2011

Characterization of Hpk2-Rrp2, two-component regulatory system in Treponema denticola

Sarkar Juni
Virginia Commonwealth University

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CHARACTERIZATION OF THE HPK2-RRP2, TWO-COMPONENT REGULATORY SYSTEM OF *TREPONEMA DENTICOLA*

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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sacrifices for me and always wishing for my happiness. I thank my dad for being the idol of my life helping me become a better human being and for fighting the odds to let me flourish. I dedicate my dissertation to you. I would also like to thank my brother Soumayajit Sarkar for helping me get settled at VCU and his guidance and support throughout my PhD. I would especially like to thank my husband, Kunal Chaniary for always being there, as a friend and as a partner with his unconditional love, patience, and most of all making my stay at VCU a fun trip along his side, by always bringing a smile to my face.

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<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>α</td>
<td>anti</td>
</tr>
<tr>
<td>γ</td>
<td>Gamma</td>
</tr>
<tr>
<td>σ</td>
<td>Sigma</td>
</tr>
<tr>
<td>$^{32}$P</td>
<td>A Beta emitter Isotope of Phosphate</td>
</tr>
<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>TMBZ</td>
<td>3, 3', 5, 5' Tetra Methyl Benzidine</td>
</tr>
<tr>
<td>T.</td>
<td>Treponema</td>
</tr>
<tr>
<td>B.</td>
<td>Borrelia</td>
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<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>NOS</td>
<td>New Oral Spirochete Media</td>
</tr>
<tr>
<td>C</td>
<td>Cysteine</td>
</tr>
<tr>
<td>D</td>
<td>Aspartate</td>
</tr>
<tr>
<td>N</td>
<td>Asparagine</td>
</tr>
<tr>
<td>H</td>
<td>Histidine</td>
</tr>
<tr>
<td>Asp</td>
<td>Aspartate</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>C terminus</td>
<td>carboxyl terminus</td>
</tr>
<tr>
<td>CA</td>
<td>Catalytic Domain</td>
</tr>
<tr>
<td>DHp</td>
<td>Dimerization Histidine Phosphate Domain</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>cp</td>
<td>circular plasmid</td>
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<tr>
<td>dATP</td>
<td>deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>DbpA</td>
<td>decorin binding protein A</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytosine triphosphate</td>
</tr>
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<td>DEPC</td>
<td>diethylpyrocarbonate</td>
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<tr>
<td>DGC</td>
<td>diguanylate cyclase</td>
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<tr>
<td>dGTP</td>
<td>deoxyguanosine triphosphate</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>dTTP</td>
<td>deoxythymidine triphosphate</td>
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<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
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<tr>
<td>PAS</td>
<td>Per-Arnt-Sim</td>
</tr>
<tr>
<td>EAL</td>
<td>glutamate-alanine-leucine</td>
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<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
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</table>
ELISA  enzyme-linked immunosorbent assay
FH    factor H
FhbB  factor H binding protein B
FHBP  factor H binding protein
FHL-1 factor H-like protein 1
G     glycine
GGDEF glycine-glycine-aspartate-glutamate-phenylalanine
hr    hour (s)
HD-GYP histidine-aspartate-glycine-tyrosine-phenylalanine
HIS   heat-inactivated serum
HRP   horse radish peroxidase
IgG   immunoglobulin G
IPTG  isopropyl-β-D-thiogalactopyranoside
Kb    kilobase
kDa   kilodalton
KO    knockout
L.    Leptospira
LIC   ligase-independent cloning
lp    linear plasmid
LPS   lipopolysaccharide
Mb    megabases
min   minute(s)
N terminus amino terminus
ORF   open reading frame
Osp   outer surface protein
PAGE  polyacrylamide gel electrophoresis
PBS   phosphate buffered saline
PBS-T PBS plus Tween
PCI   phenol/chloroform/isoamyl alcohol
PCR   polymerase chain reaction
PDE   phosphodiesterase
PF    protein family
EMSA  Electromobility Gel Shift electrophoresis
PVDF  polyvinylidene difluoride
qPCR  quantitative polymerase chain reaction
r-protein recombinant protein
RFLP  restriction fragment length polyorphism
RNA   ribonucleic acid
rpm   revolutions per minute
Hpk2  Histidine protein kinase 2
<table>
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<tr>
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<tr>
<td>Rrp2</td>
<td>Response regulatory protein 2</td>
</tr>
<tr>
<td>HK</td>
<td>Histidine kinase</td>
</tr>
<tr>
<td>RR</td>
<td>Response regulator</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>S</td>
<td>second(s)</td>
</tr>
<tr>
<td>SSC</td>
<td>salt, sodium citrate</td>
</tr>
<tr>
<td>T</td>
<td>thiamine</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetic acid-EDTA</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-boric acid-EDTA</td>
</tr>
<tr>
<td>TCS</td>
<td>two-component system</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>µl</td>
<td>Micro liter</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>Ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>Mb</td>
<td>Megabases</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobases</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>Az</td>
<td>Antizyme</td>
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<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
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Abstract

CHARACTERIZATION OF THE HPK2-RRP2, TWO-COMPONENT REGULATORY SYSTEM OF TREPONEMA DENTICOLA

By Juni Sarkar

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2011.

Major Director: Dr. Richard Marconi, Ph.D.
Professor, Department of Microbiology and Immunology.

Treponema denticola levels in the gingival crevice become elevated as periodontal disease develops. Oral treponemes may account for as much as 40% of the total bacterial population in the periodontal pocket. The stimuli that trigger enhanced growth of T. denticola and the mechanisms associated with the transmission of these signals remain to be defined. A hypothesis was set that the T. denticola ORFs tde1970 (histidine kinase) and tde1969 (response regulator) constitute a functional two component regulatory system that regulates, at least
in part, responses to the changing environmental conditions associated with the
development of periodontal disease. The results presented demonstrate that
_tde1970_ and _tde1969_ are conserved, universal among _T. denticola_ isolates and
transcribed as part of a 7 gene operon in a growth phase dependent manner.
_Tde1970_ undergoes autophosphorylation and transfers phosphate to _Tde1969_.
Henceforth the proteins encoded by these ORFs are designated as _Hpk2_ and
_Rrp2_ respectively. _Hpk2_ autophosphorylation kinetics was influenced by
environmental conditions and by the presence or absence of a _Per Arnt Sim_ (PAS)
domain. It can be concluded that _Hpk2_ and _Rrp2_ constitute a functional
two-component system that contributes to environmental sensing. This study
also sought to determine the molecular basis of _Hpk2_ function in response to
environmental stimuli. _Hpk2_ was shown to bind hemin via a putative heme-
binding domain within the PAS domain. Hemin binding to _Hpk2_ positively
regulated its autokinase ability under anaerobic conditions, suggesting that _Hpk2_
activation may play a role in the migration of _T. denticola_ away from the aerobic
zone deeper into developing periodontal pockets. In this study we have
generated point mutations of conserved amino acid residues in the sensor PAS
domain of _Hpk2_ and assessed their role in kinase activation under both aerobic
and anaerobic conditions depending on their oligomeric state, hence providing a
strong basis to correlate ligand binding, kinase activity and oligomeric states of
the protein that may provide stability of these complex interactions. Ultimately this study provides a comparative linkage between the responses of PAS domain to sensory inputs controlling access to its kinase domain within which is contained the dimerization domain, which ultimately leads to fine-tuned control of interactions between Hpk2 dimerization and catalytic domain.
Chapter 1: Introduction

1.1 Periodontitis

Periodontal disease is one of the most common chronic inflammatory diseases in humans (62). It occurs when infection of the gums (gingivitis) is untreated or treatment is delayed. As a result, infection and inflammation spreads from the gums (gingivium) to the ligaments and bone that support the teeth. The inflammation is often followed by pulpal tissue necrosis, leading to chronic infection and spread of inflammation to the tooth apex resulting in bone resorption (63). Periodontitis is caused by bacterial growth around the tooth’s surfaces and within the subgingival crevice (in the dental plaque), along with an overly aggressive immune response against these microorganisms. The bacteriology of periodontal disease is complex, due to the presence of nearly 700 bacterial species in the oral cavity, with ~400 found in association with subgingival plaque (129). Bacteria in the dental plaque release enzymes and metabolites that can damage and erode the gum tissues leading to severe infection of the periodontum. In addition to plaque, other factors that can contribute to periodontal disease are hormonal changes, esophageal cancer, certain medications that can affect the oral health, smoking, and poor oral hygiene habits (http://www.mayoclinic.com/health/periodontitis).
Periodontitis is a complex multifactorial disease involving many cell types, cell products and interactions (62). It has been aptly defined as a “microbial-shift disease” during the transition from periodontal health to periodontal disease (102). The subgingival crevice in particular is a suitable niche for biofilm formation by the oral microbial flora due to its warm, moist environment. In periodontitis, co-aggregation (a process in which genetically distinct bacteria become interconnected by specific adhesins) is central to the formation of complex multispecies biofilms (3, 133). Figure 1 depicts the model of an oral biofilm indicating the early colonizers such as *Streptococcus gordonii*, and other oral *Streptococci* that express adhesins which provide a film on which late bacterial colonizers which include the ‘red-microbial complex’ periopathogens *Porphomonas gingivalis, Treponema denticola, and Tannerella forsythia* (150) assemble to form a biofilm. Elevated numbers of the members of the red-microbial complex is highly associated with gingivitis, chronic periodontitis, acute necrotizing ulcerative gingivitis, and endodontic lesions. Although periodontal disease is not a life threatening disease, a strong association has been found between socioeconomic status and prevalence of periodontitis (15). This disease is also associated with long term consequences such as increasing risk of type 2 diabetes (155), obesity (22), metabolic syndrome (28), cardiovascular and pulmonary diseases (56, 157), and adverse pregnancy outcomes (165).
Figure 1. A model of oral biofilm formation. This figure displays the pellicle tooth surface that is recognized by primary colonizing bacteria (*Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus gordonii* and *Streptococcus sanguis*); these bacteria express receptors for salivary agglutinin glycoprotein. Late colonizers like the “Red-microbial complex” bacteria (*Treponema denticola*, *Tannerella forsythia* and *Porphyromonas gingivalis*) use these receptors and adhesins to form a co-aggregated biofilm.
The bacterial growth at the site of subgingival crevice triggers an array of host immune responses that majorly include innate immune response cells which attributes to the severity and progress of periodontal disease. Toll-like receptors (TLRs) activate the innate immune response binding to various microbial components (i.e. di-acyl lipopeptides, peptidoglycan, LPS, flagellin, bacterial DNA, etc) (98). The TLRs then activate signaling cascade leading to the activation of other components of the innate immune system (2, 30, 31, 98).

Oral treponemes and in particular, Treponema denticola, are identified as important contributors to periodontal disease (40). In healthy individuals, T. denticola is present in the subgingival crevice at low numbers (<1% of the total bacteria). However, as disease develops, T. denticola and other oral spirochetes thrive and ultimately represent as much as 40% of the total bacterial population in the periodontal pocket (40, 95). Significant advances have been made in recent years in understanding the complex mechanisms of communication that occur between organisms in oral biofilms (69, 84, 85, 147). However, the communication strategies and global regulatory mechanisms of oral spirochetes associated with periodontal disease have been largely unexplored.

1.2 Oral Treponemes
As stated before, oral *Treponemes*, along with over 700 other bacterial species exist as part of a polymicrobial biofilm accreted to the tooth surface in the gingival crevice (32, 33, 40, 84). The dominance of *Treponemes* clearly indicates that these unique bacteria are able to capitalize on the physiochemical conditions that develop in periodontal pockets. *Treponemes* play a significant role in the etiology of periodontal diseases including chronic periodontitis, acute necrotizing ulcerative gingivitis, endodontic infections and some acute dental abscesses caused by *Treponema denticola, Treponema lecithinolyticum, Treponema socranskii, Treponema vincentii* and others (48, 75, 135, 140). *Treponemes*, a member of the phylum spirochetes are believed to be have undergone extensive horizontal gene transfer with Archae and possibly with eukaryotic organisms (13, 17, 71, 77, 129, 166). The sequencing of the complete genome of *T. denticola* ATCC 35405, *T. lecithinolyticum* OMZ684T and *T. vincentii* ATCC 35580 have already proven invaluable to *treponeme* research and in understanding the evolution and virulence characteristics of these species (32, 141); (http://www.jcvi.org/); (www.homd.org/). Genomic comparison with other *Treponemes* and spirochetes facilitates identification of targeted selection of potential *T. denticola* virulence factors for detailed investigation. There are currently 49 species of oral *Treponema* listed on the Human Oral Microbiome Database (32); the best-characterized being *T. denticola, T. amylovorum, T. 
lecithinolyticum, T. maltophilum, T. medium, T. parvum, T. pectinovorum, T. socranskii, and T. vincentii (www.homd.org/). Within the oral Treponemes, T. denticola in particular is by far the most well characterized of the six currently cultivable oral spirochete species. Figure 2 represents an in-vitro aggregated T. denticola culture captured by electron microscopy.

1.3 Phylogenetics and genome composition of oral Treponemes

Seventy six phylotypes of treponemal species have been identified to be a part of the human oral microbiome (128). Of these treponemal species present in the subgingival crevice, complete genome sequence analysis has been determined for oral Treponemes including T. denticola, T. vincentii and T. lecithinolyticum. Genome sequence analysis revealed that T. denticola possess a 2.84 Mb circular chromosome (2786 ORFs), amongst that approximately 1200 of T. denticola’s ORFs have assigned functions, which makes identification of gene regulation in T. denticola easier (141). Along with T. denticola, T. vincentii and T. lecithinolyticum complete genome sequences have also been determined. T. vincentii and T. lecithinolyticum harbor a 2.51 Mb (2559 ORFs) and a 1.47 Mb (2059 ORFs) chromosome, respectively (www.homd.org/). In contrast to other spirochetes like Lyme disease and relapsing fever spirochetes, Borrelia
Figure 2. Electron microscopic analysis of *T. denticola*. Transmission (A) and scanning (B) electron micrographs of *T. denticola* ATCC 35405. The arrows in panel A indicate the endoflagella bundles.
Adapted from Frederick et al.; J Dent Res. 2010.
*burgdorferi* and *B. hermsii* respectively, plasmids are not a significant genomic component of oral *Treponemes* (4, 23). Amongst the oral spirochetes even though *T. denticola* has been reported to have a 4.2Kb plasmid, characterization of the same revealed that this plasmid do not contribute to any extent to the virulence of this periopathogen (23).

1.4 *T. denticola* virulence factors

Bacterial virulence factors include mechanisms and products of pathogens that promote their ability to cause infection and disease. Investigations underlying the identification of *T.denticola* virulence factors have exhibited numerous behaviors and products present in *T. denticola* that is consistent with its important role in periodontal disease etiology. An overview of the range of classical virulence factors of *T. denticola* is provided in this section. As mentioned, *T. denticola* exist as part of a complex, structured multispecies biofilm in the periodontal pocket, which is largely protected from the external shear forces. Interspecies bacterial binding plays a vital role in biofilm development and in co-location of bacterial species. *T. denticola* has been shown to bind other bacteria in the oral biofilm via the carbohydrate moiety of its major sheath protein (Msp) and its galactose-binding lectin receptor (81, 86, 136).
Dentilisin production is one of the major *T. denticola* virulence factors, since it is an active cell-surface located protease that cleaves at phenylalanyl/ alanyl and prolyl/ alanyl bonds (6, 64, 79, 100, 159, 160). It contributes to disease progression by disrupting or modulating intercellular host signaling pathways and degrading host cell matrix proteins. Dentilisin allows *T. denticola* to penetrate epithelial cell layers by degradating intercellular adhesion proteins (26) and modulate host cell immune responses by degradation of interleukin-1β (IL-1β), IL-6, TNF-α and monocyte chemoattractant protein 1 (112, 125). *T. denticola* is also predicted to have 166 membrane-associated lipoproteins, the highest number for any of the sequenced spirochetes (142). Factor H binding protein B (FhbB) is a small surface exposed lipoprotein in *T. denticola* that binds complement regulatory proteins of the factor H family. This protein plays a significant role in epithelial cell binding, invasion of the complement cascade, and tissue invasion (104-107). Interestingly, a novel interaction was demonstrated between dentilisin and FhbB in *T. denticola*, where full-length FH is preferentially bound to FhbB and then cleaved by the serine protease dentilisin to yeild an FH fragment that remains bound to the cell surface (106). This elucidates a correlation between dentilisin and FH cleavage which is unique to *T. denticola*. The paradigm behind this phenomenon may be, when dentilisin protease begins to degrade tissue, it presumably also cleaves cell-bound FH, facilitating tissue
invasion and further progression of periodontal disease. OppA is another cell-surface, membrane-associated lipoprotein that can bind soluble host proteins such as plasminogen and fibrinogen, but not immobilized insoluble host proteins or epithelial cells (44). The interplay of gene regulation in response to varied environmental condition is very well displayed in the expressions of lipoproteins in *T. denticola*.

Major Sheath Proteins (Msp) is one of the most abundant proteins in the *T. denticola* outer membrane (67). It is a member of the *T. pallidum* repeat (Tpr) protein family, and is one of the most-studied *T. denticola* proteins. The proposed mechanistic action of Msp, as assessed in cultured epithelial cells is to mediate colonization of host tissues and cytopathic pore-forming activity attributing towards *T. denticola* virulence in host tissue damage (39, 42). Spirochetes are unique in which the spirochetal flagella are located within the periplasmic space between the outer and cytoplasmic membrane (20, 74) (Figure 2A). Up to 15 periplasmic flagella originate from each pole of the cell in treponemes. These flagella interwind around the cytoplasmic cylinder, and overlap at the cell center (164). Flagella facilitate translocation of these bacteria through cell matrix inducing tissue penetration (45). Genome sequencing and bioinformatics analyses predicted that *T. denticola* posses a complete set of chemotaxis proteins required for signal perception, transduction, and adaptation (96).
Environmental signals are transduced to the cytosolic chemotaxis proteins CheA, CheW, and CheY in *T. denticola* via the methyl accepting protein to modulate the direction of flagellar motor rotation (146). Chemoattractants for *T. denticola* include glucose, serum, and albumin, which are possible indicators of damaged host tissues (137, 162). Other virulence determinants identified in *T. denticola* genome are the leucine rich repeats (Lrr) and the trypsin-like protease (120). Lrr mediates species-species interaction in the oral cavity (141) and binding to epithelial cells to promote swarming (78, 144). Trypsin-like protease are prolyl oligopeptidase that cleaves C-terminal to Arg residues (43, 91). Even though a substantial amount is known about the virulence determinants in *T. denticola*, however, the communication strategies and global regulatory mechanisms that may control the expression of these determinants in the spirochete when associated with periodontitis remains largely unexplored.

