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The *Treponema denticola* AtcR LytTR domain-containing response regulator interacts with three architecturally distinct promoter elements: implications for understanding the molecular signaling mechanisms that drive the progression of periodontal disease

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Abstract

Treponema denticola is an oral spirochete and periopathogen that transitions from low abundance in healthy subgingival crevices to high abundance in periodontal pockets. The *T. denticola* response regulator AtcR harbors the relatively rare, LytTR DNA binding domain. LytTR domain containing response regulators control critical transcriptional responses required for environmental adaptation. Using a multi-step bioinformatics approach, 26 strong lytTR recognition motifs were identified in the genome of *T. denticola* strain 35405. Electrophoretic mobility shift assays (EMSA) demonstrated that AtcR binds to these recognition motifs. High specificity-high affinity complexes formed with phosphorylated AtcR. The LytTR recognition sequences were found to exist in three distinct promoter architectures designated as LytTR1, LytTR2 and LytTR3 promoters. LytTR1 and LytTR2 promoters harbor σ^{54} binding sites. The functional diversity of the proteins encoded by the putative AtcR regulon suggests that AtcR sits at the top of a regulatory cascade that plays a central role in facilitating *T. denticola*'s ability to adapt to changing environmental conditions and thrive in periodontal pockets.

INTRODUCTION

Treponema denticola, *Porphyromonas gingivalis* and *Tannerella forsythia* form the “red microbial complex”, a consortium of bacteria whose abundance correlates with periodontal disease severity in humans (Socransky *et al.*, 1998). As periodontal disease progresses, the environmental and immunological properties of the subgingival crevice change dramatically. It has been hypothesized that *T. denticola* influences disease progression and

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severity by disrupting the control of complement activation mechanisms (McDowell *et al.*, 2011; Miller *et al.*, 2012). *T. denticola* transitions from low to high abundance as periodontal disease progresses (Ellen and Galimanas, 2005). The ability of *T. denticola* to thrive is a reflection of its ability to adapt to changing environmental conditions. While most bacteria, including spirochetes, employ two component regulatory (TCR) systems and cyclic nucleotides to regulate adaptive responses (Bian *et al.*, 2013; Galperin, 2004; Hengge, 2009; Kostick *et al.*, 2011; Samuels, 2011), little is known about the TCR systems of *T. denticola* and other oral spirochetes. To date, only the AtcRS and Hpk2-Rrp2 TCR systems have been studied to any degree (Frederick *et al.*, 2008; Frederick *et al.*, 2011; Sarkar *et al.*, 2010). The regulons controlled by these two systems remain undefined. A recent review, based on genomic sequence information, detailed the *T. denticola* genetic regulatory systems and their potential signalling mechanisms (Frederick *et al.*, 2011)

This study is focused on the *T. denticola* response regulator AtcR (Frederick *et al.*, 2008). AtcR is unique among spirochetal response regulators in that it possesses a putative LytTR DNA binding domain (Galperin, 2008; Nikolskaya and Galperin, 2002). LytTR domains are found in only a small subset of response regulators with 1 or 2 per bacterial genome (Galperin, 2006). Several spirochetal genome sequences have now been determined (Dewhirst *et al.*, 2010). *T. denticola* is the only spirochete found to encode a LytTR domain. LytTR domain containing response regulators contribute to the regulation of biofilm formation (Lizewski *et al.*, 2004), toxin production (Ba-Thein *et al.*, 1996; Lyrstis *et al.*, 1994), type IV pili synthesis (Belete *et al.*, 2008), antimicrobial peptide production (Risoen *et al.*, 1998), flagellar assembly, flagellar function (Martin *et al.*, 2013), overall fitness and virulence gene expression (Abdelnour *et al.*, 1993; Martin *et al.*, 2013).

The LytTR domain-DNA interaction is unique and differs from the DNA -protein interactions described for other DNA binding proteins (Galperin, 2008; Gao *et al.*, 2007; Sidote *et al.*, 2008). The *Staphylococcus aureus* AgrA response regulator serves as a model for LytTR domain interactions with DNA (Garcia *et al.*, 2004; Sidote *et al.*, 2008). The atomic structure of the AgrA LytTR domain in complex with its DNA recognition sequence has been determined (Koenig *et al.*, 2004; Rode *et al.*, 2007; Sidote *et al.*, 2008; Traber and Novick, 2006). The recognition sequence consists of 9 bp imperfect direct repeats that harbor a conserved GTT motif. AgrA binds DNA as a dimer with six positively charged amino acid loops of each monomer inserting into two successive major grooves within the DNA recognition sequence (Sidote *et al.*, 2008). This interaction also allows the side chains of the highly conserved residues to insert within the intervening minor groove. The interaction is further stabilized by salt bridges that form between AgrA residues and the DNA phosphate backbone. AgrA binds specifically along a single face of the DNA double helix triggering DNA bending which regulates transcription of genes harboring the LytTR recognition sequence.

