Bacterial Xanthine Dehydrogenase Regulators

Smitha Sivapragasam

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BACTERIAL XANTHINE DEHYDROGENASE REGULATORS

A Dissertation

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

in

The Department of Biological Sciences

by

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August 2015
DEDICATED TO MY HUSBAND AND KID...
ACKNOWLEDGEMENTS

First and foremost I would like to thank my advisor Dr. Anne Grove, for giving me an opportunity to work in this lab. She is that kind of an advisor a student would ever dream of. She would set an example of being a wonderful human being with sincerity, hard work and dedication to her profession. I would always want to have her as a role model in my scientific career. I have seen the utmost simplicity and kindness from such a knowledgeable person.

I thank my committee members Dr. Gregg Pettis, Dr. Marcia Newcomer and Dr. William Doerrler for being very supportive committee members. I specially thank Dr. Gregg Pettis for his encouraging words when I was facing the utmost challenges in my Ph.D. I was so lucky to have Dr. Megan Macnaughtan as my Dean’s representative. She is very encouraging and was readily available whenever I had to discuss with her about Science (NMR).

I would like to thank all my lab mates for encouraging me and helping me with my experiments. I thank the past members Dr. Kavitha, Dr. Hao, Dr. Ambuj and Dr. Khoa. I would specially thank Ashish, Dinesh and Anuja for being my best buddies when I joined this lab. I also thank Nabanita, Afsana and Arvind. I thank Kavitha and Nabanita for being very supportive and motivating me whenever I felt depressed. Thanks to Dante’ Johnson for helping me out with the ‘Ralstonia’ Project. I have always cherished her company in research.

I thank everybody in 5th Floor Choppin Hall. I specially thank Sunayana Mitra and Dr. Matthew Kobe for helping me with HPLC. I thank Dr. Battista and his wife for showering their love during my initial years at LSU. I thank Dr. Huangen Ding for all his help during my initial years of Ph.D. I thank everyone in 6th floor LSB. I thank Dr. Mike Hellberg, Miss. Prissy and Miss.
Chimene for being very helpful in all my dealings with the graduate school. I would like to thank all my friends Shafi, Geeta, Manish, Murali, Nitin and Supriya for morally supporting me during my stay here at LSU. I also thank Dr. Nithya Mariappan and her family for being very friendly neighbors. I thank Dr. Sreelatha Balamurugan for constantly encouraging me to reach heights in my profession.

I am so grateful to my mother, Vani and my mother-in-law, Jayalakshmi for taking care of my daughter and the whole family during my Ph.D. I am gifted to have such wonderful women in my life. I feel wonderful to have my nieces Thanishkaa and Krishanthika who have always made me forget my stress by their innocent smiles and talks. I thank my father (Sivapragasam), sister (Geevanjaly), brother-in-law (Partibane), my brothers (Balaguru and Narendran), sister-in-laws, my cousins and all my family back in India who were constantly praying for my success. I also thank all my friends Bhuvana, Azhagamma (Late), Sangeetha, Parthiban and Jerome from Pondicherry and also other friends from Bareilly who have been with me through my hardships.

I thank God for giving me such a wonderful husband, Kathir and daughter, Kanishkha. They have always let me dedicate my time to research and showered their pure love even after spending very meagre time with them during the busiest stages of my research. Without their unceasing love and tolerance my dream would not have been fulfilled to this level.

I thank Louisiana State University for providing me a very good platform to enrich my scientific knowledge and strengthening me to reach my dreams in my scientific career. I thank each and every good heart that supported me throughout my stay at LSU.
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ABSTRACT

Bacteria are resilient organisms that tend to evolve rapidly in order to acquire abilities to survive in a constantly changing environment. Transcriptional regulators play a vital role in regulating the function of various essential proteins of the cell. The gene encoding Xanthine Dehydrogenase Regulator (XdhR), a transcriptional regulator, is divergently oriented to the gene cluster \((\textit{xdhABC})\) encoding Xanthine Dehydrogenase (Xdh) protein in \textit{Streptomyces coelicolor}, \textit{Agrobacterium tumefaciens} and \textit{Ralstonia solanacearum}. Xdh participates in purine salvage pathway in converting adenine to guanine. My research here is aimed at identifying the novel function of Xdh and XdhR in the above mentioned bacteria. XdhR binds to the intergenic region between \(\textit{xdhR}\) and the \(\textit{xdhABC}\) gene cluster in all these bacteria. The small molecules GTP and ppGpp are ligands of \textit{S. coelicolor} XdhR, and intergenic DNA-XdhR binding is attenuated in the presence of these ligands. Gene expression studies in \textit{S. coelicolor} reveal that \(\textit{xdhB}\) gene expression is induced during stationary phase and stringent response. The accumulation of \((p)\text{ppGpp}\) is highest during stationary phase and stringent response. Therefore, this study sets forth a model in which XdhR responds to cellular levels of GTP and \((p)\text{ppGpp}\) to ensure production of Xdh, which biases purine salvage pathway towards generation of guanine for maintaining GTP levels in order to sustain \((p)\text{ppGpp}\) synthesis during starvation and stringent conditions.

The signaling molecules ppGpp and c-di-GMP are both ligands of XdhR from \textit{A. tumefaciens} and \textit{R. solanacearum}, attenuating binding of the XdhR proteins to their respective gene promoters. It is shown that \((p)\text{ppGpp}\) accumulates during stationary phase in \textit{A. tumefaciens}, and that \(\textit{xdhB}\) expression is repressed when Xdh activity is inhibited, consistent with guanosine nucleotides.
being ligands for XdhR. The secondary messenger c-di-GMP has been attributed to signal
exopolysaccharide production and biofilm formation in several pathogenic bacteria. The
findings of this research suggest that XdhR plays an important role during starvation and biofilm
formation in *A. tumefaciens* and *R. solanacearum*.

Since XdhR is identified to play a critical role during starvation, exopolysaccharide production
and biofilm formation in bacteria, this protein could act as a potential drug target for curtailing
bacterial infections.
CHAPTER 1
INTRODUCTION

Bacteria are microbial organisms that are omnipresent. This is because of their versatility in adapting themselves to thrive in various environmental conditions. Either as soil organisms or as infectious agents, bacteria mount defenses against the stress conditions they encounter and survive by using several proficient cell machineries and signaling molecules. Transcriptional regulators play a vital role in bestowing bacterial cells with such ability. Transcriptional regulators can sense various signals from the environment and aid the cell machineries in responding to changes in the environment. Transcriptional regulators bind to DNA and govern the expression of genes that are part of their regulons. They can either be repressors or activators or sometimes both, depending on conditions. They indirectly control the cell function by regulating the genes essential for particular cellular functions. Transcriptional regulators control the expression of genes involved in metabolism, stress survival, antibiotic resistance, expression of drug efflux pumps, virulence and so on. The aim of the work described in this dissertation was to characterize transcriptional regulators involved in regulating expression of genes encoding the enzyme xanthine dehydrogenase, which catalyzes the rate-limiting step in purine salvage.

**Xanthine oxidoreductase**

Xanthine oxidoreductase belongs to the molybdo-flavoenzyme family present in both prokaryotes and eukaryotes. Xanthine oxidoreductase exists in two forms, namely xanthine oxidase (XO) and xanthine dehydrogenase (Xdh). In eukaryotes, xanthine oxidase is one of the enzymes that produce reactive oxygen species and reactive nitrogen species under specific
physiological and pathological conditions (Cantu-Medellin & Kelley, 2013). Such reactive oxygen or nitrogen species are important in inflammation and host defenses against infection (Crane et al., 2013, Crane, 2013, Ciragil et al., 2014, Martin et al., 2004). Xanthine oxidoreductase participates in purine metabolism, where it catalyzes the conversion of hypoxanthine to xanthine and xanthine to uric acid. Though the function of XO and Xdh is the same in the above-mentioned steps of purine metabolism, XO produces reactive oxygen species or reactive nitrogen species in the process while Xdh produces NADH. The basis for this difference is that Xdh transfers electrons from the substrates hypoxanthine and xanthine to NAD\(^+\), while XO has little reactivity towards NAD\(^+\) and delivers the electrons to molecular oxygen to generate superoxide anion and hydrogen peroxide (Nishino et al., 2008, Ishikita et al., 2012, Pauff et al., 2008). The enzyme generally exists in its xanthine dehydrogenase form in eukaryotes and it may be converted to the xanthine oxidase form by proteolysis (irreversible) or by oxidation of cysteine residues (reversible) (Nishino et al., 2008). Since XO generates reactive oxygen and nitrogen species, as well as the antioxidant urate, it is important in maintaining the cellular redox state. Owing to its function in purine metabolism, any sort of enzyme dysfunction causes hyperuricemia or gout in human beings due to the poor solubility of urate and hence Xdh is an important drug target. Allopurinol, an analog of hypoxanthine, is a potent inhibitor of Xdh and it is therefore used for treatment of gout (Borges et al., 2002, Pacher et al., 2006, Nishino & Okamoto, 2015).

The mammalian xanthine oxidoreductase enzymes are homodimers and each subunit consists of three domains. The C-terminal domain has a molydopterin cofactor, the N-terminal domain has two 2Fe-2S centers (non-identical) and the intermediate domain has an FAD-
cofactor (Nishino & Okamoto, 2015, Enroth et al., 2000). Substrate oxidation occurs at the molybdopterin center, following which electrons are transferred to the Fe-S clusters, and reduction of NAD$^+$ requires the FAD cofactor. Consistent with this flow of electrons and the observed difference in affinity for NAD$^+$ between Xdh and XO, conversion of Xdh to XO is associated with structural changes primarily at the FAD site (Enroth et al., 2000, Nishino & Okamoto, 2015).

This enzyme also plays an important role in the growth and development of plants (Nakagawa et al., 2007). The plant enzyme exists exclusively as Xdh, yet it is able to use O$_2$ as an electron acceptor and produce reactive oxygen species (Yesbergenova et al., 2005, Zarepour et al., 2010, Hesberg et al., 2004). Inactivation of plant Xdh (using allopurinol or RNAi) results in an attenuated ability to mount efficient defenses against infection on account of reduced production of reactive oxygen species (Yesbergenova et al., 2005, Watanabe et al., 2010, Berner & Van der Westhuizen, 2010, Silvestri et al., 2008). These observations indicate that plant Xdh is likewise important for production of reactive oxygen species in response to stress or infection.

In bacteria, Xdh is the predominant form identified, and it is also inhibited by allopurinol (Dietzel et al., 2009, Truglio et al., 2002). The bacterial Xdh enzymes vary in their subunit composition. In *Rhodobacter capsulatus*, Xdh is found to be an \((\alpha\beta)_2\) heterotetrameric protein that possesses two subunits, namely XdhA and XdhB. XdhA carries the two [2Fe-2S] clusters and the FAD co-factor while the XdhB subunit binds the Moco molybdenum cofactor. *R. capsulatus* also encodes an XdhC subunit, which is not part of the functional Xdh enzyme, but is required for sulfuration of Moco and transfer of the cofactor to Xdh (Neumann et al., 2007). In bacteria,
the structural *xdhAB* genes are sometimes clustered in operons with the chaperone *xdhC*, whose sequence is not highly conserved (<30% amino acid sequence identity) (Neumann & Leimkuhler, 2011). Despite being encoded as separate subunits, the overall fold of *R. capsulatus* Xdh is similar to the mammalian enzyme, with the bacterial enzyme featuring an FAD-binding domain that resembles mammalian Xdh (that produces NADH). This structure also demonstrated that the inhibitor allopurinol is oxidized to alloxanthine, which coordinates directly to the molybdenum (Truglio et al., 2002). While bacterial enzymes are therefore thought to exist in the Xdh form, *Enterococcus faecalis* Xdh has been implicated in extracellular peroxide production, but only in presence of selenium. Whether Xdh is directly responsible for oxidant production, however, remains unresolved (Srivastava et al., 2011). By comparison, *E. coli* and some other species have been suggested to encode a heterotrimeric Xdh in which different subunits bind Moco, FAD, and the [2Fe-2S] clusters (Xi et al., 2000, Schultz et al., 2001, Iobbi-Nivol & Leimkuhler, 2013). Though Xdh is associated with purine metabolism, the precise function in different bacterial species has not yet been fully elucidated. The regulation of *xdh* under various conditions might provide a clue regarding the role of this protein in bacterial physiology.

**Proteins involved in purine salvage pathway and their physiological significance**

Synthesis of purines is essential, as the products provide bases for DNA and RNA, a number of cofactors and signaling molecules, and carriers of energy. Purine synthesis may occur by *de novo* pathways and salvage pathways. In the *de novo* pathway, the purine ring is synthesized from scratch, starting with 5-phosphoribosyl-1-pyrophosphate (PRPP) and glutamine. After production of 5-phosphoribosyl-1-amine, the purine ring is assembled on the
ribose phosphate in a series of steps to generate inosine monophosphate (IMP). Subsequent steps generate AMP and GMP (Rolfes, 2006).

Purine bases are released from metabolism or catabolism of nucleic acids and the cofactors containing purines and also from turnover of mRNA in the cells. In salvage pathway, these purines are recycled and interconverted depending upon need and this ensures conservation of energy investment of cell towards generation of these bases. While purine de novo synthesis pathway demands energy/ATP at five different steps, salvage pathway consumes ATP only at one step (Moffatt & Ashihara, 2002). Thus purine salvage pathway is essential for cells to judiciously use energy available especially in times of need.

The synthesis of nucleotides by salvage pathways include conversion of adenine to AMP by adenosine phosphoribosyl transferase (Aprt) and conversion of hypoxanthine and guanine to IMP and GMP, respectively, by hypoxanthine guanine phosphoribosyl transferases (Hgprt). Enzymes GMP synthase (which converts XMP to GMP and is encoded by guaA) and IMP dehydrogenase (which converts IMP to XMP and is encoded by guaB) participate in both de novo synthesis and purine salvage and deletion of genes encoding these enzymes has been reported to result in decreased growth in several bacterial species, including Salmonella enterica serotype Typhimurium, Yersinia pestis, and Shigella flexneri (Liechti & Goldberg, 2012). Mutating the guaB gene in the disease- (fireblight-) causing plant pathogen Erwinia amylovora renders the bacterium incapable of surviving and infecting plants (apples and pears) (Eastgate et al., 1997). Also, GuaA and GuaB proteins are essential for survival of the bacterium Borrelia burgdorferi which causes lyme disease in humans; B. burgdorferi lacks genes encoding enzymes involved in de novo nucleotide synthesis and depends on salvage of both purines and
pyrimidines from the host. In addition, this bacterium lacks genes encoding classical purine salvage proteins such as Hgp rt and the gene encoding ribonucleotide reductase (Pettersson et al., 2007). Purine salvage proteins allow this bacterium to utilize the high level of hypoxanthine present in mammalian blood. Though the hgp rt gene is absent, a low level of hypoxanthine is transported and incorporated into the bacterial cell (Jewett et al., 2009). This suggests that other enzymes (perhaps Xdh) could be functioning in the utilization of hypoxanthine. The stomach dwelling bacterium Helicobacter pylori is also devoid of the genes responsible for the de novo purine pathway and its growth on adenine requires the salvage enzyme Hgp rt, but not Aprt (Liechti & Goldberg, 2012). Though a plethora of evidence is available for understanding the physiological significance of most of the proteins participating in purine salvage pathways, very little study has been conducted to identify the role of Xdh in bacteria during purine salvage.

The role of E. coli Xdh in purine salvage is to bias the flow of purines towards synthesis of guanine from adenine (Figure 5.1). Therefore, loss of Xdh function in xdh mutant strains of E. coli results in cells that cannot convert adenine to guanine and therefore become sensitive to the presence of adenine in medium. The sensitivity to adenine here is due to imbalanced levels of ATP in the cell (Xi et al., 2000, Levine & Taylor, 1982).

In the root nodular bacterium Sinorhizobium meliloti, starvation for amino acids and nitrogen leads to alteration of almost the entire transcriptome of the cell. While most genes are down-regulated, only a handful of genes get upregulated and xdh is one of them(Krol & Becker, 2011). This observation implicates xdh as one of those genes whose expression increases upon starvation. In Streptomyces coelicolor, it has also been reported that expression of xdh goes up
during stationary phase (Ochi, 1987, Hillerich & Westpheling, 2008). Considering that Xdh has been reported to be rate-limiting in purine salvage, Xdh may be particularly important in purine salvage during nutrient limitation or starvation.

Figure 1.1. The proposed role of xanthine dehydrogenase is to bias the purine salvage towards the synthesis of guanine. While every enzyme catalyzing purine salvage (those indicated in black are inhibited by ppGpp) is geared towards inhibition of abnormal level of GTP synthesis, Xdh functions to maintain the basal level synthesis of GTP required for (p)ppGpp production. Xdh – xanthine dehydrogenase; Hgprt – Hypoxanthine guanine phosphoribosyl transferase; Gua B-IMP dehydrogenase; Gmk – Guanylate kinase

The magic spot / alarmone (p)ppGpp

When *E. coli* is starved for amino acids, it produces compounds that were originally identified as two spots in thin layer chromatography, spots that were associated with inhibition of transcript formation during amino acid starvation and hence were termed the magic spots (Cashel, 1975). These compounds were subsequently identified as guanosine pentaphosphate (pppGpp) and guanosine tetraphosphate (ppGpp), together referred-to as (p)ppGpp, the global regulators of transcription. Considering their accumulation during stress and starvation and role
in responding to such conditions, the term alarmone was also associated with these compounds. The so-called stringent response elicited in response to stress and starvation involves global physiological changes, but the most highly conserved is repression of rRNA synthesis and diminished ribosome content. The objective is to divert resources away from growth and cell division until conditions improve.

In *E. coli*, RelA protein synthesizes (p)ppGpp from ATP and either GDP or GTP whereas SpoT protein can produce (p)ppGpp and also degrade it to form GDP/GTP and pyrophosphate. RelA (named for the “relaxed” phenotype associated with its deletion) is activated by amino acid starvation whereas SpoT (named for the “spotless” appearance of TLC plates in its absence) is activated by other nutrient limitation or stress. *E. coli* RelA and SpoT appear to be paralogs that evolved from an ancestral gene. RSH (Rel SpoT homolog) proteins comprise the superfamily of enzymes involved in the synthesis and degradation of (p)ppGpp in bacteria. There are several homologs of this protein present in bacteria (Atkinson *et al.*, 2011).

**Role of the global regulator (p)ppGpp is different in Gram positive bacteria and Gram negative bacteria**

(p)ppGpp is called an ‘alarmone’ because of its function during nutrient limitation in bacteria. Its function has been most extensively characterized in *E. coli*. When an uncharged tRNA enters the ribosome, RelA protein is activated and synthesizes (p)ppGpp. This synthesis continues until the deacylated tRNA dissociates from the ribosomal A site (Agirrezabala *et al.*, 2013, Wendrich *et al.*, 2002, Haseltine & Block, 1973, Haseltine, 1972). Global changes in gene transcription occur upon accumulation of (p)ppGpp, but until recently, its binding site on RNA polymerase remained elusive. In Gram negative bacteria, ppGpp binds to the β’ and ω subunits of RNA polymerase (RNAP) and exerts a negative effect on σ^{70}-dependent promoters in the
presence of DksA protein, a transcription factor that acts in concert with ppGpp (Figure 1.2) (Lennon et al., 2012, Ross et al., 2013). Therefore RNAP becomes available to the alternative sigma factors essential for transcribing stress response genes. Thus ppGpp directly regulates cellular transcription during stress or the stringent response. In addition, (p)ppGpp accumulation has indirect effects as a consequence of inhibition of stable RNA synthesis. For example, reduced production of rRNA results in accumulation of ribosomal proteins, which in turn inhibit their own synthesis (Serganov et al., 2003, Yates et al., 1980).