1.5 Two-component regulatory systems (TCS)

The prototypical two-component system is composed of a transmembrane-spanning histidine kinase and a cytoplasmic response regulator (152). The mechanistic model of a prototypical TCS signal transduction pathway is shown in Figure 3. The signal transduction pathway begins when the N-
Figure 3. Two-component system signal transduction. The signal transduction pathway begins when an environmental stimulus (1) is sensed by the extracytoplasmic domain of the histidine kinase (2). This induces ATP-dependent autophosphorylation at a conserved histidine residue (3). The phosphoryl group can then be transferred to a conserved aspartate of the response regulator (4). Once phosphorylated, the response regulator may dimerize (5) and function as either: a DNA binding, RNA binding, protein binding, or enzymatic protein (6). Once the phosphoryl group is lost, the response regulator is inactivated (7), which allows for tight regulation of this two-component system.
Adapted from http://www.user.gwdg.de/~genmibio/nnascher/rcsi.jpg.
terminal extracytoplasmic domain of the histidine kinase senses an environmental stimulus, which can include but is not limited to ions, light, dissolved oxygen, carbon, turgor pressure, and amino acids (36, 54, 83). Activation of the histidine kinase leads to an ATP-dependent autophosphorylation at a histidine residue in the C-terminal cytoplasmic domain. The phosphoryl group is then transferred from the histidine kinase to an aspartate in the response regulator. Upon phosphorylation, the output domain of the response regulator is activated and can function as a DNA-binding, RNA-binding, or protein-binding domain, or as an enzyme (41, 52, 109, 145).

Almost all bacteria possess two-component systems, with most species containing 20-30 response regulator-histidine kinase pairs (53, 148). *T. denticola* strain 35405 encodes 6 histidine kinases, 7 response regulators, and 2 kinase-response regulator hybrids that have potential global regulatory ability (Table 1). *T. lecithinilyticum* and *T. vincentii* encode considerably fewer histidine kinases and response regulators suggesting *T. denticola* TCS may offer a biological advantage to this periopathogen. A speculative schematic model depicting putative regulatory mechanisms and networks from *T. denticola* 35405 genome sequence is shown in Figure 4 (50). Two *T. denticola* TCS have been identified that appears to have potential global regulatory capability; AtcRS (49) and Hpk2-Rrp2 (138). The AtcS sensor kinase and AtcR response regulator are encoded
Table 1. Histidine kinases and response regulators of *T. denticola* strain 35405

<table>
<thead>
<tr>
<th>ORF</th>
<th>Notes</th>
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<tbody>
<tr>
<td><strong>Histidine Kinases</strong></td>
<td></td>
</tr>
<tr>
<td><em>Tde0032</em> (AtcS)</td>
<td>Forms a two component system with the response regulator, AtcR; transcriptional upregulated during late stage.</td>
</tr>
<tr>
<td><em>Tde0148</em></td>
<td>Harbors a single transmembrane (TM) spanning domain, Co-transcribed with <em>Tde149</em> (see the list of regulators) and <em>Tde0150</em>: a ribonuclease containing a cyclic nucleotide binding domain.</td>
</tr>
<tr>
<td><em>Tde0656</em></td>
<td>Harbors 2 TM domains (residues 23-45 and 49-70). <em>Tde0656</em> may function as a possible intramembrane sensor. Most likely transcribed with <em>Tde0655</em> May be part of a larger operon consisting of <em>Tde0658</em>- <em>Tde0653</em>. The complete locus is conserved in <em>Bacillus</em>.</td>
</tr>
<tr>
<td><em>Tde1970</em> (Hpk2)</td>
<td>Lacks a TM domain; harbors an N terminal PAS domain; significant homology with Hpk2 of <em>B. burgdorferi</em> (Sarkar <em>et al.</em>, 2010).</td>
</tr>
<tr>
<td><em>Tde0817</em></td>
<td>Orphan kinase lacks a TM domain, possesses an N terminal winged helix turn helix (HTH) DNA binding domain. Homologous proteins are found in <em>Francisella</em>.</td>
</tr>
<tr>
<td><em>Tde2381</em></td>
<td>Orphan kinase, has 5 TM domains; kinase domain may project into the periplasm located upstream of two genes involved in cobalamine metabolism <em>cobU</em>(<em>Tde2382</em>) and <em>cobS</em>(<em>Tde2383</em>)</td>
</tr>
</tbody>
</table>
Homologous proteins found in *Clostridia* where it is found in conjunction with a response regulator

**Response Regulators**

*Tde0033 (AtcR)*
- Forms a two component system with AtcS; only spirochetal protein with a LytTR DNA binding domain

*Tde0149*
- Forms a two component system with *Tde0148* Helix-turn-helix DNA binding domain LuxR Family

*Tde0655*
- Forms a two component system with

*Tde0656*
- Helix-turn-helix DNA binding domain

*Tde1969(Rrp2)*
- Forms a two component system with Hpk2, RpoN interaction domain Fis DNA-Binding domain.

*Tde2501*
- Forms a possible two component system with *Tde2502*. Unknown effector domain

*Tde0855*
- Orphan response regulator. Excisionase DNA binding domain, homologous proteins (74% similar) found in *Spirochaeta*. It may be co-transcribed with *Tde0856-Tde0859* including a lipoprotein, membrane protein, and hypothetical.

*Tde2324*
- Orphan response regulator-LuxR DNA binding domain Located upstream of an 8 TM domain containing hypothetical protein (*Tde2325*) and a cobalamine synthesis protein cobQ (*Tde2326*)
<table>
<thead>
<tr>
<th><strong>Hybrids</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tde0492</em></td>
<td>No TM -Kinase domain and receiver domain are homologs of a <em>S. aureus</em> TCS involved in virulence and biofilm formation</td>
</tr>
<tr>
<td><em>Tde2502</em></td>
<td>No TM Kinase domain and two receiver domains possibly cotranscribed with <em>Tde2501</em></td>
</tr>
</tbody>
</table>

Figure 4. Cellular architecture and genetic regulatory and signaling networks of *T. denticola* 35405. The figure depicts the cellular architecture of *T. denticola* and the likely cellular localization of many of its genetic regulatory and signaling proteins. Both the inner and outer membrane are shown. TCS and sigma factors of *T. denticola* are shown schematically. Histidine kinase (HK), Response regulator receiver (RR), DNA binding domain within the RR are indicated in Green, Yellow and Pink respectively. DNA binding domains are labeled with the subfamily designation based on blast searching (HTH-helix-turn-helix, WHTH- Winged Helix-turn-Helix, or lytTR). Other domains are also shown (3Y- Two component 3Y motif GAF- GAF domain, PAS- PAS S fold, TTP- tetratricopeptide repeat domain, HAMP- HAMP dimerization domain, AAA- AAA ATPase domain and RpoN interaction domain).
Adapted from Frederick et al.; J Dent Res. 2010.
by *Tde0032* and *Tde0033*, respectively. The Hpk2 sensor kinase and Rrp2 response regulator are encoded by *Tde1970* and *Tde1969*, respectively. Interestingly, orthologs of AtcR and AtcS are not found in other spirochetes for which genome sequences are known. The AtcRS regulatory system therefore possibly defines unique biological properties of *T. denticola* (49) as opposed to the Hpk2-Rrp2 system that may have a more universal function, since it is present in several other spirochete species, including *Borrelia*.

Hpk2 is a 46KDa protein that harbors an N-terminal Per-ARNT-Sim (PAS) domain. PAS domains have been demonstrated to detect specific environmental stimuli (61, 113), and are discussed in detail below in view of this project. Rrp2 in *T. denticola* is a 53KDa protein and harbors a σ^54^-interaction domain which is critical to interact with σ^54^ bound RNA polymerase holoenzyme to regulate gene transcription, hence this protein family is termed as σ^54^-dependent transcription regulators. These σ^54^-dependent transcription regulators are also known as Enhancer-binding proteins (EBPs). EBPs are a unique class of prokaryotic transcriptional activators that interact with the RNA polymerase and sigma factor (σ^54^) (Figure 5) (35). This sigma factor is also known as RpoN. Interaction of the EBP with the σ^54^-RNA polymerase holoenzyme is facilitated by the binding of the EBP activator to DNA sequences (upstream activator sequences) usually located
Figure 5. Mechanistic of action of σ\textsuperscript{54} transcription activators. σ\textsuperscript{54} reversibly associates with the core RNA polymerase to recognize promoters with consensus sequences at –12 and –24 bp relative to the transcription start site and transcription initiation is dependent on interaction with Enhancer Binding Proteins (EBP) that interact with the σ\textsuperscript{54} RNA polymerase holoenzyme after it is associated with DNA sequence (Upstream activator sequences, UAS) usually located 100bp upstream to the target gene (Part a). DNA looping is results in the interactions between the DNA-bound activator and the polymerase which in most cases is assisted by other DNA-binding proteins, such as integration host factor (IHF) (Part b). Nucleotide hydrolysis by the activator promotes remodelling of the closed complex through a series of protein–protein and protein–DNA interactions that favor isomerization to an open promoter complex from a closed complex (Part c). Part d indicate the modular domain structure of a typical σ\textsuperscript{54} transcription activator which frequently comprises of three domains: Regulatory domain that accepts the phosphate group from the histidine of HK, a highly conserved central domain that is required for the nucleotide-dependent interactions that drive open complex formation by σ\textsuperscript{54} RNA polymerase, and a carboxy-terminal DNA-binding domain with a helix–turn–helix motif that is required for recognition of upstream activator sequences.
at least 100 bp upstream of the transcription initiation site (Figure 5A). σ^{54} reversibly associates with the core RNA polymerase to recognize promoter sequences at −12 and −24 bp relative to the transcription start site and transcription initiation is dependent on interaction with a member of the EBP family (18). DNA looping is required to establish productive interactions between the DNA-bound activator and the polymerase which is initiated or assisted by DNA-binding proteins, such as integration host factor (Figure 5B). In the absence of the EBP, σ^{54} RNA polymerase holoenzyme forms closed promoter complex that does not undergo isomerization to form open complex (in which the DNA strands surrounding the transcription start site are locally denatured). Nucleotide hydrolysis (usually ATP) by the activator promotes remodeling of the closed complex through a series of protein–protein and protein–DNA interactions that favor conversion to the open promoter complex (Figure 5C) (18). The conserved central domain, also known as the σ^{54}-interaction module of EBPs, belongs to the AAA+ superfamily of ATPases that function as molecular machines to remodel their substrates. Members of the AAA+ family commonly function as oligomers, frequently as hexameric ring structures in which switches in conformation are promoted by nucleotide dependent interactions (163, 176). Nucleotide binding influences promoter-promoter interactions, consistent with the observation that the presence of nucleotides alters the oligomerization state of EBPs. In addition
to other conserved features found in the AAA+ superfamily, the EBPs contain a signature motif, GAFTGA, which has a direct role in the interaction with σ^{54} and the coupling of ATP hydrolysis to open-complex formation (Figure 5D) (14). The Crystal structure of *Aquifex aeolicus* EBP, NtrC1 AAA+ domain reveals that this protein is a ring-shaped heptamer in which the GAFTGA motif is located in an ordered loop in each subunit, projecting out into a central pore (90). These loops might form an interaction surface that couples ATP hydrolysis to interactions with the σ^{54} RNA polymerase (35).

The Rrp2 ortholog in *B. burgdorferi* is a σ^{54} dependent response regulator that carries out functions essential for viability of this lyme disease spirochete by activating the RpoN-RpoS regulatory pathway (172). In *B. burgdorferi*, replacement of wild type *rrp2* with a site-directed mutant deficient in ATP binding abolished the transcriptional expression of several important, plasmid-encoded virulence factors. Many of the genes regulated by Rrp2 in *Borrelia* had been demonstrated to be regulated by environmental variables including temperature and pH (122, 132). Rrp2 in the *B. burgdorferi* genome is a pivotal regulator governing the expression of major membrane lipoproteins such as OspC, DbpA, and Mlp8, as well as many other mammalian infection-associated immunogens (173).
In view of the critical role of Rrp2 in the *Borrelia* it is likely that Rrp2 in *T. denticola* also plays a pivotal role in regulating gene transcription in response to environmental changes. Interestingly, *T. denticola* lacks RpoS (141) suggesting that the molecular pathways for Rrp2 mediated gene regulation differ mechanistically in these two organisms.

### 1.6 PAS domain

The bacterial cell is dependent on its ability to sense and adapt to changes in the physicochemical makeup of its environment. It therefore expresses a variety of sensory and signal transduction proteins, among which the PAS domain is widely utilized (161). PAS domains are found in many signaling proteins where it functions as a signal sensor (70, 130). A PAS domain typically has a predictable α/β three-dimensional fold and is ~130 residues long (57, 61, 92). PAS domains perform a variety of functions within sensory proteins by promoting protein/protein interaction (89, 97, 118) or signal transfer (124) as well as by directly sensing perceived stimuli (154). The utility of PAS domains in performing these functions as a direct cellular sensor is in part due to their plasticity in binding different substrates (113). Functionally, the PAS domain is a protein module generally correlated with sensing of a dazzling variety of environmental signals like light, oxygen, Carbon monoxide, Nitrogen, redox
state, energy (ATP), blue light, coumaric acid, 2-ketoglutarate etc. (9, 24, 27, 29, 34, 58, 72, 73, 80, 153).

Oxygen concentration in the subgingival crevice undergoes significant change as periodontal disease progresses. The most common physiological strategy in prokaryotes for detecting oxygen is signal transduction by heme-based protein sensors (59) which governs the activity of the neighboring transmitter domain. PAS domain is the one most commonly encountered heme-based oxygen sensor (113). Heme-binding PAS (heme-PAS) domains can accomplish ligand-dependent switching of a variety of partner domains, including histidine kinase, phosphodiesterase, and basic helix loop helix DNA-binding modules (59). In case of Hpk2, the N-terminal PAS domain is linked to a C-terminal kinase domain. Considering the complex environment of oral cavity that T. denticola survives and outgrows in, the PAS domain must play a vital role in sensing the constant change in the oral environment and hence assisting Hpk2-Rrp2 in regulating the downstream gene transcription in response to changes in environmental conditions.

1.7 Research Objectives

Significant advances have been made in recent years in understanding the complex mechanisms of communication that occurs between organisms in
oral biofilms. However, global regulatory network in spirochetes and their adaptive response mechanisms associated with periodontal disease are highly unknown. This project was focused at characterizing a σ54-dependent TCS, Hpk2-Rrp2 in *T. denticola* that is believed to function in environmental sensing and regulating important genes in the biology of *T. denticola*. The current study was focused at characterizing the role of Hpk2-Rrp2 in *T. denticola*, identifying the role of PAS domain in environmental sensing, and determining if Rrp2 could prove to be a critical transcription regulator in responses to the changing environmental conditions associated with progression of periodontitis.

1.7.1 **Demonstrate the functional activity of the Hpk2-Rrp2, two-component regulatory system in *T. denticola***. In this study, the hypothesis that the putative TCS in *T. denticola*, Hpk2-Rrp2 constitutes a functional TCS was tested.

1.7.2 **Determine the role of PAS domain in activation of Hpk2-Rrp2 TCS and identifying the molecular mechanism of environmental adaptation by the PAS domain**. The PAS domain is associated with sensing a dazzling variety of environmental signals. Considering the rapid changes on-going in the oral cavity the hypothesis that the Hpk2 PAS domain may play a role in sensing environmental changes was tested. Further, the molecular mechanism of the
PAS domain in sensing and responding to environmental signals in *T. denticola* was identified.

1.7.3 Identification of the Rrp2 regulon through whole genome transcriptional analyses. To identify genes regulated by Rrp2, whole genome transcriptional analyses approach was attempted to compare gene regulation between the wild type *T. denticola* and *T. denticola* (Rrp2) mutant.
Chapter 2: Materials and Methods

2.1 Bacterial isolates and cultivation

Table 2 lists and describes the *Treponema* isolates analyzed in this report. All isolates were cultivated in New Oral Spirochete (NOS) media supplemented with 2% rabbit serum (Sigma-Aldrich) in an anaerobic chamber (5% H₂, 20% CO₂, 75% N₂; 37°C). Growth was monitored by dark field microscopy using a microscope contained within the anaerobic chamber. All strains were obtained from ATCC or kindly provided by Dr. Peter Greenberg (Univ. Washington). Strains were harvested by centrifugation (14,000 × g; 4°C; 20 min).

2.2 DNA isolation

For DNA isolation, *Treponema* cells (~7x10⁸ cells) were harvested by centrifugation (14,000 × g; 4°C; 20 min). Pellets were resuspended in 250 µl PBS and lysed by incubation with 3 ml Tissue and Cell Lysis Solution containing 500 µg proteinase K at 65°C for 20 min, with vortexing every 5 min. Bacterial DNA was isolated using the MasterPure DNA purification kit (Epicenter). The samples were cooled to 37°C, and RNA was degraded by incubation at 37°C for 40 min with 50 µg RNase A. After incubation on ice for 10 min, 1.75 ml MCP.
Table 2. *Treponema* isolates analysed in this study.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. denticola</em></td>
<td>35405</td>
<td>Human periodontal pocket</td>
</tr>
<tr>
<td></td>
<td>N17A1</td>
<td>Human periodontal pocket</td>
</tr>
<tr>
<td></td>
<td>GM1</td>
<td>Human periodontal pocket</td>
</tr>
<tr>
<td></td>
<td>33521</td>
<td>Human periodontal pocket</td>
</tr>
<tr>
<td></td>
<td>MS25</td>
<td>Human periodontal pocket</td>
</tr>
<tr>
<td><em>T. vincentii</em></td>
<td>33580</td>
<td>Human periodontal pocket</td>
</tr>
<tr>
<td><em>T. socranskii</em></td>
<td>33534</td>
<td>Human periodontal pocket</td>
</tr>
<tr>
<td></td>
<td>33535</td>
<td>Human periodontal pocket</td>
</tr>
<tr>
<td></td>
<td>N3B1A</td>
<td>Human periodontal pocket</td>
</tr>
<tr>
<td></td>
<td>DN71A</td>
<td>Human periodontal pocket</td>
</tr>
<tr>
<td></td>
<td>N5B</td>
<td>Human periodontal pocket</td>
</tr>
<tr>
<td><em>B. burgdorferi</em></td>
<td>BB31</td>
<td>Skin lesion</td>
</tr>
</tbody>
</table>

**Strains used in bioinformatics analysis**

*T. pallidum*  
NCBI database

*B. garinii*  
NCBI database

*B. afzelii*  
NCBI database

*B. turicatae*  
NCBI database

*B. hermsii*  
NCBI database

*B. duttonii*  
NCBI database

*B. recurrentis*  
NCBI database

*Leptospira interrogans*  
NCBI database

*L. borgpetersenii*  
NCBI database
Protein Precipitation Reagent was added, and the mixture was vortexed for 10 sec. Cellular debris was pelleted by centrifugation at 10,000 x g for 20 min. To the supernatant, 5 ml isopropanol was added and mixed by inverting the tube 30-40 times. The DNA was pelleted by centrifugation at 14,000 x g at 4°C for 20 min. The pellet was rinsed twice with 75% ethanol and resuspended in 50 µl water and quantified by measurement of UV absorbance (A260/A280).

