# Evolutionary root defines the structural basis of high and low level vancomycin resistance in enterococci

**Mushtaq Hussain[1], Nusrat Jabeen[1], Abdul Wajid[2], Rafiq Khanani[1], Syed Khaqan Hasan[1] and Sheikh Ajaz Rasool[3]***

[1]Department of Molecular Pathology, DDRRL, Dow University of Health Sciences, Karachi, Pakistan
[2]HEJ Research Institute of Chemistry, ICCBS, University of Karachi, Karachi, Pakistan
[3]Department of Microbiology, University of Karachi, Karachi, Pakistan

**Abstract**: D-alanyl-D-lactate (Dlac) and D-alanyl-D-serine (Dser) ligases respectively mediates high and low level vancomycin resistance among enterococci. To date, the evolutionary relationship of both ligases is largely unaddressed. Also poorly understood are the molecular differences in the magnitude of vancomycin resistance. To address the mention, we constructed the phylogenetic tree of all vancomycin resistance conferring ligases with the wild type ligases (Dala). Multiple sequence alignment and tertiary structures of the structurally unresolved proteins were constructed by homology modeling. Phylogenetic tree revealed that both Dlac and Dser are profoundly different from Dala as a result of continuous selection pressure. Separate clustering of Dlac and Dser also highlighted the structural basis of molecule in maintaining different level of resistance as exhibited by the bacteria. This notion was further augmented as the functionally key region, omega loop (ω-loop), was found relatively more structured in only Dlac. Moreover, the critically active residue, His-243/244, was also noticed to be restricted in Dlac and found replaced by non polar residues in Dser. The present study not only provides protein structural explanation of the different intensities of vancomycin resistance among enterococci, but also presents yet another example for the scope of evolutionary science in biomedicine.

**Keywords**: Enterococcus, antibiotic resistance, protein homology modeling, vancomycin, microbial evolution.

## INTRODUCTION

Enterococci are second to only Staphylococci as an etiological agent of nosocomial (hospital acquired) infections (Foulquie Moreno *et al*., 2006; Otter *et al*., 2011). Presence of intrinsic resistance against several classes of antibiotics ensued great impediment to the treatment of infections associated with enterococci. Nevertheless, glycopeptide antibiotics like vancomycin and teicoplanin are considered as last line of therapeutic defense against enterococcal infections (Klare *et al*., 2003). Vancomycin exhibit selective binding to D-ala-D-ala termini (a bacterial cell wall component) and consequently inhibits the establishment of important cross bridges with the adjacent strand(s) during cell wall synthesis. Lack of such interactions weakens the cell wall integrity and leads to osmotic lysis of the cell (Barna and Williams, 1984; Reynolds, 1989). Nevertheless, after 30 years of the safe use of vancomycin, the first report of vancomycin resistance in enterococci appeared in 1988 (Leclercq *et al*., 1988), and ever since many resistant strains have been reported globally at alarming pace (Courvalin, 2005; Werner *et al.,* 2008; Souli *et al.,* 2009; Lanthier *et al*., 2011).

Mechanistically, vancomycin resistance among enterococci exists in two major forms namely high and low level resistance. Genetically, each of these is further classified into four types (Klare *et al*., 2003 Courvalin, 2005). All high level vancomycin resistance (VanA, VanB, VanD and VanM) are mediated by an enzyme, D-alanyl-D-lactate ligases (Dlac) that replaces the terminal alanine residue with lactate in peptide precursor of cell wall (Courvallin, 2005; Xu *et al*., 2010). Alternatively, all forms of low level resistance (VanC, VanE, VanG and VanL) are the function of protein named, D-alanyl-D-serine ligases (Dser) (Courvallin, 2005; Boyd *et al*., 2008). The very enzyme replaces the alanine residue with serine in the pentapeptide precursor. Both these modifications results in the profound decrease for the affinity of the bacterial cell wall precursor to vancomycin without impairing the ability to form cross bridges and cell wall synthesis. As a result, enterococci develop the resistance against vancomycin (Reynolds *et al*., 1994; Roper *et al*., 2000). Although vancomycin resistance(s) is extensively characterized in terms of its genetic architecture and regulation, the structural information regarding the resistance mechanism is noticeably scarce. Up till now, only one enzyme, VanA has been structurally resolved by crystallography (Roper *et al*., 2000). Additionally, some site directed mutation studies are also reported in order to explain the mode of action of VanA and VanC molecules (Healy *et al*., 1998; Dutta *et al*., 2002; Depardieu *et al*., 2003).

