2013

MarR Family Transcriptional Regulators from Streptomyces coelicolor

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MARR FAMILY TRANSCRIPTIONAL REGULATORS FROM
STREPTOMYCES COELICOLOR

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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December 2013
Dedicated to my parents, my grandparents and brother
for their selfless love
ACKNOWLEDGMENTS

The first and most important acknowledgements are to my advisor and mentor Dr. Anne Grove who plays a critical role in my research growth. Dr. Grove gives critical guidance in my scientific research with her patience and dedication. Her enthusiasm in science also affects me deeply. Without her encouragement and help, I would not be able to complete this dissertation.

I would like to sincerely acknowledge my committee, Dr. Joomyeong ‘Joo’ Kim, Dr. Yong-Hwan Lee, Dr. William T. Doerrler and Dr. Shane Stadler. My committee provides important guidance, discussion and encouragement.

I sincerely thank Brian J. Mackel who cooperated with me in research about PecS. I sincerely thank all my labmates who have provided strong support and sincere friendship. They include Dr. Khoa Huynh Nguyen, Ambuj Kumar Kushwaha, Dinesh Kumar, Ashish Gupta, Dr. Kavitha Srinivasa, Anuja Pande, Smitha Sivapragasam and Tiffany L. Lemon.

I sincerely thank all other persons who have given me help in this period of study and research. In fact, a lot of people helped me directly or indirectly during my research and study at LSU.

My parents, Lingyan Huang and Dafang Chen, always supported and encouraged me in different stages of my life. The love from my parents, my grandparents and my younger brother will definitely inspire me all my life.
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ABSTRACT

A family of transcriptional regulators that ubiquitously exists in prokaryotes is the multiple antibiotic resistance regulator (MarR) family. These transcriptional regulators participate in many cellular processes and can provide valuable knowledge about transcriptional regulation in response to specific conditions. The closely related MarR homologs TamR (trans-aconitate methyltransferase regulator) and PecS in Streptomyces coelicolor were studied to investigate their potential role in this bacterium.

In Streptomyces coelicolor, the gene (SCO3133), which encodes TamR, is oriented divergently from the tam gene, which encodes trans-aconitate methyltransferase. TamR was found to regulate several target genes, which encode several enzymes closely related to the citric acid cycle, by binding to their promoter regions. TamR can regulate the transcription of tamR (encoding TamR), tam (encoding trans-aconitate methyltransferase), sacA (encoding aconitase), aceB1 (encoding malate synthase), mdh (encoding malate dehydrogenase) and idh (encoding isocitrate dehydrogenase). Moreover, the divergent tam-tamR gene pairs and the predicted TamR binding sites are highly conserved in the promoter regions of its target genes in different Streptomyces species. Trans-aconitate can attenuate DNA-binding by TamR, as can citrate, cis-aconitate and isocitrate, which are the substrate, intermediate and product of aconitase, respectively. In vivo results also showed that citrate and hydrogen peroxide can induce upregulation of these target genes. The collected information in this study suggests that TamR plays an important and conserved role in promoting metabolic flux through the citric acid cycle under some stress conditions.

S. coelicolor also encodes a PecS homolog (SCO2647) that regulates a pecM gene (SCO2646). S. coelicolor PecS, which exists as a homodimer, binds the intergenic region between
pecS and pecM genes with high affinity. The binding of PecS to its target DNA can be efficiently attenuated by the ligand urate, which also quenches the intrinsic fluorescence of PecS, indicating a direct interaction between urate and PecS. In vivo measurement of gene expression showed that activity of pecS and pecM genes is significantly elevated after exposure of S. coelicolor cultures to urate. These results indicate that S. coelicolor PecS responds to the ligand urate by attenuated DNA binding in vitro and upregulation of gene activity in vivo.
CHAPTER 1
INTRODUCTION

MarR family transcriptional regulators

Multiple antibiotic resistance regulator (MarR) family members ubiquitously exist among different bacteria and archaea. MarR family transcriptional regulators are involved in a variety of biological processes in the cell through transcriptional regulation, which includes but is not limited to metabolism, environmental chemical response (such as phenolic compound degradation), pathogenicity (for instance virulence factor synthesis), and environmental stress response (e.g., oxidative stress) (Sulavik et al. 1994; Martin & Rosner 1995; Alekshun & Levy 1999a; Wilkinson & Grove 2006; Perera & Grove 2010a; Grove 2013). Because of the ubiquity and functional variety, MarR family transcriptional regulators in different bacteria and archaea are widely investigated.

MarR was first discovered in E. coli (George & Levy 1983). In E. coli, MarR regulates the operon marRAB, which is involved in the multiple antibiotic resistance (George & Levy 1983; Sulavik et al. 1994; Martin & Rosner 1995; Seoane & Levy 1995; Alekshun & Levy 1997, 1999c, a, b) (Figure 1.1). Therefore, it was named as multiple antibiotic resistance regulator. A marC gene is located adjacent to this operon. MarR can regulate the expression of marRAB by binding to the intergenic region (marO) of marC-marRAB. Two MarR binding sites (site I and site II) are located in marO. One binding site is within the region of -35 and -10 hexamers. One binding site is located in the region which overlaps the putative ribosome binding site (Martin & Rosner 1995; Alekshun & Levy 1997, 1999c). Since the discovery of MarR in E. coli, many MarR family transcriptional regulators have been identified and studied in different bacteria and archaea.
Most MarR family transcriptional regulators repress gene expression when they bind to their binding site in the promoter of target genes, although the activation of a target gene by binding also occurs. The autoregulation of its own gene and an adjacent divergently oriented gene by MarR through binding and dissociating from the binding sites in the intergenic region of these two genes is a common design to these regulators. Responding to specific ligand for regulation is also a common characteristic of MarR family regulators. Usually, when a MarR family regulator binds to its target intergenic region, it can repress the expression of both genes (Figure 2.1A). If this MarR family regulator binds to its specific ligand, which occurs when the concentration of this
ligand becomes elevated for some reason, DNA binding will be attenuated, which will result in the derepression of both genes. The derepression of both genes will increase the expression of the MarR homolog, which can again bind to its binding sites if the ligand concentration is not high enough to bind the newly expressed regulator. The expression of both genes will be repressed by MarR again. Therefore, excessive expression of unnecessary proteins will be prevented by this regulatory mechanism. In addition to genes adjacent to the gene encoding MarR, additional target genes located in other positions can also be regulated by the MarR homolog (Figure 1.2B).

![Figure 1.2. A common regulatory mechanism of MarR family transcriptional regulator responding to specific ligand.](image)

MarR family regulators usually exist as homodimers, which contain two intertwined subunits that interact mainly in the dimerization domain in each subunit. In addition to the dimerization domain, MarR family members contain the DNA-binding domain, which is involved...
in DNA recognition and interaction. In each subunit, the whole structure is usually organized by the following order of secondary structures: H1-H2-S1-H3-H4-S2-W1-S3-H5-H6, where “H” represents α-helix, “S” represents β-strand, and “W” represents loop. H1, H5 and H6 usually participate in the dimerization of protein (Alekshun et al. 2000; Alekshun et al. 2001) (Figure 1.1A). The DNA-binding motif of MarR family proteins is a common DNA binding motif. For DNA binding proteins, some common motifs include helix-turn-helix (HTH), zinc finger, leucine zipper, and helix-loop-helix, which all play central roles in DNA and protein interaction (Luscombe et al. 2000; Pabo & Sauer 1992; Huffman & Brennan 2002). In prokaryotes, a very important DNA binding motif is the HTH motif, which is found in the structure of many different kinds of transcriptional regulators (Huffman & Brennan 2002). MarR family proteins belong to the winged helix DNA-binding protein family, which is a subset of the HTH protein family (Pabo & Sauer 1992; Luscombe et al. 2000; Perez-Rueda et al. 2004). In MarR family transcriptional regulators, the most common arrangement of the secondary structure elements in each DNA-binding domain shows the typical winged-helix DNA motif: H2-S1-H3-H4-S2-W1-S3. Both the wing and the DNA recognition helix (H4) are involved in the direct interaction with DNA, although other secondary structures also play important role in this motif (Figure 1.3). The recognition helix usually interacts with the major groove of DNA in its binding position while the wing usually interacts with the minor groove of DNA. Since the dimerization domain can affect the distance between two DNA recognition helices in the dimeric structure of MarR protein, this domain can also affect the DNA binding ability of protein because this distance is especially important for DNA binding. The binding sites of MarR family regulators are generally 16–20 bp inverted repeats.
Figure 1.3. Crystal structure of SlyA from Salmonella enterica in DNA-bound state. This MarR-DNA complex shows the interaction between MarR homolog and target DNA. A, B, C show the structure in different view directions. Figures are generated from SlyA (PDB ID: 3Q5F).

For MarR family regulators, ligand binding usually causes some conformational change to the structure of the protein, which results in the switch from DNA binding conformation to a relatively incompatible conformation. The ligand-binding pocket is usually located at a specific position, which can affect the DNA binding ability of the protein. The cleft between the dimer interface and the wHTH DNA binding lobe is a common position to which ligand binds (Grove 2013). The binding between MarR family regulator and ligand usually involves the interaction between specific amino acid residues in the MarR homolog and several specific groups in the
ligand. For MarR family members that can sense oxidative stress or related stresses, the conformational change usually can be caused by oxidation of specific residues in the protein (usually Cys residues) by related stresses. The physiological ligands and the regulated genes of a MarR family regulator usually provide important information about the regulatory role of this MarR member because physiological ligands reveal the signal this MarR family regulator senses and the regulated genes reveal the response to the sensed signal. In addition, some MarR family members are global regulators, which can regulate the transcription of a variety of genes.

In many cases, it has proven difficult to identify the natural ligand for a given MarR homolog. However, a subset of MarR proteins was characterized that shared certain features of the ligand-binding pocket. In this urate-responsive transcriptional regulator subfamily (UrtR subfamily) of MarR, an extra N-terminal α-helix is contained in each subunit of the dimer (Bordelon et al. 2006; Perera & Grove 2011) (Figure 1.4A). The HucR (hypothetical urate regulator) from *Deinococcus radiodurans* represents a UrtR subfamily member, which can bind urate as a ligand for transcriptional regulation (Bordelon et al. 2006; Perera & Grove 2011; Wilkinson & Grove 2004, 2005; Perera et al. 2009; Perera & Grove 2010a, b). The gene encoding HucR (*dr1159*) and a uricase encoding gene (*dr1160*) are located adjacent to each other with divergent orientation (Figure 1.4B). HucR can specifically bind to its binding site in the intergenic region between *dr1159* and *dr1160* to repress the transcription of both genes (Wilkinson & Grove 2004). Urate can work as a ligand to cause the derepression effect because urate can attenuate the DNA binding of HucR to this intergenic DNA (Wilkinson & Grove 2004). In HucR, three amino acid residues directly take part in urate coordination, which includes Trp20, Arg80 and Asp73 (Perera & Grove 2010a). The attenuating effect of urate on the DNA binding of HucR mutants in which these residues are substituted (HucR W20F, HucR D73S, HucR R80S) decreases
dramatically. In addition, Arg106 also plays important role for modification of DNA binding ability after binding of urate. This is because Arg106, which is in the DNA recognition helix, forms a salt bridge with Asp73 and is displaced on ligand binding.

Figure 1.4. *D. radiodurans* HucR. A. Crystal structure of *D. radiodurans* HucR. Four highly conserved amino acid residues involved in response to urate are shown in red. Figure is generated with HucR (PDB ID: 2FBK). B. The regulation of *D. radiodurans* HucR.

HucR from *D. radiodurans* regulates a gene that encodes uricase. Urate, which is the substrate of uricase, is a ligand that can attenuate the DNA binding by HucR. Based on the conservation of the N-terminal extension and the ligand-binding residues, different UrtR subfamily homologs were identified. However, the target genes of UrtR subfamily members are usually not
urate-encoding genes; instead, many UrtR homologs regulate a target gene, which encodes an efﬂux pump (ref Inoka’s Protein Science paper). The regulation by a urate-sensitive transcription factor suggests a possible role for these efﬂux pumps. The following is one possible reason why urate needs be sensed by some UrtR subfamily members. Generation of ROS (reactive oxygen species) is an important mechanism for plants and many other organisms to defend against invading pathogens. Xanthine oxidase, which also takes part in purine degradation, catalyzes important ROS generation reactions to kill invading pathogens (Figure 1.5). Xanthine oxidase can catalyze reactions using hypoxanthine or xanthine as a substrate to produce hydrogen peroxide. Uric acid is another final product of these reactions. In some pathogens, urate, the ﬁnal product of xanthine oxidase, may be sensed by this UrtR subfamily regulator as a signal, which indicates the generation of ROS by the host. The response to this stress, which can directly come from the regulation by UrtR of the transcription of target genes, can be triggered in order to survive the host defense process.

Figure 1.5. Reactions catalyzed by xanthine oxidase.

The diverse roles of different MarR family members in bacteria

In the Gram-negative pathogenic bacterium Pseudomonas aeruginosa, MexR, a MarR family member, plays a critical role in intrinsic multidrug resistance. As a well characterized MarR family member, MexR was shown to regulate the expression of the multiple antibiotic resistance
efflux operon \textit{mexAB-oprM}, which encodes an important multidrug efflux pump system in \textit{P. aeruginosa} (Poole \textit{et al.} 1996; Li & Poole 1999; Srikumar \textit{et al.} 2000; Evans \textit{et al.} 2001). This efflux pump system can export antibiotics and some other chemical compounds; therefore it can increase the resistance of this bacterium to some antibiotics (Poole \textit{et al.} 1996; Li & Poole 1999; Srikumar \textit{et al.} 2000; Evans \textit{et al.} 2001). Both MexR and a TetR family regulator NalD can bind to the promoter region of the \textit{mexAB-oprM} operon and negatively regulate the transcription of this operon (Sobel \textit{et al.} 2005; Morita \textit{et al.} 2006). In addition, an antirepressor ArmR can bind to MexR and prevent the binding of MexR to this region of DNA (Starr \textit{et al.} 2012). This antirepression can be inhibited by NalC because NalC can repress the expression of ArmR by binding to the promoter region of the \textit{armR} gene (Cao \textit{et al.} 2004b; Ghosh \textit{et al.} 2011; Sadeghifard \textit{et al.} 2012; Starr \textit{et al.} 2012).

The crystal structure of MexR is consistent with the typical structure of MarR family proteins (Lim \textit{et al.} 2002; Wilke \textit{et al.} 2008), although four different conformational states can be observed. Multiple conformational states may partly result from the high flexibility of the dimer interface. The crystal structure of MexR in complex with its antirepressor ArmR reveals that the C-terminal residues of ArmR form a kinked \(\alpha\)-helix. This \(\alpha\)-helix can bind to a cleft located at the centre of the MexR dimer, resulting in the conformational rearrangement of MexR. The conformational changes in the complex include shrinkage of the distance between two DNA binding helices (between \(\alpha'4\) and \(\alpha4\)), a twist of DNA binding domain, and bend of \(\alpha5\).

In fact, MexR can regulate this operon by responding to oxidative stress (Chen \textit{et al.} 2008; Wilke \textit{et al.} 2008; Chen \textit{et al.} 2010). The formation of intermonomer disulphide bonds between Cys 30 in \(\alpha1\) and Cys 62 in the loop between \(\alpha'3\) and \(\alpha'4\) causes conformational modification, which results in incompatibility with target DNA (Chen \textit{et al.} 2008; Wilke \textit{et al.} 2008; Chen \textit{et al.}
Therefore, the regulation by sensing oxidative stress is achieved to derepress the transcription of \textit{mexAB-oprM}. This oxidation of MexR results in a clear conformational change of the DNA binding domain although the distance between two DNA binding helices (between $\alpha'4$ and $\alpha 4$) does not clearly change (Wilke \textit{et al.} 2008; Chen \textit{et al.} 2010).

A MarR family regulator in the plant pathogen \textit{Dickeya dadantii} (\textit{Erwinia chrysanthemi}), PecS, plays the role of global regulator for the expression of some virulence factors (Reverchon \textit{et al.} 1994; Praillet \textit{et al.} 1996; Praillet \textit{et al.} 1997a; Praillet \textit{et al.} 1997b; Reverchon \textit{et al.} 2002; Rouanet \textit{et al.} 2004; Hommais \textit{et al.} 2008; Mhedbi-Hajri \textit{et al.} 2011). PecS in \textit{D. dadantii} regulates the transcription of genes or operons related to flagellum biogenesis ($fliE$ and $fliFGHIJKLMNOPQR$), indigoidine biogenesis ($indA$ and $indC$), pectinase ($pelB$, $pelC$, $pelD$, $pelE$, $pem$), cellulase ($celZ$) and the type III effector HrpN ($hrp$) in addition to pec\textit{S}-pec\textit{M} (Reverchon \textit{et al.} 1994; Praillet \textit{et al.} 1996; Praillet \textit{et al.} 1997a; Praillet \textit{et al.} 1997b; Reverchon \textit{et al.} 2002; Rouanet \textit{et al.} 2004; Hommais \textit{et al.} 2008; Mhedbi-Hajri \textit{et al.} 2011). PecS plays an important role for the regulation of virulence factors in \textit{D. dadantii}, which is responsible for the soft-rot disease of a wide range of plants. Virulence factors, such as pectinases and cellulases, are expressed constitutively in a Pec\textit{S} mutant strain. Moreover, elevated expression of these proteins in the mutant can be reversed by introducing a low-copy-number plasmid with recombinant wild-type pec\textit{S} gene (Praillet \textit{et al.} 1997a; Praillet \textit{et al.} 1997b). In \textit{Rhizobium radiobacter} (\textit{Agrobacterium tumefaciens}), a Pec\textit{S} homolog can respond to urate as ligand \textit{in vivo} and \textit{in vitro} (Perera & Grove 2010b). In both \textit{R. radiobacter} and \textit{D. dadantii}, a membrane transporter-encoding gene, pec\textit{M}, which is located adjacent to pec\textit{S} with divergent direction was regulated by Pec\textit{S}. Pec\textit{M} from \textit{D. dadantii} was previously shown to be involved in indigoidine excretion as a membrane transporter (Praillet \textit{et al.} 1997a; Praillet \textit{et al.} 1997b). This excreted antioxidant
indigoidine, which is important for bacterial virulence can function in defense against reactive oxygen species (ROS) during invasion.

*Sphingobium* sp. strain SYK-6 is a bacterium that can degrade various lignin-derived aromatic compounds. In this bacterium, a MarR family member FerC is involved in the transcriptional regulation of lignin-derived aromatic compounds ferulate (Kasai *et al.* 2012). In this bacterium, *ferA* and *ferB* encode feruloyl-CoA synthetase and feruloyl-CoA hydratase/lyase, respectively. These enzymes catalyze the reactions, which can convert ferulate to vanillin and acetyl-coenzyme A as two important steps during the degradation of ferulate. FerC can bind to the intergenic region of *ferC-ferB* and regulate the transcription of *ferBA* operon,(Kasai *et al.* 2012). This binding can be attenuated by feruloyl-CoA (Kasai *et al.* 2012).

The research about SarZ in the Gram-positive human pathogen *Staphylococcus aureus* provides information about MarR family transcriptional regulator members, which can use a single Cys residue to sense oxidative stress (Chen *et al.* 2009; Poor *et al.* 2009; Tamber & Cheung 2009). SarZ, which can sense oxidative stress by oxidation of Cys13, belongs to the single-cysteine class of OhrR/MgrA proteins. SarZ, which can regulate the expression of more than eighty genes in *S. aureus*, is a global regulator. SarZ takes part in the regulation of peroxide response, biofilm formation, virulence, and metabolic processes. Oxidation of Cys13 can attenuate the binding between SarZ and its target promoter DNA. Crystal structure information reveals that mixed disulfide bond formation between Cys13 sulfenic acid and an external thiol results in an allosteric change in the DNA-binding domains, which may play a critical role in oxidative stress sensing. When Cys13 is reduced or in sulfenic acid modification state, the conformation of SarZ is similar.

*Comamonas testosteroni* KH122-3s is a Gram-negative soil-dwelling bacterium, which can utilize the isomer of salicylate (2-hydroxybenzoate) 3-hydroxybenzoate (3-HB) in its metabolic
system (Hiromoto et al. 2006; Yoshida et al. 2007; Chang & Zylstra 2008). MobR from C. testosteroni KH122-3s is a MarR family transcriptional regulator, which plays an important role in the metabolism of 3-hydroxybenzoate. MobR can negatively regulate the transcription of the mobA gene, which encodes a 3-hydroxybenzoate 4-hydroxylase (Hiromoto et al. 2006; Yoshida et al. 2007). 3-hydroxybenzoate 4-hydroxylase can catalyze the first reaction step for the catabolism of 3-hydroxybenzoate. 3-hydroxybenzoate, the substrate of 3-hydroxybenzoate 4-hydroxylase, works as the ligand, which can efficiently induce the expression of mobA after binding to MobR.