In Gram positive bacteria no direct interaction of ppGpp with RNA polymerase has been demonstrated. Instead, changes in cellular GTP levels are attributed to the response during stress. For example, it was proposed that the identity of the initiating nucleotide determines if a gene is under stringent control, as evidenced by the observation that substitution of GTP for another nucleotide as the initiating nucleotide in rDNA transcription in B. subtilis removes regulation by ppGpp (Krasny et al., 2008). In B. subtilis, the levels of GTP drastically drop upon accumulation of (p)ppGpp. Thus in Gram positive bacteria, the ppGpp levels are inversely correlated with the GTP levels in the cells, suggesting regulation of GTP levels by (p)ppGpp. In a mutant strain of B. subtilis devoid of ppGpp synthesis, proper GTP levels are not maintained, and this failure to control GTP levels leads to cell death. The reason for the cell death has yet to be identified (Kriel et al., 2012).
Figure 1.2. Targets of (p)ppGpp in Gram positive and Gram negative bacteria during stress/starvation induced response (modified from Figure 1 of (Gaca et al., 2015)). Blue arrow and blue box indicates the proposed model of (p)ppGpp-driven upregulation of xdh during stress response in bacteria. Black boxes are the targets inhibited by (p)ppGpp.

In addition, the level of (p)ppGpp has been shown to govern cell growth in various bacteria such as *E. coli* and *B. subtilis* by inhibiting enzymes other than RNAP. ppGpp inhibits DNA replication in both Gram positive and Gram negative organisms by inhibiting the enzyme DNA primase. It inhibits translation by binding to Initiation factor-2 and small GTPases (Gaca, 2015). It directly inhibits the enzymes GuaB, Gmk and Hgprt that are involved in GTP biosynthesis (Kriel et al., 2012). In *B. subtilis*, CodY protein binds to GTP and represses the CodY regulon during nutrient sufficient condition. Upon nutrient limitation, CodY cannot bind DNA, leading to the derepression of the regulon. CodY participates in transcribing genes responsible
for adaptation to nutrient limitation in low GC rich Gram positive organisms (Geiger & Wolz, 2014).

**Purine salvage linked to ppGpp and GTP homeostasis**

It has been found that in *Listeria monocytogenes*, an *hgprt* mutant strain fails to induce virulence in mice and also is defective in the synthesis of ppGpp (Taylor et al., 2002). Therefore there is also a link between the purine salvage pathway and production of the global regulator (p)ppGpp. In *B. subtilis*, GTP homeostasis is essential for the cell survival during starvation conditions. A critical regulatory network exists for the regulation of GTP homeostasis via a ppGpp-dependent feedback loop. Failure of this feedback loop leads to aberrant synthesis of GTP and cell death. The small molecule (p)ppGpp inhibits the enzymes Hypoxanthine phosphoribosyl transferase (Hpgrt), Guanosine mononucleotide kinase (Gmk), GMP synthase (GuaA) and IMP dehydrogenase (GuaB) of the purine metabolic pathway by directly binding to them and thus preventing accumulation of GTP. An increase in GTP levels above a certain level negatively impacts the growth of cells and decreasing GTP levels helps survival of cells during amino acid starvation. In cells lacking (p)ppGpp, adenine could alleviate the accumulation of GTP and thus aid in survival during nutritional stress. Low GTP levels in *B. subtilis* leads to the inactivation of the transcriptional repressor CodY aiding in the transcription of genes essential for amino acid biosynthesis (Kriel et al., 2012, Bittner et al., 2014). In *E. coli*, ppGpp inhibits IMP dehydrogenase (GuaB) thereby inhibiting GTP synthesis. Similarly, enzymes involved in GTP synthesis are inhibited by (p)ppGpp in *Enterococcus faecalis*, and low levels of (p)ppGpp during balanced growth were found to exert an important regulatory control, with complete loss of (p)ppGpp resulting in failure to control GTP homeostasis (Gaca et al., 2013). These observations
suggest that (p)ppGpp is important for controlling cellular GTP levels both during the stringent response and during balanced growth, primarily by inhibiting activity of enzymes involved in GTP synthesis. By contrast, Xdh has been implicated in promoting (p)ppGpp synthesis, and the genes encoding this enzyme have been shown to be upregulated during the stringent response, as noted above. This suggests complex regulation of purine salvage pathways. Thus the enzymes involved in purine salvage play an important role in regulating GTP homeostasis in bacteria (Figure 1.1).

The cellular GTP pool also regulates morphological differentiation in many microorganisms including *Bacillus*, *Streptomyces* and *Saccharomyces* spp. (Pall, 1988). For example, decoyinine inhibits the enzyme GMP synthetase and therefore decreases the GTP pool in *Streptomyces griseus*, a decrease that is accompanied by initiation of morphological differentiation (Ochi, 1987). In *S. coelicolor*, inactivation of the (p)ppGpp synthetase gene *relA* results in dysregulation of GTP homeostasis and delayed morphological differentiation (Sun *et al.*, 2001), and the cyanobacterium *Anabaena* depends on ppGpp for heterocyst formation (Zhang *et al.*, 2013).

**The secondary messenger cyclic-di-GMP**

Another intracellular messenger that is derived from GTP is cyclic-di-GMP (c-di-GMP). C-di-GMP plays an important role in controlling interaction of bacterial cells with surfaces and is for example important for inducing biofilm formation in various bacteria. In this process c-di-GMP participates in adhesion and matrix formation and also controls genes essential for virulence. Diguanylate cyclases synthesize c-di-GMP from GTP and phosphodiesterases degrade them (Schirmer & Jenal, 2009). The accumulation of c-di-GMP depends on the balance between
these two groups of enzymes (Povolotsky & Hengge, 2012). While enzymes involved in synthesis and degradation of c-di-GMP are readily identifiable based on conserved sequence motifs, receptors for c-di-GMP do not appear to bear sequence or structural similarity, hampering identification of such proteins. In addition, c-di-GMP may exist either as a monomer, as a dimer in which guanines are stacked, or higher order assemblies.

In *P. aeruginosa*, *V. cholerae*, *Salmonella enterica* serotype Typhimurium and *E. coli*, c-di-GMP has been shown to regulate motility. Also in *V. cholerae*, the extracellular polysaccharide production is dependent on the availability of c-di-GMP. The presence of c-di-GMP aids the production of *Vibrio* exopolysaccharide (VPS) (Beyhan *et al*., 2006, Srivastava *et al*., 2013, Tischler & Camilli, 2004). A similar function of c-di-GMP is also found in *P. aeruginosa* and *Y. pestis* (Tamayo *et al*., 2005). In *E. coli*, ribosomal stress caused by various antibiotics leads to decreased levels of ppGpp, which in turn causes derepression of *pgaA*, essential for the biosynthesis of polysaccharide adhesion poly-β-1,6-N-acetyl-glucosamine. Also synthesis of PgdA, another component of the polysaccharide adhesion, matrix requires c-di-GMP. Thus, a fine regulatory network exists between the alarmone ppGpp and the secondary messenger c-di-GMP in regulating the synthesis of the matrix for biofilm formation in *E. coli* (Boehm *et al*., 2009). Also, GTP being the precursor for synthesis of pppGpp and c-di-GMP gives us a clue regarding a common regulatory network existing in the production of these signaling molecules. An altered level of GTP in the bacterial cell might cause a difference in the generation of these critical signaling molecules.
**TetR family transcriptional regulators**

Little is known about the mechanisms by which expression of genes encoding Xdh is regulated. One clue is the recent characterization of a genomic locus in *S. coelicolor* consisting of divergently oriented genes encoding Xdh and a TetR family transcriptional regulator (Hillerich & Westpheling, 2008). Such gene orientation is common and typically associated with regulation of both genes by the encoded transcription factor (Figure 1.3). Analysis of gene expression revealed upregulation of *xdh* in stationary phase, as noted above. Inactivation of the gene encoding the transcription factor, which was named XdhR for xanthine dehydrogenase regulator, resulted in elevated and constitutive expression of *xdh* genes. This indicates that XdhR functions as a repressor. However, the inducer of *xdh* expression was not identified.

![Gene Orientation](image)

Figure 1.3. The gene orientation of *xdhABC* and *xdhR* genes in *S. coelicolor*.

TetR family transcriptional regulators, the third most abundant family of transcriptional regulators, are widely present in both Gram positive and Gram negative bacteria. These transcriptional regulators regulate biosynthesis of antibiotics, efflux pumps involved in drug resistance, osmotic stress and pathogenicity in certain bacteria. They usually bind DNA as homodimers leading to repression of gene expression. They possess a C-terminal signal transduction domain that senses signal (a ligand or a small molecule). Ligand binding leads to changes in the N-terminal helix-turn-helix domain that is involved in DNA binding. A typical example is the TetR protein of *E. coli* that binds DNA to repress the *tetA* gene (Hinrichs *et al.*, 1994). Upon encountering the antibiotic tetracycline, the TetR protein is structurally modified.
and dissociates from the DNA leading to expression of the tetA gene whose product degrades tetracycline (Ramos et al., 2005). While the sequence of the DNA binding domain is conserved in TetR family members, there is no significant sequence similarity in the ligand-binding domain, allowing TetR family members to bind structurally distinct ligands. Regardless of the nature of the ligand, the allosteric changes induced in the DNA binding domain from the DNA-bound form to the ligand-bound form lead to abrogation of DNA binding. The alpha helix 4 of TetR protein relaxes and a pendular motion of helix 6 leads to the separation of the DNA recognition helices, which leads to the release of protein from DNA (Yu et al., 2010).

As described in Chapters 2 and 3, the Gram positive bacterium S. coelicolor and the Gram negative A. tumefaciens both contain a genomic locus consisting of divergently oriented xdh and xdhR genes, where xdhR encodes a TetR family transcriptional regulator (Figure 1.3). Given the distinct regulatory roles of (p)ppGpp in Gram positive and Gram negative species and the implication of Xdh as an essential enzyme in purine salvage and (p)ppGpp synthesis, I was interested in determining whether expression of xdh might be similar or different in these species. The indirect role of XdhR with regard to maintaining the levels of ppGpp and GTP in Gram positive and Gram negative bacteria could reveal the uniqueness of regulatory mechanisms found in bacteria.

LysR family transcriptional regulators

A second clue to the mechanism by which xdh expression is regulated came from analysis of the plant pathogen Ralstonia solanacearum. It was observed that xdh is upregulated upon R. solanacearum infection of tomato plant as well as on exposure of R. solanacearum cultures to hydrogen peroxide (Flores-Cruz & Allen, 2009). While the transcription factor
responsible for hydrogen peroxide-mediated upregulation of \textit{xdh} was not identified, it is intriguing that the \textit{xdh} genes in this species are divergently oriented from a transcription factor that belongs to the LysR protein family. This gene orientation points to a possible role of the LysR protein in regulation of \textit{xdh} expression.

LysR is the largest family of transcriptional regulators found in prokaryotes. LysR proteins are widely distributed in a variety of organism including archaea and eukaryotes. Their target genes are diverse including genes involved in metabolism, virulence, quorum sensing, and oxidative stress response. They are usually found as tetramers and are shown to bind the DNA in the presence of a co-inducer. They possess an N-terminal HTH DNA-binding domain and C-terminal co-factor binding region (Maddocks & Oyston, 2008). The binding of the co-factor induces conformational change in the N-terminal DNA-binding region that alleviates repression or induces transcriptional activation. While most members of this family act as activators (e.g., OxyR, AmpR, BenM) some of them are repressors (CatR, RovM) and others act both as repressors and activators (LeuO, CrgA) (Schell, 1993, Maddocks & Oyston, 2008). \textit{R. solanacearum} thus possesses a unique putative regulator that could be a repressor, an activator or both. While conservation of the \textit{xdhR-xdhABC} locus in \textit{S. coelicolor} and \textit{A. tumefaciens} suggests conservation of function, regulation of \textit{xdhABC} in \textit{R. solanacearum} may be distinct since the transcription factor belongs to a different family.

\textbf{References}


CHAPTER 2
XANTHINE DEHYDROGENASE REGULATOR PROTEIN OF *Streptomyces coelicolor*

Introduction

*Streptomyces coelicolor* is a Gram positive bacterium belonging to the phylum actinobacteria. It responds to nutritional and environmental stresses in the soil by a complex morphological differentiation, changing its life cycle from mycelial to sporulating morphology. Streptomycetes are known for their abundant production of secondary metabolites, some of which are antibiotics or other pharmaceutically active compounds. The production of secondary metabolites is generally linked to nutrient limitation and morphological differentiation, and the phosphorylated guanosine nucleosides 5′-triphosphate-3′-diphosphate (pppGpp) and 5′-diphosphate-3′-diphosphate (ppGpp) commonly referred to as (p)ppGpp have been implicated in these processes. For example, production of the antibiotic streptomycin in *Streptomyces griseus* is decreased in a mutant strain that is defective in synthesis of (p)ppGpp (Ochi, 1987). Similarly, an inability of *S. coelicolor* to produce (p)ppGpp resulted in a strain that is deficient in production of the antibiotic actinorhodin, along with a delayed onset of morphological differentiation (Chakraburtty & Bibb, 1997, Kang *et al.*, 1998, Ryu *et al.*, 2007).

The alarmone (p)ppGpp is a global regulator of gene expression in bacteria. The synthesis of (p)ppGpp by paralogous enzymes RelA and SpoT occurs as a result of amino acid, carbon, fatty acid, phosphate or iron limitation to initiate the stringent response. Under amino acid starvation, uncharged tRNA entering the ribosomal A site hinders translation, which initiates the synthesis of (p)ppGpp by RelA, whereas SpoT is reported to be activated by other starvation conditions in a ribosome-independent manner (Potrykus & Cashel, 2008, Wu & Xie,
In *S. coelicolor*, RelA and RshA (named for RelA/SpoT homology) participate in (p)ppGpp production under amino acid/glucose starvation and phosphate starvation, respectively (Ryu et al., 2007). In Gram negative bacteria, (p)ppGpp binds to the β’ subunit of RNA polymerase in concert with DksA to negatively regulate transcription (Ross et al., 2013). In addition, (p)ppGpp positively regulates certain genes such as those encoding alternative sigma factors and proteins involved in stress responses (Magnusson et al., 2005, Traxler et al., 2011, Haugen et al., 2008). In Gram positive species, (p)ppGpp appears to act indirectly by controlling cellular GTP levels (Geiger et al., 2012, Kriel et al., 2012). The stringent response involves global changes in gene expression that allow cells to utilize scarce resources more efficiently while down-regulating genes generally associated with growth. Because (p)ppGpp also plays a central role in coupling metabolism to virulence, bacteria exert careful control over the activity of enzymes involved in (p)ppGpp metabolism, and inactivation of these enzymes has been shown to reduce virulence of pathogenic species (Bowden et al., 2013, Taylor et al., 2002).

There is evidence to suggest that purine salvage pathways are required for (p)ppGpp production. In *Listeria monocytogenes*, both relA and hpt mutants fail to produce (p)ppGpp and mutant strains are avirulent in mice. The hpt gene encodes hypoxanthine guanine phosphoribosyl transferase (Hgprt), which functions in purine salvage pathways, for example by converting guanine to GMP (Fig. 1.1A). The inability of the hpt mutant to synthesize (p)ppGpp therefore suggests that purine salvage is required to generate sufficient GTP, the substrate for (p)ppGpp synthetases (Taylor et al., 2002). Hgprt also participates in salvage of adenine nucleotides by converting hypoxanthine to IMP; (p)ppGpp synthetases transfer pyrophosphate from ATP to either GDP or GTP, thus (p)ppGpp production also occurs at the expense of ATP.
Salvage of purine bases or nucleosides deriving from cellular turnover involves the conversion of guanosine to guanine, which is then converted to GMP, either directly or via a xanthine intermediate. Adenosine is typically converted to hypoxanthine, either via adenine or inosine. Purine nucleobases are then converted to the corresponding mononucleotides, as illustrated for HgpRt. The final step in purine salvage is the conversion of hypoxanthine to xanthine by xanthine dehydrogenase (Xdh); Xdh also oxidizes xanthine to urate, thereby diverting purines away from salvage pathways (Xi et al., 2000). Notably, Xdh biases purine salvage pathways towards the formation of guanine nucleotides. Consistent with a role for Xdh in GTP synthesis, upregulation of the gene encoding Xdh has been reported as part of the stringent response; in the nitrogen-fixing bacterium Sinorhizobium meliloti, the gene encoding Xdh is one of a few that are upregulated in response to starvation (Krol & Becker, 2011). Also, the transcript level of xdh is increased during morphological differentiation in surface-grown S. coelicolor (Hillerich & Westpheling, 2008). Such observations further reinforce the requirement for a functional purine salvage pathway during the stringent response.

Mechanisms by which xdh is regulated largely remain unclear. The only clue derives from the observation that a gene encoding a member of the TetR family of transcriptional regulators is encoded divergently from the xdhABC gene cluster in S. coelicolor (Fig. 1.1B). This transcription factor, named XdhR for xanthine dehydrogenase regulator, represses both xdhABC and xdhR expression as evidenced by elevated and constitutive xdhA and xdhR expression in an xdhR− strain with the downstream part of xdhR disrupted while allowing detection of expression of the 5′-end of the residual transcript (Hillerich & Westpheling, 2008). TetR family proteins participate in various functions of the cell including multidrug resistance,
biosynthesis of antibiotics, establishing pathogenicity, and catabolic pathways (Cuthbertson & Nodwell, 2013, Ramos et al., 2005). They typically function as repressors, with induction of gene expression observed on binding of a specific ligand. The ligand for XdhR (the inducer of the xdhABC gene cluster) was not identified. We show here that S. coelicolor XdhR responds directly to ppGpp and GTP by attenuated DNA binding in vitro and that xdhABC expression is repressed when Xdh is inhibited. We propose that XdhR is a direct target for ppGpp and GTP, with ppGpp eliciting upregulation of xdhABC expression in stationary phase to optimize purine salvage pathways and ensure that sufficient GTP concentrations exist for synthesis of (p)ppGpp, which in turn maintains GTP homeostasis.

**Experimental procedures**

**Cloning and protein purification**

The open reading frame corresponding to SCO1135 was amplified from Streptomyces coelicolor A3(2) M145 genomic DNA using primers FP 5’-AATAGTCATATGCGCGAGGCAAAGGA-3’ and RP 5’-CTTGTACTCGAGTCACCGGCCCCGA-3’ (restriction sites underlined). The purified PCR product was digested and cloned into pET28b between Ndel and XhoI restriction sites such that an N-terminal His<sub>6</sub>-tag is introduced, and the recombinant plasmid was transformed into E. coli TOP10 cells. The construct was confirmed by sequencing and plasmid was transformed into E. coli BL21(DE3) for overexpression. Overexpression of XdhR was accomplished by growing cells in LB containing 30 µg mL⁻¹ kanamycin and inducing expression with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 5 hours. The cells were pelleted and stored at -80°C. The cells were thawed on ice and suspended in lysis buffer (20 mM sodium phosphate pH 8.0, 150 mM sodium chloride, 5%
glycerol, 0.15 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM β-mercaptoethanol) and lysozyme was added to 200 µg mL⁻¹. The cell pellets were incubated on ice for 30 minutes and the cells were lysed by sonication (5 cycles with 20 seconds intervals). The sonication step was carried out on ice. The lysate was centrifuged at 15,000 rpm for 1 hour and the supernatant was mixed with 1 mL of HIS Select Nickel affinity gel. The His₆-tagged protein was purified according to the manufacturer’s instructions (Sigma). The salt was removed by buffer exchange using the storage buffer (20 mM sodium phosphate pH 8.0, 150 mM NaCl, 20% glycerol, 0.15 mM PMSF and 10 mM β-mercaptoethanol and the protein was concentrated using 10K centrifugal filter units (Millipore). The protein concentration was estimated using MicroBCA protein assay kit. For protein oxidation, 30 µM XdhR was mixed with 8.3 mM DTT and incubated for 10 minutes to reduce the dimers to monomers and then 15 mM of each oxidant was mixed with the protein and incubated for 10 minutes before analysis by SDS-PAGE.