In the present work we have attempted to establish the evolutionary root of vancomycin resistance in a composite phylogenetic tree using both versions of wild

**Corresponding author:* e-mail: ajazrasool@hotmail.com

type cell wall ligases (DdlA and DdlB) and resistance conferring ligases. In addition to the evolutionary history of the enzyme, the phylogenetic tree also illustrates some protein structure-function related aspects of the resistance (discussed later). To validate the hypothesis regarding structural basis of mediating high and low level vancomycin resistance, we have developed the multiple sequence alignment and also constructed 3D *in silico* models of all representatives of the ligase enzymes. Conclusively, our findings suggest that the difference in the level of vancomycin resistance is primarily, if not completely, governed by the structural attributes of the protein rather than the regulatory features of the genes.

## MATERIALS AND METHODS

### *Sequence analysis*

Amino acid sequences of vancomycin resistance conferring (Dlac and Dser) and wild type ligases (DdlA and DdlB) of selected representatives to all major eubacterial taxonomic groups were retrieved from NCBI database and Comprehensive Microbial Resource (CMR) data base (Davidsen *et al*., 2010). Multiple sequence alignment was generated using the Dala and representatives of vancomycin resistance ligases of enterococcal origin. The sequence alignment was observed using CLC sequence viewer (Hussain *et al*., 2010).

### *Phylogenetic tree*

Multiple sequence alignment and phylogenetic inference were developed using MEGA5 (Tamura *et al*., 2011). The alignment was subjected to jModelTest (Posada, 2008) in order to deduce the best evolutionary model. In short, the evolutionary tree was developed using Maximum likelihood method based on Whelan and Goldman model of evolution (Whelan and Goldman, 2001). Gamma distribution of the data was invoked to reject the molecular clock hypothesis; however, the rate variation was set to allow some sites to remain invariant.

### *Homology modeling*

The atomic coordinates of D-ala-D-lac ligase (PDBid: 1E4E; 2.5Å; Roper *et al*., 2000) were retrieved from RCSB protein databank (Berstein *et al*., 2000) and the 3D models of selected proteins were developed. Briefly, the programs SWISS-MODEL (Kiefer *et al*., 2009) and Modeller 9.9 (Marti-Renom *et al*., 2000) were used to construct the models with manual input of PDBid.

### *Tertiary structure analysis*

The structural coordinates of the proteins were viewed using Swiss-Pdb viewer (Guex *et al*., 1997) and Accelrys Discovery Studio visualizer 3.0 (http:/accelrys.com/products/discoverystudio). The structures were analyzed for structural and thermodynamic stability using Swiss-Pdb viewer, PROCHECK, (Laskowaski and Kato, 1980), ANOLEA (Melo and Feytman, 1998) and Verify3D (Elsenberg *et al.,* 1997).

## RESULTS

### *Phylogenetic Analysis*

Phylogenetic tree (fig. 1) developed by Maximum

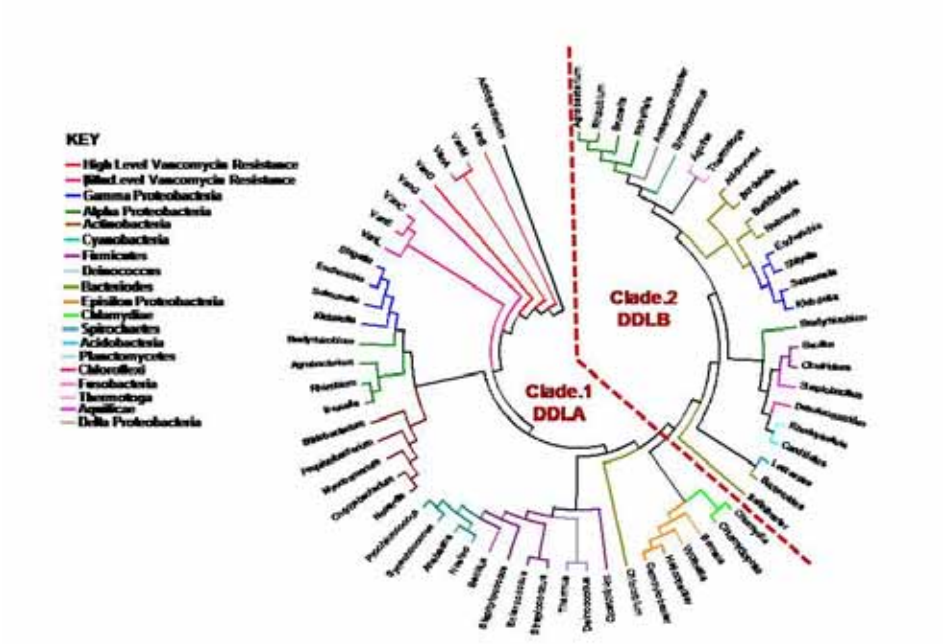


**Fig. 1**: *Evolutionary relationship of Dlac, Dser and Dala:* Phylogenetic tree was constructed using Maximum likelihood method. Branches and/or clades are colored (key) on the basis of taxonomic position of bacteria.