YetL, a MarR family member in Bacillus subtilis can regulate the transcription of an adjacent gene yetM (Leelakriangsak et al. 2008; Hirooka et al. 2009). yetM encodes a putative flavin adenine dinucleotide-dependent monooxygenase. Disruption of the yetL results in the derepression of yetM. YetL can bind to the intergenic region between yetM and yetL and repress the expression of yetM. Some flavonoids (kaempferol, apigenin, and luteolin) can effectively attenuate the binding between YetL and the promoter region of yetM.

YodB, another MarR family transcriptional regulator in B. subtilis can regulate the quinone detoxification by responding to thiol stress. YodB can regulate gene yocJ (azoR1), which encodes an azoreductase in B. subtilis, by responding to thiol-specific conditions (Leelakriangsak et al. 2008; Hirooka et al. 2009). The derepression of yocJ gene is distinct after YodB responds to thiol stress. In this regulation, several Cys residues in YodB play important roles in thiol stress responding.

In the halophilic aerobic methylotrophic bacterium Methylophaga thalassica, which can synthesize ectoine from aspartate, a MarR family transcriptional regulator EctR plays a role in the regulation of ectoine biosynthesis (Mustakhimov et al. 2012). Ectoine is involved in the tolerance of hyperosmotic stress as a compatible solute (Galinski et al. 1985; Ono et al. 1999; Reshetnikov
et al. 2011; Mustakhimov et al. 2012). The synthesis of ectoine requires specific enzymes, which are encoded by the *ectABC-ask* operon (Galinski et al. 1985; Reshetnikov et al. 2011). Enzymes encoded by this operon include diaminobutyric acid (DABA) acetyl-transferase (EctA), DABA aminotransferase (EctB), ectoine synthase (EctC) and aspartokinase isozyme (*ask*). EcrR can bind to the promoter region of *ectABC-ask* operon to regulate this operon.

NadR (*NmFarR*, encoded by NMB1843), a MarR family transcriptional regulator in the human pathogen *Neisseria meningitides*, which can cause septicemia and meningitis can regulate the transcription of adhesin encoding gene NadA (Schielke et al. 2009; Brier et al. 2012; Fagnocchi et al. 2012). NadR can bind to a 16 bp palindromic binding site in the promoter region of *nadA* and repress *nadA* expression. Because NadR is involved in the transcriptional regulation of adhesin NadA, which is related to the adhesive properties and host colonization of *N. meningitides*, the strain with insertional mutation in NMB1843 showed considerably more adhesion compared with the wild-type strain.

TcaR regulates biosynthesis of poly-N-acetylglucosamine (PNAG) and the formation of biofilm in *Staphylococcus epidermidis* (Jefferson et al. 2004; Chang et al. 2010; Chang et al. 2012). TacR and another transcriptional regulator IcaR regulate the transcription of the *ica* operon (Jefferson et al. 2004; Chang et al. 2010; Chang et al. 2012). Multiple TcaR can specifically bind to the promoter of the *ica* operon to regulate its transcription. Salicylate and some other ligands can attenuate the binding between TcaR and DNA. The crystal structure of apo form TcaR and TcaR with ligand indicates that ligand regulation may result from structural changes in the wHTH DNA-binding domain (Chang et al. 2010). Recent research showed that TcaR can even interact strongly with single-strand DNA. TcaR was shown to even prefer to bind ssDNA over a double-stranded DNA during in vitro competition experiments (Jefferson et al. 2004; Chang et al. 2012).
MarR family transcriptional regulators play important roles in the zinc homeostasis in *Streptococcus pneumoniae*. In *S. pneumoniae*, AdcR, a MarR family transcriptional regulator, regulates the transcription of operon *adcCBA*, which encodes a high-affinity ABC uptake system involved in uptake of Zn(II) (Guerra *et al*. 2011; Guerra & Giedroc 2012; Reyes-Caballero *et al*. 2010). AdcR can respond to the intracellular Zn(II) concentration by binding to Zn(II) and regulating the expression of *adcCBA* according to Zn(II) concentration.

All the above mentioned examples reflect widely diverse roles of MarR family transcriptional regulators in events from stress response and virulence gene expression to regulation of metabolic pathways. These examples also reflect that MarR family members can respond to diverse signals, which can be small molecule ligands, metal ion concentration or Cys oxidation stress. While different MarR family transcriptional regulators have diverse functional roles, the response to an environmental signal is usually a common characteristic. While some MarR homologs that can regulate metabolic enzymes during the metabolism of specific compounds (such as metabolism of 3-hydroxybenzoate by MobR or urate by HucR) have been characterized, MarR homologs regulating expression of enzymes involved in central metabolic pathways have not been reported.

**MarR family transcriptional regulators in archaean**

In addition to different bacteria, MarR transcriptional regulators also exist in archaean. Until now, the crystal structure of MarR family members in some archaean were reported, which include EmrR (Miyazono *et al*. 2007) and ST1710 (Kumarevel *et al*. 2009) from *Sulfolobus tokodaii* strain 7 and BldR (Di Fiore *et al*. 2009) from *Sulfolobus solfataricus*.

The research about the MarR family transcriptional regulators in *S. solfataricus* lead to the discovery of two MarR family regulators: BldR (Fiorentino *et al*. 2007) and BldR2 (Fiorentino *et
BldR can bind to the promoter region of a gene (Sso2536), which encodes alcohol dehydrogenase and the promoter region of an adjacent gene (Sso1352), which putatively encodes a multidrug transporter (Fiorentino et al. 2007). BldR can activate the expression of these genes by binding to the binding sites in the promoter region of these genes. The in vivo expression of these genes and the gene encoding BldR (Sso1351) is dramatically elevated in response to several aromatic aldehydes, which include cinnamaldehyde, veratraldehyde and benzaldehyde. The concentration of BldR in the cell is distinctly elevated in the presence of those aromatic aldehydes. In addition, the in vitro binding between BldR and promoter region of Sso2536 or Sso1352 can be stimulated by benzaldehyde, which is the substrate of alcohol dehydrogenase. The crystal structure of BldR shows that this archaeal MarR family member contains a typical structure of MarR family member (Di Fiore et al. 2009). BldR exists as dimer and contains a winged helix-turn-helix in its secondary structure organization. Ser65 and Arg90 are two residues, which are mainly responsible for binding specificity.

**Citric acid cycle**

As one part of central metabolism, the citric acid cycle plays a critical role in energy production in different aerobic organisms. It is a cyclic pathway, which contains eight major steps catalyzed by different enzymes (Figure 1.6). Acetyl-CoA from glycolysis or other sources (fatty acid degradation, amino acid degradation, etc.) can enter into the citric acid cycle and get oxidized to produce energy. In addition to catabolism, the citric acid cycle also plays an important role in anabolism and provides various metabolic intermediates as precursors for biosynthesis of various bimolecules. In addition to the connection to glycolysis and gluconeogenesis, the citric acid cycle (or TCA cycle) is connected to a variety of important metabolic processes through different intermediates. The following reaction is the overall net reaction of the TCA cycle: 3NAD$^+$ + FAD
\[ \text{GDP + Pi + acetyl-CoA + 2 H}_2\text{O} \rightarrow 3 \text{NADH} + \text{FADH}_2 + \text{GTP} + \text{CoA} + 2 \text{CO}_2 + 3\text{H}^+ \]

The entire citric acid cycle involves eight central enzymes: citrate synthase (EC 4.1.3.7), aconitase (EC 4.2.1.3), isocitrate dehydrogenase (EC 1.1.1.42), 2-oxoglutarate dehydrogenase (EC 1.2.4.2), succinyl-CoA synthetase (EC 6.2.1.6), succinate dehydrogenase (EC 1.3.99.1), fumarase (EC 4.2.1.2), and malate dehydrogenase (EC 1.1.1.37). Because the flux and efficiency of the citric acid cycle can be influenced by its intermediate pool, replenishing the citric acid cycle intermediate pool is important for maintaining the flux of this cycle if depletion of the intermediate pool occurs for any reasons.

The glyoxylate cycle is a variation of the citric acid cycle, which plays an important role in the synthesis of carbohydrates and in some other metabolic processes. In the glyoxylate cycle, isocitrate lyase (EC 4.1.3.1), instead of isocitrate dehydrogenase, uses isocitrate as a substrate and catalyzes a cleavage reaction to produce one molecule each of glyoxylate and succinate. Glyoxylate can be converted to malate by malate synthase. Succinate can be converted to another molecule of malate through the corresponding reactions in the citric acid cycle. Malate then can be converted to oxaloacetate by malate dehydrogenase. In this pathway, the two reaction steps of the TCA that are associated with the loss of carbon are bypassed. In the citric acid cycle, one acetyl-CoA is degraded to produce carbon dioxide and energy, whereas in the glyoxylate cycle, carbohydrate (succinate) is produced from two acetyl-CoA molecules. The following reaction is the overall net reaction of the glyoxylate cycle:

\[ 2 \text{acetyl-CoA} + 3\text{NAD}^+ = \text{succinate} + 2 \text{CoA} + \text{NADH} + \text{H}^+ \]
Figure 1.6. Brief overview of citric acid cycle and glyoxylate cycle.

For the transcriptional regulation of genes encoding enzymes in the citric acid cycle, carbon catabolite repression is usually an important mode of regulation. In Gram-positive bacteria, cAMP receptor protein (CRP) and the catabolite control protein (CcpA) usually play important roles in carbon catabolite repression (Saier et al. 1996; Warner & Lolkema 2003; Deutscher 2008). In
addition to carbon catabolite repression, expression of citric acid cycle enzymes can also be regulated by other transcriptional regulators. For instance, in *E. coli*, isocitrate lyase regulator (iclR) takes part in the transcriptional regulation of the glyoxylate bypass operon (aceBAKK), which encodes three important enzymes (malate synthase, isocitrate lyase and isocitrate dehydrogenase kinase/phosphatase) required for glyoxylate bypass (Gui *et al.* 1996; Pan *et al.* 1996; Resnik *et al.* 1996).

In the citric acid cycle, three enzymes (aconitase, fumarase and succinate dehydrogenase) contain iron-sulfur clusters, which are important for their enzyme activity. As iron-sulfur clusters are readily modified by oxidation, these enzymes are vulnerable to oxidative stress (King *et al.* 1976; Ohnishi *et al.* 1976; Robbins & Stout 1989; Flint *et al.* 1992). The inactivation of any of these iron-sulfur cluster-containing enzymes can block the respective reaction, which may result in the accumulation of intermediates before this reaction step. Since aconitase is particularly sensitive to oxidative stress because of its vulnerable [4Fe-4S] in the catalytic center, this enzyme can be a key position, which links the citric acid cycle to environmental oxidative stress influence (Robbins & Stout 1989; Verniquet *et al.* 1991; Gardner & Fridovich 1992).

**Streptomyces coelicolor and its MarR family homologs**

*Streptomyces coelicolor* belongs to the genus *Streptomyces*. Streptomycetes are mostly soil-dwelling bacteria. Streptomycetes are Gram-positive bacteria that belong to the order Actinomycetales. Streptomycetes produce a large number of natural antibiotics used in medicine. Different streptomycetes usually have some common characteristics, which include a complex lifecycle, spore generation, and abundant secondary metabolite production.

Being the model organism of *Streptomyces* species, *S. coelicolor* is studied widely. In *S. coelicolor* A3(2), the genome is composed of one linear chromosome and two plasmids. The
chromosome (with high GC-content) of *S. coelicolor* is relatively large (8,667,507 bp) compared to other bacteria (Bentley *et al.* 2002). In this large chromosome, about 7,825 predicted genes are encoded (Bentley *et al.* 2002). For bacteria, the adaptation to a changing environment is important for survival. For *S. coelicolor*, the adaptation to its living environment involves the function of many different transcriptional regulators, transporters and other proteins. Among proteins encoded in the chromosome of *S. coelicolor* A3(2), 965 proteins (12.3%) and 614 proteins (7.8%) are predicted to have regulatory function and transport function, respectively (Bentley *et al.* 2002). Two-component regulatory systems are also abundant, as are sigma factors (Bentley *et al.* 2002). Among transcriptional regulators, more than 30 proteins are predicted to be MarR family transcriptional regulators. The abundance of MarR family transcriptional regulators in *S. coelicolor* speaks to the importance of this transcription factor family to this bacterium.

Most *Streptomyces* species live in soil. Since soil exhibits variable physical conditions (such as temperature and moisture), nutritional conditions and other conditions, the competition for limited nutritional or other resources in the soil environment can be fierce. The relationships among different soil-dwelling organisms are also complex. Some *Streptomyces* species form complex relationships with plants, fungi and other organisms. The plant-streptomycete interaction is an important relationship studied widely because of its significance. Many *Streptomyces* species can colonize the rhizosphere or the plant root (Crawford *et al.* 1993; Yuan & Crawford 1995; Tokala *et al.* 2002; Cao *et al.* 2004a; Getha *et al.* 2005). In fact, streptomycetes are among the most important organisms, which play significant roles in the rhizosphere (Sardi *et al.* 1992; Crawford *et al.* 1993; Yuan & Crawford 1995; Tokala *et al.* 2002; Cao *et al.* 2004a; Getha *et al.* 2005). For instance, some *Streptomyces* species in the rhizosphere can produce and excrete secondary metabolites and enzymes to defend against fungi, therefore they protect the plant root
from invasion by some potential pathogens (Crawford et al. 1993; Yuan & Crawford 1995; Tokala et al. 2002; Cao et al. 2004a; Getha et al. 2005). Some streptomycetes can form symbiotic relationships with plants in the rhizosphere. In addition, some Streptomyces species are plant growth-promoting bacteria (Tokala et al. 2002; Cao et al. 2004a). They can produce some metabolites, which are beneficial to plant growth (Tokala et al. 2002; Cao et al. 2004a). In fact, the plant-streptomycete interaction has resulted in the potential application of some Streptomyces species in agriculture. For instance, some Streptomyces species are studied as biological control agents against soil-borne diseases.

In S. coelicolor, which contains more than 30 MarR homologs, only few MarR family members have been studied in detail. OhrR from S. coelicolor can bind to the OhrR binding sites in the intergenic region of between ohrR (SCO2987) and an adjacent gene ohrA (SCO2986) when it is in its reduced state (Oh et al. 2007). Many different organic hydroperoxides can induce the expression of both ohrA and ohrR genes. A proposed model for this regulation showed that reduced OhrR represses the transcription of both genes, whereas oxidized OhrR will result in elevated transcription (Oh et al. 2007).

PcaV, a MarR family regulator in S. coelicolor, can regulate a gene which is involved in the regulation of enzymes in the β-ketoadipate pathway, a lignin-derived aromatic compound degradation pathway (Harwood & Parales 1996). The crystal structures of apo PcaV and PcaV with the ligand protocatechuate provide rich information about the ligand response of MarR family regulators (Davis et al. 2013) (Figure 1.7). The structure of PcaV is a classic MarR structure (H1-H2-S1-H3-H4-S2-W1-S3-H5-H6, where “H” represents α-helix, “S” represents β-strand, and “W” represents loop). Ligand binding causes some conformational change of PcaV. Protocatechuate binding results in about 15° rotation of the wHTH domains towards the dimerization interface,
which is beneficial to ligand binding pocket formation. This wHTH domain position is stabilized by Arg15 and N-terminal residues. Arg15 was shown to be important for both ligand binding and DNA interaction. The 3-hydroxyl group in phenolic compounds, which can interact with His21 in PcaV, is important for ligand-PcaV interaction. Protocatechuate and some other ligands can attenuate the DNA binding by PcaV.

Figure 1.7. Crystal structure of PcaV from *Streptomyces coelicolor* with its ligand (protocatechuate). This structure shows the general ligand-binding region of MarR homologs. Figures are generated with the crystal structure of PcaV from *Streptomyces coelicolor* (PDB ID: 4FHT).

MarR homologs are particularly well suited to sense environmental changes and convert this signal into changes in gene expression. With so many MarR homologs encoded by *S. coelicolor*, they are likely to be important for its lifecycle and adaptation to environmental changes. However, it can be difficult to predict which ligands they respond to, which is a challenge in delineating their physiological role. The remainder of this dissertation will fill part of this knowledge gap by describing the function of two MarR homologs.
References


CHAPTER 2*
THE TRANSCRIPTIONAL REGULATOR TamR FROM STREPTOMYCES COELICOLOR CONTROLS A KEY STEP IN CENTRAL METABOLISM DURING OXIDATIVE STRESS

Introduction

Members of the multiple antibiotic resistance regulator (MarR) family of transcriptional regulators are involved in a variety of important biological processes, including aromatic compound catabolism, virulence factor biogenesis and stress responses (for review, see Wilkinson & Grove, 2006; Perera & Grove, 2010b). Responding to specific ligands is a characteristic of many MarR family proteins, which often repress the genes under their control until ligand is bound (Cohen et al. 1993; Ariza et al. 1994; Buchmeier et al. 1997; Wilkinson & Grove, 2004; Perera & Grove, 2010a; Davis & Sello, 2010). When the MarR homolog controls transcription of a gene encoding a metabolic enzyme, the ligand may be the substrate for this enzyme as exemplified by MobR from Comamonas testosteroni. MobR regulates transcription of the mobA gene, which encodes 3-hydroxybenzoate 4-hydroxylase, and repression of gene activity by MobR is relieved on binding 3-hydroxybenzoate (Hiromoto et al. 2006). Another example is HucR from Deinococcus radiodurans, which belongs to a subfamily of MarR homologs that respond to the ligand urate by attenuated DNA binding in vitro and increased gene activity in vivo; since HucR functions as a repressor by binding a cognate site that overlaps core promoter elements, attenuated DNA binding by HucR results in more efficient recruitment of RNA polymerase and therefore increased transcription. HucR regulates the transcription of a gene encoding uricase, an enzyme that participates in purine degradation by converting urate into 5-hydroxyisourate (Wilkinson & Grove, 2004, 2005; Perera et al. 2009).

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HucR differs from canonical MarR homologs by having an N-terminal extension, which adopts a helical conformation (Bordelon et al. 2006). Biochemical mapping of the urate-binding site in HucR identified four residues required for urate-mediated attenuation of DNA binding, with one of these residues deriving from the unique N-terminal extension (Perera et al. 2009). Using the sequence of HucR as a query to Blast bacterial genomes revealed the existence of potential urate-responsive transcriptional regulators (UrtR) in other bacterial species, two of which were experimentally confirmed; *Agrobacterium tumefaciens* PecS and *Burkholderia thailandensis* MftR respond to the ligand urate, but they do not regulate a gene encoding uricase. Instead, these transcription factors have been speculated to participate in responses to oxidative stress by regulating transcription of a divergently oriented gene encoding an efflux pump involved in secretion of antioxidants (Perera & Grove, 2010a; Grove, 2010). The latter inference was based on experimental reports that *Dickeya dadantii* (*Erwinia chrysanthemi*) PecS regulates expression of a gene encoding the efflux pump PecM, which transports the antioxidant indigoidine (Rouanet & Nasser, 2001).

Another potential urate-responsive MarR homolog was predicted in the genome of *Streptomyces coelicolor* based on bioinformatics analyses (Perera & Grove, 2011). *S. coelicolor* is a soil-dwelling bacterium and the model organism of *Streptomyces* species, characterized by a complex lifecycle and production of an extensive set of secondary metabolites (Hopwood, 1999; Bentley et al. 2002; Challis & Hopwood, 2003; Gehring et al. 2004). Notably, a gene encoding a putative homolog of HucR was identified that is not oriented divergently from a gene encoding an efflux pump, but instead a gene annotated as a putative trans-aconitate methyltransferase (Figure 2.1A). A genomic organization involving divergently encoded genes is common for loci encoding MarR homologs and predicts regulation of the divergently oriented gene by the transcription
factor, a prediction based on the frequent reports of such mode of regulation (e.g., Ariza et al. 1994; Perera & Grove, 2010a; Davis & Sello, 2010).

