ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

INVESTIGATION OF MUTATIONS IN PENICILLIN BINDING PROTEINS IN VANCOMYCIN-RESISTANT ENTEROCOCCI AND DEVELOPMENT OF NEW INHIBITORS AS DRUG ALTERNATIVES

NEŞE ÇAĞLAYAN

PH.D. THESIS

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DECLARATION

I declare that this thesis work is my own work, I had no unethical behavior at any stages from the planning to the writing of the thesis, I obtained all the information in this thesis in accordance with academic and ethical rules, I cited all the information and comments that were not obtained with this thesis work, and I provided resources in the list of references. I also declare that there was no violation of any patents and copyrights during the study and writing of this thesis.

27.12.2024
Neşe ÇAĞLAYAN

PREFACE AND ACKNOWLEDGEMENT

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To my mother, whose dreams have always been my compass, and to all daughters who honor their mothers by making their dreams come true.

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ABBREVIATIONS AND SYMBOLS

ATCC	American Type Culture Collection
CDC	Centers for Disease Control and Prevention
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EARS-Net	European Antimicrobial Resistance Surveillance Network
IC50	Half maximal inhibitory concentration
LAP	Leucine aminopeptidase
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry
MDR	Multi drug resistant
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
NCBI	National Center for Biotechnology Information
NHSN	National HealthCare Safety Network
NMR	Nuclear magnetic resonance
PASTA	Serine-threonine kinase-associated domain
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PFGE	Pulsed-field Gel Electrophoresis
PYR	Pyrrolidonyl arylamidase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TLC	Thin-layer chromatography
VRE	Vancomycin-resistant *enterococcus*
VRSA	Vancomycin-resistant *Staphylococcus aureus*
VSE	Vancomycin-susceptible *enterococcus*
VSSA	Vancomycin-susceptible *Staphylococcus aureus*
WHO	World Health Organization

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ABSTRACT

Investigation of Mutations in Penicillin Binding Proteins in Vancomycin-resistant Enterococci and Development of New Inhibitors as Drug Alternatives

The rise of vancomycin-resistant *Enterococci* (VRE) in recent years has become a global health burden due to their resistance to vancomycin. Penicillin-binding proteins (PBPs) are vital for cell wall synthesis and play a crucial role in resistance. This study investigated the changes in PBPs, play a role in adaptation to the changes of D-Ala-D-Lac in pentapeptides responsible for cross-links in peptidoglycan synthesis. For this purpose, computational and experimental analyses of PBPs in vancomycin-susceptible (VSE) and VRE strains were investigated to identify mutations and their impact. Sequence data of PBPs from GenBank was collected, aligned, and the results were compared with PBP gene sequences from clinical isolates that were amplified and sequenced using the Sanger method. Three of the six PBPs exhibited amino acid substitutions, including a previously unidentified mutation within the active site of PBP1B. Homology modeling and affinity analysis revealed that this mutation is located inside the substrate-binding site of the enzyme "active cleft", resulting in a shift in substrate preference from native D-Ala-D-Ala to the altered D-Ala-D-Lac. This is the first study of an active-site mutation in a PBP associated with vancomycin resistance, which demonstrates an adaptive enzyme preference favoring resistance-associated ligands. Free D-Ala-D-Lac molecules were synthesized to inhibit PBPs by mimicking their substrate. Despite the challenges encountered during the synthesis, the preliminary antimicrobial tests exhibited promising results. These findings contribute to our understanding of molecular mechanisms behind vancomycin-resistance, suggest a potential direction for alternative drugs against VRE, and provide a basis for further research.

Keywords: VRE, Penicillin-binding protein, PBP, Vancomycin, Vancomycin-resistance.

ÖZET

Vankomisine Dirençli Enterokoklarda Penisilin Bağlayan Proteinlerdeki Mutasyonların Araştırılması ve İlaç Alternatifi Olarak Yeni İnhibitörlerin Geliştirilmesi