Gel filtration

The purified His₆-tagged protein (in buffer containing 10 mM β-mercaptoethanol) was run on a Superose column (GE Healthcare) that was equilibrated with buffer pH 8.0 (50 mM Tris and 150 mM NaCl) using a fast protein liquid chromatography system. Gel filtration standards (bovine serum albumin (66.0 kDa), ovalbumin (44.0 kDa), myoglobin (17.0 kDa), and vitamin B12 (1350 Da); Bio-Rad) were run on the column and elution volumes were plotted to obtain the standard curve. The average molecular weight of the protein was calculated using the formula \( K_{av} = \frac{(VE-VO)}{(VT-VO)} \) where VE is retention volume of the protein, VO is void volume of the column and VT is bed volume of the column.
Circular dichroism spectroscopy

A far UV circular dichroism spectrum of XdhR was measured using a Jasco J-815 circular dichroism spectrometer (Jasco, Inc). Approximately 0.2 mg mL\(^{-1}\) of XdhR was prepared in a dilution buffer of 50 mM sodium phosphate (pH 8.0), 100 mM NaCl and 2.5% glycerol and added to a quartz cuvette of 0.1 cm path length. Ellipticity measurements in triplicate were taken over a wavelength range of 190-250 nm at 1 nM steps. The data obtained from the buffer was subtracted from that of the protein. Secondary structure composition was calculated from Dichroweb (Whitmore and Wallace, 2004; 2008) using K2d analysis program, and the maximum error was 0.122.

Electrophoretic Mobility Shift Assay

The intergenic region between the xdhABC gene cluster and the xdhR gene was amplified by PCR using primers Strepintergenic-FP 5’- A G C C T T C T T T G T G T C T G T C T G G A -3’ and Strepintergenic-RP 5’- G A C C T T G C T A A G C G G A C A A C -3’ to generate a 152 bp product. Synthetic oligonucleotides representing the 18 bp palindromic sequences flanked by 7 nt on either side were purchased and purified by denaturing gel electrophoresis. Equimolar amounts of complementary oligonucleotides were heated to 90°C in TE containing 50 mM NaCl and annealed by slow cooling to room temperature. The DNA was labeled with \(\gamma\)-\(^{32}\)P-ATP using T4 polynucleotide kinase. The labeled product (0.05 nM) was incubated with increasing concentrations of XdhR in binding buffer (25 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.05% Brij58, 5 mM DTT and 2% glycerol) for 20 minutes. The samples were run on 8% polyacrylamide gels (39:1 (w/w) acrylamide:bisacrylamide) with 0.5X Tris Borate EDTA (45 mM Tris-borate, 1 mM EDTA; TBE) running buffer for one hour at 100 volts after prerunning the gels.
for 30 min at room temperature. The gels were dried and exposed to phosphor screens. The image was scanned using Storm 840 scanner (GE Healthcare) and the quantifications were performed using ImageQuant 5.1 software. The region on the gels between complex and free DNA were considered as complex. The plots were created using KaleidaGraph software and the data were fitted to the Hill equation $f = \frac{f_{\text{max}} [X]^{n_H}}{K_d + [X]^{n_H}}$ where $f$ is fractional saturation, $n_H$ is Hill coefficient; $K_d$ is the equilibrium dissociation constant reflecting half-maximal saturation of the DNA and $X$ is the protein concentration. For binding to 18 bp DNA containing a single site, data were fitted to a single site binding isotherm ($n_H = 1$). Results are represented as mean ± SD of two replicates. Specificity of XdhR binding was assessed by titrating binding reactions with increasing concentrations of either non-specific plasmid pRAD1 (6.3 kb) or specific unlabeled 152 bp DNA.

To determine the effect of ligands, increasing concentration of ligands ppGpp (TriLink), GTP, GDP and GMP were used in individual experiments using the buffers described above. When xanthine, hypoxanthine, adenine, guanosine and urate were used as ligands for the binding assays, 500 mM Tris pH 8.0 was used in the binding buffer to avoid pH changes upon adding these ligands, which were dissolved in 0.4 N NaOH. Protein was added at the end to the mixed intergenic DNA and the ligands and the mixture was incubated for 20 minutes before loading onto the gel. The gel was run and processed as described above. $IC_{50}$ was calculated as the concentration of the ligand at which 50% of complex formation is inhibited. This was calculated using the equation $f = A + B \times e^{-kL}$ where $f$ is fractional saturation, $k$ is the decay constant, $L$ is ligand concentration, $A$ is the saturation plateau and $B$ is the decay amplitude. At least two independent experiments were performed. Inhibition constants ($K_i$) were calculated
from the equation $K_i = \text{IC}_{50}/([\text{DNA}]_{50}/K_d + [\text{XdhR}]_0/K_d + 1)$ where $[\text{DNA}]_{50}$ is the concentration of DNA at 50% inhibition and $[\text{XdhR}]_0$ is the protein concentration at 0% inhibition (Cheng & Prusoff, 1973).

**Thermal stability assay**

Sypro Orange dye (7.5X; Invitrogen) was mixed with 10 µM XdhR in assay buffer composed of 50 mM NaCl and 50 mM Tris pH 8.0. An Applied Biosystems 7500 real time PCR machine was used for measuring the fluorescence emission. A thermal profile of 5-94°C with 1°C increments every 40 seconds was used. A blank without protein was used as a negative control. The results were analyzed using Sigma Plot 9 and a sigmoidal four-parameter curve fitting was used to obtain the melting temperature. The results were from three independent replicates.

**RNA isolation and reverse transcriptase assay**

*S. coelicolor* cultures were grown in ISP1 medium for 24 hours before being treated with either 30 mM serine hydroxamate (Sigma) for 15 minutes or 10 mM allopurinol for 30 minutes. The exponential phase cultures were pelleted at 24 hours and the stationary phase cultures were pelleted after about 48 hours. RNA was isolated from the pelleted cells using illustra RNAspin Mini kit (GE Healthcare) according to the manufacturer’s protocol. AMV reverse transcriptase was used for the preparation of cDNA. One µg and 600 ng of RNA were used for cDNA preparation. Expression of the *rpoA* gene was used for normalizing the *xdhB* gene expression. The cDNA was used as template for all the PCR reactions. The *xdhB* transcripts were analyzed using primers xdh2FP 5′-GCT ACT TCA CCG ACC TGA GCA AGC-3′ and xdh2RP 5′-GGGACCGTCGAGGGTTTC-3′ and the *rpoA* transcripts were analyzed using the primers rpoAF 5′-
AAGCTGGAGATGGAGCTGAC-3’ and rpoAR 5’-TTGAGAACGCCGAGTAGAT-3’. The products were run on an agarose gel and the bands were quantified using ImageJ software. The results were obtained from three replicates.

DNaseI footprinting using automated capillary sequencing

The intergenic region was amplified using primers SCFP 5’-(6-FAM) CCGTTGATGTTCAGGGTGAC-3’ and SCRP 5’-GTCCGGCTTGCTCTCTC-3’ (6-FAM denoting 5'-end labeling with 6-carboxyfluorescein). The protein XdhR was mixed in increasing concentrations with the labeled DNA (50 ng) and incubated for 10 minutes before carrying out the DNaseI digestion. The digested product was extracted twice using phenol:chloroform and ethanol precipitated. The pellet was washed with 70% ethanol and dissolved in 10 µL formamide. Approximately 1.0 µL digested sample (diluted in formamide to ensure fluorescence intensity compatible with the analyzer) and 1 µL of the 1:10 diluted LiZ 500 standards (ABI – Life Technologies) were brought to 25 µL final volume with formamide. An aliquot of 0.05 ng of undigested DNA (to maintain fluorescence intensity compatible with the analyzer) and 0.2 ng of digested DNA was used for fragment analysis (Sivapragasam et al., 2015). The samples were boiled for 3 minutes and loaded on to the ABI 3130 analyzer with the default settings of 1.6 kV injection voltage and 15 seconds injection time. At least duplicates were run each time. The data were analyzed using GeneMapper version 4. The electropherogram traces of digested DNA without protein were overlayed with those of the digested DNA that was incubated with protein. The protected region was identified by comparing the digested fragments with that of products generated from a Thermosequenase PCR cycle sequencing reaction using the same 6-FAM-labeled primer and all four dideoxynucleotides in separate tubes.
Determination of (p)ppGpp levels by thin layer chromatography

*S. coelicolor* cells were grown for 36 hours in ISP1 media and diluted in modified MOPS media at a ratio of 1:5. Modified MOPS media contained 100 mM MOPS, 10% sucrose, 1% glucose, 2 mM MgSO₄, 0.15 % casamino acids, 0.5% yeast extract, 0.2% peptone, and 0.5% K₂HPO₄. Fifty mg of the amino acids proline, histidine, tryptophan, and tyrosine were added to 1L buffer. Carrier-free ³²P-labeled orthophosphate was added to the culture, which was grown for 5-6 hours. The cells were then treated with 30 mM serine hydroxamate for 15 minutes or with 10mM allopurinol for 30 minutes. The cells were pelleted and resuspended in 13 M formic acid. Three to five freeze-thaw cycles were carried out using a dry ice and ethanol bath. The suspension was kept on ice for at least 30 minutes and pelleted at 13,000 rpm for 10-15 minutes. The supernatant was spotted on to PEI-cellulose TLC plates (Sigma-Aldrich). The plates were developed using 1.5 M KH₂PO₄ for at least 2 hours and dried and exposed to phosphor screens. The images were scanned using Storm 840 scanner (GE Healthcare). The migration pattern of pppGpp, ppGpp and GTP were identified from the Rf published previously using this buffer system (Calderon-Flores et al., 2005). The migration of GTP and GMP was also verified using purified nucleotides.

**Results**

Divergent orientation of xdhR and the xdhABC gene cluster

The *S. coelicolor* xdhABC gene cluster (SCO1132-1134) is divergently oriented from the xdhR gene (SCO1135) (Fig. 2.1B). The xdhABC gene cluster encodes a functional Xdh (Hillerich & Westpheling, 2008), annotated as a molybdenum-containing oxidoreductase composed of a molybdopterin-binding subunit (XdhC), an FAD-binding subunit (XdhB), and XdhA, which binds a
2Fe-2S cluster. The \textit{xdhR} gene encodes the TetR family transcriptional regulator XdhR. The intergenic region between these genes is 188 bp from the start codon of \textit{xdhA} to the start codon of \textit{xdhR}. This intergenic region has two imperfect palindromes, which are potential binding sites for XdhR (Fig. 2.1B); the site in the \textit{xdhR} promoter consists of 9 bp half-sites of which seven base pairs are conserved in each half-site. The site in the \textit{xdhABC} promoter is more divergent, conserving five base pairs in each half-site. Both palindromes overlap the respective -35 promoter elements identified based on mapping of transcriptional start sites (Hillerich & Westpheling, 2008), consistent with serving as the cognate sites for a transcriptional repressor.

Figure 2.1. Xdh participates in purine salvage. (A) Outline of steps in the purine salvage pathway. Xdh converts hypoxanthine to xanthine and xanthine to urate. GTP is the substrate for (p)ppGpp synthetases. (B) Divergent gene orientation of the \textit{xdhR} gene and the \textit{xdhABC} gene cluster with the palindromes (bold and underlined) in the intergenic DNA.
Figure 2.2. Characterization of *S. coelicolor* XdhR. (A) SDS-PAGE showing purified His\textsubscript{6}-tagged XdhR and dimerization of XdhR upon addition of H\textsubscript{2}O\textsubscript{2}, cumene hydroperoxide (CHP) and tertiary butyl hydroperoxide (TBH). (B) Molecular weight of XdhR (arrow) identified by gel filtration with $K_{av}$ of molecular weight standards plotted against log\textsubscript{10} molecular weight. Standard curve was generated using BioRad gel filtration standards. (C) Thermal melting curve of XdhR, using Sypro Orange as a fluorescent reporter of protein unfolding. (D) Far UV-CD spectrum of XdhR.

XdhR is a tetramer

The gene encoding XdhR was cloned from *S. coelicolor* genomic DNA and the protein was expressed in *E. coli* with an N-terminal His\textsubscript{6}-tag. XdhR was purified to apparent homogeneity (Fig. 2.2A). TetR family transcription factors usually exist as dimers in absence of DNA, and gel filtration chromatography was performed to determine oligomeric state. Results revealed that XdhR exists as a tetramer (80 kDa) (Fig. 2.2B); a stable tetrameric assembly in absence of DNA is unusual and has only been reported for *Pseudomonas aeruginosa* MexL,
which regulates expression of the mexJK efflux pump (Chuanchuen et al., 2005). Far-UV circular dichroism (CD) spectroscopy showed that the secondary structure composition of XdhR is about 57% α-helix, 11% β-sheet and 33% random coil (Fig. 2.2D), with the secondary structure composition estimated using DichroWeb (Whitmore & Wallace, 2004). By comparison, the structures of TetR proteins, for example Staphylococcus aureus QacR, reveals largely (~75%) α-helical content (Schumacher et al., 2002); CD spectroscopy may underestimate helical content, as illustrated by the ~60% helical content of QacR estimated by this method (Hoffmann et al., 2005). Thermal stability of XdhR was analyzed using Sypro Orange dye as a reporter of protein unfolding. XdhR unfolding followed a two-state model with no evidence of an unfolding intermediate, and XdhR has a T_m of 46.2 ± 0.1 (Fig. 2.2C). These data indicate that XdhR exists as a stable tetramer in solution in absence of DNA at physiologically relevant temperatures.

TetR family transcription factors consist of a conserved helix-turn-helix DNA binding motif at the N-terminus and a C-terminal domain whose sequence is not conserved, likely reflecting the need to respond to different types of signaling molecules (Ramos et al., 2005). Despite the lack of sequence conservation, structural conservation is observed, and TetR proteins exist as α-helical homodimers that adopt an Ω-shaped structure in which α1-α3 of each monomer form the DNA-binding domain and α4 connects this domain to the regulatory domain that is responsible for dimerization and ligand binding. The tetrameric XdhR assembly therefore likely represents a dimer of dimers (we will refer to subunits within one dimer as a cis-dimer). Modeling of ScXdhR was performed using SwissModel and template 2Q24 (~41% identity to XdhR; template selected based on the highest sequence identity), which is a TetR protein of unknown function encoded by S. coelicolor (SCO0520) (Filippova et al., 2011) (Fig.
While this model reflects the conserved $\Omega$-shape, an unusual feature is the significant distance between conserved tyrosine residues in the DNA recognition helices (63 Å between C$\alpha$ carbons). As this distance far exceeds the ~34 Å between adjacent DNA major grooves, this conformation may more closely reflect the ligand-bound form that is incompatible with DNA binding.

Figure 2.3. Structure-based model of ScXdhR. (A) Model created using SwissModel in automated mode using 2Q24 as the template. Each monomer is colored from the N-terminus (blue) to the C-terminus (red). Each monomer contains a single cysteine (magenta stick representation). Conserved tyrosine residues in the DNA recognition helices are shown in stick representation. (B) Electrostatic surface potential estimated using PyMol. Positive electrostatic potential (blue), negative (red), and neutral (white).

Each XdhR monomer contains a single cysteine, predicted to be located at the end of helix one, thus placing the cysteines from each monomer in a cis-dimer far apart and on opposite faces of the protein dimer (Fig. 2.3). The 21 kDa XdhR monomer was seen to form dimeric species upon addition of oxidants $\text{H}_2\text{O}_2$, cumene hydroperoxide and tertiary-butyl hydroperoxide (Fig. 2.2A). This dimerization was reversed upon addition of DTT or $\beta$-mercaptoethanol (data not shown). Based on the predicted location of cysteine residues, we infer that the observed dimeric species reflects formation of a trans-dimer between subunits in adjacent cis-dimers. Thus, disulfide bond formation between XdhR monomers confirms the tetrameric assembly identified based on gel filtration analyses.
XdhR binds specifically to the intergenic region between \textit{xdhR} and \textit{xdhABC}

XdhR was previously shown to bind the \textit{xdhABC}-\textit{xdhR} intergenic region (Hillerich & Westpheling, 2008). Further analysis of this interaction based on electrophoretic mobility shift assays (EMSAs) revealed that XdhR bound to this intergenic region with high affinity, $K_d = 0.5 \pm 0.2$ nM, forming two discrete complexes (Fig. 2.4A). This would be consistent with the presence of two palindromes in the intergenic region that may serve as cognate sites for XdhR. Fits of the data to the Hill equation yielded a Hill coefficient $n_H = 0.7 \pm 0.1$, indicating modest negative cooperativity (Fig. 2.4A,C); negative cooperativity is somewhat surprising, however, if the observed complexes correspond to XdhR binding to cognate sites that are far apart. The binding of XdhR to the intergenic DNA remained unaffected by the addition of non-specific DNA, indicating that both complexes reflect specific binding; in contrast, addition of specific
unlabeled intergenic DNA effectively reduced complex formation (Fig. 2.4C). This shows that XdhR specifically binds the intergenic DNA between xdhR and xdhABC.

DNaseI footprinting was performed to identify the XdhR sites. A DNA fragment representing intergenic DNA between the xdhA gene and the xdhR gene as well as part of each coding sequence was amplified using a primer that introduces 6-FAM at the 5'-end of the top strand (the xdhR coding strand; 271 bp). An automated capillary sequencer was used to separate the digested fragments and the fragments were analyzed using GeneMapper software; protected sequences were identified by comparison to dideoxy cycle sequencing reactions performed with the same 6-FAM-labeled primer. XdhR caused altered DNaseI cleavage patterns across a region just upstream of the transcriptional start of the XdhR gene, spanning from 96 bp to 152 bp relative to the translational start of the xdhA gene defined as position 1 (Fig. 2.5). This region appears to represent two adjacent XdhR operator sites; a preferred Site 1 showing protection from positions 128 to 149, a site that overlaps the identified 18 bp palindromic sequence, with protection followed by a hypersensitive site 7 bp downstream of the palindrome. An adjacent Site 2 showed partial protection at lower protein concentration (Fig. 2.5A) and complete protection when the protein concentration was doubled (from 0.14 µM to 0.29 µM; Fig. 2.5B). Site 2 protection spanned positions 98 to 119, and the protection was immediately followed by another hypersensitive site, also 7 bp downstream of an 18 bp sequence that vaguely resembles the Site 1 palindrome. That protection of Site 2 gradually increased with increasing protein concentration suggests that a second XdhR is recruited.
Figure 2.5. XdhR protects several sites in the xdhA-xdhR intergenic DNA. (A)-(C) Electropherogram traces of DNaseI digestion of fluorescently labelled DNA without protein (blue) overlayed with those of DNA incubated with XdhR (red). (A) 0.14 µM XdhR. The region encompassing sites in the xdhR promoter is shown, with the identified palindromes marked in red. (B) 0.29 µM XdhR. The region encompassing sites in the xdhR promoter is shown; the inset shows the entire intergenic DNA with preferred Site 1 and Site 2 underlined. The identified palindromes are in red. (C) 1.43 µM XdhR. The entire intergenic region is shown. The translational start of xdhA is defined as position 1. The transcriptional start sites are indicated by blue arrows at positions 19 (xdhA) and 171 (xdhR). Blue stars mark every 10 bp in (A) and (B) and every 50 bp in (C). The underlined regions are the XdhR protected sites.