2.3 Polymerase chain reaction (PCR)

The DNA templates used for PCR analyses were either isolated genomic DNA or purified plasmid or live bacterial cells. PCR was performed in the Eppendorf Master cycler Gradient and the following cycle conditions were used with GoTaq (promega) 95°C for 2 minutes followed by 30 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 7 min. Each PCR reaction included 0.75 units of Taq polymerase (Promega), 1X polymerase buffer (1.5 mM MgCl₂, 0.75 pmol µl⁻¹ of each primer, 200 µM each of dATP, dGTP, dCTP, and dTTP), and the DNA template (either 1-10 ng genomic DNA or 0.1-1 ng plasmid DNA). PCR reaction volumes were 30 µl with a final primer concentration of 0.75 pmol µl⁻¹. The condition used for Phusion Taq (Finnzymes) slightly varied, 98°C for 2 minutes followed by 30 cycles of 98°C for 15 sec, +3°C of the lowest primer Tm for 30 sec, 72°C for 1 min, and a final
extension at 72°C for 7 min. Primers used in this study are listed in Table 3 and were designed based on the genome sequence of *T. denticola* 35405. PCR amplicons were analyzed by electrophoresis in 1% agarose gels with 1X Tris-acetate-EDTA (TAE) buffer and visualized by staining with ethidium bromide.

2.4 Site-directed mutagenesis

Site-directed mutagenesis was conducted by using a two-step PCR-based approach with mutagenic primers (Table 3). The template for the first round of PCR reactions was genomic *T. denticola* 35405 DNA. The gene of interest (either *hpk2* or *rrp2*) was amplified as two separate amplicons. For all constructs the 5' portion of the gene was PCR amplified using a forward primer with tails to allow for LIC cloning and a reverse mutagenic primer that harbored the desired sequence changes. To amplify the 3' portion of the gene, the forward primer also contained the desired sequence changes while the reverse primer contained LIC tails. The amplicons derived from each half of the gene were purified using the QIAquick gel extraction kit (Qiagen). The second round of PCR contained both halves of the genes used as template as well as the LIC forward and reverse primers. The resulting amplicons were purified and cloned into the pET-46 Ek/LIC vector as described below. The resulting plasmid inserts were sequenced.
Table 3. Oligonucleotides used in this study

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<td>Rrp2 Fwd</td>
<td>GACGACGACAAGATTATGAAATTCGATATTTTGGTTATGATGACGAAAAAAATATTCG</td>
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<tr>
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35
Hpk2 (D156E) Fwd
Hpk2 (D156E) Rvs
Hpk2 (D156K) Fwd
Hpk2 (D156K) Rvs
Tde1974 5' Rvs
Tde1974 5' (1) Rvs
Tde1973 5' Rvs
Tde1973 5' (1) Rvs
Tde1972 5' Rvs
Tde1972 5' (1) Rvs
Tde1971 5' Rvs
Tde1971 5' (1) Rvs
Tde1970 5' Rvs
Tde1970 5' (1) Rvs
Tde1969 5' Rvs
Tde1969 5' (1) Rvs
Tde1968 5' Rvs
Tde1968 5' (1) Rvs
AUP Fwd
Tde1969 for pt mut Fwd
Tde1969 (G236&7C) Fwd
Tde1969 (G236&7C) Rvs
Tde1968 Rvs w/ AgeI & AatII
Tde1967 Fwd w/ AatII
Tde1966 Rvs w/ AgeI
Erm F Fwd w/ AatII
Erm AM Rvs w/ AatII
Erm F 5' Rvs
Erm AM 3' Fwd
Outside Tde1969 Fwd

TCTT
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AAGAA
TTCTTCAATTCTTTTTTCGGTAATTCTCGCAATCATAA
TTAT
ATAATTATGATTGCGGAAATTACCGAAAAAAGAATTG
AAGAA
TTCTTCAATTCTTTTTTCGGTAATTCTCGCAATCATAA
TTAT
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CAACGGCAAGTCCGGGAAAAATATGC
CAAAAAAACTATATCGGCACTTCCTATCCGCAT
CGTCATAAAAACCGGTACGGAACCTTAATAACAA
AAAAAC
CGAACTGTTTATATACCCCTCTATAAAAACTTTTTCGAA
CGACGGATAATTGGTGCAAGGTTTTTTTTTG
GGACGCAGGATAATTTTCGGCAAAGGCTTTTTTTTG
GGCCACGCGTCGACTAGTAC
CGTAGGATCCCGGATATAAAATGAAATTCAGTATTTTG
GTTATTGATG ACG AAA AAA AT
GCTTTGAAATTGCAAAACTGCTGCTCTTTTTTTGG
CCAAAAAAAGAGAGCAGCAGTTTGCAATTTCAAAGC
CTAGACCGGTTCGGACGTCCTAACCCTAAAAATCCA
AACCTATTTAATAGG
CTAGACGCTCAATCTCCTCTGTTGATTTATTTATCG
ATTAAACATTATAC
CTAGACCGGTAGACTAGCCTCTTATGCAAAAAACTCCC
CGTTCTTACCGGCCTC
CTTAGACGTCTTTAAAATTCTTTAAATTTAAAGCTTGGTA
TAGACCTTTTTC
GGAGGAGACGTCGAAGCTGTCAGTAGTATACC
GTAACCTTTTACAGTGAAATTTCTTGGAG
AATATTGGAAGCTATATACGTACTTTGTT
AACGTTTATTCCGATTACC GGCATGG
Outside *Tde1966 Dn*  
Rvs  
M13 (-21) Fwd  
M13 Rvs  
pET46 vector T7 Fwd  
pET46 vector T7 Rev  
Td FlaA F  
Td FlaA R  
Td FlaA RT F  
Td FlaA RT R

The sequence underlined indicates the LIC tails introduced in the primer for T4 cloning.
to verify no additional changes were introduced into the gene sequence as desired during PCR.

2.5 Ligase-independent cloning (LIC) and DNA sequencing

PCR amplicons were amplified using primers with specific tail sequences that allow for ligase-independent cloning into the predigested series of pET Ek/LIC vectors (Novagen). After amplification of the target gene with primers that contain specific tail sequences, the PCR products were purified to remove Taq, dNTPs, and template DNA using the QIAquick gel extraction kit (Qiagen). The PCR products were treated with T4 DNA polymerase in the presence of dATP. The 3'→5' exonuclease activity of the T4 DNA polymerase allows for generation of 13 base pair specific vector-compatible overhangs. The T4-treated amplicons were annealed into the pET-46 Ek/LIC vector and propagated in Novablue E. coli cells. Purified plasmids containing the desired gene or gene fragment were sequenced on a fee-for-service basis (MWG Biotech), and plasmids with the correct sequence were transformed into BL21 (DE3) cells for generation of r-protein.

2.6 Nucleotide and amino acid sequence analysis
hpk2 and rrp2 were amplified from T. denticola strains listed in Table 2 and the amplicons were annealed into the pET46Ek-LIC expression vector or pCR 2.1 TOPO vector as described above. Insert sequences were determined on a fee-for service basis (MWG Biotech, Ebersberg, Germany), translated in Expert Protein Analysis System proteomics server and aligned using BIOEDIT sequence alignment editor 7.0.9.0; percentage similarity/identity values were calculated using Matrix Global Alignment Tool.

2.7 Expression of recombinant proteins

To generate r-protein, BL21 (DE3) E. coli cells were grown at 37°C with shaking at 250 rpm in LB with 50 µg ml⁻¹ ampicillin to an OD₆₀₀ between 0.5-0.8 followed by induction with 1 mM IPTG for 3 h. Cells were harvested by centrifugation at 6,000 x g for 15 min. All proteins expressed from the pET series vectors have a hexahistidine tag that allows for nickel-affinity chromatography purification of the proteins as instructed by the supplier of the resin (Novagen).

2.8 Generation of antigen-specific antisera and infection antiserum

To generate antiserum, 50 µg r-protein in Freund's complete adjuvant was used to inoculate C3H/HeJ mice (Jackson labs) for the first injection and in incomplete Freund's adjuvant was administered for boosts (2 boosts, 2 weeks
apart). At week 6 the mice were euthanized and the blood was collected. Serum was recovered from blood by centrifuging 5 min at 14,000 x g after 3 hr incubation at room temperature. The specificity of each antiserum was confirmed through immunoblot analyses.

2.9 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Immunoblotting

Purified recombinant proteins or T. denticola cell lysates were separated in 12.5% or 15% Criterion Precast Gels (Bio-Rad) by SDS-PAGE and immunoblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore). Expression of recombinant proteins was confirmed by immunoblot analyses using monoclonal mouse anti-His Tag (1:5,000; Novagen) which recognizes the hexahistidine tag on all r-proteins used in this study, followed by detection with horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (1:40,000; Pierce). To assess for the production of specific T. denticola proteins, T. denticola cell lysates were fractionated by SDS-PAGE, transferred to immunoblots, and screened with various antisera including mouse anti-His (1:10,000), mouse anti-FhbB (1:1000), mouse anti-Hpk2 (1:500), rabbit anti-Fla (1:10,000), and mouse anti-Rrp2 (1:5,000). Detection was through the use of the appropriate HRP-conjugated secondary antibody (1:40,000; Pierce). All
antibodies were diluted in blocking buffer (1 x PBS, 0.2% Tween, 5% nonfat dry milk) and in all cases, detection was achieved through chemiluminescence using the Super Signal West Pico chemiluminescence substrate (Pierce) and exposure to X-ray film (Phoenix). Western blot band volume analysis was performed using the ChemiDoc XRS imager (Biorad).

2.10 Triton X-114 extraction and phase partitioning

To examine the sub-cellular location of Rrp2 and Hpk2, ~1.4x10⁸ Treponema denticola 35405 cells were pelleted by centrifugation at 14,000 x g, 15 min, 4°C. The cells were washed with PBS and resuspended in a 1% Triton X-114 solution in 1X PBS and incubated overnight with gentle rocking at 4°C. The detergent insoluble material (protoplasmic cylinder) was pelleted by centrifugation (14,000 x g; 15 min; 4°C). The supernatant was incubated at 37°C for 15 min to separate the detergent soluble and aqueous phases which contain the hydrophobic outer membrane and hydrophilic periplasmic proteins, respectively. The phases were separated by centrifugation at 14,000 x g for 15 min. To collect residual aqueous proteins from the detergent soluble phase, 1 ml of PBS was added; and to collect residual detergent soluble proteins from the aqueous phase, 1 ml of 1% Triton X-114 was added, and both were incubated at 37°C for 15 min, and centrifuged again. SDS-PAGE sample buffer was added to
each sample and SDS-PAGE was performed using 12.5% Criterion Precast Gels (Biorad). For immunoblot analyses, proteins were transferred to PVDF membranes (Millipore) and screened with mouse anti-FhbB (1:1000), mouse anti-Hpk2 (1:1,000), rabbit anti-Fla (1:10,000), or mouse anti-Rrp2 (1:1,000) followed by detection with the appropriate HRP-conjugated secondary antibody (1:40,000; Pierce) and chemiluminescence.

2.11 Autophosphorylation analysis

Autophosphorylation of Hpk2 was assessed using recombinant protein (20 ng μl⁻¹) under aerobic (room atmosphere) or anaerobic (5% CO₂, 10% H₂, and 85% N₂) conditions in kinase buffer (50 μl volume, 30mM HEPES, pH 8.0, 50mM KCl, 10mM MgCl₂, 0.5mM EDTA, 2mM DTT, 40 nM γ⁻³²P ATP, 6000 Ci mmol⁻¹, at room temperature). Recombinant wild type Hpk2 or Hpk2 mutant proteins (20 ng μl⁻¹) were incubated in 1X kinase buffer with 40 nM γ⁻³²P ATP. Aliquots from each reaction (0, 5, 10, 30 min) were mixed with 2X SDS sample buffer, fractionated by SDS-PAGE and transferred to PVDF membranes. The membranes were exposed to film at -80º C for 4 hr with intensifying screens. For assays under anaerobic condition, all reagents were equilibrated in an anaerobic chamber for 3 days. For aerobic condition, the reaction was performed on bench top.
2.12 Phosphotransfer analysis

For phosphotransfer, recombinant Hpk2 (generated as described above), (20 ng μl⁻¹) was incubated with radiolabeled 40 nM γ⁻³²P ATP in 1X kinase buffer for 30min. After 30min pre-incubation equal amount of recombinant Rrp2 (20 ng μl⁻¹) protein was added to the reaction. Aliquots from the reaction mixture (0, 5, 10, and 30 min) were mixed with 2X SDS sample buffer, fractionated by SDS-PAGE, electroblotted and exposed to film as above. Phosphotransfer was also assessed under aerobic or anaerobic conditions.

2.13 Quantification of PO₄ incorporation

To quantitate autophosphorylation, the reactions were repeated as above, fractionated by SDS-PAGE, transferred to PDVF membranes, and membranes were stained with Coomassie blue stain. The bands corresponding to Hpk2 were excised, transferred to glass vials with 20 ml liquid scintilant to determine the amount of incorporated phosphate in a liquid scintillation counter.

2.14 Statistical analysis of PO₄ incorporation in Hpk2

Statistical tests were done to determine the mean difference among autophosphorylation of two groups under aerobic and anaerobic conditions. Using the normal quantile plot, the data can be assumed to be normally
distributed. Since the p-value for the Brown-Forsythe test in all analysis was more than 0.05, equal variance was assumed. Since the data was normally distributed and showed equal variance, the groups were compared using ANOVA test (Analysis of Variance) and significant difference was considered with a p-value < 0.05.

2.15 Hemin binding assay

3', 3', 5', 5' Tetramethylbenzidiño (TMBZ) staining approach was used to assess the ability of Hpk2 to bind hemin. Heme staining of SDS-PAGE gels by TMBZ/H2O2 detects heme/hemin bound to proteins. 3,000 ng of recombinant Hpk2 was incubated with hemin (20 μg/ml) at 37° C for 2 hr and diluted 4:1 with conventional SDS-PAGE reducing buffer without boiling before loading onto Criterion 15% Polyacrylamide gel. The proteins were electrophoresed at 200 V. Detection of hemin-protein complexes was accomplished by staining the gel with TMBZ (a chromogen that forms a blue product by hemin- peroxidase activity). After electrophoresis, gels were fixed in dark for 1 hour in a pre-chilled solution of sodium acetate (250mM, pH 5.0)-methanol-H2O at a ratio of 6:3:1 (v:v). Gels were stained with 7:2:1 (v:v) of sodium acetate (250mM, pH 5.0)-TMBZ (6.3 mM in methanol)-H2O for 30mins, followed by color development with 30 mM H2O2 for 30 min at 4° C in the dark.
2.16 Quantitative hemin binding assay

To assess the binding of hemin to r-proteins, 96-well plates (Costar 3590; Corning) were coated with 2 µg per well of r-protein in carbonate buffer (pH 9.6; 16 h at 4°C). All samples were assessed for hemin binding in triplicates (n=3). After protein immobilization, the plates were incubated in blocking buffer (1%PBST) for 1 h. 200 µl of Hemin (20 µg/ml) diluted in 0.1mM Tris-HCl (pH 8.0) was added to each well and plates were further incubated for 2 hr. Plates were then washed with PBS-T. To assess for hemin binding, plates were further incubated with 7:2:1 (v:v) of sodium acetate (250mM, pH 5.0)- TMBZ (6.3 mM in methanol)-H₂O for 30 min, followed by color development with 30mM H₂O₂ for 30 mins at 4°C in the dark. Since the color developed was comparatively lighter for absorbance at 450nm, 0.1M H₂SO₄ was added to each well, where the blue color developed due to hemin binding to protein changed into dark yellow to brownish depending on the intensity of hemin/protein association. This change in color was measured as absorbance at 450nm. All plates were read in an ELx 808 ELISA plate reader (Biotek).

2.17 Analysis of the oligomeric state of r-Hpk2

The oligomeric state of Hpk2 was assessed by blue native PAGE (BN-PAGE). Fifty nanograms of r-Hpk2 (wt) or r-Hpk2 (G44D, D49E/K, N58D and
D156E/K) substitution mutants were diluted in BN-PAGE sample buffer (50 mM Bis-Tris [pH 7.0], 15% glycerol, 0.02% Coomassie brilliant blue G-250 [CBB-G250]) without β-mercaptoethanol and separated on a Bis-Tris acrylamide gel (4 to 16% Native-PAGE; Invitrogen) using 50 mM Bis-Tris (pH 7.0) and 50 mM Tricine-15 mM Bis-Tris (pH 7.0) as anode and cathode buffers, respectively. The gel was run under cooled conditions (100 V for 20 min followed by 200 V for 40 min) with cathode buffer containing 0.002% CBB-G250. The gel was then run at 200 V for an additional 40 min with cathode buffer with no dye. Following electrophoresis, the gel was electroblotted to polyvinylidene difluoride (PVDF), and the blot was probed with anti-Hpk2 antiserum and goat-anti-mouse IgG-HRP. Molecular masses of the Hpk2 bands were estimated by extrapolation from a standard curve generated with NativeMark (Invitrogen) and bovine and chicken egg albumin.

