binding sites. 3 mL MOPS with 20 mM pyruvate and 5 mM glucose cultures were inoculated from LB plates and grown on a rotary wheel at 37^{o}C. These overnights were pelleted in a table top centrifuge and washed once in 1 mL MOPS media with 20 mM pyruvate and resuspended in 1 mL of the same. OD_{600} measurements were taken so that induction cultures could be inoculated at a starting OD_{600} of 0.3, and these were grown for 4 hours at 37^{o}C on a rotary wheel. Inductions were pelleted and resuspended in 800 µL of 95^{o}C RNAzol RT (Sigma) whereupon they were frozen at -80^{o}C after five minutes at room temperature.

To continue the RNA extraction, samples were thawed and 400 µL water was added to each sample and vortexed vigorously, followed by a room temperature incubation for fifteen minutes. Samples were centrifuged at 10,000 x g for fifteen minutes at 4^{o}C, and the supernatant was transferred to a clean microcentrifuge tube. The supernatant was then combined with 100% molecular grade ethanol at a 1:1 ratio, mixed, and applied to Zymo-Spin IIC columns (Zymo Research (ZR)). Columns were centrifuged for thirty seconds at 10,000 rpm, washed with 400 µL direct-zol prewash (ZR), then washed with 700 µL and 400 µL of wash buffer (ZR). The columns were further centrifuged to dryness for two minutes at 10,000 rpm, then RNA was eluted using 100 µL RNase-free water into a clean microcentrifuge tube.

In order to remove DNA, 11 µL of 10x DNase I Buffer and 1 µL of RNase-free DNase I (New England BioLabs) were added to each sample and incubated at 37^{o}C for

twenty minutes. An additional 1 µL of RNase-free DNase I was added to each sample and the incubation at 37^{o}C was continued for another twenty minutes. Samples were then further processed using RNeasy mini kit (Qiagen) per the manufacturer's protocol sans further DNase I steps. Resulting RNA was quantified by NanoDrop (Thermo, ND-1000).

3.5.8 Microarray analysis of constitutive mutants.

Prior to microarray analysis, RNA was quantified using Qubit (Thermofisher) and assayed for quality on a Bioanalyzer using the RNA 6000 pico kit (Agilent). cDNA was prepared from total RNA using the Ovation Pico WTA System V2 (NuGEN). Samples were applied to custom GeneChips (Affymetrix) designed for *P. aeruginosa* PA14, *B. thailandensis* E264, *Klebsiella pneumoniae*, and *Stenotrophomonas maltophilia* genomes. Runs were analyzed with Expression Console and Transcriptome Analysis Console v3.0 provided by Affymetrix.

3.5.9 cDNA synthesis and quantitative real-time PCR.

cDNA was generated using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen), following the manufacturer's protocol for transcripts with high GC content. Transcripts were amplified using 1 µL NS5 primer and 2.5 µL of 100 ng/µL RNA per sample.

Reactions for qRT-PCR consisted of 7.5 µL Q5 High-Fidelity 2x Master mix (NEB), 1.6 µL 1x SYBR Green I (Thermofisher), 0.2 µL of each of the two 20 µM

primers used, 2.75 µL water, 0.75 µL DMSO, and 2 µL of cDNA. qPCR was performed using a BioRad QFX96 qPCR system. Samples were compared by calculating ΔΔCt values.

3.5.10 Growth of *I0685* deletion and complemented strains in the presence of formaldehyde.

Δ*I0685*, Δ*I0685 att*::*I0685* WT, and Δ*I0685 att*::*I0685* N32G strains were constructed in a *B*. *thailandensis* Δ*II1870* background to minimize the compensatory effects of other formaldehyde detoxification systems. Strains were grown overnight in MOPS minimal media supplemented with 20 mM pyruvate and 5 mM glucose at 37^{o}C. Overnights were pelleted and washed 1x with 1 mL MOPS with 20 mM pyruvate, and resuspended in the same. Resuspended cells were diluted to an OD_{600} of 0.05 in either MOPS plus 20 mM pyruvate or MOPS plus 20 mM pyruvate at 0.75 mM formaldehyde. Cells were incubated at 37^{o}C for 24 hours in a 24 well plate, 500 µL per well.

3.6 Chapter 3 Figures

Figure 3.1 – GATR phylogeny based on full-length.

All full-length GATR amino acid sequences from *B. thailandensis* E264, *B. pseudomallei*, *B. mallei*, *B. cenocepacia*, PyrG (CTP synthetase) from *E. coli*, and FtrA orthologs from *E. coli*, *C. crescentus*, and *Y. enterocolitica* were aligned and a maximum likelihood, unrooted phylogenetic tree was constructed using 500 bootstrap replicates. Bootstrap confidence values are displayed at branches.

Figure 3.2 – GATR phylogeny based on GATase1-like domain.

The amino acid sequences for bioinformatically predicted GATase1-like domains of the GATRs of *B. thailandensis* E264, *B. pseudomallei*, *B. mallei*, *B. cenocepacia*, PyrG (CTP synthetase) from *E. coli*, and FtrA orthologs from *E. coli*, *C. crescentus*, and *Y. enterocolitica* were aligned and a maximum likelihood, unrooted phylogenetic tree was constructed using 500 bootstrap replicates. Bootstrap confidence values are displayed at branches.

Figure 3.3 – Growth of deletion strains on sole carbon sources.

The growth of WT and deletions strains on sole carbon sources at 37^{o}C and was monitored by measuring OD_{600}. The heat map represents averaged readings between the 24 and 48 hour time points. Darker purple indicates a higher optical density.

Figure 3.4 – Growth of deletion strains on sole nitrogen sources.

The growth of WT and deletions strains on sole nitrogen sources at 37^{o}C and was monitored by measuring OD_{600}. The heat map represents averaged readings between the 24 and 48 hour time points. Darker purple indicates a higher optical density.

Figure 3.5 –*II1850* (*cdhR*) regulates carnitine catabolism.

(A) The synteny of genes divergent from *II1850* in *B*. *thailandensis* E264 and *cdhR* in *P*. *aeruginosa* PAO1 is compared. The divergent genes represent clear orthologs when sequences are compared and their arrangement is conserved between the two species. (B) *B*. *thailandensis* E264 *att*::vector (WT) is able to grow on carnitine as a sole carbon source on MOPS minimal media plates. Δ*II1850 att*::vector is unable to utilize carnitine as a sole carbon source under the same conditions. The complementation of *II1850* at the *attTn7* site, under the control of its native promoter, restores the ability to grow on carnitine as a sole carbon source. This suggests that II1850 is an ortholog of CdhR and regulates the divergent genes in a similar manner.

Figure 3.6 – *I1774* (*argR*) regulates arginine catabolism.

(A) Genes in the neighborhood of *I1774* in *B*. *thailandensis* E264 are bioinformatically predicted to be involved in the catabolism and transport of arginine, similarly to those in the neighborhood of *argR* in *P*. *aeruginosa* PAO1. The synteny of genes is not perfectly

conserved between the species, but the similarity of predicted function is suggestive that ArgR and I1774 are orthologous. (B) *B. thailandensis* E264 *att*::vector (WT) is able to grow on arginine as a sole carbon source on MOPS minimal media plates. Δ*I1774* *att*::vector is unable to utilize arginine as a sole carbon source under the same conditions. This defect may be complemented by placing the *I1771-4* operon at the *attTn7* site.

Figure 3.7 – Constitutive mutations in *P. aeruginosa* SouR overlaid on 3GRA.

The amino acid sequence of SouR from *P. aeruginosa* was overlaid on the crystal structure of its ortholog in *P. putida*, 3GRA. Constitutive mutants of SouR were sequenced and cognate amino acids in 3GRA were identified based on the overlay. Highlighted mutations are as follows: P41S,T (red), D48G (orange), D76N (green), and P185S (blue).

Figure 3.8 – Constitutive GbdR rescues growth on choline.

Similarly to the previously published ability of wild type *gbdR* to complement growth defects of Δ*gbdR* in *P. aeruginosa* on choline as a sole carbon source, Δ*gbdR att*::*gbdR* E36G was able to rescue growth. This suggests that GbdR E36G is capable of folding properly and inducing GbdR controlled promoters.

Figure 3.9 – I0685 represses resistance to formaldehyde.

Δ*II1870 att*::vector is capable of growing in the presence of 0.75 mM formaldehyde. Deletion of Δ*I0685* causes a slightly increased amount of growth, while complementation of the deletion with WT *I0685* slightly reduces the amount of growth compared to wild type. Complementation of the deletion with *I0685* N32G results in a striking decrease in the ability to tolerate formaldehyde compared to any of the other strains. Data is representative of three independent experiments.

Figure 3.10 – *II1912* synteny in other *Burkholderia*.

Comparison of synteny for *II1912* orthologs in multiple strains of *B. thailandensis* and one representative strain of *B. pseudomallei*. Schematic is approximate.

Figure 3.11 – FtrA is a potential regulator of cyanide detoxification.

(A) Alignment of amino acid sequences done as described for Figure 3.1, but nodes other than FtrA are collapsed for clarity. The FtrA node is highlighted in red. (B) Gene arrangement of *II1760* and *II1761*. Synteny is identical for other Burkholderia species containing obvious *II1760* orthologs. (C) Cyanide detoxification reaction catalyzed by rhodanese.

3.7 Chapter 3 Tables

Table 3.1 – Strain list.

Table 3.2 – Plasmid list.

Table 3.3 – Primer list.

Table 3.4 – *Burkholderia* GATR homologs based on gene synteny.

This chart uses observed synteny and bioinformatic predictions of genes adjacent to GATRs in *B. thailandensis*, *B. pseudomallei*, *B. mallei*, and *B. cenocepacia* in an attempt to predict potential orthologs. It also denotes if the divergent gene is predicted to be a DJ-1 family member protein, a common feature of many putative GATRs.

Table 3.5 – Predicted constitutive mutants.

Predictions of residues that, when altered, may perturb the dimerization of the GATR and lead to constitutive activity.

Table 3.6 –GbdR E36G microarray results.

GbdR E36G induces expression of known GbdR regulon members in the absence of ligand. Cutoffs for data displayed are: fold effect ≥ 2 or ≤ -2, p value ≤ 0.05. Genes related to GB transport or catabolism are highlighted.

Table 3.7 – I0685 N32G microarray results.

I0685 N32G downregulates expression of the glutathione independent formaldehyde dehydrogenase and its associated esterase. Cutoffs for data displayed are: fold effect ≥ 5 or ≤ -5, p value ≤ 0.05.

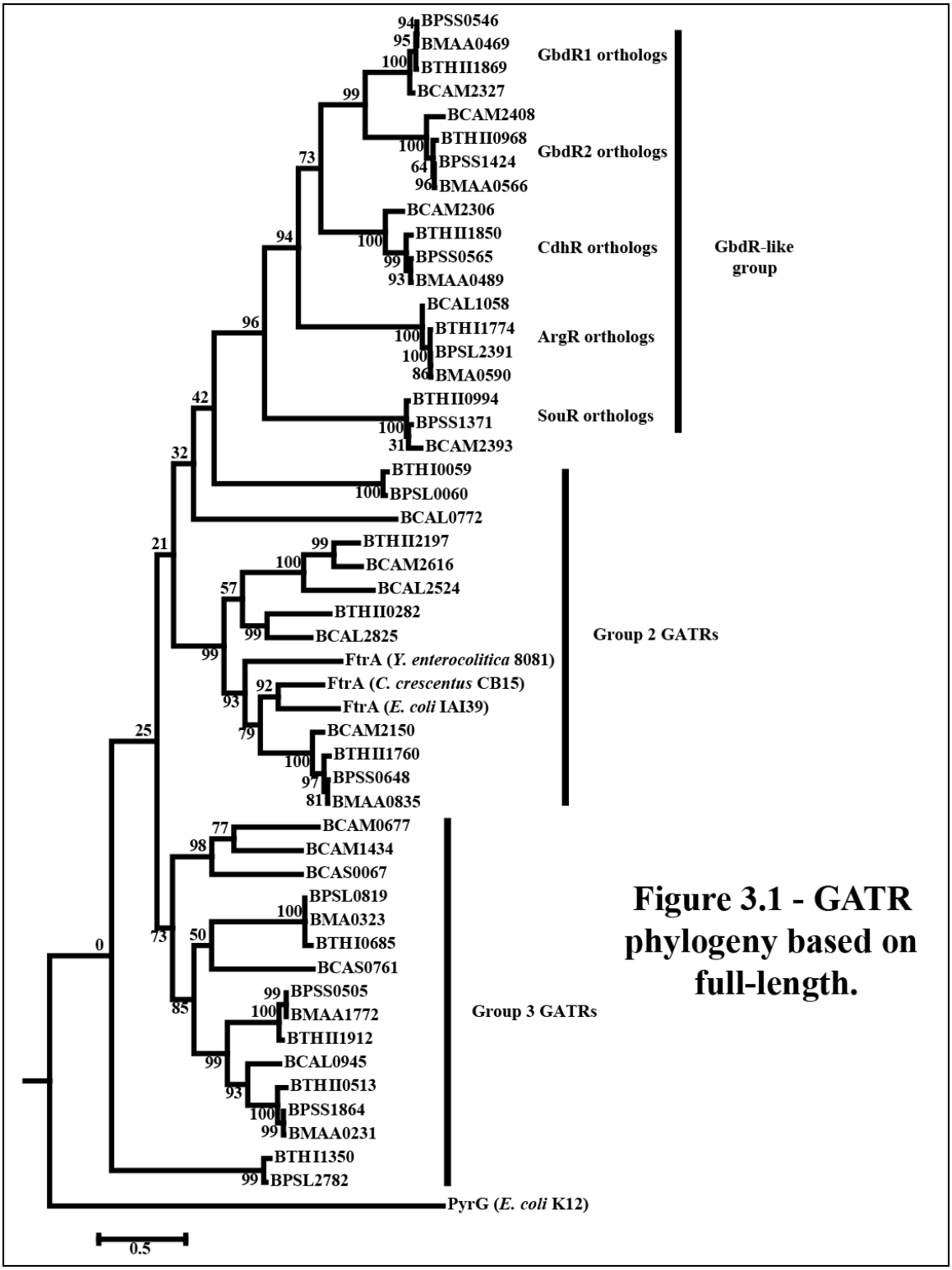


Figure 3.1 - GATR phylogeny based on full-length.

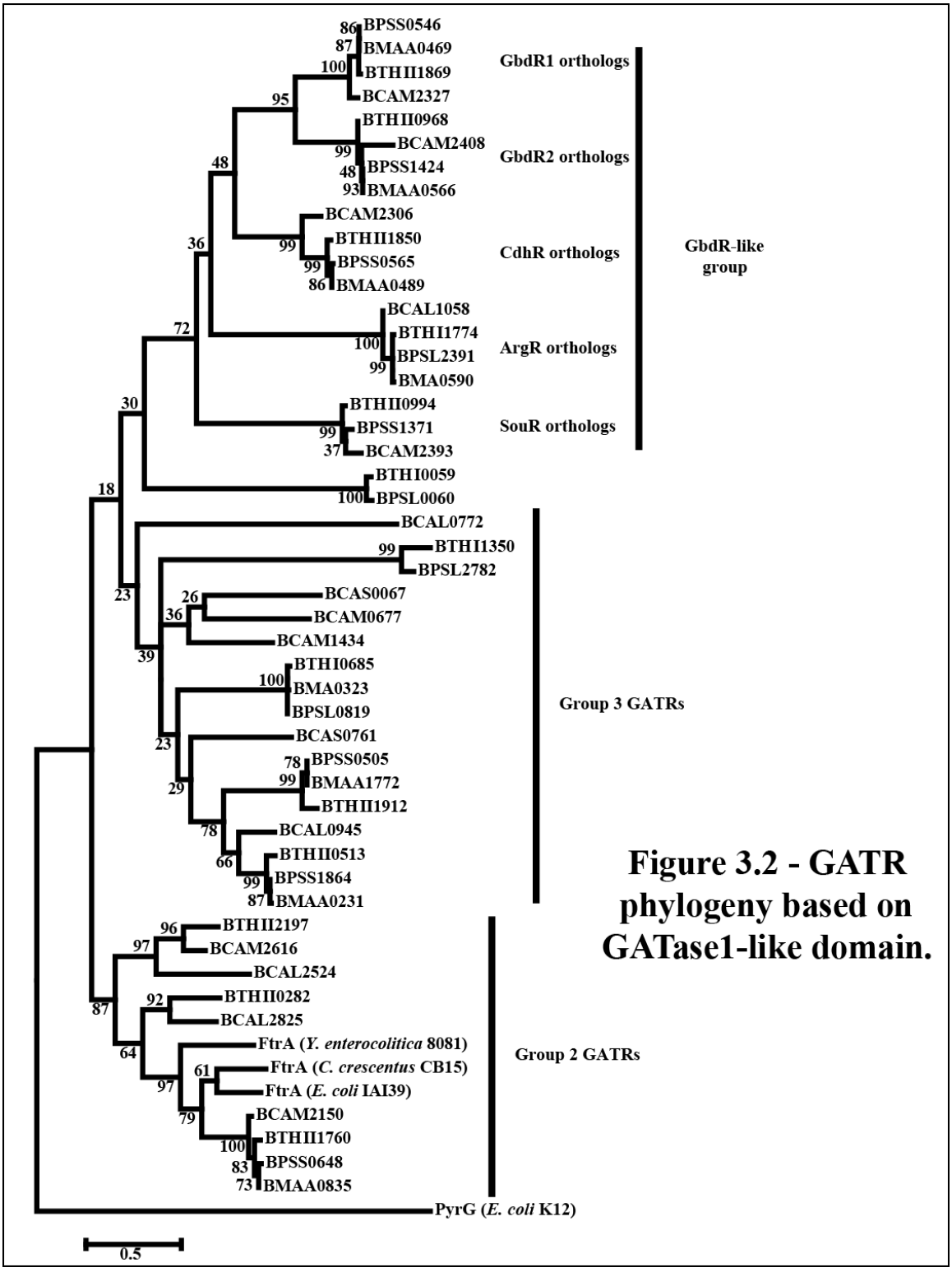


Figure 3.2 - GATR phylogeny based on GATase1-like domain.

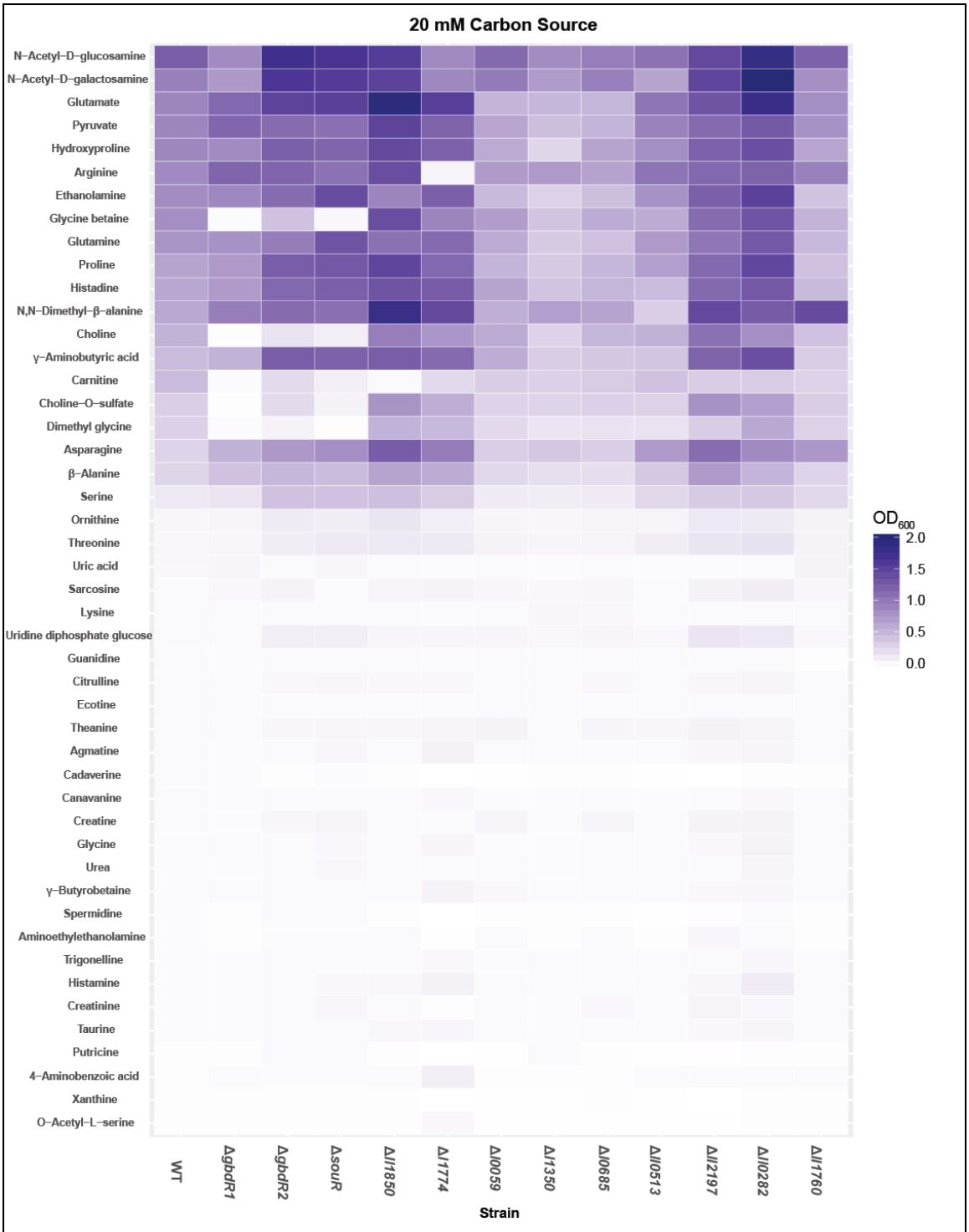


Figure 3.3 - Growth of deletion strains on sole carbon sources.

Figure 3.4 - Growth of deletion strains on sole nitrogen sources.

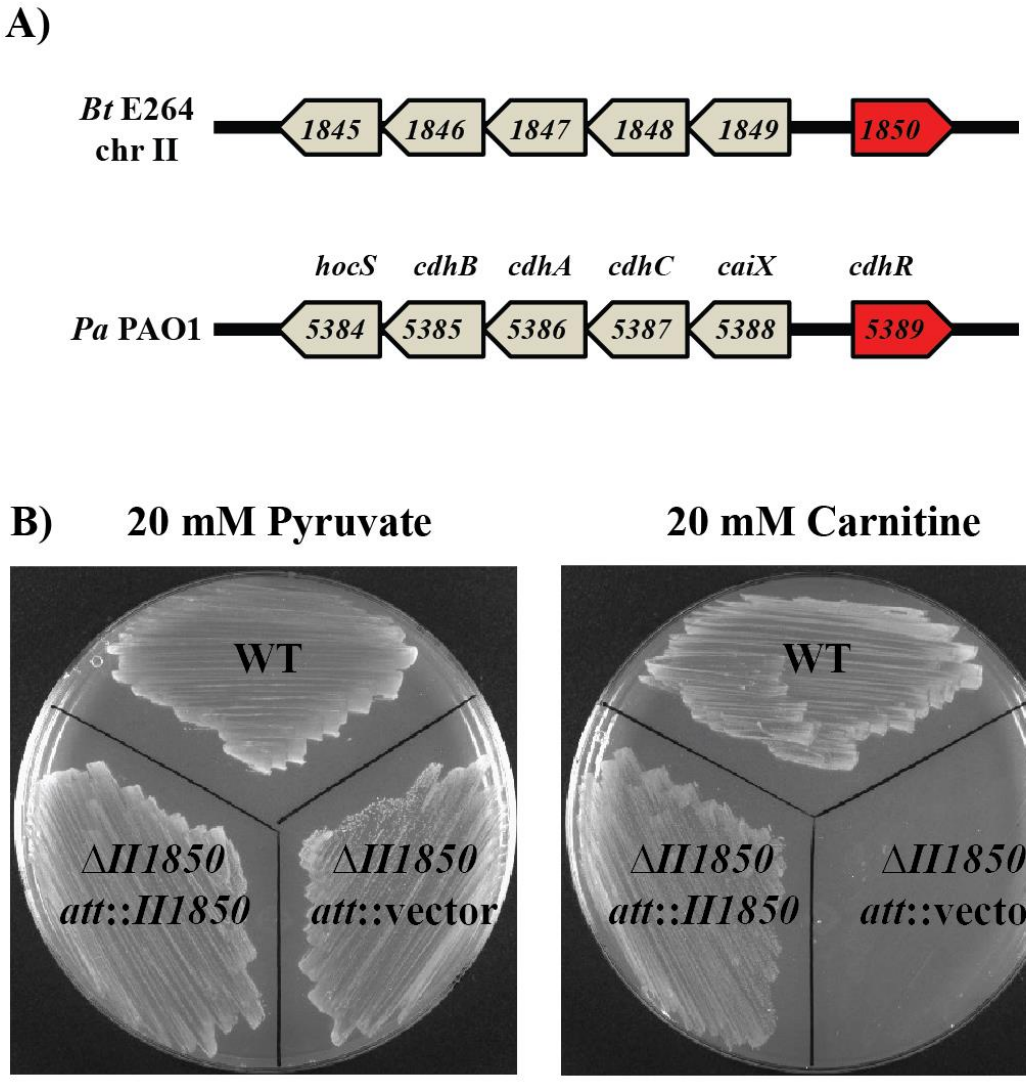


Figure 3.5 - *II1850* (*cdhR*) regulates carnitine catabolism.

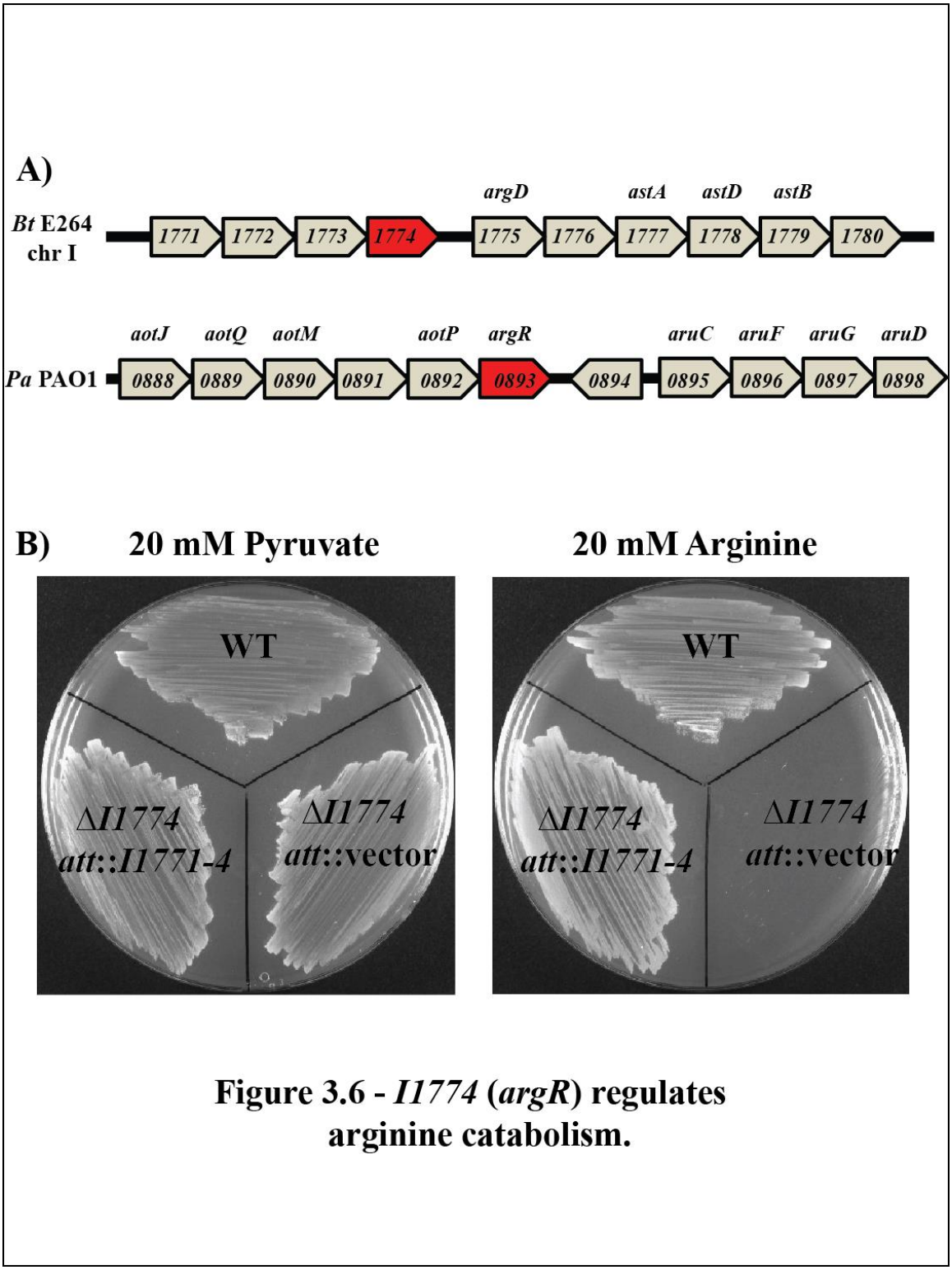


Figure 3.6 - *I1774* (*argR*) regulates arginine catabolism.