Son yıllarda yükseliş gösteren vankomisine-dirençli enterokoklar (VRE), vankomisine direnç göstermeleri nedeniyle küresel bir sağlık sorunu haline gelmiştir. Penisilin-bağlayan proteinler (PBP'ler) bakteriyel hücre duvarı biyosentezi için gereklidir. Bu çalışmada, vankomisine karşı dirence yol açan hücre duvarında çapraz bağlardan sorumlu olan peptitlerdeki D-Ala-D-Lak dönüşümlerine uyum için PBP'lerde bir değişiklik olup olmadığını araştırdık. Bu amaçla, vankomisine-duyarlı (VSE) ve VRE *E. faecalis* PBP'lerindeki mutasyonları ve bunların enzim işlevi üzerindeki etkileri hesaplamalı ve deneysel analizlerle araştırılmıştır. PBP'lerin nükleotit dizileri GenBank veri tabanından alınmış, hizalanmış ve sonuçlar PCR ile çoğaltılan ve Sanger yöntemi ile dizilenen klinik izolatlardan elde edilen verilerle karşılaştırılmıştır. Altı YMA PBP'den üçü, PBP1B'nin aktif bölgesinde daha önce tanımlanmamış bir mutasyon da dahil olmak üzere amino asit ikameleri göstermiştir. Homoloji modelleme ve afinite incelemeleri, bu mutasyonun doğrudan enzimin substrat-bağlanma bölgesini barındıran "aktif yarık" içinde konumlandığını ve substrat tercihinde doğal D-Ala-D-Ala'dan değişmiş D-Ala-D-Lak'a bir kayma ile sonuçlandığını ortaya koymuştur. Bu, vankomisin direnci ile ilişkili bir PBP'deki aktif bölge mutasyonunu gösteren ilk çalışma ve dirençle ilişkili bağlaçlara ilgi gösteren, adaptif bir enzim tercihini göstermektedir. Bu adaptasyona yanıt vermek amacıyla, afinitesinin arttığı değişen substratı taklit ederek PBP'leri inhibe etmek için, serbest D-Ala-D-Lak dipeptit tuzakları sentezlenmiştir. Antimikrobiyal duyarlılık ön deneylerinin sonuçları, sentez sürecinde karşılaşılan zorluklara rağmen umut vericidir. Bu bulgular, vankomisin direncinin arkasındaki moleküler mekanizmaları anlamamıza katkıda bulunmakla beraber, VRE'ye karşı alternatif ilaç geliştirme çalışmaları için alternatif bir yol göstermekte ve daha sonraki araştırmalar için bir temel oluşturmaktadır.

Anahtar Sözcükler: VRE, Penisilin-bağlayan proteinler, PBP, Vankomisin, Vankomisin-direnci.

1 INTRODUCTION AND AIM

1.1 Overview of Vancomycin-Resistant Enterococci (VRE)

Enterococci are typically harmless bacteria that occur naturally in the intestines of humans and animals. However, certain strains including *Enterococcus faecalis* and *Enterococcus faecium*, can cause serious infections, particularly in hospitalized patients undergoing treatment and in individuals with compromised immune systems (1-4).

Antimicrobial resistance, particularly vancomycin-resistant enterococci (VRE), is a significant and growing public health concern due to their capacity to cause severe infections and their ability to resist multiple antibiotics. VRE infections are associated with high morbidity, cost of care, longer length of hospital stays, and mortality compared to vancomycin-susceptible enterococci (VSE) infections (5,6). The first case of VRE was reported in France in 1986, which was a plasmid-mediated vancomycin- and teicoplanin-resistant *E. faecium* (7,8). This was shortly followed by reports in the United Kingdom and the United States, where it subsequently emerged as a major cause of nosocomial infections on a global scale (8,9). The emergence of vancomycin resistance in enterococci has been a troubling development in the field of infectious diseases. The resistance mechanisms in VRE which alter the peptidoglycan precursors, target site of vancomycin are primarily due to the acquisition of nine gene clusters identified to date (10). The two major circulating gene clusters are *vanA* and *vanB* in humans both worldwide (9,11).

The treatment options of VRE are limited and typically include antibiotics like linezolid, daptomycin, or tigecycline, although resistance to these antibiotics is also reported (12-16). Vancomycin, one of the last resort antibiotics, which has been a reliable option for treating infections caused by *Enterococcus* species, presents a challenge due to its occurring resistance in the last 3 decades (17-19). As might be expected, the World Health Organization (WHO) identified VRE as a high-priority

pathogen in a “priority pathogens” list for research and development of new antibiotics in 2017 (20).

The mechanism of action of vancomycin is to inhibit cell wall synthesis by binding to the terminal D-Alanyl-D-Alanine (D-Ala-D-Ala) moiety of stem pentapeptide, which serves as a substrate of Penicillin-binding proteins (PBPs) involved in the formation of peptidoglycan layers, polymerization of glycan chains and cross-linking of adjacent glycan chains in bacterial cell wall biosynthesis. However, the development of resistance to vancomycin occurs when the terminal amino acid D-Ala of the stem pentapeptide is replaced by D-Lactate (D-Lac), resulting in blocking of vancomycin binding site (**Figure 1**). However, this modified pentapeptide is still metabolized by PBPs (21, 22).