In this report, using multi-step bioinformatic and experimental approaches, a *T. denticola* AtcR recognition motif was identified and refined allowing for the partial identification of the AtcR regulon. Phosphorylated recombinant AtcR was demonstrated to interact with LytTR recognition sequences found upstream of 26 *T. denticola* genes. The LytTR containing promoters were found to exist in three distinct promoter architectures suggesting

the potential for differential or potentially multi-layered transcriptional regulation. This study is a significant step forward in defining the genetic regulatory mechanisms of *T. denticola* that may contribute to its unique ability to thrive and dominate in periodontal pockets. In addition, it represents the first characterization of a LytTR domain containing response regulator in a spirochete.

MATERIALS AND METHODS

Bacterial strains and culture conditions

T. denticola strain 35405 was cultivated in new oral spirochete (NOS) medium supplemented with thiamine pyrophosphate (4 mg L⁻¹) under anaerobic conditions (5% H₂, 20% CO₂, 75% N₂, 37 °C). Growth was monitored by dark-field microscopy using a microscope contained within the anaerobic chamber. Note that the ORF designations used in this study are those assigned to the 35405 genome sequence as described by (Seshadri *et al.*, 2004)(NCBI database; AE017226.1)

Structural modeling of the LytTR domain of AtcR

A structural model for the LytTR domain of AtcR was generated using the previously determined atomic structure of the *S. aureus* AgrA LytTR domain bound to its LytTR recognition sequence (a 15bp duplexed oligonucleotide) (Sidote *et al.*, 2008). AtcR residues 126 through 224, which contain the putative LytTR domain, were threaded onto the AgrA structure using Deep View (Guex and Peitsch, 1997) and side chains optimized to decrease unfavorable interactions. Statistical analysis was performed by querying the Dali database (Holm and Rosenstrom) with the AtcR structural prediction (http://ekhidna.biocenter.helsinki.fi/dali_server). The percentage of residues with favorable bond angles was determined by Ramachandran plotting using Deep View (Guex and Peitsch, 1997).

Generation of recombinant proteins

Recombinant AtcR and OspE (a *B. burgdorferi* outer surface protein that does not bind to DNA) were generated as N-terminal his-tag fusions (1.7 kDa) as previously described (Frederick *et al.*, 2008). The his-tags were incorporated to allow for subsequent purification. Briefly, *E. coli* BL21(DE3) cells (New England Biolabs) harboring the full length *atcR* or *ospE* (*B. burgdorferi* strain B31; ORF BBL39; lacking the signal peptide) coding sequence in the pET46 Ek/LIC vector (Novagen) were induced with IPTG. Cells were lysed using lysozyme and sonication (standard methods). The r-proteins were purified by immobilized metal ion affinity chromatography using a HisTrap Ni-NTA column on an AKTA purifier system (GE Biosciences) as previously detailed (Frederick *et al.*, 2008). A HisTrap desalting column (GE Biosciences) was used to remove imidazole and protein concentration was determined (BCA assay, Thermo Scientific, Pierce). Phosphorylated AtcR was generated by incubation with acetyl phosphate (25mM, lithium salt; Sigma) in electrophoretic mobility shift assay buffer (EMSA) buffer (10 mM Tris pH7.5, 50 mM KCl, 1 mM DTT, 1 mM MgCl₂, 1 µg ml⁻¹ poly dI-C, 5% glycerol; 37 °C, 30 min).

EMSA analyses with AtcR and DNA oligonucleotide duplexes

Biotinylated-oligonucleotides (Table 1) (Integrated DNA Technologies) were annealed by heating to 80 °C followed by cooling to 25 °C (1 °C min⁻¹). The oligonucleotide duplexes (OD) (10 fmol) were incubated with AtcR (from 0 to 9 μM) in EMSA buffer (25 °C, 30 min). Unlabeled OD served as the specific inhibitor (SI) and an unlabelled 60 bp DNA OD consisting of sequence from the coding region of *atcR* was used as a nonspecific inhibitor (NSI). SI and NSI were added at 400 fold molar excess (4 pmol). The protein-DNA complexes (C-OD) were analyzed in 5% acrylamide-0.5X Tris-Borate-EDTA (TBE) Criterion gels (~ 75 min; 100V; Bio-Rad), transferred to Hybond N+ membranes (GE Healthcare) using a transfer chamber (30 min; 360mA; Bio-Rad) and the biotinylated OD detected using HRP-conjugated streptavidin and the Pierce Chemiluminescent Nucleotide Detection kit (Thermo Scientific, Pierce).

EMSAs were also conducted using larger DNA fragments (~500 bp) harboring the *lytTR* recognition motifs. The target sequences of interest were PCR amplified from *T. denticola* 35405 using 5' biotinylated primers (Table 1) and the amplicons purified using Qiaquick PCR Purification kits (Qiagen). Non-biotinylated amplicons of the target sequences served as SI and a PCR product consisting of the *T. denticola*-factor H binding protein (*fhbB*) and its promoter region served as NSI (McDowell *et al.*, 2007). The amplicons were incubated with AtcR and EMSAs conducted as detailed above.