SouR N-terminal domain dimer

Dimer interface

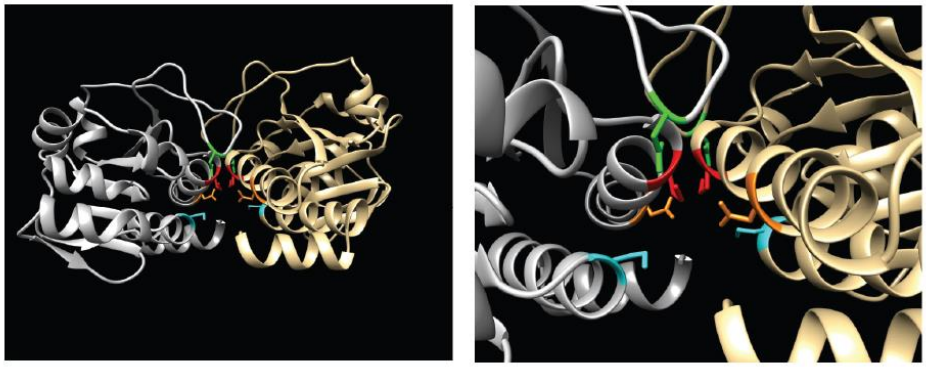


Figure 3.7 - Constitutive mutations in *P. aeruginosa* SouR overlaid on 3GRA.

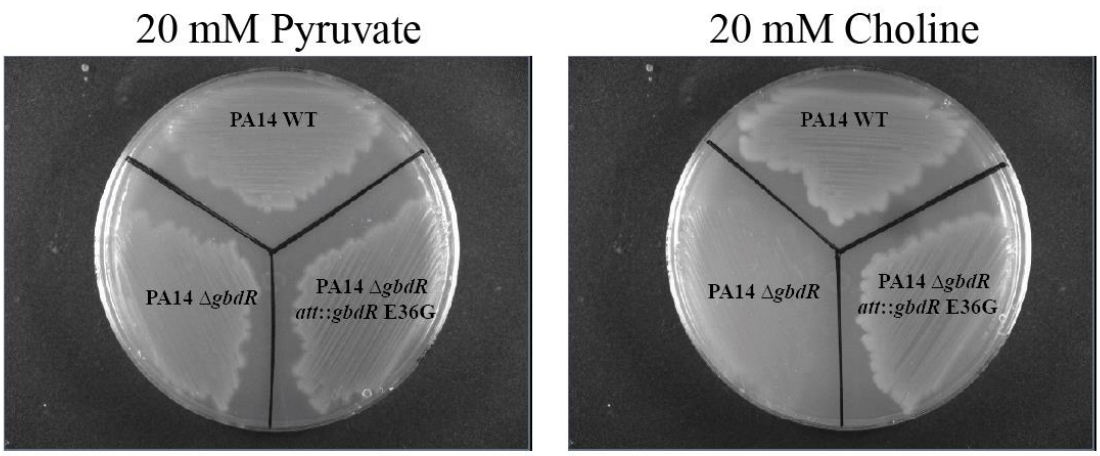


Figure 3.8 - Constitutive GbdR rescues growth on choline.

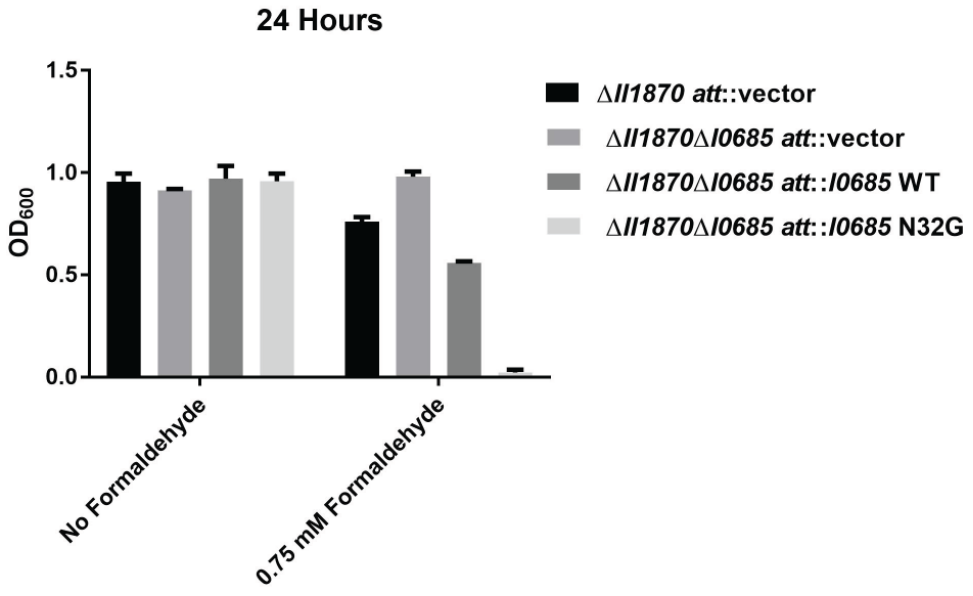


Figure 3.9 - I0685 represses resistence to formaldhyde.

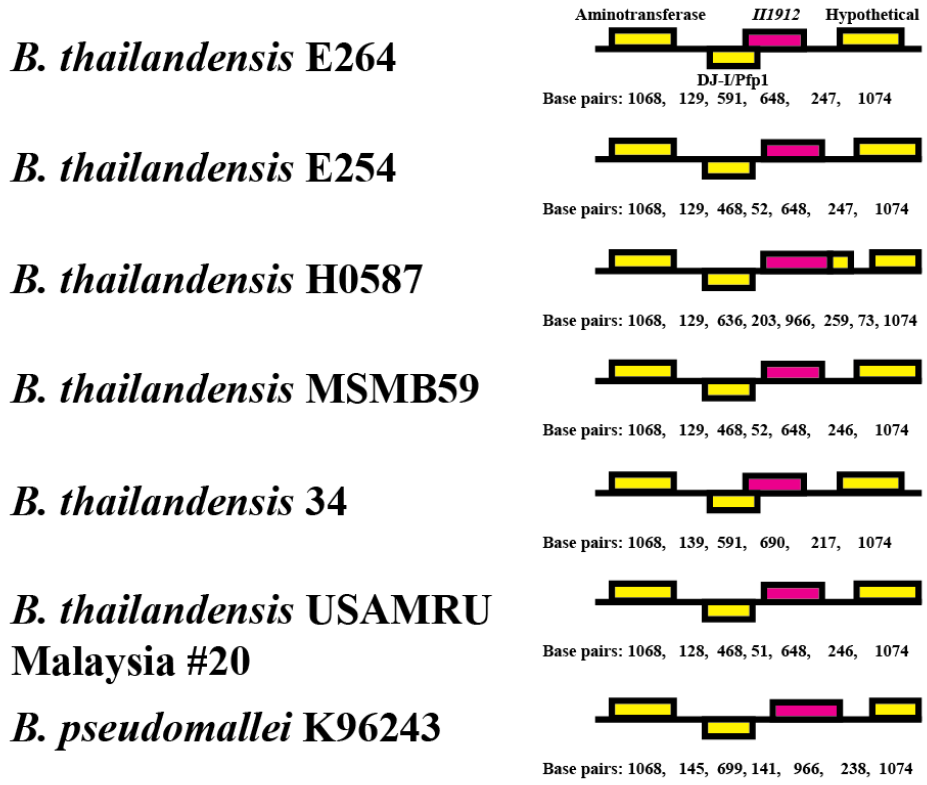


Figure 3.10 - *II1912* synteny in other *Burkholderia*.

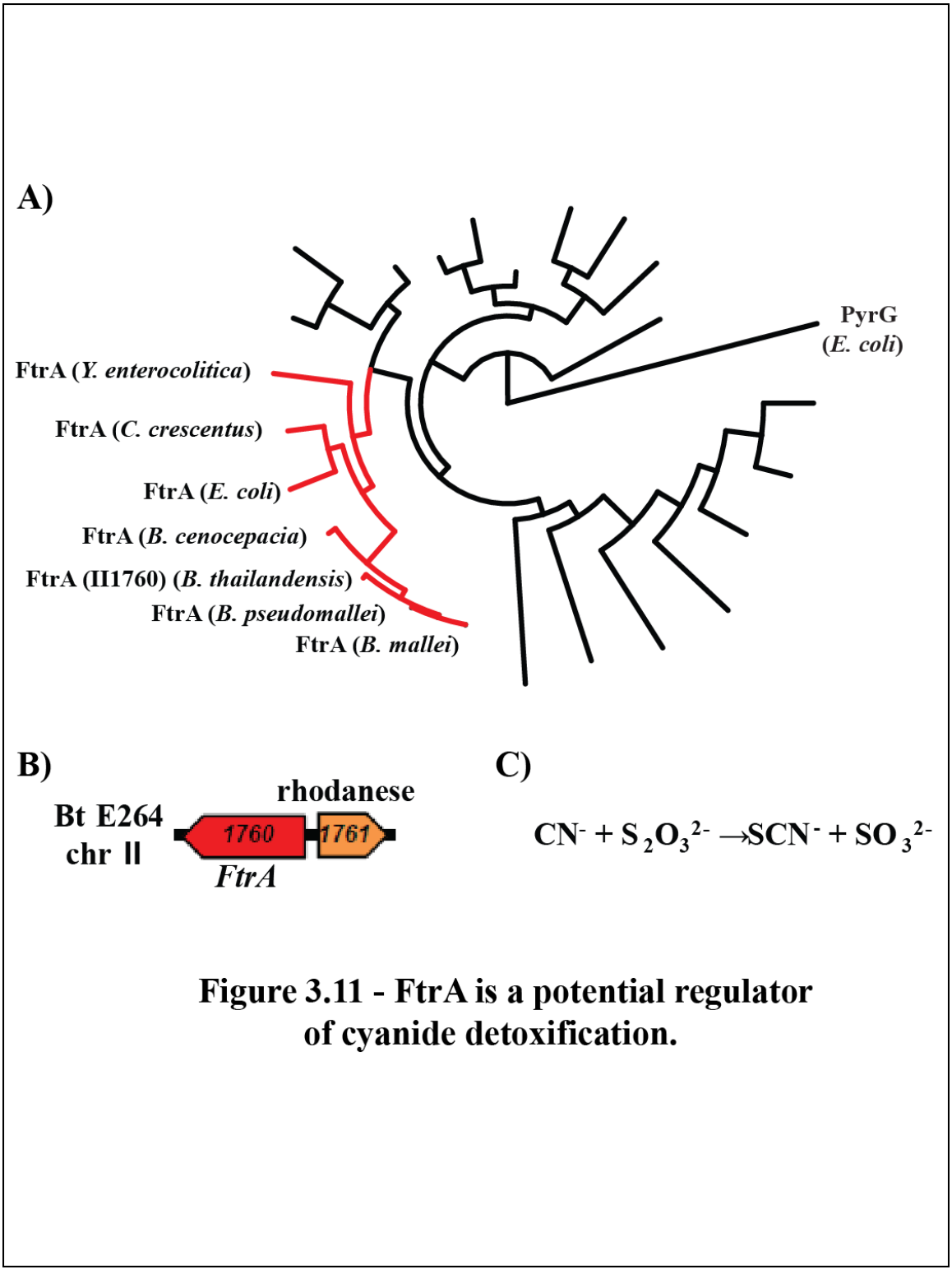


Figure 3.11 - FtrA is a potential regulator of cyanide detoxification.

Strains	Genotype	Notes
B. thailandensis E264 (ATCC 700388)	WT	
AN3	Δ*gbdR1*	Derived from ATCC700388
AN19	Δ*gbdR2*	Derived from ATCC700388
AN54	Δ*I1774*	Derived from ATCC700388
AN56	Δ*II1850*	Derived from ATCC700388
AN124	Δ*I1350*	Derived from ATCC700388
AN126	Δ*II0994*	Derived from ATCC700388
AN153	Δ*gbdR1 att*::vector	Derived from AN3, Zeo^r
AN242	Δ*II1760*	Derived from ATCC700388
AN244	Δ*II0282*	Derived from ATCC700388
AN246	Δ*II0513*	Derived from ATCC700388
AN248	Δ*I0059*	Derived from ATCC700388
AN250	Δ*II2197*	Derived from ATCC700388
AN257	Δ*II1850 att*::vector	Derived from AN56, Zeo^r
AN259	Δ*II1850 att*::*II1850*	Derived from AN56, Zeo^r
AN271	Δ*I0685*	Derived from ATCC700388
AN277	Δ*I1774v.2* (version 2)	Derived from ATCC700388
AN281	Δ*II1912*	Derived from ATCC700388
AN361	Δ*I0685 att*::*I0685* N32G	Derived from AN271, Tp^r
AN363	Δ*I0685 att*::vector	Derived from AN271, Tp^r
AN358	Δ*I1774v.2 att*::*I1771-4*	Derived from AN277, Zeo^r
AN366	Δ*I1774v.2 att*::vector	Derived from AN277, Zeo^r
AN367	*att*::vector	Derived from ATCC700388, Zeo^r
AN419	Δ*gbdR1 att*::*gbdR1* I23G	Derived from AN3, Zeo^r
P. aeruginosa PA14	WT	
AN440	Δ*gbdR att*::*gbdR* E36G	Derived from PA14, Gm^r
AN446	ΔgbdR att::vector	Derived from PA14, Gm^r
E. coli S17-1		
AN177	pTNS2	Amp^r
AN444	pUC18-mini-TN7T-Gm	Gm^r
MJ692	pAN78	Gm^r

Table 3.1 - Strain list.

Plasmids		
pUC18-mini-TN7T-Gm	*att*Tn7 integration vector, Gm^r	Used to generate vector control strains and other plasmids.
pUC18-mini-TN7T-TP	*att*Tn7 integration vector, Tp^r	Used to generate vector control strains and other plasmids.
pUC18-mini-TN7T-ZEO	*att*Tn7 integration vector, Zeo^r	Used to generate vector control strains and other plasmids.
pTNS2	carries the *att*Tn7 transposease, Amp^r	
pAN34	pUC18-mini-TN7T-ZEO-*II1850* complement, Zeo^r	Used with pTNS2 to generate AN259 by electroporation.
pAN54	pUC18-mini-TN7T-ZEO-*I1771-4* complement, Zeo^r	Used with pTNS2 to generate AN358 by electroporation.
pAN57	pUC18-mini-TN7T-TP-*I0685* constitutive complement N32G, Tp^r	Used with pTNS2 to generate AN361 by electroporation.
pAN76	pUC18-mini-TN7T-ZEO-*gbdR1* I23G constitutive complement, Zeo^r	Used with pTNS2 to generate AN419 by electroporation.
pAN78	pUC18-mini-TN7T-Gm-*gbdR* E36G constitutive complement, Gm^r	Used with pTNS2 to generate AN440 by triparental mating.

Table 3.2 - Plasmid list.

Primer Database #	Primer Name	Primer Sequence	Notes
Generation of deletion strains.			
656	I1774proxF	GACGTACATGGCGTTCCAGT	Generation of AN54.
657	I1774proxR	GCTGGACGATTCACGGACAT	Generation of AN54.
658	I1774postF	TTCCAATGCCTATCGGGAGC	Generation of AN54.
659	I1774postR	CGCGTCTTTTTGTCGGTTGT	Generation of AN54.
660	I1774tpF	GGCCGACGCCGCAGGTCGTGATGTCCGTGAATCGTCCAGCgagctcgaattagcttcaaa	Generation of AN54.
661	I1774tpR	GCGCGGCGTGACGCCGAACCGCTCCCGATAGGCATTGGAAgagctcgaattggggatctt	Generation of AN54.
650	II1850proxF	GAGGAACACGACCCACTTCT	Generation of AN56.
651	II1850proxR	CCGGGCATCAGCATGAAATG	Generation of AN56.
652	II1850postF	ATCGCCTTCGCATTTTTCCC	Generation of AN56.
653	II1850postR	GTCTTGTCCGCCTTTTCCGA	Generation of AN56.
654	II1850tpF	CGCCCATGCCGGAAGAATTCCATTTCATGCTGATGCCCGGgagctcgaattagcttcaaa	Generation of AN56.
655	II1850tpR	GAATTGCGCGCGATAAGTGCGGGAAAAATGCGAAGGCGATgagctcgaattggggatctt	Generation of AN56.
1205	I1350proxFalternate	GTGCGTCATTTGTCCGATCC	Generation of AN124.
1180	I1350prox R	GCAATGTCTATTCTGCGGCG	Generation of AN124.
1181	I1350tpF	CGATTTGAAGGAATATGATGCGCCGCAGAATAGACATTGCgagctcgaattagcttcaaa	Generation of AN124.
1182	I1350tpR	CGGACGCGGCGTGCCGCTTGTTATGAAACCGATCCGCTCAgagctcgaattggggatctt	Generation of AN124.
1183	I1350post F	TGAGCGGATCGGTTTCATAACA	Generation of AN124.
1206	I1350postRalternate	CTTGACGATGCGCTTTCCG	Generation of AN124.
1131	BtII1760proxF	GAACGGTCTGACGGATGTGA	Generation of AN242.
1132	BtII1760proxR	GTTTCGATGCGGGTTGATGC	Generation of AN242.
1133	BtII1760postF	CGAATCTTCTTGAGCAGCGG	Generation of AN242.
1134	BtII1760postR	CGCCATGCACAATCATCTCG	Generation of AN242.
1135	BtII1760tpF	CGCCCGCCACGCGCCGATGCGCATCAACCCGCATCGAAACgagctcgaattagcttcaaa	Generation of AN242.
1136	BtII1760tpR	CGGACGAAACGCCGCCCGAGCCGCTGCTCAAGAAGATTCGgagctcgaattggggatctt	Generation of AN242.
1499	II0282prox2F	AAACAAATTAGCGAGGCGGC	Generation of AN244.
1500	II0282prox2R	GCCTATTCGATTTCGCCTGG	Generation of AN244.
1501	II0282post2F	GTTGTCTCTCGGGCCACTTT	Generation of AN244.
1502	II0282post2R	AAAGACGGCGAATCAGCTTG	Generation of AN244.
1503	II0282tp2F	CCAGGCGAAATCGAATAGGCgagctcgaattagcttcaaa	Generation of AN244.
1504	I0282tp2R	AAAGTGGCCCGAGAGACAACgagctcgaattggggatctt	Generation of AN244.
1273	II0513proxF	TCGAGTCGTCCCTTCCGTATC	Generation of AN246.
1274	II0513proxR	AAAACCGAAAATGTGCGACCC	Generation of AN246.
1275	II0513postF	GAATCGTGGGGACTTTGGCA	Generation of AN246.
1276	II0513postR	CGGTGGCGGTCGAGAAGTAG	Generation of AN246.
1277	II0513tpF	GGTCGCACATTTTCGGTTTTgagctcgaattagcttcaaa	Generation of AN246.
1278	II0513tpF	TGCCAAAGTCCCCACGATTCgagctcgaattggggatctt	Generation of AN246.
1536	I0059proxF2	TTGAGAGGGGAAATGGCGAC	Generation of AN248.
1537	I0059proxR2	CCGGATGACGCTCGACCATA	Generation of AN248.
1538	I0059postF2	CGGATCAACGCAGATTGTCCCGG	Generation of AN248.
1539	I0059postR2	GCGCGAAAAATCGATCCGACCTCA	Generation of AN248.
1540	I0059tpF2	TATGGTCGAGCGTCATCCGGgagctcgaattagcttcaaa	Generation of AN248.
1541	I0059tpR2	CCGGGACAATCTGCGTTGATCCGgagctcgaattggggatctt	Generation of AN248.
1525	II2197proxF2	GAAAGCAAACCGACCGGAGA	Generation of AN250.
1526	II2197proxR2	CGTTCAGGATGCAGAGTGAC	Generation of AN250.
1527	II2197postF2	GACAATAGGCGCTCTTCGGT	Generation of AN250.
1528	II2197postR2	CTGCGCGAACCGACCTATC	Generation of AN250.
1529	II2197tpF2	TCTGAACGGCATCGTGATCGgagctcgaattagcttcaaa	Generation of AN250.
1530	II2197tpR2	ACCGAAGAGCGCCTATTGTCgagctcgaattggggatctt	Generation of AN250.
1700	I0685proxFset3	GTATCGACGGGCCGAAGGGTG	Generation of AN271.
1701	I0685proxRset3	GCAGCGGGTTTCACTCCGAC	Generation of AN271.
1702	I0685postFset3	CATCGACGGCCGTCGTCC	Generation of AN271.
1703	I0685postRset3	GGAACGTTGTCAGGCTCG	Generation of AN271.
1704	I0685tpFset3	GTCGGAGTGAAACCCGCTGCgagctcgaattagcttcaaa	Generation of AN271.
1705	I0685tpRset3	GTCGATGGTGAAACGGCATGgagctcgaattggggatctt	Generation of AN271.
1780	I1774proxF2	CTGTCGGGCAGCCTCAAATA	Generation of AN277. Use with 658, 659, and 661.
1781	I1774proxR2	AACCGCTCGCTAATGACGAT	Generation of AN277. Use with 658, 659, and 661.
1782	I1774tpF2	ATCGTCATTAGCGAGCGGTTgagctcgaattagcttcaaa	Generation of AN277. Use with 658, 659, and 661.
1795	II1912new proxF	GCACATTCGCTTCTTTCCGC	Generation of AN281.
1796	II1912new proxR	GCGCGACGACCAATTTCC	Generation of AN281.
1797	II1912new postF	GCGTGAACATGAGCGAGC	Generation of AN281.
1798	II1912new postR	AGTGCGGAGGGATCGTTTG	Generation of AN281.
1799	II1912new tpF	GTGGAAATTGGTCGTCGCGCgagctcgaattagcttcaaa	Generation of AN281.
1800	II1912new tpR	GCGCTCGCTCATGTTCACGCgagctcgaattggggatctt	Generation of AN281.
Construction of plasmids.			
1716	cdhRcompTempF	CGACGGCGACCTTTTCTTGCCT	Generation of pAN34.
1717	cdhRcompTempR	AGCATACGATCTTGCACCGCCG	Generation of pAN34.
1718	HindIIIcdhRcompF	CCTAAGCTTCGACGGCGACCTTTTCTTGCCT	Generation of pAN34.
1719	KpnIcdhRcompR	CTTGGTACCAGCATACGATCTTGCACCGCCG	Generation of pAN34.
1996	argRcompProxF	GCAATGTCCGGGATCGACG	Generation of pAN54.
1997	argRcompProxR	GACTGGAACGCCATGTACGTC	Generation of pAN54.
1903	argRoperon amp R	ACATCACAGGCCATTCACCA	Generation of pAN54.
656	I1774proxF	GACGTACATGGCGTTCCAGT	Generation of pAN54.
2033	argRcompProxFHindIIIc	CCT ttccaa GCAATGTCCGGGATCGACG	Generation of pAN54.
1999	argRcompPostRKpnI	CCT GGTACC ACATCACAGGCCATTCACCA	Generation of pAN54.
692	gbdR1compFHindIII	CCTAAGCTTGAGCGACATCCCGAGCACCG	Generation of pAN76.
693	gbdR1compRKpnI	CTTGGTACCCGGGGGCCGGTTTCGAGTTC	Generation of pAN76.
2260	gbdR1 I23G postF	GCCCAACTTCACGATGggCGCGTTC	Generation of pAN76.
2261	gbdR1 I23G proxR	GAACGCGccCATCGTGAAGTTGGGC	Generation of pAN76.
1990	I0685N32GproxR	GTCGAACACGCCGATGACGCCG	Generation of pAN57.
1991	I0685N32GpostF	CGGCGTCATCGGCGTGTTCGAC	Generation of pAN57.
1992	I0685ampFBamHI	CCG GGATCC GGAACGTTGTCAGGCTCG	Generation of pAN57.
1993	I0685ampRKpnI	CCG GGTACC GACTGGCGGACGGATTACG	Generation of pAN57.
1845	gbdR-GOI-eng-F	CTGCCTACGACTTCTCCAG	Generation of pAN78.
1846	gbdR-SOE-eng-R-HindIII	CTTCCgaattcCTTaagCTTTAGCCCGGCATTTCC	Generation of pAN78.
1847	gbdR-SOE-eng-F-AgeI	CTTCCgaattcAATaccggtAGCGTGCGGATCTGACC	Generation of pAN78.
1848	gbdR-GOI-eng-R	CCACCTGCATTACCTGGAAG	Generation of pAN78.

Table 3.3 - Primer list.

Species	*B. thailandensis*	*B. pseudomallei*	*B. mallei*	*B. cenocepacia*	
Strain	E264	K96243	ATCC 23344	J2315	
#	13	11	8	15	Divergent DJ-1/PfpI Partner?
gbdR1	*BTH_II1869*	*BPSS0546*	*BMAA0469*	*BCAM2327*	No
gbdR2	*BTH_II0698*	*BPSS1424*	*BMAA0566*	*BCAM2408*	No
argR	*BTH_I1774*	*BPSL2391*	*BMA0590*	*BCAL1058*	No
cdhR	*BTH_II1850*	*BPSS0565*	*BMAA0489*	*BCAM2306*	No
souR	*BTH_II0994*	*BPSS1371*	N/A	*BCAM2393*	No
	BTH_II2197	N/A	N/A	*BCAM2616*	No
	BTH_II1760	*BPSS0648*	*BMAA0835*	*BCAM2150*	No
	BTH_II0282	N/A	N/A	*BCAL2825*	No
	BTH_I0685	*BPSL0819*	*BMA0323*	N/A	No
	BTH_II0513	*BPSS1864*	*BMAA0231*	N/A	Yes
	BTH_II1912	*BPSS0505*	*BMAA1772*	N/A	Yes
	BTH_I0059	*BPSL0060*	N/A	N/A	No
	BTH_I1350	*BPSL2782*	N/A	N/A	No
	N/A	N/A	N/A	*BCAM0677*	*Yes (essentially adjacent)
	N/A	N/A	N/A	*BCAM1434*	No
	N/A	N/A	N/A	*BCAS0761*	No
	N/A	N/A	N/A	*BCAL0772*	No
	N/A	N/A	N/A	*BCAS0067*	No
	N/A	N/A	N/A	*BCAL0945*	Yes
	N/A	N/A	N/A	*BCAL2524*	No

Table 3.4 - *Burkholderia* GATR homologs based on gene synteny.

Pseudomonas putida			
3GRA (SouR)	A22	D29	P169
Pseudomonas aeruginosa			
SouR	P41	D48	P185
GbdR	I29	E36	G172
Burkholderia thailandensis			
III1869 (GbdR1)	I23	E30	G164
II0968 (GbdR2)	I33	E40	G175
I1774 (ArgR)	S26	D33	G169
II1850 (CdhR)	L16	E23	G159
II0994 (SouR)	L31	D39	G183
II2197	F15	D22	A157
II1760	F17	E24	S161
II0282	F15	I22	T159
I0685	V28, no helix	N32	V175
II0513	L39	Q46	V188
I0059	G23, helix break	E31	poorly aligned
I1350	M40	E47	E175

Table 3.5 - Predicted constitutive mutants.