likelihood method clearly separated the vancomycin resistance conferring ligases (Dlac and Dser) of enterococcal origin with the wild type ligases (DalaA and DalaB) of the bacterial kingdom. Topologically, the clade constituted by Dala ligases was further dissociated into two clades, for the sake of convenience we named it as clade I and II. Though clade I congregates the DalaA of α and γ Proteobacteria and Actinobacteria, the clade II is made up of orderly arranged DalaA of Cyanobacteria, Firmicutes, Dienococcus, Bacteriodes, Chlamydiae and all representatives of DalaB. Topologically, the distinct positions of DalaB from all DalaA suggest that both genes have been evolved from a common ancestral gene as a result of gene duplication. Interestingly, the Dala of Aquifex, reportedly the most ancient split of eubacteria (Barns *et al*., 1996), suits itself with the DalaB clade. In order to support the notion regarding the point of origin of DalaA from the DalaB like ancestral gene, we have conducted data mining of both homologs among bacterial kingdom. No species of Aquificae and Thermotoga were found to have DalaA but the next immediate split of Bacteriodes (Kearns, 2010) has shown both forms of ligases. However, it is important to note here that no single Bacteriodes species has found to contain both forms of ligases concomitantly (fig. 1). In the present phylogenetic tree, all vancomycin resistance mediating ligases are topologically very distant from the DalaA (wild type) of enterococci suggesting that enterococci may have acquired the progenitor of vancomycin resistance genes via horizontal gene transfer. The spatial positions of Dlac (high level vancomycin resistance) and Dser (low level vancomycin resistance) also facilitate us to develop another inference regarding the difference in the magnitude of the vancomycin resistance (fig. 1).

***Multiple sequence alignment***

At N-terminal, the substrate binding site Glu (14/17), was found conserved in both Dlac and Dser. However, conspicuous differences were observed in the immediate pre and proceeding adjacent amino acids as Glu-15 and His-16/18 that were respectively found fixed in all Dlac but replaced by Ser/Thr/Pro and Tyr in Dser. With reference to substrate binding stabilization site namely His-98/107 no difference in the site and adjacent amino acids was noticed. Stronger sequence identity was observed in the physiologically active domain (240/242 to 255/260), ω-loop, of all proteins (fig. 2). His-243/244 was found conserved in all Dlac. However, in all low level vancomycin resistance ligase, in place of His-243/244, characteristically opposite amino acid Glu or Thr were appeared at the corresponding position. This pattern was exactly reciprocated in one residue down to that position, where low level resistance proteins showed characteristically similar to His a positively charged