The enzyme trans-aconitate methyltransferase functions to regulate key metabolic pathways. Citrate isomerization is an important reaction in both the citric acid and glyoxylate cycles, in which citrate is converted to isocitrate by aconitate hydratase (aconitase) via the intermediate cis-aconitate (Figure 2.1B). If cis-aconitate is released from the enzyme-substrate complex, it can spontaneously be converted to the more stable isomer trans-aconitate (Ambler & Roberts, 1948). Trans-aconitate is an efficient inhibitor of aconitase and if this toxic byproduct accumulates, it inhibits the citrate isomerization step (Saffran & Prado, 1949; Lauble et al. 1994; Cai & Clarke, 1999; Cai et al. 2001). One circumstance under which trans-aconitate may accumulate is oxidative stress; aconitase contains a [4Fe-4S] cluster that is essential for catalytic activity, and this cofactor is disassembled under oxidative stress conditions rendering the enzyme non-functional (Verniquet et al. 1991; Gardner & Fridovich, 1992). The enzyme trans-aconitate methyltransferase functions to prevent accumulation of trans-aconitate by using it as a substrate to catalyze a methyl group transfer from S-adenosyl-methionine (SAM), resulting in formation of the trans-aconitate methylester. Esterification of trans-aconitate significantly attenuates its ability to inhibit aconitase and to interfere with key steps in central metabolism (Cai & Clarke, 1999; Cai et al. 2001). The fate of the trans-aconitate methyl ester is unclear.

In this chapter, we report that the predicted HucR homolog (named TamR for trans-aconitate methyltransferase regulator) functions to control expression of the genes encoding trans-aconitate methyltransferase (tam) and TamR. Consistent with attenuated DNA binding in vitro, gene activity is increased under oxidative stress conditions under which accumulation of citrate
and trans-aconitate occurs. Thus, TamR functions in oxidative stress responses to alleviate the consequences of aconitase inactivation.

**Experimental Procedures**

**Sequence alignment and phylogenetic analysis**

Amino acid sequences of selected MarR homologs were aligned using the MUSCLE sequence alignment server (Edgar, 2004). Residues were shaded according to their identity and similarity using BOXSHADE v3.21. The sequence of HucR (*D. radiodurans*) was used to identify secondary structure elements (Bordelon et al. 2006). Phylogenetic tree was generated with MEGA4 using Neighbor-Joining method and pre-aligned sequences (Saitou & Nei, 1987; Tamura et al. 2007). Five hundred bootstrap replicates were analyzed to generate bootstrap consensus tree and to estimate the statistical confidence values. Positions that contain gaps were eliminated during calculation. The tree was drawn to scale. The evolutionary distances are in units of number of amino acid substitutions per site. The sequence logo, which shows the consensus sequence of the TamR binding site was generated using WebLogo (Crooks et al. 2004) at http://weblogo.berkeley.edu/.

**Cloning and purification of TamR**

*S. coelicolor* A3(2) M145 strain was kindly provided by Gregg Pettis, LSU. After *S. coelicolor* was grown in tryptone yeast extract broth (ISP medium 1), the genomic DNA was isolated using the salting out method (Kieser et al. 2000). Forward primer 5'-CACTACACTGATCCATATGGAGGAC-3' and reverse primer 5'-GACCTGGACGGGAATTCCAGCC-3' were used to amplify the gene encoding TamR (*SCO3133*; restriction sites underlined). The PCR product was cloned into the NdeI-EcoRI sites of pET28b (Novagen), which introduces an N-terminal His$_6$-tag, and the recombinant plasmid transformed
into *E. coli* TOP10 (Invitrogen). After the correct construct was confirmed by sequencing, it was transformed into *E. coli* BL21(DE3)pLysS for protein expression. A single colony was used to inoculate an overnight culture. For overexpression, the overnight culture, which was grown at 37°C (250 rpm) in Luria-Bertani media (with 50 μg/ml kanamycin) was diluted 1:500 with LB media containing 50 μg/ml kanamycin. Cultures were grown at 37°C (250 rpm). When the OD$_{600}$ reached about 0.6, overexpression of protein was induced with 0.2 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 3 hours. The induced cultures were chilled on ice. Cells were harvested by centrifugation and stored at -80°C.

After cell pellets were thawed on ice, cells were resuspended in ice-cold lysis buffer (50 mM potassium phosphate buffer (pH 7.0), 400 mM NaCl, 10% glycerol, 10 mM imidazole, 0.15 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM 2-mercaptoethanol). Sonication was used to disrupt cells. DNase I was added to digest nucleic acids. This solution was centrifuged at 28,000 × g for 60 min. The supernatant was filtered through filter paper and loaded onto a HIS-Select Nickel Affinity column (Sigma), previously equilibrated with lysis buffer. The column was washed by gravity flow with 10 volumes of wash buffer (50 mM potassium phosphate buffer (pH 7.0), 400 mM NaCl, 10% glycerol, 20 mM imidazole, 0.15 mM PMSF, 1 mM 2-mercaptoethanol). Elution buffer (50 mM potassium phosphate buffer (pH 7.0), 400 mM NaCl, 10% glycerol, 250 mM imidazole, 0.15 mM PMSF, 1 mM 2-mercaptoethanol) was used to elute proteins. Peak fractions were pooled and dialyzed against 2L dialysis buffer (50 mM potassium phosphate buffer (pH 7.0), 400 mM NaCl, 10% glycerol, 0.15 mM PMSF, 1 mM 2-mercaptoethanol). The purity of protein preparations was ascertained using SDS-PAGE, followed by staining of gels with Coomassie brilliant-blue. TamR concentration was determined based on its absorbance at 280 nm.
using the calculated extinction coefficient (12,490 M^{-1}cm^{-1}). All experiments were performed with His6-tagged TamR.

**DNA binding assays**

The intergenic segment between *S. coelicolor* tamR (SCO1133) and tam (SCO1132) genes was amplified using primers tamO-Fw (5’-TCCGGCGTGCGAGGTACT-3’) and tamO-Rv (5’-GCGACCAGCCGATCGACCT-3’). This 247 bp tamO DNA contains the entire intergenic region and extends 54 bp into the coding region of SCO1132 and 29 bp into the coding region of SCO1133. The 247 bp DNA was 32P-labeled at the 5’-ends using T4-polynucleotide kinase (T4-PNK). 32P-labeled tamO (0.015 nM) was incubated with TamR in binding buffer (20 mM Tris (pH 8.0), 50 mM NaCl, 0.06% detergent BRIJ58 (Pierce), 20 μg/ml bovine serum albumin (BSA), 2% glycerol) at 25°C for 30 min. Complex and free DNA were resolved using 6% non-denaturing polyacrylamide gels (39:1(w/w) acrylamide:bisacrylamide). After the gel was pre-run for 20 minutes in 0.5×Tris–borate–EDTA (TBE) buffer at 4°C, the samples were loaded and run at 10 V/cm for 2 hours in 0.5×TBE buffer. The gel was dried and exposed to phosphor screens. Results were visualized using a Storm 840 phosphorimager (GE Healthcare), and analyzed using ImageQuant 5.1. Data were fitted to the Hill equation: 

\[ f = \frac{f_{\text{max}}[\text{TamR}]^n}{K_d + [\text{TamR}]^n} \]

where [TamR] is the protein concentration, \( f \) is fractional saturation, \( K_d \) is the apparent equilibrium dissociation constant, and \( n \) is the Hill coefficient. Specificity of interaction between TamR and tamO was measured using competition assay in which unlabeled non-specific plasmid DNA (pGEM5) or unlabeled 247 bp tamO were used as competitor, with competitor DNA added prior to labeled tamO DNA. The binding buffer for these assays contained 0.5 M Tris. Binding of TamR to DNA representing the sacA promoter was performed as described for binding to tamO, except that the binding buffer contained 0.5 M Tris and that 0.5 nM labeled sacA DNA was used.
179 bp sacA promoter DNA (comprising four base pairs of the coding region and 175 bp upstream) was amplified from S. coelicolor genomic DNA using primers 5'-CCCCATGTACTAGTTATCT-3' and 5'-ACACGACAGTCTCCTTCA-3'.

To determine the effect of ligand, the binding buffer used was 0.5 M Tris (pH 8.0), 50 mM NaCl, 0.06% BRIJ58, 20 μg/ml BSA, 2% glycerol. Note that the higher buffer concentration was necessitated to prevent pH changes on addition of urate, xanthine, and hypoxanthine, which were dissolved in 0.4 M NaOH, and that this higher ionic strength reduces the affinity of TamR for both DNA and ligands. Other ligands were dissolved in distilled water and added before addition of TamR to DNA. The concentrations of TamR were 0.09 nM and 0.73 nM, respectively, when measuring the effect of a ligand on complex 1 and complex 2. After 30 min incubation at 25°C, samples were analyzed using EMSA under the conditions described above. For quantification, the gel region considered as complex 1 included C1 and the region between C1 and free DNA, while the gel region considered as complex 2 included C2 and the gel region between C2 and C1 to account for complex dissociation during electrophoresis. Data were analyzed by fitting to exponential decay equation: $f=Ae^{-kL}$, where $f$ is fractional saturation, $L$ is the ligand concentration, $A$ is the saturation plateau, and $k$ represents the exponential decay constant. Quantification results derive from at least three independent experiments.

**Gel filtration**

A Bio-Gel P-100 (GE Healthcare) column (0.7×100 cm) was pre-equilibrated and eluted with mobile phase buffer (50 mM potassium phosphate buffer (pH 7.0), 150 mM NaCl). Markers used to create the standard curve include bovine serum albumin (66.0 kDa), ovalbumin (44.0 kDa), myoglobin (17.0 kDa), and vitamin B12 (1350 Da). The equation $Kav= (VE – VO)/(VT – VO)$ was used to calculated the Kaverage (Kav) of a protein. In this equation, VE, VO, and VT represent
the retention volume of the protein, void volume of the column and the geometric bed volume of
the column, respectively.

**Circular Dichroism spectroscopy**

A Jasco J-815 circular dichroism spectrometer (Jasco, Inc.) was used to measure the far
UV circular dichroism spectrum of 10 μM TamR in CD buffer (12.5 mM potassium phosphate
buffer (pH 7.0), 100 mM NaCl, 2.5% glycerol, 0.25 mM 2-mercaptoethanol) at 20°C. Where
added, trans-aconitate was included at a final concentration of 100 mM. Measurements were
conducted at 1-nm steps in triplicate. A quartz cuvette with 0.1-cm path length was used.
Secondary structure composition was calculated using the analysis K2d Program from the website
DichroWeb (Whitmore & Wallace, 2004, 2008). The maximum error for this K2d analysis was
0.097 and the goodness of fit was determined from the NRMSD value of 0.105.

**Thermal stability**

TamR (8 μM) in a measurement buffer (200 μM Tris (pH 8.0), 200 mM NaCl) was mixed
with reference fluorescent dye 5×SYPRO Orange (Invitrogen). When added, ligands were
included at a final concentration of 50 mM after adjusting the pH of ligand solutions to 8.0 with
NaOH. Fluorescence emission was measured over a temperature range of 5–94°C in 1°C
increments for 45 s using an Applied Biosystems 7500 Real-Time PCR System. SYBR green filter
was used for detection. Total fluorescence yield was corrected using a measured result from a
reaction without protein. The sigmoidal part of the melting curve was fit to a four-parameter
sigmoidal equation using Sigma Plot 9. At least three independent experiments were performed.

**In vivo regulation of gene activity**

*S. coelicolor* cultures, which were germinated from spores in yeast extract-malt extract
media (YEME media, with 10.3% sucrose) were grown for 22 hours and then treated with either
citrate (100 mM), citrate (100 mM) combined with Fe$^{3+}$ or Ca$^{2+}$ (5 mM), or H$_2$O$_2$ (10 mM) for 2 hours before cells were harvested by centrifugation. For determination of sacA transcript level, S. coelicolor cultures were grown in ISP medium 1 (without glucose or sucrose to avoid potential catabolite repression) before incubation with citrate (100 mM) and Ca$^{2+}$ (10 mM) or H$_2$O$_2$ (10 mM) for 2 hours. The total RNA was isolated using illustra RNAspin Mini Isolation Kit (GE Healthcare) after the pellet was quickly washed with 50 mM sodium phosphate buffer (pH 6.4) twice. AMV reverse transcriptase (New England BioLabs) was used to generate cDNA for quantitative PCR (qPCR). Applied Biosystems 7500 Real-Time PCR system was used to carry out qPCR with SYBR green I as fluorescent dye and gene rpoA (house-keeping gene encoding RNA polymerase alpha subunit whose expression is not expected to vary under the experimental conditions) as internal control. Comparative CT ($2^{-\Delta\Delta CT}$) method was used for data analysis after data validations (Schmittgen & Livak, 2008).

Results

The genomic locus containing tamR and tam genes is conserved among Actinomycetales

S. coelicolor gene SCO3133 encodes a predicted MarR homolog that is divergently oriented from gene SCO3132 annotated as a putative trans-aconitate methyltransferase (tam; Figure 2.1A). The intergenic region would be predicted to contain -10 and -35 promoter elements of both genes. Based on the function of this MarR homolog described below, we propose the name TamR (trans-aconitate methyltransferase regulator). In addition to what is seen in the S. coelicolor genome, the tam-tamR locus organization consisting of divergently oriented tam and tamR genes is conserved in the genomes of several other bacteria that belong to the order Actinomycetales.
Figure 2.1 Genetic locus organization of tam–tamR and related metabolic reactions.

Comparing the amino acid sequence of TamR proteins with that of canonical MarR homologs revealed that all TamR homologs contain the N-terminal extension that is characteristic of urate-responsive MarR homologs and that they harbor the four residues shown to be involved in urate binding (Figure 2.2A). TamR homologs were found to be highly conserved among the species in which they are encoded, including a wide region (boxed in Figure 2.2A), which covers the turn between α4 and α5 and the N-terminal half of α5. Since α5 corresponds to the DNA recognition helix identified from structures of MarR homologs complexed with DNA (e.g., Hong et al. 2005), conservation of DNA binding sites for TamR would be predicted.
Figure 2.2 Conservation of TamR. A. Sequence alignment of TamR proteins and other MarR homologs. Secondary structure elements are from the structure of HucR and aligned with the sequence of HucR (Bordelon et al. 2006). The N-terminal helix α1 is absent in *E. coli* MarR and *Methanobacterium thermoautotrophicum* MTH313. In addition to the TamR homolog from *S. coelicolor* (Q9K3T1), *Streptomyces avermitilis* (Q82HD8), *Kribbella flavida* (D2PPC7), and *Thermomonospora curvata* (D1A7I8), the alignment includes *D. radiodurans* HucR (Q9RV71), *E. coli* MarR (P27245), *M. thermoautotrophicum* MTH313 (O26413), and *A. tumefaciens* PecS (Q7D1T4). TamR proteins, *D. radiodurans* HucR and *A. tumefaciens* PecS all belong to the urate-responsive UrtR subfamily of MarR homologs. Red frame highlights conservation of residues near and within the DNA recognition helix (α5). B. Phylogenetic analysis of selected MarR homologs from the information of amino acid sequences. Phylogenetic tree was generated with MEGA4 using Neighbor-Joining method with 500 bootstrap replicates.
TamR homologs were found to be encoded by bacterial species that belong to the order Actinomycetales, particularly the genus *Streptomyces*. Phylogenetic analysis of TamR and other MarR homologs revealed clustering of TamR homologs, with the most closely related homologs being the identified urate-responsive proteins (e.g., *A. tumefaciens* PecS and *D. radiodurans* HucR) while homologs such as *E. coli* MarR are more distantly related (Figure 2.2B). Thus, bacterial species that encode TamR homologs are evolutionarily closely related, raising the possibility that a common ancestor may have adopted the genomic locus containing the *tam* and *tamR* genes, perhaps by a gene duplication of an existing *marR* homolog followed by selection for the novel function (i.e., responding to ligands associated with aconitase function).

Consistent with sequence conservation of the DNA-binding helices of the analyzed TamR homologs, all corresponding genomic loci encoding the *tam-tamR* gene pair were seen to feature an 18 bp conserved palindromic site in the *tam-tamR* intergenic region that is predicted to constitute the TamR binding site (Figure 2.3A). Sequence comparison and WebLogo revealed the consensus sequence of TamR binding sites (Figure 2.3A-B). In particular, residues at the center of each half-site of the binding motif (positions 3-8 and 11-16) were found to be highly conserved. In analogy with other MarR homologs, the layout of the genomic locus and the presence of conserved palindromic sequences in the intergenic region also leads to the prediction that TamR autoregulates its own gene transcription. Examination of the *S. coelicolor* *tam-tamR* intergenic region did not reveal other sequence repeats likely to function as binding sites for transcription factors.
Figure 2.3 Sequence consensus of TamR binding site. A. Predicted TamR binding sequences (18 bp inverted repeat sequences) from the intergenic region of tam-tamR loci from 12 different bacteria. The respective tamR genes are named at the right, preceded by the distance (in bp) between the start codon and the nearest identified binding motif. B. WebLogo (http://weblogo.berkeley.edu/logo.cgi) representing the consensus TamR binding site. The relative frequency of base pairs at each position is represented by the height of each nucleotide. C. Predicted TamR binding site (underlined) in the promoter region of aconitase encoding genes. Asterisks indicate positions where nucleotides are identical in all sequences.
Figure 2.4. *S. coelicolor* tam-tamR intergenic region. The *tam* and *tamR* genes are oriented divergently. The sequence of the intergenic region is shown with predicted binding sites. The conserved TamR binding site is site 1. Five additional TamR binding sites are located adjacent to the conserved binding site (sites 2 to 6). Each TamR binding site overlaps the preceding site by three base pairs. Bottom panel shows alignment of all six cognate sites. Asterisks indicate positions where nucleotides are identical in all sequences.

All analyzed tam-tamR intergenic regions were found to contain a palindromic site that matches the consensus sequence (Figure 2.3A). However, the tam-tamR intergenic region in the chromosome of *S. coelicolor* has some special characteristics. In addition to the conserved TamR binding site (site 1), five additional predicted TamR binding sites were identified (sites 2-6; Figure 2.4). These five putative TamR binding sites are similar to each other, but more divergent from the identified consensus sequence compared to site 1. All six sites are adjacent to each other, overlapping by three base pairs.

A weight matrix based on the frequencies of individual bases occurring at each position within the 18 bp consensus binding motif was applied in a genome-scale screen of the *S. coelicolor* genome using PATSER ([http://rsat.ulb.ac.be/genome-scale-patser_form.cgi](http://rsat.ulb.ac.be/genome-scale-patser_form.cgi)) to find other genes potentially regulated by TamR. The promoter regions of genes identified by this method were examined, revealing putative TamR binding sites in the promoters of two genes that encode...
enzymes of the citric acid cycle, aconitase (SCO5999) and malate synthase (SCO6243). Notably, the TamR binding site was conserved in the promoters of aconitase genes in all 12 bacterial species seen to feature the tam-tamR genomic locus (some species encode two aconitase genes, only one of which containing the conserved TamR binding site) (Figure 2.3C). The presence of predicted TamR binding sites in the promoters of genes encoding aconitase in all these bacterial species further points to a link between TamR and citrate isomerization.

**TamR binds to the tam-tamR intergenic region**

The tamR gene was cloned from *S. coelicolor* genomic DNA and the His6-tagged protein expressed in *E. coli* and purified to apparent homogeneity (Figure 2.5A). Since MarR proteins are expected to exist as dimers, size-exclusion chromatography was used to determinate the oligomeric state of purified TamR, which was found to exist as single species by native gel electrophoresis (data not shown). TamR eluted from the gel filtration column at 41.4 kDa (Figure 2.5B), which is consistent with the molecular mass of the expected dimer (theoretical molecular mass of recombinant TamR dimer is 41.7 kDa). Dimeric TamR could also be detected in SDS-PAGE gels following crosslinking with glutaraldehyde (data not shown).

Far-UV circular dichroism spectroscopy showed that the secondary structure composition of TamR is about 57% α-helix, 10% β-sheet and 33% random coil (Figure 2.5C), with the secondary structure composition estimated based on the CD spectrum using DichroWeb (Whitmore & Wallace, 2004, 2008). This is similar to the composition of HucR, which contains about 55% α-helix and 5% β-sheet (Bordelon *et al.* 2006). TamR was quite stable with a melting temperature (Tm) of 59.9±0.3°C (Figure 2.5D). This is comparable to other MarR homologs, which also have relatively high melting temperatures (Wilkinson & Grove, 2004; Perera & Grove,
Thus, TamR exists as a stable dimer at physiologically relevant temperatures and features the secondary structure content expected for a MarR homolog.