Sites 1 and 2 are immediately upstream of the xdhR transcriptional start at position 171 (Fig. 2.5C, blue arrow), consistent with XdhR repressing transcription of xdhR. When the protein concentration was increased 10-fold (to 1.43 µM), a partially protected area appeared,
spanning a wide region that includes the transcriptional (position 19; indicated by blue arrow) and translational (defined as position 1) start sites of the \( xdhA \) gene (Fig. 2.5C). Consistent with additional regions of DNA protection at a greater ratio of XdhR:DNA, a third complex was detected in EMSAs at higher protein concentrations (Figure 2.6), with both observations indicating lower affinity binding to this site. We also verified XdhR binding to the individual operator sites identified by footprinting; EMSA with 32 bp DNA constructs representing the 18 bp palindromic sequences flanked by 7 bp on either side revealed a single complex and equivalent affinity for Sites 1 and 2 (Kd = 0.4 ± 0.1 nM and 0.4 ± 0.1 nM, respectively; Figure 2.7). Evidently, the affinity of XdhR for each site is identical. This suggests that preferential protection of Site 1 is due to the previously noted negative cooperativity of binding.

Figure 2.6. EMSA showing formation of a third complex with increasing concentration of XdhR in lanes 2 through 15 – 1 pM, 10 pM, 100 pM, 250 pM, 500 pM, 750 pM, 1 nM, 2 nM, 5 nM, 10 nM, 50 nM, 100 nM, 500 nM and 1 μM titrated with labeled full length intergenic DNA (0.05 nM).

XdhR binding to intergenic DNA is attenuated by ppGpp

Xdh participates in the purine metabolic pathway (Fig. 2.1A). Considering that it is very common for transcriptional regulators that control expression of genes encoding metabolic enzymes to respond to intermediates in the corresponding metabolic pathways, we examined the ability of compounds associated with purine metabolism to function as ligands for XdhR. GTP, GDP, GMP, guanosine, xanthine, hypoxanthine, urate, and adenine were used in EMSA in order to observe their effect on DNA-protein binding. GTP was found to be a very effective ligand of XdhR; GDP,
GMP and xanthine very modestly affected the DNA-protein binding complex (Fig. 2.9A-C and Fig. 2.10), whereas no effect of guanosine, hypoxanthine, urate or adenine was observed (data not shown).

Figure 2.7. EMSA showing binding affinity of XdhR to (A) site 1 and (B) site 2 of the protected region near the xdhR gene. Lane 1 – labeled DNA only; XdhR concentration in lanes 1 through 14 – 0, 1 pM, 5 pM, 7.5 pM, 10 pM, 20 pM, 40 pM, 60 pM, 80 pM, 100 pM, 200 pM, 1 nM, 5 nM, 10 nM. (C) and (D). % complex formation plotted against XdhR concentration for site 1 (C) and site 2 (D).

The $K_i$ of GTP was $2.0 \pm 0.1$ mM, for GMP $IC_{50}$ was $11.4 \pm 1.3$ mM and xanthine inhibited complex formation with $K_i$ of $17.5 \pm 3.2$ mM (Fig. 2.9 and Table 1). We note that the effect of xanthine was estimated in high ionic strength buffer (0.5 mM Tris), precluding a direct comparison with the other ligands. This caveat notwithstanding, our data suggest that the substrates for Xdh,
hypoxanthine and xanthine, are unlikely to serve as ligands for XdhR and as inducers of \textit{xdhABC}

Based on the observation that the oxidants \textit{H}_2\textit{O}_2, cumene hydroperoxide and tertiary
butyl hydroperoxide induced dimerization of XdhR \textit{in vitro} (Fig. 2.2A), we examined binding of
XdhR to its cognate DNA after oxidation. EMSA showed that complex formation with oxidized
XdhR was only very modestly attenuated (Fig. 2.8). This suggests that XdhR functions to repress
gene \textit{xdhABC} regardless of redox state.

![Figure 2.8. EMSA showing modest attenuation of DNA-XdhR binding by (A) Hydrogen peroxide
\textit{(H}_2\textit{O}_2\textit{)} and (B) Cumene hydroperoxide. DNA concentration – 0.05nM; XdhR concentration – 9nM. Attenuation of DNA-protein could be seen only at a concentration of 120\textmu M \textit{H}_2\textit{O}_2 (Panel A last lane) and very modest attenuation of DNA-protein binding even with 90\textmu M cumene hydroperoxide (Panel B last lane).](image)

Considering that GTP was most efficient at attenuating DNA binding by XdhR, with no effect of
guanosine and a very modest effect of GDP and GMP, we surmised that a highly
phosphorylated ligand is preferred. Secondly, given the previous observation that \textit{xdhABC} is
upregulated during stationary phase when GTP levels are reduced, we also reasoned that GTP
cannot be the physiologically relevant ligand during stationary phase growth. We therefore
wondered if (p)ppGpp might serve as a ligand for XdhR; (p)ppGpp structurally resembles GTP,
and it has been documented that it accumulates during stationary phase growth in \textit{S. coelicolor}
while GTP pools decrease (Ochi, 1987, Strauch \textit{et al.}, 1991). Notably, we found that ppGpp
attenuated XdhR-DNA complex formation modestly better than GTP with an \textit{K}_i of 2.1±0.6 \textmu M.
That ppGpp attenuated complex formation much more efficiently than GDP also indicates that a 3'-phosphate is important for interaction with XdhR.

Figure 2.9. Attenuation of XdhR-DNA binding by ligands. The first lane of each gel has DNA only. Remaining reactions contained a constant protein concentration: (A), (D) – 0.5 nM; (B), (C) – 1.5 nM. (A) Increasing concentrations of xanthine (0 – 30 mM). (B) Increasing concentrations of GMP (0 – 20 mM). (C) Increasing concentrations of GTP (0 – 6 mM). (D) Increasing concentrations of ppGpp (0 – 5 mM). (E) Percent complex as a function of ppGpp concentration.

Figure 2.10. EMSA with increasing concentration of GDP titrated with DNA-XdhR complex. [XdhR] – 0.5 nM and [DNA] – 0.05 nM.
In vivo regulation of the $xdhABC$ gene cluster under conditions of ppGpp accumulation

Of the ligands tested in vitro for their ability to attenuate DNA binding by XdhR, ppGpp was the most efficient. We therefore wanted to examine expression of the $xdhABC$ gene cluster under conditions of (p)ppGpp accumulation. Serine hydroxamate has been used to mimic amino acid starvation in $S. coelicolor$ by inhibiting charging of seryl-tRNA synthetase, and its addition has been shown to lead to ppGpp accumulation (Strauch et al., 1991, Takano et al., 1992). To examine if expression of the $xdhABC$ gene cluster is altered on addition of serine hydroxamate, an exponential phase culture of $S. coelicolor$ was treated with 30 mM serine hydroxamate and RNA was isolated. Approximately 1 µg of RNA was converted to cDNA, and primers amplifying a region of the $xdhB$ gene were chosen for the semi-quantitative PCR reaction. The $rpoA$ gene ($SCO4929$), encoding the $\alpha$-subunit of RNA polymerase, was used as a control; $rpoA$ is transcribed as part of an operon containing ribosomal protein genes (Charaniya et al., 2007). In cultures treated with serine hydroxamate for 15 min, expression of $rpoA$ was reduced

Table 1. $K_i$ of ligands that attenuate DNA-XdhR binding. Values are mean ± standard deviation of two experiments.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K_i$ (mM)</th>
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<tbody>
<tr>
<td>ppGpp</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>GTP</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>GMP</td>
<td>11.4 ± 1.3</td>
</tr>
<tr>
<td>Xanthine</td>
<td>17.5 ± 3.2</td>
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approximately nine-fold compared to control cultures (Fig. 2.11). This is consistent with the observation from several bacterial species that rpoA expression is reduced when the stringent response is induced either by entry into stationary phase or by addition of serine hydroxamate or norvaline that mimic specific amino acid starvation (Chang et al., 2002, Eymann et al., 2002, Brockmann-Gretza & Kalinowski, 2006, Kazmierczak et al., 2009). By comparison, expression of xdhB was only reduced ~2.8-fold in presence of serine hydroxamate. Relative to the expression of rpoA, expression of xdhB was therefore upregulated approximately $4.7 \pm 1.4$ fold in the serine hydroxamate-treated cells (Fig. 2.11).

![Figure 2.11](image)

Figure 2.11. Induction of xdhB expression relative to rpoA expression upon exposure to serine hydroxamate and during stationary phase. (A) Change in expression level of xdhB in exponentially growing cells exposed to serine hydroxamate (SH) and during stationary phase (SP) from three replicates. (B)-(C) Amplification of target regions from the xdhB gene and the rpoA gene using cDNA templates obtained from unsupplemented exponential phase cultures (EP), serine hydroxamate (SH)-treated exponential phase culture, and unsupplemented stationary phase (SP) culture. Gels were stained with ethidium bromide.

It was previously reported that expression of xdhA in surface-grown S. coelicolor cultures varies with morphological development, peaking at times coinciding with aerial hyphae formation (Hillerich & Westpheling, 2008). A comparison of gene expression in exponential
phase and stationary phase cultures revealed that the \textit{xdhB} gene was overexpressed 27.2 ± 5.6 fold (Fig. 2.11) during stationary phase (48 hours) compared to its expression during exponential phase. That \textit{rpoA} expression did not appear reduced in stationary phase may be a consequence of having saturated the amplification; assuming reduced \textit{rpoA} expression in stationary phase, the observed overexpression of \textit{xdhB} may therefore be underestimated.

Nutritional starvation, a hallmark of stationary phase growth, induces the stringent response due to the accumulation of (p)ppGpp (Ochi, 1987, Strauch \textit{et al.}, 1991). The observed increase in \textit{xdhB} expression during stationary phase is therefore consistent with (p)ppGpp being an inducer of \textit{xdhABC} gene expression.

Expression of \textit{xdhABC} is decreased in cells exposed to the Xdh inhibitor allopurinol

Genes encoding Xdh are upregulated during the stringent response in \textit{S. coelicolor} and \textit{Sinorhizobium meliloti} (Hillerich \& Westpheling, 2008, Krol \& Becker, 2011). It has also been reported that inactivation of an enzyme in the purine salvage pathway in the human pathogen \textit{L. monocytogenes} resulted in failure to accumulate (p)ppGpp, suggesting that purine salvage pathways are essential for (p)ppGpp production (Taylor \textit{et al.}, 2002). This is consistent with inferences derived from analyses in \textit{E. coli} that Xdh biases purine salvage towards GMP (and hence GTP, the substrate for (p)ppGpp synthetases; Fig. 2.1A) and away from AMP (Xi \textit{et al.}, 2000). We therefore reasoned that inhibition of Xdh might attenuate GTP (and (p)ppGpp) production, which in turn should lead to reduced \textit{xdhABC} expression. We investigated the expression of \textit{xdhABC} upon treatment with allopurinol, which is oxidized by Xdh to alloxanthine (oxypurinol) that in turn acts as a tight binding inhibitor of the enzyme (Truglio \textit{et al.}, 2002). \textit{S. coelicolor} cells were grown until exponential phase and treated with allopurinol for 30 minutes.
RNA was isolated and the transcript level of the *xdhB* gene was monitored using semi-quantitative PCR. Our results revealed that the *xdhB* gene was down-regulated 6.7-fold compared to its expression during exponential phase (Fig. 2.11). Evidently, interfering with the purine salvage pathway resulted in significant repression of *xdhABC*, an outcome that would be expected if the inducer of *xdhABC* expression (the ligand for XdhR) were depleted. The reported requirement for purine salvage pathways for (p)ppGpp production would be consistent with depletion of GTP and (p)ppGpp under conditions of reduced Xdh activity.

**Figure 2.12.** Expression of *xdhB* upon allopurinol treatment. (A) Change in *xdhB* transcript levels upon treating exponentially growing *S. coelicolor* cells with allopurinol. Mean and standard deviation were obtained from three experiments. (B) Agarose gels showing amplification of targets from the *xdhB* gene and the *rpoA* gene in unsupplemented exponential phase culture (EP) and allopurinol-supplemented exponential phase culture (AP). Gels were stained with ethidium bromide.

Expression of *xdhB* correlates with (p)ppGpp accumulation

The level of (p)ppGpp *in vivo* under various experimental conditions was assessed using thin layer chromatography. In exponentially growing *S. coelicolor* cells treated with serine hydroxamate, the expected accumulation of (p)ppGpp was seen (Fig. 2.13A). Similarly, accumulation of (p)ppGpp was confirmed during stationary phase (Fig. 2.13B). Examination of levels of pppGpp and ppGpp suggests that serine hydroxamate induces a lower level of pppGpp
(~10% of total (p)ppGpp) compared to stationary phase, in which ~30% of total (p)ppGpp is pppGpp. By contrast, the level of (p)ppGpp was reduced in cells treated with 10 mM allopurinol for 30 minutes compared to cells to which the solvent 0.4 M NaOH was added (Fig. 2.13C).

![Figure 2.13. Accumulation of (p)ppGpp. (A) Exponential phase culture (lane 1) and after serine hydroxamate treatment (30 mM for 15 minutes; lane 2). (B) Exponential (lane 1) and stationary phase (96 hours growth in 2XLB; lane 2). (C) Exponential phase culture supplemented with NaOH (lane 1) and allopurinol treatment (10 mM for 30 minutes; lane 2)]]

Discussion

XdhR binds preferentially to the xdhR gene promoter

TetR family proteins typically function as homodimers in which each monomer contributes a helix-turn-helix DNA binding domain. This binding mode entails interaction of DNA recognition helices with consecutive DNA major grooves as exemplified for *E. coli* TetR, which regulates expression of a gene encoding a tetracycline efflux pump. Binding of tetracycline results in a movement of helix four that creates a greater separation between DNA-binding domains that is incompatible with DNA binding (Orth *et al.*, 2000). While proteins such
as *E. coli* TetR bind 15-17 bp cognate sites as a dimer, several TetR proteins bind DNA as a dimer of dimers. One example is *S. aureus* QacR, which binds a longer 28 bp cognate site with two dimers docking to opposite faces of the DNA duplex. In absence of DNA, QacR exists as a dimer, and even when bound to DNA, there are no direct contacts between individual dimers (Schumacher et al., 2002). Considering the identification of 18 bp palindromic sequences in the *xdhABC-xdhR* intergenic region and the extent of the footprint, we find it most likely that the XdhR binding mode resembles that of *E. coli* TetR, with one XdhR dimer binding the DNA and the *trans*-dimer “piggybacking” (Figure 2.14). In addition, the identified association of QacR dimers on opposite faces of the DNA, and the existence of QacR as a dimer in absence of DNA, does not correspond to the observed XdhR tetramer; the predicted location of cysteine residues and the efficient formation of disulfide-bonded *trans*-dimers argues against a tetramer in which dimers associate with opposing DNA binding domains. That XdhR oxidation does not significantly affect DNA binding is also consistent with this interpretation. We also note that binding of a single TetR dimer introduces more significant DNA bending, while TetR proteins that bind as a dimer of dimer to opposite faces of the duplex generally introduce more subtle DNA bends (Orth et al., 2000, Schumacher et al., 2002, Miller et al., 2010, Le et al., 2011). The pronounced hypersensitive site induced on XdhR binding suggests DNA bending or distortion.

![Figure 2.14. Model of XdhR tetramer binding to DNA upstream of xdhABC and xdhR genes in *S. coelicolor* involving looping of the bound DNA.](image)

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Only *P. aeruginosa* MexL was reported to exist as a stable tetramer in absence of DNA, and DNaseI footprinting revealed the concerted protection of an approximately 60 bp region, suggesting side-by-side binding of two MexL dimers (Chuanchuen et al., 2005). By contrast, XdhR protects its preferred site in the *xdhR* gene promoter in a step-wise fashion, suggesting that Site 1 corresponds to a preferred site. Since XdhR has equivalent affinity for Sites 1 and 2, each site would have an equal probability of binding XdhR; that we observe preferential protection of Site 1 may be due to the previously observed negative cooperativity of binding. Assuming that an XdhR dimer binds each palindrome with the “piggybacked” dimer imposing asymmetry, and since both palindromes are also asymmetrical, we propose that binding of XdhR to Site 1 renders binding of a second XdhR binding to Site 2 less favorable, whereas binding of XdhR to Site 2 still allows a second XdhR access to Site 1. This scenario would rationalize both the observed negative cooperativity and the preferred protection of Site 1. The 32 bp distance between the centers of the Site 1 and Site 2 palindromes suggests that two XdhR tetramers bind on the same face of the DNA duplex, an inference that is also supported by the symmetrical induction of hypersensitive sites downstream of each protected region (Fig. 2.5).

XdhR represses its own expression (Hillerich & Westpheling, 2008), and the location of preferred Sites 1 and 2 in the *xdhR* gene promoter is consistent with XdhR repressing expression by preventing RNA polymerase binding to the promoter. This site, however, is ~75 bp upstream of the transcriptional start site of *xdhABC*, and XdhR binding to this site would not be consistent with repression of *xdhABC* expression. With increasing protein concentration, an extended region upstream of the preferred Sites 1 and 2 is protected that overlaps the transcription and translation start sites of *xdhABC* (Fig. 2.5C). The observation that XdhR-
mediated protection spreads only in one direction relative to the preferred Site 1 offers a possible rationale for XdhR existing as a tetramer. If XdhR bound as a symmetrical homodimer, directed accretion of additional protein dimers would require the presence of cognate DNA sites, whereas the formation of a tetrameric protein assembly would break the symmetry and permit recruitment of XdhR only upstream of the preferred site.

Alternatively, it is tempting to speculate that DNA looping \textit{in vivo} may result in tetrameric XdhR simultaneously binding cognate sites in both \textit{xdhABC} and \textit{xdhR} promoters; such looping would require the assistance of a DNA-bending protein such as HU (akin to formation of the Gal repressosome (Roy \textit{et al.}, 2005). DNA looping would increase the local concentration of XdhR and perhaps explain the failure of XdhR expressed in \textit{trans} to rescue the \textit{xdh}R\textit{Δ} phenotype (Hillerich & Westpheling, 2008), and it would facilitate concerted regulation of \textit{xdhABC} and \textit{xdhR} genes.

XdhR ligand accumulates during stationary phase growth

Xdh is required for purine salvage pathways regardless of growth phase. Since the reaction catalyzed by Xdh is considered to be the rate-limiting step in purine salvage, upregulation of \textit{xdh} would be expected to promote this pathway. It was previously observed that expression of the \textit{S. coelicolor xdhA} and \textit{xdhR} genes is low at 24 hours, then gradually increases and peaks around 48 hours in cells grown on solid MYM agar, corresponding to morphological development and formation of aerial hyphae (Hillerich & Westpheling, 2008). It was further reported that \textit{xdhA} expression is constitutive in the \textit{xdhRΔ} strain, indicating that XdhR is responsible for mediating the differential expression during exponential and stationary phase growth and that \textit{xdhABC} expression is unlikely to be regulated by other transcription
factors under these conditions. Consistent with this observation, we found that \textit{xdhB} expression is increased \textasciitilde27-fold during stationary phase growth in liquid culture compared to its expression during exponential phase (Fig. 2.11). These observations clearly indicate that the ligand for XdhR and the inducer of \textit{xdhABC} expression accumulates in stationary phase.