### 2.18 RNA purification

Cells were lysed with diethylpyrocarbonate-treated 1% SDS and RNA was recovered using the RNeasy Midi kit as instructed by the manufacturer (Qiagen). Residual DNA was removed by treatment with DNase I (Invitrogen) for 1 h at 37°C followed by inactivation with 2.5 mM EDTA (70°C; 10 min).
2.19 Quantitative real time reverse transcriptase-polymerase chain reaction (qPCR)

*T. denticola* cells were cultivated for either 4 or 13 days and RNA was extracted using the RNEasy Extraction Kit (Qiagen). qPCR was performed in triplicate using the DNA Engine Opticon 2 System (MJ Research) and the SYBR Green PCR Master Mix (Applied Biosystems) as instructed by the supplier. Primer pairs (final concentration, 0.5 μM) were designed to amplify a 100- to 150-bp fragment of each target. To generate standard curves, amplicons of each gene were cloned into the pCR2.1 TOPO vector (Invitrogen) and serial dilutions of the purified plasmid were used as the PCR template. *flaA* transcript levels, a constitutively expressed gene, served as the standardization-normalization control for real time RT-PCR analyses. PCR was performed by using the following cycling parameters: 1 cycle of 10 min at 95°C, followed by 40 cycles of 10 s at 94°C, 20 s at 60°C, and 20 s at 72°C. PCR product was quantified, and the data were analyzed by using software provided by the thermocycler manufacturer (MJ Research).

2.20 Reverse transcriptase-polymerase chain reaction (RT-PCR)

For RT-PCR analyses, the absence of DNA in the RNA preparation was verified by PCR using a *flaA* primer set and *Taq* DNA polymerase. cDNA was
generated by using Superscript II RT enzyme (Invitrogen) with 1 μg of RNA, 1.5 pmol of each specific primer (Table 2), and reagents supplied by the manufacturer (42°C; 50 min). The RT was inactivated by incubation at 70°C for 15 min. As a negative control, reactions were also run without reverse transcriptase. The cDNA was used as the template for amplification in PCR with Taq polymerase with the following cycle parameters: 40 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 1.5 min. The resulting amplicons were analyzed in 2% agarose gels. Genomic DNA was also used as template to test each primer set.

2.21 Transcription Start Site identification using 5’ RACE

Transcriptional start sites (TSS) were identified using 5’ rapid amplification of cDNA ends (RACE) (Invitrogen). cDNA was generated using primers specific for the 5’ regions of Tde1968 (ftsJ), Tde1969 (Rrp2), Tde1970 (Hpk2), Tde1971, Tde1972, Tde1973 and Tde1974 as listed in Table 2 and purified using Snap columns (Invitrogen). Purified cDNA were 3’ poly(C) tailed using terminal deoxynucleotidyl transferase and dCTP. The amplicons were then PCR amplified using the abridged anchor primer (Invitrogen), which anneals to the poly(C) tail, and nested gene-specific primers. A second round of PCR was then performed using another set of gene specific primer closer to the 5’end of each
gene and the universal anchor primer (Invitrogen). The resulting amplicons were cloned into the pCR2.1 TOPO vector and the inserts sequenced on a fee-for-service basis (MWG Biotech).

2.22 Allelic exchange mutagenesis

To further characterize the role that Rrp2 plays in the pathogenesis of the T. denticola, an rrp2 mutant construct to be used in T. denticola for allelic exchange mutagenesis was made (Figure 6). Mutation in Rrp2 at position G236 and G237 to cystine was introduced using overlapping PCR as explained in site directed mutagenesis text. Rrp2 (Tde1969) and FtsJ (Tde1968) were fused as the upstream region and Tde1967 onwards made up the downstream. After an erythromycin resistance cassette was inserted between the upstream and downstream regions using AatII sites. All these genes were brought together in pCR2.1 vector as described in Figure 6. All circular plasmids werepropogated in E. coli Novablue cells (Invitrogen). The resultant circular plasmid was digested with SpeI and NotI to remove the insert from the pCR2.1 vector backbone as shown in Figure 6 and DNA was electroporated into T. denticola competent cells.

2.23 T. denticola transformation and plating
Figure 6. Generation of an Rrp\textsuperscript{G236&7C} construct. In brief, a point mutation was introduced into Tde1969 using overlapping PCR as described in the protocol. Tde1969 PCR product was generated keeping Tde1968 flanking to Tde1969 using the Tde1968 R (described in the primer list) when amplifying from Tde genomic DNA. The Tde1969+Tde1968 (upstream) was cloned in pCR2.1-TOPO vector, approximately 1Kb downstream gene to Tde1968 was cloned into the vector as well. Upstream and downstream sequences flanking rrp2 were amplified with primers possessing unique restriction sites, and cloned into the pCR2.1-TOPO vector. The plasmids were propagated in E. coli, purified, and digested with AgeI and AatII simultaneously. The digested fragments were purified and ligated together yielding a plasmid with the upstream and downstream sequences separated by an AatII restriction site. This plasmid was propagated in E. coli, purified, and digested with AatII. Simultaneously, plasmid containing the Erm cassette flanked by AatII restriction sites, was also cut with AatII. The fragments were purified and ligated together. The final construct carries an insert consisting of 1 kb of sequence downstream of Tde1968, the Erythromycin resistance cassette, and Tde1968+ Rrp\textsuperscript{G236&7C} sequence upstream. The resultant knockout plasmid (pJS Rrp\textsuperscript{G236&7C}) was linearized with NotI and SpeI; electroporated into T. denticola strains 35405.
Fresh-passaged *T. denticola* cells were grown to the log-phase (5x10^6 colony forming units/ml), harvested by centrifugation at 4000 g for 10 min at 4°C, washed three times with ice-cold 10% glycerol and resuspended in 10% glycerol. Aliquots of cells were then stored at -70°C. Before electroporation, 80 µl of cell suspension was mixed with 1-5 µl of highly concentrated (15-20 µg) plasmid preparations on ice, and then transferred to a 0.1 mm electroporation cuvette (Bio-Rad Laboratories). One pulse was delivered from a Gene Pulser (Bio-Rad Laboratories) with the Pulse Controller set at 1.8 kV, 25 µF and 200 Ω, producing a time constant of 4-4.5ms. One milliliter of pre-reduced NOS medium was immediately added to the cuvette and the cells were transferred to a culture tube and placed into the anaerobic chamber. After overnight incubation without antibiotics, the culture was then mixed with 35 ml of pre-cooled NOS medium supplemented with 1% GTG agarose (Sigma-Aldrich) and 50 µg/ml Erm (final concentration), and poured into petri dishes. After 1-2 weeks of anaerobic incubation, single colonies emerging on Erm plates were picked up and inoculated into NOS + 50 µg/ml Erm broth. The broth culture was continuously incubated in the anaerobic chamber until a log-phase growth was obtained. The purity of the culture was checked under phase-contrast microscopy before harvesting. To verify allelic exchange, PCR and sequencing was performed on a clonal population of spirochetes. This PCR data should indicate that wt *rrp2* has
been successfully replaced and an Erm resistance marker is inserted using allelic exchange mutagenesis.
Chapter 3: Distribution, sequence conservation and properties of Hpk2 and Rrp2

3.1 Analysis of functional domains of Hpk2 and Rrp2

BLAST searches over conserved domain databases provided significant information about structure and function of the annotated \textit{tde1970} and \textit{tde1969} two component regulatory system in the \textit{T. denticola} genome. Due to their homology with Hpk2 (Histidine protein kinase 2) and Rrp2 (Response regulator protein 2) of \textit{Borrelia burgdorferi} (172), \textit{tde1970} and \textit{tde1969} were designated as Hpk2 and Rrp2, respectively.

The domain architecture of Hpk2 and Rrp2 is depicted in Figure 7. Hpk2, a predicted sensor kinase protein is around 46kDa. TIGRFAM and pFAM matches indicated that Hpk2 comprises of 3 conserved functional domains, details of which are discussed below. The Hpk2 as accessed in Tmap and TMpred algorithms lacks any transmembrane spanning domain. Instead this sensor kinase possesses a PAS domain S-box at its N-terminal (139 residues) (Figure 7A). Within the PAS domain is a predicted putative heme-binding pocket, extending from amino acid 92-131. The association of heme binding-PAS domains with oxygen sensing suggests that Hpk2 could play an important role in responding to changing oxygen concentration in the subgingival crevice as
Figure 7. Sequence and functional domains of Hpk2 and Rrp2. Amino acid sequences are shown for the Hpk2 (panel A) and Rrp2 (panel B) proteins of *T. denticola* strain 35405. Predicted functional domains for both proteins are indicated. The bolded amino acids of Rrp2 indicate the σ54-interaction domain. Additional functional domains that reside within the σ54-interaction domain are highlighted by boxing. Residues that may undergo autophosphorylation in Hpk2 or serve as phosphoacceptor residues in Rrp2 are indicated by asterisks.
The disease progresses (51, 154). The PAS domain is followed by an H-Box domain (with 3 conserved putative His autophosphorylation sites at H185, H197 and H219) and an H-ATPase domain (ATP-Mg²⁺ binding sites) that spans the C-terminal of the protein as determined by structural modeling based on the HMMSTR/Rosetta algorithm.

Rrp2, on the other hand harbors a receiver-phosphorylation domain (with 3 highly conserved Asp residues at positions 48, 53 and 99), a σ⁵⁴-interaction domain and a helix-turn-helix DNA-binding domain (Figure 7B). The putative functional domains of Rrp2 share significant sequence similarity with domains present in response regulators of the NtrC-fis family (σ⁵⁴-RNA polymerase transcriptional activators). These activators function with σ⁵⁴-RNA polymerase holoenzyme and stimulate isomerization of the closed promoter complex to an open complex in a reaction that requires ATP hydrolysis as described above (7). NtrC regulator domains are found in approximately 8.5% of response regulators with generally only 1 or 2 per bacterial genome (53). Orthologs of this TCS are found in other spirochetes including the Relapsing fever and Lyme disease spirochete, Borrelia. In B. burgdorferi, Rrp2 (a σ⁵⁴ transcriptional activator) regulates a large number of genes encoding virulence factors and membrane proteins (11, 12, 126, 172).
3.2 Genetic distribution and sequence conservation of *hpk2* and *rrp2*

To assess sequence conservation of *hpk2-rrp2*, PCR analyses on *T. denticola* strains (35405, N171A, GM1, MS25 and 33521), *T. socranskii* strains (33535, 33535, N3B1A, DN71A and N5B), *T. vincentii* and *B. burgdorferi* Bb31 was performed using gene specific primer sets listed in table 3. Obtaining amplicons of predicted size suggested that this TCS is universal in oral *Treponemes* (Figure 8). These amplicons were then cloned into pET46 vector and subsequent sequence analysis was done as described in the text. Comparison of DNA sequences revealed that both *hpk2* and *rrp2* are highly conserved in *T. denticola* species. MatGAT (Matrix Global Alignment Tool) was used to generate similarity/identity distance matrix for Hpk2 and Rrp2, refer tables 4 and 5. Amino acid sequence comparison of Hpk2 from a panel of *T. denticola* isolates showed similarity values >97% and identity values >96% (Table 4). Similarly amino acid sequence comparison for Rrp2 from the panel of *T. denticola* isolates showed similarity values >99% and identity values >98% (Table 5). *Td* Hpk2 homology with other *treponemes* was found to be only 65% similarity and 45% identity. The average homology values between Hpk2 of *T. denticola* and Hpk2 of *Borrelia* species was 54% similarity and 28% identity. Hpk2 sequence of *Leptospira* species seemed highly divergent from Hpk2 of *T. denticola* with similarity value as low as 36% and identity value approximately
**Figure 8.** *rrp2* and *hpk2* are universally distributed among oral treponemes. To determine if the genes encoding the Rrp2-Hpk2 two-component system are widely distributed among oral spirochetes, PCR analyses were performed. All isolates except *B. burgdorferi* yielded an amplicon of similar sizes with the *rrp2* and *hpk2* primer set. Both *hpk2* and *rrp2* primer set yielded product for all 11 oral *Treponeme* isolates tested. *T. denticola*: Tde; and *T. socranskii*: Tsac.
Tde35405 Tdel(N17A1) Tdel(GM1) Tdel(33521) Tsac(33534) Tsac(33535) Tsac(N3B1A) Tsac(DN71A) Tsac(N5B) T. vincentii B. burgdorferi

$rrp2$

$hpk2$
Table 4. Distance matrix comparison of Hpk2 orthologs of spirochetes

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Table 5. Distance matrix comparison of Rrp2 orthologs of spirochetes

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</table>

The top right hand and lower left hand quadrants lists the the percent amino acid identity and similarity values for each protein pair. The following abbreviations were used for the species of origin of each sequence: Tde- T. denticola; TREV0001- T. vincentii; Tp- T. pallidum; Bb- Borrelia burgdorferi; BG-Borrelia garinii; BA-Borrelia afzelii; BT-Borrelia turicatae; BH-Borrelia hermsii; BDU-Borrelia duttonii; BRE-Borrelia recurrentis; LA-Leptospira interrogans; LBL-Leptospira borgpetersenii. Note that for the T. denticola sequences the strain of origin is indicated after the species abbreviation while for other species, the assigned ORF number follows the species abbreviation.
20%. It was observed that the predicted phosphorylation sites in Hpk2, the 3 Histidine residues at H-185, H-197 and H-219 are conserved in all *T. denticola* isolates and so are the predicted phosphorylation sites in Rrp2, the 3 Asp residues at positions 48, 53, and 99 as analyzed by sequence alignment using BIOEDIT sequence alignment editor 7.0.9.0. Rrp2 homologs from other spirochete: *T. pallidum; B. burgdorferi; B. garinii; B. afzelii; B. hermsii; B. turicatae; B. duttonii; B. recurrentis; Leptospira interrogans; and L. borgpetersenii* were also compared for identity and similarity values. Around 81.6% similarity and 66.6% identity was found in Rrp2 of *T. denticola* and Rrp2 of *T. pallidum*. Around 73% similarity and 52% identity was found between Rrp2 of *T. denticola* and both *B. hermsii* and *B. turicatae*. A similar range of homology values were obtained between Rrp2 of *T. denticola* and other *Borrelia* species. The least homology was found between Rrp2 of *T. denticola* and Rrp2 of *Leptospira* species (36.6% similarity and 41% identity). The sequence similarity searches showed that the region of Rrp2 comprising of the receiver and σ^{54} interaction domain (aa 3-364) is highly homologous (data not shown) amongst all Rrp2 orthologs. The conserved motifs identified in σ^{54} activators were nucleotide binding “Walker A”, ATP hydrolysis domain “Walker B”, GAFTGA and AAA consensus site. The putative phosphate acceptors in Rrp2, 3 Asp residues at 48, 53 and 99 were also conserved in all these bacterial response regulators. From
the above data it can be concluded that Rrp2 sequence homology within its orthologs are much higher than Hpk2 homology. This higher degree of homology in Rrp2 orthologs is attributed by the highly conserved $\sigma^{54}$-interaction domain within all Rrp2 orthologs.

3.3 Analysis of transcriptional expression pattern of \textit{hpk2} and \textit{rrp2}

Sensor kinase and response regulator pairs of TCS are typically co-transcribed as a polycistronic mRNA. \textit{hpk2} and \textit{rrp2} are overlapping genes and are separated by 4 base pairs, hence may also be co-transcribed. To determine if \textit{hpk2} and \textit{rrp2} are expressed together during \textit{in-vitro} cultivation as a part of an operon, mRNA was extracted from 4 and 13 day old cultures grown under standard physiological conditions. Internal specific primers were designed to amplify across the intergenic spacer region between \textit{tde1970-69} indicated in table 3. RT-PCR analysis showed that both \textit{hpk2} and \textit{rrp2} are expressed \textit{in-vitro} and are co-transcribed. Visually these genes looked to be transcribed more on day 13 (late phase) as oppose day 4 (early phase). FlaA that was used as positive control for the RT-PCR reaction showed same level of expression at day 4 and 13 (Figure 9A). To confirm if transcription of the \textit{hpk2-rrp2} operon is influenced by growth stage, real-time qPCR was performed using the same
Figure 9. Demonstration of co-transcription and growth phase dependent expression of Hpk2 and Rrp2. To determine if *rrp2* and *hpk2* are co-transcribed, reverse transcriptase PCR was performed using RNA harvested from *T. denticola* 35405 cultures grown for 4 or 13 days (Panel A). The (-RT) stands for absence of the RT enzyme. To determine if growth phase affected *hpk2-rrp2* transcript levels, real time RT-PCR was performed using *hpk2-rrp2* specific intergenic primers and data were normalized using the levels of the constitutively and highly transcribed *flaA* gene. (Panel B).
mRNA from 4 and 13 days old cultures. Relative to day 4, a 100-fold induction in *hpk2-rrp2* transcript levels was observed at day 13 (Figure 9B). The data was normalized against the numbers of *flaA* transcript detected and are presented as the ratio of the number of *hpk2-rrp2* transcripts to the number of *flaA* transcripts.

### 3.4 Analysis of growth-phase dependent expression of Hpk2 and Rrp2

Rrp2 homolog in *E. coli* (AtoC) is shown to be expressed at later stage of growth cycle as it posses secondary functions as antizymes (Az) (47, 103). Az is a non competitive protein inhibitor of ornithine decarboxylase that is induced by polyamines, the end products of the biosynthetic pathways (47, 94). To confirm if Hpk2-Rrp2 protein production were influenced by growth-phase, immunoblots were generated and screened with antiserum to Hpk2 and Rrp2 respectively. Immunoblot analyses of *T. denticola* cell lysates obtained from cultures harvested at 4, 9 and 13 days confirmed increased production of Hpk2 and Rrp2 with growth-phase (Figure 10). The levels of FlaA protein, a constitutively produced protein, remained unchanged.