DNase Protection Assays

The upstream region of genes of interest were PCR amplified. DNase protection assays were conducted by incubating AtcR or the negative control protein (OspE) (concentration range of 0.2 μM to 6.2 μM) with 150 ng (492 fmol) of the 500 bp purified PCR products and RQ1 nuclease (Promega, 0.001 U μL⁻¹; EMSA buffer with 1 mM CaCl₂; 25 °C, 30 min). Reactions were stopped by addition of RQ1 Stop buffer (Promega) and heat treatment (65 °C; 10 min). Samples were analyzed in 2% agarose gels (80 V; 90 min) and the DNA visualized by ethidium bromide staining.

Nucleotide sequence analyses

Initial pattern searching was performed using the fuzznuc algorithm (<http://www.proteomics.leeds.ac.uk/emboss/fuzznuc.html>) with the degenerate nucleotide sequence (TAC)(AC)(AC)GTTN(TAG)(TG). Refinement of the putative AtcR binding site was achieved using the MEME (Bailey and Elkan, 1994), FIMO (Grant *et al.*, 2011), and Virtual Footprinting algorithms (prodoric.de) (Munch *et al.*, 2005). RNA structural predictions were generated with the mfold algorithm (mfold.rna.albany.edu) (Zuker, 2003).

Transcription start site (TSS) identification using 5' RACE

TSS were identified using 5' rapid amplification of cDNA ends (5' RACE; Life Technologies, Invitrogen) as previously described (Zhang and Marconi, 2005). In brief, cDNA was generated with Superscript III reverse transcriptase (Life technologies, Invitrogen) with primers specific for the 5' region of each target gene (Table 1), purified using Snap columns (Life Technologies, Invitrogen) and 3' poly-cytidine (poly C) tailed

using terminal deoxyribonucleoside transferase and dCTP. The cDNA was PCR amplified using the “abridged anchor primer” (Life technologies, Invitrogen), which anneals to the poly C tail, and a gene-specific primer. The amplicons served as template in second PCR reaction using the universal anchor primer (Life technologies, Invitrogen) and a nested gene specific primer. The resulting amplicons were cloned into pCR2.1 TOPO (Life Technologies, Invitrogen) and the inserts sequenced on a fee-for-service basis (MWG Biotech).

RESULTS and DISCUSSION

Structural modeling of AtcR

To determine if the putative *T. denticola* AtcR lytTR domain is structurally similar to the *S. aureus* AgrA lytTR domain, modeling analyses were performed. The AtcR LytTR domain sequence was threaded onto the known AgrA-DNA structure (Sidote *et al.*, 2008) (Figure 1, panel A). Ramachandran plotting indicated that 89.36% of the AtcR residues in the predicted structure have favorable bond angles indicating a good fit for the deduced model. Query of the Dali structural database indicated the average positional variance of each residue to be 1.6 μ. The potential for AtcR to insert amino acid loops of its putative LytTR binding domain into two consecutive major grooves of its DNA recognition sequence in a manner analogous to AgrA is evident in the model presented in Figure 1 (panel B). Sequence alignment identified AtcR residues that are positionally analogous to AgrA residues H169, N201 and R233. Each of these residues has been demonstrated to be important in binding DNA (Figure 1, Panel C) (Sidote *et al.*, 2008). H169 and R233 make direct base specific interactions while AgrA N201 makes a side-chain water mediated contact with DNA. The AtcR amino acid counterparts to AgrA residues H169, N201 and R233 are H157, R191 and R221, respectively. It is noteworthy, that AtcR H157 resides within a conserved 4 amino acid motif (SHNL) that is strictly conserved in *T. denticola* with only a single conservative difference in AgrA (SHRL). A second semi-conservative motif that has not yet been demonstrated to participate in DNA interactions is found 13 aa downstream of the SHRL motif spanning AtcR positions 173–176 (NLL/S/KE). R221 (the analogous residue to AgrA R233) is a stand alone conserved residue that does not sit within a conserved motif. AgrA R233 has been demonstrated to insert within a major groove of its DNA recognition sequence. AgrA N201 makes a side chain-water mediated interaction with DNA. AtcR R191 is the positionally analogous residue of AgrA N201. However, due to differences in properties of N and R side chains it is not clear if R191 plays an analogous role in the LytTR domain-DNA interaction.