Fold Change	P value	Gene (PA14 / PAO1)	Description
14.48	0.026	*gbcA*	putative ring hydroxylating dioxygenase
10.06	0.007	*PA14_71250 / PA5397*	conserved hypothetical protein
8.31	0.034	*BetX*	putative glycine betaine-binding protein precursor
6.81	0.007	*PA14_71240 / PA5396*	putative dipeptidase
4.44	0.034	*PA14_64910 / PA4915*	putative transcriptional regulator
4.21	0.005	*soxA*	sarcosine oxidase alpha subunit
4.07	0.032	*soxB*	sarcosine oxidase
3.8	0.026	*PA14_70860 / PA5369*	putative phosphate ABC transporter
3.79	0.002	*fdhA*	glutathione-independent formaldehyde dehydrogenase
3.13	0.003	*PA14_71310 / PA5401*	putative electron transfer flavoprotein
2.61	0.046	*PA14_43730 / PA1606*	hypothetical protein
2.44	0.009	*PA14_39700* / not present	conserved hypothetical protein
2.44	0.036	*PA14_66350 / PA5020*	putative acyl-CoA dehydrogenase
2.42	0.028	*glyA1*	serine hydroxymethyltransferase
2.26	0.008	*fapC*	conserved hypothetical protein
2.06	0.044	*PA14_19590 / PA3441*	putative molybdopterin-binding protein
2.03	0.000	*PA14_61260 / PA4629*	putative membrane protein
2.01	0.043	*cbcW*	putative BC-type proline/glycine betaine transport system
-2.26	0.011	*PA14_52800 / PA0887*	acetyl-coenzyme A synthetase
-2.28	0.000	*PA14_55750 / PA4290*	putative chemotaxis transducer
-2.49	0.017	*PA14_22340 / PA3235*	putative membrane protein
-2.78	0.014	*PA14_22350 / PA3234*	putative sodium/proline:solute symporter
-2.89	0.037	*PA14_22370 /* PA3233	putative signal-transduction protein

Table 3.6 - GbdR E36G microarray results.

Fold Change	P value	Gene	Description
-7.89	0.002	*BTH_I0686*	glutathione-dependent formaldehyde dehydrogenase
-6.32	0.026	*BTH_I0687*	esterase
-9.59	0.003	*BTH_I1829*	aminotransferase
-5.02	0.032	*BTH_I3305*	cyclohexadienyl dehydratase
-12.65	0.004	*BTH_II0269*	transposase
-6.51	0.048	*BTH_II0472*	benzoate 1
-5.04	0.012	*BTH_II1039*	hypothetical protein
-7.58	0.048	*BTH_II1048*	hypothetical protein
-5.87	0.036	*BTH_II1430*	hypothetical protein

Table 3.7 - I0685 N32G microarray results.

3.8 Chapter 3 References

1. **Gallegos MT, Schleif R, Bairoch A, Hofmann K, Ramos JL.** 1997. Arac/XylS family of transcriptional regulators. Microbiol Mol Biol Rev **61:**393-410.
2. **Egan SM.** 2002. Growing repertoire of AraC/XylS activators. J Bacteriol **184:**5529-5532.
3. **Yang J, Tauschek M, Robins-Browne RM.** 2011. Control of bacterial virulence by AraC-like regulators that respond to chemical signals. Trends Microbiol **19:**128-135.
4. **Schleif R.** 2010. AraC protein, regulation of the l-arabinose operon in Escherichia coli, and the light switch mechanism of AraC action. FEMS Microbiol Rev **34:**779-796.
5. **Ghosh M, Schleif RF.** 2001. Biophysical evidence of arm-domain interactions in AraC. Anal Biochem **295:**107-112.
6. **Hart E, Yang J, Tauschek M, Kelly M, Wakefield MJ, Frankel G, Hartland EL, Robins-Browne RM.** 2008. RegA, an AraC-like protein, is a global transcriptional regulator that controls virulence gene expression in Citrobacter rodentium. Infect Immun **76:**5247-5256.
7. **Bustos SA, Schleif RF.** 1993. Functional domains of the AraC protein. Proc Natl Acad Sci U S A **90:**5638-5642.
8. **Mouilleron S, Golinelli-Pimpaneau B.** 2007. Conformational changes in ammonia-channeling glutamine amidotransferases. Curr Opin Struct Biol **17:**653-664.
9. **Massiere F, Badet-Denisot MA.** 1998. The mechanism of glutamine-dependent amidotransferases. Cell Mol Life Sci **54:**205-222.
10. **Weng ML, Zalkin H.** 1987. Structural role for a conserved region in the CTP synthetase glutamine amide transfer domain. J Bacteriol **169:**3023-3028.
11. **Tesmer JJ, Klem TJ, Deras ML, Davisson VJ, Smith JL.** 1996. The crystal structure of GMP synthetase reveals a novel catalytic triad and is a structural paradigm for two enzyme families. Nat Struct Biol **3:**74-86.
12. **Li QA, Mavrodi DV, Thomashow LS, Roessle M, Blankenfeldt W.** 2011. Ligand binding induces an ammonia channel in 2-amino-2-desoxyisochorismate (ADIC) synthase PhzE. J Biol Chem **286:**18213-18221.
13. **Barry RM, Bitbol AF, Lorestani A, Charles EJ, Habrian CH, Hansen JM, Li HJ, Baldwin EP, Wingreen NS, Kollman JM, Gitai Z.** 2014. Large-scale filament formation inhibits the activity of CTP synthetase. Elife **3:**e03638.
14. **Geer LY, Domrachev M, Lipman DJ, Bryant SH.** 2002. CDART: protein homology by domain architecture. Genome Res **12:**1619-1623.
15. **Winsor GL, Griffiths EJ, Lo R, Dhillon BK, Shay JA, Brinkman FS.** 2016. Enhanced annotations and features for comparing thousands of Pseudomonas genomes in the Pseudomonas genome database. Nucleic Acids Res **44:**D646-653.
16. **Winsor GL, Khaira B, Van Rossum T, Lo R, Whiteside MD, Brinkman FS.** 2008. The Burkholderia Genome Database: facilitating flexible queries and comparative analyses. Bioinformatics **24:**2803-2804.
17. **Thongdee M, Gallagher LA, Schell M, Dharakul T, Songsivilai S, Manoil C.** 2008. Targeted mutagenesis of Burkholderia thailandensis and Burkholderia

pseudomallei through natural transformation of PCR fragments. Appl Environ Microbiol **74:**2985-2989.
18. **Choi KH, Mima T, Casart Y, Rholl D, Kumar A, Beacham IR, Schweizer HP.** 2008. Genetic tools for select-agent-compliant manipulation of Burkholderia pseudomallei. Appl Environ Microbiol **74:**1064-1075.
19. **Choi KH, DeShazer D, Schweizer HP.** 2006. mini-Tn7 insertion in bacteria with multiple glmS-linked attTn7 sites: example Burkholderia mallei ATCC 23344. Nat Protoc **1:**162-169.
20. **Choi KH, Gaynor JB, White KG, Lopez C, Bosio CM, Karkhoff-Schweizer RR, Schweizer HP.** 2005. A Tn7-based broad-range bacterial cloning and expression system. Nat Methods **2:**443-448.
21. **Perumal Samy R, Stiles BG, Sethi G, Lim LHK.** 2017. Melioidosis: Clinical impact and public health threat in the tropics. PLoS Negl Trop Dis **11:**e0004738.
22. **Van Zandt KE, Greer MT, Gelhaus HC.** 2013. Glanders: an overview of infection in humans. Orphanet J Rare Dis **8:**131.
23. **Hampel KJ, LaBauve AE, Meadows JA, Fitzsimmons LF, Nock AM, Wargo MJ.** 2014. Characterization of the GbdR regulon in Pseudomonas aeruginosa. J Bacteriol **196:**7-15.
24. **Wargo MJ, Szwergold BS, Hogan DA.** 2008. Identification of two gene clusters and a transcriptional regulator required for Pseudomonas aeruginosa glycine betaine catabolism. J Bacteriol **190:**2690-2699.
25. **Nishijyo T, Park SM, Lu CD, Itoh Y, Abdelal AT.** 1998. Molecular characterization and regulation of an operon encoding a system for transport of arginine and ornithine and the ArgR regulatory protein in Pseudomonas aeruginosa. J Bacteriol **180:**5559-5566.
26. **Park SM, Lu CD, Abdelal AT.** 1997. Cloning and characterization of argR, a gene that participates in regulation of arginine biosynthesis and catabolism in Pseudomonas aeruginosa PAO1. J Bacteriol **179:**5300-5308.
27. **Wargo MJ, Ho TC, Gross MJ, Whittaker LA, Hogan DA.** 2009. GbdR regulates Pseudomonas aeruginosa plcH and pchP transcription in response to choline catabolites. Infect Immun **77:**1103-1111.
28. **Willsey GG, Wargo MJ.** 2015. Sarcosine catabolism in Pseudomonas aeruginosa is transcriptionally regulated by SouR. J Bacteriol doi:10.1128/JB.00739-15.
29. **Meadows JA, Wargo MJ.** 2018. Transcriptional Regulation of Carnitine Catabolism in Pseudomonas aeruginosa by CdhR. mSphere **3**.
30. **Kalashnikov NV.** 1986. [Characteristics of the damage to bacteria by formaldehyde]. Mol Gen Mikrobiol Virusol**:**35-41.
31. **Marx CJ, Van Dien SJ, Lidstrom ME.** 2005. Flux analysis uncovers key role of functional redundancy in formaldehyde metabolism. PLoS Biol **3:**e16.
32. **Nock AM, Wargo MJ.** 2016. Choline Catabolism in Burkholderia thailandensis Is Regulated by Multiple Glutamine Amidotransferase 1-Containing AraC Family Transcriptional Regulators. J Bacteriol **198:**2503-2514.
33. **Chen NH, Djoko KY, Veyrier FJ, McEwan AG.** 2016. Formaldehyde Stress Responses in Bacterial Pathogens. Front Microbiol **7:**257.

34. **Crosson S, McGrath PT, Stephens C, McAdams HH, Shapiro L.** 2005. Conserved modular design of an oxygen sensory/signaling network with species-specific output. Proc Natl Acad Sci U S A **102:**8018-8023.
35. **Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, Huerta-Cepas J, Simonovic M, Roth A, Santos A, Tsafou KP, Kuhn M, Bork P, Jensen LJ, von Mering C.** 2015. STRING v10: protein-protein interaction networks, integrated over the tree of life. Nucleic Acids Res **43:**D447-452.
36. **Wargo MJ.** 2013. Homeostasis and catabolism of choline and glycine betaine: lessons from Pseudomonas aeruginosa. Appl Environ Microbiol **79:**2112-2120.
37. **Nissen P, Benson AA.** 1961. Choline Sulfate in Higher Plants. Science **134:**1759.
38. **Hussey C, Orsi BA, Scott J, Spencer B.** 1965. Mechanism of choline sulphate utilization in fungi. Nature **207:**632-634.
39. **Nau-Wagner G, Boch J, Le Good JA, Bremer E.** 1999. High-affinity transport of choline-O-sulfate and its use as a compatible solute in Bacillus subtilis. Appl Environ Microbiol **65:**560-568.
40. **Wargo MJ, Hogan DA.** 2009. Identification of genes required for Pseudomonas aeruginosa carnitine catabolism. Microbiology **155:**2411-2419.
41. **Chen C, Malek AA, Wargo MJ, Hogan DA, Beattie GA.** 2010. The ATP-binding cassette transporter Cbc (choline/betaine/carnitine) recruits multiple substrate-binding proteins with strong specificity for distinct quaternary ammonium compounds. Mol Microbiol **75:**29-45.
42. **Meadows JA, Wargo MJ.** 2013. Characterization of Pseudomonas aeruginosa growth on O-acylcarnitines and identification of a short-chain acylcarnitine hydrolase. Appl Environ Microbiol **79:**3355-3363.
43. **Marx CJ, Miller JA, Chistoserdova L, Lidstrom ME.** 2004. Multiple formaldehyde oxidation/detoxification pathways in Burkholderia fungorum LB400. J Bacteriol **186:**2173-2178.
44. **Wu M, Schleif R.** 2001. Strengthened arm-dimerization domain interactions in AraC. J Biol Chem **276:**2562-2564.
45. **Dirla S, Chien JY, Schleif R.** 2009. Constitutive mutations in the Escherichia coli AraC protein. J Bacteriol **191:**2668-2674.
46. **List F, Bocola M, Haeger MC, Sterner R.** 2012. Constitutively active glutaminase variants provide insights into the activation mechanism of anthranilate synthase. Biochemistry **51:**2812-2818.
47. **Park SM, Lu CD, Abdelal AT.** 1997. Purification and characterization of an arginine regulatory protein, ArgR, from Pseudomonas aeruginosa and its interactions with the control regions for the car, argF, and aru operons. J Bacteriol **179:**5309-5317.
48. **Mitsui R, Kusano Y, Yurimoto H, Sakai Y, Kato N, Tanaka M.** 2003. Formaldehyde fixation contributes to detoxification for growth of a nonmethylotroph, Burkholderia cepacia TM1, on vanillic acid. Appl Environ Microbiol **69:**6128-6132.
49. **Mitsui R, Hirota M, Tsuno T, Tanaka M.** 2010. Purification and characterization of vanillin dehydrogenases from alkaliphile Micrococcus sp. TA1 and neutrophile Burkholderia cepacia TM1. FEMS Microbiol Lett **303:**41-47.

50. **Liao Y, Chen S, Wang D, Zhang W, Wang S, Ding J, Wang Y, Cai L, Ran X, Wang X, Zhu H.** 2013. Structure of formaldehyde dehydrogenase from Pseudomonas aeruginosa: the binary complex with the cofactor NAD+. Acta Crystallogr Sect F Struct Biol Cryst Commun **69:**967-972.
51. **Mason RP, Sanders JKM, Crawford A, Hunter BK.** 1986. Formaldehyde Metabolism by Escherichia-Coli - Detection by Invivo C-13 Nmr-Spectroscopy of S-(Hydroxymethyl)Glutathione as a Transient Intracellular Intermediate. Biochemistry **25:**4504-4507.
52. **Uotila L, Koivusalo M.** 1974. Formaldehyde dehydrogenase from human liver. Purification, properties, and evidence for the formation of glutathione thiol esters by the enzyme. J Biol Chem **249:**7653-7663.
53. **Uotila L, Koivusalo M.** 1974. Purification and properties of S-formylglutathione hydrolase from human liver. J Biol Chem **249:**7664-7672.
54. **Beck CF, Warren RA.** 1988. Divergent promoters, a common form of gene organization. Microbiol Rev **52:**318-326.
55. **Ibarra JA, Perez-Rueda E, Segovia L, Puente JL.** 2008. The DNA-binding domain as a functional indicator: the case of the AraC/XylS family of transcription factors. Genetica **133:**65-76.
56. **Meadows JA, Wargo MJ.** 2015. Carnitine in bacterial physiology and metabolism. Microbiology **161:**1161-1174.
57. **Cave MC, Hurt RT, Frazier TH, Matheson PJ, Garrison RN, McClain CJ, McClave SA.** 2008. Obesity, inflammation, and the potential application of pharmaconutrition. Nutr Clin Pract **23:**16-34.
58. **Bourdin B, Adenier H, Perrin Y.** 2007. Carnitine is associated with fatty acid metabolism in plants. Plant Physiol Biochem **45:**926-931.
59. **Reddy JK, Hashimoto T.** 2001. Peroxisomal beta-oxidation and peroxisome proliferator-activated receptor alpha: an adaptive metabolic system. Annu Rev Nutr **21:**193-230.
60. **Steiber A, Kerner J, Hoppel CL.** 2004. Carnitine: a nutritional, biosynthetic, and functional perspective. Mol Aspects Med **25:**455-473.
61. **Wanders RJ, Waterham HR.** 2006. Biochemistry of mammalian peroxisomes revisited. Annu Rev Biochem **75:**295-332.
62. **Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, Feldstein AE, Britt EB, Fu X, Chung YM, Wu Y, Schauer P, Smith JD, Allayee H, Tang WH, DiDonato JA, Lusis AJ, Hazen SL.** 2011. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature **472:**57-63.
63. **Koeth RA, Levison BS, Culley MK, Buffa JA, Wang ZN, Gregory JC, Org E, Wu YP, Li L, Smith JD, Tang WHW, DiDonato JA, Lusis AJ, Hazen SL.** 2014. gamma-Butyrobetaine Is a Proatherogenic Intermediate in Gut Microbial Metabolism of L-Carnitine to TMAO. Cell Metabolism **20:**799-812.
64. **Aurich H, Kleber HP, Schopp WD.** 1967. An inducible carnitine dehydrogenase from Pseudomonas aeruginosa. Biochim Biophys Acta **139:**505-507.
65. **Kets EPW, Galinski EA, Debont JAM.** 1994. Carnitine - a Novel Compatible Solute in Lactobacillus-Plantarum. Archives of Microbiology **162:**243-248.
66. **Robert H, Le Marrec C, Blanco C, Jebbar M.** 2000. Glycine betaine, carnitine, and choline enhance salinity tolerance and prevent the accumulation of sodium to

a level inhibiting growth of Tetragenococcus halophila. Applied and Environmental Microbiology **66:**509-517.
67. **Park S, Smith LT, Smith GM.** 1995. Role of Glycine Betaine and Related Osmolytes in Osmotic-Stress Adaptation in Yersinia-Enterocolitica Atcc-9610. Applied and Environmental Microbiology **61:**4378-4381.
68. **Cunin R, Eckhardt T, Piette J, Boyen A, Pierard A, Glansdorff N.** 1983. Molecular basis for modulated regulation of gene expression in the arginine regulon of Escherichia coli K-12. Nucleic Acids Res **11:**5007-5019.
69. **Charlier D, Roovers M, Van Vliet F, Boyen A, Cunin R, Nakamura Y, Glansdorff N, Pierard A.** 1992. Arginine regulon of Escherichia coli K-12. A study of repressor-operator interactions and of in vitro binding affinities versus in vivo repression. J Mol Biol **226:**367-386.
70. **A.-M. Chakrabarty BI, and S. Kaplan.** 1990. Arginine network of Pseudomonas aeruginosa: specific and global controls., p p. 303-316. *In* Silver S (ed), Pseudomonas : biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
71. **Chou HT, Hegazy M, Lu CD.** 2010. L-lysine catabolism is controlled by L-arginine and ArgR in Pseudomonas aeruginosa PAO1. J Bacteriol **192:**5874-5880.
72. **Lang K.** 1933. Die rhodanbildung in tierkorper. Biochem Z **259:**243-256.
73. **Solomonson LP.** 1981. Cyanide as a metabolic inhibitor. Academic Press, London.
74. **Cipollone R, Ascenzi P, Visca P.** 2007. Common themes and variations in the rhodanese superfamily. IUBMB Life **59:**51-59.
75. **Baxter J, Cummings SP.** 2006. The current and future applications of microorganism in the bioremediation of cyanide contamination. Antonie Van Leeuwenhoek **90:**1-17.
76. **Cipollone R, Ascenzi P, Frangipani E, Visca P.** 2006. Cyanide detoxification by recombinant bacterial rhodanese. Chemosphere **63:**942-949.
77. **Adjei MD, Ohta Y.** 2000. Factors affecting the biodegradation of cyanide by Burkholderia cepacia strain C-3. Journal of Bioscience and Bioengineering **89:**274-277.
78. **Broderick KE, Chan A, Balasubramanian M, Feala J, Reed SL, Panda M, Sharma VS, Pilz RB, Bigby TD, Boss GR.** 2008. Cyanide produced by human isolates of Pseudomonas aeruginosa contributes to lethality in Drosophila melanogaster. J Infect Dis **197:**457-464.
79. **Bernier SP, Workentine ML, Li X, Magarvey NA, O'Toole GA, Surette MG.** 2016. Cyanide Toxicity to Burkholderia cenocepacia Is Modulated by Polymicrobial Communities and Environmental Factors. Frontiers in Microbiology **7**.
80. **Hanson AD, Rathinasabapathi B, Rivoal J, Burnet M, Dillon MO, Gage DA.** 1994. Osmoprotective compounds in the Plumbaginaceae: a natural experiment in metabolic engineering of stress tolerance. Proc Natl Acad Sci U S A **91:**306-310.
81. **Nissen P.** 1968. Choline sulfate permease. Transfer of information from bacteria to higher plants? Biochem Biophys Res Commun **32:**696-703.

82. **Wagner MA, Jorns MS.** 2000. Monomeric sarcosine oxidase: 2. Kinetic studies with sarcosine, alternate substrates, and a substrate analogue. Biochemistry **39:**8825-8829.
83. **Wagner MA, Trickey P, Chen ZW, Mathews FS, Jorns MS.** 2000. Monomeric sarcosine oxidase: 1. Flavin reactivity and active site binding determinants. Biochemistry **39:**8813-8824.
84. **Edgar RC.** 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res **32:**1792-1797.
85. **Tamura K, Stecher G, Peterson D, Filipski A, Kumar S.** 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol **30:**2725-2729.
86. **Neidhardt FC, Bloch PL, Smith DF.** 1974. Culture medium for enterobacteria. J Bacteriol **119:**736-747.
87. **LaBauve AE, Wargo MJ.** 2012. Growth and laboratory maintenance of Pseudomonas aeruginosa. Curr Protoc Microbiol **Chapter 6:**Unit 6E 1.
88. **Bagaria A, Kumaran, D., Burley, S.K., Swaminathan, S.** 2009. Crystal structure of AraC family transcriptional regulator from Pseudomonas putida doi:10.2210/pdb3gra/pdb.
89. **Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE.** 2000. The Protein Data Bank. Nucleic Acids Res **28:**235-242.
90. **Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE.** 2004. UCSF chimera - A visualization system for exploratory research and analysis. Journal of Computational Chemistry **25:**1605-1612.
91. **Choi KH, Schweizer HP.** 2006. mini-Tn7 insertion in bacteria with single attTn7 sites: example Pseudomonas aeruginosa. Nat Protoc **1:**153-161.

CHAPTER 4:

DISCUSSION AND FUTURE DIRECTIONS

4.1 A brief summary of progress.

The first contribution of this work is the generation of an alternative model for the regulation of glycine betaine (GB) catabolism. In this new model, we identified multiple AraC-family transcriptional regulators (ATRs) and characterized their phenotypes and target operons. The development of a model complementary to our original *P. aeruginosa* model provides the opportunity for to compare and contrast in order to gain new information about each. As part of this process, we identified and characterized three ATRs in this pathway that are structurally related to the GATase1 enzymes, and represent a subfamily of ATRs referred to as Glutamine amidotransferase1 (GATase1)-containing AraC-family transcriptional regulators (GATRs). The new regulatory model represents a scheme that better reflect the regulation found in *Burkholderia*, and may allow for assumptions of more distantly related bacteria that also possess these GATRs.

The second major contribution is the further characterization of GATRs as a family by presenting a methodology for perturbing the dimerization domain to yield constitutively active, ligand-independent mutants. This allows for the identification of target genes, whose potential functional predictions for which may inform us about the metabolic process that an unknown GATR is involved in and potentially ligands to which the GATR responds. Utilizing this method we identified I0685, a GATR that regulates

formaldehyde detoxification. The discovery of a GATR that functions as a repressor challenges our initial hypothesis that GATRs function strictly as activators, and opens the possibility that ligands for these regulators may not all be amine-containing compounds.

Simultaneously, these advancements have directly expanded our knowledge of the basic biology *B. thailandensis*. Several questions remain and our work has led to additional questions.

4.2 Remaining questions about the regulation of glycine betaine catabolism in *B. thailandensis*.

4.2.1 What are the possibilities for GbdR1/GbdR2 interaction?

We observed that GbdR1 and GbdR2 are both required for optimal induction of genes required for the transport and catabolism of GB. Furthermore, both GATRs can bind to the promoter region of the large GB catabolic operon (Figures 2.1, 2.5) (1). The presence of GbdR1 is an absolute requirement for expression of GbdR2 and GbdR2 increases the expression of GbdR1 (Table 2.1). This suggests that there is some manner of interaction between these two GATRs. However, the experiments described here were not sufficient to determine the particular nature of this relationship.

There are several possible explanations for the presence of two orthologs in this system. It is possible that there are unidentified half sites or overlapping binding sites for GbdR2 within the portion of promoter used in our experiments. Such sites might allow GbdR2 to regulate expression of GbdR1, as opposed to other ATRs which have been shown to autoregulate their own expression (2-4). Multiple binding sites might provide

an explanation for the banding pattern seen in electromobility shift assays incorporating GbdR2 (Figure 2.5B).

We also observed that the N-terminal arm of GbdR2 is slightly longer than other GATR N-terminal sequences. The presence of a longer N-terminal arm might offer an opportunity for regulation either through protein degradation or perhaps as a mechanical sensor for osmotic stress where its structure may be influenced by the physiological state of the cell (5, 6). Perhaps the arm affects the dimerization state by altering structural conformation as it does in the case of AraC (7). These possibilities might allow GbdR2 to act as a licensing factor for catabolism of GB under favorable conditions while preventing its breakdown during osmotic stress.

In order to determine the relationship between GbdR1 and GbdR2, there are several relatively simple approaches we could take. The first is to more carefully determine the binding site of each regulator. Our initial attempts to achieve this through DNAase footprinting assays were unsuccessful, but there are many adjustments on the technique that could be applied. Alternatively, ChIP-seq might provide insights by identifying new target promoters, cumulatively from which binding sites and half sites which we were not able to detect might be derived (8).

The challenge in working with ATRs biochemically is thought to be due to a number of factors including their ability to oligomerize, potential folding issues when overexpressed protein is unbound, and potential non-specific binding to DNA that disrupts cell function during overexpression (9). However, much of this difficulty can be abrogated by expressing the domains individually, and focusing on just the C-terminal domain to determine the target binding site (10, 11). We could also repeat our RNA-seq

experiments under osmotically stressful conditions to discern if the apparent regulons of these regulators changes.

4.2.2 Likely influences on the regulation of GB catabolism in *B. thailandensis* that remain unaddressed.

Although the focus of our study has been on the GATRs involved in the regulation of GB catabolism, there are additional points of regulation in the pathway based on the model in *P. aeruginosa* that are likely to impact it. The breakdown of choline into GB is accomplished by the products of the *betBA* genes in *P. aeruginosa*, which are regulated by the TetR-family repressor, BetI (12, 13). These genes have also been identified in *B. thailandensis*, but it is not known if BetI impacts genes beyond *betBA* in this organism (14). Recently the expression of *gbdR* in *P. aeruginosa* was found to be under the direct control of BetI, as well as the global regulators NtrC and CrbB responsible for accessory nitrogen and carbon catabolism respectively (15). Notably, CrbB has also been shown to be necessary for the expression of the operon containing the GATR *argR* in *P. aeruginosa* (16). Nucleoid-associated proteins like H-NS, which are functionally similar to histones, are increasingly appreciated to play an important regulatory role in gene expression and have been shown to compete with ATRs for binding sites (17-19). Though not described, this is most certainly the case for *B. thailandensis* as well and it is possible that nucleoid-associated proteins impact the regulation of the GB catabolic pathway.

Exploration of the additional regulators impacting this pathway could benefit from several approaches. First, the individual characterization of the binding sites of these regulators to enable the bioinformatic prediction of target promoters. Alternatively, deletion strains could be generated and used in combination with reporter plasmids similarly to our previous strategy to determine if they impact the expression of the GB catabolic operon (1). The findings could then be verified through ChIP to confirm physical interaction between the predicted binding sites and regulator. However, this approach might be hindered by the degree to which these global regulators can impact the cell and that may obfuscate the results.

4.2.3 How important is GB as an osmolyte and are there pools of related molecules in *B. thailandensis*?

Despite our focus on the importance of the GB catabolic pathway and our lab's past interest in osmoprotection and molecular pools of osmolytes, we have not focused specifically on osmoprotection in *B. thailandensis*. The particular characteristics that predispose a given molecule to lend the cell resistance to osmotic stress, or for that matter barometric or thermal stress, are somewhat abstract. It is clear that such a compound must be a compatible solute, that is, the cell must be able to accumulate the molecule to high levels without disrupting the other biological processes and pathways necessary for life (20). Ideally, the amount of these molecules would be readily controlled by means of transport or metabolic flux to compensate for a rapidly changing environment.