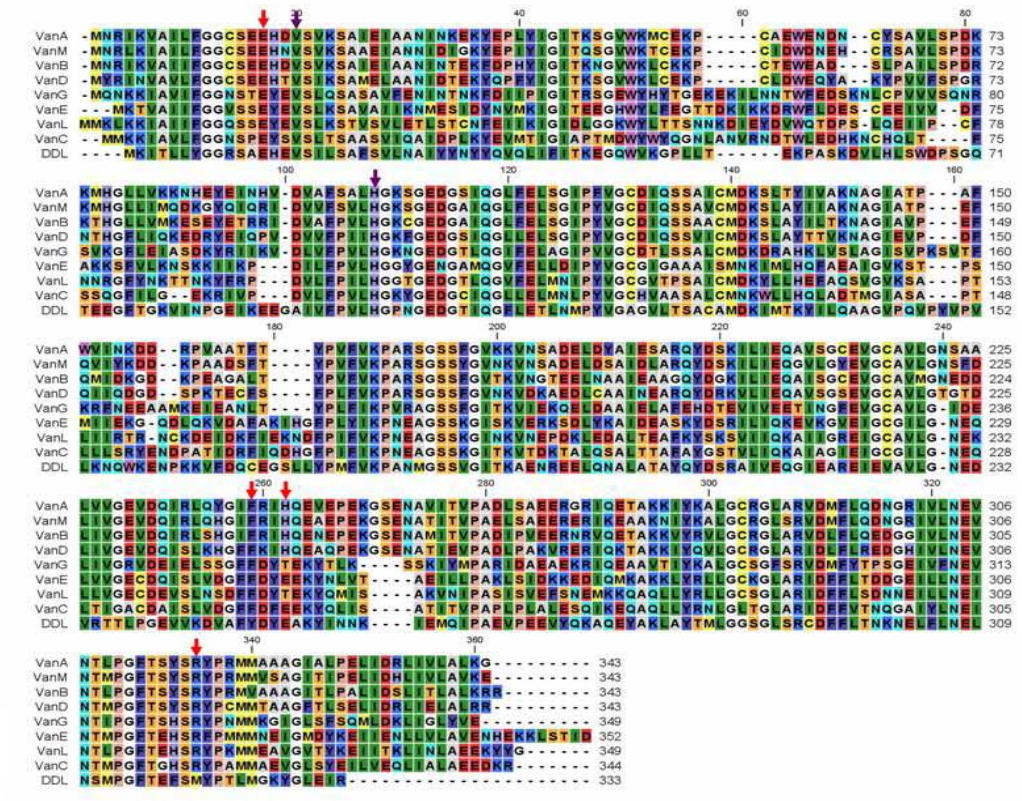

**Fig. 2**: *Mutiple seqeuence alignment of different ligases of enterococci:* Functionally important residues are annotated with colored arrows. Substrate binding sites (red arrows), substrate binding stabilization sites (purple arrows).

residue Lys while high level resistance proteins possessed Glu (fig. 2). Phe-250, another substrate binding site, was found conserved in alignment in all resistance conferring ligases with positional variation range of 246-261. Iso-functional variations (Ileu/Phe) were observed in preceding residues while significant variations were found in downstream positions as Arg/Lys was present in most ligases of Dlac in contrast to Asp in all Dser ligases. The other substrate binding site as indicated by site directed mutagenesis is Arg-322, interestingly all Dlac and Dser ligases have this residue at the same place in alignment with Ser as preceding and Phe/Tyr residues at the proceeding sites.

***Three dimensional structures***

The predicted tertiary structures of vancomycin resistance conferring ligases, revealed substantial accord with crystallographically determined 3D structure of VanA. The over all topology and tertiary structure architecture were found considerably conserved to the known VanA structure. Furthermore, the residue placements in Ramachandran plot and negative value of total energy ranges from -4485 KJ/mole to -15607 KJ/mole emphasize the thermodynamic acceptability of predicted structures. The root mean square deviation (RMSD) values of C-α backbone between template and modeled structures range from 0.04Å to 0.35 Å suggesting core structure congruencies among the structures (table 1). However, these values could be conspicuously segregated into two ranges. All high level vancomycin resistance conferring ligases (Dlac) deviates from each other from 0.04Å to 0.07Å, in contrast to this the low level resistance mediating ligase (Dser) RMSD values varies from 0.22Å to 0.34Å. Roper *et al*. (2000) structurally cleaves the VanA enzyme into three domains namely N-terminal domain, central domain and C-terminal domain. Similar patterns were observed in all the predicted ligase models and all domains contain relatively balanced and uniform density of α-helices and β-pleated sheets. However, noticeable dissimilarities were observed with the central domain of wild type (Dala) ligases of enterococci, where the central domain adopts the confirmation of extended downward loop instead of becoming a part of central core structure (fig. 3A). Additionally, micro-heterogeneities were also observed in the spatial placements and length of loops, helices & sheets in all molecules.

***Catalytic sites***

It is reported that that the most important substrate binding sites (His244/245) are stationed in ω-loop in Dlac and Dser molecules. Site directed mutation studies proved that replacement of these residues result in loss of activity of resistance conferring ligases. The ω-loop has been initially described by Fan *et al*. (1995) as a catalytic active region of *E.coli* ligase (DdlB). Same loop has been observed in both template and modeled structure indicating its physiological significance. Analysis of the electrostatic surface potential and hydrophobic profile reveals conspicuous differences between ω-loop of Dlac and Dser. Along with the difference in the key amino acids (His-244/245) such difference is also due to the reciprocated adjacent residual heterogeneity as mentioned previously. In all Dlac molecules, the ω-loop is relatively more structured in comparison to Dser molecules. Furthermore, the key residue His244/245 is also oriented downward towards the central pocket of the molecule where other substrate binding and stabilization residues are oriented. In contrast to this among all compared Dser molecules the residues corresponding to His244/245 that is Glu/Thr side chains are oriented extreriorly (fig. 3B). The spatial orientation of ω-loop in Dlac along with the negative side chain residues allow a ditch to be formed having pith of positive residue His243-244. Beside the ω-loop, there are other loops present but the coordinates at which they are placed make them catalytically irrelevant. The Arg at the C-terminal remains conserved in all Dser and Dlac ligase suggesting their generalized role in conferring vancomycin resistance. This notion could be further verified by conserved Ser and Tyr at preceding and proceeding of the mentioned residue in all the ligases.