Identification of a putative AtcR DNA recognition motif

A search of the *T. denticola* 35405 genome sequence (Seshadri *et al.*, 2004) with a consensus *S. aureus* LytTR recognition sequence did not detect hits with significant identity. In light of the phylogenetic divergence between spirochetes and Gram-positive bacteria this is not entirely surprising. To identify potential LytTR domain recognition sequences, the *T. denticola* 35405 genome sequence was queried with a degenerate LytTR consensus sequence, (TAC)(AC)(AC)GTTN(TAG)(TG) using the fuzznuc algorithm. This degenerate sequence was derived from analyses of other known LytTR recognition sequences

(Nikolskaya and Galperin, 2002; Risoen *et al.*, 1998; Sidote *et al.*, 2008). One hundred and fifty eight hits were identified in which the recognition sequence existed as a direct repeat with a intervening spacer of 4 to 20 nucleotides. Eighty-one of the hits mapped upstream of ORFs in positions consistent with potential transcriptional regulatory elements.

Oligonucleotides (60 bp) duplexes (OD) corresponding to 6 of the 81 hits were synthesized and tested for AtcR binding using EMSA (Figure 2). AtcR bound to the ODs forming a slowly migrating complex designated complex OD-1 (C-OD1). Complex formation was inhibited by the addition of SI but not NSI, demonstrating that the interaction of AtcR with DNA is sequence specific. The influence of AtcR concentration and its phosphorylation state on complex formation was then assessed using ODs derived from sequences upstream of ORFs *tde1514*, *tde1221*, *tde0025*, *tde1971*, *tde1998* and *tde2000*. The potential function of the proteins encoded by sequences analyzed in this study are listed in Table 2. A constant amount of each OD was incubated with increasing amounts (0 to 9 μ M) of non-phosphorylated or phosphorylated AtcR and EMSAs were performed (Figure 3). When ODs were incubated with 9 μ M of AtcR, both phosphorylated and non-phosphorylated AtcR formed large (i.e., slowly migrating) DNA binding complexes (C-OD1). Other response regulators have also been demonstrated to form slowly migrating, phosphorylation-independent complexes at high protein concentrations (Leech *et al.*, 2008; Sun *et al.*, 2010). The migration position of C-OD1 suggests high stoichiometry AtcR binding along the DNA backbone. An additional phosphorylation independent complex (C-OD4) was observed with duplexes derived from ORFs *tde1514* and *tde0025*. Phosphorylated AtcR formed additional complexes (C-OD2 and C-OD3) at low protein concentrations with duplexes derived from ORFs *tde1514* and *tde1971*. The two distinct phosphorylation dependent complexes observed most likely reflect differences in AtcR binding stoichiometry to the duplexes.

Refinement of consensus AtcR recognition sequence

To allow for the identification of the broader AtcR regulon, the DNA sequences upstream of ORFs *tde1514* and *tde1971*, which formed AtcR phosphorylation dependent complexes, were analyzed using the MEME algorithm to generate a “seed” nucleotide motif. MEME is a pattern searching tool that can identify conserved motifs in DNA sequences and generate an alignment scoring matrix. The resulting position specific scoring matrix of the seed motif was then analyzed with the FIMO algorithm to search for degenerate but related motifs in the *T. denticola* genome. Hits were then assessed for orientation and distance from potential start codons. Sites found on coding strands within 500 bp of a start codon were selected for further analysis. The 500 bp upstream regions of candidate genes were retrieved and reassessed by MEME to identify shared motifs 15 to 80 nucleotides in length. Candidate AtcR recognition sequences that did not have the conserved LytTR recognition motif core GTT sequence were excluded. The resulting data set was then reassessed by MEME and FIMO. After three rounds of analysis, the ACAGTT(CG)GGN₁₅(TA)CAGTTTA(GT) motif (designated as motif 1) was identified. This motif possesses an imperfect 9 bp repeat linked by a 15 base spacer. The organization of motif 1 is consistent with the organization of DNA recognition sequences of other LytTR domain-containing transcription factors (Nikolskaya and Galperin, 2002). It is important to note that the FIMO algorithm assigns weight to residues contained within spacer regions. However, this can artificially skew prediction of

the potential of a sequence to bind a LytTR domain containing protein because sequence variation within the spacer region may have no impact on the interaction. Thus potentially significant LytTR DNA recognition sequences could fall below the significance threshold. To address this, motif 1 was used as the query for the Virtual Footprinting algorithm (prodoric.de), which in contrast to FIMO, does not weight spacer residues. A total of 25 hits were identified that harbor putative AtcR binding motifs that reside upstream of a predicted ORF or operon start codon (Table 2). It is important to note that LytTR recognition sequences identified through fuzznuc analysis that did not exhibit phosphorylated AtcR dependent shifting in the EMSAs were not found in the final MEME analysis. Hence this multi-step approach proved successful in refining the identified AtcR binding motif.