### 3.5 Sub-cellular localization of Hpk2 and Rrp2

To determine the subcellular localization of Hpk2 and Rrp2, Triton X-114 extraction and phase partitioning was performed. The resulting fractions and the
Figure 10. Demonstration of *in-vitro* growth phase dependent production of Hpk2 and Rrp2. Antiserum to Hpk2 and Rrp2 was generated using r-Hpk2 and r-Rrp2 derived from isolate *T. denticola* 35405. The data presents immunoblot analyses in which the relative production of Hpk2, Rrp2 and FlaA (a constitutive control) were measured in *T. denticola* 35405 cells cultivated for 4, 9 or 13 days. All methods are described in the text.
whole cell lysate were screened by immunoblotting (Figure 11). The Rrp2 antiserum detected Rrp2 and Hpk2 antiserum detected Hpk2 in the whole cell lysate as well as in the detergent insoluble phase which includes all proteins in the protoplasmic cylinder. As controls, additional blots were screened with antiserum against FhbB (a known outer membrane lipoprotein) and FlaA (a periplasmic protein). The control proteins partitioned as expected. The FhbB partitioned exclusively to the detergent soluble phase and FlaA was detected in the detergent insoluble phase. These analyses indicate that Hpk2 and Rrp2 are associated with the protoplasmic cylinder which includes inner membrane and cytoplasmic proteins. Hpk2 contains no export signals or predicted transmembrane-spanning domains, thus it is most likely a cytoplasmic protein along with its cognate response regulator Rrp2 consistent with other response regulators.

3.6 Discussion

Amongst 7 response regulators encoded by T. denticola 35405 (141), Rrp2 is the only $\sigma^{54}$-dependent transcription activator found in its genome. Going with the consensus that Rrp2 in Borrelia regulates important virulence factor genes, a hypothesis was set that Rrp2 in T. denticola must play important role in biology of T. denticola. Previous to this study, no work had been done on the
Figure 11. Hpk2 and Rrp2 localizes to the protoplasmic cylinder. To determine the cellular location of Hpk2 and Rrp2, Triton X-114 extraction and phase partitioning was performed and the resulting fractions were analyzed by screening immunoblots with antiserum to Hpk2 and Rrp2. As controls, identical immunoblots were screened with antiserum to FhbB (an established outer membrane lipoprotein) and FlaA (an inner periplasmic protein).
Hpk2-Rrp2 TCS in *T. denticola*. The goals of these analyses were to assess a diverse panel of oral *Treponemes* for the distribution, conservation, and functional domains of the Hpk2- Rrp2 TCS. PCR and bioinformatics analyses revealed that *hpk2* and *rrp2* were detected in all oral *treponemes* and that the organization of these genes was conserved. *hpk2-rrp2* are separated by a short intergenic spacer and consistent with other TCS, *rrp2* and *hpk2* were found to be co-transcribed. Transcript levels were 100 fold higher in late-stage cultures indicating that expression of the operon responds to stimuli associated with growth phase and or cell density.

To assess the molecular properties of Hpk2 and Rrp2, PCR, DNA sequence and database analyses were conducted. These genes are universal and the putative functional domains and residues of *hpk2* and *rrp2* are conserved among all *T. denticola* isolates that have been analyzed. The sequence analyses detailed above revealed that the N-terminal PAS domain of Hpk2 harbors a 15 amino acid insertion that is not found in PAS domains of other bacteria (http://cmr.jcvi.org/). This insertion was detected in all *T. denticola* isolates but not in Hpk2 orthologs of other spirochetes including *T. vincentii*, *T. pallidum*, *Borrelia* species, and the *Leptospira*. It remains to be determined if this insert imparts unique biological characteristics or influences the activity of Hpk2. Conserved within the Hpk2 PAS domain is a putative heme-binding pocket.
Heme-binding domains allow for the sensing of redox potential and or oxygen levels (51, 53, 154). The features of Hpk2 and Rrp2 suggest that this putative two-component regulatory system may be an important contributor to the sensing of environmental changes that occur in the subgingival crevice as periodontal disease progresses.
Chapter 4: Hpk2-Rrp2, a functional TCS

4.1 Transcriptional analysis of *hpk2* and *rrp2*.

In the *T. denticola* isolate 35405, *hpk2* and *rrp2* are surrounded by ORFs that overlap or have short intergenic spacers (Figure 12A). This arrangement suggests that these genes may be transcribed as a polycistronic messenger RNA that initiates with *tde1974* and extends through *tde1968*. To determine if *hpk2-rrp2* are also co-transcribed with its flanking genes, RT-PCR analyses were conducted using gene spanning primers. RT-PCR analyses identified a transcriptional unit consisting of ORFs *tde1968* through *tde1974* (Figure 12B). All the genes present in the same orientation in the *T. denticola* genome extending from *tde1968-74* co-transcribed as a part of a larger seven gene operon. As a negative control, RT-PCR was performed using a primer set spanning ORFs *tde1975* and *tde1974*. No product was obtained, consistent with the opposite orientation of *tde1975*. RT-PCR of *fhbB*, a constitutively expressed membrane protein that binds to the complement regulatory protein factor H (106), served as a positive control for RT-PCR. Finally all primers were tested using genomic DNA as template to verify that all primers were functional. Henceforth this 7 gene operon is refered as the *hpk2-rrp2* operon. Contained within this operon are genes encoding proteins critical for DNA replication, cell wall synthesis and
Figure 12. Schematic of the *T. denticola* 35405 *hpk2-rrp2* locus: demonstration of co-transcription of *hpk2-rrp2* as a part of a 7 gene operon. The organization of *hpk2*, *rrp2* and adjacent genes are shown in the figure (ORF designations and the direction of transcription are indicated). Intergenic spacer lengths (in base pairs) are listed below the schematic in parentheses with negative numbers indicating coding sequence overlap. RT-PCR analyses, using primers that amplify across the intergenic spacer region, are presented in panel B (cDNA panel). To verify that the primers were functional, each was tested with genomic DNA (gDNA) as template. Detection of the constitutively expressed *fhbB* (Factor H binding protein B) gene served as a positive control for RT-PCR. Nt indicates that no template was added (negative control). The size standards (in base pairs) are indicated to the left.
translational efficiency. The putative functions of each of these genes are listed in table 6. These above data suggest that **hpk2-rrp2** operon is possibly expressed with genes significant for growth and survival of *T. denticola*. This analysis can be continued into a transcription start site analysis to determine the number of possible transcript combinations co-transcribed within this 7 gene operon. This can provide insights into the regulation of genetic co-transcription of the **hpk2-rrp2** operon, based on the requirement of the respective protein product of genes at any given time or condition by the bacteria.

### 4.2 Identification of Transcription start site (TSS) for **hpk2-rrp2** operon

To identify the TSS for the genes and operons in the **hpk2-rrp2** gene cluster (extended from **tde1974** through **tde1968**) and to further localize the transcriptional control elements, 5’RACE analyses was performed. The amplicons obtained from these analyses were TA cloned and sequenced. Two TSS were mapped as shown in Figure 13. One TSS mapped upstream to **tde1971**, presumably may serve as the primary TSS for **hpk2-rrp2**, **ftsJ**, and **dnaK** operon. A second TSS was mapped upstream to **tde1974**. This must be an initiation site for genes through **tde1974** to **tde1972** or for the whole **hpk2-rrp2** gene cluster (**tde1974** through **tde1968**) depending on the growth or environmental conditions. Analyses for putative promoter sequence or sigma
<table>
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<th>Gene name</th>
<th>paralogs</th>
<th>Function and notes</th>
</tr>
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<tbody>
<tr>
<td>Tde1968</td>
<td>ftsJ (rrmJ)</td>
<td>none</td>
<td>23S rRNA methyltransferase; 2’-O methylates residue U2552 of the A loop of 23S rRNA; methylation stabilizes the 50S subunit within the 70S ribosome (Hager <em>et al.</em>, 2002); inactivation of this gene in <em>B. burgdorferi</em> impaired growth rate and morphology (Morozova <em>et al.</em>, 2005)</td>
</tr>
<tr>
<td>Tde1969</td>
<td>rrp2*</td>
<td>2079, 2309, 2593, 0492, 1494, 2324, 2501, 2502, 0033, 0648, 0655, 0149, 0855</td>
<td>σ^54^ dependent transcriptional regulator/response regulatory protein; In <em>B. burgdorferi</em> (Bb), Rrp2 is required for survival as deletion of <em>rrp2</em> appears to be lethal (Yang <em>et al.</em>, 2003); In Bb Rrp2 directly or indirectly controls a regulon consisting primarily of plasmid carried genes involved in virulence (Blevins <em>et al.</em>, 2009; Burtnick <em>et al.</em>, 2007; Caimano <em>et al.</em>, 2007; Lybecker and Samuels, 2007);</td>
</tr>
<tr>
<td>Tde1970</td>
<td>hpk2*</td>
<td>2502, 0492, 0656</td>
<td>histidine kinase, possibly involved in oxygen sensing; In Bb, it is presumed to be the cognate kinase for Rrp2 however this has not been directly demonstrated</td>
</tr>
<tr>
<td>Tde1971</td>
<td>dnaX</td>
<td>2586</td>
<td>gamma/tau subunit of DNA polymerase III, DNA replication</td>
</tr>
<tr>
<td>Tde1972</td>
<td>HP</td>
<td>2397, 1131, 2094, 0692, 2223, 1235, 1005</td>
<td>40 aa peptide with a possible toxin BmKK4 domain (a member of the sub-family α-KTx17) (Zhang <em>et al.</em>, 2004a; Zhang <em>et al.</em>, 2004b);</td>
</tr>
<tr>
<td>Tde1973</td>
<td>cvpA</td>
<td>none</td>
<td>colicin V (anti-bacterial) production factor</td>
</tr>
<tr>
<td>Tde1974</td>
<td>murG</td>
<td>none</td>
<td>a glycosyltransferase that catalyzes the last intracellular step of peptidoglycan synthesis; it is required for cell growth and survival; interacts via hydrophobic interactions with the inner membrane; may be part of the divisome (Mohammadi <em>et al.</em>, 2007)</td>
</tr>
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</table>
Figure 13. Analysis of the upstream promoter region for *hpk2-rrp2* operon.
The nucleotide sequences of the upstream regions of *Tde1971* and *Tde1974* are shown. The start of transcription is indicated by an arrow, with +1 denoting the first base of the transcript. The -24 and -12 sites (RpoN) binding site upstream to *Tde1971*, -35 and -10 sites upstream to *Tde1974* and ribosome binding site (RBS) are shown. The start codon is indicated by the star.
**tde1971**

```
CTTTGTTCATTGATAAAAATATTAAGTGAAAGCATTCTTTTGCGAAAATCCTTTCTCCATTTATTT
AGATATTACCAAGCAGTTTTAGGAGTTGGATAAAACCTTGCAAAGAAAGTTTTTATAGGGG
ACATGCATTATTGTATGTAGTTTTGCATAAAAGCAAAAACTACTTGAAAACTTTTTTCGGAAA
TTGATATTTTCTGCAAAGAAGTTTTTATAGGAGTTGATTTTGAA
```

**tde1974**

```
CATCCTTTTCTTCATTGTTCCAATACTCTTTTTAAAGCGTACATTCTGGAAGAGTTACTTT
ACATTTTTTCACTTTTCAATAAAATATTCTTTTCAAGAGAAGCTTTTCTCCTTCCAT
AATTATGATGAATTGATTTTGACTATGATGTTTTTCTGAAAAATGGATGTTGACTGACCA
AATTATCATTATTTGAATATTCTTTTTTTCTGACTAAAAGATATTAAACTTAA
CCAAAGGAATGCTGAAAGTCTTTTGCAGCTTTTCAAAAAATTTGTGATACACTTACA
TTATGAAATG
```

**RBS**

```
M F E
```

**RBS**

```
M K C
```
factor binding consensus showed a possible RpoN or σ^{54} binding sequence 5’
GG\_\text{NNNNNNNNNNNNN}GC 3’ upstream to the \text{tde1971} TSS (5). GC at position -12 from
the start site was found conserved and was separated by GG at position -24.
This analysis further confirmed that Hpk2-Rrp2 regulation is also σ^{54}-dependent.
The other TSS found upstream to \text{tde1974} did not appear to be σ^{54}-binding
consensus; instead it showed a consensus sequence similarity to the σ^{70} binding
site with TATA at the -10 position and TTGACA at -35 position respectively.

4.3 Autophosphorylation and phosphotransfer capabilities of Hpk2

To demonstrate the autophosphorylation ability of Hpk2, r-Hpk2 was
incubated with γ-^{32}P ATP in kinase buffer. Aliquots from the sample mixture was
removed at different time points (1, 5, 10 and 30 min) and added to the loading
buffer. Excessive EDTA was added to the loading buffer in order to stop the
enzymatic reaction. The tubes were also immediately freezed in a chilled ethanol
bath at -80°C for the same. Samples were ran on an SDS-PAGE gel
electrophoresis setup, blotted on PVDF and \text{^{32}P} incorporation in Hpk2 was
assessed by autoradiography. Hpk2 demonstrated to autophosphorylate in a
time-dependent manner (Figure 14; left panel). As a control for the specificity of
autophosphorylation, Rrp2, which is not expected to autophosphorylate, was
incubated alone with γ-^{32}P ATP. No labeling was observed (data not shown).
Figure 14. Demonstration of Hpk2 autophosphorylation and phosphotransfer to Rrp2. Autophosphorylation of Hpk2 (left panel) and phosphotransfer to Rrp2 (middle panel) was assessed over time (as indicated above each lane in minutes) using recombinant proteins and protocols detailed in the text. To verify that equal molar amounts of Hpk2 and Rrp2 were used in the assay, an identical blot was screened with anti-his antibody (right panel). Note that for the phosphotransfer analyses, Hpk2$^+$ was preloaded with phosphate prior to mixing with Rrp2 and then aliquots were removed at the time points indicated. The migration position of each protein is indicated to the right.
Phosphorylated Hpk2 was then demonstrated to transfer phosphate to Rrp2 (Figure 14). A plateau or equilibrium in Rrp2 phosphorylation was reached by 5 min. This rapid transfer and plateau is consistent with use of a 1:1 ratio of Hpk2 to Rrp2 in the reaction. The 1:1 ratio of Hpk2 to Rrp2 was verified by immunoblotting using anti-His antiserum (Figure 14; right panel). It was noted that phosphotransfer requires preloading of Hpk2 with phosphate. When non-phosphorylated Hpk2 and Rrp2 were combined prior to the addition of γ-32P ATP, phosphotransfer did not occur.

4.4 Discussion

Analyses of a panel of *T. denticola* isolates demonstrated that the genes up and downstream of the *hpk2-rrp2* genes of *T. denticola* 35405 are conserved in sequence, gene order and orientation. Several of these genes have short intergenic spacers or have overlapping coding sequence. Consistent with this, transcriptional analyses revealed that *hpk2-rrp2* are co-transcribed as part of a larger polycistronic mRNA that includes FtsJ (23S rRNA methyltransferase), DnaK (DNA polymerase III tau/gamma subunit), CvpA (colicin V production factor), a hypothetical ORF and MurG (peptidoglycan synthesis).

To further understand the molecular basis of the transcriptional regulation of the *hpk2-rrp2* operon and its flanking genes, TSS analyses were performed.
TSS were identified upstream of *tde1971* (DnaK), and upstream of *tde1974*. The predicted -12 and -24 regions associated with the *Tde1971* TSS are consistent with σ^{54} binding sites (based on the RpoN binding consensus sequence analysis) (5). Another TSS upstream to *tde1974* was found to have -10 and -35 consensus promoter sequence for σ^{70} binding site (based on the conserved database analysis for σ^{70} binding site) (16). Having 2 divergent promoter sequence and two different start site for a operon with genes co-transcribed suggests that the bacteria switches between the use of σ^{70}-RNA polymerase or σ^{54}-RNA polymerase, to fine tune the expression or repression of the genes within the operon as and when they are required as seen in *Desulfovibrio vulgaris* (46). In an *in-silico* approach used to determine AtcR regulated operons (unpublished data), AtcR was analysed for DNA binding of putative regulon genes via the LytR domain using Electrophoresis Mobility Shift Assay. In this analysis one of the DNA motifs identified to bind AtcR was upstream to the σ^{54} binding promoter sequence of *Tde1971* within the *hpk2-rrp2* operon. This indicates a possiblility of a regulatory pathway where AtcR might be a regulator of the *hpk2-rrp2* operon by repressing the *hpk2-rrp2* primary transcript to promote the full length transcription of the secondary larger transcript via the σ^{70} promoter sequence. Like the *hpk2-rrp2* operon, the transcription of the *atcRS* operon is also growth phase regulated.
suggesting that they may go hand in hand for an interconnected gene regulation pathways.

The ability of the Hpk2-Rrp2 two-component system to function as a cognate kinase-response regulator pair was demonstrated in vitro using recombinant Hpk2 and Rrp2. Autophosphorylation progressed in a linear fashion out to 30 min and then reached a plateau. Phosphotransfer from Hpk2 to Rrp2 occurred rapidly but was completely dependent on the preloading of Hpk2 with phosphate. This is consistent with that previously demonstrated for the *T. denticola* AtcRS system (49). The basis for this phenomenon may lie in the requirement for kinase dimerization to transfer phosphate. Dimerization of histidine kinases generates a stable structure that is required for the response regulator interaction and subsequent phosphotransfer (108, 121). Unphosphorylated response regulator may interact with kinase monomers preventing dimerization and thus inhibiting formation of the necessary structural elements required for phosphotransfer.

While the regulon controlled by the Hpk2-Rrp2 two-component regulatory system remains to be defined, the putative functions of the other proteins encoded by the *hpk2-rrp2* operon suggests that they may play an important role in facilitating the outgrowth of *T. denticola*. Contained within this operon are genes encoding proteins critical for DNA replication, cell wall synthesis and
translational efficiency. A paradigm supporting these data can be, as environmental conditions associated with the progression of periodontal disease develop, the *hpk2-rrp2* operon becomes transcriptionally activated. This presumably leads to a significant increase in the production of *murG* (peptidoglycan biosynthesis), *DnaX* (DNA replication) and *FtsJ* (translational efficiency) all of which could play a key role in facilitating the rapid outgrowth of *T. denticola*. The presence of *CvpA* (colicin V production factor) and *tde1972* (a potential toxin of the α-KTx17 toxin sub-family) (174, 175) within this operon is intriguing. These proteins are not known to contribute to core functions but it is possible that as *T. denticola* growth becomes stimulated by physiochemical changes in the periodontal pocket, the production of *CvpA* and *tde1972* could contribute to inhibiting the growth of competitors or inhibit host immune effector cells allowing *T. denticola* to become a dominant organism. Future analyses will test these hypotheses. In conclusion, the results presented here, coupled with the homology of the Hpk2-Rrp2 to two-component systems of other bacteria, suggest potential involvement of this TCS in regulating adaptive responses associated with changing environmental conditions.
Chapter 5: Functional role of Hpk2 PAS domain

5.1 The Hpk2 PAS domain senses *in-vitro* environmental conditions and influences the kinetics of autophosphorylation and phosphotransfer.