**Table 1**: ***Structural Identity Matrix:*** Root mean square deviation was compared of each molecule with another. Note the strong similarities between Dlac (high level resistance conferring ligases) in comparison to Dser.

| Root Mean Square Deviation of Cα atoms in Å | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Molecules | | High Level Resistance (Dlac) | | | | Low Level Resistance (Dser) | | | |
| | | VanA | VanB | VanD | VanM | VanC | VanE | VanG | VanL |
| Dlac | VanA | 0 | | | | | | | |
| | VanB | 0.06 | 0 | | | | | | |
| | VanD | 0.06 | 0.07 | 0 | | | | | |
| | VanM | 0.04 | 0.07 | 0.06 | 0 | | | | |
| Dser | VanC | 0.24 | 0.22 | 0.21 | 0.21 | 0 | | | |
| | VanE | 0.27 | 0.27 | 0.27 | 0.27 | 0.29 | 0 | | |
| | VanG | 0.22 | 0.22 | 0.22 | 0.24 | 0.35 | 0.34 | 0 | |
| | VanL | 0.23 | 0.24 | 0.25 | 0.22 | 0.34 | 0.19 | 0.34 | 0 |

## DISCUSSION

The Dala ligase of relatively anciently diverged bacteria like Aquifex clades with the DalaB, this implies that ancestral bacterial cell wall ligases is likely to be more similar to DalaB than DalaA. Additionally, the common node of *Aquifex* and *Thermotoga* also strengthen the earlier findings of Boussau *et al*., 2008 suggesting the shared ancestral root of both groups. Concomitantly, it also implies that the relationship between Aquifex and ε-proteobacteria, as otherwise found, is possibly be the result of large scale horizontal gene transfer rather than the vertical descent (Boussau *et al*., 2008). The presence of DalaA in the later splits of bacteria may suggest the point of origin of DalaA but conversely may indicate the possibility of inter genera horizontal gene transfer from some recently evolved bacterial group. This notion is further supported by the fact that no single species of Bacteriodes have both DalaA and DalaB at same time, as *Chlorobium* has DalaA and *Bacteriodes* and *Salinibacter* have DalaB . Moreover, later split of Acidobacteria and Plantomycetes have shown only DalaB supporting the notion of intergeneric horizontal gene transfer in Bacteriodes. Furthermore, The presence of only DalaA in Chlamydiae and in most of cyanobacteria suggest that the event of gene duplication may have occurred at the time when proteobacterial ancestors split from the remaining extant bacterial groups. Alternatively, the absence of DalaB in most of the compared cyanoacterial and firmicutes species (where DalaA is frequently found) could be inferred in terms of Birth and Death model of gene evolution. The model describes that new genes may have evolved by gene duplication of progenitor gene, but some descendants may lost or converted into pseudogenes during the evolutionary course of the species (Nei and Ronney, 2005).

As suggested earlier that enterococci may have acquired the vancomycin resistance gene via intergenic horizontal gene transfer. In this connection. It is proposed that