Typically for many bacteria, a sudden increase in hypertonic osmotic stress results

in the rapid uptake of K^+ and production of glutamate, an often less than ideal countermeasure (21). The cell then accumulates and switches to the preferred osmolytes, while reducing the intracellular level of glutamate (22). Beyond GB and closely related compounds, other osmolytes found to be used by bacteria include trehalose, proline, ectoine, dimethylsulfoniopropionate, and others (23-28).

Previously our lab has utilized ^{13}C nuclear magnetic resonance to examine the presence of molecular pools of choline and GB present during conditions of osmotic stress in different proteobacteria. As part of these experiments *Burkholderia cepacia* was observed to maintain a small choline pool and a larger GB pool, possibly functioning as a hedge against osmotic stress (29). *B. thailandensis* almost certainly maintains similar pools due to this being shown in *B. cepacia* and in addition to many other proteobacteria, but it has not been shown empirically. Preliminary experiments have also shown that *B. thailandensis* grown on choline is more tolerant to salt stress than when grown on pyruvate (unpublished). Presumably, these findings and the significant conservation of pathways resulting in the production of GB in *Burkholderia* indicate that examining GB induced osmoprotection in *B. thailandensis* would yield the same results. However, understanding the molecular mechanisms behind osmoprotection in *B. thailandensis*, and by extension its close relatives *B. pseudomallei* and *B. mallei*, will address pertinent biological questions. For instance, how does *B. pseudomallei* manage to endure environmental stress as the seasons changes from rainy to dry where it is endemic (30, 31)? Additionally, might GB related metabolism be important for interaction with plants (33)?

4.2.4 How closely is the transport and catabolism of choline-O-sulfate linked to GbdR1 and GbdR2?

Choline-O-Sulfate (CS) catabolic operon is expressed in the presence of choline, dependent on the presence of both GbdR1 and GbdR2 (1). CS is used as a mechanism for sulfur storage in fungi, as well as an osmolyte by many plants (34, 35). Certain members of the Plumbaginaceae (a family of salt tolerant plants) preferentially utilize CS and β-alanine betaine as osmoprotectants over GB, likely due to the utility of using the generation of CS as a means to detoxify sulfur in the salt marsh environment (36). Many bacteria can utilize CS as an osmolyte or indirectly by converting it into GB, and in the case of *B. thailandensis*, subsequently using it as a carbon and nitrogen source (37, 38). *B. thailandensis* possess genes for the transport and catabolism of CS located near the genes involved in the catabolism of GB. These genes encode a choline sulfatase (*betC*), a CS substrate binding periplasmic transport protein (*betD*), a sulfate transporter (*betE*), and a divergently transcribed LysR-family transcriptional regulator (*betR*). This gene arrangement is broadly conserved in *Burkholderia*, particularly in the closely related *B. pseudomallei* and *B. mallei*. In *Pseudomonas* species, BetR has been shown to positively activate transcription of this operon in the presence of CS, but not choline or GB, and to negatively repress its own transcription in the absence of CS (39).

We have shown that *B. thailandensis* can utilize CS as either a sole carbon or nitrogen source (Figures 3.3 and 3.4). Studies from our own lab on the regulons of GbdR-like GATRs in *P. aeruginosa* have not suggested any such regulatory link exists between the CS catabolism and any of the GATRs (40, 41). It is conceivable that the upregulation of CS-related genes is due to read through transcription of the large GB catabolic operon,

which might continue to include the CS catabolic operon despite being interrupted by *purU-2* and *betR* being transcribed in the opposite direction between the two (Figure 2.1C). Another possibility is that the CS catabolic operon or *betR* are regulated directly by either GbdR1 or GbdR2. The answer to this could be tackled by simple genetic techniques already utilized in our lab and would make an excellent project for an undergraduate.

The data obtained thus far suggest that *B. thailandensis* and other *Burkholderia* may have a more complex regulatory response to GB than initially appreciated, in a sense anticipating the environmental presence of CS through the presence of a downstream metabolite. The presence of pathways that utilize CS and related metabolites is logical in a bacterium that often resides in soil with an association to plants and fungi. The utilization of CS may provide an additional clue to the particular environmental niche where *B. thailandensis* and *B. pseudomallei* reside, given that its particular environmental reservoir is still unknown as discussed in Chapter 1.

4.2.5 The absence of SouR in *B. mallei* and potential effects on GB catabolism.

B. mallei lacks a sequence ortholog for SouR, the GATR that is responsible for the regulation of sarcosine catabolism, a downstream portion of the glycine betaine utilization pathway in *B. thailandensis* and *P. aeruginosa* (1, 40). An ortholog for the α-subunit of sarcosine oxidase is present in twenty seven out of forty two sequenced *B. mallei* strains while the γ-subunit ortholog can only be found in four of those strains (42). Traces of the remaining sarcosine oxidase subunits and *sdaA* cannot be identified at all.

Tellingly, putative transposases are uniquely located adjacent to the α-subunit suggesting that the sarcosine oxidase operon was truncated in the process of genetic reduction. This suggests that unlike *B. pseudomallei* and *B. thailandensis*, *B. mallei* is unable to utilize GB or its catabolites as a carbon or nitrogen source due to a probable inability to degrade sarcosine.

Despite missing this piece of the pathway, *B. mallei* maintains orthologs for GbdR1, GbdR2, and CdhR (Table 3.4, Figures 3.1 and 3.2). Also maintained are the operons that these GATRs regulate. *B. mallei* is considered to be derived directly from a particular isolate of *B. pseudomallei* that underwent significant genetic reduction due the proliferation of insertion sequence (IS) elements from transposons and phages (43-45). This evolution and reduction continue through genomic recombination due to homology between IS elements (46). This genomic reduction likely led to *B. mallei* becoming an obligate parasite of mammalian cells, particular equines (47). It is possible that the transport systems for carnitine, choline, and GB retain importance for survival due to the role of GB as an osmoprotectant, but the acquisition of these compounds for usage as carbon and nitrogen sources is diminished due to the host environment being rich in other nutrient sources. It is possible that *B. mallei* possesses an alternative pathway for sarcosine degradation that would otherwise complete the larger pathway, but none are obvious.

Although *B. mallei* has never been isolated from the environment and is considered an obligate pathogen, it is possible to cultivate it under laboratory condition in minimal media (48). Thus it may be surprisingly simple to test if sarcosine may be utilized. Methods for genetic manipulation have been developed for *B. mallei (49)*, and

many animal models for virulence have been published (50-54). It may be possible to use these methods to discern if carnitine or GB transport and catabolism play a role in fitness during *B*. *mallei* infection.

4.3 Deciphering the mechanism of GATRs

4.3.1 How do GATRs interact with RNA polymerase?

While most ATRs have been shown to function as activators, it remains unclear how GATRs that function as activators interact with RNA polymerase, if at all. Activators may function through a number of mechanisms including class I and II activation, which involve direct interaction with RNA polymerase (55). In class I activation, the activator interacts with the α subunit C-terminal domain of RNA polymerase. In class II activation, the activator sits adjacent to the -35 site and interacts with the σ subunit. Additional mechanism include class III activation where multiple activators interact with different subunits of RNA polymerase and activators that change the promoter conformation such that RNA polymerase can recognize the -10 and -35 elements (56). Different ATRs have previously shown to act as class I or class II activators (57-60). Based on the position of the CdhR binding site relative to the *caiX* promoter in *P*. *aeruginosa* (61), it is most likely that GATRs are class I activators.

The type of activation may be ascertained in several different ways. Showing interaction by immunoprecipitation of RNA polymerase components would be informative. Improvements in our understanding of promoters targeted by GATRs may aid our understanding by determining the positioning of various GATRs with respect to

other elements of the promoter. Performing 5' rapid amplification of cDNA ends (RACE) to determine the actual transcriptional start sites of GATR targeted promoters and determining GATR binding sites as previously mentioned would readily accomplish this.

Because we already have some evidence that I0685 functions as a repressor (Figure 3.8, Table 3.7), it is possible that not all GATRs actually interact with RNA polymerase. However, it is still possible that I0685 N32G is simply locked in a repressive conformation. The location of binding sites within promoters often dictates whether a regulator functions as a repressor or an activator and can differ for the same regulator between promoters as with the ATR, MarA (62). However, it is possible that some findings will be transferable among more closely related GATRs, particularly those within the GbdR1 clade that appear to function specifically as activators (Figures 3.1 and 3.2).

4.3.2 A novel mechanism for ATR function.

The mechanism of GATRs, similar to other ATRs, likely revolves around a conformational change in the dimer to achieve either activation or de-repression. For GATRs this conformational change is potentially derived from homology to GATases, which depend on dimerization for creation of an ammonia channel and subsequent conformation change to open the channel and transfer the ammonia to an associated synthase (63). Additional analysis of the 3GRA structure and the generation of new crystal structures in the presence and absence of ligand may provide insight into whether conformational changes from GATases are likely to be conserved in GATRs. We could

also incorporate our constitutive GATR mutants into these experiments to determine if the crystal structures, and subsequently the conformation of the predicted dimer, are altered.

In other ATRs such as AraC, changes in conformation arise in part from interaction of the N-terminal arm with bound ligand (7, 64-66). Due to the unconserved nature of the N-terminal arm in GATRs, it is unclear if it serves a similar function. N-terminally affinity tagged GATRs have been shown to bind DNA targets *in vitro* (1, 40, 41, 61), which might be expected to impact the functionality of the arm and subsequently the DNA binding properties of the protein. Future studies should employ strains with deletions of the N-terminal arm to help determine if the arms is a critical component of known GATRs. These apparent differences between GATR mechanism from previously described mechanisms of ATRs offers a new, additional model for ATR function.

4.3.3 The potential functions of unknown GATRs.

Several GATRs are divergently transcribed from putative DJ-1/Pfp1family proteins (Figure 1.7, *II0513* and *II1912*), a family which includes GATase1 domain containing proteins. It is tempting to speculate if homology between the GATR and adjacent protein product might reflect commonalities between the ligand for the regulator and the substrate for the enzyme. One of these adjacent genes in *B. thailandensis*, *II0514*, has recently been bioinformatically predicted to be involved in the degredation of caprolactam (42). Caprolactam is a cyclic amide used in the production of Nylon 6 and may be used as a carbon source by some bacteria (67).

Other GATRs of unknown function are also divergently transcribed from their likely regulatory targets. *II2197* is divergently transcribed from a predicted O-acetylserine sulfhydrylase, an enzyme that generates L-cysteine and acetate from bisulfide and O-acetyl-L-serine (68). Although our carbon and nitrogen screens included O-acetyl-L-serine (Figure 3.3, 3.4), it is possible that there is no efficient transporter specific for O-acetly-L-serine and some bacteria are known to contain multiple O-acetylserine sulfhydrylases (69). *II0282* is divergently transcribed from a gene encoding a predicted isochorismatase-family protein. Isochorismatase family proteins include a GATase1 domain and catalyze the conversion of chorismate to 2,3-dihydroxybenzoate and pyruvate. Chorismate itself is an important precursor for aromatic amino acids, folate, vitamin K, salicylic acid, and siderophores (70, 71). A description of the relationship between *II1760* and rhodanese is described in chapter 3 and between *I1350* and capsule genes in Appendix A.

These potential functions greatly broaden the scope of metabolic processes which GATRs are involved in. These insights also point to additional compounds to test the GATR deletion strains against in future variations of our carbon- and nitrogen-source screens (Figures 3.3, 3.4).

4.4 Concluding remarks.

B. thailandensis has proven to be an excellent model for the exploration of GATRs and we have identified the function of roughly half the GATRs in this organism. Importantly, we have gained insight into a GATR outside of the GbdR clade (Figure 4.1).

This has challenged some of our initial assumptions about this family of regulators, as we now understand they may act as either activators or repressors, and may not be limited to amine-containing compounds as ligands. With this new information, we can refine our methodology for generating constitutive mutants, gain understanding of the remaining GATRs in *B*. *thailandensis*, and hopefully unravel the molecular mechanisms behind GATR function.

4.5 Chapter 4 Figures

Figure 4.1 – GATRs of known function in *B. thailandensis*.

This phylogenetic tree was constructed as described in chapter 3 of this thesis. GATRs with known functions are highlighted in purple. While most of the described GATRs are clustered together, notably I0685 is located on the far side of the tree. Thus, perhaps new insights into the remaining GATRs may be gleaned from the differences we find between the GbdR1-like clade and I0685.

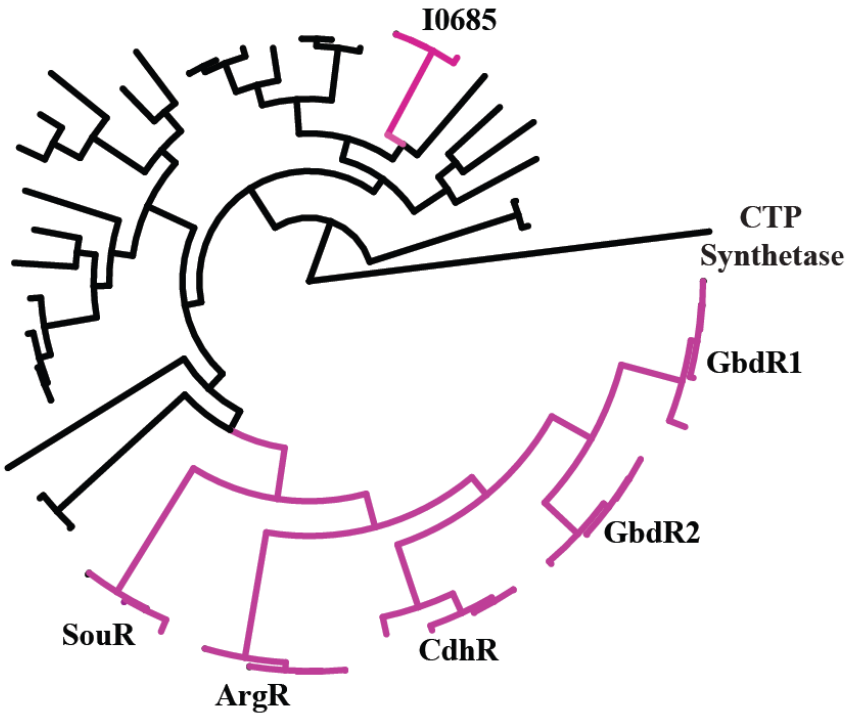


Figure 4.1 - GATRs of known function in *B. thailandensis*.

4.6 References

1. **Nock AM, Wargo MJ.** 2016. Choline Catabolism in Burkholderia thailandensis Is Regulated by Multiple Glutamine Amidotransferase 1-Containing AraC Family Transcriptional Regulators. J Bacteriol **198:**2503-2514.
2. **Lee NL, Gielow WO, Wallace RG.** 1981. Mechanism of araC autoregulation and the domains of two overlapping promoters, Pc and PBAD, in the L-arabinose regulatory region of Escherichia coli. Proc Natl Acad Sci U S A **78:**752-756.
3. **Nunoshiba T, Hidalgo E, Li Z, Demple B.** 1993. Negative autoregulation by the Escherichia coli SoxS protein: a dampening mechanism for the soxRS redox stress response. J Bacteriol **175:**7492-7494.
4. **Martin RG, Jair KW, Wolf RE, Jr., Rosner JL.** 1996. Autoactivation of the marRAB multiple antibiotic resistance operon by the MarA transcriptional activator in Escherichia coli. J Bacteriol **178:**2216-2223.
5. **Baker TA, Sauer RT.** 2006. ATP-dependent proteases of bacteria: recognition logic and operating principles. Trends Biochem Sci **31:**647-653.
6. **Mansouri AL, Grese LN, Rowe EL, Pino JC, Chennubhotla SC, Ramanathan A, O'Neill HM, Berthelier V, Stanley CB.** 2016. Folding propensity of intrinsically disordered proteins by osmotic stress. Mol Biosyst **12:**3695-3701.
7. **Weldon JE, Schleif RF.** 2006. Specific interactions by the N-terminal arm inhibit self-association of the AraC dimerization domain. Protein Sci **15:**2828-2835.
8. **Johnson DS, Mortazavi A, Myers RM, Wold B.** 2007. Genome-wide mapping of in vivo protein-DNA interactions. Science **316:**1497-1502.
9. **Schleif R.** 2003. AraC protein: a love-hate relationship. Bioessays **25:**274-282.
10. **Brutinel ED, Vakulskas CA, Yahr TL.** 2009. Functional domains of ExsA, the transcriptional activator of the Pseudomonas aeruginosa type III secretion system. J Bacteriol **191:**3811-3821.
11. **Bustos SA, Schleif RF.** 1993. Functional domains of the AraC protein. Proc Natl Acad Sci U S A **90:**5638-5642.
12. **Rkenes TP, Lamark T, Strom AR.** 1996. DNA-binding properties of the BetI repressor protein of Escherichia coli: the inducer choline stimulates BetI-DNA complex formation. J Bacteriol **178:**1663-1670.
13. **Lamark T, Rokenes TP, McDougall J, Strom AR.** 1996. The complex bet promoters of Escherichia coli: regulation by oxygen (ArcA), choline (BetI), and osmotic stress. J Bacteriol **178:**1655-1662.
14. **Kang Y, Norris MH, Barrett AR, Wilcox BA, Hoang TT.** 2009. Engineering of tellurite-resistant genetic tools for single-copy chromosomal analysis of Burkholderia spp. and characterization of the Burkholderia thailandensis betBA operon. Appl Environ Microbiol **75:**4015-4027.
15. **Sanchez DG, Primo ED, Damiani MT, Lisa AT.** 2017. Pseudomonas aeruginosa gbdR gene is transcribed from a sigma54-dependent promoter under the control of NtrC/CbrB, IHF and BetI. Microbiology **163:**1343-1354.
16. **Nishijyo T, Haas D, Itoh Y.** 2001. The CbrA-CbrB two-component regulatory system controls the utilization of multiple carbon and nitrogen sources in Pseudomonas aeruginosa. Mol Microbiol **40:**917-931.

17. **Grainger DC.** 2016. Structure and function of bacterial H-NS protein. Biochemical Society Transactions **44:**1561-1569.
18. **Giangrossi M, Zattoni S, Tramonti A, De Biase D, Falconi M.** 2005. Antagonistic role of H-NS and GadX in the regulation of the glutamate decarboxylase-dependent acid resistance system in Escherichia coli. J Biol Chem **280:**21498-21505.
19. **Santiago AE, Yan MB, Hazen TH, Sauder B, Meza-Segura M, Rasko DA, Kendall MM, Ruiz-Perez F, Nataro JP.** 2017. The AraC Negative Regulator family modulates the activity of histone-like proteins in pathogenic bacteria. PLoS Pathog **13:**e1006545.
20. **Miller KJ, Wood JM.** 1996. Osmoadaptation by rhizosphere bacteria. Annu Rev Microbiol **50:**101-136.
21. **Csonka LN, Hanson AD.** 1991. Prokaryotic osmoregulation: genetics and physiology. Annu Rev Microbiol **45:**569-606.
22. **Wood JM.** 2011. Bacterial osmoregulation: a paradigm for the study of cellular homeostasis. Annu Rev Microbiol **65:**215-238.
23. **Bernard T, Jebbar M, Rassouli Y, Himdikabbab S, Hamelin J, Blanco C.** 1993. Ectoine Accumulation and Osmotic Regulation in Brevibacterium-Linens. Journal of General Microbiology **139:**129-136.
24. **Wood JM.** 1988. Proline Porters Effect the Utilization of Proline as Nutrient or Osmoprotectant for Bacteria. Journal of Membrane Biology **106:**183-202.
25. **Csonka LN.** 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol Rev **53:**121-147.
26. **Iturriaga G, Suarez R, Nova-Franco B.** 2009. Trehalose Metabolism: From Osmoprotection to Signaling. International Journal of Molecular Sciences **10:**3793-3810.
27. **Cosquer A, Pichereau V, Pocard JA, Minet J, Cormier M, Bernard T.** 1999. Nanomolar levels of dimethylsulfoniopropionate, dimethylsulfonioacetate, and glycine betaine are sufficient to confer osmoprotection to Escherichia coli. Appl Environ Microbiol **65:**3304-3311.
28. **Sleator RD, Hill C.** 2002. Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. FEMS Microbiol Rev **26:**49-71.
29. **Fitzsimmons LF, Hampel KJ, Wargo MJ.** 2012. Cellular choline and glycine betaine pools impact osmoprotection and phospholipase C production in Pseudomonas aeruginosa. J Bacteriol **194:**4718-4726.
30. **Baker AL, Ezzahir J, Gardiner C, Shipton W, Warner JM.** 2015. Environmental Attributes Influencing the Distribution of Burkholderia pseudomallei in Northern Australia. PLoS One **10:**e0138953.
31. **Kaestli M, Harrington G, Mayo M, Chatfield MD, Harrington I, Hill A, Munksgaard N, Gibb K, Currie BJ.** 2015. What Drives the Occurrence of the Melioidosis Bacterium Burkholderia pseudomallei in Domestic Gardens? Plos Neglected Tropical Diseases **9**.
32. **Rolim DB, Rocha MF, Brilhante RS, Cordeiro RA, Leitao NP, Jr., Inglis TJ, Sidrim JJ.** 2009. Environmental isolates of Burkholderia pseudomallei in Ceara State, northeastern Brazil. Appl Environ Microbiol **75:**1215-1218.

33. **Kaestli M, Schmid M, Mayo M, Rothballer M, Harrington G, Richardson L, Hill A, Hill J, Tuanyok A, Keim P, Hartmann A, Currie BJ.** 2012. Out of the ground: aerial and exotic habitats of the melioidosis bacterium Burkholderia pseudomallei in grasses in Australia. Environ Microbiol **14:**2058-2070.
34. **Spencer B, Harada T.** 1960. The role of choline sulphate in the sulphur metabolism of fungi. Biochem J **77:**305-315.
35. **Rhodes D, Hanson AD.** 1993. Quaternary Ammonium and Tertiary Sulfonium Compounds in Higher-Plants. Annual Review of Plant Physiology and Plant Molecular Biology **44:**357-384.
36. **Hanson AD, Rathinasabapathi B, Chamberlin B, Gage DA.** 1991. Comparative Physiological Evidence That Beta-Alanine Betaine and Choline-O-Sulfate Act as Compatible Osmolytes in Halophytic Limonium Species. Plant Physiology **97:**1199-1205.
37. **Nau-Wagner G, Boch J, Le Good JA, Bremer E.** 1999. High-affinity transport of choline-O-sulfate and its use as a compatible solute in Bacillus subtilis. Appl Environ Microbiol **65:**560-568.
38. **Canovas D, Vargas C, Csonka LN, Ventosa A, Nieto JJ.** 1996. Osmoprotectants in Halomonas elongata: high-affinity betaine transport system and choline-betaine pathway. J Bacteriol **178:**7221-7226.
39. **Jovcic B, Venturi V, Topisirovic L, Kojic M.** 2011. Inducible expression of choline sulfatase and its regulator BetR in Pseudomonas sp. ATCC19151. Arch Microbiol **193:**399-405.
40. **Willsey GG, Wargo MJ.** 2015. Sarcosine catabolism in Pseudomonas aeruginosa is transcriptionally regulated by SouR. J Bacteriol doi:10.1128/JB.00739-15.
41. **Hampel KJ, LaBauve AE, Meadows JA, Fitzsimmons LF, Nock AM, Wargo MJ.** 2014. Characterization of the GbdR regulon in Pseudomonas aeruginosa. J Bacteriol **196:**7-15.
42. **Winsor GL, Khaira B, Van Rossum T, Lo R, Whiteside MD, Brinkman FS.** 2008. The Burkholderia Genome Database: facilitating flexible queries and comparative analyses. Bioinformatics **24:**2803-2804.
43. **Ronning CM, Losada L, Brinkac L, Inman J, Ulrich RL, Schell M, Nierman WC, Deshazer D.** 2010. Genetic and phenotypic diversity in Burkholderia: contributions by prophage and phage-like elements. BMC Microbiol **10:**202.
44. **Moore RA, Reckseidler-Zenteno S, Kim H, Nierman W, Yu Y, Tuanyok A, Warawa J, DeShazer D, Woods DE.** 2004. Contribution of gene loss to the pathogenic evolution of Burkholderia pseudomallei and Burkholderia mallei. Infect Immun **72:**4172-4187.
45. **Nierman WC, DeShazer D, Kim HS, Tettelin H, Nelson KE, Feldblyum T, Ulrich RL, Ronning CM, Brinkac LM, Daugherty SC, Davidsen TD, Deboy RT, Dimitrov G, Dodson RJ, Durkin AS, Gwinn ML, Haft DH, Khouri H, Kolonay JF, Madupu R, Mohammoud Y, Nelson WC, Radune D, Romero CM, Sarria S, Selengut J, Shamblin C, Sullivan SA, White O, Yu Y, Zafar N, Zhou LW, Fraser CM.** 2004. Structural flexibility in the Burkholderia mallei genome. Proceedings of the National Academy of Sciences of the United States of America **101:**14246-14251.

46. **Losada L, Ronning CM, DeShazer D, Woods D, Fedorova N, Kim HS, Shabalina SA, Pearson TR, Brinkac L, Tan P, Nandi T, Crabtree J, Badger J, Beckstrom-Sternberg S, Saqib M, Schutzer SE, Keim P, Nierman WC.** 2010. Continuing Evolution of Burkholderia mallei Through Genome Reduction and Large-Scale Rearrangements. Genome Biology and Evolution **2:**102-116.
47. **Godoy D, Randle G, Simpson AJ, Aanensen DM, Pitt TL, Kinoshita R, Spratt BG.** 2003. Multilocus sequence typing and evolutionary relationships among the causative agents of melioidosis and glanders, Burkholderia pseudomallei and Burkholdefia mallei (vol 41, pg 2068, 2003). Journal of Clinical Microbiology **41:**4913-4913.
48. **Burtnick MN, Brett PJ.** 2013. Burkholderia mallei and Burkholderia pseudomallei Cluster 1 Type VI Secretion System Gene Expression Is Negatively Regulated by Iron and Zinc. Plos One **8**.
49. **Hamad MA, Zajdowicz SL, Holmes RK, Voskuil MI.** 2009. An allelic exchange system for compliant genetic manipulation of the select agents Burkholderia pseudomallei and Burkholderia mallei. Gene **430:**123-131.
50. **Schully KL, Bell MG, Ward JM, Keane-Myers AM.** 2014. Oropharyngeal aspiration of Burkholderia mallei and Burkholderia pseudomallei in BALB/c mice. PLoS One **9:**e115066.
51. **Jelesijevic T, Zimmerman SM, Harvey SB, Mead DG, Shaffer TL, Estes DM, Michel F, Quinn FD, Hogan RJ, Lafontaine ER.** 2015. Use of the Common Marmoset to Study Burkholderia mallei Infection. Plos One **10**.
52. **Fisher NA, Ribot WJ, Applefeld W, DeShazer D.** 2012. The Madagascar hissing cockroach as a novel surrogate host for Burkholderia pseudomallei, B. mallei and B. thailandensis. BMC Microbiol **12:**117.
53. **Fritz DL, Vogel P, Brown DR, Waag DM.** 1999. The hamster model of intraperitoneal Burkholderia mallei (glanders). Vet Pathol **36:**276-291.
54. **Lopez J, Copps J, Wilhelmsen C, Moore R, Kubay J, St-Jacques M, Halayko S, Kranendonk C, Toback S, DeShazer D, Fritz DL, Tom M, Woods DE.** 2003. Characterization of experimental equine glanders. Microbes Infect **5:**1125-1131.
55. **Browning DF, Busby SJ.** 2004. The regulation of bacterial transcription initiation. Nat Rev Microbiol **2:**57-65.
56. **Lee DJ, Minchin SD, Busby SJ.** 2012. Activating transcription in bacteria. Annu Rev Microbiol **66:**125-152.
57. **Yang J, Tauschek M, Robins-Browne RM.** 2011. Control of bacterial virulence by AraC-like regulators that respond to chemical signals. Trends Microbiol **19:**128-135.
58. **Vakulskas CA, Brutinel ED, Yahr TL.** 2010. ExsA recruits RNA polymerase to an extended -10 promoter by contacting region 4.2 of sigma-70. J Bacteriol **192:**3597-3607.
59. **Taliaferro LP, Keen EF, Sanchez-Alberola N, Wolf RE.** 2012. Transcription Activation by Escherichia coli Rob at Class II Promoters: Protein-Protein Interactions between Rob's N-Terminal Domain and the sigma(70) Subunit of RNA Polymerase. Journal of Molecular Biology **419:**139-157.

