Chapter 9

Summary of thesis

AMPs or bacteriocins production by bacteria follows the phenomenon, 'survival of the fittest' as they compete to survive in competitive environments. In the effect of this phenomenon, microbial diversity evolved to fight against closely and distantly related organisms. At the same time during the process of survival drug resistance is emerged in pathogenic and opportunistic pathogenic bacteria towards diverse antibiotics. Therefore, in the present study we have made an attempt to understand the phenomenon of AMPs or bacteriocins production and inhibition of closely and distantly related bacterial strains by molecular and genetic characterization of AMPs from rhizosphere subsurface soil samples.

The antimicrobial substance producing strains isolated in this study were identified as *Bacillus* or *Bacillus* related genera using phenotypic and genotypic characterization. A total of seven isolates were selected for purification and characterization of antimicrobial substance. Amongst them, strains, SKDU4 and SKDU12 revealed high identity with *B. subtilis* and *B. endophyticus*. Strains SKDU4 produced a bacteriocin like AMP (5.3 kDa) along with an iturin like bio-surfactant (1056 Da) with antibacterial activities. These peptides displayed additive effect on antimicrobial activity when used in combination. Antimicrobial substance produced by strain SKDU12, showed selective activity against eukaryotic cell membranes like *C. albicans*. The AMP confirmed to be a new member of surfactin like bio-surfactants (1079 Da) that is designated as endophysin. Endophysin further revealed as potential anticancer agent and did not affect normal cells.

Two strains designated as SKDU10 and SKR3 showed high identity with *B. laterosporus*. The bacteriocins produced were showed high identities with laterosporulin (produced by *Brevibacillus* sp. GI-9) but differences in inhibitory activity spectrum observed. Thus, the bacteriocins were produced by strain SKDU10 and SKR3 designated as laterosporulin10 and laterosporulin3, respectively. Interestingly, all these AMPs contained six cysteine residues at conversed positions as observed in eukaryotic defensins. Laterosporulin10 (6.0 kDa) showed

potent and selective inhibition against pathogenic strain of *M. tuberculosis* (MtbH37Rv). Additionally, being a defensin like AMP, laterosporulin10 found to have selective anticancer properties with non-cytotoxic effects against normal human cells and no hemolysis. Potent apoptosis and LDH release was detected at short and longer treatment time points, respectively, as killing mechanism of laterosporulin10 against cancer cells. Although, molecular weight of laterosporulin3 (5.6 kDa) was found identical to laterosporulin, but it differed in two amino acids composition and found to kill drug resistant bacteria (like MRSA and VRE) efficiently in comparison to laterosporulin, laterosporulin10 or any other standard antibiotics. The crystal structure of laterosporulin3 revealed the distribution of distinct positive charge on surface as Asn24 replaced with Lys24, which suggested its potent antimicrobial activities in comparison to laterosporulin and laterosporulin10.

One strain SEHII also confirmed as member of genus *Brevibacillus*, showing high identity with type species of the genus *B. brevis*. Strain SEHII produced a class I lantibiotic with inhibitory activities against bacterial and yeast test strains, hence designated as bacilin. Interestingly, bacilin (2.3 kDa) revealed to have potent inhibitory activities against an intra-cellular protozoan parasite *L. donovani*. Bacilin confirmed to have randomic structures in solution studies, which is a property of lantibiotics.

Two strains designated as A3 and A4 showed high identity with different species of the genus *Paenibacillus*. The bacteriocins produced by these strains were confirmed as class I lantibiotics and designated as penisin and penisin4, respectively. Interestingly, both bacteriocins contained cysteine rich amino acid sequence like eukaryotic defensins. Penisin (4.5 kDa) showed potent killing against drug resistant bacteria (MRSA) in comparison to standard antibiotics. Penisin revealed to have non-cytotoxic properties against mammalian cells and did not show hemolysis. Moreover, penisin efficiently reduced bacterial load in animal system and protected the mice at 80 mg/kg dose. Although molecular weight of penisin4 (4.6 kDa) was very close to penisin, the sequence alignment revealed significant differences. However, cysteine residues were found at conserved positions, like eukaryotic defensins. Penisin4 inhibited the growth of plant pathogens (like *X. campestris*) efficiently in comparison to penisin and standard antibiotics. Both the peptides confirmed to have random structures in solution. Interestingly, genome mining for lantibiotic among the genome sequences of genus *Paenibacillus* along with known literature

confirmed penisin and penisin4 as largest known lantibiotics till date. Further diversity of AMPs is much more diverse than anticipated as more than 50% of the *Paenibacillus* genomes revealed to have lantibiotic gene clusters. Interestingly, more than 45% of *Paenibacillus* genomes found to have novel lantibiotic gene cluster out of all lantibiotic gene clusters having genome sequences. All AMPs reported in this study exhibited to have membrane specific killing mechanism as revealed by fluorescence and electron microscopy studies of bacteriocins against drug resistant and pathogenic bacteria as well as yeast, cancer cells and parasites. Further, membrane disruption of membrane potential, di-nucleotide pool and membrane permeabilizing properties analyzed by FACS also suggested the membrane specific killing mechanisms of these AMPs.

Interestingly, selective inhibitory activities against multiple organisms suggested distinct evolution of these AMPs. Although differences in inhibitory activities against different organisms distinguished these bacteriocins form each other, significant similarities were observed in their biosynthetic gene cluster organizations. More strikingly, all these bacteriocins revealed to have cysteine rich amino acid sequences where cysteine residues are found to be conserved like eukaryotic defensins, hence all these bacteriocins designated as bacterial defensin like peptides (BDLPs).. Here in this study, we are for the first time reporting the evolutionary link between leader less AMPs (laterosporulin like prokaryotic defensins) and lantibiotics (penisin like). Further, BDLPs confirmed to have high conserve ness of cysteine residues when aligned with eukaryotic defensins. Additionally, defensin specific γ-core motif was found conserved in all BDLPs along with eukaryotic defensins and the same was also supported by phylogenetic analysis. Finally, molecular modeling and simulation analysis of BDLPs was in accordance of conserved γ-core motif among all defensin like peptides throughout the all forms of life.

In summary, seven AMPs characterized in this study were found to be novel with potential biotechnological applications. Since biosynthetic genes of these AMPs can be expressed in recombinant systems using suitable vectors, these molecules can be taken forward for recombinant production with improved and selective inhibitory activities. Further, BDLPs could provide new insight into understanding the evolution process of human defensin molecules which are playing important role in innate immunity.