Chapter-6
Summary & Conclusion
In the present thesis, BAS1213-1214 and BAS2108-2109 TCSs of \textit{B. anthracis} have been characterized and their response regulators have been elucidated as important transcriptional regulators modulating expression of different genes thereby affecting various physiological processes in \textit{B. anthracis}.

**The main findings of the characterization of BAS1213-1214 TCS include:**

- The genes encoding BAS1213 and BAS1214 of \textit{B. anthracis} Sterne strain were identified as putative TCS.
- SMART database predicted BAS1213 as the 223 amino acid RR with C terminal receiver domain and N terminal DNA binding domain. BAS1214 was predicted as 466 amino acid HK having two transmembrane regions, HAMP domain, HisKA domain and HATPase\_c domains.
- Both the genes were found to exist in an operon with a common promoter region.
- \textit{BAS1213} and \textit{BAS1214} ORFs were cloned in pET28a (+) and pMALc2x vectors respectively and expressed in \textit{E. coli} BL21 (λDE3) cells.
- Recombinant BAS1213 and BAS1214 proteins were purified by Ni-NTA and amylose affinity chromatography respectively.
- The purified recombinant BAS1213 and BAS1214 proteins were tested for their biochemical activities. BAS1214 showed autophosphorylation \textit{in vitro} confirming it as an active HK. Phosphotransfer between BAS1214 and BAS1213 was observed, confirming BAS1213 as cognate RR and thus, established BAS1214-1213 as a functional TCS.
- Although acetyl phosphate was found to phosphorylate BAS1213, phosphorylation was not efficient.
- Divalent cations were found to be essential for phosphorylation of BAS1213-1214 TCS. Among the tested divalent cations, Mg\textsuperscript{2+} ions were the most efficient cations for phosphorylation.
- The phosphorylation of BAS1213 was acid stable and base labile indicating phosphoaspartate bond at the phosphorylation site. MSA with different RRs predicted Asp-54 as the residue essential for phosphorylation.
- The phosphorylation of BAS1214 was base stable and acid labile indicating histidine residue at the phosphorylation site. MSA with different HKs predicted His-248 as the residue essential for phosphorylation.
In silico analysis of BAS1213 regulon identified 187 genes as its target genes in *B. anthracis*. After COG database analysis, 117 genes were predicted to be associated with metabolic pathways, 26 were transporters, and nine were implicated in sporulation while only three were virulence related genes and one was enterotoxin gene.

Being a transcriptional regulator, BAS1213 was able to bind to the promoter regions of *BAS0803* (Catalase), *BAS1252* (Sporulation kinase D), and *BAS1752* (Manganese transporter, mntH).

Like other transcription factors, BAS1213 was shown to regulate its own transcription by binding to its promoter region.

BAS1213-1214 and PhoPR TCS of *B. anthracis* were found to transcriptionally regulate each other suggesting cross-talk between the two TCSs.

MSA and site directed mutagenesis revealed Val-171, Asp-194, Thr-213 and Val-214 as the critical residues essential for the DNA binding activity of BAS1213.

qRT-PCR demonstrated that BAS1213 expression increases during stationary phase of growth, oxidative and bicarbonate stress.

BAS1213 was overexpressed in *B. anthracis* by cloning in pHCMC05, *E.coli-Bacillus*, shuttle vector. qRT-PCR and western blotting confirmed overexpression of BAS1213 upon induction with 1mM IPTG.

Overexpression of BAS1213 in *B. anthracis* resulted in slower growth rate and reduced cell size.

Regulatory effect of BAS1213 in *B. anthracis* on its target genes (*BAS0803, BAS1252, BAS1743*) was evaluated in *B. anthracis* overexpressing BAS1213 by qRT-PCR and increase in expression of the transcript of these genes was observed.

BAS213 was found to be important in sporulation process as the spore titre decreased in *B. anthracis* overexpressing BAS1213.

BAS1213 was further implicated in its role in oxidative stress due to its regulation of catalase and mntH along with augmented expression during oxidative stress.