Figure 2.5. Characterization of TamR. A. Purified TamR in 15% SDS-PAGE gel. Lane 1, TamR; Lane 2, molecular mass marker (BioRad; Mw indicated at right). B. Gel-filtration analysis of TamR. The standard curve was generated from the $K_{av}$ of molecular weight standards (grey squares) versus $\log_{10}(\text{Mw})$ of these standards. The $K_{av}$ of TamR is indicated as the black circle. C. Far-UV CD spectrum of TamR. Ellipticity measurements are represented in units of millidegrees (mdeg; machine units). D. Thermal stability of TamR. Fluorescence emission resulting from the binding of SYPRO Orange to denatured protein was measured as a function of temperature.
To determine whether TamR binds the \textit{tam-tamR} intergenic region, a 247 bp sequence named \textit{tamO} representing this intergenic region was used in electrophoretic mobility shift assays (EMSA). TamR bound to \textit{tamO} forming two clearly distinguishable complexes (Figure 2.6A). TamR bound with high affinity as evidenced by an apparent dissociation constant (Kd) of 16.5±1.2 pM (Figure 2.6B). This Kd likely represents an upper limit, as conditions for formation of complex 1 are nearly stoichiometric ([DNA] ~ Kd). A TamR-\textit{tamO} complex (identified as C1 in Figure 2.6A) was formed at relatively low TamR concentrations (Kd ~ 16 pM) and with increasing concentrations, another TamR-\textit{tamO} complex (C2) appeared; consistent with the observation that DNA is effectively saturated to form complex 1 before additional sites are filled, formation of complex 1 and 2 is not cooperative, as evidenced by a Hill coefficient of 1.0±0.0.

At sufficiently high TamR concentrations (~1.5 nM), complex 1 disappeared and all DNA was bound as complex 2, with 50% conversion of complex 1 to complex 2 at a >10-fold higher TamR concentration than that required for half-maximal conversion of free DNA to complex 1. The difference in migration between complex 1 and complex 2 indicates that there are more than two TamR dimers bound to \textit{tamO} in complex 2 (Figure 2.6A). This is consistent with the prediction that there are six TamR binding sites in the intergenic region, one (conserved TamR binding site 1) with relatively high TamR binding affinity and five sites with lower affinity for TamR (Figure 2.4). Indeed, six bands corresponding to TamR-\textit{tamO} complexes were detected when electrophoresis was performed under different conditions (inset to Figure 2.6B). Quantitation of complex 2 formation, considering complex 1 as “free DNA”, yielded a Hill coefficient of 1.8±0.1 (Figure 2.6B), suggesting cooperativity of binding to sites 2-6 (Figure 2.4).
Figure 2.6. Binding of TamR to tam-tamR intergenic region tamO. A. EMSA showing binding of TamR. DNA (0.015 nM) was titrated with TamR (lanes 1-15 representing reactions with 0, 1.4 pM, 2.9 pM, 5.7 pM, 11.4 pM, 22.9 pM, 45.7 pM, 91.4 pM, 0.18 nM, 0.37 nM, 0.73 nM, 1.46 nM, 2.92 nM, 5.85 nM and 11.7 nM TamR, respectively). Two relatively stable complexes (C1 and C2) were detectable. Complexes (C1 and C2) and free DNA (F) are identified by arrows. B. Fractional complex formation was plotted as a function of TamR concentration. Data represented by filled circles considers the sum of complex 1 and complex 2 as complex (Kd = 16.5±1.2 pM; nH = 1.0±0.0), while data represented by filled squares considers only complex 2 as complex and the sum of free DNA and complex 1 as “free DNA” (Kd = 4.5±1.0 nM; nH = 1.8±0.1; note that Kd does not represent the affinity for a single site). Error bars represent standard deviation from three independent repeats. Inset shows example of electrophoresis at room temperature, revealing six TamR-tamO complexes (lane 2; other experimental conditions are unaltered). Bands corresponding to C1 and C2 in panel (A) are indicated, with bands corresponding to additional TamR-DNA complexes visible at intermediate electrophoretic mobilities. Lane 1 contains DNA only. C. Binding of 0.015 nM labeled tamO to 0.18 nM TamR was challenged with increasing concentration of unlabeled 247 bp tamO DNA (lanes 3-6: 0.038, 0.075, 0.15, 0.225 nM) or the same concentration of 3,000 bp plasmid pGEM5 (lanes 7-10). Reaction in lane 1 contained labeled DNA only. Reaction in lane 2 contained no competitor DNA. D. Challenge of complex 2 formation by competition assay. All conditions are the same as (C) except 0.73 nM TamR is used. Note that reactions in (C) and (D) were carried out at higher ionic strength (0.5 M Tris) compared to reactions in (A) (50 mM Tris).
Specificity of TamR binding to \textit{tamO} was assessed by EMSA experiments in which unlabeled specific DNA (\textit{tamO}) or nonspecific DNA (plasmid pGEM5) were combined with labeled \textit{tamO} DNA and TamR. Two different concentrations of TamR were used to examine specificity of complex 1 and complex 2 separately. Using a concentration of TamR where only complex 1 is seen (0.18 nM TamR; Figure 2.6C), only addition of unlabeled \textit{tamO} could compete with labeled \textit{tamO} for binding to TamR. That not all labeled DNA was unbound at the highest concentration of competitor reflects that total \textit{tamO} DNA (0.24 nM) was only in modest excess over TamR. By contrast, complex formation between TamR and \textit{tamO} was not significantly affected by addition of non-specific pGEM5 DNA (up to 15-fold molar excess of the 3,000 bp pGEM5 compared to the concentration of 247 bp \textit{tamO}). Using a concentration of TamR where complex 2 is formed (0.73 nM TamR; Figure 2.6D), unlabeled \textit{tamO} DNA efficiently competed for formation of complex 2 despite TamR being in excess over unlabeled \textit{tamO}, with all DNA converted to complex 1; this is consistent with the inference that the affinity of TamR for site 1 (Figure 2.4) is higher than the affinity for adjacent sites. As for complex 1 formation, addition of nonspecific pGEM5 DNA had no effect on formation of complex 2. These experiments show that TamR binds sequence-specifically to \textit{tamO}.

**TamR responds to \textit{trans-aconitate}, \textit{cis-aconitate}, citrate and isocitrate by attenuated DNA binding**

As \textit{trans-aconitate} is the substrate of \textit{trans-aconitate} methyltransferase, its effect on TamR-\textit{tamO} binding was measured. In addition, effects of three related compounds, citrate, isocitrate and \textit{cis-aconitate}, which are the substrate, product and intermediate of the citrate isomerization reaction, respectively, were determined. All these four compounds have close structural and metabolic relationships (Figure 2.1B).
With increasing concentration of trans-aconitate, the binding of TamR to *tamO* was significantly attenuated. Formation of both TamR-*tamO* complex 1 (with an IC$_{50}$ of 70.5±1.9 mM) and TamR-*tamO* complex 2 (with an IC$_{50}$ of 35.8±0.4 mM) was attenuated by trans-aconitate (Figure 2.7A, 2.7E). Cis-aconitate, citrate and isocitrate also attenuated the binding of TamR to *tamO* (Figure 2.7B-2.7D, 2.7F-2.7H). For complex 1, the binding of TamR to *tamO* was attenuated by cis-aconitate, citrate and isocitrate with IC$_{50}$ values of 62.6±1.4, 88.2±3.1 and 99.1±2.5 mM, respectively (Figure 2.7F-2.7H). For complex 2, the IC$_{50}$ values were 53.0±1.2, 71.5±2.0 and 61.3±1.5 mM for cis-aconitate, citrate and isocitrate, respectively (Figure 2.7F-2.7H). Evidently, TamR exhibits little discrimination between these structurally related compounds, which more efficiently attenuated formation of complex 2 than complex 1. To confirm that attenuation of DNA binding was not due to compromised structural integrity of TamR on ligand binding, CD spectra and thermal stabilities of TamR were measured in presence of ligand. The CD spectrum of TamR in presence of 100 mM trans-aconitate did not indicate significant changes in secondary structure content (data not shown). Addition of ligand had no effect on protein stability, even at the highest concentration of 50 mM (Table 2.1); this could perhaps reflect changes in protein flexibility that compensate for stabilizing effects of ligand binding or that ligand binding perturbs the energy landscape of the native state ensemble by preferred binding to a less stable substate.

Since TamR was identified based on similarity to urate-responsive MarR homologs, the effect of urate on DNA binding of TamR was also investigated. Other intermediates in the purine degradation pathway (including xanthine, hypoxanthine and allantoin), which are structurally similar and metabolically related to urate were also examined. EMSA results revealed that urate
Figure 2.7. Effect of different ligands on the binding of TamR to \textit{tamO}. A-D. Reactions in lanes 1, 8, and 9 contained labeled \textit{tamO} DNA only. Ligand concentrations in lanes 2-7 and 10-15 are 0, 10, 20, 50, 75, 100 mM, respectively. DNA (0.015 nM) was incubated with 0.73 nM TamR (lanes 2-7) or 0.09 nM TamR (lanes 10-15). Complexes (C1 and C2) and free DNA (F) are identified. E-H. Normalized complex 1 and complex 2 formation as a function of ligand concentration. Error bars represent the standard deviation of three independent repeats.
and related ligands have little or no effect on DNA binding by TamR (data not shown), consistent with TamR responding to distinct ligands.

**In vivo effect of hydrogen peroxide and citrate on TamR-mediated gene regulation**

To investigate gene regulation by TamR *in vivo*, transcript levels of *S. coelicolor tam* and *tamR* were measured under conditions in which intracellular citrate concentrations are increased, either by uptake of citrate or by inactivating aconitase with hydrogen peroxide, an event associated with accumulation of both citrate and *trans*-aconitate. In *S. coelicolor* and other Gram-positive bacteria, citrate can be transported across cell membranes by the CitMHS family of transporters when citrate forms complexes with specific metal ions (Lensbouer *et al.* 2008, 2010). In *S. coelicolor*, the function of one member of the CitMHS family of transporters (*SCO1710; CitSc*) has been experimentally documented, and the protein was found to transport citrate efficiently when it forms complexes with Fe$^{3+}$ or Ca$^{2+}$, but not with Mg$^{2+}$, Ni$^{2+}$ or Co$^{2+}$. Exogenous citrate cannot be transported by the CitMHS family of citrate transporters unless it is complexed with metal; consistent with this observation, exposing *S. coelicolor* cultures to exogenous citrate did not have any effect on transcript levels of *tam* (relative expression level 1.1±0.4 for cultures grown in presence of citrate relative to cultures to which no citrate was added) or *tamR* (relative level 1.3±0.1) (Figure 2.8A). However, if the media was supplemented with 100 mM citrate and 5 mM Fe$^{3+}$, qRT-PCR results showed that the transcript level of *tam* is elevated 11.3±1.9 fold. In contrast, the transcript level of *tamR* was only modestly increased (1.7 ±0.3 fold). Consistent with the uptake of only citrate complexed with appropriate metals, supplementing the media with citrate and Ca$^{2+}$ lead to similarly increased transcript levels (14.5±3.2 fold for *tam* and 2.7±0.4 for *tamR*; Figure 2.8A). These data suggest that citrate functions as a TamR ligand *in vivo*, and that expression of the *tam* gene is more sensitive to citrate compared to that of *tamR*. 

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In *S. coelicolor*, the activity of aconitase is closely linked to intracellular citrate concentrations; inactivating the gene encoding aconitase dramatically increases intracellular citrate concentrations from nearly undetectable to >14 mM (Viollier *et al.* 2001). Aconitase contains a [4Fe-4S] cluster, which plays a critical role in catalysis, and its conversion to a [3Fe-4S] cluster results in loss of enzymatic activity (Beinert *et al.* 1983; Robbins & Stout, 1989; Verniquet *et al.* 1991; Gardner & Fridovich, 1992; Flint *et al.* 1993). The in vivo effect of hydrogen peroxide on the transcript level of *tam* and *tamR* was therefore investigated, as it can cause accumulation of intracellular citrate by inactivating aconitase as well as cause release of *cis*-aconitate from inactivated enzyme (Viollier *et al.* 2001). Quantitative RT-PCR results demonstrated that expression of *tam* and *tamR* genes is significantly elevated in response to hydrogen peroxide, with transcript levels of *tam* and *tamR* increasing by 27.3±3.9 fold and 3.0±0.9 fold, respectively (Figure 2.8A).

Because a TamR binding site is predicted in the promoter region of the aconitase gene *sacA*, transcription of *sacA* was also investigated. Incubation of *S. coelicolor* cultures with citrate (100 mM) plus Ca^{2+} (10 mM) or with H_{2}O_{2} (10 mM) results in increased expression of *sacA* by

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Table 2.1 Thermal stability of TamR in presence of ligands.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Tm (°C)</th>
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<tbody>
<tr>
<td>None</td>
<td>59.9 ± 0.3</td>
</tr>
<tr>
<td><em>Trans</em>-aconitate</td>
<td>59.7 ± 0.4</td>
</tr>
<tr>
<td><em>Cis</em>-aconitate</td>
<td>59.9 ± 0.2</td>
</tr>
<tr>
<td>Citrate</td>
<td>59.6 ± 0.2</td>
</tr>
<tr>
<td>Isocitrate</td>
<td>60.2 ± 0.5</td>
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1.9±0.1 fold and 2.5±0.1 fold, respectively (Figure 2.8B). To examine further the role of TamR in mediating regulation of the sacA gene, EMSA experiments were performed using a DNA fragment representing the sacA promoter. As shown in Figure 2.9A, TamR bound this DNA forming one distinct complex, consistent with the presence of a single conserved palindromic binding motif in the sacA promoter. The affinity of TamR for this DNA was 1.9 ± 0.2 nM. These data also corroborate the validity of the consensus TamR binding motif. As for TamR binding to the tam-tamR intergenic region, binding to the sacA promoter was attenuated in the presence of trans-aconitate, cis-aconitate, citrate and isocitrate (Figure 2.9B-C and data not shown). Collectively, these data suggest that TamR responds to intracellular accumulation of citrate by differentially upregulating tam and tamR genes, and they are consistent with TamR contributing to regulation of sacA gene activity.

Discussion

Aconitase function is coupled to oxidative stress

Both eukaryotic and prokaryotic aconitases are bifunctional proteins (Haile et al. 1992; Alén & Sonenshein, 1999). In the presence of iron, their [4Fe-4S] clusters are assembled, and the proteins function as aconitases. When iron is limiting and [4Fe-4S] cluster assembly is compromised, the proteins lose aconitase function and serve instead as RNA-binding proteins to regulate expression of genes associated with iron metabolism by binding iron response elements (IREs) in the mRNA. This dual function permits the cells to sense and respond to changes in cellular iron concentration. When aconitase catalytic function is compromised and citrate cannot
Figure 2.8. *In vivo* gene regulation. A. Relative abundance of *tam* and *tamR* transcripts after exposure to 100 mM citrate, 100 mM citrate+5 mM Fe\(^{3+}\), 100 mM citrate+5 mM Ca\(^{2+}\) or 10 mM H\(_2\)O\(_2\). Quantitative RT-PCR was used to measure the relative mRNA levels of *tam* and *tamR* genes and reference control gene (*rpoA*). B. Relative abundance of *sacA* transcript after exposure to 100 mM citrate+10 mM Ca\(^{2+}\) or 10 mM H\(_2\)O\(_2\). Note that y-axis is expanded compared to graph in panel (A). Error bars represent standard deviation of three repeats.
Figure 2.9. Binding of TamR to sacA promoter DNA. A. EMSA showing binding of TamR. Labeled sacA promoter DNA (0.5 nM) was titrated with TamR; the TamR concentrations in lanes 1-9 are 0.04, 0.08, 0.16 ,0.31, 0.63, 1.25, 2.50, 5.0 and 10.0 nM, respectively. Reaction in lane 10 contained labeled DNA only. Complexes (C) and free DNA (F) are identified. B-C. Effect of transaconitate (B) and citrate (C) on binding of TamR to the promoter region of sacA. Ligand concentrations in lanes 2-6 are 0, 25, 50, 75, 100 mM, respectively. Reactions contained 0.63 nM TamR and 0.5 nM DNA. Reaction in lanes 1 contained DNA only. Reactions in all panels were performed at high ionic strength (0.5 M Tris).

be metabolized, secreted citrate can in turn function as an iron-chelator, facilitating uptake of iron to reassemble [4Fe-4S] clusters and restore catalytic activity.

The [4Fe-4S] cluster of aconitase is disassembled by peroxide stress. Consistent with this observation, the mRNA-binding property of aconitase has also been associated with regulation of genes involved in oxidative stress responses (e.g., Tang et al. 2002). Such function is also likely for aconitase encoded by Streptomyces (Michta et al. 2012). Under these conditions, TamR may contribute to restoring the catalytic function of aconitase by participating in regulating its gene activity and by preventing inhibition of functional enzyme by removing the inhibitor transaconitate.

**TamR promotes metabolic flux through the citric acid and glyoxylate cycles**

Reactive oxygen species cause inactivation of aconitase due to disruption of its [4Fe-4S] cluster, resulting in elevated citrate and trans-aconitate concentrations. Under these conditions, tam, tamR and aconitase genes are upregulated (Figure 2.8). Citrate has been shown to accumulate
to >14 mM concentration on inactivation of aconitase (SCO5999), which was experimentally shown to be the primary vegetative aconitase in *S. coelicolor* (Viollier *et al.* 2001). We observe increased gene activity not only on inactivation of aconitase by H$_2$O$_2$, but also after uptake of iron-citrate or calcium-citrate complexes. For iron-citrate complexes, the most relevant species has been shown to be a monoiron-dicitrate complex (Silva *et al.* 2009). Using 5 mM Fe$^{3+}$, and considering a monoiron-dicitrate complex as the most relevant species, the extracellular concentration of transportable citrate would be 10 mM. Under these conditions, an ~11-fold upregulation of *tam* was observed, while an increase of ~27-fold was seen on exposure to H$_2$O$_2$. Given that intracellular citrate concentrations may rise to >14 mM on inactivation of aconitase, this suggests that we observe increased gene activity under physiologically relevant concentrations of citrate. That exposure to H$_2$O$_2$ results in a greater increase in gene expression may be a consequence of a higher intracellular citrate concentration compared to that obtained after uptake of exogenous citrate, or it may be due to contributing effects of *trans*-aconitate formed as a result of *cis*-aconitate released from inactivated aconitase. TamR contains no cysteines (oxidation of which could be associated with conformational changes in the protein and possible modification of DNA-binding activity) and would not be expected to respond directly to oxidative stress. Thus, our data suggest that TamR serves an important function in ensuring metabolic flux through the citric acid and glyoxylate cycles by responding to the increased cellular levels of citrate and *trans*-aconitate that occur under oxidative stress conditions to effect upregulation of key metabolic enzymes.

**Ligand-binding by TamR**

TamR was identified based on homology to UrtR, specifically conservation of the N-terminal helix and residues shown to be involved in urate-mediated attenuation of DNA binding (Perera & Grove, 2011). Yet, TamR does not bind urate or other intermediates of purine
metabolism, but compounds associated with aconitase function. TamR exhibits little preference for any of these ligands, all of which share significant negative charge. The IC\textsubscript{50} is also comparable for disruption of complex 1 and for conversion of complex 2 to complex 1, despite the binding of five additional TamR dimers in complex 2 (Figs. 2.6B and 2.7). If each additional TamR dimer in complex 2 must bind ligand for complex 2 to be converted to complex 1, a higher ligand concentration would be required compared to conversion of complex 1 to free DNA. This is not observed. It is possible that differences in the DNA sites may impose distinct conformational changes in the ligand binding pockets on DNA binding, causing high-affinity bound TamR to be less sensitive to ligand, or that ligand binding to a single TamR dimer that is part of complex 2 leads to cooperative disassembly of this complex. That saturation of all six TamR sites requires >10-fold higher TamR concentration compared to that required to saturate site one is consistent with lower affinity binding to the adjacent sites. We also note that the arrangement of cognate sites with a 3 bp overlap would place the centers of each palindrome about 15 bp apart, predicting that adjacent TamR dimers bind on opposite faces of the DNA duplex. In vivo, however, tam gene expression is more sensitive to ligand than that of tamR (Figure 2.8). This may reflect more efficient repression of tam gene expression by TamR, perhaps due to TamR protein concentrations that are insufficient to saturate the adjacent sites, resulting in a greater net increase in tam gene expression when ligand is present compared to the increase in tamR gene expression. In addition, differential promoter strength or the participation of other regulatory proteins may contribute to the observed differences.