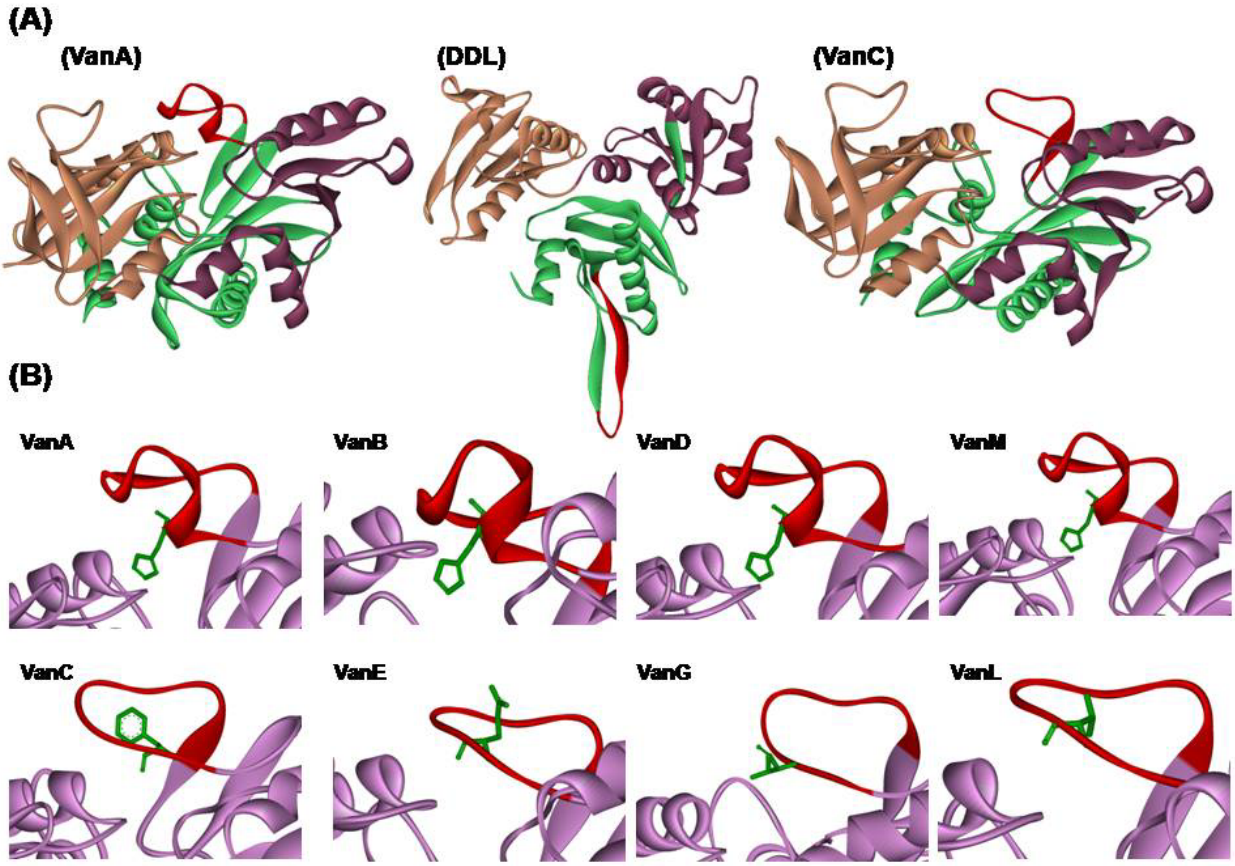


**Fig. 3**: *Structure-Function analysis of vancomycin resistance conferring ligases:* 3A. Three dimensional structure of Dala (DDL), Dlac (VanA) and Dser(VanC). The three main domains and ω-loop are differently colored. N-terminal domain (purple), Central domain (green) and C-terminal domain (brown). Functionally active region (ω-loop) is represented by red color. **3B**. ω-loop is represented in red color, note the difference in the structure conformation between high and low level ligases. Also mentioned in the spatial orientation of the key catalytic residue (His) in green.

glycopeptides producing actinomycetes (Hong *et al*., 2005; 2008) could be the source of these genes to enterococci. Interestingly, the topological difference between the branches of high and low level vancomycin resistance could be inferred mechanistically. As if the high and low level resistance against vancomycin is mediated by the expression of the aberrant ligases the topology may not separate the Dlac and Dser. Conversely, the clear separation as found strongly implies that the level of vancomycin resistance in enterococci is related to the structural conformation of the ligase. In the later part of this script we have tried to prove this very intriguing notion with inductive reasoning using primary and tertiary structural analysis of Dlac and Dser.

The ω-loops has shown stronger sequence identity between high and low level vancomycin resistance. Indeed, site directed mutagenesis studies have shown that the substrate binding residue (His-243/244) and (Phe-250) are present in ω-loop of the Dlac and Dser molecules respectively (Healy *et al*., 1998; Roper *et al*., 2000; Dutta *et al*., 2002; Depardieu *et al*., 2003). In short, at the primary structure level stronger N-terminal conservations in all ligases (resistance conferring) not only imply uniformity in the mode of action of the enzymes but also exclude the possibility of their involvement in substrate specificity at least with reference to substrate stabilization. However, the cumulative discrepancies which have been observed in pre and/or proceeding residues of the substrate binding site might render different affinity for the ligands. Conserved presence of His-243/244 in ω-loop of Dlac represent adequate arsenal for the shift from peptide to catalytically mediated ester bond formation on binding D-lactate (Fan *et al*., 1995; Artymiuk *et al*., 1996; Galperin and Koonin, 1997; Roper *et al*., 2000). Contrarily, in ω-loop, presence of Glu or Thr in place of the His in Dser explains the substrate specificity for low level resistance protein, as other substrate binding residues, Phe-250 and Arg-322, have been found strongly conserved in both Dlac and Dser ligases. Moreover, downstream to the Phe, characteristic differences between residues (Arg/Lys) and Gln in Dlac and Dser respectively are also likely to impose some electrostatic constrains on Dlac/Dser molecules to establish difference in substrate affinity and consequently determines the magnitude of resistance.

Though the tertiary structure of different ligases is holistically similar but the differences in RMSD values indicate considerable structural variations between Dlac and Dser and within Dser which could be trickled down to their functions and consequently the phenotypic characteristics of the organism in terms of antibiotic resistance. Importantly, as ligases are intracellular enzymes the presence of reducing environment halts establishment of Cys-disulphide bond; on the contrary, the domains are primarily stabilized by the cumulative effect of non-covalent interactions i.e. hydrogen bonding and salt bridges. Additionally, the presence of disulphide bond in template molecule has also been referred as artifact which appears during crystal formation (Huyton and Roper, 1999).