MEME analyses uncovered a second unique sequence motif (motif 2) that was found in association with some putative AtcR regulated genes (Figure 4). Motif 2 harbors a direct repeat of the sequence AAAAAA(CG)TTTTTT. Logo plots for motifs 1 and 2 are presented in Figure 4 (panel A). Motifs 1 and 2 exist in three distinct promoter architectures (Figure 4, panel B). Promoter type 1 (designated as LytTR1) harbors motif 1 only whereas promoter type 2 (LytTR2) harbors both motifs 1 and 2. In LytTR2 associated genes, the positioning of motifs 1 and 2 relative to each other is conserved. The third promoter architecture (LytTR3) is similar in organization to LytTR2 except that motif 2 is interrupted by a conserved insertion sequence of 65 bp. The impact of this insertion on transcriptional regulation is yet unknown. It remains to be determined if promoter architecture influences AtcR mediated transcriptional regulation.

Transcriptional start sites (TSS) for ORFs associated with LytTR1, LytTR2 and LytTR3 promoters (*tde1210*, *tde1971* and *tde1132*, respectively) were identified using the 5' RACE approach (Figure 4, panel B). The *tde1210* TSS mapped within the 3' region of the AtcR binding motif. Ten nucleotides upstream from the TSS is a consensus σ^{54} recognition site sequence. σ^{54} regulated genes can also be regulated by other response regulators (Dasgupta *et al.*, 2003; Kiupakis and Reitzer, 2002; Penaloza-Vazquez *et al.*, 2004; Samuels, 2011). In some cases, response regulators binds to the σ^{54} binding site repressing σ^{54} -dependent signaling (Porrua *et al.*, 2009). Conversely, others bind upstream and influence interactions with enhancer binding proteins resulting in either activation or repression of σ^{54} mediated signaling (Feng *et al.*, 1995). The *tde1971* TSS mapped ~80 nucleotides downstream from AtcR binding motif 2. A σ^{54} binding site is located between the AtcR binding motif 1 and the TSS located at nucleotide -15. The TSS for *tde1132* is similar to *tde1971* with respect to its location relative to the AtcR binding motif. However, LytTR3 promoters lack a putative σ^{54} binding site.

It is important to note that motif 2, which is found in LytTR2 and LytTR3 promoters, shows no homology with any known RNA or DNA motif. Secondary structure analyses of the 5' UTR of *tde1971* (LytTR2) and *tde1132* (LytTR3) predict a stem loop that includes the Shine-Dalgarno sequence (Figure 5). A double stem loop structure is predicted in the 5' UTR of *tde1971*, whereas upstream of *tde1132*, a more complex structure is predicted. In this case, the conserved insertion sequence present in LytTR3 promoters is predicted to form a large loop containing two internal stem loops at the end of a 27 bp stem structure. Similar RNA structures have been implicated in post-transcriptional regulation (Arthur *et al.*, 2011;

Irie *et al.*, ; Ringner and Krogh, 2005; Schuck *et al.*, 2009). The secondary structure elements in the 5' UTRs of LytTR2 and LytTR3 derived mRNAs may possibly serve as RNA regulatory motifs.

Promoter architecture and AtcR binding

To determine if promoter architecture influences AtcR binding, EMSAs were conducted using 500 bp DNA segments that reside upstream of ORFs *tde1210* (LytTR1), *tde2220* (LytTR1), *tde2770* (LytTR2), *tde2496* (LytTR2), *tde1971* (LytTR2), and *tde1132* (LytTR3). AtcR bound to all promoters and formed a single complex designated as “B” (bound) (Figure 6A). The binding was inhibited by 400 fold excess of identical-unlabeled DNA but not by unrelated sequences indicating binding specificity. Hence, while the *in vitro* analyses conducted here did not reveal an obvious influence of promoter architecture on AtcR binding, *in vivo* there may be other trans-acting factors that could differentially interact with or influence AtcR binding to the three different promoter types.

The large complexes observed in Figure 6 (panel A) are consistent with high stoichiometry AtcR binding. If these larger complexes result from AtcR binding to the DNA backbone, then AtcR should protect the DNA from cleavage by DNase. This was tested by incubating the 500 bp *tde2496* upstream region with increasing amounts of phosphorylated AtcR or OspE (a negative control protein from *B. burgdorferi* with no known DNA binding activity) and RQ1 DNase. High concentrations of AtcR protected DNA from DNase digestion while OspE did not (Figure 6B). The protection of DNA by AtcR is consistent with the formation of a high stoichiometry-low affinity interaction of AtcR with the DNA backbone. This interaction may function as a scanning mechanism that ultimately allows for more specific interactions. Interestingly, this low affinity interaction is inhibited by specific competitor but not non-specific inhibitor. Initial binding of AtcR to the LytTR domain recognition sequence may initiate further interaction of AtcR monomers and thus stabilize DNA binding as has been reported for other DNA binding proteins (Burns *et al.*, 2010; Lang *et al.*, 2007).