The TamR ligand-binding pocket is distinct from that of urate-responsive MarR homologs. Sequence alignments reveal that TamR proteins share residues not otherwise conserved among urate-responsive transcriptional regulators, particularly in helix two, which lines the predicted
ligand-binding pocket (Figure 2.2A). A model of *S. coelicolor* TamR based on the structure of HucR is shown in Figure 2.10. The residues previously shown to participate in urate binding to HucR occupy equivalent positions in TamR (identified in cyan). One of these residues is a tryptophan from helix one, which is absent from other MarR proteins for which a structure has been reported. Since this helix would be expected to block access to the ligand-binding pocket from this direction, a more likely access route for ligands is from the underside of the protein near the dimer interface. Notably, two arginine residues from helix two, which are conserved only among TamR proteins are seen to face the ligand-binding pocket (shown in magenta), perhaps affording selectivity for highly negatively charged ligands. In addition, a tryptophan at the beginning of helix three is seen to form a lid at the bottom of the binding pocket. While the predicted role of these residues in ligand binding to TamR awaits confirmation, their presence only in TamR proteins suggests a mechanism for discrimination between urate and ligands associated with aconitase function.

Figure 2.10. Model of TamR. Model based on structure of HucR (2fbk), using SwissModel. Arginine 41 and tryptophan 53 from one subunit and arginine 35 from the other are identified in magenta. Residues previously shown to participate in urate binding to HucR are shown in cyan. Secondary structure elements α1 and α2 forming one edge of the ligand-binding pocket are identified, along with the DNA recognition helix α5. Figure generated with PyMOL.
Model for expression of the TamR regulon

A possible model for gene regulation by TamR is shown in Figure 2.11. Conserved TamR sites are located in the promoters of *tam* and *sacA*, with more divergent sites near the start of the *tamR* gene. At low concentrations of TamR, the high-affinity site in the *tam* gene promoter is filled, and *tam* gene expression is repressed. Occupancy of the site in the *sacA* promoter would attenuate *sacA* gene expression; however, *sacA* regulation is likely complex and predicted to include the ferric uptake regulator Fur, as a site for this transcription factor is predicted to overlap the TamR site (Muschko *et al.* 2002). Based on this model, a *tamR* mutant would be predicted to exhibit constitutive *tam* gene expression. With no TamR bound to divergent sites in the *tamR* gene promoter, *tamR* is expressed. As TamR accumulates, it can fill the cognate sites in the *tamR* gene promoter, resulting in repression of gene expression. We note that the observed mode of TamR binding to the *tam*-*tamR* intergenic region involving cooperative binding to low-affinity sites near the *tamR* gene, but no cooperativity of binding to the high affinity site near the *tam* gene and the adjacent low-affinity sites is consistent with differential TamR-mediated regulation of the *tam* and *tamR* genes. The *tam*-*tamR* intergenic regions contains six adjoining DNA sites of which cooperative binding is only observed for the five low-affinity sites; this may imply that occupancy of high- and low-affinity sites leads to differential structural changes in DNA or protein and that only occupancy of low-affinity sites is compatible with cooperative binding. Based on this model of repression, the negative autoregulation of *tamR* prevents excessive accumulation of TamR, yet ensures adequate levels of TamR for repression of *tam* gene expression and a more sensitive response to ligands. Thus, even if physiological conditions do not change, cellular concentrations of TamR would be predicted to fluctuate within a range that ensures sensitive control of the *tam* gene.
If aconitase is inactivated, intracellular citrate concentrations will increase. In addition, if the intermediate cis-aconitate is released, trans-aconitate concentrations will increase because cis-aconitate will be converted to the more stable isomer. These events are intertwined and may happen at the same time. Both citrate and trans-aconitate will attenuate the binding of TamR to its cognate sites, resulting in increased gene transcription; upregulation of sacA ensures production of functional aconitase, and production of trans-aconitate methyltransferase results in esterification of trans-aconitate, preventing its inhibition of aconitase. The concomitant upregulation of tamR ensures that sufficient TamR is available to restore the repressed state of tam gene expression once citrate and trans-aconitate concentrations return to normal levels.

Figure 2.11. Proposed model for regulation of tam, tamR and sacA by TamR. Elliptical symbols represent TamR, small filled circles represent ligand. Genes are denoted by large arrows. Ligand concentrations may be increased by uptake of citrate or by inactivation of aconitase, resulting in accumulation of both citrate and trans-aconitate.

Taken together, we propose that TamR constitutes a divergent member of the urate-responsive transcriptional regulator (UrtR) family that mediates a novel regulatory function that
ensures metabolic flux through the citric acid and glyoxylate cycles during oxidative stress. While TamR is unique in binding ligands associated with aconitase function and in regulating a key step in central metabolism, it shares with other characterized members of the UrtR family a primary function in oxidative stress responses.

References


CHAPTER 3
THE REGULATORY ROLE OF STREPTOMYCES COELICOLOR TamR IN CENTRAL METABOLISM

Introduction

Members of the multiple antibiotic resistance regulator (MarR) family of transcriptional regulators are involved in the regulation of a variety of important biological processes. As part of its regulatory mechanism, a MarR family member often responds to specific ligands (Cohen et al. 1993; Buchmeier et al. 1997; Wilkinson & Grove 2006; Davis & Sello 2010; Perera & Grove 2010a; Grove 2013). For instance, HucR from Deinococcus radiodurans can regulate the expression of the adjacent uricase-encoding gene by responding to the ligand urate, the substrate for uricase (Wilkinson & Grove 2004; Perera et al. 2009). In absence of ligand, gene expression is repressed, while binding of urate to HucR attenuates DNA binding, resulting in increased gene activity. HucR is a founding member of a subfamily of MarR proteins (named UrtR for urate-responsive transcriptional regulator). Compared to canonical MarR homologs, these proteins are characterized by an N-terminal extension containing a conserved tryptophan as well as three other residues inferred from biochemical analyses to be involved in ligand binding (Perera et al. 2009; Perera & Grove 2011).

Trans-aconitate methyltransferase regulator (TamR), a MarR homolog from Streptomyces coelicolor, was previously shown to belong to this subfamily. TamR regulates the transcription of \( \text{tamR} \), \( \text{tam} \) (encoding trans-aconitate methyltransferase; Figure 3.1A) and \( \text{sacA} \) (encoding aconitase) (Huang & Grove 2013). During the aconitase-catalyzed citrate isomerization step of the citric acid cycle (Figure 3.1B), the reaction intermediate \( \text{cis}-\text{aconitate} \) may be released from the enzyme-substrate complex; if this occurs, it will be spontaneously converted to the more stable isomer \( \text{trans}-\text{aconitate} \) (Ambler & Roberts 1948). Unlike \( \text{cis}-\text{aconitate} \), \( \text{trans}-\text{aconitate} \) is an
efficient inhibitor of aconitase, thus blocking the conversion of citrate to isocitrate (Saffran & Prado 1949; Lauble et al. 1994; Cai & Clarke 1999; Cai et al. 2001). Trans-aconitate methyltransferase can use trans-aconitate as a substrate and catalyze a methyl group transfer from S-adenosyl-methionine (SAM) to form the trans-aconitate methylester, whose ability to function as an inhibitor is significantly attenuated. Therefore, TamR-mediated upregulation of trans-aconitate methyltransferase prevents the accumulation of trans-aconitate and ensures metabolic flux through the citric acid cycle (Cai & Clarke 1999; Cai et al. 2001). This is further reinforced by the simultaneous regulation of an aconitase-encoding gene by TamR (Huang & Grove 2013). Upregulation of both genes is accomplished by binding of the structurally similar ligands citrate, trans-aconitate, cis-aconitate and isocitrate to TamR, resulting in attenuation of DNA binding; these ligands are all closely related to the citrate isomerization step catalyzed by aconitase (Huang & Grove 2013). As a circumstance under which citrate and trans-aconitate may accumulate involves the inactivation of aconitase by oxidative disassembly of its iron-sulfur cluster, one important function of TamR may be to restore metabolic flux through the citric acid cycle under conditions of oxidative stress. That the tam-tamR locus organization and the TamR binding sites in the tam-tamR intergenic region and in the promoter of sacA are conserved in different Streptomyces species emphasizes the importance of this regulatory mechanism (Huang & Grove 2013).

We show here that S. coelicolor TamR regulates additional genes whose products are involved in maintaining flux through the citric acid cycle. These genes encode malate synthase, malate dehydrogenase, and isocitrate dehydrogenase. Malate dehydrogenase and isocitrate dehydrogenase function in the citric acid cycle. Malate synthase, which participates in the glyoxylate cycle, can provide malate to the citric acid cycle by synthesizing malate from glyoxylate
and acetyl-CoA, thus replenishing the intermediate pool (Figure 3.1B). We also find that predicted TamR binding sites are highly conserved in the promoters of these genes from different Streptomyces species, indicating that the contribution of TamR to regulation of enzymes involved in the citric acid and glyoxylate cycles is conserved among streptomycetes.

**Experimental Procedures**

**TamR binding site identification and logo**

The sequence logo, which shows the consensus sequence of the TamR binding sites was generated using WebLogo ([http://weblogo.berkeley.edu/](http://weblogo.berkeley.edu/)) (Crooks *et al.* 2004). The relative frequency of base pairs at each position is represented by the height of each nucleotide. To identify other potential target genes, a weight matrix corresponding to the 18 bp consensus TamR binding site in the *tam-tamR* intergenic region was used in genome-scale screens of *Streptomyces* genomes using PATSER. The sequences of *S. coelicolor* TamR (*SCO3133*), trans-aconitate methyltransferase (*SCO3132*), malate synthase (*SCO6243*), malate dehydrogenase (*SCO4827*), and isocitrate dehydrogenase (*SCO7000*) were used as queries using BlastP on the NCBI website for identification of homologs in other streptomycetes. TamR binding sites were identified by inspection of the respective promoters.

**Cloning and purification of TamR**

Cloning and purification of TamR was carried out as described previously (Huang & Grove 2013). Briefly, *tamR* (*SCO3133*) was amplified from genomic DNA of *S. coelicolor* A3(2) M145 strain and cloned into NdeI-EcoRI sites of pET28b (Novagen). Recombinant plasmid was transformed into *E. coli* BL21(DE3) for overexpression of protein. TamR with N-terminal His$_6$-tag was purified using HIS-Select Nickel Affinity column (Sigma). Protein concentration was
determined from its absorbance at 280 nm using the extinction coefficient calculated based on the amino acid sequence.

**DNA binding assays**

A 193 bp DNA segment, which contains the promoter region of the *aceB1* (*SCO6243*) gene was amplified from *S. coelicolor* genomic DNA using primers *aceB1*-Fw (5'-TTGAGTGAGCGAGGTGG-3') and *aceB1*-Rv (5'-TGAGCTGTCACCTCCTTCA-3'). DNA concentration was determined by its absorbance at 260 nm. This DNA segment was $^{32}$P-labeled at the 5'-ends using T4-polynucleotide kinase (T4-PNK). For electrophoretic mobility shift assay, $^{32}$P-labeled pACEB1 (0.07 nM; stoichiometric conditions) was incubated with TamR in binding buffer (0.5 M Tris (pH 8.0), 50 mM NaCl, 0.06% detergent BRIJ58, 20 μg/ml bovine serum albumin (BSA), 2% glycerol) at 25°C for 30 min before reactions were loaded onto 6% non-denaturing polyacrylamide gels (39:1(w/w) acrylamide:bisacrylamide). The gel was pre-run for 20 minutes in 0.5×Tris–borate–EDTA (TBE) buffer at 4°C before the samples were loaded and run at 10 V/cm for 2 hours in 0.5×TBE buffer to separate free DNA and complex. After the gel was dried, it was exposed to phosphor screens. Data were obtained using a Storm 840 phosphorimager (GE Healthcare). The densitometric result was obtained using ImageQuant 5.1 (Molecular Dynamics). The quantitative data were fitted to the Hill equation: $f=f_{\text{max}}\frac{[\text{TamR}]^n}{(K+[\text{TamR}]^n)}$, where [TamR] is the protein concentration, $f$ is fractional saturation, $K$ is a constant, and $n$ is the Hill coefficient. Tangents were drawn to the initial rise and the final plateau and their intercept used to calculate the stoichiometry of binding. KaleidaGraph 4.0 (Synergy Software) was used for this analysis.

To determine the effect of ligands on the formation of TamR-DNA complex, reactions with different concentration of ligand were prepared. Ligands were dissolved in distilled water and
added before addition of TamR (0.16 nM) to DNA (pACEB1). After 30 min incubation at 25°C, samples were analyzed using EMSA under the conditions described above. Data were analyzed by fitting to exponential decay equation: $f=Ae^{-kL}$, where $f$ represents fractional saturation, $L$ represents the ligand concentration, $A$ represents the saturation plateau, and $k$ represents the exponential decay constant. Quantification results derive from at least three independent experiments.

Primers $mdh$-Fw (5'-CCTTCTTTTGCCTGCC-3') and $mdh$-Rv (5'-GCGTTCTCCGTATGACAGC-3') were used to amplify 184 bp DNA containing the promoter region of $mdh$. Primers $idh$-Fw (5'-TCCCGCTCCCCTGA-3') and $idh$-Rv (5'-TGGTCGAGTCAGTCACCG-3') were used to amplify 85 bp DNA covering the promoter region of $idh$. For EMSA, 0.13 nM $^{32}$P-labeled pMDH or 0.46 nM $^{32}$P-labeled pIDH was used in each reaction. All other conditions were as described above.

**In vivo regulation of gene activity**

*S. coelicolor* cultures, which were germinated from spores in tryptone yeast extract broth (ISP medium 1) were grown 36 hours at 30°C (280 rpm) before they were treated with either citrate (100 mM) combined with Ca$^{2+}$ (10 mM) or H$_2$O$_2$ (10 mM) for 2 hours. Then, the *S. coelicolor* cells were harvested by centrifugation and immediately washed twice using 50 mM sodium phosphate buffer (pH 6.4). The total RNA was then immediately isolated using Illustra RNAspin Mini Isolation Kit (GE Healthcare). AMV reverse transcriptase (New England BioLabs) was used to generate cDNA for quantitative PCR using designed primers and total RNA.

Quantitative PCR was performed on an Applied Biosystems 7500 Real-Time PCR system with gene rpoA (house-keeping gene encoding RNA polymerase alpha subunit) as internal control using SYBR green I as fluorescent dye. After data was validated, comparative CT ($2^{-\Delta\Delta CT}$) method was used for data analysis (Schmittgen & Livak 2008). The primers used in this experiment are:
Results

Predicted TamR binding sites in the promoters of three target genes

*S. coelicolor* TamR regulates transcription of its own gene (*tamR, SCO3133*) and that of a divergent gene encoding *trans*-aconitate methyltransferase (*tam, SCO3132*). A consensus binding site was identified based on a comparison of the intergenic region between these two genes in select *Streptomyces* species (Figure 3.1A) (Huang & Grove 2013). TamR also binds the promoter of a gene encoding aconitase (*sacA, SCO5999*) and controls its expression (Huang & Grove 2013). In order to find additional genes potentially regulated by TamR, a weight matrix based on the frequencies of individual bases occurring at each position within the 18 bp consensus binding motif was applied in a genome-scale screen of the *S. coelicolor* genome using the program PATSER (http://rsat.ulb.ac.be/genome-scale-patser_form.cgi). Features with a score greater than or equal to 10, corresponding to sites in gene promoters, were examined and found to include the expected sites in the tam-tamR intergenic region and in the sacA promoter. Equivalent sites were also found on examination of other Streptomyces genomes (e.g., *S. avermitilis* and *S. griseus*). This is consistent with the previously reported conservation of TamR in various Streptomyces species (Huang & Grove 2013). In addition, three genes were found to contain predicted TamR sites in both *S. coelicolor* and most other Streptomyces genomes available on the PATSER website; these genes encode malate synthase (*aceB1, SCO6243*), malate dehydrogenase (*mdh, SCO4827*) and
isocitrate dehydrogenase (idh, SCO7000). No candidate sites were found in promoters for genes encoding other enzymes in the citric acid cycle.

Figure 3.1. Summary of metabolic processes related to the target genes of TamR. A. Genomic locus encoding tamR and tam genes with the TamR consensus site identified. Gene SCO3131, which encodes a putative glyoxylase, overlaps the tam gene by 3 bp. B. Overview of citric acid and glyoxylate cycles. Enzymes indicated in red are encoded by genes regulated by TamR.
To examine more rigorously whether TamR binding sites are generally conserved in the promoters of these genes among species that encode TamR, the sequences of \textit{S. coelicolor} TamR and \textit{trans}-aconitate methyltransferase were used as queries with BlastP on the NCBI website. Examination of all available \textit{Streptomyces} sequences revealed a subset of species that encode a homolog of \textit{S. coelicolor} \textit{trans}-aconitate methyltransferase (shown in Table 3.1); notably, all species that encode this homolog also encode TamR. For all species but \textit{S. ambofaciens}, the \textit{tamR} gene is encoded divergently from the \textit{tam} gene. A BlastP of species that encode \textit{tam-tamR} gene pairs with \textit{S. coelicolor} malate synthase, malate dehydrogenase, and isocitrate dehydrogenase revealed significant conservation of these enzymes as well as conservation of predicted TamR binding sites in their promoters (Table 3.1).

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</tbody>
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Not found; gene not found by BlastP using *S. coelicolor* homolog as query or by search for gene name. No site; no TamR consensus site found in promoter. *The tam gene is not divergent from a tamR gene.* Gene encoding pyrimidine utilization protein C upstream of tam gene. Gene encoding endoribonuclease upstream of tam gene. IDH partial sequence found, but truncated at the N-terminus. Protein sequence divergent; not found by BlastP, only by search for gene name.
In the promoter region of *aceB1*, encoding malate synthase (Figure 3.2A), two predicted TamR binding sites are located near each other with 3 bp overlap (Figure 3.3A; overlapping base pairs in positions 16-18). These two binding sites and their organization are conserved in several *Streptomyces* species, which encode the *tam-tamR* gene pairs (Table 3.1). The sequence of these sites is highly conserved, particularly site 1 (positions 16-33; Figure 3.3A). Malate synthase uses acetyl-CoA and glyoxylate as substrates to produce malate (Figure 3.1B); activation of *aceB1* gene expression would therefore be expected to promote formation of malate for entry into the citric acid cycle. The presence of two potential TamR sites in the gene promoter predicts association of two TamR dimers.

One predicted TamR binding site is found in the promoters of *S. coelicolor* *mdh* (Figure 3.2B) and *idh* (Figure 3.2C). Isocitrate dehydrogenase catalyzes the conversion of isocitrate to α-ketoglutarate and malate dehydrogenase converts malate to oxaloacetate, both key steps in the citric acid cycle. The predicted TamR binding sites are almost invariably present in *Streptomyces* species that encode *tam-tamR* gene pairs, with the site in the *idh* promoter being particularly well conserved (Figure 3.3B-C and Table 1). The significant conservation of predicted TamR binding sites in the promoter regions of *mdh* and *idh* suggests that regulation of these genes by TamR is evolutionarily conserved. In addition, alignment of the predicted TamR binding sites from different *S. coelicolor* target genes reveals significant conservation (Figure 3.4).
Figure 3.2. Potential TamR target genes with predicted TamR binding sites in the promoter region. A. The promoter region of aceB1 (SCO6243) contains two predicted TamR binding sites (underlined). Genetic locus organization of aceB1 and surrounding region in Streptomyces coelicolor is shown. The relative orientations of the genes are represented by open arrows (note that sequence is shown from 3’ to 5’). B. A predicted TamR binding site is shown in the promoter region of mdh (SCO4827). C. The promoter region of idh (SCO7000) contains a predicted TamR binding site.
Figure 3.3. Sequence consensus of predicted TamR binding sites. A. Weblogo (http://weblogo.berkeley.edu/logo.cgi) representing the consensus binding site in the promoter region of aceB1. The relative frequency of base pairs at each position is represented by the height of each nucleotide. The two sites overlap by 3 bp (positions 16-18). B. Weblogo representing the consensus binding site in the promoter region of mdh. C. Weblogo representing the consensus binding site in the promoter region of idh.

Figure 3.4. The sequence consensus of predicted TamR binding site in the promoter region of different target genes in S. coelicolor. The sequence of predicted binding site related to tamO (site 1), (Huang & Grove 2013) SCO5999, (Huang & Grove 2013) SCO6243 (site 1), SCO4827, SCO7000 were aligned and compared.
TamR binds to the promoter region of predicted target genes

To determine whether TamR can bind to the promoter regions of *aceB1*, *mdh* and *idh*, labeled DNA fragments representing the target gene promoters were used in electrophoretic mobility shift assays (EMSA). The TamR used in EMSA was overexpressed in *E. coli* and purified to apparent homogeneity, as described previously (Huang & Grove 2013). As expected for MarR homologs, TamR exists as a dimer under physiological conditions, as determined by gel filtration chromatography (Huang & Grove 2013).