60. **Ruiz R, Ramos JL, Egan SM.** 2001. Interactions of the XylS regulators with the C-terminal domain of the RNA polymerase alpha subunit influence the expression level from the cognate Pm promoter. FEBS Lett **491:**207-211.
61. **Meadows JA, Wargo MJ.** 2018. Transcriptional Regulation of Carnitine Catabolism in Pseudomonas aeruginosa by CdhR. mSphere **3**.
62. **Duval V, Lister IM.** 2013. MarA, SoxS and Rob of Escherichia coli - Global regulators of multidrug resistance, virulence and stress response. Int J Biotechnol Wellness Ind **2:**101-124.
63. **Wang XS, Roitberg AE, Richards NG.** 2009. Computational studies of ammonia channel function in glutamine 5'-phosphoribosylpyrophosphate amidotransferase. Biochemistry **48:**12272-12282.
64. **Ghosh M, Schleif RF.** 2001. Biophysical evidence of arm-domain interactions in AraC. Anal Biochem **295:**107-112.
65. **Wu M, Schleif R.** 2001. Strengthened arm-dimerization domain interactions in AraC. J Biol Chem **276:**2562-2564.
66. **Saviola B, Seabold R, Schleif RF.** 1998. Arm-domain interactions in AraC. J Mol Biol **278:**539-548.
67. **Baxi NN.** 2013. Influence of epsilon-caprolactam on growth and physiology of environmental bacteria. Annals of Microbiology **63:**1471-1476.
68. **Rabeh WM, Cook PF.** 2004. Structure and mechanism of O-acetylserine sulfhydrylase. J Biol Chem **279:**26803-26806.
69. **Chattopadhyay A, Meier M, Ivaninskii S, Burkhard P, Speroni F, Campanini B, Bettati S, Mozzarelli A, Rabeh WM, Li L, Cook PF.** 2007. Structure, mechanism, and conformational dynamics of O-acetylserine sulfhydrylase from Salmonella typhimurium: comparison of A and B isozymes. Biochemistry **46:**8315-8330.
70. **Li QA, Mavrodi DV, Thomashow LS, Roessle M, Blankenfeldt W.** 2011. Ligand binding induces an ammonia channel in 2-amino-2-desoxyisochorismate (ADIC) synthase PhzE. J Biol Chem **286:**18213-18221.
71. **Wildermuth MC, Dewdney J, Wu G, Ausubel FM.** 2001. Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature **414:**562-565.

Comprehensive References

A.-M. Chakrabarty, B.I., and S. Kaplan. 1990. Arginine network of Pseudomonas aeruginosa: specific and global controls. *In* Pseudomonas : biotransformations, pathogenesis, and evolving biotechnology. S. Silver, editor. American Society for Microbiology, Washington, D.C. p. 303-316.

Adjei, M.D., and Y. Ohta. 2000. Factors affecting the biodegradation of cyanide by Burkholderia cepacia strain C-3. *J Biosci Bioeng*. 89:274-277.

Allwood, E.M., R.J. Devenish, M. Prescott, B. Adler, and J.D. Boyce. 2011. Strategies for Intracellular Survival of Burkholderia pseudomallei. *Frontiers in microbiology*. 2:170.

Anders, S., and W. Huber. 2010. Differential expression analysis for sequence count data. *Genome Biol*. 11:R106.

Andresen, P.A., I. Kaasen, O.B. Styrvold, G. Boulnois, and A.R. Strom. 1988. Molecular cloning, physical mapping and expression of the bet genes governing the osmoregulatory choline-glycine betaine pathway of Escherichia coli. *J Gen Microbiol*. 134:1737-1746.

Andrews, S. 2009. FastQC: a quality control tool for high throughput sequence data.

Angus, A.A., C.M. Agapakis, S. Fong, S. Yerrapragada, P. Estrada-de los Santos, P. Yang, N. Song, S. Kano, J. Caballero-Mellado, S.M. de Faria, F.D. Dakora, G. Weinstock, and A.M. Hirsch. 2014. Plant-associated symbiotic Burkholderia species lack hallmark strategies required in mammalian pathogenesis. *PLoS One*. 9:e83779.

Ariga, H., K. Takahashi-Niki, I. Kato, H. Maita, T. Niki, and S.M. Iguchi-Ariga. 2013. Neuroprotective function of DJ-1 in Parkinson's disease. *Oxid Med Cell Longev*. 2013:683920.

Ashraf, M., and M.R. Foolad. 2007. Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environ Exp Bot*. 59:206-216.

Aurich, H., H.P. Kleber, and W.D. Schopp. 1967. An inducible carnitine dehydrogenase from Pseudomonas aeruginosa. *Biochimica et biophysica acta*. 139:505-507.

Bagaria, A., Kumaran, D., Burley, S.K., Swaminathan, S. 2009. Crystal structure of AraC family transcriptional regulator from Pseudomonas putida.

Baker, A.L., J. Ezzahir, C. Gardiner, W. Shipton, and J.M. Warner. 2015. Environmental Attributes Influencing the Distribution of Burkholderia pseudomallei in Northern Australia. *PLoS One*. 10:e0138953.

Baker, T.A., and R.T. Sauer. 2006. ATP-dependent proteases of bacteria: recognition logic and operating principles. *Trends in biochemical sciences*. 31:647-653.

Bandyopadhyay, S., and M.R. Cookson. 2004. Evolutionary and functional relationships within the DJ1 superfamily. *BMC evolutionary biology*. 4:6.

Bankapalli, K., S. Saladi, S.S. Awadia, A.V. Goswami, M. Samaddar, and P. D'Silva. 2015. Robust glyoxalase activity of Hsp31, a ThiJ/DJ-1/PfpI family member protein, is critical for oxidative stress resistance in Saccharomyces cerevisiae. *The Journal of biological chemistry*. 290:26491-26507.

Barry, R.M., A.F. Bitbol, A. Lorestani, E.J. Charles, C.H. Habrian, J.M. Hansen, H.J. Li, E.P. Baldwin, N.S. Wingreen, J.M. Kollman, and Z. Gitai. 2014. Large-scale filament formation inhibits the activity of CTP synthetase. *Elife*. 3:e03638.

Baxi, N.N. 2013. Influence of epsilon-caprolactam on growth and physiology of environmental bacteria. *Ann Microbiol*. 63:1471-1476.
Baxter, J., and S.P. Cummings. 2006. The current and future applications of microorganism in the bioremediation of cyanide contamination. *Antonie Van Leeuwenhoek*. 90:1-17.
Beck, C.F., and R.A. Warren. 1988. Divergent promoters, a common form of gene organization. *Microbiol Rev*. 52:318-326.
Berman, H.M., J. Westbrook, Z. Feng, G. Gilliland, T.N. Bhat, H. Weissig, I.N. Shindyalov, and P.E. Bourne. 2000. The Protein Data Bank. *Nucleic acids research*. 28:235-242.
Bernard, T., M. Jebbar, Y. Rassouli, S. Himdikabbab, J. Hamelin, and C. Blanco. 1993. Ectoine Accumulation and Osmotic Regulation in Brevibacterium-Linens. *Journal of General Microbiology*. 139:129-136.
Bernhard, W., S. Hoffmann, H. Dombrowsky, G.A. Rau, A. Kamlage, M. Kappler, J.J. Haitsma, J. Freihorst, H. von der Hardt, and C.F. Poets. 2001. Phosphatidylcholine molecular species in lung surfactant: composition in relation to respiratory rate and lung development. *American journal of respiratory cell and molecular biology*. 25:725-731.
Bernier, S.P., M.L. Workentine, X. Li, N.A. Magarvey, G.A. O'Toole, and M.G. Surette. 2016. Cyanide Toxicity to Burkholderia cenocepacia Is Modulated by Polymicrobial Communities and Environmental Factors. *Frontiers in microbiology*. 7.
Bonifati, V., P. Rizzu, M.J. van Baren, O. Schaap, G.J. Breedveld, E. Krieger, M.C. Dekker, F. Squitieri, P. Ibanez, M. Joosse, J.W. van Dongen, N. Vanacore, J.C. van Swieten, A. Brice, G. Meco, C.M. van Duijn, B.A. Oostra, and P. Heutink. 2003. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. *Science*. 299:256-259.
Borlee, G.I., B.A. Plumley, K.H. Martin, N. Somprasong, M.R. Mangalea, M.N. Islam, M.N. Burtnick, P.J. Brett, I. Steinmetz, D.P. AuCoin, J.T. Belisle, D.C. Crick, H.P. Schweizer, and B.R. Borlee. 2017. Genome-scale analysis of the genes that contribute to Burkholderia pseudomallei biofilm formation identifies a crucial exopolysaccharide biosynthesis gene cluster. *PLoS neglected tropical diseases*. 11.
Bourdin, B., H. Adenier, and Y. Perrin. 2007. Carnitine is associated with fatty acid metabolism in plants. *Plant Physiol Biochem*. 45:926-931.
Brett, P.J., D. DeShazer, and D.E. Woods. 1998. Burkholderia thailandensis sp. nov., a Burkholderia pseudomallei-like species. *International journal of systematic bacteriology*. 48 Pt 1:317-320.
Broderick, K.E., A. Chan, M. Balasubramanian, J. Feala, S.L. Reed, M. Panda, V.S. Sharma, R.B. Pilz, T.D. Bigby, and G.R. Boss. 2008. Cyanide produced by human isolates of Pseudomonas aeruginosa contributes to lethality in Drosophila melanogaster. *The Journal of infectious diseases*. 197:457-464.
Browning, D.F., and S.J. Busby. 2004. The regulation of bacterial transcription initiation. *Nature reviews. Microbiology*. 2:57-65.
Brutinel, E.D., C.A. Vakulskas, K.M. Brady, and T.L. Yahr. 2008. Characterization of ExsA and of ExsA-dependent promoters required for expression of the

Pseudomonas aeruginosa type III secretion system. *Molecular microbiology*. 68:657-671.
Brutinel, E.D., C.A. Vakulskas, and T.L. Yahr. 2009. Functional domains of ExsA, the transcriptional activator of the Pseudomonas aeruginosa type III secretion system. *J Bacteriol*. 191:3811-3821.
Burkholder, W.H. 1950. Sour skin, a bacterial rot of onion bulbs. *Phytopathology*. 40:115-117.
Burtnick, M.N., and P.J. Brett. 2013. Burkholderia mallei and Burkholderia pseudomallei Cluster 1 Type VI Secretion System Gene Expression Is Negatively Regulated by Iron and Zinc. *Plos One*. 8.
Bustos, S.A., and R.F. Schleif. 1993. Functional domains of the AraC protein. *Proceedings of the National Academy of Sciences of the United States of America*. 90:5638-5642.
Canovas, D., C. Vargas, L.N. Csonka, A. Ventosa, and J.J. Nieto. 1996. Osmoprotectants in Halomonas elongata: high-affinity betaine transport system and choline-betaine pathway. *J Bacteriol*. 178:7221-7226.
Cass, L.G., and G. Wilcox. 1986. Mutations in the araC regulatory gene of Escherichia coli B/r that affect repressor and activator functions of AraC protein. *J Bacteriol*. 166:892-900.
Cave, M.C., R.T. Hurt, T.H. Frazier, P.J. Matheson, R.N. Garrison, C.J. McClain, and S.A. McClave. 2008. Obesity, inflammation, and the potential application of pharmaconutrition. *Nutr Clin Pract*. 23:16-34.
Chambers, S., and C.M. Kunin. 1985. The osmoprotective properties of urine for bacteria: the protective effect of betaine and human urine against low pH and high concentrations of electrolytes, sugars, and urea. *The Journal of infectious diseases*. 152:1308-1316.
Chang, K., J. Luo, H. Xu, M. Li, F. Zhang, J. Li, D. Gu, S. Deng, M. Chen, and W. Lu. 2017. Human Infection with Burkholderia thailandensis, China, 2013. *Emerging infectious diseases*. 23:1416-1418.
Charlier, D., M. Roovers, F. Van Vliet, A. Boyen, R. Cunin, Y. Nakamura, N. Glansdorff, and A. Pierard. 1992. Arginine regulon of Escherichia coli K-12. A study of repressor-operator interactions and of in vitro binding affinities versus in vivo repression. *Journal of molecular biology*. 226:367-386.
Chattopadhyay, A., M. Meier, S. Ivaninskii, P. Burkhard, F. Speroni, B. Campanini, S. Bettati, A. Mozzarelli, W.M. Rabeh, L. Li, and P.F. Cook. 2007. Structure, mechanism, and conformational dynamics of O-acetylserine sulfhydrylase from Salmonella typhimurium: comparison of A and B isozymes. *Biochemistry*. 46:8315-8330.
Chen, C., and G.A. Beattie. 2007. Characterization of the osmoprotectant transporter OpuC from Pseudomonas syringae and demonstration that cystathionine-beta-synthase domains are required for its osmoregulatory function. *J Bacteriol*. 189:6901-6912.
Chen, C., A.A. Malek, M.J. Wargo, D.A. Hogan, and G.A. Beattie. 2010. The ATP-binding cassette transporter Cbc (choline/betaine/carnitine) recruits multiple substrate-binding proteins with strong specificity for distinct quaternary ammonium compounds. *Molecular microbiology*. 75:29-45.

Chen, N.H., K.Y. Djoko, F.J. Veyrier, and A.G. McEwan. 2016. Formaldehyde Stress Responses in Bacterial Pathogens. *Frontiers in microbiology*. 7:257.
Choi, K.H., D. DeShazer, and H.P. Schweizer. 2006. mini-Tn7 insertion in bacteria with multiple glmS-linked attTn7 sites: example Burkholderia mallei ATCC 23344. *Nature protocols*. 1:162-169.
Choi, K.H., J.B. Gaynor, K.G. White, C. Lopez, C.M. Bosio, R.R. Karkhoff-Schweizer, and H.P. Schweizer. 2005. A Tn7-based broad-range bacterial cloning and expression system. *Nature methods*. 2:443-448.
Choi, K.H., T. Mima, Y. Casart, D. Rholl, A. Kumar, I.R. Beacham, and H.P. Schweizer. 2008. Genetic tools for select-agent-compliant manipulation of Burkholderia pseudomallei. *Applied and environmental microbiology*. 74:1064-1075.
Choi, K.H., and H.P. Schweizer. 2006. mini-Tn7 insertion in bacteria with single attTn7 sites: example Pseudomonas aeruginosa. *Nature protocols*. 1:153-161.
Chou, H.T., M. Hegazy, and C.D. Lu. 2010. L-lysine catabolism is controlled by L-arginine and ArgR in Pseudomonas aeruginosa PAO1. *J Bacteriol*. 192:5874-5880.
Cipollone, R., P. Ascenzi, E. Frangipani, and P. Visca. 2006. Cyanide detoxification by recombinant bacterial rhodanese. *Chemosphere*. 63:942-949.
Cipollone, R., P. Ascenzi, and P. Visca. 2007. Common themes and variations in the rhodanese superfamily. *Iubmb Life*. 59:51-59.
Coca-Cola Company Collection (Library of Congress), and LC Purchase Collection (Library of Congress). 1974. Monty Python and the holy grail. Almi Cinema 5,

Columbia Pictures Home Entertainment,, S.l.

United States.
Collins, R.E., and J.W. Deming. 2013. An inter-order horizontal gene transfer event enables the catabolism of compatible solutes by Colwellia psychrerythraea 34H. *Extremophiles*. 17:601-610.
Cosquer, A., V. Pichereau, J.A. Pocard, J. Minet, M. Cormier, and T. Bernard. 1999. Nanomolar levels of dimethylsulfoniopropionate, dimethylsulfonioacetate, and glycine betaine are sufficient to confer osmoprotection to Escherichia coli. *Applied and environmental microbiology*. 65:3304-3311.
Craig, S.A. 2004. Betaine in human nutrition. *Am J Clin Nutr*. 80:539-549.
Crosson, S., P.T. McGrath, C. Stephens, H.H. McAdams, and L. Shapiro. 2005. Conserved modular design of an oxygen sensory/signaling network with species-specific output. *Proceedings of the National Academy of Sciences of the United States of America*. 102:8018-8023.
Csonka, L.N. 1989. Physiological and genetic responses of bacteria to osmotic stress. *Microbiol Rev*. 53:121-147.
Csonka, L.N., and A.D. Hanson. 1991. Prokaryotic osmoregulation: genetics and physiology. *Annual review of microbiology*. 45:569-606.
Cunin, R., T. Eckhardt, J. Piette, A. Boyen, A. Pierard, and N. Glansdorff. 1983. Molecular basis for modulated regulation of gene expression in the arginine regulon of Escherichia coli K-12. *Nucleic acids research*. 11:5007-5019.
Currie, B.J., and S.P. Jacups. 2003. Intensity of rainfall and severity of melioidosis, Australia. *Emerging infectious diseases*. 9:1538-1542.

D'Orazio, S.E., V. Thomas, and C.M. Collins. 1996. Activation of transcription at divergent urea-dependent promoters by the urease gene regulator UreR. *Molecular microbiology*. 21:643-655.
de Rudder, K.E., C. Sohlenkamp, and O. Geiger. 1999. Plant-exuded choline is used for rhizobial membrane lipid biosynthesis by phosphatidylcholine synthase. *The Journal of biological chemistry*. 274:20011-20016.
DeShazer, D., D.M. Waag, D.L. Fritz, and D.E. Woods. 2001. Identification of a Burkholderia mallei polysaccharide gene cluster by subtractive hybridization and demonstration that the encoded capsule is an essential virulence determinant. *Microbial pathogenesis*. 30:253-269.
Dhiman, A., and R. Schleif. 2000. Recognition of overlapping nucleotides by AraC and the sigma subunit of RNA polymerase. *J Bacteriol*. 182:5076-5081.
Diab, F., T. Bernard, A. Bazire, D. Haras, C. Blanco, and M. Jebbar. 2006. Succinate-mediated catabolite repression control on the production of glycine betaine catabolic enzymes in Pseudomonas aeruginosa PAO1 under low and elevated salinities. *Microbiology*. 152:1395-1406.
Dirla, S., J.Y. Chien, and R. Schleif. 2009. Constitutive mutations in the Escherichia coli AraC protein. *J Bacteriol*. 191:2668-2674.
DiSalvo, S., T.S. Haselkorn, U. Bashir, D. Jimenez, D.A. Brock, D.C. Queller, and J.E. Strassmann. 2015. Burkholderia bacteria infectiously induce the proto-farming symbiosis of Dictyostelium amoebae and food bacteria. *Proceedings of the National Academy of Sciences of the United States of America*. 112:E5029-5037.
Domenech, C.E., M.N. Garrido, and T.A. Lisa. 1991. Pseudomonas aeruginosa cholinesterase and phosphorylcholine phosphatase: two enzymes contributing to corneal infection. *FEMS Microbiol Lett*. 66:131-135.
Dominguez-Cuevas, P., P. Marin, S. Busby, J.L. Ramos, and S. Marques. 2008. Roles of effectors in XylS-dependent transcription activation: intramolecular domain derepression and DNA binding. *J Bacteriol*. 190:3118-3128.
Du, Y., A. Holtel, J. Reizer, and M.H. Saier, Jr. 1996. sigma54-dependent transcription of the Pseudomonas putida xylS operon is influenced by the IIANtr protein of the phosphotransferase system in Escherichia coli. *Research in microbiology*. 147:129-132.
Duval, V., and I.M. Lister. 2013. MarA, SoxS and Rob of Escherichia coli - Global regulators of multidrug resistance, virulence and stress response. *Int J Biotechnol Wellness Ind*. 2:101-124.
Edgar, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic acids research*. 32:1792-1797.
Egan, S.M. 2002. Growing repertoire of AraC/XylS activators. *J Bacteriol*. 184:5529-5532.
El-Halfawy, O.M., and M.A. Valvano. 2014. Putrescine reduces antibiotic-induced oxidative stress as a mechanism of modulation of antibiotic resistance in Burkholderia cenocepacia. *Antimicrobial agents and chemotherapy*. 58:4162-4171.
Englesberg, E., J. Irr, J. Power, and N. Lee. 1965. Positive control of enzyme synthesis by gene C in the L-arabinose system. *J Bacteriol*. 90:946-957.

Eustance, R.J., S.A. Bustos, and R.F. Schleif. 1994. Reaching out. Locating and lengthening the interdomain linker in AraC protein. *Journal of molecular biology*. 242:330-338.
Fazli, M., H. Almblad, M.L. Rybtke, M. Givskov, L. Eberl, and T. Tolker-Nielsen. 2014. Regulation of biofilm formation in Pseudomonas and Burkholderia species. *Environ Microbiol*. 16:1961-1981.
Fisher, N.A., W.J. Ribot, W. Applefeld, and D. DeShazer. 2012. The Madagascar hissing cockroach as a novel surrogate host for Burkholderia pseudomallei, B. mallei and B. thailandensis. *BMC microbiology*. 12:117.
Fitzsimmons, L.F., S. Flemer, Jr., A.S. Wurthmann, P.B. Deker, I.N. Sarkar, and M.J. Wargo. 2011. Small-molecule inhibition of choline catabolism in Pseudomonas aeruginosa and other aerobic choline-catabolizing bacteria. *Applied and environmental microbiology*. 77:4383-4389.
Fitzsimmons, L.F., K.J. Hampel, and M.J. Wargo. 2012. Cellular choline and glycine betaine pools impact osmoprotection and phospholipase C production in Pseudomonas aeruginosa. *J Bacteriol*. 194:4718-4726.
Flemming, H.C., J. Wingender, U. Szewzyk, P. Steinberg, S.A. Rice, and S. Kjelleberg. 2016. Biofilms: an emergent form of bacterial life. *Nature reviews. Microbiology*. 14:563-575.
Fritz, D.L., P. Vogel, D.R. Brown, and D.M. Waag. 1999. The hamster model of intraperitoneal Burkholderia mallei (glanders). *Veterinary pathology*. 36:276-291.
Gallegos, M.T., C. Michan, and J.L. Ramos. 1993. The XylS/AraC family of regulators. *Nucleic acids research*. 21:807-810.
Gallegos, M.T., R. Schleif, A. Bairoch, K. Hofmann, and J.L. Ramos. 1997. Arac/XylS family of transcriptional regulators. *Microbiology and molecular biology reviews : MMBR*. 61:393-410.
Galyov, E.E., P.J. Brett, and D. DeShazer. 2010. Molecular insights into Burkholderia pseudomallei and Burkholderia mallei pathogenesis. *Annual review of microbiology*. 64:495-517.
Geer, L.Y., M. Domrachev, D.J. Lipman, and S.H. Bryant. 2002. CDART: protein homology by domain architecture. *Genome research*. 12:1619-1623.
Gerhart, J.C., and H.K. Schachman. 1965. Distinct subunits for the regulation and catalytic activity of aspartate transcarbamylase. *Biochemistry*. 4:1054-1062.
Ghosh, M., and R.F. Schleif. 2001. Biophysical evidence of arm-domain interactions in AraC. *Anal Biochem*. 295:107-112.
Giangrossi, M., S. Zattoni, A. Tramonti, D. De Biase, and M. Falconi. 2005. Antagonistic role of H-NS and GadX in the regulation of the glutamate decarboxylase-dependent acid resistance system in Escherichia coli. *The Journal of biological chemistry*. 280:21498-21505.
Gibney, K.B., A.C. Cheng, and B.J. Currie. 2008. Cutaneous melioidosis in the tropical top end of Australia: a prospective study and review of the literature. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 47:603-609.
Glass, M.B., J.E. Gee, A.G. Steigerwalt, D. Cavuoti, T. Barton, R.D. Hardy, D. Godoy, B.G. Spratt, T.A. Clark, and P.P. Wilkins. 2006. Pneumonia and septicemia

caused by Burkholderia thailandensis in the United States. *Journal of clinical microbiology*. 44:4601-4604.
Godoy, D., G. Randle, A.J. Simpson, D.M. Aanensen, T.L. Pitt, R. Kinoshita, and B.G. Spratt. 2003. Multilocus sequence typing and evolutionary relationships among the causative agents of melioidosis and glanders, Burkholderia pseudomallei and Burkholdefia mallei (vol 41, pg 2068, 2003). *Journal of clinical microbiology*. 41:4913-4913.
Grainger, D.C. 2016. Structure and function of bacterial H-NS protein. *Biochem Soc T*. 44:1561-1569.
Griffith, K.L., I.M. Shah, T.E. Myers, M.C. O'Neill, and R.E. Wolf, Jr. 2002. Evidence for "pre-recruitment" as a new mechanism of transcription activation in Escherichia coli: the large excess of SoxS binding sites per cell relative to the number of SoxS molecules per cell. *Biochem Biophys Res Commun*. 291:979-986.
Griffith, K.L., I.M. Shah, and R.E. Wolf, Jr. 2004. Proteolytic degradation of Escherichia coli transcription activators SoxS and MarA as the mechanism for reversing the induction of the superoxide (SoxRS) and multiple antibiotic resistance (Mar) regulons. *Molecular microbiology*. 51:1801-1816.
Guang-Han, O., C. Leang-Chung, K.M. Vellasamy, V. Mariappan, C. Li-Yen, and J. Vadivelu. 2016. Experimental Phage Therapy for Burkholderia pseudomallei Infection. *PLoS One*. 11:e0158213.
Halio, S.B., Blumentals, II, S.A. Short, B.M. Merrill, and R.M. Kelly. 1996. Sequence, expression in Escherichia coli, and analysis of the gene encoding a novel intracellular protease (PfpI) from the hyperthermophilic archaeon Pyrococcus furiosus. *J Bacteriol*. 178:2605-2612.
Hamad, M.A., S.L. Zajdowicz, R.K. Holmes, and M.I. Voskuil. 2009. An allelic exchange system for compliant genetic manipulation of the select agents Burkholderia pseudomallei and Burkholderia mallei. *Gene*. 430:123-131.
Hampel, K.J., A.E. Labauve, J.A. Meadows, L.F. Fitzsimmons, A.M. Nock, and M.J. Wargo. 2013. Characterization of the GbdR regulon in Pseudomonas aeruginosa. *J Bacteriol*.
Hampel, K.J., A.E. LaBauve, J.A. Meadows, L.F. Fitzsimmons, A.M. Nock, and M.J. Wargo. 2014. Characterization of the GbdR regulon in Pseudomonas aeruginosa. *J Bacteriol*. 196:7-15.
Hanson, A.D., B. Rathinasabapathi, B. Chamberlin, and D.A. Gage. 1991. Comparative Physiological Evidence That Beta-Alanine Betaine and Choline-O-Sulfate Act as Compatible Osmolytes in Halophytic Limonium Species. *Plant Physiology*. 97:1199-1205.
Hanson, A.D., B. Rathinasabapathi, J. Rivoal, M. Burnet, M.O. Dillon, and D.A. Gage. 1994. Osmoprotective compounds in the Plumbaginaceae: a natural experiment in metabolic engineering of stress tolerance. *Proceedings of the National Academy of Sciences of the United States of America*. 91:306-310.
Hanukoglu, I. 2015. Proteopedia: Rossmann fold: A beta-alpha-beta fold at dinucleotide binding sites. *Biochem Mol Biol Educ*. 43:206-209.
Haraga, A., T.E. West, M.J. Brittnacher, S.J. Skerrett, and S.I. Miller. 2008. Burkholderia thailandensis as a Model System for the Study of the Virulence-Associated Type

III Secretion System of Burkholderia pseudomallei. *Infection and Immunity*. 76:5402-5411.
Hart, E., J. Yang, M. Tauschek, M. Kelly, M.J. Wakefield, G. Frankel, E.L. Hartland, and R.M. Robins-Browne. 2008. RegA, an AraC-like protein, is a global transcriptional regulator that controls virulence gene expression in Citrobacter rodentium. *Infect Immun*. 76:5247-5256.
Hashim, S., D.H. Kwon, A. Abdelal, and C.D. Lu. 2004. The arginine regulatory protein mediates repression by arginine of the operons encoding glutamate synthase and anabolic glutamate dehydrogenase in Pseudomonas aeruginosa. *J Bacteriol*. 186:3848-3854.
Henderson, E.J., H. Zalkin, and L.H. Hwang. 1970. The anthranilate synthetase-anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase aggregate. Catalytic and regulatory properties of aggregated and unaggregated forms of anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase. *The Journal of biological chemistry*. 245:1424-1431.
Horvath, M.M., and N.V. Grishin. 2001. The C-terminal domain of HPII catalase is a member of the type I glutamine amidotransferase superfamily. *Proteins-Structure Function and Genetics*. 42:230-236.
Hou, Y.B., and S.J. Lin. 2009. Distinct Gene Number-Genome Size Relationships for Eukaryotes and Non-Eukaryotes: Gene Content Estimation for Dinoflagellate Genomes. *Plos One*. 4.
Howe, C., A. Sampath, and M. Spotnitz. 1971. The pseudomallei group: a review. *The Journal of infectious diseases*. 124:598-606.
Hussey, C., B.A. Orsi, J. Scott, and B. Spencer. 1965. Mechanism of choline sulphate utilization in fungi. *Nature*. 207:632-634.
Ibarra, J.A., E. Perez-Rueda, L. Segovia, and J.L. Puente. 2008. The DNA-binding domain as a functional indicator: the case of the AraC/XylS family of transcription factors. *Genetica*. 133:65-76.
Inglis, T.J.J., P. Rigby, T.A. Robertson, N.S. Dutton, M. Henderson, and B.J. Chang. 2000. Interaction between Burkholderia pseudomallei and Acanthamoeba species results in coiling phagocytosis, endamebic bacterial survival, and escape. *Infection and Immunity*. 68:1681-1686.
Iturriaga, G., R. Suarez, and B. Nova-Franco. 2009. Trehalose Metabolism: From Osmoprotection to Signaling. *International Journal of Molecular Sciences*. 10:3793-3810.
Jahn, D., Y.C. Kim, Y. Ishino, M.W. Chen, and D. Soll. 1990. Purification and functional characterization of the Glu-tRNA(Gln) amidotransferase from Chlamydomonas reinhardtii. *The Journal of biological chemistry*. 265:8059-8064.
Jelesijevic, T., S.M. Zimmerman, S.B. Harvey, D.G. Mead, T.L. Shaffer, D.M. Estes, F. Michel, F.D. Quinn, R.J. Hogan, and E.R. Lafontaine. 2015. Use of the Common Marmoset to Study Burkholderia mallei Infection. *Plos One*. 10.
Johnson, D.S., A. Mortazavi, R.M. Myers, and B. Wold. 2007. Genome-wide mapping of in vivo protein-DNA interactions. *Science*. 316:1497-1502.
Jovcic, B., V. Venturi, L. Topisirovic, and M. Kojic. 2011. Inducible expression of choline sulfatase and its regulator BetR in Pseudomonas sp. ATCC19151. *Archives of microbiology*. 193:399-405.