Efforts to generate an AtcR null mutant in *T. denticola* as part of this study were unsuccessful. Hence a limitation of this study is that the wider set of AtcR regulated genes in *T. denticola* could not be investigated *in vivo*. However, data from other organisms provide strong support for the putative AtcR regulon identified here (Garmyn *et al.*, 2012; Martin *et al.*, 2013). Many of the *T. denticola* genes identified in this study that harbor LytTR recognition sequences have been demonstrated in other bacteria to be regulated by LytTR domain containing proteins. Examples include genes regulated by the AgrA proteins of *Clostridium difficile* and *Listeria monocytogenes* (Garmyn *et al.*, 2012; Martin *et al.*, 2013). While the number of genes identified in this study as potential targets of LytTR mediated regulation is less than that reported for *C. difficile* and *L. monocytogenes* (Garmyn *et al.*, 2012; Martin *et al.*, 2013), it is important to note that we employed a conservative bioinformatics approach to identify and refine the LytTR recognition sequence. It is likely that additional genes not identified here are also controlled by AtcR. In spite of this inherent limitation, there are advantages to the approach employed in this study in that only genes that are directly regulated by AtcR would not be detected.

Biological implications of AtcR mediated transcriptional regulation

It is noteworthy that the *T. denticola* Hpk2-Rrp2 operon, which encodes a TCR system, harbors a LytTR2 promoter. Similar to AgrA of *C. difficile* (Martin *et al.*, 2013), both AtcSR and Hpk2-Rrp2 are upregulated during late log phase (Frederick *et al.*, 2008; Sarkar *et al.*, 2010). Interestingly, *hpk2* and *rrp2* are transcribed as a bicistronic mRNA and as a larger operon that includes genes encoding proteins that function in peptidoglycan synthesis, DNA replication and cell division (Sarkar *et al.*, 2010). Hence, log phase induced production of AtcR and its potential upregulation of the larger *rrp2*, in and of itself, could play a key role in regulating genes required for the rapid expansion of the *T. denticola* population in periodontal pockets. The Rrp2 regulon of *T. denticola* has not been identified but it is noteworthy that Rrp2 harbors a PAS domain. PAS domains function in sensing various environmental variables including oxygen levels (Gilles-Gonzalez and Gonzalez, 2004; Moglich *et al.*, 2009). As anaerobic periodontal pockets expand as disease progresses, the increased expression of the *hpk2-rrp2* operon mediated by AtcR, could play a critical role in allowing the *T. denticola* population to thrive.

This study suggests that AtcR may integrate into a complex regulatory network that regulates genes that encode proteins of diverse functional categories. The most significant overlap between the set of potential LytTR domain regulated genes of *T. denticola* and those of *C. difficile* (Martin *et al.*, 2013) is in the flagellar regulon. This regulon includes gene involved in flagellar assembly, structure and function (Table 2). In *C. difficile*, the flagellar regulon is downregulated in a null *agrA* mutant indicating that AgrA serves as a transcriptional activator for flagella synthesis and activity. In this study we found that orthologs of 14 of the flagellar regulon genes identified in *C. difficile* (indicated by asterisks in Table 2) are flanked by promoters with LytTR recognition sequences and thus potentially regulated by AtcR. In addition, to the flagellar regulon, other genes that overlap with those regulated by the LytTR domain containing AgrA protein of *C. difficile* includes genes that encode proteins involved in various aspects of metabolism, transport and gene regulation (i.e., TCR systems). The significant overlap between the *T. denticola* genes that harbor upstream LytTR recognition sequences with the *C. difficile* AgrA regulated genes serves to indirectly validate the putative AtcR regulon identified here. Future studies will seek to test the hypothesis that the AtcR regulon may be controlled by a highly complex signaling network that integrates multiple signaling pathways including post-transcriptional mechanisms.

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Figure 1.

Modeling of the AtcR LytTR Domain-DNA interaction. In panel A, a predictive model of the AtcR LytTR domain-DNA interaction is shown. Positively charged residues of AtcR are indicated in blue (space fill model). The conserved tri-nucleotide GTT motif of the LytTR DNA recognition sequence is indicated in red. The position of AtcR R191, which is positionally analogous to AgrA N201, is indicated with an arrow. Panel B presents a ribbon model of AtcR bound to DNA (space fill model). The alpha helices (red), beta sheets (yellow) and loop domains (blue) of AtcR are indicated. The putative DNA interacting loops of AtcR are labelled L1 through L6 progressing from the N to C terminus of the protein. The side chains of AtcR residues H157 and R221, which are analogous to AgrA residues H169

and R233 and predicted to interact directly DNA, are shown. The positive and negative charge regions of DNA are indicated by red and blue respectively. The DNA structure is presented as a space fill model. An alignment of the LytTR domain of several *T. denticola* AtcR with the LytTR domain of AgrA is shown in panel C. Residue numbering for AgrA and AtcR are shown along the top and bottom of the alignment, respectively. Highly conserved residues or motifs are indicated by red boxing. The position of residues demonstrated to be of central importance in the AgrA-DNA interaction that are discussed in detail in the text are indicated by asterisks.

Figure 2.