Relatively more structured configuration of ω-loop may provide more rigidity and consequently more affinity for the docking to the substrate to Dlac in comparison to Dser. More over the spatial orientation of the substrate binding residues also differs considerably. It is our understanding that difference in the nature of residues at the ω-loop determines the substrate specificity of the molecule. Additionally, the strength of resistance is governed by collectively by the orientation of the key residues and structural features of the ω-loop. This architecture not only enables to direct any negative entity, in this case lactate, towards positively charged His-244 pith but ensures halting their escape concomitantly. Additionally, this orientation of ω-loop may consequently the minimum inhibitory concentration (MIC) conferred by the ligase enzyme. Summarily, the structural differences primarily at the functionally important region, ω-loop, between Dlac and Dser strengthen the inference deduced from the phylogenetic tree that the (MIC) of vancomycin resistance among enterococci is defined by the structural–functional aspects of ligase.

## CONCLUSION

Enteorococci exhibits two different forms of vancomycin resistance, which varies from each other in terms of magnitude. The key enzymes responsible for the high and low level resistance are respectively Dlac or Dser ligase. Our phylogenetic tree suggests that both enzymes have a common evolutionary root which is xenologus to the wild type enterococci. Topology of both Dlac and Dser in the evolutionary tree also implied the structural differences in both forms of enzymes that may consequently lead to difference in the substrate specificity and affinity. Both multiple sequence alignment and structural analysis corroborate this notion as key functional regions are similar within Dlac and Dser but different when both groups are compared with each other. To the best of our knowledge, the present study provides the first set of data towards the structural based derivation of the intensity of vancomycin resistance. Further our findings are also novel in establishing the evolutionary relationship of orhtologs and paralogs of Dala ligases

## REFERENCES

Artymiuk PJ, Poirrette AR, Rice DW and Willett P (1996). Biotin carboxylase comes into the fold. *Nat. Struct. Biol.*, **3**: 128-132.

Barna JCJ and Williams DH (1984). The structure and mode of action of glycopeptide antibiotics of the

vancomycin group. *Annu. Rev. Microbiol.*, **38**: 339-357.

Barns SM, Delwiche CF, Palmer JD and Pace NR (1996). Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. *Proc. Natl. Acad. Sci.* USA, **93**: 9188-9193.

Berstein FC, Koetzzle TF, Williams GJ, Meyer EE, Brice MD, Rodgers JR, Kennard O, Schimanouchi T and Tasumi M (1997). Protein data bank. *J. Mol. Biol.*, **112**: 535-542.

Boussau B, Guéguen L and Gouy M (2008). Accounting for horizontal gene transfers explains conflicting hypotheses regarding the position of aquificales in the phylogeny of Bacteria. *BMC Evol. Biol*., **8**: 272.

Boyd DA, Willey BM, Fawcett D, Gillani N and Mulvey MR (1998). Molecular characterization of *Enterococcus faecalis* N06-0364 with low-level vancomycin resistance harboring a novel D-Ala-D-Ser gene cluster, vanL. *J. Antimicrob. Agents Chemother*., **52**: 2667-2672.

Courvalin P (2005). Genetics of glycopeptide resistance in gram positive pathogens. *Int. J. Med. Microbiol*., **294**: 479-486.

Davidsen T, Beck E, Ganapathy A, Montgomery R, Zafar N, Yang Q, Madupu R, Goetz P, Galinsky K, White O and Sutton G (2010). The comprehensive microbial resource. *Nucleic Acids Res*., **38**: D340-345.

Depardieu F, Bonora MG, Reynolds PE and Courvalin P (2003). The vanG glycopeptide resistance operon from *Enterococcus faecalis* revisited. *Mol. Microbiol*., **50**: 931-948.

Dutta I and Reynolds PE (2002). Biochemical and genetic characterization of the vanC-2 vancomycin resistanse gene clusters of *Enterococcus casseliflavus* ATCC 25788. *Antimicrob. Agents Chemother.*, **46**: 3125-3132.

Elsenberg D, Luthy R, and Bowie JU (1997). VERIFY3D: assessment of protein models with three-dimensional profiles. *Methods Enzymol*., **277**: 396-404.

Fan C, Moews PC, Shi Y, Walsh CT and Knox JR (1995). A common fold for peptide synthetases cleaving ATP to ADP: Glutathione synthetase and D-Alanine: D-Alanine ligase. *Proc. Natl. Acad. Sci.* USA, **92**: 1172-1176.

Foulquie Moreno MR, Sarantinopoulos P, Tsakalidou E and De Vuyst L (2006). The role of Enterococci in food and health. *Int. J. Food Microbiol.*, **106**: 1-24.

Galperin MY and Koonin EV (1997). A diverse superfamily of enzymes with ATP-dependent carboxylate-amine/thiol ligase activity. *Protein Sci*., **6**: 2639-2643.

Guex N and Peitsch MC (1997). SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modeling. *Electrophoresis,* **18**: 2714-2723.