The signaling molecule ppGpp is a ligand for XdhR

In \textit{S. coelicolor}, morphological differentiation in surface-grown cultures and stationary phase growth in liquid media is linked to secondary metabolism and to the production of the diffusible messenger (p)ppGpp. The correlation between timings of (p)ppGpp production and \textit{xdhABC} expression motivated an analysis of the interaction between ppGpp and XdhR. Consistent with the interpretation that ppGpp is a ligand for XdhR, we find that DNA binding is attenuated \textit{in vitro} in the presence of ppGpp. Cellular accumulation of ppGpp in Streptomyces has been reported to reach \textasciitilde500 pmol mg\(^{-1}\) dry weight (Chakraburty \& Bibb, 1997, Hesketh \textit{et al.}, 2007, Ochi, 1987). \textit{E. coli} accumulates mM concentrations of ppGpp during the stringent response, and estimates based on cell volume and mass suggest that 1 mM ppGpp corresponds to \textasciitilde3,000 pmol mg\(^{-1}\) dry weight (Cashel, 1975, Riesenberg \textit{et al.}, 1984). The mass of the average \textit{S. coelicolor} cell is not known, mainly because it is difficult to define dimensions of a cell that propagates by filamentous growth. Assuming comparable volumes, \textit{S. coelicolor} may accumulate lower levels of (p)ppGpp compared to \textit{E. coli}, although it has also been suggested that local concentrations may be higher (Gatewood \& Jones, 2010, Riesenberg \textit{et al.}, 1984). These considerations suggest that an IC\(_{50}\) of \textasciitilde2 mM for ppGpp is physiologically relevant.

\textit{In vivo}, expression of \textit{xdhB} is upregulated under conditions of (p)ppGpp accumulation. Serine hydroxamate induces the stringent response in \textit{S. coelicolor} by accumulating ppGpp to
its highest level within 15 minutes of its addition (Strauch et al., 1991). Our results revealed that $xdhB$ gene expression is increased ~5-fold upon addition of serine hydroxamate when compared to expression of the $rpoA$ gene. Expression of $rpoA$ was significantly repressed on addition of serine hydroxamate, which is consistent with the $rpoA$ gene being encoded as part of an operon that includes ribosomal protein genes and with the significant repression of ribosomal protein gene expression observed in several bacterial species during the stringent response (Chang et al., 2002, Eymann et al., 2002, Strauch et al., 1991, Brockmann-Gretza & Kalinowski, 2006, Kazmierczak et al., 2009). Notably, accumulation of ppGpp was reported to be ~5-fold higher during stationary phase than during a stringent response initiated by addition of serine hydroxamate (Strauch et al., 1991). This is in accord with our finding that the expression of $xdhB$ increases an additional ~6-fold during stationary phase compared to treatment with serine hydroxamate (Fig. 2.11).

Proteins that are direct targets for ppGpp include cellular GTPases, metabolic proteins involved in nucleotide and lipid metabolism and PLP-dependent basic aliphatic amino acid decarboxylases. In most instances, the binding of ppGpp causes enzyme inhibition. Most of the GTPases that are potential binding targets of ppGpp bind better to GTP than ppGpp in order to make the inhibitory effect of ppGpp reversible (Kanjee et al., 2012). This is in contrast to XdhR, for which GTP and ppGpp appear to serve equivalent roles. Although increased ppGpp levels lead to expression of genes under the control of CodY protein in $B. subtilis$, this regulation was attributed to the decrease in the GTP pool (Geiger & Wolz, 2014). FadR is a transcriptional repressor that binds ppGpp, however, in this case ppGpp/DksA function to inhibit FadR-activated promoters encoding proteins involved in fatty acid synthesis in $E. coli$ (My et al.,
Although several proteins have been found to bind ppGpp, there are no examples so far of direct binding of ppGpp to a transcriptional repressor serving to induce gene activity. Our data suggest that ppGpp binds to XdhR and thereby relieves its repression of the xdhABC gene cluster.

Regulation of xdhABC links purine salvage pathways to stringent response

Regulating expression of genes encoding enzymes of the purine metabolic pathway by regulators that respond to pathway intermediate is not uncommon. For example, Deinococcus radiodurans contains divergently oriented genes encoding uricase and the transcriptional regulator HucR, with HucR tightly controlling uricase gene expression; upon binding of urate to HucR, derepression of the divergently oriented genes is observed (Wilkinson & Grove, 2004). In E. coli, guanine and hypoxanthine function as corepressors of the purine repressor PurR to repress the pur operon (Meng & Nygaard, 1990). However, we found no evidence for Xdh substrates or their precursors significantly affecting DNA binding by XdhR. This suggests that xdhABC activity is not regulated simply to ensure degradation of excess purines.

While ppGpp most efficiently attenuates DNA binding by XdhR, GTP also reduces DNA binding. During exponential growth, cellular levels of GTP reach mM concentrations, whereas (p)ppGpp levels are almost negligible. By contrast, entry into stationary phase is associated with a significant reduction in GTP concentrations as (p)ppGpp levels increase (Ochi, 1987, Strauch et al., 1991). The observed Kᵢ for GTP of 2.0 mM is therefore physiologically relevant during exponential growth. As noted above, however, the significantly increased xdhB expression in stationary phase argues against GTP serving as the ligand under these conditions. It is therefore conceivable that GTP functions as an XdhR ligand during exponential phase growth to induce
basal levels of $\textit{xdhABC}$ expression and maintain a functional purine salvage pathway. When (p)ppGpp accumulates during the stringent response and GTP levels are reduced, the higher affinity ligand more efficiently induces $\textit{xdhABC}$ expression. Considering that GTP attenuates DNA binding much more efficiently than GMP and GDP (Fig. 2.7B & 2.9B), suggesting that the presence of a triphosphate enhances affinity, we also entertain the possibility that pppGpp may induce gene expression more efficiently than ppGpp. Thin layer chromatography results revealed that accumulation of pppGpp is approximately three fold higher in stationary phase than upon treatment with serine hydroxamate (Fig. 2.11). This suggests that it is not surprising to see increased $\textit{xdhB}$ gene expression during stationary phase when compared to stringent response (Fig. 2.11).

GTP is the substrate for (p)ppGpp synthetases, rationalizing the role of purine salvage pathways in sustaining (p)ppGpp synthesis. Allopurinol is widely used for inhibiting Xdh, including bacterial homologs (Truglio et al., 2002). Therefore, inhibiting this enzyme would be expected to attenuate purine salvage and in turn GTP production. Consistent with a role for GTP as an XdhR ligand during exponential phase, we observed significant repression of $\textit{xdhB}$ gene activity on addition of allopurinol (Fig. 2.12). Taken together, our data suggest that the purine salvage pathway is required to maintain the GTP pool during both exponential and stationary phase growth.

The roles of (p)ppGpp in regulating gene activity have been extensively characterized in $\textit{E. coli}$, where the alarmone has multiple direct targets. However, in Gram positive bacteria, (p)ppGpp may function indirectly by inhibiting enzymes required for GTP synthesis, thereby controlling GTP homeostasis. For example, $\textit{Bacillus subtilis}$ that is deficient in (p)ppGpp
synthesis experiences perturbed GTP homeostasis and may suffer a rise in intracellular GTP that leads to cell death (Kriel et al., 2012). It was also observed that deletion of S. coelicolor xdhR results not only in constitutive xdhA expression, but in a bld phenotype, proposed to be due to abnormal GTP levels (Hillerich & Westpheling, 2008). Xdh activity is required for purine salvage pathways, and it biases the pathway towards formation of guanine (Xi et al., 2000). The significant upregulation of S. coelicolor xdhABC during the stringent response may therefore serve to ensure that sufficient GTP is available for continued (p)ppGpp synthesis and to maintain GTP homeostasis.

Conclusion

S. coelicolor XdhR is the first transcriptional repressor shown to bind to either GTP or ppGpp to cause attenuated DNA binding in vitro and depression of gene activity in vivo. We propose that during exponential growth, GTP elicits basal levels of xdhABC expression, while accumulation of the higher affinity ligand (p)ppGpp during the stringent response results in significant upregulation. The depletion of GTP as a consequence of (p)ppGpp synthesis rationalizes the need to promote the purine salvage pathway; our data reinforce the link between purine salvage and GTP homeostasis and implicate both GTP and (p)ppGpp in promoting this pathway.

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CHAPTER 3
XANTHINE DEHYDROGENASE REGULATOR PROTEIN OF Agrobacterium tumefaciens

Introduction

Species in the Agrobacterium genus are members of the class α-proteobacteria within the phylum proteobacteria that includes several nitrogen fixing symbionts of leguminous plants. A. tumefaciens (A. fabrum) causes the disease crown gall (tumorous nodules) in various economically important plants such as grapes, cherries, walnuts, sugarbeets, shrubs (rose plants) and others. Though this organism has been studied for decades, its pathogenicity remains uncontrolled and it is therefore considered the third most significant plant pathogen (Mansfield et al., 2012).

There are several unique features of this bacterium that enable it to thrive in a host under stress and starvation conditions. Transfer of a large segment of the tumor-inducing Ti plasmid (the T-DNA) into the plant genome such that the transformed host cell expresses T-DNA genes is among those (Platt et al., 2014). This organism can sense plant-derived molecules or other environmental conditions and can fine-tune its cellular machineries to ensure expression of Ti- or chromosomally-encoded virulence genes only upon host infection. Environmental conditions that contribute to virulence gene expression are acidic pH (~5.5) and limiting inorganic phosphate (Li et al., 2002, Xu et al., 2012).

In bacteria, unfavorable conditions also trigger the stringent response, which is associated with production of the phosphorylated guanosine nucleosides 5′-triphosphate-3′-diphosphate (pppGpp) and 5′-diphosphate-3′-diphosphate (ppGpp), together referred-to as (p)ppGpp.
Production of (p)ppGpp has been mainly characterized in *Escherichia coli*, where it is produced by the paralogous enzymes RelA and SpoT. Less is known about (p)ppGpp synthesis and regulatory roles in α-proteobacteria, which appear to encode a single dual-function Rsh (RelA/SpoT homology) enzyme that can both synthesize and degrade (p)ppGpp (Mittenhuber, 2001). In *Sinorhizobium meliloti*, a nitrogen fixing α-proteobacterium that forms root nodules on leguminous plants, (p)ppGpp has been shown to exert global roles on transcription and to be required for formation of root nodules (Vercruysse et al., 2011, Wells & Long, 2002). Notably, induction of (p)ppGpp is most effectively induced by starvation for carbon or nitrogen and not by starvation for a few amino acids or by addition of serine hydroxamate, which mimics starvation for serine by inhibiting charging of tRNA (Belitsky & Kari, 1982, Krol & Becker, 2011).

The stringent response includes inhibition of transcription, translation, replication and other cellular functions. Depending on species, this inhibitory effect is achieved by the binding of (p)ppGpp to various proteins including RNA polymerase, initiation and elongation factors involved in translation, enzymes involved in DNA replication, enzymes involved in biosynthesis of guanosine nucleotides, and transcriptional repressor proteins such as CodY and FadR (My et al., 2013, Geiger & Wolz, 2014, Kriel et al., 2014, Gaca, 2015). In *S. meliloti*, suppressors of the (p)ppGpp⁰ phenotype map to *rpoB* and *rpoC*, which encode the β and β' subunits of RNA polymerase, suggesting direct interaction with RNA polymerase (Wells & Long, 2002).

Transcriptional regulators are vital in communicating environmental cues to cellular machineries and enabling bacteria to overcome inhospitable situations. The TetR family of proteins comprises such transcriptional regulators and they are widely present in prokaryotes. They regulate various functions of the cell including metabolism, antibiotic resistance, oxidative
stress, and virulence (Cuthbertson & Nodwell, 2013, Ramos et al., 2005). In this chapter, I will present the characterization of one such TetR family regulator, the xanthine dehydrogenase regulator (XdhR). In *Streptomyces coelicolor*, which belongs to the phylum actinobacteria, the gene encoding XdhR is divergently oriented to the *xdhABC* gene cluster encoding xanthine dehydrogenase (Xdh) (Hillerich & Westpheling, 2008). A similar gene orientation is also found in *A. tumefaciens*, suggesting conservation of function. Xdh participates in purine metabolism in the conversion of hypoxanthine to xanthine and xanthine to urate (Enroth et al., 2000). In *S. coelicolor*, XdhR functions as a repressor of both *xdhR* and *xdhABC* expression (Hillerich & Westpheling, 2008) and as described in Chapter 2, DNA binding by *S. coelicolor* XdhR is attenuated most efficiently by highly phosphorylated guanosine nucleotides, and *xdhABC* is most highly induced in stationary phase.

The purine salvage pathway serves to recycle purines (adenine and guanine) that are released during cell metabolism. This is important for energy conservation since *de novo* purine synthesis requires bacterial cells to utilize more energy. The purine salvage pathway is essential for interconversion of purines. In *E. coli*, a mutant of *xdh* is impaired in converting adenine to guanine during purine salvage (Xi et al., 2000). Though other enzymes of purine metabolism such as hypoxanthine phosphoribosyl transferase (Hprt), guanine phosphoribosyl transferase (Gprt), adenine phosphoribosyl transferase (Aprt), IMP dehydrogenase (GuaB) and GMP synthase (GuaA) participate in the *de novo* pathway, their role in salvaging purines is also critical in several bacteria. *Erwinia amylovora* a Gram negative plant pathogen requires the enzyme GuaB for its pathogenicity. Lack of *guaB* gene activity by mutation gives rise to a non-pathogenic strain that is incapable of causing fireblight disease (Eastgate et al., 1997). Human
pathogens such as *Borrelia burgdorferi* (Fraser et al., 1997) and *Helicobacter pylori* (Liechti & Goldberg, 2012) lack enzymes of the *de novo* purine synthesis pathway and rely on purine salvage enzymes GuaA, GuaB and Gprt, respectively, for utilization of purines from the host. Thus purine salvage enzymes become essential for these bacterial pathogens infecting either plants or animals.

In *S. meliloti*, expression of the gene encoding Xdh is increased upon carbon or nitrogen starvation, conditions that are associated with accumulation of (p)ppGpp. Moreover, the *xdh* gene is upregulated in a RelA- and DksA-dependent manner (Krol & Becker, 2011). Also, in *S. coelicolor*, the *xdh* gene is upregulated during stationary phase when (p)ppGpp levels are highest (Hillerich & Westpheling, 2008, Ochi, 1987). Since *xdh* expression is induced during stringent response in both *S. meliloti* and *S. coelicolor*, and since *A. tumefaciens* conserves the genomic locus consisting of divergently oriented *xdhABC* and *xdhR* genes, I examined the link between the stringent response signal (p)ppGpp with XdhR function in *A. tumefaciens*.

Production of exopolysaccharides is associated with virulence in terms of cell adhesion and biofilm formation. It becomes critically important to conserve nutrients under starvation or stringent conditions during exopolysaccharide synthesis since this requires a significant amount of the cell’s resources. In *Vibrio cholerae*, production of exopolysaccharides is induced by c-di-GMP signaling (Tischler & Camilli, 2004, Beyhan et al., 2006, Lim *et al.*, 2007, Srivastava *et al.*, 2013). In *Agrobacterium* sp. ATCC 31749, production of the exopolysaccharide curdlan is dependent on (p)ppGpp and c-di-GMP signaling. Mutants incapable of generating these two signaling molecules show a sharp decrease in the production of curdlan (Ruffing & Chen, 2012). Owing to a similar phenotype that is produced in the absence of (p)ppGpp and c-di-GMP it is
possible that both of these signaling molecules share a function that is essential during starvation. Generation of these signaling molecules requires GTP in a situation when the cells are already starving. Thus increasing the activity of enzymes of the purine salvage pathway might facilitate an efficient response to the stress or starvation situation. Therefore I also wanted to determine if there is link between the purine salvage pathway and c-di-GMP signaling in A. tumefaciens.

**Experimental procedures**

Cloning and protein purification

The Atu5496 (xdhR) gene from Agrobacterium tumefaciens C48 was amplified from genomic DNA using primers 5'-AGCTATCATGACTTCATGCTGACTGAC-3' and 5'-CAGCTTAAGCTCTAAGCTACGCT-3' (restriction sites underlined). The PCR product was cloned into pET28b between NdeI and XhoI restriction sites and the resultant plasmid was transformed into E. coli TOP10 cells. Plasmid isolated from E. coli TOP10 was sequenced to confirm integrity of the clone. This plasmid was then transformed into E. coli BL21(DE3) for overexpression of protein with an N-terminal His<sub>6</sub>-tag. These cells were grown in LB media containing 30 µg mL<sup>-1</sup> kanamycin and XdhR expression was induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). Six hours after induction, cells were pelleted and stored at -80°C. The cells were resuspended in lysis buffer (20 mM sodium phosphate pH 8.0, 150 mM sodium chloride, 5% glycerol, 0.15 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM β-mercaptoethanol) and lysozyme was added to 200 µg mL<sup>-1</sup>. The cells were disrupted by sonication on ice. The lysed cells were centrifuged at 15,000 rpm for 1 hour and the lysate was incubated with 1 mL of HIS Select Nickel affinity gel for 30 minutes. The His<sub>6</sub>-tagged protein was purified according to the
manufacturer’s instructions (Sigma). Buffer exchange into storage buffer (20 mM sodium phosphate pH 8.0, 150 mM NaCl, 20% glycerol, 0.15 mM PMSF and 10 mM β-mercaptoethanol) and concentration of the sample was achieved using 10K centrifugal filter units (Millipore). Purified XdhR concentration was estimated using MicroBCA protein assay kit.

Gel filtration

A Superose column (GE Healthcare) was pre-equilibrated with buffer pH 8.0 (50 mM Tris and 150 mM NaCl). The protein sample was diluted in the same buffer and loaded on to the fast protein liquid chromatography system. BioRad gel filtration standards bovine serum albumin (66.0 kDa), ovalbumin (44.0 kDa), myoglobin (17.0 kDa), and vitamin B12 (1350 Da) were run on the same column and a standard curve was plotted using the respective elution volumes obtained for each standard. The formula $K_{av} = (VE-VO)/(VT-VO)$ was used to calculate the average molecular weight of the protein where VE is retention volume of the protein, VO is void volume of the column and VT is bed volume of the column.

Electrophoretic Mobility Shift Assay (EMSA)

A PCR product of 214 bp corresponding to the intergenic region between the xdhABC gene cluster and the xdhR gene was amplified using primers Agrointergenic-FP 5'-GAAGGGCACTCCATGAGAAA–3' and Agrointergenic-RP 5'-CTCCCCTGCTGGTCAGAAT-3'. T4 polynucleotide kinase was used to label the PCR product with γ-32P-ATP. A. tumefaciens XdhR was titrated into the labelled DNA in binding buffer (25 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.05% Brij58, 5 mM DTT and 2% glycerol) and incubated at room temperature for 20 minutes. 8% polyacrylamide gels (39:1 (w/w) acrylamide:bisacrylamide) were prerun for 30 min at room temperature in 0.5X TBE (45 mM Tris-borate, 1 mM EDTA) running buffer. The samples
were loaded onto the gels and run for an hour at 100 volts. The gels were dried and exposed to phosphor screens. A Storm 840 scanner (GE Healthcare) was used to scan the image and the bands were quantified using ImageQuant 5.1 software. The region on the gels between complex and free DNA were considered as complex. KaleidaGraph software was used to create the plots and the data were fitted to a single site binding isotherm \( f = f_{\text{max}} \times [X] / (K_d + [X]) \) where \( f \) is fractional saturation \( K_d \) is the equilibrium dissociation constant, and \([X]\) is the protein concentration. To assess specificity of XdhR, EMSAs were carried out in which non-specific plasmid DNA or specific unlabeled DNA was titrated against labeled DNA in the binding buffer described above.

