The increased use of antibiotics enables the clinically significant pathogen to gain tolerance to the life-saving drugs and in the current scenario, which is quite alarming, there is an upsurge in the incidence of drug-resistant infections and antibiotic treatment has been a failure for many severe bacterial infections. In 2008, some of the most notorious pathogens were grouped together by Rice et al., and given an acronym "ESKAPE" which includes *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter spp.* (Rice, 2008). One of the ESKAPE pathogens which are emerging as a major cause of nosocomial infection especially in clinical settings is *Acinetobacter baumannii*.

A. baumannii is a potent human pathogen which has shown resistance to even the last line of drugs because of its versatile arsenal of resistance determinants. There are many reports throughout the world where MDR *A. baumannii* has been shown to cause various diseases in immunocompromised patients. The emergence of this pathogen and the scarcity of new antibiotics is leading us again to the pre-antibiotic era where antibiotics won't be effective against any type of infections leading to the increase in mortality and morbidity. Therefore, there is a pressing need to identify new promising drug targets and also keep a check on the changing resistance profile of the emerging *Acinetobacter* strains. TCS shows a remarkable potential to be considered as novel drug targets, surprisingly though many *A. baumannii* genomes have been sequenced, the true biological functions of signaling pairs in cellular physiology in general and antimicrobial resistance in particular; have remained unexplored so far. One such TCS which has been characterized is the GacAS in *A. baumannii*, though preliminary reports exist, what remains unknown is their involvement in regulating the antimicrobial resistance phenomenon and also the possible mechanisms with which it can mediate the same. Given the dearth of the situation, a systematic study was initiated by asking the following questions:

- What are the differences in the antibiogram and resistome pattern of Indian *A. baumannii* isolates presently isolated from medical centers?
- Does GacAS have any role in conferring antimicrobial resistance in *A. baumannii* – the red alert pathogen?
- Is the function of GacA, the response regulator, residue or domain specific with respect to its impact on modulating resistance determinants?
- Does GacA have any sequence specificity in DNA binding while executing its function?

Results obtained for each objective were described in chapters as mentioned in this thesis.

To begin with, our study with the clinical *Acinetobacter* strains from Chennai, India has shown that most of the isolates showed MDR phenotype which was much higher as compared to reports from other areas like Bengal and Odisha. These strains were showing tolerance to antibiotics like colistin which is commonly used for the treatment of *A. baumannii* infections. Resistance to meropenem was comparatively less and least resistance was observed against tigecycline which is the latest member of our antibiotic arsenal. Another antibiotic which is not commonly used and showed least resistance was polymyxin B. Our study showed that in case of highly MDR *Acinetobacter* strains, polymyxin B and tigecycline are the drug of choice in treating these infections. Further, it supports the notion that continuous surveillance is needed to monitor the antibiotic resistance profile in hospital settings. We also found that MDR strains were showing higher oxidative stress tolerance which strengthens the observations that oxidative stress tolerance is related to antibiotic resistance. One of the factors for drug resistance is the production of biofilm and we also found a strong correlation between biofilm production and MDR phenotype in *Acinetobacter spp*. This observation matches with other reports where people have shown a strong correlation between antibiotic resistance and biofilm production. Since antibiotic resistance has become a persistent problem with increasing numbers of MDR *A. baumannii*, there is a need to search for new targets for drug development.

One such target which is gaining attention is the two-component signaling systems. In *A. baumannii* AYE strain we identified one such TCS, i.e., GacA-GacS TCS, which is a global regulator and known to control diverse functions like virulence, secondary metabolite production and even drug resistance. It was observed that this TCS was conserved in clinical isolates and further studies can authenticate it being considered a suitable candidate for drug discovery programs. It was here, for the first time, the importance of GacA-GacS TCS has been described in *A. baumannii* with the help of small-scale epidemiological analysis. Various factors are responsible for the development of resistance in *A. baumannii* including the presence of antibiotic degrading/modifying enzymes, efflux pumps, porin protein, etc. *A. baumannii* AYE strain genome sequence has shown the prevalence of all these resistance determinants so we went on to dissect the role of this TCS in regulating different resistance determinants. With the help of EMSA experiments, we were able to show that GacA is a direct regulator of β-lactamases, efflux pumps, and porins. This further suggested that this

TCS is not only a globar regulator of virulence but is also a regulator of antibiotic resistance in *A. baumannii*. *Overall results from this first chapter summarize to conclude that gacAS though annotated as putative TCS, is a functional signaling pair with magnesium dependency. The domains found essential for the activity indicates the precise nature of the cascade. Increased expression coupled together with the lack of sense mutation in the collection of Acinetobacter indicates the functioning of an additional regulatory hierarchy in the biology of this notorious human pathogen.*