![Figure 3.5](image)

Figure 3.5. Binding of TamR to the promoter region of *aceB1*. A. Electrophoretic mobility shift assay showing binding of TamR to the promoter region of *aceB1*. DNA (0.07 nM) was titrated with TamR (lanes 1-11 representing reactions with 9.8 pM, 19.5 pM, 39.0 pM, 78.1 pM, 156.2 pM, 312.5 pM, 625.0 pM, 1.25 nM, 2.50 nM, 5.00 nM, 10.0 nM TamR dimer). Complexes C and free DNA F are identified by arrows. Reaction in lane 12 contained labeled DNA only. B. Fractional complex formation plotted against the ratio of TamR and pACEB1 concentrations. The intercept of tangents yields a stoichiometric ratio of TamR:DNA of 1.8:1. Error bars represent standard deviation from three independent repeats.
Titration of 0.07 nM pACEB1 DNA, which contains the promoter region of aceB1, with TamR revealed formation of one predominant complex, with a second complex of an electrophoretic mobility intermediate between that of free DNA and the predominant complex barely detectable (Figure 3.5A). The formation of two distinct complexes is consistent with the predicted existence of two TamR sites. The data also suggest that formation of a complex with a protein:DNA stoichiometry of 2:1 is favored over a 1:1 complex. Considering that TamR binds the tam-tamR

Figure 3.6. Binding of TamR to the promoter region of mdh. A. Electrophoretic mobility shift assay showing binding of TamR to the promoter region of mdh. Labeled pMDH DNA (0.13 nM) was titrated with TamR (lanes 2-12 representing reactions with 4.9 pM, 9.8 pM, 19.5 pM, 39.0 pM, 78.1 pM, 156.2 pM, 312.5 pM, 625.0 pM, 1.25 nM, 2.50 nM, 5.00 nM TamR dimer). Complexes C and free DNA F are identified by arrows. Reaction in lane 1 contained labeled DNA only. B. Fractional complex formation plotted against the ratio of TamR and pMDH concentrations. The intercept of tangents yields a stoichiometric ratio of TamR:DNA of 0.8:1. Error bars represent standard deviation from three independent repeats.
intergenic region with a $K_d\sim 17$ pM (Huang & Grove 2013), and the significant conservation of its predicted binding sites (Figure 3.4) we surmised that reaction conditions used were likely stoichiometric ([DNA]$>K_d$). Quantitation of EMSA data indicated saturation of pACEB1 DNA at a protein:DNA ratio of 1.8:1, consistent with the presence of two TamR sites (Figure 3.5B).

A titration of TamR with pMDH DNA, which contains the promoter region of mdh showed that TamR forms a single complex with this DNA (Figure 3.6A). Using 0.13 nM DNA, quantitation of EMSA data indicates saturation at a protein:DNA ratio of 0.8:1, consistent with the

![Electrophoretic mobility shift assay](image.png)  
Figure 3.7. Binding of TamR to the promoter region of idh. A. Electrophoretic mobility shift assay showing binding of TamR to the promoter region of idh. Labeled pIDH DNA (0.46 nM) was titrated with TamR dimer (lanes 2-11 representing reactions with 9.8 pM, 19.5 pM, 39.0 pM, 78.1 pM, 156.2 pM, 312.5 pM, 625.0 pM, 1.25 nM, 2.50 nM, 5.00 nM TamR dimer). Complexes C and free DNA F are identified by arrows. Reaction in lane 1 contained labeled DNA only. B. Fractional complex formation plotted against the ration of TamR and pIDH concentrations. The intercept of tangents yields a stoichiometric ratio of TamR:DNA of 1.0:1. Error bars represent standard deviation from three independent repeats.
presence of a single TamR site (Figure 3.6B). Similarly, titration of pIDH DNA, which contains the idh promoter region yields a single complex (Figure 3.7A). TamR saturates this DNA (0.46 nM) at a protein:DNA ratio of 1:1 (Figure 3.7B). That the latter gene promoters with a single predicted TamR site are saturated at a protein:DNA ratio of 1:1 also indicates that the TamR preparation is fully active, and that saturation of the pACEB1 DNA at a ratio of approximately 2:1 indeed reflects the accommodation of two TamR molecules on this DNA and not a partially inactive protein preparation.

**The binding of TamR to aceB1 promoter DNA is attenuated by trans-aconitate, cis-aconitate, citrate and isocitrate**

Since binding of TamR to tam-tamR intergenic DNA is attenuated by citrate, trans-aconitate, cis-aconitate and isocitrate (Huang & Grove 2013), the effect of these ligands on the binding of TamR to pACEB1 DNA was also investigated. All four ligands significantly attenuated the binding of TamR to this DNA (Figure 3.8A-D). The ability of these ligands to attenuate TamR-pACEB1 complex formation was reflected by IC$_{50}$ values; citrate, trans-aconitate, and cis-aconitate showed comparable efficiency with IC$_{50}$ values of 50.6±3.5 mM, 48.0±1.9 mM and 32.1±0.7 mM respectively (Figure 3.8E-F) while isocitrate was somewhat less efficient at attenuating DNA binding, with an IC$_{50}$ of 87.0±2.0 mM (Figure 3.8E). By comparison, succinate and α-ketoglutarate, which are also intermediates in the citric acid cycle, do not affect DNA binding by TamR (data not shown).

**In vivo effect of citrate and hydrogen peroxide on transcription**

To investigate the transcriptional regulation of the three target genes (aceB1, mdh and idh) by TamR, transcript levels were measured in *S. coelicolor* under conditions in which the TamR ligands accumulate. In *S. coelicolor*, the transport of exogenous citrate across cell membranes is
Figure 3.8. Effect of ligands on the binding of TamR to the promoter region of *aceB1*. A-D. Ligand concentrations in lanes 2-7 are 0, 12.5, 25, 50, 75, 100 mM, respectively. In lanes 2-7, 0.16 nM DNA and 312.5 pM TamR dimer was included in each reaction. Reaction in lane 1 contained labeled pACEB1 DNA only. Complexes C and free DNA F are identified. E-F. Normalized TamR-pACEB1 complexes formation as a function of ligand concentration. Error bars represent the standard deviation of three independent repeats.

mainly accomplished by the CitMHS family of transporters (Lensbouer et al. 2008; Lensbouer et al. 2010). Citrate needs to form metal complex with specific metals (with Ca$^{2+}$ or Fe$^{3+}$) for this transport to occur, otherwise exogenous citrate cannot be transported by this family of transporters. Therefore, *S. coelicolor* was cultured in medium with 100 mM citrate and 10 mM CaCl$_2$. As
reported previously, supplementing the culture medium with citrate only has no effect on \textit{tam}, \textit{tamR}, or \textit{sacA} transcript levels whereas the addition of both citrate and Ca\textsuperscript{2+} leads to significantly increased transcript levels (Huang & Grove 2013). Quantitating transcripts using qRT-PCR showed that the transcript level of \textit{aceB1}, \textit{mdh}, and \textit{idh} increased by 3.7±0.8 fold, 1.4±0.1 fold and 2.8±0.9 fold, respectively, when citrate and Ca\textsuperscript{2+} was supplemented in the medium (Figure 3.9).

Aconitase contains an iron-sulfur cluster that is susceptible to oxidation, leading to inactivation of enzymatic activity (Beinert \textit{et al.} 1983; Robbins & Stout 1989; Verniquet \textit{et al.} 1991; Gardner & Fridovich 1992). Under such conditions, intracellular levels of the substrate citrate accumulate and the intermediate \textit{cis}-aconitate may be released and converted to \textit{trans}-aconitate. While accumulation of \textit{trans}-aconitate would be expected to mirror the number of inactivated copies of aconitase, citrate was reported to accumulate to greater than 14 mM on inactivation of the enzyme (Viollier \textit{et al.} 2001). Addition of hydrogen peroxide, which inactivates aconitase and therefore causes an increase in intracellular citrate concentrations, generally elevated the transcript levels of \textit{aceB1}, \textit{mdh} and \textit{idh} comparably to the addition of citrate, except for the \textit{mdh} transcript, which was produced more efficiently on addition of H\textsubscript{2}O\textsubscript{2} (Figure 3.9). The transcript levels of \textit{aceB1}, \textit{mdh} and \textit{idh} increased by 2.7±0.3 fold, 5.7±1.2 fold and 4.4±2.0 fold, respectively. By comparison, transcript levels of \textit{tam}, \textit{tamR}, and \textit{sacA} were generally increased somewhat more efficiently on treatment of cultures with H\textsubscript{2}O\textsubscript{2} compared to uptake of citrate, perhaps due to a combined effect of greater intracellular citrate levels on inactivation of aconitase with H\textsubscript{2}O\textsubscript{2} and the associated accumulation of \textit{trans}-aconitate (Huang & Grove 2013).
Figure 3.9. *In vivo* gene regulation of TamR target genes. Relative abundance of gene transcripts (*aceB1, mdh* or *idh*) after exposure to 100 mM citrate+10 mM Ca^{2+} or 10 mM H_{2}O_{2}. The relative mRNA levels of target genes (*aceB1, mdh* or *idh*) and reference control gene (*rpoA*) were measured by qRT-PCR. Error bars represent standard deviation of three repeats.

**Discussion**

The TamR regulon is conserved among *Streptomyces* species

Enzymes involved in the citric acid cycle are expected to be present at some level regardless of growth conditions. However, their levels have been shown to fluctuate significantly depending on circumstances such as carbon source and oxygen level (Holms 1987; Gibala et al. 1998; Han *et al.* 2008; Tannler *et al.* 2008; Eoh & Rhee 2013). Our data suggest that *S. coelicolor* TamR regulates *aceB1, mdh* and *idh* in addition to three target genes described previously (*tam, tamR* and *sacA*). These genes either encode enzymes in the citric acid cycle or proteins with the capacity to affect flux through this metabolic pathway.

The gene locus consisting of divergently oriented *tamR* and *tam* genes is highly conserved among the *Streptomyces* species in which this gene pair is encoded. Exceptions include a few *Streptomyces* species in which the *tam* gene is not immediately adjacent to and divergent from *tamR* but preceded by another gene and *S. ambofaciens* in which the *tam* gene is not divergent
from \textit{tamR} (Table 3.1). The frequent occurrence of divergent \textit{tam-tamR} gene pairs suggests a shared evolutionary origin, while the absence of these genes in many other \textit{Streptomyces} species may reflect gene loss. That TamR binding sites are highly conserved in the intergenic region between these two genes as well as in the promoters of genes encoding aconitase, malate synthase, malate dehydrogenase, and isocitrate dehydrogenase further indicates that the regulatory role of TamR is evolutionarily conserved.

The \textit{tam-tamR} gene locus often includes a gene encoding a predicted glyoxalase (Figure 3.1A; the predicted glyoxalase gene overlaps the \textit{tam} gene by 3 bp). While this particular homolog remains uncharacterized, we note that glyoxalases are generally involved in detoxification of the metabolite methylglyoxal, which is formed when dihydroxyacetone phosphate and glyceraldehyde-3-phosphate accumulate during glycolysis (Weber \textit{et al.} 2005). This situation may pertain if flux through the citric acid cycle is compromised. It is conceivable that TamR-mediated upregulation of glyoxalase may alleviate the toxicity associated with methylglyoxal production.

The promoters for aconitase, malate dehydrogenase, and isocitrate dehydrogenase contain a single TamR site, while the malate synthase gene promoter features two sites that overlap by three base pairs. The latter is reminiscent of the \textit{tam-tamR} intergenic region, which contains six sites that all overlap each other by three base pairs (Huang & Grove 2013). This organization of cognate sites places the centers of each site on opposite faces of the double helix, predicting that two TamR dimers bind on opposite faces of the duplex. A similar organization of two overlapping cognate sites is seen in the promoter for \textit{Agrobacterium tumefaciens} PecS, where the extent of protection from DNase I digestion is much greater than the protection of a single site, consistent with occupancy by two PecS molecules (Perera & Grove 2010b). The stoichiometry of TamR binding to the malate synthase gene promoter and the formation of a single predominant complex
suggests that two TamR dimers effectively bind simultaneously to this promoter. Despite the difference in stoichiometry, changes in gene expression on accumulation of ligand are comparable (Figure 3.9), and the concentrations of ligand associated with 50% reduction in complex formation \textit{in vitro} is comparable to that seen for TamR binding to the six sites in the \textit{tam-tamR} intergenic region. Since the number of TamR sites does not appear to correlate with either levels of gene expression or sensitivity to ligand \textit{in vitro}, it is conceivable that the existence of accessory sites may contribute to the exclusion of other regulatory factors by occluding the promoter.

\textbf{TamR promotes flux through the citric acid cycle}

All TamR target genes are related to the citric acid cycle (Figure 3.1). Genes \textit{sacA}, \textit{mdh} and \textit{idh} encode enzymes (aconitase, malate dehydrogenase, and isocitrate dehydrogenase, respectively) in the citric acid cycle. Gene \textit{tam} encodes \textit{trans}-aconitate methyltransferase, which is important for preventing the accumulation of a toxic byproduct of the citrate isomerization step, \textit{trans}-aconitate (Cai & Clarke 1999; Cai et al. 2001). Gene \textit{aceB1} encodes a malate synthase, which can provide malate for the citric acid cycle from glyoxylate and acetyl-CoA. This indicates that TamR plays an important role in restoring metabolic flux through the citric acid cycle.

The roles of proteins encoded by TamR target genes reflect an interesting relationship and provide a rationale for concerted regulation (Figure 3.1). In the citric acid cycle, one acetyl-CoA is degraded per cycle to produce NADH (or NADPH) and ATP. Replenishing the intermediate pool is important to maintain the efficiency of the cycle as many intermediates function as precursors for other biosynthetic reactions. If aconitase is inactivated, \textit{cis}-aconitate may be released. In this circumstance, \textit{trans}-aconitate methyltransferase becomes important for preventing accumulation of the inhibitory metabolite \textit{trans}-aconitate (Figure 3.1B). Secondly, inactivation of aconitase can result in depletion of other citric acid cycle intermediates. Upregulation of the gene
encoding aconitase will alleviate this situation as well as consume accumulated citrate (which is also inhibitory), and malate synthase will synthesize malate from glyoxylate and acetyl-CoA, thereby providing malate to the citric acid cycle. It is therefore reasonable for TamR to regulate genes encoding trans-aconitate methyltransferase, aconitase, and malate synthase to restore cycle flux. Malate dehydrogenase and isocitrate dehydrogenase both catalyze a dehydrogenation step that produces energy, therefore, the elevated expression of these two enzymes would be beneficial to the efficiency of the citric acid cycle and its energy production.

If inactivation of aconitase is the primary circumstance in which TamR-mediated gene regulation becomes important, then this scenario corresponds to a situation in which the metabolites citrate and trans-aconitate accumulate, as discussed above. What would then be the rationale for TamR-mediated upregulation of the gene encoding isocitrate dehydrogenase, considering that isocitrate would likely be depleted on inactivation of aconitase? Converting isocitrate to succinate and glyoxylate by isocitrate lyase via the glyoxylate bypass circumvents energy-producing steps of the citric acid cycle (Figure 3.1B); upregulation of the gene encoding isocitrate dehydrogenase would therefore favor the citric acid cycle and prevent glyoxylate bypass once isocitrate becomes available.

The glyoxylate bypass allows cells to utilize simple carbon compounds when carbohydrates such as glucose are scarce by synthesizing malate from glyoxylate and acetyl-CoA. In *E. coli*, there are two types of malate synthase, named malate synthase A and malate synthase G (Molina *et al.* 1994; Pellicer *et al.* 1996; Pellicer *et al.* 1999). Both can synthesize malate from glyoxylate and acetyl-CoA. In *E. coli*, the *aceB-aceA-aceK* operon is very important to the glyoxylate cycle, encoding malate synthase A (*aceB*), isocitrate lyase (*aceA*), and isocitrate dehydrogenase kinase/phosphatase (*aceK*) (Maloy & Nunn 1982; el-Mansi *et al.* 1987; Chung *et
Isocitrate lyase and malate synthase A carry out the glyoxylate bypass, and isocitrate dehydrogenase kinase/phosphatase is a bifunctional enzyme, which can phosphorylate or dephosphorylate isocitrate dehydrogenase to regulate its activity (Nimmo et al. 1984; Nimmo & Nimmo 1984; LaPorte & Chung 1985; Stueland et al. 1987; Laporte et al. 1989). Malate synthase G, which is encoded by the glcB gene, can be induced by glycolate and synthesizes malate from glyoxylate produced by the glycolate pathway (Molina et al. 1994; Pellicer et al. 1996; Pellicer et al. 1999).

*S. coelicolor* also harbors two genes encoding malate synthase, aceB1 and aceB2 (SCO0983). A gene encoding isocitrate lyase (SCO0982) is adjacent to aceB2, encoding malate synthase A. The aceB1 gene encodes a homolog of malate synthase G, and surrounding genes encode allatoinase (SCO6247), allantoicase (SCO6248) and an IclR homolog (SCO6246) (Figure 3.2A). Allantoinase (EC. 3.5.2.5) and allantoicase (EC. 3.5.3.4) catalyze consecutive reactions that convert allantoin to ureidoglycolate and urea as part of the purine degradation pathway. Ureidoglycolate is then converted to urea and glyoxylate, the latter a substrate for malate synthase. The gene organization suggests that malate synthase G may replenish the citric acid cycle intermediate pool with malate, which is converted from the glyoxylate produced by purine degradation or other metabolic processes.

The glyoxylate cycle bypasses two iron-sulfur proteins (fumarase and succinate dehydrogenase) in the citric acid cycle, both of which contain iron-sulfur clusters that are vulnerable to oxidative stress. If the citric acid cycle is compromised due to oxidative stress, the glyoxylate cycle may be able to sustain the cell until damaged enzymes in the citric acid cycle are regenerated. TamR-mediated upregulation of malate synthase gene expression would also be beneficial from this point of view.
References


CHAPTER 4

STREPTOMYCES COELICOLOR ENCODES A URATE-RESPONSIVE TRANSCRIPTIONAL REGULATOR WITH HOMOLOGY TO PecS FROM PLANT PATHOGENS

Introduction

Members of the multiple antibiotic resistance regulator (MarR) family of transcriptional regulators are involved in a variety of important biological processes. MarR proteins are particularly well suited to sense environmental changes as they can regulate target gene expression in response to the binding of specific ligands (Perera & Grove 2010a; Grove 2013). Therefore, many MarR family regulators mediate bacterial responses to environmental stress. A common regulatory mechanism involves the MarR homolog binding to the intergenic region between the marR gene and a divergently oriented gene (or operon), repressing the transcription of both. Upon binding of a specific ligand, DNA binding is attenuated, causing derepression of both genes (Ariza et al. 1994; Reverchon et al. 1994; Perera & Grove 2010a; Grove 2013).

A subfamily of MarR proteins, termed urate responsive transcriptional regulators (UrtR), has been described (Perera & Grove 2011). The transcriptional regulators in this subfamily are characterized by an N-terminal extension not found in “classical” MarR proteins such as Escherichia coli MarR. In addition, they conserve four amino acid residues, which have been implicated in urate coordination and the attendant attenuation of DNA binding, and conservation of sequence within the DNA recognition helices correlates with similarity of their cognate DNA binding sites in target promoters (Wilkinson & Grove 2004; Bordelon et al. 2006; Perera et al. 2009; Grove 2010; Perera & Grove 2010b, 2011). Several members of this subfamily have been shown to respond to urate as a ligand; the founding member, HucR from Deinococcus radiodurans, regulates expression of a gene encoding a uricase (Wilkinson & Grove 2004). However, PecS from Agrobacterium tumefaciens (Rhizobium radiobacter) and MftR from
*Burkholderia thailandensis* regulate expression of a predicted membrane transporter (Grove 2010; Perera & Grove 2010b). *R. radiobacter* PecS and PecM (the membrane transporter) are homologous to proteins previously described in the plant pathogen *Erwinia chrysanthemi* (*Dickeya dadantii*) (Reverchon et al. 1994; Praillet et al. 1997; Rouanet & Nasser 2001; Hommais et al. 2008). *D. dadantii pecM*, which is also regulated by PecS, was shown to encode a transporter involved in indigoidine excretion (Praillet et al. 1997). The antioxidant indigoidine functions in defense against reactive oxygen species (ROS) and is important for bacterial virulence (Rouanet & Nasser 2001).