Kaestli, M., G. Harrington, M. Mayo, M.D. Chatfield, I. Harrington, A. Hill, N. Munksgaard, K. Gibb, and B.J. Currie. 2015. What Drives the Occurrence of the Melioidosis Bacterium Burkholderia pseudomallei in Domestic Gardens? *PLoS neglected tropical diseases*. 9.

Kaestli, M., M. Schmid, M. Mayo, M. Rothballer, G. Harrington, L. Richardson, A. Hill, J. Hill, A. Tuanyok, P. Keim, A. Hartmann, and B.J. Currie. 2012. Out of the ground: aerial and exotic habitats of the melioidosis bacterium Burkholderia pseudomallei in grasses in Australia. *Environ Microbiol*. 14:2058-2070.

Kalashnikov, N.V. 1986. [Characteristics of the damage to bacteria by formaldehyde]. *Mol Gen Mikrobiol Virusol*:35-41.

Kang, Y., M.H. Norris, A.R. Barrett, B.A. Wilcox, and T.T. Hoang. 2009. Engineering of tellurite-resistant genetic tools for single-copy chromosomal analysis of Burkholderia spp. and characterization of the Burkholderia thailandensis betBA operon. *Applied and environmental microbiology*. 75:4015-4027.

Karam, J.D., and E.S. Miller. 2010. Bacteriophage T4 and its relatives. *Virol J*. 7:293.

Kespichayawattana, W., P. Intachote, P. Utaisincharoen, and S. Sirisinha. 2004. Virulent Burkholderia pseudomallei is more efficient than avirulent Burkholderia thailandensis in invasion of and adherence to cultured human epithelial cells. *Microbial pathogenesis*. 36:287-292.

Kets, E.P.W., E.A. Galinski, and J.A.M. Debont. 1994. Carnitine - a Novel Compatible Solute in Lactobacillus-Plantarum. *Archives of microbiology*. 162:243-248.

Kim, H.S., M.A. Schell, Y. Yu, R.L. Ulrich, S.H. Sarria, W.C. Nierman, and D. DeShazer. 2005. Bacterial genome adaptation to niches: divergence of the potential virulence genes in three Burkholderia species of different survival strategies. *BMC genomics*. 6:174.

Koeth, R.A., B.S. Levison, M.K. Culley, J.A. Buffa, Z.N. Wang, J.C. Gregory, E. Org, Y.P. Wu, L. Li, J.D. Smith, W.H.W. Tang, J.A. DiDonato, A.J. Lusis, and S.L. Hazen. 2014. gamma-Butyrobetaine Is a Proatherogenic Intermediate in Gut Microbial Metabolism of L-Carnitine to TMAO. *Cell Metab*. 20:799-812.

Kolodrubetz, D., and R. Schleif. 1981. Identification of araC protein and two-dimensional gels, its in vivo instability and normal level. *Journal of molecular biology*. 149:133-139.

Kortstee, G.J. 1970. The aerobic decomposition of choline by microorganisms. I. The ability of aerobic organisms, particularly coryneform bacteria, to utilize choline as the sole carbon and nitrogen source. *Arch Mikrobiol*. 71:235-244.

Krahn, J.M., J.H. Kim, M.R. Burns, R.J. Parry, H. Zalkin, and J.L. Smith. 1997. Coupled formation of an amidotransferase interdomain ammonia channel and a phosphoribosyltransferase active site. *Biochemistry*. 36:11061-11068.

Kunin, C.M., T.H. Hua, L.V. White, and M. Villarejo. 1992. Growth of Escherichia-Coli in Human Urine - Role of Salt Tolerance and Accumulation of Glycine Betaine. *Journal of Infectious Diseases*. 166:1311-1315.

LaBauve, A.E., and M.J. Wargo. 2012. Growth and laboratory maintenance of Pseudomonas aeruginosa. *Current protocols in microbiology*. Chapter 6:Unit 6E 1.

Lai, M.C., and R.P. Gunsalus. 1992. Glycine Betaine and Potassium-Ion Are the Major Compatible Solutes in the Extremely Halophilic Methanogen Methanohalophilus Strain Z7302. *Journal of Bacteriology*. 174:7474-7477.
Lai, M.C., K.R. Sowers, D.E. Robertson, M.F. Roberts, and R.P. Gunsalus. 1991. Distribution of Compatible Solutes in the Halophilic Methanogenic Archaebacteria. *Journal of Bacteriology*. 173:5352-5358.
Lamark, T., T.P. Rokenes, J. McDougall, and A.R. Strom. 1996. The complex bet promoters of Escherichia coli: regulation by oxygen (ArcA), choline (BetI), and osmotic stress. *J Bacteriol*. 178:1655-1662.
Land, M., L. Hauser, S.R. Jun, I. Nookaew, M.R. Leuze, T.H. Ahn, T. Karpinets, O. Lund, G. Kora, T. Wassenaar, S. Poudel, and D.W. Ussery. 2015. Insights from 20 years of bacterial genome sequencing. *Funct Integr Genomics*. 15:141-161.
Lang, K. 1933. Die rhodanbildung in tierkorper. *Biochem. Z*. 259:243-256.
Lau, S.K., S. Sridhar, C.C. Ho, W.N. Chow, K.C. Lee, C.W. Lam, K.Y. Yuen, and P.C. Woo. 2015. Laboratory diagnosis of melioidosis: past, present and future. *Exp Biol Med (Maywood)*. 240:742-751.
Lawrence, M., W. Huber, H. Pages, P. Aboyoun, M. Carlson, R. Gentleman, M.T. Morgan, and V.J. Carey. 2013. Software for computing and annotating genomic ranges. *PLoS computational biology*. 9:e1003118.
Lee, D.J., S.D. Minchin, and S.J. Busby. 2012. Activating transcription in bacteria. *Annual review of microbiology*. 66:125-152.
Lee, N., C. Francklyn, and E.P. Hamilton. 1987. Arabinose-induced binding of AraC protein to araI2 activates the araBAD operon promoter. *Proceedings of the National Academy of Sciences of the United States of America*. 84:8814-8818.
Lee, N., W. Gielow, R. Martin, E. Hamilton, and A. Fowler. 1986. The organization of the araBAD operon of Escherichia coli. *Gene*. 47:231-244.
Lee, N.L., W.O. Gielow, and R.G. Wallace. 1981. Mechanism of araC autoregulation and the domains of two overlapping promoters, Pc and PBAD, in the L-arabinose regulatory region of Escherichia coli. *Proceedings of the National Academy of Sciences of the United States of America*. 78:752-756.
Lee, Y.H., Y.H. Chen, X.Z. Ouyang, and Y.H. Gan. 2010. Identification of tomato plant as a novel host model for Burkholderia pseudomallei. *BMC microbiology*. 10.
Leitenberg, M., R.A. Zilinskas, and J.H. Kuhn. 2012. The Soviet biological weapons program : a history. Harvard University Press, Cambridge, Massachusetts. xvi, 921 pages pp.
Lertpatanasuwan, N., K. Sermsri, A. Petkaseam, S. Trakulsomboon, V. Thamlikitkul, and Y. Suputtamongkol. 1999. Arabinose-positive Burkholderia pseudomallei infection in humans: case report. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 28:927-928.
Li, H., and R. Durbin. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 25:1754-1760.
Li, Q.A., D.V. Mavrodi, L.S. Thomashow, M. Roessle, and W. Blankenfeldt. 2011. Ligand binding induces an ammonia channel in 2-amino-2-desoxyisochorismate (ADIC) synthase PhzE. *The Journal of biological chemistry*. 286:18213-18221.
Liao, Y., S. Chen, D. Wang, W. Zhang, S. Wang, J. Ding, Y. Wang, L. Cai, X. Ran, X. Wang, and H. Zhu. 2013. Structure of formaldehyde dehydrogenase from

Pseudomonas aeruginosa: the binary complex with the cofactor NAD+. *Acta crystallographica. Section F, Structural biology and crystallization communications*. 69:967-972.
Limmathurotsakul, D., N. Golding, D.A. Dance, J.P. Messina, D.M. Pigott, C.L. Moyes, D.B. Rolim, E. Bertherat, N.P. Day, S.J. Peacock, and S.I. Hay. 2016. Predicted global distribution of Burkholderia pseudomallei and burden of melioidosis. *Nat Microbiol*. 1:15008.
List, F., M. Bocola, M.C. Haeger, and R. Sterner. 2012. Constitutively active glutaminase variants provide insights into the activation mechanism of anthranilate synthase. *Biochemistry*. 51:2812-2818.
Lobell, R.B., and R.F. Schleif. 1991. AraC-DNA looping: orientation and distance-dependent loop breaking by the cyclic AMP receptor protein. *Journal of molecular biology*. 218:45-54.
Lopez, J., J. Copps, C. Wilhelmsen, R. Moore, J. Kubay, M. St-Jacques, S. Halayko, C. Kranendonk, S. Toback, D. DeShazer, D.L. Fritz, M. Tom, and D.E. Woods. 2003. Characterization of experimental equine glanders. *Microbes and infection / Institut Pasteur*. 5:1125-1131.
Losada, L., C.M. Ronning, D. DeShazer, D. Woods, N. Fedorova, H.S. Kim, S.A. Shabalina, T.R. Pearson, L. Brinkac, P. Tan, T. Nandi, J. Crabtree, J. Badger, S. Beckstrom-Sternberg, M. Saqib, S.E. Schutzer, P. Keim, and W.C. Nierman. 2010. Continuing Evolution of Burkholderia mallei Through Genome Reduction and Large-Scale Rearrangements. *Genome Biology and Evolution*. 2:102-116.
Lu, C.D., J.E. Houghton, and A.T. Abdelal. 1992. Characterization of the arginine repressor from Salmonella typhimurium and its interactions with the carAB operator. *Journal of molecular biology*. 225:11-24.
Lynch, K.H., P. Stothard, and J.J. Dennis. 2010. Genomic analysis and relatedness of P2-like phages of the Burkholderia cepacia complex. *BMC genomics*. 11:599.
Maas, W.K. 1994. The arginine repressor of Escherichia coli. *Microbiol Rev*. 58:631-640.
Mahenthiralingam, E., T.A. Urban, and J.B. Goldberg. 2005. The multifarious, multireplicon Burkholderia cepacia complex. *Nature reviews. Microbiology*. 3:144-156.
Malek, A.A., C. Chen, M.J. Wargo, G.A. Beattie, and D.A. Hogan. 2011. Roles of three transporters, CbcXWV, BetT1, and BetT3, in Pseudomonas aeruginosa choline uptake for catabolism. *J Bacteriol*. 193:3033-3041.
Mansouri, A.L., L.N. Grese, E.L. Rowe, J.C. Pino, S.C. Chennubhotla, A. Ramanathan, H.M. O'Neill, V. Berthelier, and C.B. Stanley. 2016. Folding propensity of intrinsically disordered proteins by osmotic stress. *Mol Biosyst*. 12:3695-3701.
Martin, R.G., K.W. Jair, R.E. Wolf, Jr., and J.L. Rosner. 1996. Autoactivation of the marRAB multiple antibiotic resistance operon by the MarA transcriptional activator in Escherichia coli. *J Bacteriol*. 178:2216-2223.
Martin, R.G., and J.L. Rosner. 2001. The AraC transcriptional activators. *Curr Opin Microbiol*. 4:132-137.
Marx, C.J., J.A. Miller, L. Chistoserdova, and M.E. Lidstrom. 2004. Multiple formaldehyde oxidation/detoxification pathways in Burkholderia fungorum LB400. *J Bacteriol*. 186:2173-2178.

Marx, C.J., S.J. Van Dien, and M.E. Lidstrom. 2005. Flux analysis uncovers key role of functional redundancy in formaldehyde metabolism. *PLoS Biol*. 3:e16.
Mason, R.P., J.K.M. Sanders, A. Crawford, and B.K. Hunter. 1986. Formaldehyde Metabolism by Escherichia-Coli - Detection by Invivo C-13 Nmr-Spectroscopy of S-(Hydroxymethyl)Glutathione as a Transient Intracellular Intermediate. *Biochemistry*. 25:4504-4507.
Massiere, F., and M.A. Badet-Denisot. 1998. The mechanism of glutamine-dependent amidotransferases. *Cell Mol Life Sci*. 54:205-222.
Meadows, J.A., and M.J. Wargo. 2013. Characterization of Pseudomonas aeruginosa growth on O-acylcarnitines and identification of a short-chain acylcarnitine hydrolase. *Applied and environmental microbiology*. 79:3355-3363.
Meadows, J.A., and M.J. Wargo. 2015. Carnitine in bacterial physiology and metabolism. *Microbiology*. 161:1161-1174.
Meadows, J.A., and M.J. Wargo. 2018. Transcriptional Regulation of Carnitine Catabolism in Pseudomonas aeruginosa by CdhR. *mSphere*. 3.
Meskys, R., R.J. Harris, V. Casaite, J. Basran, and N.S. Scrutton. 2001. Organization of the genes involved in dimethylglycine and sarcosine degradation in Arthrobacter spp.: implications for glycine betaine catabolism. *European journal of biochemistry / FEBS*. 268:3390-3398.
Miller, K.J., and J.M. Wood. 1996. Osmoadaptation by rhizosphere bacteria. *Annual review of microbiology*. 50:101-136.
Mitrophanov, A.Y., and E.A. Groisman. 2008. Signal integration in bacterial two-component regulatory systems. *Genes & development*. 22:2601-2611.
Mitsui, R., M. Hirota, T. Tsuno, and M. Tanaka. 2010. Purification and characterization of vanillin dehydrogenases from alkaliphile Micrococcus sp. TA1 and neutrophile Burkholderia cepacia TM1. *FEMS Microbiol Lett*. 303:41-47.
Mitsui, R., Y. Kusano, H. Yurimoto, Y. Sakai, N. Kato, and M. Tanaka. 2003. Formaldehyde fixation contributes to detoxification for growth of a nonmethylotroph, Burkholderia cepacia TM1, on vanillic acid. *Applied and environmental microbiology*. 69:6128-6132.
Moore, R.A., S. Reckseidler-Zenteno, H. Kim, W. Nierman, Y. Yu, A. Tuanyok, J. Warawa, D. DeShazer, and D.E. Woods. 2004. Contribution of gene loss to the pathogenic evolution of Burkholderia pseudomallei and Burkholderia mallei. *Infect Immun*. 72:4172-4187.
Mouilleron, S., and B. Golinelli-Pimpaneau. 2007. Conformational changes in ammonia-channeling glutamine amidotransferases. *Current opinion in structural biology*. 17:653-664.
Mujacic, M., and F. Baneyx. 2007. Chaperone Hsp31 contributes to acid resistance in stationary-phase Escherichia coli. *Applied and environmental microbiology*. 73:1014-1018.
Murphy, J., J. Mahony, S. Ainsworth, A. Nauta, and D. van Sinderen. 2013. Bacteriophage orphan DNA methyltransferases: insights from their bacterial origin, function, and occurrence. *Applied and environmental microbiology*. 79:7547-7555.

Nau-Wagner, G., J. Boch, J.A. Le Good, and E. Bremer. 1999. High-affinity transport of choline-O-sulfate and its use as a compatible solute in Bacillus subtilis. *Applied and environmental microbiology*. 65:560-568.

Neidhardt, F.C., P.L. Bloch, and D.F. Smith. 1974. Culture medium for enterobacteria. *J Bacteriol*. 119:736-747.

Ngamdee, W., S. Tandhavanant, C. Wikraiphat, O. Reamtong, V. Wuthiekanun, J. Salje, D.A. Low, S.J. Peacock, and N. Chantratita. 2015. Competition between Burkholderia pseudomallei and B. thailandensis. *BMC microbiology*. 15.

Nicolaus, B., V. Lanzotti, A. Trincone, M. Derosa, W.D. Grant, and A. Gambacorta. 1989. Glycine Betaine and Polar Lipid-Composition in Halophilic Archaebacteria in Response to Growth in Different Salt Concentrations. *Fems Microbiology Letters*. 59:157-160.

Nierman, W.C., D. DeShazer, H.S. Kim, H. Tettelin, K.E. Nelson, T. Feldblyum, R.L. Ulrich, C.M. Ronning, L.M. Brinkac, S.C. Daugherty, T.D. Davidsen, R.T. Deboy, G. Dimitrov, R.J. Dodson, A.S. Durkin, M.L. Gwinn, D.H. Haft, H. Khouri, J.F. Kolonay, R. Madupu, Y. Mohammoud, W.C. Nelson, D. Radune, C.M. Romero, S. Sarria, J. Selengut, C. Shamblin, S.A. Sullivan, O. White, Y. Yu, N. Zafar, L.W. Zhou, and C.M. Fraser. 2004. Structural flexibility in the Burkholderia mallei genome. *Proceedings of the National Academy of Sciences of the United States of America*. 101:14246-14251.

Nikolakakis, K., S. Amber, J.S. Wilbur, E.J. Diner, S.K. Aoki, S.J. Poole, A. Tuanyok, P.S. Keim, S. Peacock, C.S. Hayes, and D.A. Low. 2012. The toxin/immunity network of Burkholderia pseudomallei contact-dependent growth inhibition (CDI) systems. *Molecular microbiology*. 84:516-529.

Nishijyo, T., D. Haas, and Y. Itoh. 2001. The CbrA-CbrB two-component regulatory system controls the utilization of multiple carbon and nitrogen sources in Pseudomonas aeruginosa. *Molecular microbiology*. 40:917-931.

Nishijyo, T., S.M. Park, C.D. Lu, Y. Itoh, and A.T. Abdelal. 1998. Molecular characterization and regulation of an operon encoding a system for transport of arginine and ornithine and the ArgR regulatory protein in Pseudomonas aeruginosa. *J Bacteriol*. 180:5559-5566.

Nissen, P. 1968. Choline sulfate permease. Transfer of information from bacteria to higher plants? *Biochem Biophys Res Commun*. 32:696-703.

Nissen, P., and A.A. Benson. 1961. Choline Sulfate in Higher Plants. *Science*. 134:1759.

Nock, A.M., and M.J. Wargo. 2016. Choline Catabolism in Burkholderia thailandensis Is Regulated by Multiple Glutamine Amidotransferase 1-Containing AraC Family Transcriptional Regulators. *J Bacteriol*. 198:2503-2514.

Noinarin, P., P. Chareonsudjai, P. Wangsomnuk, S. Wongratanacheewin, and S. Chareonsudjai. 2016. Environmental Free-Living Amoebae Isolated from Soil in Khon Kaen, Thailand, Antagonize Burkholderia pseudomallei. *PLoS One*. 11:e0167355.

Nunoshiba, T., E. Hidalgo, Z. Li, and B. Demple. 1993. Negative autoregulation by the Escherichia coli SoxS protein: a dampening mechanism for the soxRS redox stress response. *J Bacteriol*. 175:7492-7494.

Nyunoya, H., and C.J. Lusty. 1984. Sequence of the small subunit of yeast carbamyl phosphate synthetase and identification of its catalytic domain. *The Journal of biological chemistry*. 259:9790-9798.
O'Sullivan, L.A., and E. Mahenthiralingam. 2005. Biotechnological potential within the genus Burkholderia. *Letters in Applied Microbiology*. 41:8-11.
O'Toole, G.A. 2011. Microtiter dish biofilm formation assay. *J Vis Exp*.
Park, S., L.T. Smith, and G.M. Smith. 1995. Role of Glycine Betaine and Related Osmolytes in Osmotic-Stress Adaptation in Yersinia-Enterocolitica Atcc-9610. *Applied and environmental microbiology*. 61:4378-4381.
Park, S.M., C.D. Lu, and A.T. Abdelal. 1997a. Cloning and characterization of argR, a gene that participates in regulation of arginine biosynthesis and catabolism in Pseudomonas aeruginosa PAO1. *J Bacteriol*. 179:5300-5308.
Park, S.M., C.D. Lu, and A.T. Abdelal. 1997b. Purification and characterization of an arginine regulatory protein, ArgR, from Pseudomonas aeruginosa and its interactions with the control regions for the car, argF, and aru operons. *J Bacteriol*. 179:5309-5317.
Perumal Samy, R., B.G. Stiles, G. Sethi, and L.H.K. Lim. 2017. Melioidosis: Clinical impact and public health threat in the tropics. *PLoS neglected tropical diseases*. 11:e0004738.
Pettersen, E.F., T.D. Goddard, C.C. Huang, G.S. Couch, D.M. Greenblatt, E.C. Meng, and T.E. Ferrin. 2004. UCSF chimera - A visualization system for exploratory research and analysis. *J Comput Chem*. 25:1605-1612.
Pilatova, M., and M.S. Dionne. 2012. Burkholderia thailandensis Is Virulent in Drosophila melanogaster. *Plos One*. 7.
Rabeh, W.M., and P.F. Cook. 2004. Structure and mechanism of O-acetylserine sulfhydrylase. *The Journal of biological chemistry*. 279:26803-26806.
Raushel, F.M., J.B. Thoden, and H.M. Holden. 2003. Enzymes with molecular tunnels. *Acc Chem Res*. 36:539-548.
Reckseidler-Zenteno, S.L., R. DeVinney, and D.E. Woods. 2005. The capsular polysaccharide of Burkholderia pseudomallei contributes to survival in serum by reducing complement factor C3b deposition. *Infect Immun*. 73:1106-1115.
Reckseidler-Zenteno, S.L., R. Moore, and D.E. Woods. 2009. Genetics and function of the capsules of Burkholderia pseudomallei and their potential as therapeutic targets. *Mini reviews in medicinal chemistry*. 9:265-271.
Reckseidler-Zenteno, S.L., D.F. Viteri, R. Moore, E. Wong, A. Tuanyok, and D.E. Woods. 2010. Characterization of the type III capsular polysaccharide produced by Burkholderia pseudomallei. *Journal of medical microbiology*. 59:1403-1414.
Reckseidler, S.L., D. DeShazer, P.A. Sokol, and D.E. Woods. 2001. Detection of bacterial virulence genes by subtractive hybridization: identification of capsular polysaccharide of Burkholderia pseudomallei as a major virulence determinant. *Infect Immun*. 69:34-44.
Reddy, J.K., and T. Hashimoto. 2001. Peroxisomal beta-oxidation and peroxisome proliferator-activated receptor alpha: an adaptive metabolic system. *Annu Rev Nutr*. 21:193-230.
Rhodes, D., and A.D. Hanson. 1993. Quaternary Ammonium and Tertiary Sulfonium Compounds in Higher-Plants. *Annu Rev Plant Phys*. 44:357-384.

Rhodes, K.A., and H.P. Schweizer. 2016. Antibiotic resistance in Burkholderia species. *Drug Resist Updat*. 28:82-90.

Rkenes, T.P., T. Lamark, and A.R. Strom. 1996. DNA-binding properties of the BetI repressor protein of Escherichia coli: the inducer choline stimulates BetI-DNA complex formation. *J Bacteriol*. 178:1663-1670.

Robert, H., C. Le Marrec, C. Blanco, and M. Jebbar. 2000. Glycine betaine, carnitine, and choline enhance salinity tolerance and prevent the accumulation of sodium to a level inhibiting growth of Tetragenococcus halophila. *Applied and environmental microbiology*. 66:509-517.

Roitman, J.N., N.E. Mahoney, W.J. Janisiewicz, and M. Benson. 1990. A New Chlorinated Phenylpyrrole Antibiotic Produced by the Antifungal Bacterium Pseudomonas-Cepacia. *Journal of agricultural and food chemistry*. 38:538-541.

Rolim, D.B., M.F. Rocha, R.S. Brilhante, R.A. Cordeiro, N.P. Leitao, Jr., T.J. Inglis, and J.J. Sidrim. 2009. Environmental isolates of Burkholderia pseudomallei in Ceara State, northeastern Brazil. *Applied and environmental microbiology*. 75:1215-1218.

Ronning, C.M., L. Losada, L. Brinkac, J. Inman, R.L. Ulrich, M. Schell, W.C. Nierman, and D. Deshazer. 2010. Genetic and phenotypic diversity in Burkholderia: contributions by prophage and phage-like elements. *BMC microbiology*. 10:202.

Ruiz, R., J.L. Ramos, and S.M. Egan. 2001. Interactions of the XylS regulators with the C-terminal domain of the RNA polymerase alpha subunit influence the expression level from the cognate Pm promoter. *FEBS Lett*. 491:207-211.

Sanchez, D.G., E.D. Primo, M.T. Damiani, and A.T. Lisa. 2017. Pseudomonas aeruginosa gbdR gene is transcribed from a sigma54-dependent promoter under the control of NtrC/CbrB, IHF and BetI. *Microbiology*. 163:1343-1354.

Santiago, A.E., M.B. Yan, T.H. Hazen, B. Sauder, M. Meza-Segura, D.A. Rasko, M.M. Kendall, F. Ruiz-Perez, and J.P. Nataro. 2017. The AraC Negative Regulator family modulates the activity of histone-like proteins in pathogenic bacteria. *PLoS pathogens*. 13:e1006545.

Santiago, A.E., M.B. Yan, M. Tran, N. Wright, D.H. Luzader, M.M. Kendall, F. Ruiz-Perez, and J.P. Nataro. 2016. A large family of anti-activators accompanying XylS/AraC family regulatory proteins. *Molecular microbiology*. 101:314-332.

Saviola, B., R. Seabold, and R.F. Schleif. 1998. Arm-domain interactions in AraC. *Journal of molecular biology*. 278:539-548.

Sawana, A., M. Adeolu, and R.S. Gupta. 2014. Molecular signatures and phylogenomic analysis of the genus Burkholderia: proposal for division of this genus into the emended genus Burkholderia containing pathogenic organisms and a new genus Paraburkholderia gen. nov. harboring environmental species. *Frontiers in genetics*. 5:429.