To assess the contribution of the PAS domain in Hpk2 autophosphorylation and phosphotransfer, full length Hpk2 and an N-terminal 122 aa truncation variant (Hpk2ΔPAS) were generated. The N-terminal truncation removes the PAS domain but leaves other functional domains of the protein intact. Autophosphorylation reactions were set up as detailed above except the reactions were conducted under aerobic and anaerobic conditions. Assays under anaerobic condition were carried out in the anaerobic chamber with already reduced buffers. The protein samples were reduced for 2hrs before starting the reaction. Autophosphorylation was quantified by measuring the incorporation of $^{32}$P into Hpk2 and Hpk2ΔPAS. Under aerobic conditions no significant difference in phosphate incorporation was observed between Hpk2 and Hpk2ΔPAS ($p>0.05$) (Figure 15; left panel). However, under anaerobic conditions significant differences were observed. Hpk2ΔPAS displayed a reduction in phosphate incorporation relative to the full-length form of Hpk2 ($p<0.05$) (Figure 15; right panel). While Hpk2ΔPAS retained its autophosphorylation
Figure 15. Measurement of Hpk2 autophosphorylation: analysis of the contribution of the PAS domain and the influence of environmental conditions. Autophosphorylation of recombinant Hpk2 and Hpk2ΔPAS under aerobic and anaerobic conditions (as indicated above each panel) was assessed as detailed in the text with incorporation of $^{32}$P serving as the read out. All assays were conducted in triplicate and the variance determined. Asterisks (*) indicate statistically significant differences between Hpk2 and Hpk2ΔPAS.
Anaerobic

Aerobic

Time (min)

Total PO\textsubscript{4}\ incorporation (fmols)

Hpk2     Hpk2\Delta PAS

*  

Hpk2     Hpk2\Delta PAS

*  

Time (min)

Total PO\textsubscript{4}\ incorporation (fmols)
activity (albeit at a reduced level under anaerobic conditions), it was not competent to transfer phosphate to Rrp2 (data not shown).

5.2 Identification of conserved residues in the PAS domain.

There is considerable amount divergence amongst PAS domain sequences. To identify conserved residues within the PAS domain of *T. denticola* Hpk2, Hpk2 homologs from other spirochetes and *E. coli* (AtoS) from NCBI database were aligned using BioEdit sequence alignment editor 7.0.9.0. Glycine (G) at position 44, Aspartate (D) at position 49, Asparagine (N) at position 58 and Aspartate (D) at position 156 were found to be 100% conserved amongst all the Hpk2 homologs analyzed as seen in Figure 16. To test the hypothesis that these residues within the PAS domain are involved in environmental sensing and regulating the activation of Hpk2, amino acids at G44, D49, N58 and D156 were targeted for site-directed mutagenesis. The amino acid substitutions were G44D, D49E/K, N58D, D156E/K. The substitution from D to E was a change in structure of amino acid keeping the charge of the amino acid at that position intact. Substitution D to K and N to D was change the charge and structure of amino acid at that position. It was reasoned that these substitutions would alter charge distribution, a key parameter in ligand binding, without significantly affecting secondary structure of the sensor kinase. Note that wt Hpk2 with amino acid
**Figure 16. Identifying conserved residues in PAS domain.** Amino acid sequence of Hpk2 PAS domain was aligned with its homologs from other bacteria. 100% conserved residues were identified (G44, D49, N58 and D156) and are indicated with asterisks. All sequences were obtained from the databases. *Treponema denticola*, Td; *Treponema vincentii*, Tv; *Borrelia afzelii*, Ba; *Borrelia garinii*, Bg; *Borrelia burgdorferi*, Bb; *Borrelia turicatae*, Bt; *Borrelia hermsii*, Bh; *Escherichia coli*, Ec.
<table>
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<tr>
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<th>Td Hpk2 (PAS)</th>
<th>*</th>
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<tr>
<td>Td</td>
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<th>ADIFVSSVQNESEGQTSKEFNKADKPEGKNKYIEVSVP-LVNEKK--IQG</th>
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<tr>
<td>Tv</td>
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<tr>
<td>Ba</td>
<td>LDDIQIPILINLIKEVLRTEDKIGGLEVPISN-------NIYIKISFMP-YVEKKEK--LEG</td>
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<tr>
<td>Bg</td>
<td>LDDIQIPILINLIKEVLRTEDKIGGLEVPISN-------NIYIKISFMP-YVEKKEK--LEG</td>
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<td>Bb</td>
<td>LDDIQIPILINLIKEVLRTEDKIGGLEVPISN-------NIYIKISFMP-YVEKKEK--LEG</td>
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substitutions are here on addressed as Hpk2 substitution mutant throughout the text.

5.3 Role of PAS domain conserved residues in influencing the autophosphorylation ability of Hpk2

It was already demonstrated above that Hpk2 is a functional histidine kinase that undergoes time dependent autophosphorylation when incubated with $^{32}$P labeled ATP, and the rate of autophosphorylation is regulated in part by the PAS domain. To assess if the PAS domain conserved residues influenced the autophosphorylation ability of Hpk2, kinase assays were carried out using purified wt Hpk2 and all the above Hpk2 substitution mutants under both anaerobic and aerobic conditions. Since wt r-Hpk2 had shown appropriate amount of $^{32}$P incorporation within 10 min (refer section 4.3 and Figure 14), for the current study $^{32}$P incorporation in wt Hpk2 and Hpk2 substitution mutants were compared for only 2 time points, 0 and 10min. As expected, under aerobic condition, wt Hpk2 showed $^{32}$P incorporation within 10min (Figure 17). All the other Hpk2 substitution mutants also showed similar amount of $^{32}$P incorporation as wt Hpk2 within 10min except Hpk2 (D156K), which had lost its autophosphorylation ability. Under anaerobic conditions, wt Hpk2 underwent autophosphorylation as expected within 10min. Hpk2 substitution mutants: Hpk2
Figure 17. Comparison of autophosphorylation profile of wt Hpk2 and Hpk2 PAS domain substitution mutants. Autophosphorylation of Hpk2 and mutant Hpk2 was assessed using recombinant proteins under aerobic and anaerobic conditions as described in the protocol. Only 2 time points 0 and 10min were assessed. Top panel was kinase assay carried out under aerobic condition (bench top). Middle panel is kinase assay carried out under anaerobic conditions (anaerobic chamber), bottom panel represents western blot of kinase assay performed with non labeled ATP and screened with anti-his to show equal loading of proteins.
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**Aerobic**
![Aerobic blot](image)

**Anaerobic**
![Anaerobic blot](image)

**α-His**
![α-His blot](image)
(G44D), Hpk2 (D49E) and Hpk2 (N58D) showed similar levels of autophosphorylation as wt Hpk2. However under anaerobic condition Hpk2 susbstitution mutants: Hpk2 (D49K), Hpk2 (D156E) and Hpk2 (D156K) did not show any $^{32}$P incorporation. Total loss of kinase activity by Hpk2 (D156K) under both aerobic and anaerobic conditions suggests that this mutation in r-Hpk2 may have altered the PAS domain folding in a way that is improper to influence kinase activity. For conserved substitution at position D156 in Hpk2, where only the structure of the amino acid was changed (DtoE), the protein loss of autophosphorylation activity only under anaerobic condition. These data suggest that Asp at position156 is important in maintaining the structure and charge integrity of Hpk2 or the correct form of Hpk2 that is required for it to interact with ATP and hydrolyze ATP under a suitable environmental condition. Interestingly, substitution of amino acid D49 (negatively charged) to K (positively charged) also regulated the kinase activity of Hpk2 under aerobic and anaerobic conditions. Loss of $^{32}$P incorporation in Hpk2 (D49K) only under anaerobic condition suggests that having a negatively charged amino acid at position 49 in r-Hpk2 is required for the PAS domain to sense the highly reduced anaerobic environment and positively activate the kinase domain as the substitution from D to E (change in amino acid structure alone) at the same position did not alter the kinase activity of the protein under non-reduced conditions. Hence the charge of the
amino acid at position 49 in Hpk2 plays significant role in sensing oxygen levels or redox potential to influence the catalytic domain for ATP hydrolysis.

5.4 Role of PAS domain conserved residues in Hpk2 phosphotransfer ability

Previous data demonstrated that prelabeled $^{32}$P-Hpk2 can transfer the PO$_4$ to its cognate response regulator in a time dependent manner. Since Hpk2 substitution mutants showed varied kinase activity compared to the wt, it was sought to assess if these mutations in the protein had any effect on the phosphotransfer activity of Hpk2. Since all Hpk2 substitution mutants except Hpk2 (D156K) showed kinase activity under aerobic condition, the phosphotransfer assays were carried out only under aerobic condition. Phosphotransfer assay was carried out exactly as mentioned in section 4.3 with pre-labeled Hpk2 proteins and Rrp2 introduced in the reaction mixture in equal stoichiometry. Phosphotransfer by wt Hpk2 to Rrp2 was consistent as before (Figure 18). Hpk2 substitution mutants: Hpk2 (G44D), Hpk2 (D49E) and Hpk2 (N58D) showed phosphotransfer ability similar to that of wt r-Hpk2. Hpk2 (D49K) showed a delayed PO$_4$ transfer to Rrp2 starting only at 10min. Hpk2 (D156E) had completely lost its ability to transfer PO$_4$ to Rrp2, even though it showed self phosphorylation and no dephosphorylation over time (Figure 18).
Figure 18. Comparison of phosphortransfer ability of Hpk2 and Hpk2 PAS domain substitution mutants. Phosphotransfer of Hpk2 and Hpk2 substitution mutants was assessed using recombinant proteins under aerobic conditions as described in the protocol. To verify that equal molar amounts of Hpk2 and Rrp2 were used in the assay, an identical blot was screened with anti-his antibody (right panel). Hpk2 was preloaded with phosphate prior to mixing with Rrp2 and then aliquots were removed at the time points indicated. The migration position of each protein is indicated to the right.
5.5 Assessing oligomeric state of Hpk2

Histidine kinases have been demonstrated to regulate their active site via controlling dimer and oligomer forms of itself (151). To determine if regulation of Hpk2 autophosphorylation was influenced by its oligomeric state, Hpk2 and Hpk2 substitution mutants were fractionated under complete non-reduced condition. The protein samples were ran in a Blue-Native PAGE gel, transferred on PVDF and Hpk2 was detected by anti-Hpk2 anti-serum. In its native condition wt Hpk2 showed to exist in oligomeric forms. The protein showed a ladder of higher order oligomer formation as seen in Figure 19. Wt Hpk2 under complete non-reduced conditions displayed significantly low monomeric forms (M.W 47kDa), relatively higher dimeric forms (M.W 95kDa) and considerable amounts of tetrameric (M.W 190kDa) and pentameric (M.W 240kDa) forms. Hpk2 substitution mutants (G44D, D49E, D49K and N58D) showed similar ladder pattern as the wt Hpk2 under native condition. Amino acid D156 showed significantly high influence on the dimerization ability of Hpk2, where Hpk2 substitution mutant D156E showed higher amount of monomeric forms (M.W 47kDa), and less proteins in dimeric forms (M.W 95kDa) and significantly low proteins in trimeric forms (M.W 143kDa) under native condition on the other hand Hpk2 (D156K) did not show any oligomeric forms and only existed in monomeric form (M.W 47kDa) under native condition.
Figure 19. Formation of oligomers by r-Hpk2 and r-Hpk2 PAS domain mutants. Western blot of r-Hpk2 and r-Hpk2 PAS domain mutants separated by blue native PAGE under non reducing conditions, blotted to PVDF, and probed with anti-Hpk2 antiserum. Calculated molecular masses are shown on left.
5.6 Discussion

The potential contribution of the PAS domain as a whole in environmental sensing and autophosphorylation was assessed by generating a recombinant Hpk2 truncation mutant lacking the PAS domain (Hpk2ΔPAS). Precedent for assessing the function of individual domains of his-kinases was established in earlier studies (139). As an example, the role of the PhoR PAS domain was assessed by domain deletion. Deletion of the PAS domain from this histidine kinase provided information about its functional role without abolishing the activity of the autophosphorylation or phosphotransfer associated domains (168). As demonstrated here, autophosphorylation kinetics of Hpk2 differed significantly for full length recombinant Hpk2 under aerobic conditions versus under anaerobic conditions. Autophosphorylation of full length Hpk2 occurred more rapidly and reached a higher level under anaerobic conditions. Deletion of the PAS domain significantly decreased Hpk2 autophosphorylation specifically under anaerobic conditions (Figure 15). These data indicate a link between the PAS domain, Hpk2 autophosphorylation efficiency, and environmental conditions.

In the current data, it was showed that mutation in the conserved residues of PAS domain can affect the autokinase activity of Hpk2. Such effects have been seen in other sensor kinases and response regulator pairs as discussed below. For example WalRK two-component regulatory system in *Streptococcus*
*pneumonia*, plays an important role in maintaining cell wall homeostasis and responding to antibiotic stress. Mutation of Ser78 to Asp in WalK resulted in a reduction of over 90% of the catalytic kinase activity of the sensor kinase (66). Another example in the same bacterial system would be the two-component MicAB system in *S. pneumonia*, whose MicB kinase carries a PAS domain, which is involved in competence repression under oxygen limitation. In a study on MicB sensor kinase, Echenique *et al.* were able to show that, the L100 residue of PAS domain in MicB was required for its autophosphorylation. Mutational and biochemical analysis showed that although L100 in the N-terminal of the PAS domain in MciB was less conserved yet it was important to influence the C-terminal kinase activity of recombinant MciB *in-vitro* (37). All the above above established work on role of PAS domain in sensor kinase activation supports our data where a single amino acid substitution in Hpk2 drastically affected its activity.

Several structural models have been predicted of the complex formed between the PAS-containing ThkA histidine kinase and its TrrA cognate response regulator from *Thermotoga maritima* (171). The PAS domain of the ThkA does not dimerize in the complete structure, but rather forms contacts with the catalytic domain. Two interactions have been identified in the ThkA-TrrA TCS system, one is between the dimerization domain of ThkA and TrrA and the
other is an unanticipated interaction between the PAS domain and TrrA (171). These interactions predicted in *T. maritlime* can also be a possible mechanism of kinase activity regulation in the Hpk2-Rrp2 TCS system.

Consistent with observations in other sensor kinases, a similar result was obtained from mutational analysis in Hpk2 PAS domain. The results presented here can be interpreted in a slightly different manner and fit into a proposed model where mutation in Hpk2 (D156K) had totally abolished the catalytic activity of Hpk2; similarly Hpk2 (D156E) had also lost catalytic activity but only under anaerobic conditions. This phenomenon can be explained as, any mutation affecting the charge and structure of the aa at position D156 results in fall of dimerization ability of Hpk2. Since Hpk2 (D156E) still retained the autokinase ability under aerobic condition it's possible that the negative charge of this amino acid influences a conformational change in Hpk2 only under anoxygenic condition. Hence a change in only the structure of the amino acid at position 156 DtoE, under aerobic condition, did not trigger the conformational change that is instigated by encountering dissociation of oxygen at the PAS domain of Hpk2. Therefore in Hpk2 (D156K), since both the factors: dimerization influenced by negative charge at D156 and conformational change due to association/dissociation of oxygen are not matched, there is total loss of kinase activity at any given condition. Even though the putative Dimerization/Histidine
phosphoacceptor (DHp) domain in Hpk2 as annotated in NCBI is from aa 173 to 242, these data suggest that the later part of C-terminal PAS domain can also be considered as a part of this DHp domain that regulates and influences the Hpk2 catalytic activity domain (CA) depending on the oligomeric form of Hpk2. At present there are not many structures available for full-length Histidine Kinases (HK), so exact relative orientation of sensor, DHp and CA domains are not known. However clearly from the above data DHp and CA domains need to associate transiently to transfer the phosphoryl group from ATP to phosphoaccepting Histidine and the sensor PAS domain must have some mechanism to manipulate DHp-CA interaction in response to the external signal to achieve regulation of His kinase activity.

Hpk2 (D49K) had similar effect on its autokinase ability as Hpk2 (D156E). Kinase activity was lost under anaerobic condition and phosphotransfer ability was drastically affected. However the mutation at D49 in Hpk2 did not affect the oligomerization ability of this protein. This was observed by similar oligomeric forms of Hpk2 (D49K) as compared to wt Hpk2 under non-reducing conditions. Based on this information it can be speculated that the function of aspartate residue at position 49 in Hpk2 is different to that at position 156 where D49 may not be involved in influencing dimerization but the charge of the amino acid at that position may be required to bind ATP stably under anaerobic condition. This
aa may also be required to maintain the stability of the ATP-Hpk2 complex under anaerobic condition. After assessing the effect of mutation on Hpk2 autokinase ability, study was set out to assess if these mutation on PAS domain had any effect on the phosphotransfer ability of Hpk2, hence providing an insight into the role of these conserved residues in PAS domain in assisting the interaction and activation of the response regulator Rrp2. Consistent with effect on autophosphorylation, mutations in Hpk2 D49K and D156E showed altered phosphotransfer ability. Hpk2 (D156K) could not be assessed for phosphotransfer ability, as it did not show autophosphorylation under any given condition (aerobic or anaerobic).