Plants produce ROS in the early stages of pathogen invasion as part of their mechanism to defend themselves, hence production of antioxidants is important for virulence of plant pathogens (Sandalio *et al.* 1988; Bolwell *et al.* 2002; Yoshioka *et al.* 2008). One of the mechanisms by which host plants produce ROS is by activating xanthine oxidase. Since xanthine oxidase uses xanthine and hypoxanthine as substrates to produce urate and ROS, urate will be produced as a byproduct and may be detected by the invading bacterium as a signal of impending oxidative stress. If the bacterium can use urate as a signal to regulate specific genes, it might augment the defense against oxidative stress or elicit expression of other genes required for effective colonization. The observed upregulation of *R. radiobacter pecM* on exposure to urate would be consistent with this scenario (Perera & Grove 2010b).

We have recently reported that *Streptomyces coelicolor* encodes an UrtR homolog that is very similar to PecS from plant pathogens, yet differs by responding to the ligand *trans*-aconitate and related compounds (Huang & Grove 2013). This homolog, named *trans*-aconitate methyltransferase regulator (TamR), regulates expression of a gene encoding *trans*-aconitate methyltransferase and is important for metabolic flux through the citric acid cycle during oxidative
stress. *S. coelicolor* lives in different ecological environments and is one of the most widely distributed soil bacteria (Hodgson 2000). It needs to compete with other organisms (plants, fungi, or bacteria) for limited nutrients and other resources in the soil. For *Streptomycetes* spp. and other bacteria, secretion of antibiotics is thought to be one of their important strategies during this competition (Slattery *et al.* 2001; Haas & Keel 2003; Laskaris *et al.* 2010). In this complex ecological environment, many organisms (such as plants) employ the strategy of producing ROS in defense against invading pathogens (Sandalio *et al.* 1988; Bolwell *et al.* 2002; Yoshioka *et al.* 2008). For organisms that inhabit the rhizosphere, mechanisms must therefore be in place to respond to incidental exposure to ROS and other secreted compounds.

We show here that *S. coelicolor* encodes divergent pecS and pecM genes that are upregulated by exogenous urate. The sequence conservation and predicted domain architecture of PecS and TamR proteins from Streptomyces suggests that they derive from a common ancestor. However, TamR and PecS from *Streptomycetes* spp. have evolved distinct functions, with PecS homologs comparable to PecS from plant pathogens in regulating expression of a gene encoding PecM.

**Experimental Procedures**

**Sequence analyses**

Sequences were aligned using ClustalW. Pre-aligned amino acid sequences were used to generate a phylogenetic tree using the neighbor-joining method in MEGA4 (Saitou & Nei 1987; Tamura *et al.* 2007). Five hundred bootstrap replicates were analyzed to generate the bootstrap consensus tree and to estimate the statistical confidence values. Positions that contain gaps were eliminated during calculation. The evolutionary distances are in units of number of amino acid substitutions per site.
Cloning and purification of PecS

*S. coelicolor* A3(2) M145 strain was cultured in tryptone yeast extract broth (ISP medium 1) at 30°C. The genomic DNA was isolated from *S. coelicolor* using the salting out method (Kieser *et al.* 2000). Forward primer 5’-CCGATACTCGTCATATGACTGAACG-3’ and reverse primer 5’-GGGCGGATCCCTACTTCTCCG-3’ were used to amplify the gene encoding PecS (SCO2647; restriction sites underlined) using genomic DNA as template. The PCR product was cloned into the NdeI-BamHI sites of pET28b (Novagen). As a result, a N-terminal His6-tag was introduced into the recombinant PecS. The recombinant plasmid was transformed into *E. coli* TOP10 (Invitrogen). After the correct construct was confirmed by sequencing, it was transformed into *E. coli* BL21(DE3)pLysS for protein overexpression.

For overexpression, a 50 ml culture was inoculated with a single colony and grown overnight at 37°C (250 rpm) in Luria-Bertani media (with 50 μg/ml kanamycin). The overnight culture was diluted 1:500 with LB media (with 50 μg/ml kanamycin) and grown at 37°C (250 rpm). When the OD<sub>600</sub> reached about 0.6, overexpression of protein was induced with 0.2 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 3 hours. The induced cultures were then chilled on ice. Cell pellets were stored at -80°C after the cultures were centrifuged at 4000 g for 10 minutes.

For protein purification, cell pellets were thawed on ice, and cells were resuspended in ice-cold lysis buffer (50 mM sodium phosphate buffer (pH 7.2), 250 mM NaCl, 5% glycerol, 10 mM imidazole, 0.15 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM 2-mercaptoethanol). Sonication was used to disrupt cells. After sonication, DNase I was added to digest nucleic acids. Then, this solution was centrifuged at 15,000 g for 60 min at 4°C. After the supernatant was filtered through filter paper, it was loaded onto a HIS-Select Nickel Affinity column (Sigma),
which was already equilibrated with lysis buffer. Then, the column was washed by gravity flow with 10 volumes of wash buffer (50 mM sodium phosphate buffer (pH 7.2), 250 mM NaCl, 5% glycerol, 20 mM imidazole, 0.15 mM PMSF, 1 mM 2-mercaptoethanol). Elution buffer (50 mM sodium phosphate buffer (pH 7.2), 250 mM NaCl, 5% glycerol, 250 mM imidazole, 0.15 mM PMSF, 1 mM 2-mercaptoethanol) was applied to elute proteins after the washing step. Peak fractions from the elution were pooled and dialyzed against dialysis buffer (50 mM potassium phosphate buffer (pH 7.2), 250 mM NaCl, 5% glycerol, 0.15 mM PMSF, 1 mM 2-mercaptoethanol) at 4°C. All steps of purification were performed at 4°C. After dialysis, the protein was stored at -80°C. Purity was determined by Coomassie brilliant blue-stained SDS-polyacrylamide gels. PecS concentration was determined based on its absorbance at 280 nm using the calculated extinction coefficient (8250 M\(^{-1}\)cm\(^{-1}\)). All experiments were performed with His\(_6\)-tagged PecS.

**DNA binding assays**

The intergenic segment between PecS (SCO2647) and SCO2646 genes was amplified from *S. coelicolor* genomic DNA using primers pecS-Fv (5'-GTGGCGGCCATGA-3') and pecS-Rv (5'-TGCGACGAGTATCGG-3'). This 124 bp DNA contains the entire intergenic region and extends 1 bp into the coding region of SCO2647 and 11 bp into the coding region of SCO2646. Then, this DNA segment was \(^{32}\)P-labeled at the 5'-ends using T4-polynucleotide kinase (T4-PNK). In titration experiments, \(^{32}\)P-labeled pecO (0.10 nM) was incubated with PecS in binding buffer (25 mM Tris (pH 7.5), 50 mM NaCl, 0.06% detergent BRIJ58 (Pierce), 10 µg/ml bovine serum albumin (BSA), 2% glycerol) at 25°C for 20 min. Complex and free DNA was separated on 6% non-denaturing polyacrylamide gels (39:1(w/w) acrylamide:biacrylamide). The gel was pre-run for about 20 minutes in 0.5×Tris–borate–EDTA (TBE) buffer at 4°C before the samples were
loaded and run at 10 V/cm for 2 hours in 0.5×TBE buffer at 4°C. After the gel was dried, it was exposed to phosphor screens. A Storm 840 phosphorimager (GE Healthcare) was used to visualize and scan results. The densitometric result was quantified using ImageQuant 5.1 (Molecular Dynamics). The quantitative data was fitted to the Hill equation: 

\[ f = f_\text{max} \times \frac{[\text{TamR}]^n}{(K + [\text{TamR}])^n} \]

where [TamR] is the protein concentration, f is fractional saturation, K is a constant, and n is the Hill coefficient.

To determine the effect of ligand binding on PecS, the binding buffer used was 0.5 M Tris (pH 7.5), 50 mM NaCl, 0.06% BRIJ58, 10 μg/ml BSA, 2% glycerol. Note that the higher buffer concentration was necessitated to prevent pH changes by the addition of ligands, which were dissolved in 0.4 M NaOH. This higher ionic strength reduces the affinity of PecS for both DNA and ligands. Reactions contained 1.25 nM PecS and 0.10 nM labeled DNA, except for the control (DNA only). After 20 min incubation at 25°C, samples were analyzed using EMSA under the conditions described above. Data was analyzed by fitting to exponential decay equation: 

\[ f = A e^{-kL} \]

where f is fractional saturation, L is the ligand concentration, A is the saturation plateau, and k represents the exponential decay constant. All quantitative results derive from at least three independent experiments.

**Thermal stability**

PecS (10 μM) was diluted in a measurement buffer (200 μM Tris (pH 8.0), 200 mM NaCl), which contained reference fluorescent dye 5×SYPRO Orange (Invitrogen). An Applied Biosystems 7500 Real-Time PCR System was used to measure the fluorescence emission at a range of temperatures (5–94°C) in 1°C increments for 45s. SYBR green filter was used in the measurement. The result from a reaction without protein was used to correct the fluorescence yield. Then, the sigmoidal part of the melting curve was fit to a four-parameter sigmoidal equation using
Sigma Plot 9 to calculate the melting temperature. Eight independent experiments (n=8) were performed for this measurement.

**In vivo regulation of gene activity**

*S. coelicolor* cultures, which were germinated from spores in ISP medium 1, were grown for 36 hours before they were treated with urate (10 mM) for 4 hours. After the 4 hour incubation, cells were harvested by centrifugation and immediately washed twice using 50 mM sodium phosphate buffer (pH 6.4). The total RNA was then immediately isolated using Illustra RNAspin Mini Isolation Kit (GE Healthcare). Then, AMV reverse transcriptase (New England BioLabs) was used to generate cDNA for quantitative PCR (qPCR). Quantitative PCR was performed on an Applied Biosystems 7500 Real-Time PCR system using SYBR green I as the fluorescent dye. Gene *rpoA* (house-keeping gene encoding RNA polymerase alpha subunit) was used as internal control. The comparative CT \(2^{-\Delta\Delta CT}\) method was used for data analysis after necessary data validations (Schmittgen & Livak 2008). The primers used in qRT-PCR were: ScPecS-RT5: 5'-TGTCGCCACGCTGATGC-3'; ScPecS-RT3: 5'-CGGTGAGGGTGACCTGGAGTC-3'; SCO2646-RT5: 5'-CGCTGGCGTACTGGCTCTGGTT-3'; SCO2646-RT3: 5'-CCCGTCAGGTCCATCGAATCTTTT-3'; RopA-RT5: 5'-AAGCTGGAGATGGAGCTGAC-3'; RopA-RT3: 5'-TTGAGAACC CGCGAGTAGAT-3'.

**Glutaraldehyde-mediated crosslinking**

After dilution with 50 mM sodium phosphate buffer (pH 7.0), 40 µM PecS in 50 mM sodium phosphate buffer (pH 7.0) was incubated in the presence of glutaraldehyde (0.1%, 0.2% or 0.3%) in 25°C for 30 min. 4× Laemmlli sample buffer was used to terminate the reaction. Then, protein samples were loaded on a SDS-PAGE (15%) gel. The gel was stained with Coomassie brilliant-blue after electrophoresis.
Gel filtration

A Superose 12 10/300 column (inner diameter 10 mm, bed length 30 cm; GE Healthcare) was pre-equilibrated and eluted with mobile phase buffer (50 mM sodium phosphate buffer (pH 7.0), 150 mM NaCl, 2% glycerol) in an FPLC system (ÄKTA FPLC, GE Healthcare) at 4°C. The flow rate was 0.5 mL/min for measurements. Several molecular weight markers (Bio-Rad gel filtration standard) were used to create the standard curve. These markers include γ-globulin (158.0 kDa), ovalbumin (44.0 kDa), myoglobin (17.0 kDa), and vitamin B12 (1350 Da). The equation $Kav = (VE – VO)/(VT – VO)$ was used to calculated the $Kaverage$ ($Kav$) of a protein. In this equation, $VE$, $VO$, and $VT$ represent the retention volume of the protein, void volume of the column and the geometric bed volume of the column, respectively.

Circular Dichroism spectroscopy

A Jasco J-815 circular dichroism spectrometer was used to measure the far UV circular dichroism spectrum. PecS (0.19 mg/ml) was in CD buffer (50 mM potassium phosphate buffer (pH 7.0), 20mM KCl, 0.8% Glycerol) during ellipticity measurements. A quartz cuvette with 0.1-cm path length was used. All measurements were conducted at 1-nm steps in triplicate at 20°C. The results were corrected for buffer contributions to the signal. The SELCON3 Program from the website DichroWeb was used to calculate the predicted secondary structure composition from the spectrum (Sreerama et al. 1999; Whitmore & Wallace 2004, 2008). The goodness of fit was determined from the NRMSD (normalized root-mean-square deviation) value of 0.034.
Fluorescence spectrum and tryptophan fluorescence quenching

A Jasco FP-6300 spectrofluorimeter was used to measure the fluorescence emission spectra from 295 nm to 400 nm with an excitation wavelength set at 290 nm at 25°C. A 0.5 cm pathlength cuvette was used for the measurement. PecS (10 μM) was in FL buffer (40 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM MgCl₂, 0.2 mM EDTA, 0.1% Brij58). To measure effects of ligand, ligands were dissolved in 0.4 M NaOH and serially diluted with 0.4 M NaOH. Ligand solution (1μl; the control was equal volume 0.4 M NaOH) was contained in a 200 μl total volume reaction (with 10 μM PecS in FL buffer) and incubated for 2 min for fluorescence measurement. Data analysis, including the inner filter effect correction, fluorescence quenching calculation and fitting to the Hill equation, were carried out as described previously (Wilkinson & Grove 2005).

Results

The genomic locus encoding PecS

Previously characterized PecS proteins from R. radiobacter and D. dadantii are encoded divergently from pecM, which encodes a membrane transporter. This genomic locus organization is conserved in S. coelicolor (Figure 4.1A). Locus SCO2647 encodes a MarR family transcriptional regulator with homology to PecS and a divergently oriented gene encoding a putative integral membrane protein related to PecM (SCO2646) is separated from the pecS gene by an intergenic region of 112 bp. Two consecutive genes encoding another putative integral membrane protein (SCO2645) and a LysR family transcriptional regulator (SCO2644), respectively, are encoded on the same strand as SCO2646, each pair of genes separated by approximately 30 bp.

This MarR homolog is annotated in the genome as PecS since the amino acid sequence is similar to that of D. dadantii PecS (about 40% identity); by comparison, PecS from the plant pathogens D. dadantii and R. radiobacter share ~45% identity. Moreover, the sequence of the
membrane protein encoded by the divergent gene is highly similar to that of *D. dadantii* PecM (about 44% identity). The sequence of the intergenic region suggests that the common regulatory mechanism involving the transcriptional regulator binding to this region and repressing expression of the divergent genes may also apply to PecS. In the *pecM-pecS* intergenic region, several imperfect inverted repeats may be identified that could serve as binding sites for PecS (Figure 4.1A). Notably, these sites share similarity to the UrtR consensus site, whose 9 bp half-sites are most conserved in the center (the central three bases of each consensus half-site are CTT and AAG, respectively) (Perera & Grove 2011).

Amino acid sequence alignment of MarR homologs reveals that *S. coelicolor* PecS belongs to the UrtR subfamily. It contains the characteristic N-terminal extension seen in *D. radiodurans* HucR to form a helical segment as well as the four conserved amino acid residues (W22, D66, R73, R99) implicated in ligand binding (Figure 4.1B). Significant conservation of residues in $\alpha_3$ is consistent with binding to a common ligand, as this secondary structure element lines the inferred ligand-binding pocket in HucR (Bordelon et al. 2006). Conservation of residues in $\alpha_5$, the DNA recognition helix, is consistent with shared features of the cognate DNA sites.

While UrtR homologs are not universally encoded by *Streptomyces* spp. as evidenced by BlastP searches of the numerous available genomes using *R. radiobacter* PecS as a query, ~20 species that encode PecS are found, all among the more than 40 that also encode TamR; assignment of UrtR homologs as either TamR or PecS was based on identification of the divergent gene as either *tam* (*trans*-aconitate methyltransferase) or *pecM* (*EamA*, encoding protein domains previously called DUF6). Using the sequences of 10 randomly selected *Streptomyces* spp. that encode PecS, a phylogenetic tree was created to illustrate the evolutionary relationship between
Figure 4.1. A. The genetic organization of pecS and adjacent genes. Genes are represented by open arrows. An intergenic region of 112 bp separates pecS and pecM (SCO2646). The sequence of the intergenic region is shown with predicted binding sites indicated with lines. B. Sequence alignment of PecS and other MarR homologs. Secondary structure elements are based on the structure of HucR (Bordelon et al. 2006). E. coli MarR and MTH313 do not include the N-terminal helix α1 (Alekshun et al. 2001; Saridakis et al. 2008).
Figure 4.2. Phylogenetic analysis of UrtR homologs. MEGA4 was used to generate the phylogenetic tree using the neighbor-joining method with 500 bootstrap replicates. The tree is drawn to scale. The scale bar represents an evolutionary distance of 0.05. Orange background shading denotes the subtree of PecS homologs from Streptomyces. Blue background shading shows the subtree of TamR homologs.

TamR and PecS proteins (Figure 4.2). TamR and PecS proteins form two separate clusters, consistent with divergence from a common ancestor and a shared function of PecS from *Streptomyces* spp. We also note that species encoding PecS include the few characterized pathogenic species for which sequence information is available, such as *S. turgidiscabies* and *S. ipomoeae*. 
**PecS is a stable dimer**

The *pecS* gene was amplified from *S. coelicolor* genomic DNA cloned into pET28b, and overexpressed in *E. coli*. PecS with an N-terminal His$_6$-tag was purified to apparent homogeneity, and it yielded a single band in an SDS-PAGE gel with a molecular mass consistent with the monomer (theoretical molecular mass of recombinant PecS is about 21 kDa; Figure 4.3B). The UV-visible spectrum of PecS showed a single peak at a wavelength around 280 nm (Figure 4.3A). PecS migrated as a single band in native gel electrophoresis, suggesting that it exists as a single species (Figure 4.3A, inset). The far-UV circular dichroism spectroscopy revealed a secondary structure composition of PecS of about 52% α-helix, 6% β-sheet, 17% turn and 25% unordered (Figure 4.3C). This structure composition estimate is similar to the composition of HucR, which contains about 55% α-helix and 5% β-sheet (Bordelon *et al.* 2006). PecS had a melting temperature of 47.3±0.5 °C, indicating that it is stable at physiological temperatures (Figure 4.3D).

In order to determine the oligomeric state of purified PecS, size-exclusion chromatography was used. Purified PecS eluted as a single peak in size-exclusion chromatography, which is consistent with the single species seen after native gel electrophoresis. PecS eluted from the gel filtration column at about 49 kDa (Figure 4.4A, B), consistent with the molecular mass of the dimer. In addition, glutaraldehyde-mediated cross-linking of PecS resulted in a doublet at the molecular mass of the dimer in SDS-PAGE gels, probably reflecting the formation of two different crosslinked species (Figure 4.4C). These results revealed that purified PecS exists as a dimer with the secondary structure composition and stability expected for a MarR homolog.
Figure 4.3. Characterization of purified PecS. A. UV-visible absorption spectra of purified recombinant PecS. Insert: Lanes 1-2, purified PecS in 10% native PAGE. B. Purified recombinant PecS in 15% SDS-PAGE gel. Lane 1, PecS; Lane 2, molecular mass marker (BioRad; Mw indicated at the right). C. Far-UV CD spectrum of purified PecS. Ellipticity measurements are represented in units of millidegrees (mdeg; machine units). D. Melting temperature of PecS. Thermal denaturation is represented by the fluorescence emission resulting from the binding of SYPRO Orange to denatured protein as a function of temperature.
Figure 4.4. PecS mainly exists as dimer. A. Size-exclusion chromatography of purified recombinant PecS. Elution volumes of molecular weight standards are identified with arrows. B. Gel-filtration analysis of PecS and standards to determinate molecular weight. The standard curve was generated from the $K_{av}$ of molecular weight standards (grey circles) versus $\log_{10}(\text{Mw})$ of these standards. The $K_{av}$ of PecS is indicated by an arrow. C. Glutaraldehyde-mediated cross-linking of purified PecS. Lane 1, unmodified PecS; lanes 2-4, PecS crosslinked with 1%, 2% and 3% glutaraldehyde, respectively. Lane 5, molecular weight marker (Mw indicated at the right). A 15% SDS-PAGE gel was used.
**PecS binds to the pecM-pecS intergenic region**

Electrophoretic mobility shift assays (EMSA) were used to detect the binding of PecS to *pecO*, a 124 bp DNA segment representing the *pecM-pecS* intergenic region. PecS bound to this DNA segment forming multiple complexes, which indicates the presence of more than one binding site in *pecO* (Figure 4.5A). A single PecS–*pecO* complex appeared at relatively low PecS concentrations, whereas more complexes formed gradually with increasing concentrations of PecS, with complex formation saturating at high PecS concentrations. The pattern of complex formation is consistent with gradual accumulation of additional PecS molecules on the DNA. Quantitation of EMSA data revealed half-maximal saturation of *pecO* at [PecS] = 0.11 ± 0.03 nM, indicating high affinity binding (Figure 4.5B). The interaction between PecS and *pecO* is not cooperative (Hill coefficient of 1.0±0.0), consistent with the gradual formation of complexes with lower electrophoretic mobility with increasing [PecS].