Healy VL, Park IS and Walsh CT (1998). Active site mutants of Vanc-2 D alanyl: D- serine ligase,characteristic of one vancomycin-resistant phenotype, revert towards wild type D-alanyl:D-alanine ligase. *Chem. Biol.,* **5**: 197-207.

Hong HJ, Hutchings MI, Hill LM and Buttner MJ (2005). The role of the novel Fem protein VanK in vancomycin resistance in *Streptomyces coelicolor*. *J. Biol. Chem.,* **280**: 13055-13061

Hong HJ, Hutchings MI and Buttner MJ (2008). Vancomycin resistance VanS/VanR two-component systems. *Adv. Exp. Med. Biol.,* **631**: 200-213.

Hussain M, Khanani MR, Jabeen N, Shoaib SS and Mirza T (2010). Protein homology modeling and structure-function relationship of 2009 swine influenza virus hemagglutinin (HA1): More human than swine. *Biologia.*, **65**: 183-2010.

Kearns DB (2010). A field guide to bacterial swarming motility. *Nat. Rev. Microbiol.,* **8**: 634-644.

Kiefer F, Arnold K, Künzli M, Bordoli L and Schwede T (2009). The SWISS-MODEL Repository and associated resources. *Nucleic Acids Research,* **37**: D387-D392.

Klare I, Konstabel C, Badstubner D, Werner G and Witte W (2003). Occurrence and spread of antibiotic resistance in *Enterococcus faecium*. *Int. J. Food Microbiol.*, **88**: 269-290.

Lanthier M, Scott A, Zhang Y, Cloutier M, Durie D, Henderson VC, Wilkes G, Lapen DR and Topp E (2011). Distribution of selected virulence genes and antibiotic resistance in Enterococcus species isolated from the South Nation River drainage basin, Ontario, Canada. *J. Applied Microbiol*., **110**: 407–421.

Laskowaski MJ and Kato L (1980). PROCHECK. *Annu. Rev. Biochem.*, **49**: 593-626.

Leclercq R, Derlot E, Duval J and Courvalin P (1988). Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. *N. Engl. J. Med.,* **319**: 157-161.

Marti-Renom MA, Stuart A, Fiser A, Sánchez R, Melo F and Sali A (2000). Comparative protein structure modeling of genes and genomes. *Annu. Rev. Biophys. Biomol. Struct*., **29**: 291-325.

Melo F and Feytmans E (1998). Assessing protein structures with a non-local atomic interaction energy. *J. Mol. Biol.,* **277**: 1141-1152.

Nei M and Rooney AP (2005). Concerted and birth-and-death evolution of multigene families. *Annu. Rev. Genet.*, **39**: 121-152.

Posada D (2008). jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution*, **25**: 1253-1256.

Otter JA, Yezli S and French GL (2011). The role played by contaminated surfaces in the transmission of nosocomial pathogens. *Infect. Control Hosp. Epidemiol*., **32**: 687-699.

Reylonds PE (1989). Structure, biochemistry and mechanism of action of glycopeptide antibiotics. *Eur. J. Clin. Microbiol. Infect. Dis*., **8**: 943-950.

Reynolds PE, Snaith HA, Maguire AJ, Dutka-Malen S and Courvalin P (1994). Analysis of peptidoglycan precursors in vancomycin-resistant *Enterococcus gallinarum* BM4174. *Biochem. J*., **301**: 5-8.

Roper DI, Huyton T, Vagin A and Dodson G (2000). The molecular basis of vancomycin resistance in clinically relevant Enterococci: Crystal structure of D-alanyl-D-lactate ligase (VanA). *Proc. Natl. Acad. Sc.,* USA, **97**: 8921-8925.

Whelan S and Goldman N (2001). A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Mol. Biol. Evol*., **18**: 691-699.

Souli M, Sakka V, Galani I, Antoniadou A, Galani L, Siafakas N, Zerva L, Fytrou H, Tsiodras S and Giamarellou H (2009). Colonisation with vancomycin- and linezolid-resistant *Enterococcus faecium* in a university hospital: Molecular epidemiology and risk factor analysis. *Int. J. Antimicrob. Agents,* **33**: 137-142.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, and Kumar S (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution (In Press). doi: 10.1093/molbev/msr121.

Werner G, Klare I, Fleige C and Witte W (2008). Increasing rates of vancomycin resistance among *Enterococcus faecium* isolated from German hospitals between 2004 and 2006 are due to wide clonal dissemination of vancomycin-resistant enterococci and horizontal spread of vanA clusters. *Int. J. Med. Microbiol.,* **298**: 515-527.

Xu X, Lin D, Yan G, Ye X, Wu S, Guo Y, Zhu D, Hu F, Zhang Y, Wang F, Jacoby GA and Wang M (2010). vanM, a new glycopeptide resistance gene cluster found in *Enterococcus faecium*. Antimicrob. *Agents Chemother*., **54**: 4643-4647.