The ligands xanthine, hypoxanthine, adenine, guanosine and urate were used in EMSAs to analyze their effect on DNA-XdhR binding as were the phosphorylated guanosine derivatives ppGpp (TriLink), GTP and GMP. NaOH (0.4 N) was used to dissolve the former set of ligands and 500 mM Tris pH 8.0 was used in the binding buffer to avoid pH changes upon adding these ligands. The phosphorylated guanosine derivatives were included using the binding buffer described above (25 mM Tris pH 8.0). To a mixture containing DNA and ligand in the appropriate binding buffer, XdhR was added and incubated for 20 minutes. Once the samples were loaded, the gel was run for an hour at 100V. IC\(_{50}\) was calculated as the concentration of the ligand at which 50% of complex formation is inhibited. This was calculated using the equation \( f = A + B \times e^{-kL} \) where \( f \) is fractional saturation, \( k \) is the decay constant, \( L \) is ligand concentration, \( A \) is the saturation plateau and \( B \) is the decay amplitude. Inhibition constants (\( K_i \)) were calculated from the equation \( K_i = IC_{50}/([DNA]_{50}/K_d + [XdhR]_0/K_d + 1) \) where \([DNA]_{50}\) is the
concentration of DNA at 50% inhibition and \([XdhR]_0\) is the protein concentration at 0% inhibition (Cheng & Prusoff, 1973).

RNA isolation and reverse transcriptase assay

*Agrobacterium tumefaciens* was grown in 2X LB medium for 24-36 hours and then subcultured for further studies. For exponential phase studies, the cells were grown until OD$_{600}$ 0.2 and then treated with 10 mM allopurinol or separately with a combination of allopurinol and either GMP or xanthine for 30 minutes. The control cells were treated with an equal volume of 0.4 N NaOH, used to dissolve all ligands. RNA was extracted using acid phenol:chloroform, incubated with DNaseI and then purified using illustra RNASpin Mini kit (GE Healthcare) according to the manufacturer’s protocol. Two µg of RNA was used for cDNA preparation using AMV reverse transcriptase (NEB). Quantitative PCR was performed on an Applied Biosystems 7500 realtime PCR machine using SYBR green I dye. Expression of the *rim* gene was used for normalizing the *xdhB* gene expression. The expression level of *xdhB* gene was analyzed using primers *xdhFP* 5'-CCGAACTCGATACGGATGAT-3' and *xdhRP* 5'-AAACGAGAGCGAAGGCATAA-3' and the *rim* gene transcripts were analyzed using the primers *rimFP* 5'-ACCCGATACTCATGGCAAAG-3' and *RimRP* 5'-ACGACGACATTTCTTGCCTTC-3'. Data analysis was performed using the comparative threshold cycle method ($2^{\Delta\Delta C_T}$). The results were obtained from three replicates.

DNaseI footprinting using automated capillary sequencing

A 391 bp PCR product consisting of the intergenic region between *xdh* and *xdhR* was amplified using primers *AgroFP* 5'-(6-FAM) ATGCCAGCGACGAACTTCTATCAAC-3' and *AgroRP* 5'-GTCGGCATTGCCAGGCAACC-3', where 6-FAM reflects 5'-end labeling with 6-carboxyfluorescein. The labeled DNA (50 ng) was incubated with varying concentrations of *A.
*tumefaciens* XdhR for 10 minutes at room temperature. The DNasel digestion reaction was carried out with 0.08 units of enzyme for 50 ng labeled DNA and incubated at room temperature for 3.5 minutes. The reaction was stopped using 8 mM Na$_2$EDTA. The digested DNA was then extracted twice using phenol:chloroform and ethanol precipitated. The DNA pellet was washed with 70% ethanol and the DNA dissolved in 10 µL formamide. An aliquot of 0.05 ng of undigested DNA (to maintain fluorescence intensity compatible with the analyzer) and 0.2 ng of digested DNA was used for fragment analysis (Sivapragasam et al., 2015). An ABI 3130 analyzer with the default settings of 1.6 kV injection voltage and 15 seconds injection time was used for fragment analysis. LIZ 500 ladder (ABI – Life Technologies) was diluted 1:10 and added to each sample before loading on to the analyzer. GeneMapper version 4 software was used for analyzing the data. Electropherogram traces of digested DNA were overlayed with those of the digested DNA that was incubated with XdhR. A Thermosequenase PCR cycle sequencing reaction was carried out using the same 6-FAM-labeled primer and all four dideoxynucleotides in separate tubes. The products generated from these reactions were analyzed using fragment analysis technique and overlayed with that of the digested fragments to identify the XdhR protected sequence.

Adenine sensitivity test

An overnight culture of *A. tumefaciens* was subcultured to OD$_{600}$ 0.2-0.4. The cells were treated with 10 mM allopurinol for 30 minutes. Allopurinol-treated cultures and control cultures were serially diluted (until $10^{-9}$) and 10 µL of each dilution was spotted on LB plates containing 100 µg adenine per mL media (Levine & Taylor, 1982). The cells were incubated for 24-48 hours before the images were taken.
(p)ppGpp determination by thin layer chromatography (TLC)

For (p)ppGpp detection in *A. tumefaciens*, a 36-48 hours grown culture was subcultured in 2XLB media at a dilution of 0.15 with 150 µCi/mL of carrier-free $^{32}$P-labeled orthophosphate. 50 µL of the culture was harvested at 24, 48, 60, 72 and 80 hours, respectively, and mixed with an equal volume of 13 M formic acid. The samples were freeze-thawed 3-4 times and incubated on ice for 15 minutes before 5 µL of the sample was spotted on a PEI-cellulose TLC plate (Sigma-Aldrich). The plates were developed in 1.5 M KH$_2$PO$_4$ pH 3.4 for 2 hours. Migration of GTP, GMP, and ATP was verified by spotting these purified nucleotides, and spots corresponding to ppGpp and pppGpp were identified based on published Rf using this buffer system (Calderon-Flores *et al.*, 2005). The plates were dried and exposed to phosphor screens overnight. The images were scanned using Storm 840 scanner (GE Healthcare).

**Results**

*A. tumefaciens* XdhR binds to the intergenic DNA between divergently oriented *xdhR* and *xdhABC* genes

*S. coelicolor* XdhR binds to the intergenic region between *xdhABC* and *xdhR* genes to control their expression (Hillerich & Westpheling, 2008). Searching the STRING data base ([http://string-db.org/](http://string-db.org/)), which queries the genomic and functional context of target genes (Franceschini *et al.*, 2013), with the sequence of *S. coelicolor* XdhB and XdhR, predicts conservation of divergent *xdhABC-xdhR* genes in a limited set of evolutionarily distant bacterial species. Occurrence of *xdhABC-xdhR* genes was identified, for example, in *Saccharopolyspora erythraea*, *Streptomyces griseus*, *A. tumefaciens*, *A. vitis*, *A. radiobacter* and *Mesorhizobium loti*, with orthologs absent in genomes from related species. Such sporadic distribution suggests
acquisition by horizontal gene transfer (HGT). HGT would be expected to confer a fitness advantage, and it is important in the acquisition of virulence genes. A perfect palindrome

Figure 3.1. Structure-based model of *A. tumefaciens* XdhR created using SwissModel using 2Q24 as template. The N-terminal helices are identified by blue color and the C-terminal helices by red color. Tyrosine residues in DNA recognition helices (turquoise) are identified in stick representation.

(potential XdhR binding site) of 22 bp was found adjacent to the *xdhR* gene (Figure 3.1 shows XdhR model and figure 3.2 shows its binding site). The sequence of this palindrome shares similarity with the identified *S. coelicolor* XdhR binding site (Chapter 2).

*A. tumefaciens* is a dimeric protein belonging to the TetR family

A TetR protein of unknown function from *S. coelicolor* (PDB ID 2Q24) shares the greatest sequence similarity with *A. tumefaciens* XdhR (46% identity) among proteins for which structures have been reported (Filippova *et al.*, 2011), and it was used to create a protein model (Figure 3.2). The model illustrates the conserved helix-turn-helix DNA binding domains, the position of a conserved tyrosine in DNA recognition helices, and the Ω-shape that is characteristic of TetR proteins. The dimerization domain is typically variable in TetR proteins to accommodate binding of diverse ligands.

The gene encoding *A. tumefaciens* XdhR was cloned in pET28b and overexpressed in *E. coli*. The N-terminal His$_6$-tagged protein was purified to apparent homogeneity. The oligomeric
state of the protein analyzed using gel filtration indicated that XdhR was a dimer of 44 kDa (Figure 3.3), which is the typical oligomeric state of TetR family of proteins in absence of DNA.

![Diagram](image)

**Figure 3.2.** Divergent orientation of xdh and xdhR genes in *A. tumefaciens*. Palindromes found in the intergenic region are identified at the bottom. A perfect palindrome is identified adjacent to the xdhR gene (grey box). The identical sequences common to both potential binding sites are indicated in black in the palindrome near the xdh gene cluster and underlined in both boxes.

![Diagram](image)

**Figure 3.3.** *A. tumefaciens* XdhR is a dimer. Right panel shows molecular weight of XdhR indicated by arrow, determined by gel filtration. X-axis represents log₁₀ molecular weight and Y-axis represents $K_{av}$ of the molecular weight standards from BioRad. Left panel shows purified His₆-tagged XdhR.

Electrophoretic mobility shift assays revealed that XdhR bound to the xdhABC-xdhR intergenic region, forming a single complex with $K_d = 9.2 \pm 0.08$ nM (Figure 3.4 A,B). Upon addition of unlabeled specific DNA, the binding was compromised (compare Figure 3.4C, lanes 2-4), whereas addition of plasmid DNA did not affect the binding, indicating that XdhR binds to the intergenic region with high specificity (Figure 3.4).
Figure 3.4. XdhR binds specifically to the *xdhABC*-*xdhR* intergenic region. A. Electrophoretic mobility shift assay showing complex formation upon increasing [XdhR] from lanes 2 to 12 (1.4 pM, 14 pM, 70 pM, 0.14 nM, 0.7 nM, 1.4 nM, 7 nM, 14 nM, 70 nM, 140 nM, 700 nM; [DNA] = 50 pM). B. Percent XdhR-DNA complex formation as a function of [XdhR]. C. Specificity of binding; [DNA] = 50 pM, [XdhR] = 7 nM in lanes 2 through 9; lanes 3 through 6 – specific unlabeled DNA added at final concentrations of 0.05 nM, 0.1 nM, 0.5 nM and 10 nM respectively; lanes 7, 8, 9 – non-specific plasmid DNA at final concentrations of 0.1 nM, 3.0 nM, and 7.0 nM respectively.

XdhR binds to a palindrome near the translational start of *XdhR*

To identify the binding site for XdhR in the intergenic region between *xdhABC* and *xdhR*, DNaseI footprinting was carried out. The labeled intergenic DNA was incubated with protein in a ratio of 1:10 and then digested with DNaseI. The digested fragments from samples with and without protein were analyzed using an automated capillary electrophoresis genetic analyzer. The results were analyzed using GeneMapper 4 software. Traces from the sample with protein and without protein were overlayed to identify the protected sites. PCR products from Thermosequenase PCR using intergenic DNA template were analyzed by the same method, and the sequence of the protected region was identified by overlaying the fragments generated from Thermosequenase PCR and the digested intergenic DNA. XdhR protected a site that spans
Figure 3.5. XdhR protects a site in the xdhR promoter. Overlay of traces of digested intergenic DNA with (red traces) and without XdhR (blue traces). The sequence determined from overlay of Thermosequenase reaction is shown with the identified palindrome in red. Numbering is relative to the predicted translational start, defined as +1 (blue horizontal arrow).

~26 bp starting ~5 bp from the translational start of the xdhR gene (Figure 3.5). This sequence corresponds to the 22 bp perfect palindrome identified in the xdhR promoter (Figure 3.2). Protection extends an additional 3 bp beyond the palindrome (-6 to -31 bp relative to the translational start defined as +1) followed by a hypersensitive site (Figure 3.5). Protection of a single site is consistent with the identification of a single complex in EMSA.

Ligands attenuate DNA-XdhR binding

Several ligands were tested for their ability to attenuate DNA-XdhR binding using EMSA. Intermediates in purine metabolism, xanthine, hypoxanthine, adenine, guanine and urate were tested based on the role of Xdh in this metabolic pathway. Since S. coelicolor XdhR responded to GMP, GTP and ppGpp by attenuated DNA binding, these ligands were also examined. Our results indicated that xanthine ($K_i = 14.0$ mM) and GMP ($K_i = 10.6$ mM) very modestly attenuated
DNA-XdhR binding, while c-di-GMP, ppGpp and GTP more effectively dissociated the complex. For c-di-GMP, the Ki was 0.9 ± 0.0 mM; for ppGpp, the Ki was 0.9 ± 0.1 mM and for GTP, the Ki was 2.8 mM (Figure 3.6 and 3.7; Ki values were calculated based on $IC_{50}$). Thus c-di-GMP and ppGpp act as the most efficient ligands for XdhR and therefore might be important for inducing $xdhABC$ expression in vivo. The ligands adenine, guanine, hypoxanthine and urate did not affect XdhR-DNA binding (data not shown).

Figure 3.6. Ligands attenuate DNA-XdhR binding. The first lane of each gel has DNA only (D). Remaining reactions contained a constant protein concentration; the second lane has DNA and protein only (D+P). Panels A and B, [XdhR] = 15 nM; panels C and D, 20 nM and 40 nM, respectively. [DNA] was 50 pM in all reactions. Remaining lanes represent reactions with increasing concentrations of ligand. A. Xanthine from 1 mM to 30 mM. B. GMP from 0.5 mM to 30 mM. C. GTP from 0.1 mM to 6.5 mM. D. ppGpp from 0.1 mM to 5 mM. Graph on the right shows percent complex as a function of ppGpp concentration.
Allopurinol-mediated inhibition of Xdh results in repression of \( xdhB \) gene

Allopurinol, an analog of hypoxanthine, is an inhibitor of Xdh. Since Xdh participates in purine salvage to promote formation of guanosine nucleotides, inhibition of Xdh would be expected to attenuate their accumulation. Considering that XdhR responds most efficiently to highly phosphorylated guanosine nucleotides by attenuated DNA binding, we assessed if inhibition of Xdh would be associated with repression of \( xdhABC \) expression. Exponentially growing \( A. \) tumefaciens cells were treated with allopurinol for 30 minutes, and RNA was isolated and converted to cDNA and used as template for quantitative real-time PCR analysis. The results showed that \( xdhB \) gene expression decreased approximately two-fold when compared to the wild type exponential phase culture 1 hour after addition of allopurinol. This would be consistent with inhibition of Xdh and attenuated production of GTP, a ligand of XdhR. Expression studies using ligands revealed that the \( xdhB \) transcript level was restored upon addition of ligands GMP (10 mM) and xanthine (10 mM) in the presence of allopurinol (Figure 3.8). This is consistent with an attenuated production of GMP and xanthine when Xdh is inhibited.
Figure 3.8. *xdhB* gene expression at one and two hours time point after addition of allopurinol alone or in combination with ligands GMP and xanthine (10 mM each). Transcript levels are normalized to transcripts levels measured in absence of ligand.

*A. tumefaciens* cells treated with allopurinol are sensitized to adenine

Xdh plays a role in the conversion of adenine to guanine (*Xi et al.*, 2000). Inhibition of this enzyme leads to adenine accumulation in the cells that is toxic. The toxicity might be due to the depleted guanine nucleotide pool (Levine & Taylor, 1982). When exponential phase cultures of *A. tumefaciens* were treated with allopurinol for 30 minutes to inhibit the function of Xdh, sensitivity to adenine would therefore be expected. Our results revealed that allopurinol-treated cells showed reduced survival compared to untreated culture on LB plates containing adenine (Figure 3.9). This could be because of adenine accumulation due to inefficiency in the conversion of adenine to guanine by Xdh. This needs to be further tested in liquid culture for analyzing the decreased survival caused by adenine.
Ligand (p)ppGpp accumulates during stationary phase in *A. tumefaciens*

To determine if ppGpp accumulates in *A. tumefaciens* during stationary phase, cells were sub-cultured and grown in the presence of $^{32}$P-labeled orthophosphate in rich 2XLB medium. Nucleotides were extracted with formic acid and samples were spotted on to PEI-cellulose TLC plates and developed with 1.5 M $K_2HPO_4$. The images scanned revealed that the spot corresponding to ppGpp started showing from 48 hours and accumulated up to 80 hours (Figure 3.10).

Figure 3.9. Allopurinol (10 mM) treatment modestly reduces the survival of *A. tumefaciens* cells in LB plates containing adenine (100 µg per mL).

Figure 3.10. Thin layer chromatography of nucleotides extracted from *A. tumefaciens* at various time points of growth showing (p)ppGpp accumulation at later stages.
Discussion

XdhR autoregulates its own expression by binding adjacent to the translational start of \textit{xdhR}.

\textit{A. tumefaciens} XdhR binds to the intergenic region between \textit{xdhABC} and \textit{xdhR} genes (Figure 3.3). This protein belongs to the TetR family of proteins that are typically homodimers. Footprinting results revealed that XdhR binds to a palindrome near the start codon of the \textit{xdhR} gene (Figure 3.5). The position of the XdhR site suggests that XdhR binding would hinder its own expression and it would therefore be expected to be autoregulatory. \textit{S. coelicolor} XdhR also binds to its own promoter region, repressing its expression (Hillerich & Westpheling, 2008).

Our footprinting results revealed a binding site for XdhR (coinciding with the palindrome near the \textit{xdhR} gene), but no binding site could be detected near the \textit{xdhABC} gene though a potential binding site is present (Figure 3.2 and Figure 3.5). XdhR might be binding the \textit{xdhABC} gene promoter at higher protein concentrations. In \textit{S. coelicolor} a looping model has been proposed in which the XdhR tetramer binds to a site near the \textit{xdhR} gene and DNA is looped around a bending protein (perhaps HU) leading to the other dimer unit of the tetramer binding to the promoter of the \textit{xdh} gene (Chapter 2). It is conceivable that dimeric \textit{A. tumefaciens} XdhR similarly oligomerizes \textit{in vivo} to autoregulate its own expression along with that of the \textit{xdhABC} gene cluster.

Thus two different bacteria \textit{A. tumefaciens} and \textit{S. coelicolor} belonging to different phyla (\(\alpha\)-proteobacteria and actinobacteria) have a similar divergent gene orientation of conserved \textit{xdhABC} and \textit{xdhR} genes whose expression is governed by an autoregulatory transcriptional repressor XdhR. Combined with the absence of this gene pair in related species, this suggests horizontal gene transfer mechanism that had occurred during the process of evolution. It
should also be noted that the \( xdhABC \)-\( xdhR \) genes considered in this chapter are located on the AT plasmid. However, NCBI BLAST search revealed that \( A. \) \textit{tumefaciens} possesses another \( xdhABC \) gene cluster (Atu2309-2311) encoded on the chromosome that is found near a gene (Atu2312) encoding a LysR transcriptional regulator oriented in the same direction as that of \( xdhABC \). This further reinforces the interpretation that divergent \( xdhABC \)-\( xdhR \) genes have been acquired via horizontal gene transfer and may have conferred a selective advantage under stress conditions.