Specificity of PecS binding to *pecO* was assessed by EMSA experiments in which unlabeled specific DNA (*pecO*) or nonspecific DNA (plasmid pGEM5) was used to compete with labeled specific DNA (*pecO*) for PecS binding. Addition of unlabeled *pecO* could compete with labeled *pecO* for binding to PecS (Figure 4.5C). However, complex formation between PecS and *pecO* was not affected by addition of non-specific pGEM5 DNA (up to 2.5-fold molar excess of the 3,000 bp pGEM5 compared to the concentration of 124 bp *pecO*), which showed that the binding of PecS to DNA is specific.
Figure 4.5. Binding of PecS to pecS-pecM intergenic region pecO. A. Electrophoretic mobility shift assays showing binding of PecS to pecO. Labeled pecO (0.10 nM) was titrated with PecS (lanes 1-11 representing reactions with 0, 4.9 pM, 9.8 pM, 19.5 pM, 39.0 pM, 78.1 pM, 156.2 pM, 312.5 pM, 625.0 pM, 1.25 nM, 2.50 nM, 5.00 nM, 10.0 nM PecS). Free DNA (F) is identified by an arrow at the left. B. Normalized fractional complex formation as a function of PecS concentration. Error bars represent standard deviation from three independent repeats. C. Binding of labeled pecO (0.1 nM) to PecS (0.625 nM) was challenged with increasing concentration of unlabeled 124 bp pecO DNA (lanes 3-5: 0.1, 0.25, 0.5 nM) or 3,000 bp plasmid pGEM5 (lanes 6-7: 0.1, 0.25 nM). Reaction in lane 1 contained labeled DNA only. Reaction in lane 2 contained no competitor DNA.
Urate attenuates the binding of PecS to pecO

Since *S. coelicolor* PecS exhibits features that are characteristic of UrtR proteins, urate and several related intermediates in the purine degradation pathway were used to determine their effect on DNA binding by PecS. These ligands included xanthine, hypoxanthine and allantoin (Figure 4.6A). The four conserved amino acid residues, which are predicted to participate in urate binding are labeled in the structure model of PecS (Figure 4.6C). The predicted interactions between urate and these amino acids is based on the proposed model for interaction between HucR and urate, in which the Trp in α1 and Arg in α3 interact with urate through a hydrogen bond and a salt bridge, respectively (Perera et al. 2009). The occupancy of urate in the binding pocket would result in the repulsion of Asp in α3, which would then cause displacement of the DNA recognition helix (α5) as Asp forms a salt bridge with Arg in α5. According to this model, xanthine mostly contains the functional groups necessary for binding, whereas both hypoxanthine and adenine lack some features necessary for efficient interaction.

To measure the effect of ligands on DNA binding of PecS, complexes of PecS and pecO were challenged with increasing concentration of urate, xanthine, hypoxanthine or allantoin. EMSA results showed that urate significantly attenuated the binding between PecS and DNA with an IC50 value of 15.6±0.5 mM (Figure 4.7A,E). Although xanthine can attenuate the binding between PecS and DNA, the effect is somewhat weaker with an IC50 value of 29.0±0.9 mM (Figure 4.7B,E). In contrast, hypoxanthine and allantoin have no effect on binding of PecS to its target DNA pecO (Figure 4.7C-E). This result shows that DNA binding by PecS could be attenuated by urate and it is consistent with the effect of urate and related ligands on DNA binding by other UrtR subfamily members, *D. radiodurans* HucR, *R. radiobacter* PecS and *B. thailandensis* MfrR (Perera et al. 2009; Grove 2010; Perera & Grove 2010b). The comparable
ligand specificity is consistent with a shared ligand binding pocket and mechanism for attenuation of DNA binding.

PecS contains only one Trp residue per monomer, located at the predicted urate-binding site (Figure 4.6C). Ligand binding to PecS would therefore be predicted to alter the environment of this tryptophan and cause a change in intrinsic fluorescence. When excited at a wavelength of 290 nm, PecS exhibited intrinsic fluorescence with a peak around 330 nm (Figure 4.8A). A concentration-dependent fluorescence quenching was induced by urate and xanthine (Figure 4.8B). The intrinsic fluorescence was significantly quenched by urate and to a lesser extent by xanthine; this is consistent with the effect of urate and xanthine on the intrinsic fluorescence of *D. radiodurans* HucR and *R. radiobacter* PecS (Wilkinson & Grove 2005; Perera & Grove 2010b). Binding affinity of these ligands was analyzed by fitting the fluorescence quenching as a function of ligand concentration to the Hill equation. Urate bound PecS with Kd = 0.17±0.01 mM and no cooperativity (nH = 1.0±0.1), and xanthine bound PecS with Kd = 0.10±0.03 mM and nH = 1.3±0.3.

We have previously shown that incubation of *S. coelicolor* TamR with urate has little effect on DNA binding, suggesting optimization of the TamR ligand-binding pocket for association with *trans*-aconitate and structurally related molecules (Huang & Grove 2013). For PecS, we compared binding to *pecO*, for which half maximal saturation is ~0.1 nM, with its binding to two different promoters that are regulated by TamR (genes encoding malate dehydrogenase, *SCO4827*, and isocitrate dehydrogenase, *SCO7000*) and found no detectable complex formation at [PecS] up to 10 nM (data not shown). This suggests that DNA binding domains of TamR and PecS and their respective DNA sites have diverged sufficiently to prevent protein association with non-cognate DNA.
Figure 4.6. Model of PecS and ligand interaction. A. Structure of urate and related ligands used in this study. Note that urate is fully deprotonated at the pH at which experiments are performed (pKa ~ 5.5) whereas xanthine has a higher pKa (~7.7). B. Predicted interactions between urate and amino acid residues in the ligand-binding pocket. C. Homology model of PecS. The structure was generated by Phyre using HucR as template (Bordelon et al. 2006; Kelley & Sternberg 2009). The two monomers are shown in different color (blue and green, respectively). Residues predicted to be important for urate coordination and urate-mediated conformational changes are shown in red (stick representation). The recognition helices (α5) are shown in yellow.
Figure 4.7. Effect of different ligands on the binding of PecS to pecO. Free DNA (F) is identified by arrows at the left. A-D. Reactions in lane 1 contained labeled pecO DNA only. Ligand concentrations in lanes 2-7 were 0, 2.5, 5, 10, 15, 20 mM, respectively. Each reaction contained 0.10 nM DNA and 1.25 nM PecS (lanes 2-7). E. Normalized PecS-pecO complex formation as a function of ligand concentration. Error bars represent the standard deviation of three independent repeats.
Figure 4.8. Fluorescence profile of PecS and effect of ligand binding on intrinsic fluorescence. A. Intrinsic fluorescence spectrum of PecS. The excitation wavelength was 290 nm. B. Fluorescence quenching as a function of ligand concentration. The change of PecS intrinsic fluorescence at 330 nm was measured in the presence of urate (filled circles) or xanthine (filled squares). The excitation wavelength was 290 nm. Error bars represent the standard deviation of three independent repeats.

**In vivo effect of urate on PecS-mediated gene regulation**

To measure the effect of urate on the transcriptional regulation by PecS, the transcript level of *pecS* and *pecM* was detected after *S. coelicolor* was exposed to exogenous urate. qRT-PCR results showed that the transcript level of *pecS* and *pecM* increased 15.9±1.7 fold and 4.2±1.2 fold, respectively, after exposure to 10 mM urate (Figure 4.9). This significant increase in *pecS* and *pecM* mRNA levels after exposure to urate indicates that PecS can bind to the *pecS-pecM* intergenic region and regulate the transcription of these genes *in vivo.*
Figure 4.9. *In vivo* gene expression of *pecS* and *pecM*. Relative abundance of *pecS* and *pecM* transcripts level in *S. coelicolor* after exposure to 10 mM urate for 4 hours. Relative mRNA levels of *pecS* and *pecM* genes and reference control gene (*rpoA*) were measured by qRT-PCR. Error bars represent standard deviation of three repeats.

**Discussion**

**Urate is a ligand for *S. coelicolor* PecS**

*R. radiobacter* PecS controls expression of *pecM* in response to exogenous urate (Perera & Grove 2010b). *R. radiobacter* is most likely to encounter urate when a host plant produces ROS as an antibacterial defense, hence urate-mediated regulation of gene activity may contribute to host colonization. Consistent with this proposed mechanism, UrtR proteins are mainly encoded by bacterial species whose life cycle may include association with a living host (Perera & Grove 2011). The soil bacterium *S. coelicolor* is an exception. Not only does *S. coelicolor* encode TamR, an UrtR homolog with altered ligand specificity, but it also encodes a PecS protein with similarity to PecS from plant pathogens. *S. coelicolor* PecS binds urate directly, as evidenced by the ability of urate to quench its intrinsic fluorescence (Figure 4.8B). Urate binding attenuates PecS binding
to the pecS-pecM intergenic DNA (Figure 4.7A, E), and in vivo analyses show that exposure to exogenous urate causes a significantly elevated expression of pecS and pecM genes (Figure 4.9). These features indicate that urate functions as a ligand for S. coelicolor PecS.

An amino acid sequence alignment shows that PecS contains all the characteristics of UrtR proteins. PecS is predicted to contain the N-terminal helix (α1), which includes the Trp implicated in ligand binding. The coordination of urate is predicted to involve Trp22 in helix α1 and Arg73 and Asp66 in α3, with Arg99 in the DNA recognition helix α5 sensing ligand binding via its salt bridge with Asp66 (Figure 4.6B, C). That urate attenuates DNA binding more efficiently compared to xanthine is consistent with the complete deprotonation of urate and the partial deprotonation of xanthine, yielding the requisite negative charge for a salt bridge with Arg73 and repulsion of Asp66 (Figure 4.6) (Perera et al. 2009). Hypoxanthine and allantoin lack these negative charges and do not attenuate DNA binding, consistent with the proposed model.

The identified consensus sequence for UrtR proteins allowed the prediction of several binding sites in the pecS-pecM intergenic region (Figure 4.1A). EMSA confirmed the existence of multiple PecS binding sites as increasing PecS concentration lead to the formation of additional complexes with reduced electrophoretic mobility. While the observed complexes may correspond to additional PecS proteins accumulating on the DNA, perhaps nucleated by PecS binding to an optimal site, it is also possible that the electrophoretic mobility of DNA with PecS bound to different combinations of sites may differ if PecS binding induces DNA distortion. Multiple binding sites in this intergenic region may facilitate differential regulation of the divergent genes.

**Conservation of PecS in pathogenic Streptomyces species**

The presence of multiple UrtR homologs is consistent with the frequent gene duplication and horizontal gene transfer events that have been inferred to occur in Streptomyces spp. (Zhou et
Neither TamR, nor PecS homologs are ubiquitously present in *Streptomyces* species. It is notable, however, that the about 20 *Streptomyces* species presently seen to encode a PecS homolog are found only among the more than 40 that encode TamR, perhaps reflecting a gene duplication event followed by divergence to develop a novel function and ligand specificity.

Only few pathogenic *Streptomyces* species have been characterized. Notably, the pathogenic species for which sequence information is available are included among those that encode PecS, including the plant pathogens *S. ipomoeae, S. scabiei, S. acidiscabies* and *S. turgidiscabies* and the human pathogen *S. somaliensis* (Loria et al. 2006; Lerat et al. 2009; Bignell et al. 2010; Kirby et al. 2012). The pathogenicity of *Streptomyces* spp. results from a diverse set of reasons. For species that cause scab symptoms, for example, the cellulose synthesis inhibitor thaxtomin is a pivotal virulence factor (Loria et al. 2008; Guan et al. 2012), and other conserved virulence determinants include Nec1, a secreted protein, which can cause necrosis of the host cell (Bukhalid et al. 1998). The conservation of PecS in pathogenic *Streptomyces* species would be consistent with its role in promoting survival under the oxidative stress conditions elicited by the host upon bacterial infection.

*D. dadantii* PecS and *S. coelicolor* PecS share ~40% sequence identity, which is relatively high considering the phylogenetic distance between these species. In addition, the regulated gene *SCO2646* encodes a putative integral membrane protein with about 44% identity to *D. dadantii* PecM. Gene locus *SCO2645* also encodes a putative integral membrane protein with two copies of Pfam entry PF00892 (DUF6), however, this protein shares only 27% identity to *D. dadantii* PecM. *D. dadantii* PecM functions to excrete indigoidine, an antioxidant involved in ROS defense (Praillet et al. 1997; Rouanet & Nasser 2001). Considering the significant conservation of both PecS and PecM proteins, *S. coelicolor* PecS may likewise be involved in ROS defense by sensing
urate produced when xanthine oxidase generates ROS. Consistent with this inference, *Streptomyces* spp. have been shown to produce indigoidine (Takahashi et al. 2007). TamR, which is also encoded by pathogenic *Streptomyces* spp., functions to ensure metabolic flux through the citric acid cycle during oxidative stress (Huang & Grove 2013). While TamR and PecS likely derive from a common ancestor, yet have evolved distinct functions and ligand specificities, they apparently both function to control gene activity during oxidative stress. Selective pressures associated with efficient responses to ROS may therefore have formed the basis for evolution and retention of *tamR* and *pecS* genes in select *Streptomyces* species.

**Potential reasons for the existence of a urate responsive transcriptional regulator in S. coelicolor**

Most *Streptomyces* spp. are not pathogens, yet several encode divergent *pecS-pecM* genes. However, the soil is a very competitive environment for microorganisms, not only because of limited nutrition and variable physical conditions (such as temperature and moisture), but also because of fierce competition between organisms that occupy this ecological niche. Competitive strategies employed include the production of antibiotics or other bioactive metabolites, and plants secrete both ROS and other compounds in response to stress or pathogen invasion. In addition, many streptomycetes play important roles in plant-associated microbial communities. For instance, some streptomycetes form symbioses with plants in the rhizosphere and decrease the possibility of those plants being invaded by pathogenic organisms (Sardi *et al.* 1992; Tokala *et al.* 2002). In this case, if pathogens successfully invade the plant cell, both pathogens and streptomycetes may be exposed to bursts of ROS. The mechanical injury of plant roots caused by animals or other factors may also result in the exposure of streptomycetes to ROS (Razem & Bernards 2003; Sagi *et al.* 2004). In addition, plant activity such as root tip growth may result in an ROS burst in the surrounding rhizosphere (Monshausen *et al.* 2007).
Streptomycetes inhabiting the rhizosphere may use elevated exogenous urate concentrations as a signal to prepare for ROS tolerance; an advantage to detecting urate in addition to sensing the ROS directly may be the longer half-life of urate compared to the highly reactive ROS. In addition, if urate produced by xanthine oxidase is detected, the corresponding response can also defend against ROS produced by other systems (mainly NADPH oxidase).

References


CHAPTER 5
SUMMARY AND FUTURE DIRECTIONS

For bacteria and archaea, regulation of gene expression in response to different environmental conditions is very important in many respects (e.g., survival, growth, and reproduction). As a genus that is very important for the production of antibiotics and other medicinally active biomolecules, *Streptomyces* has been widely studied for a long time. Since the physical and nutritional conditions of soil can be dramatically different, the ability of the soil-dwelling bacterium *Streptomyces coelicolor* to live in this environment requires the capability to make adjustments according to different environmental condition. The abundance of transcriptional regulators, which can modify gene expression by responding to different environmental conditions, is beneficial to survival of *S. coelicolor*. As a family of transcriptional regulators that is widely distributed in different bacteria and archaea, MarR (Multiple antibiotic resistance regulator) family regulators usually play important roles in responding to environmental conditions. In *S. coelicolor*, which is the model organism of *Streptomyces*, there are more than 30 MarR family homologs. In this study, the research is focused on collecting information on two *S. coelicolor* MarR homologs, which belong to the UrtR subfamily.

In this study, TamR (*trans*-aconitate methyltransferase regulator), a MarR family homolog in *S. coelicolor*, was found to regulate several target genes, which are all closely related to the citric acid cycle. The target genes of TamR include the TamR-encoding gene *tamR* (*SCO3133*), the *trans*-aconitate methyltransferase-encoding gene *tam* (*SCO3132*), the aconitase-encoding gene *sacA* (*SCO5999*), the malate synthase-encoding gene *aceB1* (*SCO6243*), the malate dehydrogenase-encoding gene *mdh* (*SCO4827*), and the isocitrate dehydrogenase-encoding gene *idh* (*SCO7000*). The binding of TamR to these gene promoters can be attenuated by *trans*-aconitate, citrate, *cis*-aconitate and isocitrate, which are all closely related to the citrate
isomerization reaction in the citric acid cycle. The regulation of these citric acid cycle-related enzymes suggests that TamR plays an important role in protecting this central metabolism process from dysfunction under some stress conditions and maintaining the flux of this cycle. Because accumulation of citrate and trans-aconitate can occur when aconitase is inactivated by oxidative stress, TamR may be particularly important in maintaining citric acid cycle flux when these intermediates accumulate because of oxidative stress. The conservation of TamR and predicted binding sites in the promoter regions of target genes in different Streptomyces species suggest the role of TamR is relatively conserved in Streptomyces species, which also speaks to the importance of its function. Although featuring residues not otherwise conserved among UrtR transcriptional regulators, TamR homologs contain several other highly conserved residues in the ligand binding pocket. These residues may be important for binding of ligands associated with the citrate isomerization reaction. Thus, TamR homologs may be evolutionarily optimized to respond to a ligand different from urate.

Another MarR family homolog is PecS, which is encoded by SCO2647 and can respond to urate as a ligand. PecS also contains the characteristics of UrtR subfamily members that usually can bind urate as a ligand. Urate can dramatically attenuate the binding ability of PecS to its target DNA, even though the binding affinity of PecS to its target DNA is very high. In addition, the intrinsic fluorescence of PecS can be efficiently quenched by urate, which indicates a direct interaction between urate and PecS. Expression of pecS and pecM genes in vivo is also significantly elevated after exposure of S. coelicolor cultures to urate. This information suggests that PecS can respond to urate as a ligand for transcriptional regulation. Urate sensing may help this bacterium to survive under oxidative stress caused by reactive oxygen species. This inference is based on the
significant homology of *S. coelicolor* PecM to *D. dadantii* PecM, which exports the antioxidant indigoidine, and the production of urate when xanthine oxidase generates reactive oxygen species.

In the future, several mutants of PecS could be created to investigate those amino acid residues, which are predicted to be important for urate binding. For PecS, residues previously predicted to participate in responses to urate would be selected. TamR mutants could also be created to investigate the amino acid residues, which are predicted to coordinate the ligand. In this case, residues selectively conserved in TamR proteins and not other UrtR homologs should also be targeted to determine if their conservation indeed correlates with a role in ligand binding. A tamR gene knock-out mutant strain and a pecS gene knock-out mutant strain may also provide valuable information about the role of these two MarR family members. For instance, pecS knockout strain may reveal additional genes regulated by PecS. A mutant strain may also be helpful to investigate the substrate of the PecM efflux pump, which will provide very important information about PecS. The knockout of tamR can confirm the TamR regulon identified based on conservation of TamR binding sites in gene promoters. We will also try to get crystal structure of these proteins to investigate the structure of TamR and PecS. The crystal structure of TamR and PecS can provide important information. For instance, the TamR structure with ligand can reveal the exact ligand-binding scheme.
APPENDIX
PERMISSION

Title: The transcriptional regulator TamR from Streptomyces coelicolor controls a key step in central metabolism during oxidative stress
Author: Hao Huang, Anne Grove
Publication: Molecular Microbiology
Publisher: John Wiley and Sons
Date: Feb 3, 2013
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VITA

Hao Huang is the first son of Lingyan Huang and Dafang Chen. He was born in 1980. His hometown is Shangrao in Jiangxi Province of China. He received his bachelor degree in aquaculture from Jimei University in 2003. He started his graduate study in Xiamen University in 2003 and received his master degree in the major of Biochemistry and Molecular Biology in 2006. He started his graduate program in the Department of Biological Sciences at Louisiana State University in August of 2007. He expects to graduate with Doctor of Philosophy in biochemistry in December of 2013.