Successful crystallization and characterization of nucleotide bound DHp + CA versions of HKs: HK853 (HK from *Thermotoga maritima*) (21, 101); DseK (HK from *Bacillus subtilis*) (1); and ThkA (from *T. maritima*) (169) provide key insights into how these HKs function. These data show the evidence of interaction between Response regulators (RR) with DHp domain and with CA domain. Thus suggesting mutation that affects the dimerization domain can ultimately negatively influence the transient interaction between Rrp2 and Hpk2-dimerization domain that is required for phosphotransfer. Thus based on the current data, it can be concluded that, regulation of Hpk2 autokinase activity involves control of dimerization and catalytic domain interactions in response to
sensory input by controlling the oligomeric stability of Hpk2 and the access of phosphor-accepting His to ATP. This paradigm is supported by similar pathways observed with other HKs and RRs (55, 93, 101, 170).
Chapter 6: Effect of hemin binding on Hpk2 activation

6.1 Hpk2 shows hemin binding ability in SDS-PAGE with TMBZ staining

To test the hemin binding ability of r-Hpk2 and Hpk2 substitution mutants, purified wt r-Hpk2, Hpk2ΔPAS, Hpk2 (G44D, D49E, D49K, N58D, D156E, D156K) substitution mutants, and AtcS (a *T. denticola* sensor kinase that does not have a predicted heme binding pocket) were incubated with 20μg/ml hemin at 37°C for 1hr electrophoresed in an SDS-PAGE gel, and then incubated with TMBZ. Hemin binding to the proteins was detected by peroxidase activity of bound hemin in presence of TMBZ that generates a blue color. As controls, Hemin alone and Hpk2 alone was also subjected to the above treatment after running on SDS-PAGE. The r-Hpk2 incubated with hemin gave a strong reaction indicating its binding to hemin (Figure 20, lane 3), while Hpk2 without hemin did not react with TMBZ to give a blue colored band (Figure 20, lane 2). This indicates that the purified Hpk2 can bind hemin *in-vitro*. When r-Hkp2ΔPAS was also incubated with hemin, it was unable to develop the blue color band as a result of peroxidase activity (Figure 20, lane 4). Thus, suggesting that hemin binding of Hpk2 occurs at the PAS domain possibly via the putative heme binding pocket extending from amino acid 92-131. The Hpk2 substitution mutants were also tested for their hemin binding ability. All recombinant proteins (Figure 20,
Figure 20. Hemin binding by Hpk2 assessed using TMBZ staining.
Tetramethyl benzidine staining of SDS PAGE gel to detect hemin bound protein was performed. 3000ng of each protein; Hpk2, Hpk2ΔPAS, Hpk2 substitution mutants along with AtcS were incubated with Hemin (20μg/ml) at 37°C for 1hr ran in a SDS PAGE gel. Detection of hemin-protein complexes was accomplished by staining the gel with 3′, 3′, 5′, 5′ Tetramethylbenzidine; a chromogen that is turned blue by proteins with hemin associated peroxidase activity. AtcS was used as a negative control protein without a predicted heme binding domain to confirm the specificity of hemin binding to Hpk2.
Hemin alone
Hpk2 alone
Hpk2 + Hemin
Hpk2ΔPAS + Hemin
Hpk2(G44D) + Hemin
Hpk2(D49E) + Hemin
Hpk2(D49K) + Hemin
Hpk2(N58D) + Hemin
Hpk2(D156E) + Hemin
Hpk2(D156K) + Hemin
AtcS + Hemin
lane 5-10) except Hpk2 (D156K) showed hemin binding ability as detected by the blue colored band of appropriate MW observed on the gel. Considering Hpk2 (D156K) had lost kinase activity under both aerobic and anaerobic conditions, and is not able to bind hemin; it is clear that changing the charge and structure of the amino acid at position 156 has affected the structural conformation (in this case dimerization ability) of this protein that has rendered the protein into an OFF confirmation. Thus indicating that, Aspartate at position 156 of PAS domain in Hpk2 is critical residue to maintain the 'ON' integrity of the protein for a positive regulation of ligand binding (hemin and ATP). A better understanding to this data can be provided by crystallography studies to determine the structural changes in wt and Hpk2 substitution mutants.

6.2 Quantification of hemin binding to r-Hpk2

Since Hpk2 (D156K) that had lost its autokinase and hemin binding ability, it stands intriguing if Hpk2 (D49K) and (D156E) that had altered autokinase ability have any regulation in its hemin binding ability. To assess this, recombinant proteins of Hpk2, Hpk2ΔPAS and Hpk2 substitution mutants along with AtcS were immobilized to 96 well ELISA plates and incubated for 2 hr with hemin 20μg/ml, washed, reacted with TMBZ and hemin binding was quantified from the end point change in color at 450nm due to peroxidase activity of hemin
Figure 21. Quantification of hemin binding to Hpk2. A graphical representation of amount of hemin binding to Hpk2 and Hpk2 substitution mutants was determined based on its ability to show hemin-associated peroxidase activity. The color change was measured as absorbance at 450nm on ELISA plate reader. Asterisk indicates significance with p value less than 0.05.
bound to protein. r-Hpk2 as seen in Figure 21 showed hemin binding activity along with Hpk2(G44D), Hpk2(D49E), and Hpk2(N58D). Consistent with the results obtained in section 6.1, Hpk2 (D156K) had lost its hemin binding ability and there is significant reduction (p<0.05) in the endpoint color developed from hemin binding to Hpk2(D156K). Interestingly, Hpk2 (D49K) and Hpk2 (D156E) that had lost its autophosphorylation ability under anaerobic condition had a significant drop in hemin binding (p<0.05) as compared to the wt Hpk2 (Figure 21). Thus, the mutations at D49 and D156 in Hpk2 not only affect its ability to regulate kinase activity but also its hemin binding ability. These data suggest that the amino acids at these positions in Hpk2 are critical to maintain the charge and structure integrity on Hpk2 for ligand binding that is required to respond to environmental changes in the subgingival crevice.

6.3 Kinase assay of Hpk2 in presence and absence of hemin

Based on the data, rHpk2 responded to environmental signals under anaerobic conditions via its PAS domain (previous studies chapter 5), and in the current study the responsiveness of Hpk2 to in-vitro hemin binding; it can be hypothesized that, under anaerobic condition in the subgingivial crevice T. denticola may also be sensing and responding to heme available in the periodontal pocket from damaged gingival tissues and lesions as an
environmental stimulus. To test this hypothesis an experiment was set where rHpk2 was incubated with hemin at 37°C for 1 hr before carrying out kinase assays under aerobic and anaerobic conditions. For comparison Hpk2 not bound to hemin was also used to perform kinase assay under aerobic and anaerobic conditions. Autophosphorylation reactions were set up as detailed in methods and material under aerobic and anaerobic conditions. Difference in Hpk2 autophosphorylation pattern was observed on autoradiogram (Figure 22A). From the Figure 22A, it can be clearly seen that ³²P incorporation in r-Hpk2 was more under anaerobic condition in presence of hemin than in absence of hemin. r-Hpk2 autophosphorylation was relatively low under aerobic condition irrespective of presence or absence of hemin. This observation was confirmed by quantitative measurement of ³²P incorporation into Hpk2 under each condition. Under aerobic condition no significant difference in phosphate incorporation in r-Hpk2 was observed in presence or absence of hemin (p>0.005) (Figure 22B). However, under anaerobic condition in presence of hemin or when associated with hemin; r-Hpk2 showed significant increase in phosphate incorporation (p<0.05) (Figure 22B) than in absence of hemin. As observed previously in section 5.1, the phosphate incorporation in r-Hpk2 incubated without hemin under anaerobic condition, was slightly higher than phosphate incorporation in r-Hpk2 under aerobic condition, but the difference was not significant.
Figure 22. Hpk2 auto phosphorylation in presence and absence of hemin. 
Kinase assay of Hpk2 incubated with or without hemin under aerobic and anaerobic conditions were performed. A. top panel compares autophosphorylation of Hpk2 in presence and absence of hemin under anaerobic conditions. Middle panel compares autophosphorylation of Hpk2 in presence and absence of hemin under aerobic conditions. Bottom panel is an immunoblot of kinase assay performed with non labelled ATP and screened with anti-his to show equal loading of proteins. B. Autophosphorylation of Hpk2 associated with or without hemin under aerobic and anaerobic conditions was assessed with incorporation of $^{32}$P serving as the read out. All assays were conducted in triplicate and variance determined. Asterisks indicate statistically significant differences between presence and absence of hemin.
A. Wt Hpk2

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B. Hpk2 Kinase assay

PO₄ incorporation (fmols) vs. min

- An(+H)
- An(-H)
- A(+ H)
- A(- H)
6.4 Discussion

Physiological adaptation of an organism to changing environmental conditions requires molecular sensors capable of sensing and signaling specific physico-chemical parameters. PAS domain in most bacteria and archaea is involved in the perception to these external signals (65, 130, 154, 178). In the previous study it was shown that Hpk2 PAS domain responds to redox state. The response to the redox state signal was by regulating Hpk2 activation under different environmental conditions. The amino acids that governed the autokinase ability by possibly triggering a specific conformational change (oligomeric state) in the Hpk2 protein or maintain the stability of ATP-Hpk2 catalytic domain under anaerobic conditions were identified. This current study was set out to identify additional signals to the sensor PAS domain and the molecular basis to this sensing. These multiple signal sensing may be required by this two component system to fine tune the regulatory pathways of Hpk2-Rrp2 regulons. The above data showed that Hpk2 binds hemin (heme with FeIII) via its PAS domain confirming that the PAS domain heme binding pocket interacts with hemin in-vitro. Most PAS domain responding to redox states are heme-binding oxygen sensors. Consistent with this fact, the Hpk2 PAS domain which was showed to sense redox state, could also bind hemin. This data is consistent with TCS functions seen in other bacteria like Sinorhizobium meliloti. The FixL/FixJ
two-component regulatory system of *S. meliloti* controls the expression of nitrogen fixation genes in response to O$_2$. Dissociation of O$_2$ from the heme-binding PAS domain in FixL greatly accelerates the rate of its autophosphorylation (158). A similar effect was observed in hemin-bound PAS domain in Hpk2 which displayed accelerated rate of autophosphorylation under reduced anaerobic condition, supporting the hypothesis that the PAS domain in the histidine kinase of Hpk2 serves as a heme-bearing molecular oxygen sensor. Upon oxygen binding by the ferrous iron within the heme group, the PAS domain changes its confirmation and inhibits the activity of the histidine kinase domain, thus in-turn regulating the activity of Rrp2 transcription factor in an oxygen dependent manner. This mechanism of environmental sensing by Hpk2 PAS domain can be corroborated to *T. denticola* adaptation in the periodontal pocket, as the pocket depth increases the soluble O$_2$ level drops along with infiltration of heme from the blood oozing out of the lesions and tissues damaged at the periodontal infection site. This condition might set an ideal platform for *Td* Hpk2 to sense the low O$_2$ levels or reduced conditions via its heme-bound PAS domain and allow the activation of the Hpk2-Rrp2 TCS for global gene regulation in *T. denticola*.

The molecular basis of hemin binding by Hpk2 was assessed in the similar way as the molecular basis of PAS domain autokinase regulation of Hpk2 was
assessed. Hpk2 substitution mutants were tested for hemin binding using TMBZ staining. The binding was also quantified by spectrophotometric analysis of change in color developed due to the hemin-Hpk2 complex formation that showed peroxidase activity in presence of TMBZ. A similar profile of protein activity was observed as seen with autokinase activity regulation. r-Hpk2 showed binding to hemin, along with Hpk2 (G44D), Hpk2 (D49E), Hpk2 (D49K), Hpk2 (N58D), and Hpk2 (D156E) except Hpk2 (D156K). This result is consistent with the previous data where Hpk2 (D156K) was the only Hpk2 substitution mutant that had completely lost its autokinase ability. Quantitative analyses also showed that though Hpk2 (D49K) and Hpk2 (D156E) showed hemin binding, the amounts of hemin bound to these proteins were significantly low. This variation observed in the hemin binding ability of Hpk2 substitution mutants, in part explains the relationship between ligand binding and oligomerization state where ligand such as heme binding initiates dimerization of the sensor kinase. Proteins in apo form exist in monomers and in most cases as dimers in holo forms. Extracellular signals such as heme binding have been shown to regulate the activity of sensor kinase. This was observed in crystal structure studies of the sensor domain of CitA, a sensor kinase of *Klebsiella pneumonia*; involved in induction of citrate fermentation genes under anaerobic condition along with its cognate RR CitB. Comparison of crystal structures of CitA sensor domain in
citrate free and citrate-bound states showed that ligand binding caused contractions of sensor domain and activated transmembrane signaling to the receptor domain. Under citrate-bound state CitA crystal structure showed a dimeric form of this HK as oppose to monomeric form existence under citrate free condition (143). Nan B. et al; 2010 also showed a ligand-induced dimeric switch and a strong correlation between DctB’s (a membrane integrative sensor histidine kinase that transports C₄-dicarboxylic acids) kinase activation. Mutations at different sections of DctB’s dimerization interface can lock full-length DctB at either ‘on’ or ‘off’ state, independent of ligand binding suggesting that ‘ligand-induced dimeric switch’, which changes the dimeric conformations upon ligand binding are responsible for the signal transduction in DctB (117). In a similar study, comparing the apo structure and the succinate-bound one, succinate binding induces conformational changes to dimeric levels. At the dimeric level, the binding pocket is tightened by the binding of succinate (177). A similar effect was seen by hemin binding to Hpk2 PAS domain, suggesting that hemin binding to Hpk2 might initiate a tighter dimeric association that positively induces signal transduction and kinase activation of Hpk2. A better understanding to this can be gained with further crystallographic study on the Hpk2 signal transduction states in ligand-free and ligand-bound states. From the above data it is clear that mutations in Hpk2 affecting dimeric formations cannot hold the ligand, in this
case hemin to form a functional holo enzyme that initiates kinase activity and further activation of response regulator. Hpk2 may also follow a similar molecular adaptation as sensor kinases DctB and CitA, where it exists in monomeric form and upon encountering environmental stimulus (redox state and heme), the binding of ligand (heme) to Hpk2 PAS domain initiates dimeric forms of Hpk2 that can now capture ATP stably and allow the kinase domain to hydrolyze ATP for self activation. Thus dimerization of Hpk2 that is initiated in part by PAS domain ligand binding is the key element for Hpk2 to persist in an “ON” conformation via constant interaction with ATP in a stable conformation.
Chapter 7: Allelic Exchange Mutagenesis to study the Global Regulatory Capabilities of Rrp2

7.1 Generation of Rrp2 mutant.

A master allelic exchange construct pJS236C containg Rrp2 with amino acid substitutions at G236 and G237 to C was generated as shown in Figure 23A and as detailed in the text. The linear plasmid was electroporated into competent T. denticola 35405 cells. Selection of colonies with gene insertion was accomplished with an Erythromycin resistance cassette (Erm’) downstream to rrp2 and Tde1968 open reading frame. Colonies appeared within seven days of plating. Seven colonies were picked and sub cultured in NOS media with erythromycin for further analyses. As control plates, T. denticola 35405 competent cells electroporated with H2O instead of DNA were plated on NOS plates with and without Erythromycin. Colonies growing on plate without erythromycin was positive control for the electroporation conditions. No colonies on NOS plates with erythromycin showed T. denticola 35405 strain is sensitive to erythromycin and only those colonies growing in presence of erythromycin that express the Erm’ cassette are true mutants.

7.2 Screening of transformants
Figure 23. Verification of *rrp2* allelic exchange mutagenesis. To confirm allelic exchange mutagenesis was successful, PCR analyses were performed. A schematic depicting the genetic arrangement of the chromosome after allelic exchange mutagenesis is shown. The letters above each set of PCR reaction are in reference to the alphabet above the chromosome schematic indicating the specific primer set used to obtain that particular PCR product.
To assess that the transformants expressing Erm\(^\prime\) resistance have undergone allelic exchange at the right genetic location, PCR was performed as shown in Figure 23B. All seven colonies isolated showed the same PCR screening pattern as seen in Figure 23B. The positive PCR products for specific primers was considered as successful transformation.

### 7.3 Verification of sequence of the replaced rrp2

To analyze the proper replacement of wt rrp2 (Tde1969) with rrp2 (G236&7C), rrp2 was amplified from the individual colonies expressing Erm\(^\prime\) resistance. The amplicons were sequenced to ensure that the desired sequence change was introduced into rrp2 and also no other change was introduced into the rrp2 sequence other than the desired. None of the transformants had the desired amino acid substitution in its rrp2 sequence when compared with wt rrp2. Thus it can be concluded that none of the transformants that survived were mutants to be used for studying global gene regulation by Rrp2 in *T. denticola*.