The magic spot ppGpp and GTP are ligands of XdhR

Upon sensing nutrient limitation or starvation conditions, bacteria synthesize (p)ppGpp that signals cells to control overall consumption of nutrients and energy that are normally utilized for replication, transcription and translation. Thus, (p)ppGpp acts as an alarmone in cautioning cell machineries to conserve nutrients. The conserved energy is spent for production of molecules essential for cell survival. The type of response to starvation depends on the level of (p)ppGpp production in the cell. For example, while the \( lrp \) regulon is induced at low levels of ppGpp (400 picomoles ml\(^{-1}\) OD\(^{-1}\)), \( rpoS \) is strongly induced only when ppGpp levels are higher (Traxler \textit{et al.}, 2011). EMSA revealed that XdhR-DNA binding is efficiently inhibited by ppGpp with \( K_i \) of 0.9 ± 0.1 mM (Figure 3.6). Thus this level of ppGpp could correspond to a ‘famine’ situation rather than a ‘hunger’ situation in bacteria (Traxler \textit{et al.}, 2011). In Gram negative organisms, XdhR is the first identified transcriptional repressor on which ppGpp exerts its action to induce gene expression. Since GTP also attenuates DNA-XdhR binding with \( K_i \) of 2.8 mM, we propose that GTP acts as a lower affinity ligand (Figure 3.6). The abundance of GTP during exponential phase growth (mM) may result in GTP binding XdhR and therefore \( xdhABC \)
expression. GTP being a weaker ligand than ppGpp would result in a modest induction of the
\textit{xdhABC} gene cluster that is sufficient for maintaining GTP levels, since other enzymes (GuaA, GuaB, Gmk, Hprt) in the purine metabolism also contribute significantly to GTP production during the exponential growth phase. During starvation, the enzymes mentioned above may be inhibited by the stringent response alarmone, ppGpp (Kriel \textit{et al.}, 2012). Thus during starvation or stringent response, the role of Xdh in this metabolic pathway becomes critical in replenishing the GTP pool in order to maintain (p)ppGpp levels. As a higher affinity ligand, (p)ppGpp binds to XdhR and induces \textit{xdhABC} to peak levels during stationary phase. It has been found that during carbon and nitrogen starvation, the \textit{xdh} gene is upregulated in a RelA and DksA dependent manner in \textit{S. meliloti} (Krol & Becker, 2011). Therefore, we propose that Xdh is essential for salvaging adenine to produce guanine that is essential for replenishing GTP pools, either for maintaining GTP homeostasis during exponential phase or for generation of (p)ppGpp to maintain stringent response during nutrient limitation.

Allopurinol inhibits purine metabolic pathway by inhibiting the accumulation of GTP

\textit{Rhodobacter capsulatus} Xdh is inhibited by allopurinol, an analog of hypoxanthine (Truglio \textit{et al.}, 2002, Dietzel \textit{et al.}, 2009, Pauff \textit{et al.}, 2009). If Xdh function is inhibited by allopurinol, then the purine salvage pathway is hindered and in turn GTP production is reduced. Decreased GTP levels might lead to decreased expression of the \textit{xdhABC} gene cluster. When \textit{A. tumefaciens} exponential phase cultures were treated with 10 mM allopurinol for 2 hours, \textit{xdhB} gene expression was reduced \textasciitilde{}50% one hour after addition of allopurinol when compared to untreated cells (Figure 3.8). This is consistent with the interpretation that allopurinol interferes with Xdh function in \textit{A. tumefaciens} and therefore hinders GTP production. It would be
interesting to establish if (p)ppGpp production (stationary phase ligand of XdhR) is likewise reduced by allopurinol. Since in S. coelicolor, xdh gene expression during stationary phase is higher than in exponential phase (Hillerich & Westpheling, 2008, Krol & Becker, 2011), a sharp decrease in xdh expression in the presence of allopurinol would be predicted in stationary phase.

When exponentially growing A. tumefaciens cells were treated with ligands GMP or xanthine (10 mM) along with allopurinol, the decrease in xdhB gene expression was reduced to ~30% compared to the ~50% reduction observed on treatment with allopurinol only. This is consistent with reduced Xdh activity compromising synthesis of xanthine and with the ability of xanthine and guanosine nucleotides to bypass the requirement for Xdh. Similarly, it was previously reported that the requirement for Xdh in utilization of guanine precursors in Rhizobium tropici is circumvented on addition of xanthine and guanine, but not hypoxanthine and adenine (Riccillo et al., 2000). Our results also suggest that synthesis of GMP via GuaB-mediated conversion of IMP to XMP is insufficient and that purine salvage is important for synthesis of guanosine nucleotides, even during exponential phase growth.

Interference of xanthine oxidase function leads to cell death in the presence of adenine

Xdh participates in the conversion of adenine to guanine. Interference with the function of Xdh was shown to lead to an accumulation of adenine that is toxic to E. coli cells, as evidenced by xdhΔ mutants failing to survive when grown in presence of adenine (Xi et al., 2000). Therefore, A. tumefaciens cells were grown to exponential phase and treated with allopurinol for 30 minutes to inhibit Xdh. Cells treated with allopurinol and grown on adenine exhibited a modest growth defect when compared to those that were not treated with
allopurinol (Figure 3.9). This observation is consistent with reduced Xdh activity in allopurinol-treated cells.

Role of ppGpp and c-di-GMP in exopolysaccharide production in A. tumefaciens

In a relA/spoT deletion mutant of Agrobacterium sp. ATCC 31749 that is devoid of (p)ppGpp synthesis, curdlan synthesis goes down by 57-fold. It has also been shown that curdlan production drops by 57% in a mutant strain carrying a deletion of AGRO_3967 (a gene encoding a protein with a GGDEF domain, the catalytic domain responsible for c-di-GMP synthesis). Being closely related to exopolysaccharide production and biofilm formation, (p)ppGpp and c-di-GMP together might contribute to the extraordinary survival capabilities of bacteria belonging to the genus Agrobacterium. Since DNA binding by XdhR is attenuated by the signaling molecules (p)ppGpp and c-di-GMP with Ki of 1.0 ± 0.1 mM and 0.9 ± 0.0 mM, respectively (Figures 3.6 D and 3.7), we predict that XdhR plays a crucial role in exopolysaccharide production and biofilm formation in this bacterium. Accumulation of (p)ppGpp during the stationary phase (Figure 3.10) suggests that repression by XdhR would be most effectively relieved under these conditions such that xdhABC gene expression would be maximal as the growth stage progresses towards lag phase. Thus, (p)ppGpp and c-di-GMP act as ligands for XdhR to optimize purine salvage and sustain their own synthesis, in addition to acting as signaling molecules during nutrient starvation and biofilm formation.

Conclusion

The function of XdhR in S. coelicolor and A. tumefaciens are conserved in terms of intergenic DNA and ligand binding. From the experiments presented in chapters 2 and 3, a proposed function of XdhR is to actively regulate production of Xdh, which participates in
purine salvage required for GTP/(p)ppGpp/c-di-GMP synthesis. (p)ppGpp and c-di-GMP act as crucial signaling molecules for pathogenic bacteria for biofilm formation and exopolysaccharide production during infection in the host. Curdlan exopolysaccharide from *Agrobacterium* sp. ATCC 31749 is widely used in the food industry, and is well known for its anti-tumor and anti-HIV properties. Therefore, understanding the metabolism of these signaling molecules is important in terms of identifying potential mechanisms to curtail pathogenic bacteria and improve curdlan production by *Agrobacterium* sp. ATCC 31749 in industries.

**References**


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CHAPTER 4
XANTHINE DEHYDROGENASE REGULATOR PROTEIN OF Ralstonia solanacearum

Introduction

*Ralstonia solanacearum* is a soil-borne plant pathogen belonging to the class β-Proteobacteria and the order Burkholderiales. It infects a wide variety of plants causing wilting disease in more than 200 plant species, including potatoes, tomatoes, eggplant, and tobacco (Genin & Denny, 2012, Mansfield et al., 2012). This organism causes a loss of approximately $1 billion every year just on potato cultivation alone worldwide. This bacterium can withstand extremely unfavorable conditions, for example by surviving in wet soil for many years and still retaining its infectivity in its host, and it is placed as the second most important plant pathogen (Mansfield et al., 2012).

*R. solanacearum* typically enters susceptible hosts through a wound and colonizes the water-conducting xylem tissue, which is considered a nutrient-poor environment. A number of virulence genes are expressed in response to plant-derived signals (Jacobs et al., 2012), one of which is the reactive oxygen species produced as part of the plant defense mechanism. One of the genes expressed under such conditions encodes xanthine dehydrogenase (Xdh), and it was shown to be expressed both by addition of hydrogen peroxide to *R. solanacearum* culture and in tomato plants (Flores-Cruz & Allen, 2009).

Xanthine dehydrogenase and xanthine oxidase are two forms of the same enzyme collectively termed xanthine oxidoreductase. While Xdh is required for the conversion of hypoxanthine to xanthine and xanthine to urate and generally produces NADH, xanthine oxidase catalyzes the same reactions, but produces reactive oxygen species in place of NADH.
(Truglio et al., 2002). However, in *Enterococcus faecalis*, a Gram positive firmicute, a selenium-dependent xanthine dehydrogenase enzyme produces superoxide that induces biofilm formation; an *xdh* mutant strain is defective in superoxide production and biofilm formation (Srivastava et al., 2011). Xdh takes part in the purine salvage pathway, where it mediates the conversion of adenine to guanine; in *E. coli*, loss of Xdh function leads to adenine sensitivity, reflecting failure to control purine homeostasis (Levine & Taylor, 1982). In several bacteria such as *Erwinia amylovora*, *Borrelia burgdorferi*, *Listeria monocytogenes* and *Helicobacter pylori*, the enzymes required for purine biosynthesis play a vital role in establishing virulence while infecting the host (Liechti & Goldberg, 2012, Jewett et al., 2009, Eastgate et al., 1997, Taylor et al., 2002). Loss of one or more enzymes of the purine salvage pathway compromises virulence in these organisms.

The reason why a functional purine salvage pathway may be required for virulence may be linked to the synthesis of guanine-derived signaling molecules. The starvation signaling molecule (p)ppGpp is synthesized from GTP and ATP by RelA or SpoT or Rsh (RelA/SpoT homology) enzymes in bacteria, for example when uncharged aminoacyl tRNA binds to the ribosome (Gaca et al., 2015). Whenever bacteria face starvation or stringent conditions, the alarmone/magic spot (p)ppGpp is produced. (p)ppGpp is a global regulator that inhibits overall cellular functions required for growth such as replication, transcription and translation (Ochi, 1987, Magnusson et al., 2005, Potrykus & Cashel, 2008, Wu & Xie, 2009, Traxler et al., 2011, Ross et al., 2013, Gaca, 2015). In *L. monocytogenes*, a food-borne pathogen, an *hpt* (encoding hypoxanthine phosphoribosyl transferase) mutant that is defective in purine metabolism could not synthesize ppGpp and was avirulent in mice (Taylor et al., 2002).
The second messenger c-di-GMP is synthesized from two molecules of GTP by diguanylate cyclases, and it is involved in multiple cellular functions, including biofilm formation, motility, and virulence (Tamayo et al., 2007). The insect Riptortus pedestris harbors a gut symbiont, which belongs to the β-Proteobacterial genus Burkholderia. The bacterium produces an exopolysaccharide that is essential for biofilm formation; PurT and PurM are involved in purine biosynthesis, and loss of function of these proteins leads to reduced synthesis of c-di-GMP, which is essential for biofilm formation (Kim et al., 2014). In Burkholderia pseudomallei, c-di-GMP plays an important role in expression of various virulence factors such as flagellar synthesis, motility and biofilm formation (Lee et al., 2010). Exopolysaccharide is also produced in large quantities in R. solanacearum cultures and during infection in plants and this is critical for virulence. An eps mutant loses its virulence and fails to cause wilting disease in plants (Saile et al., 1997). Motility in R. solanacearum also plays an important role in invading plant tissues and mutants motN and aer2 that are devoid of optimal motility function are slightly reduced in virulence (Kang et al., 2002, Yao & Allen, 2007, Meng et al., 2011). Since enzymes of the purine biosynthetic pathway have been shown to be essential for c-di-GMP production in Burkholderiales, and since c-di-GMP has been extensively implicated in the biofilm formation and transition from sessile to motile lifestyle that is required for virulence, we investigated if the purine salvage pathway is likewise linked to c-di-GMP signaling in R. solanacearum. Since c-di-GMP signaling is universally present in many pathogenic bacteria of a wide host range, this study would address the role of Xdh and the purine salvage pathway with regard to c-di-GMP signaling.
Transcriptional regulators play a vital role in regulating various cellular functions of bacteria. The gene cluster encoding *R. solanacearum* Xdh is divergently oriented to a gene encoding a LysR family regulator protein that we named xanthine dehydrogenase regulator (XdhR). Such gene orientation predicts regulation of *xdh* by XdhR. The aim of this chapter is to characterize the function of XdhR protein and identify its ligands and oxidation properties that might play a role in regulating *xdhABC* gene cluster in *R. solanacearum*.

**Experimental Procedures**

Cloning and purification of XdhR

Primers for amplifying the *R. solanacearum* *xdhR* gene (*Rsc2094*) were designed for cloning into the pET100 TOPO vector. Primers RalxdhR FP 5’-caccatgcaatgcagctagc-3’ and RalxdhR RP 5’-tcagggccggtctccagctg-3’ were used to amplify the *xdhR* gene. The PCR product was purified using PCR purification kit (Promega) and cloned into pET100 plasmid, which was then transformed into NEBβ10 cells. Colonies were cultured in media containing ampicillin (50 µg mL⁻¹). Recombinant plasmid was sequenced to verify presence of insert and then transformed into *E. coli* BL21(DE3) for overexpression. *E. coli* BL21(DE3) carrying the recombinant plasmid was grown to exponential phase OD600 0.4-0.6 and induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). After 5 hours of induction, cells were pelleted and stored at -80 °C. Cells were resuspended in lysis buffer (20 mM sodium phosphate pH 8.0, 150 mM sodium chloride, 5% glycerol, 0.15 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM β-mercaptoethanol) to which lysozyme was added to 200 µg mL⁻¹ and the suspension incubated on ice for 30 minutes. Cells were lysed further by sonication and the lysate was centrifuged at 13,000 rpm for 1 hour to pellet debris.
The supernatant was mixed with HIS-select Nickel affinity beads for 30 minutes at 4°C. XdhR protein was purified according to the manufacturer’s protocol. The purified protein was stored in storage buffer (20 mM sodium phosphate pH 8.0, 150 mM NaCl, 20% glycerol, 0.15 mM PMSF and 10 mM β-mercaptoethanol). Purity was verified by SDS-PAGE followed by staining with Coomassie Brilliant Blue. Protein concentration was determined by MicroBCA kit (BioRad).

Glutaraldehyde crosslinking

Purified His6-tagged XdhR (8.8 μM) was incubated with 1 μL of 0.005%, 0.01% or 0.05% glutaraldehyde on ice for 20 minutes. After incubation, the reaction was stopped with Laemmli sample buffer without β-mercaptoethanol and loaded onto an SDS-PAGE gel. Gels were run at 75 volts for 2 hours and stained with Coomassie Brilliant Blue stain. Gels were destained and the images were taken using GelDoc system and alpha-imager software.

Electrophoretic mobility shift assay (EMSA)

The intergenic DNA between xdhR and xdh genes (245 bp) was amplified using primers interFP 5’- gcaggaggtaggtatcgag-3’ and interRP 5’-gcctgtcggcctgtaa-3’ using R. solanacearum genomic DNA as template. The intergenic DNA was purified and labeled at the 5’-ends with 32P using T4 Polynucleotide kinase. Labeled DNA was incubated with increasing concentration of purified XdhR protein from 10 pM to 1.3 μM in binding buffer (25 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.05% Brij58, 5 mM DTT and 2% glycerol) for 30 minutes and loaded onto a 4%, 6% or 8% native PAGE that was prerun for 30 minutes in 0.5X TBE (45 mM Tris-borate, 1 mM EDTA). The gels were run for 1 hour and 15 minutes at 100 V, dried, and exposed to a phosphor screen. The images were scanned using a Storm scanner. The bands were quantified using ImageQuant software. The data were plotted using KaleidaGraph.
software. $K_d$ was calculated using the Hill equation $f = f_{\text{max}} *[X]^{n_H} / (K_d + [X]^{n_H})$ where $f$ refers to fractional saturation, $n_H$ refers to Hill coefficient, $K_d$ refers to equilibrium dissociation constant reflecting half-maximal saturation of the DNA, and $X$ refers to protein concentration. Experiments were repeated twice and the results were indicated as mean ± standard deviation. Specificity of XdhR binding was assessed by titrating binding reactions with increasing concentrations of polydGdC.

To identify the effect of oxidants on DNA protein binding, increasing concentrations of oxidants hydrogen peroxide, cumene hydroperoxide or tertiary butyl hydroperoxide were incubated with protein for 15 minutes. The oxidized protein was then incubated with labeled DNA for 15 minutes and loaded on to native gels and gel processed as described above.

Ligands in EMSA

GMP, GTP (Sigma Aldrich), ppGpp (TriLink) and c-di-GMP (Kerafast) were used as ligands in EMSA. Increasing concentrations of these ligands were added to DNA and XdhR was added last. This was incubated for 15 minutes in the binding buffer mentioned above. After incubation the reaction mixtures were loaded onto gels and run at 100 volts for 1 hour and 15 minutes. Gels were dried and exposed to phosphor screens. The bands were quantified as mentioned above. $IC_{50}$ is defined as the concentration of the ligand at which 50% of complex formation is inhibited. The equation $f = A + B \times e^{-kL}$ where $f$ is fractional saturation, $k$ is the decay constant, $L$ is ligand concentration, $A$ is the saturation plateau and $B$ is the decay amplitude was used to calculated $IC_{50}$ concentration. At least two independent experiments were performed.
Results

*Ralstonia solanacearum* XdhR belongs to the LysR family

*R. solanacearum* XdhR belongs to the LysR family of transcriptional regulators that are usually tetramers (Maddocks & Oyston, 2008). Modeling of XdhR using 2esn, a *Pseudomonas aeruginosa* transcription factor, as template reveals a conserved N-terminal winged helix-turn-helix domain and a C-terminal ligand binding domain that is predicted to bind to inducers (Figure 4.1); these two domains are connected by a helical linker.

![Figure 4.1. *R. solanacearum* XdhR modeled using SwissModel in automated mode using 2esn as template. Each subunit is represented in different colors (lightteal, red, orange, and purple) and the helix-turn-helix domain of all monomers is represented in green. Figure was created with PyMol. Dashed lines outline the path of DNA binding to the protein.](image)

Binding to a long 50-60 bp site is predicted to occur by inducing a significant DNA bend to accommodate interaction with two paired helix-turn-helix domains (Muraoka *et al.*, 2003). While the inducer-binding domain is structurally conserved, amino acid sequence is more variable among LysR proteins compared to the DNA-binding domain to accommodate binding of diverse inducers.