EMSA analysis of AtcR binding to 60 base pair DNA duplexes. Biotinylated DNA duplexes that have potential LytTR recognition sequences (identified as described in the text) were incubated with or without purified AtcR for EMSA analyses. The *T. denticola* strain 35405 ORFs that reside downstream of the recognition sequences are indicated above each panel. Specific and non-specific inhibitors, SI and NSI, were added as indicated. The migration position of unbound DNA (UB) and AtcR complexed with DNA (C-OD1) are indicated. All methods were as detailed in the text.

Figure 3.

EMSA analysis using phosphorylated or non-phosphorylated AtcR. Biotinylated DNA duplexes derived from several genes (indicated at the top) were incubated with increasing concentrations (μM) of either non-phosphorylated AtcR (AtcR) or phosphorylated AtcR (AtcR-P) as indicated above each lane. The migration positions of the unbound DNA duplexes (UB) and AtcR-DNA complexes are indicated (C-OD1 through C-OD4).

Figure 4.

Schematic representation of AtcR target sequences and LytTR promoter types. Panel A presents a logo plot nucleotide conservation maps of motifs 1 and 2. The relative “height” of each residue(s) indicates degree of conservation. In panel B, the three distinct promoter architectures are depicted. The location of transcriptional start sites (TSS), identified as detailed in the text, are indicated by arrows (labeled as +1). σ^{54} binding sites are also indicated. All numbering is relative to the TSS. AtcR binding motif 1 is indicated by diagonal hashed boxes, motif 2 by solid black boxes and the conserved insertion sequence that resides within motif 2 denoted by a vertical dashed line. ORFs are indicated by grey arrows. Promoter designations (LytTR1, etc) are indicated above each schematic.

Figure 5.

Structural analysis of motif 2. Predicted secondary structures for the 5' UTR of *tde1971* (LytTR2) and *tde1132* (LytTR3) are presented (left and right respectively). ΔG values are given as kcal/mol.

Figure 6.

EMSA analyses using 500 bp DNA fragments and DNase protection analyses. Panel A presents EMSAs in which 500 bp DNA fragments that contain the three different *LytTR* promoter architectures, derived from three different ORFs (indicated in the figure), were incubated with or without phosphorylated AtcR in the presence of specific or non-specific inhibitor (SI and NSI, respectively). The migration positions of unbound DNA (UB) and AtcR-DNA complexes (B for bound) are indicated. Panel B presents the results of DNase protection assays. A 500 bp sequence corresponding to the upstream region of ORF *tde2496* was incubated with phosphorylated AtcR or *B. burgdorferi* OspE and RQ1 Dnase. The concentration of recombinant OspE or AtcR added in lanes 1 through 4 was 0.17 μ M, 0.56 μ M, 1.86 μ M and 6.2 μ M, respectively. The reactions were analysed by agarose gel electrophoresis and the DNA visualized by ethidium bromide staining.

Table 1

Oligonucleotides (5'-3') used in this study

a. Oligonucleotide sequences used to generate 60 bp duplexes for EMSA analyses*	
<i>ide1971</i>	GTTGGATAAACCTTGCAAAAAGTTTTATAGGGGGTATGATAAACAGTTCGGCAGAA
<i>ide2000</i>	TGCCGGGCTTGCAAGTTATGCCCTGCAAAACCTTGCAACACAGTTCGGCGTTCAAGCCG
<i>ide1514</i>	CATCGTTTAAATAGTAAAAAGTTTTATGGGAGAAGTGCTAAACAGTTCGGTGCAAATG
<i>ide0025</i>	CCACTTGGGTACGGCGCCGAACCTTGCCCAAGTATACCAAAAAATACAAACAAAGACAAGG
<i>ide1221</i>	TGCATATAATAATTCCGGCCTAACGGATTTTATAAACTTAAATCAGGTTTTAGTATTGC
<i>ide1998</i>	CCCTTGACAGAAACCTCTGACTGTGATATAATTATAAAGTTATAAACGGAGGACTTTTT
b. Primers used to amplify 500 bp promoter regions of select <i>T. denticola</i> ORFs	
<i>ide2770</i> F	TTTCTGCCTTGTGATTTTACCGT
<i>ide2770</i> R	TGAATCATTGCTTGCATATGAACCTCCATAAAAACTT
<i>ide1514</i> F	GAGCAGTTGGAAGATGATAAACTTCA
<i>ide1514</i> R	GTAATCATCATTCTCCAAAACATAAATTCTCTCTATAA
<i>ide1210</i> F	TTTCTTATGCCATTGTTTTTGG
<i>ide1210</i> R	GTAATCATCTAAAACCTCTATTCCGAT
<i>ide2220</i> F	ATATAGTTTACGCATTATGAACATGTCTC
<i>ide2220</i> R	GTAATCATAATTATTTCTCCAATAAAGATAAACAGTGA
<i>fhhB</i> F	CTCTTGACAGTACGTATAGTG
<i>fhhB</i> R	TTACTTTATCTTTTTGGGTAT
<i>ide1971</i> F	AAAGGCTGCGTTATTGTGG
<i>ide1971</i> R	GTAATCATCTACAACCTCTA
<i>ide1132</i> F	CTTGTAAGAAGGCCTCGAAC
<i>ide1132</i> R	CATAGATTACTCTATAAAAACTTTTTGC
<i>ide2496</i> F	GGCTTCCATGTCAGGTATA
<i>ide2496</i> R	CATGGACTCTTCAATAATTTTAAACG