### 7.4 Discussion

Several attempts to delete or mutate Rrp2 in *T. denticola* 35405 strain were made. The unsuccessful attempts led to the reasoning that since Rrp2 is contained in a large operon with FtsJ, a cell division gene downstream to it.
Deletion of Rrp2 may disrupt the downstream expression of this cell division gene. To overcome this hurdle, *rrp2* was fused with *ftsJ* and the resistance marker was introduced downstream to these genes in the plasmid construct to ensure expression of all the genes in the *hpk2-rrp2* operon in the *T. denticola* Rrp2 mutant and gene regulation in the mutant is only attributed to non-functional Rrp2. Successful allelic exchange of the externally introduced linear pJS236C plasmid sequence at the correct chromosomal location in *T. denticola* was achieved, keeping the Erm’ cassette downstream of tde1968, to ensure no obstruction of any gene expression in the Hpk2-Rrp2 operon. However from the screening of the colonies it was found that the insertion of the master construct at the right place. Yet, no substitution in *rrp2* sequence suggests that the homologous recombination did not occur all the way and a partial recombination of the genes occurred. It is also possible with the fact that complete homologous recombination occurred but only those spirochetes survived which had partial recombination and no replacement of wt *rrp2* with *rrp2*(G236&7C). Thus proving that deletion or mutation altering the function of Rrp2 in *T. denticola* may be lethal to the bacteria. Rrp2 may be controlling the expression of genes vital for the growth or division of *T. denticola* and inactivation of Rrp2 in this spirochete does not let the cells to survive or divide. Also considering the fact that Rrp2 is a part of an operon expressing vital genes for cell growth and survival, the
possibility of Rrp2 controlling the regulation of these genes are high, hence rendering Rrp2 non functional may result in shut down of Hpk2-Rrp2 operon and cell death. To determine the role of Rrp2 other alternative methods can be used like overexpression of Rrp2 in \textit{T. denticola} and further comparison of global gene regulatotion between wt and Rrp2 overexpressor strain.
Chapter 8: Conclusion

More than 70 Treponemal species reside in the oral cavity (127, 129), that represent a low percentage of the total bacterial mass of the sub-gingival crevice in healthy individuals. However, spirochetes become the dominant species in the periodontal pocket as disease progresses (40, 95). The molecular basis of the adaptive responses associated with successful outgrowth of spirochetes during periodontal disease has not been delineated. The T. denticola genome encodes several two-component systems, orphan kinases and orphan response regulators (49, 141) that are likely to be key mediators of adaptive responses. This project report highlights the findings that T. denticola Hpk2 and Rrp2 proteins form a functional two-component system that plays a role in sensing changes in environmental conditions. As detailed above, the rationale for studying this particular system and for the nomenclature applied, stemmed from the homology of these proteins to the B. burgdorferi Hpk2-Rrp2 two-component system, a key transducer of environmental signals (11, 12, 19, 126, 172). The molecular properties of hpk2 and rrp2 assessed using PCR, DNA sequence and database analyses revealed that these genes are universal and the putative functional domains and residues of hpk2 and rrp2 are conserved among all T. denticola isolates that were analyzed. Hpk2 is a highly conserved 46 kDa protein
that is universally distributed among *T. denticola* strains and harbors an N-terminal PAS domain which has been demonstrated to detect specific environmental stimuli (61, 113). The PAS domain is followed by an H-Box with 3 possible His autophosphorylation sites (H185, H197 and H219) and an H-ATPase catalytic domain domain (ATP-Mg\(^{2+}\) binding sites) that spans the C-terminal third of the protein. Rrp2 is a conserved 52 kDa protein that is carried by several spirochete species as well. Rrp2 possesses 3 conserved putative phosphoacceptor Asp residues (positions 45, 48 and 53) that reside within its receiver domain. The ortholog of Rrp2 in *B. burgdorferi* is a σ\(^{54}\)-dependent response regulator that carries out functions essential for viability by serving as an activator of the RpoN-RpoS regulatory pathway (172) where RpoN upregulates RpoS which in turn positively regulates genes encoding proteins involved in the spirochete transmission from ticks to mammalian hosts (76, 126, 149). In an attempt to determine the role of Rrp2, wild type *rrp2* in *B. burgdorferi* was replaced with a site-directed mutant *rrp2* deficient in ATP binding that abolished the transcriptional expression of several important, plasmid-encoded virulence factors in *B. burgdorferi*. Many of the genes regulated by Rrp2 had been demonstrated to be regulated by environmental variables including temperature and pH (123, 132). In view of the critical role of Rrp2 in the *Borrelia* it was hypothesized that Rrp2 in *T. denticola* may also play a pivotal role in
regulating transcriptional responses to environmental changes. The genes encoding histidine kinase-response regulatory pairs are commonly adjacent to one another, closely spaced and transcribed as a polycistronic mRNA (54). Consistent with that paradigm hpk2 and rrp2 were demonstrated by RT-PCR to be co-transcribed and were shown to be regulated by growth phase with a dramatic upregulation during late log phase. hpk2/rrp2 was found to be transcribed as part of larger operons consisting of ORFs Tde1974-Tde1968. It is possible that the dominant transcriptional unit could be different during growth in the human host or upon exposure to specific environmental conditions. Additional analyses of transcription start site for hpk2-rrp2 operon identified two TSS for this operon. One TSS was identified just upstream of Tde1971 and other upstream of Tde1974. The promoter sequence analysis for the hpk2-rrp2 primary transcript consisting of genes from Tde1971 to Tde1968 showed presence of $\sigma^{54}$ -12 and -24 promoter consensus sequence (5). The secondary TSS mapped upstream to Tde1974 showed that the locus is defined by $\sigma^{70}$ consensus -10 and -35 consensus sequences (167). The putative functions of these genes co-transcribed with hpk2-rrp2 provided possible insight as to how the upregulation of this operon could contribute to the rapid outgrowth of T. denticola in periodontal pockets. Tde1968 (FtsJ), Tde1971 (DnaX), and Tde1974 (MurG) all encode proteins that carry out functions that are required for rapid growth. FtsJ is a 23S
rRNA methytransferase that stabilizes the 50S subunit within the 70S ribosome allowing for efficient translation (68). In B. burgdorferi, inactivation of this gene results in impaired growth and morphological abnormalities (115). DnaX (Tde1971) encodes the gamma/tau subunit of DNA polymerase III, a protein involved in DNA replication (99). MurG (Tde1974), a glycosyltransferase, catalyzes a terminal step in peptidoglycan synthesis. MurG is essential for cell viability and may be part of the divisome (114). Lastly, ORFs Tde1972 and Tde1973 are annotated as a possible peptide toxin and colicin V production factor, respectively. While the specific role or activity of these particular proteins has not been directly demonstrated, they may function to inhibit the growth of competing organisms in the subgingival crevice. The collective up-regulation of Hpk2-Rrp2 and its adjacent genes could be envisioned as a significant contributing factor in facilitating the rapid growth of T. denticola.

The ability of Hpk2 to autophosphorylate and transfer phosphate to their cognate response regulators was demonstrated. It is clear that accessory proteins for this process are not required since transfer occurs with defined purified proteins. Interestingly, efficient phosphotransfer required that the kinase first be preloaded with phosphate prior to its incubation with response regulator. The basis for this phenomenon may lie in the requirement for kinase dimerization to transfer phosphate. Dimerization of histidine kinases generates a possible
stable structure that is required for the response regulator interaction and subsequent phosphotransfer (108, 121), this phenomenon was better explained in the later studies in this report.

*T. denticola* grows optimally under anaerobic conditions but it can survive for short periods in the presence of oxygen (111). Exposure to oxygen results in increased production of hydrogen sulfide which; further depletes oxygen restoring an anaerobic micro-environment (87). The PAS domain of Hpk2 harbors a putative heme binding pocket suggesting a possible role for the Hpk2-Rrp2 two component regulatory system in sensing and mediating responses to oxygen concentrations. To determine if the PAS domain influences autophosphorylation in the presence or absence of oxygen, Hpk2 recombinant protein with a N-terminal 122 aa truncation variant lacking the PAS domain (Hpk2ΔPAS) was generated and then assessed for its ability to autophosphorylate under anaerobic and aerobic conditions in comparison with full-length r-Hpk2. When the reactions were conducted under aerobic conditions, no significant difference in phosphate incorporation was observed between Hpk2 and Hpk2ΔPAS (p>0.05). However, under anaerobic conditions Hpk2ΔPAS displayed a significant reduction in phosphate incorporation relative to Hpk2 (p<0.05). While Hpk2ΔPAS retained its autophosphorylation activity under both aerobic and anaerobic reaction conditions, the truncated protein was not
competent to transfer phosphate to Rrp2. It was concluded that under anaerobic conditions, the autophosphorylation activity of the Hpk2 PAS is responsive to oxygen concentration.

Further analysis of molecular basis of environmental adaptation by PAS domain and its influence on regulating signal transduction, recombinant Hpk2 proteins with site-directed mutations at conserved residues in PAS domain were generated. The mutations G44D, D49E and N58D in the individual Hpk2 proteins were mutation in which the amino acid substitutions did not have any significant impact on the function of Hpk2 assessed in different experiments. D49K, D156E or D156K substitutions in Hpk2 showed variation in autokinase activity of Hpk2. Hpk2 (D156K) lost its autophosphorylation ability under both aerobic and anaerobic conditions. However Hpk2 substitution mutants D156E and D49K did not show autokinase ability only under anaerobic conditions. This data suggest that these mutations in Hpk2 altered the PAS domain orientation in such a way that the conformational change that induces activation of the Dimerization/Histidine phosphate (DHp) domain and catalytic domain (CA) for efficient kinase activity. Consistent with the above data, Hpk2 mutants G44D, D49E and N58D did not show any effect in phosphotransfer ability to the cognate response regulator Rrp2 in comparison with the wt full-length Hpk2. Since, Hpk2 (D156K) had lost its autophosphorylation ability under both aerobic and anaerobic
conditions it could not be tested for its ability to transfer phosphate. Even though Hpk2 (D49K) and Hpk2 (D156E) retained the autophosphorylation ability under aerobic conditions further analysis for effect of mutation on their phosphotransfer ability showed a reduced phosphotransfer by Hpk2 (D49K) and complete loss of phosphotransfer by Hpk2 (D156K) respectively. BN-PAGE analyses under non reduced conditions of all the Hpk2 substitution mutants and wt r-Hpk2 revealed that like the wt Hpk2 protein, Hpk2 (G44D), Hpk2 (D49E), Hpk2 (D49K) and Hpk2 (N58D) showed high levels of dimerization as well as higher order oligomerization. In the same BN-PAGE analyses, Hpk2 (D156K) on the other had showed existence only in monomeric forms along with Hpk2 (D156E) which also exclusively existed in monomeric form but still formed dimers and trimers to some extent. This phenomenon explains the changes in the autophosphorylation and phosphotransfer functions of Hpk2 due to the mutations at D156. The change in amino acid charge and structure at position D156 caused severe hindrances in oligomer formation of Hpk2. Change in the structure alone in amino acid at position 156 (D to E) could still retain dimeric and trimeric forms, however the forms may not be a stable association. The same phenomenon explains the loss of phosho transfer ability of Hpk2 (D156E), which requires stable oligomeric formation of the sensor kinase for interaction with response regulator and subsequent phosphotransfer. Since Hpk2 (D49K) substitution mutant did not
show any change in its oligomeric state compared to the wt Hpk2, the phenomenon of loss of autokinase ability under anaerobic conditions and delayed phosphotransfer ability can be explained as a lack of stronger association with ATP. The change in the charge of amino acid at the position 49 from negative to positive might have altered the total charge distribution of Hpk2 that results in weak interaction of ATP at the PAS domain that is required for sponsoring the ATP molecule to the kinase and catalytic domain of the Hpk2 sensor kinase.

The Hpk2 PAS domain possesses a putative heme binding pocket supporting its possible role in sensing changes in oxygen concentration via the bound heme-FeIII ability to associate with oxygen. Changes in oxygen concentration are pronounced in the subgingival crevice as periodontal disease progresses (110). Wt Hpk2 showed hemin binding via the PAS domain as assessed by TMBZ staining. All the Hpk2 substitution mutants except Hpk2 (D156K) showed hemin binding. Hpk2 (D156K) which did not show autokinase ability had also lost its hemin binding ability this can be attributed to its loss of exisance in oligomeric form which is a pre-requisite for ligand binding. In further analysis of quantification of hemin binding by Hpk2 and its PAS domain substitution mutants, Hpk2 (D49K) and Hpk2 (D156E) showed significant drop in hemin binding ability. This reduced interaction of the Hpk2 (D156E) mutant with
hemin might also be attributed to the lack of stable oligomer formation of this mutant. Ligand binding to kinase requires and also initiates further dimer and oligomer formations (143). All the above phenotypes imparted by Hpk2 and Hpk2 substitution and deletion variant is summarized in Table 7.

These data also present the positive impact of ligand binding on signal transduction ability of Hpk2, where hemin binding positively upregulated the autokinase ability of Hpk2 only under anaerobic conditions. These findings support the model that PAS domains which are heme-binding oxygen sensors respond to reduced condition by regulating the activation of the sensor kinases via oxygen sensing by the heme ligand present in it in a stable complex. One example is the FixL/FixJ two-component regulatory system of *Sinorhizobium meliloti* that controls the expression of nitrogen fixation genes in response to O₂. Dissociation of O₂ from a heme-bound PAS domain in FixL greatly accelerates the rate of its autophosphorylation (158). A general paradigm to explain the above phenomenon can be put together in a predicted model for Hpk2 activation based on its association with heme (Figure 24). Upon oxygen binding by the ferrous iron within the heme group, the PAS domain in Hpk2 changes its confirmation and inhibits the histidine kinase domain, further regulating the activity of Rrp2 transcription factor in an oxygen dependent manner. This mechanism of environmental sensing by Hpk2 PAS domain can be corroborated
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ND, Not Determined; +++, Higher activity; ++, reduced activity relative to wt; +, significantly reduced activity relative to wt (p value <0.5); -, No activity relative to wt.

Asterisk: + + +, Higher order oligomeric forms (Monomer, Dimer, Tetramer and Pentamer); +, reduced oligomeric forms compared to wt (Monomer, dimer and trimer); -, only monomeric forms.

Note that only autophosphorylation of Hpk2 and Hpk2 substitution and deletion variants were assessed under aerobic and anaerobic conditions, for all other assays the condition were standard lab conditions (aerobic, bench top)
Figure 24. Predicted model of Hpk2 activation. Under aerobic conditions, the heme group associates with O₂ and results in folding of the kinase and catalytic domain of the protein into an ‘OFF’ conformation. At low oxygen condition, the PAS domain association with heme tightens, followed by dimerization of Hpk2 protein resulting in release of O₂ from the heme group leading to increased activation of Hpk2 kinase domain (‘ON’ conformation). This may ultimately result in increased activation of Rrp2 and in turn regulation of Rrp2 regulon.
to *T. denticola* adaptation in the periodontal pocket, as the pocket depth increases the soluble O$_2$ level drops along with infiltration of heme from the blood oozing out of the lesions and tissues damaged at the periodontal infection site. This condition might set an ideal platform for *T. denticola* Hpk2 to sense the low O$_2$ levels or reduced conditions via its heme-bound PAS domain and allow the activation of the sensor kinase Hpk2 and response regulator Rrp2 TCS.

Finally in light of the findings in this project it can be concluded that Hpk2-Rrp2, a functional TCS in *T. denticola* that may be regulating important genes in this spirochete that attributes to the survival and virulence of the genes. The findings that Hpk2 responds to environmental signals associated with the periodontal pocket conditions further confirms that Hpk2 activation is critical for this bacteria to sense the appropriate signals that triggers the downstream processes in *T. denticola* via activating the $\sigma^{54}$-dependent response regulator, which in Lyme disease spirochete has been shown to play vital role in activation of virulence genes (11, 12, 126, 172). This work sets out a platform for further investigation to identify the genes regulated by Rrp2 transcription activator. This current research also opens the possibility of further investigation on the Hpk2 PAS domain to determine the structure model of this sensor protein and the effect of amino acid substitution on the protein structure rearrangement.
Future analyses

The current research in this project was mainly focused at indentifying the \textit{in-vitro} role and mechanistics of Hpk2 sensor kinase in response to the environmental signals associated with periodontal pocket. For better understanding of the exact \textit{in-vivo} role of Hpk2-Rrp2 TCS, further analysis of the genes regulated by the Hpk2 and Rrp2 must be determined. Due to unsuccessful attempts to mutate or inactivate Rrp2 that is required for assessment of global gene regulation by a transcription regulator, alternative approaches can be adapted. One major obstacle encountered in \textit{T. denticola} for genetic manipulation is the low transformation efficiency of this spirochete. DNA restriction and modification (R-M) systems have been described as "immune systems" to defend against invading foreign DNA (116, 119). In many prokaryotes, R-M systems serve as genetic barriers for gene transformation, conjugation, and transfection (8, 38, 116, 156). R-M systems were recently identified in the Lyme disease spirochete \textit{Borrelia burgdorferi}, and disruptions of these systems were able to increase the transformation efficiency of foreign DNA (82, 88, 131). The genome of \textit{T. denticola} ATCC 35405 encodes three putative type II R-M systems: \textit{Tde0227} (MTase)/\textit{Tde0228} (REase), \textit{Tde0909} (MTase)/\textit{Tde0911} (REase), and \textit{Tde1268} (REase) (134). The existence of these R-M systems may prevent \textit{T. denticola} 35405 from accepting foreign
DNA, such as the shuttle vectors of *T. denticola* 33520. It was also found that these R-M systems were absent in *T. denticola* 33520 and that the inactivation of *Tde0911*, a gene encoding a type II restriction endonuclease, allowed the *T. denticola* 35405 mutant to accept the unmethylated pBFC shuttle vector. This opens up the possibility to constitutively expressing Rrp2 under the dentilisin promoter which is already engineered in pBFC vector (25, 60) and hence allows us to compare gene regulation in wild type and Rrp2 overexpressor strains using microarray analysis. Further, to determine the set of direct and indirect regulon of Rrp2 gel shift assays or electromobility shift assay (EMSA) can be used. Direct binding of Rrp2 to the DNA sequence upstream of the start codon of the target gene will confirm that the target gene is a direct regulon of Rrp2 and the other set of genes that do not show direct binding to Rrp2 at the upstream DNA sequence can be concluded as its indirect regulon.

In this study hemin was identified as a direct ligand of Hpk2 PAS domain that positively activates Hpk2 under anaerobic condition; it will be interesting to compare gene regulation in *T. denticola* 35405 grown in presence and absence of hemin. The list of genes differentially regulated in presence of hemin can be putative genes regulated by Hpk2-Rrp2 TCS in response to sensing hemin. The direct relation of Rrp2 and the genes differentially regulated in presence of hemin can be again determined using EMSAs.
This report majorly focuses towards the role of PAS domain in environmental sensing. Even though significant progress have been made in understanding the molecular basis of environmental adaptation by the PAS domain the exact role of each functional domain and the interplay of conformational shifts in the protein facilitating the sensing and signal transduction of a diverse array of signals present in the periodontal pocket is highly undefined. Structural study of the PAS domain conformational shifts and interplay between signal sensing, dimerization and kinase activation will provide detailed insight into the active sites and the regulation in signaling pathway of Hpk2. Crystallography studies of dimerization domain, catalytic domain and PAS domain along with few full length kinases have provided key insights into how histidine kinases function (1, 10, 21, 101, 169).
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VITA

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PUBLICATIONS:

- **Sarkar J**, and Marconi R.T., “The PAS domain of the *Treponema denticola* histidine kinase, Hpk2, plays a key role in adaptive responses to environmental stimuli” (in preparation)
• Sarkar J, and Marconi R.T., “Role of Rrp2, a σ54-dependent response regulator in Treponema denticola, identifying direct Rrp2 regulon by microarray analysis” (in preparation)

ABSTRACTS:
• Sarkar J., Frederick J., Marconi R.T., “Identification of a two component regulatory system of the periodontal pathogen, Treponema denticola: insight into the molecular mechanisms of environmental adaptation during the


**AWARDS:**

- 2010- American society of Microbiology Meeting student travel grant, Lynchburg, Virginia.

- 2009- The 26th Annual Graduate Research Symposium and Exhibit Participation Award, Richmond, Virginia.

- 2009- Mid- Atlantic Microbial Pathogenesis Meeting student travel grant, Wintergreen, Virginia.

- 2008- The 25th Annual Graduate Research Symposium and Exhibit Participation Award, Richmond, Virginia.

- 2008- Inducted as member of the Phi Kappa Phi Honor Society