The key findings of the characterization of BAS2108-2109 TCS include:

- The genes encoding BAS2108 and BAS2109 of *B. anthracis* Sterne strain were identified as orthologs of DegUS TCS.
SMART database predicted BAS2108 as 372 amino acid HK comprising of GAF domain and HATPase_c domain. Likewise, BAS2109, a 210 amino acid RR consisting of receiver domain and DNA binding domain.

Both the genes were found to co-transcribe in an operon sharing a common promoter region.

BAS2108 and BAS2109 ORFs were cloned in pMALc2x and pET28a (+) vectors respectively and expressed in E. coli BL21 (λDE3) cells.

Recombinant BAS2108 and BAS2109 proteins were purified by amylose and Ni-NTA affinity chromatography respectively.

The purified recombinant BAS2108 and BAS2109 exhibited properties of HK and RR in vitro respectively. BAS2108 showed autophosphorylation and phosphotransfer to BAS2109, thus, conforming BAS2108-2109 as a functional TCS.

Phosphorylation of BAS2109 was observed with acetyl phosphate, however, it was not very efficient. Mg$^{2+}$ was found to be the most efficient cations for phosphorylation reaction.

The phosphorylation of BAS2108 was base stable and acid labile representing histidine residue at the phosphorylation site. MSA with different HKs predicted His-193 as the residue important for phosphorylation.

The phosphorylation of BAS2109 was acid stable and base labile indicating presence of aspartic acid at the phosphorylation site. MSA and site directed mutagenesis confirmed Asp-10 and Asp-50 as the essential residues for phosphorylation.

BAS2109 demonstrated binding with its promoter region and its activation as confirmed by EMSA and reporter gene assay respectively.

Being a transcriptional regulator, BAS2109 showed binding with promoter regions of 8 different proteases (BAS0567, BAS0638, BAS1197, BAS1859, BAS2216, BAS3198, BAS4252, and BAS4907) of B. anthracis and also with SpoVR (BAS0731), sspB (BAS0815), wapA (BAS1022), YvcA (BAS1079), and sigF (BAS3983), suggesting them to be a part of BAS2109 regulon.

MSA and site directed mutagenesis revealed Lys-167, Thr-179 and Thr-182 as the crucial residues involved in the DNA binding activity of BAS2109.
qRT-PCR revealed uniform expression of BAS2109 throughout the growth phases, however, BAS2109 expression elevated under nutritional stress, CO₂ and during pellicle formation.

BAS2109 overexpression was achieved in B. anthracis by cloning in pHCMC05 shuttle vector and overexpression was confirmed by qRT-PCR and western blotting.

Overexpression of BAS2109 in B. anthracis had no effect on growth but resulted in decrease in chain formation as compared to the wild type.

BAS2109 was implicated as a transcriptional regulator of proteases in vivo since overexpression of BAS2109 in B. anthracis resulted in altered expression of the proteases.

BAS2109 was found to affect multicellular behavior of B. anthracis such as biofilm, and pellicle formation, and swarming motility.

Overexpression of BAS2109 resulted in increase in sporulation efficiency, however, spore germination ability was reduced in comparison with B. anthracis.

Overall, our study establishes BAS1213-1214 and BAS2108-2109 as functional TCS in B. anthracis. This study reports the role of BAS1213-1214 in the regulation of metabolism and adaptive responses during oxidative stress and sporulation. Both sporulation and response to environmental oxygen are important for the maintenance of B. anthracis lifecycle, therefore, characterization of BAS1213-1214 provides a step closer towards understanding the regulatory network governing in B. anthracis. This is also the first report to identify a TCS pair for its role in the regulation of proteases and multicellular behavior of B. anthracis. Importance of proteases in the pathogenesis of B. anthracis is well documented, therefore, studying the regulatory networks governing their expression will help in identification of new drug targets. Different biochemical approaches used to decipher amino acid residues involved in protein-protein and protein-DNA interactions can be explored in future for their potential as drug targets.