*R. solanacearum* XdhR (Mw~35.6 kDa) was purified to apparent homogeneity (Figure 4.2). Glutaraldehyde cross-linking revealed the formation of dimers and tetramers as well as
multimers that fail to enter the gel. Since other LysR family proteins form tetramers, the observation that XdhR tetrameric assemblies may be detected after glutaraldehyde crosslinking is consistent with its existence as a tetramer in solution. A gel filtration experiment is needed to confirm the native state of this protein.

A gel filtration experiment is needed to confirm the native state of this protein.

Orientation of xdhABC and xdhR genes in R. solanacearum

The gene encoding XdhR is divergently oriented to an operon encoding the three Xdh subunits, XdhA, -B, and -C (Figure 4.3). The labeled intergenic DNA was incubated with increasing concentration of protein and reactions separated by native PAGE. EMSA results revealed that XdhR binds to the intergenic region forming more than one complex with Kd of 163.3 ± 23.4 nM; the Hill co-efficient of 1.0 ± 0.1 indicates that no co-operativity of binding occurs (Figure 4.4A and B).

Figure 4.3. Divergent orientation of xdhR and xdhABC genes in R. solanacearum.

Any non-specific DNA added to the DNA-XdhR protein complex inhibited DNA-XdhR complex formation (data not shown), indicating that XdhR can non-specifically bind DNA. LysR
proteins have been reported to bind to an inverted repeat of a stretch of nucleotides containing the sequence T-N\textsubscript{11}-A (Schell, 1993) and therefore LysR family proteins generally bind readily to a stretch of AT-rich DNA. In contrast, addition of poly-dGdC did not significantly interfere with DNA-XdhR binding (Figure 4.4C). We did observe the appearance of an XdhR-DNA complex of greater electrophoretic mobility with increasing concentrations of poly-dGdC; differences in electrophoretic mobility of complexes may either reflect the stoichiometry of protein to DNA or the extent of DNA bending, with complexes containing more severely bent DNA migrating slower.

**Figure 4.4.** Affinity and specificity of XdhR. A. Labeled intergenic DNA titrated with increasing concentrations of protein run on 6\% gel; labeled DNA – 0.05 nM in all lanes; lanes 2 through 15 increasing concentration of protein 0.01 nM, 0.1 nM, 1 nM, 10 nM, 20 nM, 40 nM, 60 nM, 80 nM, 100 nM, 250 nM, 440 nM, 500 nM, 1000 nM and 1,300 nM, respectively. B. Percent XdhR-DNA complex as a function of XdhR concentration. C. 4\% native gel with all lanes containing 0.05 nM labeled DNA; lanes 2-7 contain 150 nM XdhR; lanes 3 through 7 contain 10 pg, 50 pg, 100 pg, 500 pg and 1 ng of poly-dGdC.
GTP, ppGpp and c-di-GMP are ligands of XdhR

Ligands GMP, GTP, ppGpp and c-di-GMP were used as ligands to examine their ability to disrupt DNA-XdhR complex formation. EMSA results revealed that all these ligands could inhibit the DNA-XdhR complex formation. For c-di-GMP, the IC$_{50}$ was 1.3 ± 0.1 mM, ppGpp had IC$_{50}$ of 1.2 ± 0.2 mM, and GTP had IC$_{50}$ of 3.8 ± 0.6 mM, whereas GMP had the highest IC$_{50}$ of 15.8 ± 0.8 mM (Figures 4.5 and 4.6).

Figure 4.5. Ligands of XdhR. EMSA showing ligands GMP (A), GTP (B), ppGpp (C &D) attenuating DNA-XdhR binding. DNA in panels A,B,C- 0.1 nM; protein concentration in panels A, B and C-250 nM. D. Graph showing % complex as a function of [ppGpp].
Effect of oxidants on DNA-protein binding

Since *R. solanacearum* encounters reactive oxygen species upon infecting tomato plants and since *xdh* gene expression is increased both *in planta* and *in vitro* upon exposure to H$_2$O$_2$ (Flores-Cruz & Allen, 2009), we investigated the effect of oxidants on XdhR. Oxidants hydrogen peroxide, cumene hydroperoxide and tertiary butyl hydroperoxide were used to oxidize XdhR; XdhR contains two cysteine residues per monomer, both in the ligand-binding domain. Oxidized XdhR was used in EMSA with labeled intergenic DNA. EMSAs with oxidized protein revealed that as the concentration of tertiary butyl hydroperoxide and cumene hydroperoxide was increased, XdhR binding was only very modestly decreased. As determined by EMSA, hydrogen peroxide could attenuate DNA-XdhR binding only at very high concentrations (Figure 4.7). These observations argue against a regulatory mechanism in which reduced XdhR represses *xdhABC*, followed by derepression due to oxidized XdhR dissociating from the DNA.

Figure 4.6 A. EMSA showing attenuation of DNA-XdhR binding by c-di-GMP. XdhR- 100 nM; DNA-0.05 nM. B. Graph showing % complex as a function of [c-di-GMP].
Figure 4.7. EMSAs showing effect of oxidants on DNA-XdhR binding. DNA – 0.05 nM, protein concentration in A, B and C is 150 nM. A. Tertiary butyl hydroperoxide in lanes 3 through 5 – 30 µM, 3 mM and 30 mM. B. Cumene hydroperoxide – 30 µM and 3 mM. C. Hydrogen peroxide in lanes 3 through 8- 300 nM, 3 µM, 30 µM, 300 µM, 3 mM and 30 mM.

Discussion

XdhR is a tetramer belonging to the LysR family of transcriptional regulators

The LysR family of proteins constitutes the largest family of DNA-binding proteins with >800 members (Schell, 1993). They can be either activators or repressors. They regulate genes that perform diverse functions. Functions of divergently oriented gene products include metabolism, virulence, motility, toxin production, and response to oxidative stress (Kovacikova & Skorupski, 1999, Deghmane et al., 2000, Cao et al., 2001, Russell et al., 2004, Byrne et al., 2007, Lu et al., 2007, Sperandio et al., 2007). A common mode of transcriptional regulation by LysR proteins involves repression of its own gene expression, whereas activation of target genes occurs upon binding of an inducer that may be an intermediate in the corresponding pathway. Inducers bind in a cleft between the two Rossman-fold type subdomains of the inducer-binding domain of each monomer (Ezezika et al., 2006, Devesse et al., 2011).
Conformational changes that occur as a result are thought to be communicated to the DNA-binding domains via the connecting helix (Monferrer et al., 2008, Ruangprasert et al., 2010). Typically, the homotetrameric LysR protein can bind three possible DNA sites in gene promoters (Porrua et al., 2007). Simultaneous binding to a high-affinity site and one of two low-affinity sites leads to DNA bending in absence of inducer, while binding of inducer causes the protein to bind instead to the other low-affinity site in concert with the high-affinity site; this change in binding mode is driven by inducer-mediated conformational changes that change the distance between dimeric DNA-binding domains (Ezezika et al., 2007, Monferrer et al., 2010). This results in relaxation of the DNA bend and transcriptional activation. XdhR forms tetramers as detected by glutaraldehyde cross-linking and therefore might be similar to other LysR family members that usually occur as tetramers (Maddocks & Oyston, 2008). In light of the proposed mechanism for transcriptional activation by LysR proteins, the dissociation of XdhR from DNA upon binding the ligands GTP, ppGpp, and c-di-GMP may imply that XdhR instead functions to repress expression of xdhABC.

XdhR may control formation of Xdh to replenish GTP pool and maintain the synthesis of the alarmone (p)ppGpp during starvation. Xdh is involved in purine salvage during the conversion of adenine to guanine (Xi et al., 2000). In Sinorhizobium meliloti, the gene encoding Xdh is upregulated during starvation when ppGpp levels are high (Krol & Becker, 2011). Also in S. coelicolor, xdh gene transcripts are induced during stationary phase (Hillerich & Westpheling, 2008). EMSA results revealed that ppGpp acts as an efficient ligand for XdhR with an IC50 of 1.2 ± 0.2 mM (Figure 4.5C and D). This suggests that during starvation or stationary phase, ppGpp binds to XdhR thereby inducing expression of xdhR and the xdhABC gene cluster. This induction might be essential for replenishing the
guanine nucleotide pool for restoring GTP levels in order to sustain ppGpp synthesis during starvation and stringent response.

Role of Xdh in purine salvage for generation of the secondary messenger c-di-GMP

Ligand binding assays revealed that c-di-GMP attenuates XdhR binding to the intergenic region between \textit{xdhABC} and \textit{xdhR} genes (Figure 4.6 A and B). The immunosuppressive drug azathioprine inhibits 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase enzyme of purine biosynthetic pathway leading to a decrease in nucleotide pools. This leads to a decrease in c-di-GMP formation and in turn to decreased biofilm formation in \textit{E. coli} (Antoniani \textit{et al.}, 2013). AICAR is an intermediary precursor of inosine monophosphate that could be converted to GMP or AMP depending upon need of the cell. The secondary messenger c-di-GMP is essential for signaling various functions of the cell such as motility, flagellar synthesis and biofilm formation (Lee \textit{et al.}, 2010). All these functions are associated with stationary phase or starvation responses when the nutrients are very limiting. Thus restoring the levels of precursors by purine salvage becomes essential for the production of the signaling molecule c-di-GMP during starvation and stress conditions. This suggests that c-di-GMP might play a role in inducing \textit{xdhABC} gene cluster by binding to its regulator and help restore GTP levels by enhancing the guanine nucleotide pool.

Oxidation does not significantly affect xanthine dehydrogenase regulator protein binding to the intergenic DNA EMSA results reveal that oxidation of XdhR with cumene hydroperoxide, tertiary butyl hydroperoxide and hydrogen peroxide has little effect on the efficiency of XdhR binding to intergenic DNA (Figure 4.7). Since \textit{R. solanacearum} \textit{xdh} gene expression increases during oxidative stress responses (Flores-Cruz & Allen, 2009) it was predicted that oxidized XdhR
binding to the intergenic DNA might activate $xdhABC$ transcription. This would be essential during purine salvage for replenishing GTP for synthesis of (p)ppGpp or c-di-GMP, the signaling molecules that play an important role during starvation and stringent conditions experienced by bacteria in host plants. DNA footprinting would be required to ascertain if the mode of XdhR binding to DNA is altered upon oxidation. We also cannot rule out the possibility that a separate transcription factor is responsible for regulating $xdhABC$ expression in response to oxidative stress.

*R. solanacearum* cells starved for 28 months in MilliQ water could withstand treatment with 180 $\mu$M $H_2O_2$ for 90 minutes and still remain viable. This tolerance level to oxidants could not be achieved with any other stresses like heat, ethanol and osmotic stress (Stevens *et al.*, 2010). This observation might be because starved cells have induced $xdh$ gene expression that would result in (p)ppGpp and c-di-GMP production. The signaling molecules (p)ppGpp and c-di-GMP initiate starvation response and biofilm formation, respectively, and aid the organism in withstanding starvation/nutrient limitation effectively. Since the $xdhABC$ gene cluster is induced during starvation, it is also possible that Xdh function in the production of urate, which is an antioxidant, would effectively take part in neutralizing reactive oxygen species or any type of oxidative stress during starvation.

**Conclusion**

We propose a model in which *R. solanacearum* XdhR participates in regulating expression of the $xdhABC$ gene cluster depending upon the availability of its ligands GTP, (p)ppGpp and c-di-GMP and perhaps also the presence of reactive oxygen species. These signaling molecules carefully regulate the $xdhABC$ gene cluster depending upon the need. Owing to the diversity of signaling
molecules that XdhR responds to, Xdh likely fulfills an important function that needs to be constantly regulated during different growth stages of the cell. Though R. solanacearum XdhR belongs to a different family than S. coelicolor and A. tumefaciens XdhR, it is intriguing that the ligands GTP, ppGpp and c-di-GMP exert similar effects on XdhR-DNA complex formation. This suggests that Xdh functions similarly in purine salvage during starvation and oxidative stress conditions in several bacterial species to control production of GTP and in turn the signaling molecules (p)ppGpp and c-di-GMP.

References


CHAPTER 5
CONCLUSION AND FUTURE WORK

The gene cluster encoding xanthine dehydrogenase (Xdh) is divergently oriented to the gene encoding xanthine dehydrogenase regulator (XdhR) protein in S. coelicolor, A. tumefaciens and R. solanacearum. In S. coelicolor it has been shown that XdhR protein represses expression of the divergent genes by binding to the intergenic region between the xdhABC gene cluster and the xdhR gene (Hillerich & Westpheling, 2008). Since the xdh gene is upregulated during starvation conditions and stationary phase, the research here aimed at identifying the role of Xdh during stationary phase. Experiments performed indicated that the magic spot ppGpp could act as a ligand for XdhR. Another structurally similar molecule c-di-GMP was also identified to be an efficient ligand for XdhR (Figure 5.1). Gene expression studies in S. coelicolor indicated that the xdh gene is upregulated during stationary phase, and thin layer chromatography revealed that ppGpp levels are high during starvation and stringent response. During exponential phase or when abundant nutrients are present, GTP acts as a ligand of XdhR leading to the expression of xdh. Upon starvation or nutrient limited conditions, ppGpp and c-di-GMP might act as ligands for XdhR of A. tumefaciens and R. solanacearum and therefore there could be chances that under these conditions expression of xdhABC gene cluster might occur in these two organisms. Thus depending upon the need of the cell, expression of xdh is controlled by XdhR protein employing a mechanism that is co-ordinated by the ligands mentioned above (Figure 5.1). Therefore in this research it has been shown that XdhR plays an important role in regulation of an essential step in purine metabolism by Xdh, especially during starvation.
Based on these results a model is proposed (Figure 5.2) in which XdhR represses the \textit{xdhABC} gene cluster. Whenever ppGpp and c-di-GMP are produced in the cell during starvation, they bind to XdhR leading to upregulation of the \textit{xdhABC} gene cluster. The resulting Xdh can now function to salvage purines and direct them towards production of GTP. Maintaining GTP level becomes essential for sustained synthesis of ppGpp and c-di-GMP that are essential signaling molecules of the cell during times of starvation and biofilm formation, respectively.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5_1.png}
\caption{Ligands of XdhR A. Guanosine triphosphate (GTP) B. Guanosine tetraphosphate (ppGpp) C. Cyclic-di-guanosine monophosphate (c-di-GMP)}
\end{figure}

\textit{S. coelicolor} XdhR is identified to be a tetramer while \textit{A. tumefaciens} XdhR is a dimer. XdhR binding to the intergenic region near \textit{xdhR} gene was identified by footprinting, but no specific binding to the intergenic region near \textit{xdh} could be observed. Therefore, it was proposed that a
DNA lopping mechanism is involved during XdhR repression of the \textit{xdhABC} gene cluster (Figure 2.14). A DNA bending protein such as HU could be involved in this looping process. Determining the structure of \textit{S. coelicolor} XdhR in presence of ligands or DNA would give more information regarding the structural rearrangements that could happen during ligand binding and DNA binding, respectively.

![Diagram](image)

Figure 5.2. Proposed model of XdhR regulatory function in bacteria.

Regulation of \textit{xdh} gene expression by \textit{A. tumefaciens} XdhR during stationary phase and starvation conditions has yet to be demonstrated. It has been identified that RelA protein of this organism does not respond to a single amino acid starvation and serine hydroxamate (Krol & Becker, 2011, Belitsky & Kari, 1982). RelA is found to produce ppGpp upon carbon starvation.
and therefore it would be interesting to study if this gene cluster could be upregulated upon carbon starvation. Moreover in *A. tumefaciens*, it has been identified that presence of urate upregulates the genes *pecS* and *pecM* (Perera & Grove, 2010). It would be interesting to analyze expression of *pecS* and *pecM* whenever *xdh* expression goes up. Increased synthesis of Xdh would cause increased production of urate and it would not be surprising if *pecS* and *pecM* get upregulated during starvation or stringent response.

*R. solanacearum* XdhR belongs to the LysR family of transcriptional regulators that can occur both as activators and repressors (Maddocks & Oyston, 2008). It would be interesting to know the binding pattern of XdhR in the presence of ligands and oxidants, and this could be revealed in a footprinting experiment. *R. solanacearum* is a very sturdy organism that can survive in wet soil for years. This bacterium encounters reactive oxygen species upon infecting tomato plants. *In planta* expression studies revealed that the *xdh* gene is upregulated in this bacterium during infection in the host (Flores-Cruz & Allen, 2009). During its infection in the host, the bacterium colonizes xylem tissues, which have low nutrient content (Jacobs *et al.*, 2012). Therefore it is obvious that the organism needs to withstand nutrient starvation and oxidative stress for effective colonization to become a successful pathogen. With all these information it could be surmised that XdhR of *R. solanacearum* being a LysR family member could transform into an activator upon encountering reactive oxygen species. The oxidized protein could now activate transcription of *xdhABC* gene cluster that is identified observed during infection in tomato plants. It would be interesting to know if the *xdhABC* gene cluster is upregulated during starvation and stringent responses in this organism.
The divergent gene orientation of \textit{xdh} and \textit{xdhR} genes is uncommon in bacteria. XdhR of \textit{S. coelicolor} and \textit{A. tumefaciens} belong to the TetR family, whereas XdhR of \textit{R. solanacearum} belongs to the LysR family, it is surprising to note that XdhR proteins of all these bacteria have evolved a similar regulatory function. It would be fascinating to know if up-regulation of \textit{xdh} is seen during starvation and stringent conditions in other bacteria not possessing divergent gene orientation. A study of this kind would be very informative to compare the function of Xdh and XdhR of \textit{S. coelicolor}, \textit{A. tumefaciens} and \textit{R. solanacearum} with other bacteria.

Owing to the critical regulatory function of XdhR on \textit{xdh} in bacteria in order to sustain production of GTP to generate the signaling molecules ppGpp and c-di-GMP during starvation, it would make sense to consider this protein as a potential drug target to control virulence and biofilm formation in bacteria. Though Xdh is not essential for the survival of this organism, it plays an important role in virulence and biofilm formation without which pathogenic organisms could not establish infection in their host successfully. Therefore studying XdhR protein and Xdh would definitely explore an additional means to efficiently tackle disease-causing pathogenic organisms.

References


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Smitha Sivapragasam was born in Puducherry Union Territory, India. She attended Vivekanandha Higher secondary school during primary and high school education. After completing her higher secondary education at Thiruvalluvar Government Girls Higher Secondary School in 2000 she decided to pursue her Bachelor’s degree in Veterinary sciences and Animal Husbandry at Rajiv Gandhi College of Veterinary and Animal Sciences. She graduated in the year 2005 and served as a veterinary doctor at Blue cross, Auroville Branch, Puducherry momentarily after which she decided to pursue her Master’s degree in Veterinary Sciences. She successfully competed in All India Entrance Test conducted by Indian Council of Agricultural Research in the year 2006 and was awarded with Junior Research Fellowship for pursuing her Master’s degree in Veterinary Parasitology in the prestigious Indian Veterinary Research Institute. She completed her Master’s degree in 2008 and soon decided to pursue her interest in Basic Sciences by joining the Department of Biological Sciences, LSU, as a Ph.D. scholar. Twists and turns in her graduate life educated her to search for an ideal environment that could sustain and enrich her passion for Science. She then got an opportunity to pursue her Ph.D. under the guidance of Dr. Anne Grove in Fall 2012. There she worked on Bacterial Xanthine Dehydrogenase Regulator proteins and identified the novel function of these proteins during starvation conditions and biofilm formation in bacteria. She would love to pursue her career in academics and research.