* For each primer, a complementary primer was also made so that oligonucleotide duplexes could be formed. The above sequences represent the coding strand derived oligonucleotides (the complementary oligonucleotides are not shown).

Table 2The putative AtcR regulon of *T. denticola* strain 35405

Promoter Type	ORF	p-value	Annotation*
LytTR1	TDE0465	1.62×10^{-27}	twitching motility protein, PilT
LytTR1	TDE0870	2.63×10^{-24}	cyclic nucleotide phosphodiesterase
LytTR1	TDE1006	8.59×10^{-25}	flagellar basal body protein, FlgG-1 *
	TDE1007		FlgG-2 *
LytTR1	TDE1210	5.46×10^{-28}	heat shock protein, HslV
	TDE1211		heat shock protein, HslU
LytTR1	TDE1298	1.99×10^{-26}	cyclic nucleotide binding protein
	TDE1297		putative peptidoglycan hydrolase, LysM
LytTR1	TDE1625	3.82×10^{-22}	glycine cleavage system protein PI
	TDE1624		glycine cleavage system protein PII
LytTR1	TDE1655	5.73×10^{-26}	peptidoglycan synthesis, MurG
LytTR1	TDE1849	3.95×10^{-29}	conserved hypothetical protein
	TDE1848		conserved hypothetical protein
LytTR1	TDE2220	6.20×10^{-26}	prolyl-tRNA synthetase
	TDE2221		conserved hypothetical protein
	TDE2222		glycerol-3-phosphate O-acyltransferase *
LytTR1	TDE2131	4.89×10^{-26}	cobalamin biosynthesis CbiM
LytTR2	TDE0064	1.38×10^{-28}	phosphofructokinase
LytTR2	TDE0641	7.05×10^{-29}	peptidoglycan synthesis, MurA
LytTR2	TDE1514	1.95×10^{-22}	ABC transporter permease
	TDE1515		ABC transporter permease
	TDE1516		ABC transporter, ATP-binding protein
	TDE1517		conserved hypothetical protein
	TDE1518		ABC transporter permease
LytTR2	TDE1693	3.95×10^{-29}	conserved hypothetical protein
LytTR2	TDE1903	2.27×10^{-35}	conserved hypothetical protein
LytTR2	TDE1971	2.71×10^{-33}	DNA polymerase III domain protein
	TDE1970		histidine kinase, Hpk2
	TDE1969		response regulator, Rrp2
	TDE1968		cell division protein, FtsJ
LytTR2	TDE2093	1.74×10^{-33}	conserved hypothetical protein
LytTR2	TDE2392	5.30×10^{-33}	conserved hypothetical protein
LytTR2	TDE2484	1.91×10^{-24}	conserved hypothetical protein
	TDE2483		conserved hypothetical protein
LytTR2	TDE2496	2.27×10^{-35}	Methyl accepting chemotaxis protein
LytTR2	TDE2770	2.77×10^{-26}	flagella synthesis regulator, FliK *
	TDE2769		flagellar hook assembly scaffold, FlgD *

Promoter Type	ORF	p-value	Annotation [*]
	TDE2768		flagellar hook protein, FlgE [*]
	TDE2767		flagellar protein, FlbD [*]
	TDE2766		flagellar motor rotation protein A, MotA [*]
	TDE2765		flagellar motor rotation protein B, MotB [*]
	TDE2764		flagellar protein, FliL [*]
	TDE2763		flagellar motor switch protein, FliM [*]
	TDE2762		flagellar motor switch protein, FliY
	TDE2761		flagellar biosynthesis protein, FliO [*]
	TDE2760		flagellar biosynthesis protein, FliP [*]
LytTR3	TDE0522	1.79×10^{-30}	conserved hypothetical protein
LytTR3	TDE0693	2.02×10^{-26}	phosphomethylpyrimidine kinase, ThiD
LytTR3	TDE1132	1.15×10^{-24}	chorismate synthase, AroC
LytTR3	TDE2225	6.01×10^{-25}	acyl-CoA thioesterase

^{*} The asterisks indicate ORFs that encode functionally related proteins that have also been demonstrated to be regulated by a LytTR domain containing protein (AgrA) of *C. difficile* (Martin *et al.*, 2013). ORF designations listed above are derived from the *T. denticola* 35405 genome sequence annotation (Seshadri *et al.*, 2004).