Further experiments helped us to postulate the possible mechanism of regulation by GacA. It was hypothesized that the binding of GacA to their promoter region is independent of the phosphorylation status of GacA and the most possible mode of regulation involves the structural changes in the DNA bound GacA in response to a signal that is transmitted in the form of phosphorylation. These structural changes bring out the activation of the promoters that are regulated by GacA. Mutational studies highlighted the differential binding of different mutants to different promoters. Arg_{152} and Tyr_{185} binding activities were either reduced or not visible on alanine substitution to most of the promoter of efflux pumps, β-lactamase and porins suggesting the utmost importance of these residues for the functionality of GacA. *Our study in the second chapter clearly presented the first experimental evidence of GacA regulating the most popular known intrinsic resistance determinant, the beta-lactamases for the very first time. An unprecedented observation that clearly signifies the fine regulation adopted by this superbug to sustain different environmental assails. Its added impact on modulating the expression of efflux pumps and porins indicates that besides BaeRS and CpxAR, GacAS also has a definite role to play in maintaining the cell envelope integrity.*

Many studies have linked the Upstream Accessory Sequences (UAS) present in the promoter region as characteristic of GacA dependent regulation (Kay et al., 2006; Valverde et al., 2003). In line to this, our work also highlighted the presence of UAS elements in the genes encoding for efflux pumps, *β*-lactamases and porins. The presence of this element in the antibiotic-resistant determinants further strengthens our observation that GacA is an active key player in the regulation of antibiotic resistance in *A. baumannii*. The GacA/GacS system homolog VarA/VarS of *V. cholerae* is reported to regulate pathogenesis as well as quorum sensing in this bacterium. The presence of a similar UAS in the promoter regulated by those response regulators that are established as active players in the regulation of pathogenesis in different bacteria, makes this UAS site an ideal region that could be targeted by small molecules to undo or weaken the infection caused by *A. baumannii* as well as by other

virulent bacteria. The structural analysis uncovered the oligomerization status of GacA. *In silico* data suggested that GacA may exist as a homodimer and most possibly has a dimerization domain at C-terminal. Also, we found that in homodimeric form of GacA, DBD of each monomer may be splayed apart from each other, indicating the absence of intermolecular interaction between these domains of two monomers. The docking experiments aided us to unveil the structurally and functionally important sites of GacA-DNA complex. We observed that DBD of each monomer binds to the recognition site of the UAS separated by 5 to 7 bases in DNA helix. On looking back to multiple sequence alignment of UAS of *mexE*, *veb-1*, and *carO* it was found that this region is most variable region of UAS. The variation indicates that this region is not involved in making base-specific contact to GacA and thus considered as non-contacted spacer region of UAS. Further, we found that alpha helix 3 and 4 of DBD are involved in making contacts with UAS element by inserting in the major groove of the opposite faces.

In the last section of the study, *in-silico* analysis of the docked protein with DNA predicted the possible nucleotides involved in the interaction. On referring these nucleotides in web logo, we found that the most important residues for interaction are Thy_3, Ade_5, Thy_{15}, Ade/Cyt_{16}, Ade/Cyt_{17}, Ade/Cyt_{18} (position corresponding to the weblogo of UAS). These residues were not only almost conserved amongst the UAS of *mexE, veb-1* and *carO* but also shared remarkable sequence similarities with *rsmX, rsmY* and *rsmZ*. This together pointed to the utmost importance of these residues in mediating the regulation of GacA dependent genes. *Our efforts in the last chapter have been to decode the preferred region in the DNA where GacA might possibly bind and our experimental efforts together with in silico analysis indicate that possibly the flanking DNA region may also have a role in stabilizing the DNA-protein complex formed. Though the efforts are at a preliminary level, further studies to validate and authenticate this hypothesis are strictly warranted.*

Overall this study entitled "Studies on the functions of GacA/GacS two-component system in antimicrobial resistance in Acinetobacter baumannii" has provided novel insights to fill the salient gaps in this area of research. Our efforts have decoded the role of gacAS in mediating antimicrobial resistance for the very first time. The insights gained from site-directed mutagenesis strongly converge to indicate the role of this unique TCS in regulating β-lactamases, a novel highlight of this study. Subsequent data reflects on its unanimous presence in Indian origin strains, undoubtedly proving its worth as a promising drug target.