Schaper, S., W. Steinchen, E. Krol, F. Altegoer, D. Skotnicka, L. Sogaard-Andersen, G. Bange, and A. Becker. 2017. AraC-like transcriptional activator CuxR binds c-di-GMP by a PilZ-like mechanism to regulate extracellular polysaccharide production. *Proceedings of the National Academy of Sciences of the United States of America*. 114:E4822-E4831.

Schleif, R. 2003. AraC protein: a love-hate relationship. *BioEssays : news and reviews in molecular, cellular and developmental biology*. 25:274-282.

Schleif, R. 2010. AraC protein, regulation of the l-arabinose operon in Escherichia coli, and the light switch mechanism of AraC action. *FEMS microbiology reviews*. 34:779-796.
Schully, K.L., M.G. Bell, J.M. Ward, and A.M. Keane-Myers. 2014. Oropharyngeal aspiration of Burkholderia mallei and Burkholderia pseudomallei in BALB/c mice. *PLoS One*. 9:e115066.
Schwartz, H.F., and S.K. Mohan. 2008. Compendium of onion and garlic diseases and pests. American Phytopathological Society, St. Paul, MN. 127 p. pp.
Schwarz, S., T.E. West, F. Boyer, W.C. Chiang, M.A. Carl, R.D. Hood, L. Rohmer, T. Tolker-Nielsen, S.J. Skerrett, and J.D. Mougous. 2010. Burkholderia Type VI Secretion Systems Have Distinct Roles in Eukaryotic and Bacterial Cell Interactions. *PLoS pathogens*. 6.
Shanks, R.M., D.E. Kadouri, D.P. MacEachran, and G.A. O'Toole. 2009. New yeast recombineering tools for bacteria. *Plasmid*. 62:88-97.
Shortridge, V.D., A. Lazdunski, and M.L. Vasil. 1992a. Osmoprotectants and Phosphate Regulate Expression of Phospholipase-C in Pseudomonas-Aeruginosa. *Molecular microbiology*. 6:863-871.
Shortridge, V.D., A. Lazdunski, and M.L. Vasil. 1992b. Osmoprotectants and phosphate regulate expression of phospholipase C in Pseudomonas aeruginosa. *Molecular microbiology*. 6:863-871.
Silver, S., and American Society for Microbiology. 1990. Pseudomonas : biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C. xxiv, 423 p. pp.
Sleator, R.D., and C. Hill. 2002. Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. *FEMS microbiology reviews*. 26:49-71.
Smith, L.T., J.A. Pocard, T. Bernard, and D. Le Rudulier. 1988. Osmotic control of glycine betaine biosynthesis and degradation in Rhizobium meliloti. *J Bacteriol*. 170:3142-3149.
Smith, M.D., B.J. Angus, V. Wuthiekanun, and N.J. White. 1997. Arabinose assimilation defines a nonvirulent biotype of Burkholderia pseudomallei. *Infect Immun*. 65:4319-4321.
Solomonson, L.P. 1981. Cyanide as a metabolic inhibitor. Academic Press, London.
Spencer, B., and T. Harada. 1960. The role of choline sulphate in the sulphur metabolism of fungi. *The Biochemical journal*. 77:305-315.
Steiber, A., J. Kerner, and C.L. Hoppel. 2004. Carnitine: a nutritional, biosynthetic, and functional perspective. *Mol Aspects Med*. 25:455-473.
Sun, Z.X., Y. Kang, M.H. Norris, R.M. Troyer, M.S. Son, H.P. Schweizer, S.W. Dow, and T.T. Hoang. 2014. Blocking Phosphatidylcholine Utilization in Pseudomonas aeruginosa, via Mutagenesis of Fatty Acid, Glycerol and Choline Degradation Pathways, Confirms the Importance of This Nutrient Source In Vivo. *Plos One*. 9.
Szklarczyk, D., A. Franceschini, S. Wyder, K. Forslund, D. Heller, J. Huerta-Cepas, M. Simonovic, A. Roth, A. Santos, K.P. Tsafou, M. Kuhn, P. Bork, L.J. Jensen, and C. von Mering. 2015. STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic acids research*. 43:D447-452.
Taliaferro, L.P., E.F. Keen, N. Sanchez-Alberola, and R.E. Wolf. 2012. Transcription Activation by Escherichia coli Rob at Class II Promoters: Protein-Protein

Interactions between Rob's N-Terminal Domain and the sigma(70) Subunit of RNA Polymerase. *Journal of molecular biology*. 419:139-157.
Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular biology and evolution*. 30:2725-2729.
Tesmer, J.J., T.J. Klem, M.L. Deras, V.J. Davisson, and J.L. Smith. 1996. The crystal structure of GMP synthetase reveals a novel catalytic triad and is a structural paradigm for two enzyme families. *Nat Struct Biol*. 3:74-86.
Thoden, J.B., F.M. Raushel, M.M. Benning, I. Rayment, and H.M. Holden. 1999. The structure of carbamoyl phosphate synthetase determined to 2.1 A resolution. *Acta crystallographica*. *Section D, Biological crystallography*. 55:8-24.
Thomas, A.D., J. Forbesfaulkner, and M. Parker. 1979. Isolation of Pseudomonas-Pseudomallei from Clay Layers at Defined Depths. *Am J Epidemiol*. 110:515-521.
Thongdee, M., L.A. Gallagher, M. Schell, T. Dharakul, S. Songsivilai, and C. Manoil. 2008. Targeted mutagenesis of Burkholderia thailandensis and Burkholderia pseudomallei through natural transformation of PCR fragments. *Applied and environmental microbiology*. 74:2985-2989.
Tocheva, E.I., D.R. Ortega, and G.J. Jensen. 2016. Sporulation, bacterial cell envelopes and the origin of life. *Nature reviews*. *Microbiology*. 14:535-542.
Tomich, M., and C.D. Mohr. 2003. Adherence and autoaggregation phenotypes of a Burkholderia cenocepacia cable pilus mutant. *FEMS Microbiol Lett*. 228:287-297.
Trakulsomboon, S., V. Vuddhakul, P. Tharavichitkul, N. Na-Gnam, Y. Suputtamongkol, and V. Thamlikitkul. 1999. Epidemiology of arabinose assimilation in burkholderia pseudomallei isolated from patients and soil in Thailand. *Southeast Asian J Trop Med Public Health*. 30:756-759.
Tseng, B.S., C.D. Majerczyk, D. Passos da Silva, J.R. Chandler, E.P. Greenberg, and M.R. Parsek. 2016. Quorum Sensing Influences Burkholderia thailandensis Biofilm Development and Matrix Production. *J Bacteriol*. 198:2643-2650.
Ulrich, L.E., E.V. Koonin, and I.B. Zhulin. 2005. One-component systems dominate signal transduction in prokaryotes. *Trends in microbiology*. 13:52-56.
Uotila, L., and M. Koivusalo. 1974a. Formaldehyde dehydrogenase from human liver. Purification, properties, and evidence for the formation of glutathione thiol esters by the enzyme. *The Journal of biological chemistry*. 249:7653-7663.
Uotila, L., and M. Koivusalo. 1974b. Purification and properties of S-formylglutathione hydrolase from human liver. *The Journal of biological chemistry*. 249:7664-7672.
Vakulskas, C.A., E.D. Brutinel, and T.L. Yahr. 2010. ExsA recruits RNA polymerase to an extended -10 promoter by contacting region 4.2 of sigma-70. *J Bacteriol*. 192:3597-3607.
van Meer, G., D.R. Voelker, and G.W. Feigenson. 2008. Membrane lipids: where they are and how they behave. *Nat Rev Mol Cell Bio*. 9:112-124.
Van Zandt, K.E., M.T. Greer, and H.C. Gelhaus. 2013. Glanders: an overview of infection in humans. *Orphanet J Rare Dis*. 8:131.
Velasco-Garcia, R., M.A. Villalobos, M.A. Ramirez-Romero, C. Mujica-Jimenez, G. Iturriaga, and R.A. Munoz-Clares. 2006. Betaine aldehyde dehydrogenase from Pseudomonas aeruginosa: cloning, over-expression in Escherichia coli, and regulation by choline and salt. *Archives of microbiology*. 185:14-22.

Vial, L., F. Lepine, S. Milot, M.C. Groleau, V. Dekimpe, D.E. Woods, and E. Deziel. 2008. Burkholderia pseudomallei, B. thailandensis, and B. ambifaria produce 4-hydroxy-2-alkylquinoline analogues with a methyl group at the 3 position that is required for quorum-sensing regulation. *Journal of Bacteriology*. 190:5339-5352.

Wagner, M.A., and M.S. Jorns. 2000. Monomeric sarcosine oxidase: 2. Kinetic studies with sarcosine, alternate substrates, and a substrate analogue. *Biochemistry*. 39:8825-8829.

Wagner, M.A., P. Trickey, Z.W. Chen, F.S. Mathews, and M.S. Jorns. 2000. Monomeric sarcosine oxidase: 1. Flavin reactivity and active site binding determinants. *Biochemistry*. 39:8813-8824.

Wanders, R.J., and H.R. Waterham. 2006. Biochemistry of mammalian peroxisomes revisited. *Annu Rev Biochem*. 75:295-332.

Wang, X.S., A.E. Roitberg, and N.G. Richards. 2009. Computational studies of ammonia channel function in glutamine 5'-phosphoribosylpyrophosphate amidotransferase. *Biochemistry*. 48:12272-12282.

Wang, Z., E. Klipfell, B.J. Bennett, R. Koeth, B.S. Levison, B. Dugar, A.E. Feldstein, E.B. Britt, X. Fu, Y.M. Chung, Y. Wu, P. Schauer, J.D. Smith, H. Allayee, W.H. Tang, J.A. DiDonato, A.J. Lusis, and S.L. Hazen. 2011. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature*. 472:57-63.

Warawa, J.M., D. Long, R. Rosenke, D. Gardner, and F.C. Gherardini. 2009. Role for the Burkholderia pseudomallei capsular polysaccharide encoded by the wcb operon in acute disseminated melioidosis. *Infect Immun*. 77:5252-5261.

Wargo, M.J. 2013a. Choline catabolism to glycine betaine contributes to Pseudomonas aeruginosa survival during murine lung infection. *PLoS One*. 8:e56850.

Wargo, M.J. 2013b. Homeostasis and catabolism of choline and glycine betaine: lessons from Pseudomonas aeruginosa. *Applied and environmental microbiology*. 79:2112-2120.

Wargo, M.J., M.J. Gross, S. Rajamani, J.L. Allard, L.K. Lundblad, G.B. Allen, M.L. Vasil, L.W. Leclair, and D.A. Hogan. 2011. Hemolytic phospholipase C inhibition protects lung function during Pseudomonas aeruginosa infection. *American journal of respiratory and critical care medicine*. 184:345-354.

Wargo, M.J., T.C. Ho, M.J. Gross, L.A. Whittaker, and D.A. Hogan. 2009. GbdR regulates Pseudomonas aeruginosa plcH and pchP transcription in response to choline catabolites. *Infect Immun*. 77:1103-1111.

Wargo, M.J., and D.A. Hogan. 2009. Identification of genes required for Pseudomonas aeruginosa carnitine catabolism. *Microbiology*. 155:2411-2419.

Wargo, M.J., B.S. Szwergold, and D.A. Hogan. 2008. Identification of two gene clusters and a transcriptional regulator required for Pseudomonas aeruginosa glycine betaine catabolism. *J Bacteriol*. 190:2690-2699.

Wattam, A.R., D. Abraham, O. Dalay, T.L. Disz, T. Driscoll, J.L. Gabbard, J.J. Gillespie, R. Gough, D. Hix, R. Kenyon, D. Machi, C. Mao, E.K. Nordberg, R. Olson, R. Overbeek, G.D. Pusch, M. Shukla, J. Schulman, R.L. Stevens, D.E. Sullivan, V. Vonstein, A. Warren, R. Will, M.J. Wilson, H.S. Yoo, C. Zhang, Y. Zhang, and B.W. Sobral. 2014. PATRIC, the bacterial bioinformatics database and analysis resource. *Nucleic acids research*. 42:D581-591.

Weldon, J.E., and R.F. Schleif. 2006. Specific interactions by the N-terminal arm inhibit self-association of the AraC dimerization domain. *Protein science : a publication of the Protein Society*. 15:2828-2835.
Weng, M.L., and H. Zalkin. 1987. Structural role for a conserved region in the CTP synthetase glutamine amide transfer domain. *J Bacteriol*. 169:3023-3028.
Wheelis, M. 1998. First shots fired in biological warfare. *Nature*. 395:213.
Wheelis, M., L. Rózsa, and M. Dando. 2006. Deadly cultures : biological weapons since 1945. Harvard University Press, Cambridge, Mass. xi, 479 p. pp.
White, R.F., L. Kaplan, and J. Birnbaum. 1973. Betaine-homocysteine transmethylase in Pseudomonas denitrificans, a vitamin B 12 overproducer. *J Bacteriol*. 113:218-223.
Whiteley, L., T. Meffert, M. Haug, C. Weidenmaier, V. Hopf, K. Bitschar, B. Schittek, C. Kohler, I. Steinmetz, T.E. West, and S. Schwarz. 2017. Entry, intracellular survival and multinucleated giant cell-forming activity of Burkholderia pseudomallei in human primary phagocytic and non-phagocytic cells. *Infect Immun*.
Whitmore, A.K., CS. 1912. An account of the discovery of a hitherto undescribed infectious disease among the population of Rangoon. *Ind Med Gaz*. . 46:262–267.
Wilcox, G., and P. Meuris. 1976. Stabilization and size of araC protein. *Molecular & general genetics : MGG*. 145:97-100.
Wildermuth, M.C., J. Dewdney, G. Wu, and F.M. Ausubel. 2001. Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature*. 414:562-565.
Willsey, G.G., and M.J. Wargo. 2015. Sarcosine catabolism in Pseudomonas aeruginosa is transcriptionally regulated by SouR. *J Bacteriol*.
Winsor, G.L., E.J. Griffiths, R. Lo, B.K. Dhillon, J.A. Shay, and F.S. Brinkman. 2016. Enhanced annotations and features for comparing thousands of Pseudomonas genomes in the Pseudomonas genome database. *Nucleic acids research*. 44:D646-653.
Winsor, G.L., B. Khaira, T. Van Rossum, R. Lo, M.D. Whiteside, and F.S. Brinkman. 2008. The Burkholderia Genome Database: facilitating flexible queries and comparative analyses. *Bioinformatics*. 24:2803-2804.
Withatanung, P., N. Chantratita, V. Muangsombut, N. Saiprom, G. Lertmemongkolchai, J. Klumpp, M.R. Clokie, E.E. Galyov, and S. Korbsrisate. 2016. Analyses of the Distribution Patterns of Burkholderia pseudomallei and Associated Phages in Soil Samples in Thailand Suggest That Phage Presence Reduces the Frequency of Bacterial Isolation. *PLoS neglected tropical diseases*. 10:e0005005.
Wood, J.M. 1988. Proline Porters Effect the Utilization of Proline as Nutrient or Osmoprotectant for Bacteria. *J Membrane Biol*. 106:183-202.
Wood, J.M. 2011. Bacterial osmoregulation: a paradigm for the study of cellular homeostasis. *Annual review of microbiology*. 65:215-238.
Woods, D.E., J.A. Jeddeloh, D.L. Fritz, and D. DeShazer. 2002. Burkholderia thailandensis E125 harbors a temperate bacteriophage specific for Burkholderia mallei. *J Bacteriol*. 184:4003-4017.
Wu, M., and R. Schleif. 2001. Strengthened arm-dimerization domain interactions in AraC. *The Journal of biological chemistry*. 276:2562-2564.

Wuthiekanun, V., M.D. Smith, D.A. Dance, A.L. Walsh, T.L. Pitt, and N.J. White. 1996. Biochemical characteristics of clinical and environmental isolates of Burkholderia pseudomallei. *Journal of medical microbiology*. 45:408-412.

Yabuuchi, E., Y. Kosako, H. Oyaizu, I. Yano, H. Hotta, Y. Hashimoto, T. Ezaki, and M. Arakawa. 1992. Proposal of Burkholderia gen. nov. and transfer of seven species of the genus Pseudomonas homology group II to the new genus, with the type species Burkholderia cepacia (Palleroni and Holmes 1981) comb. nov. *Microbiol Immunol*. 36:1251-1275.

Yang, J., M. Tauschek, and R.M. Robins-Browne. 2011. Control of bacterial virulence by AraC-like regulators that respond to chemical signals. *Trends in microbiology*. 19:128-135.

Yu, Y.T., H.S. Kim, H.H. Chua, C.H. Lin, S.H. Sim, D.X. Lin, A. Derr, R. Engels, D. DeShazer, B. Birren, W.C. Nierman, and P. Tan. 2006. Genomic patterns of pathogen evolution revealed by comparison of Burkholderia pseudomallei, the causative agent of melioidosis, to avirulent Burkholderia thailandensis. *BMC microbiology*. 6.

Zachowski, A. 1993. Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. *The Biochemical journal*. 294 (Pt 1):1-14.

Zalkin, H. 1993. The amidotransferases. *Adv Enzymol Relat Areas Mol Biol*. 66:203-309.

Appendix A - Biofilm formation in *Burkholderia thailandensis*.

A.1 Introduction.

Our previous investigation into the GATRs GbdR1, GbdR2, and SouR included an RNAseq, which included a tantalizing change in expression of yet another GATR and the adjacent operon (1). This GATR, *I1350*, exhibited changes in expression in the same range as the adjacent operon, which it may be a part of and we hypothesized that it might regulate. This particular operon is one of the major capsule operons in *B. thailandensis*, and the potential for a GATR to impact capsule expression and a possible connection with other GATRs piqued our curiosity.

B. pseudomallei possesses four major capsular polysaccharide gene clusters (CPS I-IV), of which it shares three with *B. thailandensis*. Notably missing from *B. thailandensis* but present in *B. mallei*, is CPS-I. CPS-I is considered an important virulence determinant for both *B. pseudomallei* and *B. mallei* because it reduces the efficiency of opsonization in the blood stream, thus reducing phagocytosis (2-4). Expression of CPS-III is increased in water, and may be associated with the ability of *B. pseudomallei* and *B. mallei* to persist in pure water for extended durations (5). CPS-II and CPS-IV (which is adjacent to *I1350* and the focus of our interest here) have no obvious effect on virulence and their roles are currently unknown (6). In addition to these, there are several smaller gene clusters relating to exopolysaccharide production, modification, and transport, some of which have been shown to correspond to biofilm production in *B. pseudomallei* (7). Thus far, current studies of the major CPS and potential link to biofilm

formation are currently inconclusive since they may provide some redundant functionality (8).

The perturbation of genes that affect the outer-most layer of the cell and consequently biofilm formation was an attractive thread to pull. Like many other bacteria, *B. thailandensis* and its relatives are known to form biofilms that contribute to resistance against drugs, desiccation, and predation (9). Therefore, we performed a series of experiments to examine potential morphological and biofilm phenotypes in our deletion strains.

A.2 Methods.

Construction of strains. The method of construction for these strains has been previously published (1), or described elsewhere in this thesis. Briefly, as per Thongdee et al. (10), primers were designed to amplify regions upstream and downstream of the region targeted for deletion. In tandem, a trimethoprim resistance marker enclosed by FLP recombinase sites was amplified with twenty base pair regions on the ends with homology to the amplified upstream and downstream fragments. Splice overlap extension PCR was used to combine these fragments. Wild type *B. thailandensis* was grown overnight in DM media to induce natural competence, and were pelleted and combined with the splice overlap extension amplified product. Homologous recombination resulted in the generation of deletion strains that were selected on LB plates containing 100 μg/mL trimethoprim. The trimethoprim resistance marker was removed by transforming

the trimethoprim resistant deletion strains with pFLPe2 by electroporation. pFLPe2 is a plasmid expressing FLP recombinase under the control of a rhamnose-inducible promoter as described in Choi et al. (11). After selecting transformants on LSLB plates with 0.2% rhamnose and 250 µg/mL zeocin incubated at 30°C, and subculturing them a second time on the same type of plate, they were struck for isolation on standard LB plates and grown at 42°C overnight in order to cure pFLPe2, which possesses a temperature sensitive origin. Colonies were then plated onto LB incubated at 37°C, LSLB with 250 µg/mL zeocin incubated at 30°C overnight to test for curing of pFLPe2, and LB with 100 µg/mL trimethoprim incubated at 37°C overnight to test for absence of the trimethoprim resistance marker. Trimethoprim sensitive deletion strains were confirmed by PCR and saved.

Spot plating and microscopy. Strains were struck out onto LB agar plates from -80°C stocks and incubated overnight at 37°C. For each strain, 3 mL LB broth was inoculated and incubated overnight at 37°C. Strains were diluted to an OD600 of 0.25 using LB broth, then 5 µL of each was spotted onto an R2B plate. Spots were allowed to dry for up to thirty minutes at room temperature before flipping the plate and incubating overnight at 37°C. Photographs of resulting macro-colonies were taken using a Leica MZ16 F stereomicroscope and Leica DC500 CCD camera at the University of Vermont Microscopy Imaging Center.

Biofilm assay. The methodology for this assay was adapted from the popular microtiter

biofilm assay popularized by George O'Toole's lab at Dartmouth (12). Strains were struck out from -80^{o}C stocks onto LB agar plates and incubated overnight at 37^{o}C. For each strain, 3 mL LB broth aliquots were inoculated from the plates and incubated overnight at 37^{o}C. At stationary phase, the overnights were diluted one to one hundred in LB. Added 150 µL of diluted bacteria to five technical replicate wells each on a Costar Serocluster 96 well "U" bottom, vinyl plate (REF 2797). Place plate in a small container with a moistened paper towel to act as a humidity chamber. Incubate at 37^{o}C overnight. Invert plate over waste container and shake gently. Rinse twice by gently submerging in distilled water. Tamp dry on paper towels. Add 180 µL of 0.1% crystal violet to each well and let sit for ten minutes. Invert plate over waste tray and shake gently. Rinse plate twice gently in distilled water, invert and tamp down on paper towels to dry. add 150 µL 80% ethanol to each well, wait five minutes for the crystal violet to solubilize, and read plate at OD_{550} on a Biotek Synergy 2.

RNAseq analysis. In previous work (1), *B. thailandensis* strains were examined to elucidate the putative regulons for GbdR1 and GbdR2. WT. Δ*gbdR1*, Δ*gbdR2*, and Δ*gbdR1*Δ*gbdR2* strains were grown in MOPS media with 20 mM pyruvate in the presence or absence of 1 mM choline. Inductions were done in duplicate and RNAseq was performed as previously described. Comparisons were filtered as follows: 1) WT + choline versus WT no choline log fold change (expressed as $\log_2(X)$) ≥ -2 or ≤ 2, WT + choline versus Δ*gbdR2* + choline ≤-2 or ≥2 and significance ≤ 0.05, 3) WT + choline versus Δ*gbdR1*Δ*gbdR2* + choline ≤-2 or ≥2 and significance ≤ 0.05. Applying these

filters results in a comparison of genes that are up or down regulated in Δ*gbdR2* and Δ*gbdR1*Δ*gbdR2* compared to the WT, but not due to the presence or absence of choline. Presumably, the results include genes that might be responsible for the colony morphology and biofilm phenotypes. Data is available at NCBI's Gene Expression Omnibus, accession GSE81652.

A.3 Results and Discussion.

Our previous work investigating the regulons of GbdR1, GbdR2, and SouR, as well as their role in glycine betaine catabolism, hinted at some relationship between strains containing a deletion of *gbdR2* and the uncharacterized GATR, *I1350* (1). However the nature of this putative relationship remained unclear and did not seem to directly relate the presence or absence of choline. In other instances, GATRs have been shown to impinge on the regulation of promoters primarily ascribed to another GATR, as in the case of GbdR and SouR in *P. aeruginosa* (13), as well as for GbdR and CdhR (unpublished, Jamie Meadows). Thus, it was tempting to speculate that there might be some regulatory cross-talk between GbdR2 and I1350 in *B. thailandensis*. I1350 is a particularly interesting GATR because of synteny with a major capsule operon in *B. thailandensis*, possibly being transcribed as part of the same operon. The proximity of this GATR to CPS-IV (*I1351* to *I1362*), as well as previous RNAseq data which showed changes in expression being relatively uniform across *I1350* to *I1362*, make it attractive to consider that I1350 might be involved in the regulation of this capsule. This particular

capsule operon is homologous to capsule IV in *B. pseudomallei*. Although capsule I of *B. pseudomallei* is primarily responsible for virulence, experiments in the Golden Syrian Hamster model suggested that CPS-IV may have some contribution to virulence. However, the role of CPS-IV with respect to the biology of *B. pseudomallei* or *B. thailandensis* is not well understood or particularly well studied due to being overshadowed by the clear importance of CPS-I.

In the course of working with *B. thailandensis* strains, it was noticed that some of the deletion mutants exhibited differential colony morphologies. This difference was particularly notable when a number of cells were spotted onto plates as opposed to colonies arising from single colony forming units. Spotting bacteria onto R2B plates consistently yielded a striking 'stained glass' morphology for Δ*gbdR2* and Δ*gbdR1*Δ*gbdR2* strains, but not for WT or Δ*gbdR1* strains (Figure 5.1 A). This morphological phenotype was also present in the Δ*I1350*. Unfortunately, none of the various GATR complementation strains we constructed altered this morphological phenotype (data not shown). This was true despite the GbdR1 and GbdR2 complementation strains having been shown to phenotypically complement defect in growth of *gbdR1* and *gbdR2* deletion strains when grown on choline or its derivative metabolites as sole carbon sources (1). The inability of clearly functional catabolic complementation to alter this morphological phenotype suggests that the hypothetical relationship between *gbdR2* is incorrect, but the possibility existed that other explanations could account for the disparity.

Because of the potential of capsule modification to impinge upon biofilm

formation due to alterations of the cell's exterior surface, we opted to utilize the microtiter plate biofilm assay popularized by the O'Toole lab (12). Coincident with this project, many other GATR deletion strains had been produced (Table 5.1). We included the GATR deletion strains available to us because of the simplicity of the screen and achieved surprising results (Figure 5.1 B). Seven out of the eleven deletion strains appeared to have some level of defect in biofilm formation. Barring a massive level of cross-talk between these regulators, it became evident that the source of the biofilm defect was more likely due to other mutations, perhaps selected for by the process involved for subculturing and creating these strains.

Another possible explanation for the apparent biofilm defect in our various deletion strains was that the phenotype is due to a subpopulation within the wild type stock that contains the responsible mutation or that the unknown factor regulated by phase variation. In order to untangle these possible explanations, the wild type stock was struck on LB agar plates and isolated colonies were selected and subjected to our biofilm assay (Figure 5.2 A). This resulted in many clones exhibiting typical levels of biofilm formation, and several that exhibited a defect. Clones 9 and 10 were further subcultured and struck for isolation on LB agar plates. These subclones were again subjected to the biofilm assay and were found to yield phenotypes consistent with their parental clone (Figure 5.2 B). Additional experimentation was consistent with these results (data not shown). The absence of any biofilm phenotype revertants in either direction makes it unlikely that phase variation is the cause of the phenotypes. Rather, the most likely explanation is that our original stock of wild type *B. thailandensis E264* contains a

subpopulation that exhibits a biofilm formation defect. This is critical to note as we intend to further investigate many of the GATR deletion strains we created for as of yet unknown phenotypes and were this not known, it could convolute our future results.

In order to derive some explanation for our observed phenotypes, we delved back into our old RNAseq data (Table 5.2). Because of the strains and conditions used, it was possible to eliminate genes expression changes that were specifically due to choline, which the *gbdR1* and *gbdR2* deletions impinge directly on. Also because both Δ*gbdR2* and Δ*gbdR1*Δ*gbdR2* expressed the colony morphology and biofilm deficient phenotypes, we could eliminate disparities between the two strains unrelated to these particular phenotypes and compare both to wild type. Doing so reveals two large operons that may be responsible for these phenotypes. The first, *I1350-I1357*, contains the capsule operon linked with the putative GATR regulating it, *I1350*, which initially captured our interest. The second putative operon, *I2674-I268*, contains a putative two-component regulatory response system and genes that are bioinformatically predicted to be involved with the assembly of fimbriae. The altered expression of capsule and fimbriae genes is a logical source for the observed phenotypes, as they could both easily be imagined to affect cell to cell or cell to surface interactions. Both of these clusters are present in *B. pseudomallei*, although neither has been directly tied to virulence. There are other fimbriae genes that have been associated with both virulence in *B. pseudomallei* and biofilm formation in *B. cenocepacia* (14, 15). Our experiments with these strains utilizing the Madagascar hissing cockroach infection model did not suggest any impact on virulence (data not shown) (16). However, the question of why these genes are expressed differentially

remains. Further investigation would require the construction of deletion and complementation strains to explain the phenotype at a minimum. At present, we are content with this explanation and subsequent genetic manipulation for use in our research in *B*. *thailandensis* E264 has been with a WT subclone confirmed for biofilm production.

A.4 Appendix A Figures

Figure A.1 – Searching for a link between phenotype and genotype.

(A) Deletion strains of *B*. *thailandensis* E264 on R2B plates exhibited consistent morphological phenotypes. WT and Δ*gbdR1* have topologically flatter, filled in centers. Δ*gbdR2*, Δ*gbdR1*Δ*gbdR2*, and Δ*I1350* exhibited an unusual 'stained glass' morphology. (B) Available GATR deletion strains were tested for biofilm formation utilizing crystal violet staining. Surprisingly, seven out of the twelve strains tested exhibited a defect in biofilm formation.

Figure A.2 – Biofilm phenotype subculturing in *B*. *thailandensis* E264.

(A) Subclones of WT *B*. *thailandensis* E264 from our stock vile were isolated and subjected to crystal violet biofilm assay. Both biofilm positive and biofilm negative subclones were present. (B) Selected subclones nine and ten were struck out for isolation and secondarily subclones. These subclones were also subjected to crystal violet biofilm assaying, and their phenotypes were consistent with the subpopulation from which each was derived.

A.5 Appendix A Tables

Table A.1 – Strain Utilized in Study.

A list of strains derived from *Burkholderia thailandensis* E264 utilized in the experiments described in this chapter. AN3, AN16, AN19, and AN126 were first described in previously published work (1).

Table A.2 – RNAseq Analysis Correlation.

Previously obtained RNAseq results were differentially filtered in order to seek a potential explanation for the phenotypes seen in Δ*gbdR2* (AN19) and Δ*gbdR1*Δ*gbdR2* (AN16) strains, but do not include changes in gene expression that are due to the presence of choline. Two sets of interesting genes were downregulated in relation to the WT strain (MJ358). These included an operon involving capsule production and its presumptive GATR regulator, as well as an operon bioinformatically predicted to be involved in the assembly and transport of fimbriae. 'm' in between strain names signifies subtractive comparison. '.lfc' signifies log fold change expressed as either positive or negative $\log_2(x)$. '.p' signifies the calculated significance of the correlation.

A)

WT ΔgbdR1 ΔgbdR2 ΔgbdR1ΔgbdR2 ΔI1350

B)

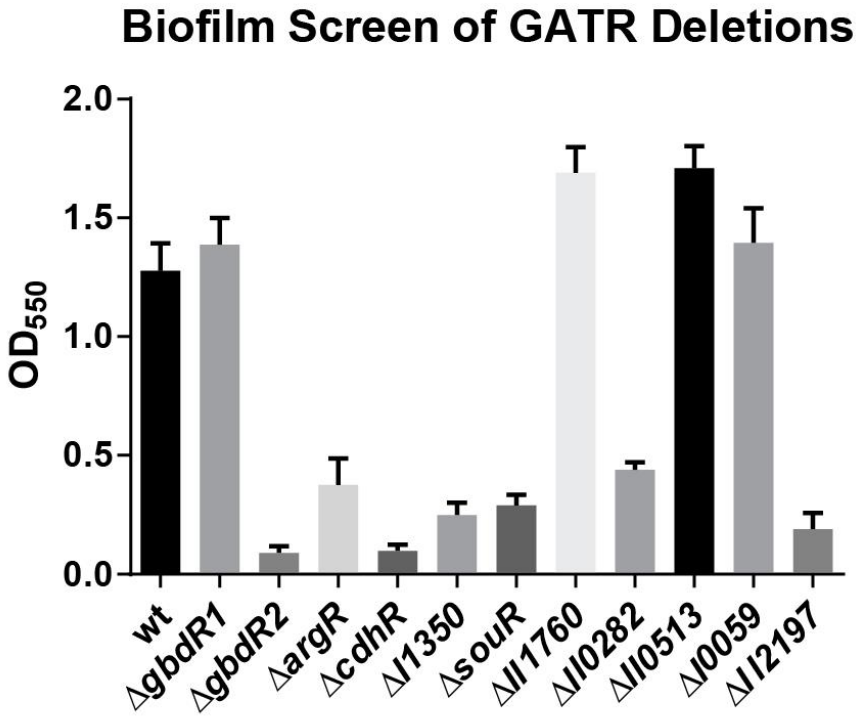


Figure A.1 - Searching for a link between pheotype and genotype.

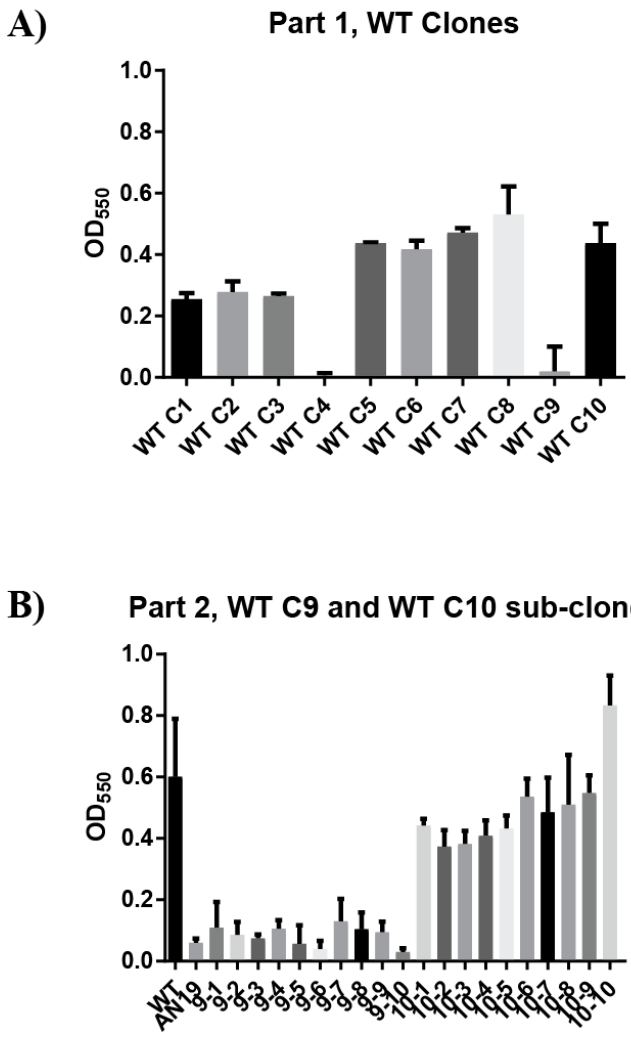


Figure A.2 - Biofilm pheotype subculturing of *B. thailandensis* E264

Strain Collection Designation	Genotype
MJ358	WT
AN3	Δ*gbdR1* (*II1869*)
AN16	Δ*gbdR1*Δ*gbdR2* (*II1869*, *II0868*)
AN19	Δ*gbdR2* (*II0868)*
AN54	Δ*argR* (*I1774*)
AN56	Δ*cdhR* (*II1850*)
AN124	Δ*I1350*
AN126	Δ*souR* (*II0994*)
AN242	Δ*II1760*
AN244	Δ*II0282*
AN246	Δ*II0513*
AN248	Δ*I0059*
AN250	Δ*II2197*

Table A.1 - Strains Utilized in Study

			MJ358_YmAN19_Y.lfc	MJ358_YmAN19_Y.p	MJ358_YmAN16_Y.lfc	MJ358_YmAN16_Y.p
BTH_I1350	YP_441897.1	AraC family transcriptional regulator	2.267908437	4.02E-05	3.844427347	4.50E-09
BTH_I1351	YP_441898.1	undecaprenyl-phosphate galactosephosphotransferase	2.21225889	0.000660081	3.672716689	1.78E-06
BTH_I1352	YP_441899.1	capsular polysaccharide biosynthesis/export periplasmic protein	2.39755144	0.000547756	3.106806194	3.55E-05
BTH_I1353	YP_441900.1	low molecular weight protein-tyrosine-phosphatase	2.692060837	0.029752724	30.53059618	0.000709903
BTH_I1355	YP_441902.1	hypothetical protein	2.86682667	0.001075217	30.61472225	2.87E-07
BTH_I1356	YP_441903.1	capsule polysaccharide biosynthesis protein	2.416901435	0.000130544	3.532337097	9.25E-07
BTH_I1357	YP_441904.1	satase isoform II	2.525794412	0.00673205	2.220857814	0.014369564
BTH_I1359	YP_441906.1	glycosyltransferase	2.373722473	0.000547789	3.056916468	4.07E-05
BTH_I1999	YP_442523.1	ATP-dependent RNA helicase RhlE	2.213005882	0.014114202	3.144211018	0.000798655
BTH_I2072	YP_442596.1	transthyretin family protein	-2.995856416	0.002663341	-3.78163591	0.000192067
BTH_I2430	YP_442951.1	short chain dehydrogenase	-2.599282926	0.037341698	-2.837991139	0.020783743
BTH_I2536	YP_443053.1	hypothetical protein	-2.829593027	0.015325872	-2.602937877	0.028757799
BTH_I2674	YP_443189.1	DNA-binding response regulator	2.427373074	5.89E-10	2.720705423	8.52E-12
BTH_I2675	YP_443190.1	sensor histidine kinase/response regulator	2.857338893	1.75E-13	3.455473432	0
BTH_I2676	YP_443191.1	spore coat protein U domain-contain protein	2.840061678	6.43E-13	3.646516911	0
BTH_I2677	YP_443192.1	fimbrial usher protein	2.803898132	2.18E-13	3.735230799	0
BTH_I2678	YP_443193.1	fimbrial assembly chaperone	2.94252092	3.46E-14	3.471508785	0
BTH_I2679	YP_443194.1	spore coat protein U domain-contain protein	2.664183438	3.92E-12	3.173436886	7.77E-16
BTH_I2680	YP_443195.1	hypothetical protein	2.821608479	1.05E-13	3.628438582	0
BTH_I2681	YP_443196.1	hypothetical protein	2.812624852	1.29E-13	3.50592449	0
BTH_I2686	YP_443201.1	H-NS histone family protein	2.75191578	5.10E-12	2.775996795	3.58E-12
BTH_II0137	YP_438339.1	hypothetical protein	2.720104084	0.00383327	2.140711644	0.015231593
BTH_II0240	YP_438442.1	L-lactate dehydrogenase	-3.209435213	0.003786591	-4.216801967	5.10E-05
BTH_II1002	YP_439199.1	cold-shock domain-contain protein	2.230017959	0.018010514	2.88480468	0.002888176
BTH_II1170	YP_439367.1	nitrite reductase [NAD(P)H] large subunit	-2.047667876	3.44E-06	-2.678162461	1.38E-09
BTH_II1581	YP_439776.1	hypothetical protein	2.864724782	0.048758456	2.859985518	0.04919747
BTH_II1795	YP_439989.1	long-chain-fatty-acid--CoA ligase	2.993486002	0.004116112	2.393781851	0.012992194
BTH_II2357	YP_440544.1	glutamine ABC transporter periplasmic protein	2.286953617	0.003050142	2.270202623	0.0032847

Table A.2 - RNAseq Analysis Correlation

A.6 References

1. **Nock AM, Wargo MJ.** 2016. Choline Catabolism in Burkholderia thailandensis Is Regulated by Multiple Glutamine Amidotransferase 1-Containing AraC Family Transcriptional Regulators. J Bacteriol **198:**2503-2514.
2. **DeShazer D, Waag DM, Fritz DL, Woods DE.** 2001. Identification of a Burkholderia mallei polysaccharide gene cluster by subtractive hybridization and demonstration that the encoded capsule is an essential virulence determinant. Microb Pathog **30:**253-269.
3. **Reckseidler SL, DeShazer D, Sokol PA, Woods DE.** 2001. Detection of bacterial virulence genes by subtractive hybridization: identification of capsular polysaccharide of Burkholderia pseudomallei as a major virulence determinant. Infect Immun **69:**34-44.
4. **Reckseidler-Zenteno SL, DeVinney R, Woods DE.** 2005. The capsular polysaccharide of Burkholderia pseudomallei contributes to survival in serum by reducing complement factor C3b deposition. Infect Immun **73:**1106-1115.
5. **Reckseidler-Zenteno SL, Viteri DF, Moore R, Wong E, Tuanyok A, Woods DE.** 2010. Characterization of the type III capsular polysaccharide produced by Burkholderia pseudomallei. J Med Microbiol **59:**1403-1414.
6. **Reckseidler-Zenteno SL, Moore R, Woods DE.** 2009. Genetics and function of the capsules of Burkholderia pseudomallei and their potential as therapeutic targets. Mini Rev Med Chem **9:**265-271.
7. **Borlee GI, Plumley BA, Martin KH, Somprasong N, Mangalea MR, Islam MN, Burtnick MN, Brett PJ, Steinmetz I, AuCoin DP, Belisle JT, Crick DC, Schweizer HP, Borlee BR.** 2017. Genome-scale analysis of the genes that contribute to Burkholderia pseudomallei biofilm formation identifies a crucial exopolysaccharide biosynthesis gene cluster. Plos Neglected Tropical Diseases **11**.
8. **Tseng BS, Majerczyk CD, Passos da Silva D, Chandler JR, Greenberg EP, Parsek MR.** 2016. Quorum Sensing Influences Burkholderia thailandensis Biofilm Development and Matrix Production. J Bacteriol **198:**2643-2650.
9. **Fazli M, Almblad H, Rybtke ML, Givskov M, Eberl L, Tolker-Nielsen T.** 2014. Regulation of biofilm formation in Pseudomonas and Burkholderia species. Environ Microbiol **16:**1961-1981.
10. **Thongdee M, Gallagher LA, Schell M, Dharakul T, Songsivilai S, Manoil C.** 2008. Targeted mutagenesis of Burkholderia thailandensis and Burkholderia pseudomallei through natural transformation of PCR fragments. Appl Environ Microbiol **74:**2985-2989.
11. **Choi KH, Mima T, Casart Y, Rholl D, Kumar A, Beacham IR, Schweizer HP.** 2008. Genetic tools for select-agent-compliant manipulation of Burkholderia pseudomallei. Appl Environ Microbiol **74:**1064-1075.
12. **O'Toole GA.** 2011. Microtiter dish biofilm formation assay. J Vis Exp doi:10.3791/2437.

13. **Willsey GG, Wargo MJ.** 2015. Sarcosine catabolism in Pseudomonas aeruginosa is transcriptionally regulated by SouR. J Bacteriol doi:10.1128/JB.00739-15.
14. **Allwood EM, Devenish RJ, Prescott M, Adler B, Boyce JD.** 2011. Strategies for Intracellular Survival of Burkholderia pseudomallei. Front Microbiol **2:**170.
15. **Tomich M, Mohr CD.** 2003. Adherence and autoaggregation phenotypes of a Burkholderia cenocepacia cable pilus mutant. FEMS Microbiol Lett **228:**287-297.
16. **Fisher NA, Ribot WJ, Applefeld W, DeShazer D.** 2012. The Madagascar hissing cockroach as a novel surrogate host for Burkholderia pseudomallei, B. mallei and B. thailandensis. BMC Microbiol **12:**117.

Appendix B – A prophage affecting *B. thailandensis* E264.

B.1 Introduction.

B. thailandensis strains, including E264, are known to harbor various phages that are capable of infecting *B. mallei*, *B. pseudomallei*, and other bacteria (1). The degree to which these phages have been categorized often is dependent on their immediate experimental utility. The status of *B. mallei* and *B. pseudomallei* as select agents has stimulated interest in the identification of phages which might potentially infect them, either as diagnostic tools or potentially as therapeutics due to their capacity for drug resistance (2). Additionally, the identification of phages capable of infecting *B. pseudomallei* in soil samples could be used as an indicator of the likely presence of *B. pseudomallei*, which is not always consistently isolated from soil where it is known to be present (3).

While generating *B. thailandensis* E264 Δ*gbdR1*Δ*gbdR2*Δ*souR* during the course of exploration of glycine betaine catabolism, we noticed the emergence of a lytic phage in one of the two clones, AN206 (Figure B.1 A). Bacteria from the plate were re-struck unto two new LB plates, with one being struck using inoculum including presumptive plaques and the other struck using inoculum seemingly clear of any plaques. The plate struck using inoculum was clearly riddled with phage (left) while the other plate contained no evidence of plaques (right) (Figure B.1 B). The initial preparation of phage was collected from this left plate by adding 10 mL of 10 mM Tris pH = 7.4 to the plate to rinse its surface, then filter sterilizing the collected liquid with a 0.22 μ filter. Adding 5 μL drops to an LB plate inoculated with a fresh lawn of wild type *B. thailandensis* E264

resulted in a large, totally cleared zone after an overnight incubation at 37°C (Figure B.1 C). These findings suggested the presence of a phage, which we have designated as "There are some who call me Tim" or φTim for short (4). Because of the interest in phages affecting Burkholderiales, we endeavored to identify the phage.

B.2 Methods.

Amplification of bacteriophage. *B. thailandensis* E264 was grown overnight at 37°C in 3 mL LB media. It was then diluted by adding 1 mL of overnight to 50 mL LB, add 10 μL of phage, and incubated overnight at 37°C. Cells and debris were pelleted by spinning at 2500 rpm for 15 minutes in a 4.2 rotor. The supernatant was filter sterilized using a .22 μm filter.

Rough bacteriophage DNA isolation. To 5 mL of filtered bacteriophage, add 10 μL DNase I (NEB) and incubate on ice for 1 hour to eliminate the majority of carry-over bacterial DNA. The filtrate was aliquoted to ten 1.5 mL microcentrifuge tubes, 300 μL each. To each tube, 0.5 μL glycogen, 33 μL 3 M NaOAc pH = 5.2, and 300 μL phenol chloroform isoamyl alcohol (50:49:1) were added. The tubes were vortexed thoroughly, then centrifuged for four minutes at 14,000 rpm in a table top microcentrifuge at room temperature. The aqueous phase was saved to a clean 1.5 mL microcentrifuge tube, to which 900 μL ethanol was added. Tubes were mixed by inversion and stored at -80°C overnight. Thawed samples were then spun at 14,000 rpm for 30 minutes at 4°C. The pellets were washed with 800 μL 80% ethanol, then air dried for five minutes. Each pellet was resuspended in 25 μL of water and pooled.

Cloning of phage DNA into pGEM-T-Easy vector. 10 µL of phage DNA was combined with 5 µL cutsmart buffer (New England Biolabs (NEB)), 33 µL water, and 2 µL AluI (NEB #R0137S) for 40 minutes at 37^{o}C. The digestion was stopped by incubating at 80^{o}C for 5 minutes. 0.5 µL Taq polymerase and 1 uL 10 mM dATP were added and the reaction, and it was incubated for 20 minutes at 72^{o}C. The reaction was cleaned up using a GeneJET Gel Extraction and DNA cleanup Micro Kit following the standard protocol for reaction cleanup (Thermo Scientific #K0832). The AluI cut, adenylated phage DNA was then ligated into pGEM-T-Easy (Promega #A1360) following the manufacturers protocol. The ligation reaction was used to transform chemically competent *Escherichia coli* DH5α cells and selected on LB plates with 100 µg/mL carbenicillin. Plasmid DNA from clones was isolated using a GeneJET Plasmid Miniprep Kit (Thermo Scientific #K0503). Plasmid DNA was screened for inserts by digesting with EcoRI-HF (NEB #R3101), and five clones positive for inserts were sent for sequencing using the M13F primer.

B.3 Results and Discussion.

Sequencing inserts derived from a crude preparation of phage DNA resulted in nBLAST hits exactly matching sections of *II1331*, *II1332*, and *II1364* found in *B. thailandensis* E264 (Table B.1), indicating that we have isolated the lytic form of a prophage. These genes are part of a previously recognized prophage-like genomic island that was not observed to be active in E264, but would be categorized as a member of the Myoviridae (5). The Myoviridae are a group of non-enveloped bacteriophages with

contractile tails, the most common example being the famous bacteriophage T4 (6). This particular genomic island was present in roughly 25% of a survey of 29 strains of environmentally isolated *B*. *thailandensis* (7). BLAST results for the inserts homologous to *II1331* and *II1332* suggested close similarity to phages in *B*. *pseudomallei* ((φE122, accession CP000624), (φX216, accession JX681814), (Burkholderia phage BEK, accession CP008753), (φ52237, accession DQ087285)) and in *B*. *thailandensis* E202 (φE202, accession CP000623), and *B*. *cenocepacia* C6433 (Burkholderia phage KS14, accession HM461982 (8)).. The size of this phage genome is predicted to be between 35 and 38 kilobases depending on the bioinformatics parameters, and in our hands the prepared genomic DNA appeared as a single prominent band in this approximate size range (data not shown).

Interestingly, these similar phages do not contain sequences homologous to our *II1364* insert, although this gene falls within the confines of the bioinformatically predicted prophage-like island (5). *II1364* is predicted to code for a DNA methyltransferase, which are often used to protect phage DNA from cleavage by host endonucleases (9). Although it is possible this phage may have been previously incidentally isolated and tested for its ability to infect *B*. *pseudomallei* or *B*. *mallei* without being further analyzed and annotated, it does not appear that this phage has been previously sequenced and submitted to NCBI apart from being a part of the *B*. *thailandensis* E264 genome. Further characterization of this phage may reveal it to be another useful tool in the study of Burkholderiales.

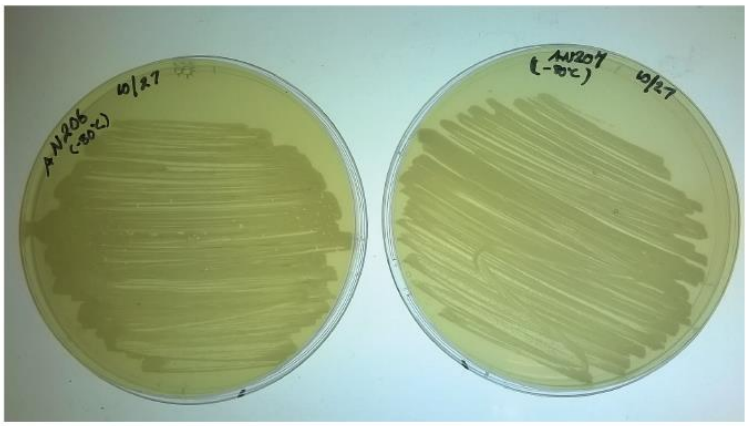
B.4 Appendix B Figure

Figure B.1 – A bacteriophage infecting *B*. *thailandensis* E264. (A) Plaques were initially observed when plating a particular clone of a deletion strain, the plate on the left side of the panel. (B) The plate with apparent plaques was re-struck onto two new LB plates, one with inoculum including the plaque (left) and one with inoculum seemingly free of plaques (right). (C) An LB plate was struck for confluency with *B*. *thailandensis* E264, and a 5 µL drop of filter sterilized phage preparation was added with a 5 µL drop of filtered 10 mM Tris pH = 7.4 as a negative control. The plate was then incubated overnight at 37^{o}C.

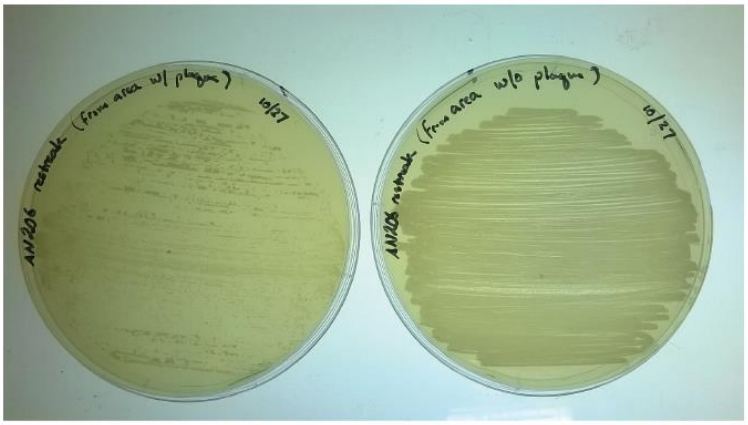
B.5 Appendix B Table

Table B.1 – Sequencing results for cloned inserts. Sequencing results were subjected to nBLAST and bioinformatic predictions were added to the table. Highlighted entries indicate genes that may be phage-associated.

A)



B)



C)

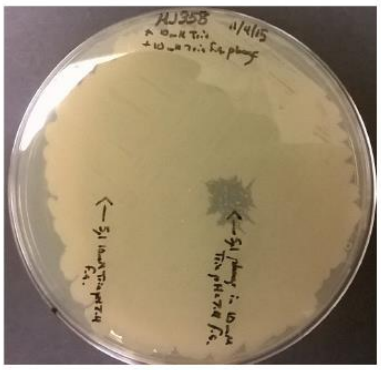


Figure B.1 - A bacteriophage infecting *B. thailandensis* E264

Clone	Sequencing results for insert.
pGEM-T-Easy-Tim-C1	*II1331*, phage tail sheath protein
pGEM-T-Easy-Tim-C2	*I0650*, O-antigen polymerase family protein
pGEM-T-Easy-Tim-C3	*II1364*, DNA methyltransferase
pGEM-T-Easy-Tim-C4	Failed sequencing reaction.
pGEM-T-Easy-Tim-C5	*II1332*, tail fiber assembly protein from lambdoid prophage e14

Table B.1 - Sequencing results for cloned inserts.

B.6 References

1. **Woods DE, Jeddeloh JA, Fritz DL, DeShazer D.** 2002. Burkholderia thailandensis E125 harbors a temperate bacteriophage specific for Burkholderia mallei. J Bacteriol **184:**4003-4017.
2. **Guang-Han O, Leang-Chung C, Vellasamy KM, Mariappan V, Li-Yen C, Vadivelu J.** 2016. Experimental Phage Therapy for Burkholderia pseudomallei Infection. PLoS One **11:**e0158213.
3. **Withatanung P, Chantratita N, Muangsombut V, Saiprom N, Lertmemongkolchai G, Klumpp J, Clokie MR, Galyov EE, Korbsrisate S.** 2016. Analyses of the Distribution Patterns of Burkholderia pseudomallei and Associated Phages in Soil Samples in Thailand Suggest That Phage Presence Reduces the Frequency of Bacterial Isolation. PLoS Negl Trop Dis **10:**e0005005.
4. **Coca-Cola Company Collection (Library of Congress), LC Purchase Collection (Library of Congress).** 1974. Monty Python and the holy grail. Almi Cinema 5, Columbia Pictures Home Entertainment, S.l. United States.
5. **Ronning CM, Losada L, Brinkac L, Inman J, Ulrich RL, Schell M, Nierman WC, Deshazer D.** 2010. Genetic and phenotypic diversity in Burkholderia: contributions by prophage and phage-like elements. BMC Microbiol **10:**202.
6. **Karam JD, Miller ES.** 2010. Bacteriophage T4 and its relatives. Virol J **7:**293.
7. **Yu YT, Kim HS, Chua HH, Lin CH, Sim SH, Lin DX, Derr A, Engels R, DeShazer D, Birren B, Nierman WC, Tan P.** 2006. Genomic patterns of pathogen evolution revealed by comparison of Burkholderia pseudomallei, the causative agent of melioidosis, to avirulent Burkholderia thailandensis. Bmc Microbiology **6**.
8. **Lynch KH, Stothard P, Dennis JJ.** 2010. Genomic analysis and relatedness of P2-like phages of the Burkholderia cepacia complex. BMC Genomics **11:**599.
9. **Murphy J, Mahony J, Ainsworth S, Nauta A, van Sinderen D.** 2013. Bacteriophage orphan DNA methyltransferases: insights from their bacterial origin, function, and occurrence. Appl Environ Microbiol **79:**7547-7555.