In this study, we aimed to investigate the possible changes in *Enterococcus faecalis* PBPs, visualize their three-dimensional (3D) structures, and analyze how these modified PBPs interact with both the native D-Ala-D-Ala and the altered D-Ala-D-Lac substrates, which contribute to the acquisition of vancomycin resistance. Additionally, we synthesized a peptide-based antimicrobial candidate, the D-Ala-D-Lac, and tested its antimicrobial activity. Our goal was to see if introducing free D-Ala-D-Lac molecules into the environment could act as a kind of competitive inhibitor by occupying enzyme binding sites, or if they might work synergistically with vancomycin to enhance its antimicrobial effects.

2 BACKGROUND

2.1 Classification and Identification of Enterococci

The enterococci are Gram-positive, facultative anaerobe, catalase-negative, non-motile cocci, that form various lengths of chains (2). The term "*Enterococcus*" (originally "entérocoque") was firstly proposed by Thiercelin in 1899 to describe a group of gut commensal bacteria with capacity to become pathogenic when conditions are favorable (23). Enterococci form a significant part of the normal flora in the human gastrointestinal tract, playing a dual role: being both harmless commensals and opportunistic pathogens (24). Historically, enterococci were classified under the genus *Streptococcus,* due to their morphological similarities and certain biochemical characteristics until the 1980s (25,26). Despite the identification of four separate groups of streptococci, namely, the viridans, pyogenic, lactic streptococci and the enterococci as group D streptococci, this nomenclature was more often regarded as a "Gram-positive cocci isolated from the gut or feces", instead of a monophyletic group (11). However, detailed research on biochemical and cultural characteristics of *Enterococcus* in 1970 (27), led to the reclassification of enterococci according to their genetic and phenotypic differences, and this resulted in the establishment of a separate genus, *Enterococcus,* in 1984 (28,29). The *Enterococcaceae* family was firstly suggested as a taxonomic level in 2009, based on similarities in the 16S rRNA gene and included the genera *Enterococcus, Tetragenococcus, Vagococcus,* and *Melissococcus* (30). Nevertheless, the precise phylogenetic position of *Tetragenococcus, Melissococcus* and the other presumptive genera, *Catellicoccus* (31) and *Pilibacter* (32), remains uncertain according to the limited number of described species and the lack of certain separation of branches in the phylogenetic tree (33,34). This family is now classified within the order *Lactobacillales*, which also includes other medically and economically important families, such as *Lactobacillaceae* and *Streptococcaceae*, class *Bacilli* in the phylum *Firmicutes* (35).

The genus *Enterococcus* has currently more than 50 species, among which *Enterococcus faecalis* and *Enterococcus faecium* are clinically relevant (2,36-47).

These two species represent not only common commensals in the human gastrointestinal tract but also opportunistic pathogens, which should be considered especially in individuals with compromised immune systems (1-4, 48). Other species, such as *E. gallinarium*, *E. casseliflavus*, *E. raffinosus,* and *E. avium* exhibit a relatively lower prevalence in human disease and represent less than 5% of the clinical isolates (49-54).

It is further observed that *Enterococcus faecalis* is the most common species implicated in human infections, responsible for approximately 80-90% of all enterococcal infections (49-53,55). This bacterium is known to cause a wide range of infections, including urinary tract infections (UTIs), bacteremia, endocarditis, and intra-abdominal infections. While *Enterococcus faecium* is comparatively less observed, its high resistance to most antibiotics, especially to vancomycin, makes it a major nosocomial pathogen (56-58).

For classification purposes, enterococci are identified based on their capacity to produce certain enzymes, such as the production of pyrrolidonyl arylamidase (PYR) and leucine aminopeptidase (LAP), aside from their growth capability in presence of sodium azide and bile salts. These biochemical characteristics are frequently applied in clinical laboratories for the differentiation of enterococcal species from other Gram-positive cocci (55,57).

Additionally, the most commercial systems for microbial identification able to differentiate *E. faecalis* from other *Enterococcus* species, but still additional pigment production and motility tests are required to distinguish *E. gallinarum* (nonpigmented and motile) and *E. casseliflavus* (pigmented and motile) from *E. faecium* (nonpigmented and nonmotile) (59- 61).

In clinical settings, *Enterococcus faecalis* and *Enterococcus faecium* are the most prevalent species, being particularly notable for their multidrug-resistant (MDR) strains, including VRE (58). These MDR strains also cause serious challenges in hospitals, where they have often been associated with high morbidity and mortality

rates (62,63). Reservations about the ability of enterococci to survive under harsh conditions, such as high salt concentrations, extreme pH, and in the presence of bile, have been made based on their role as commensals and pathogens. Their resilience allows them to persist in the hospital environment and colonize patients, particularly those who are critically ill or immunocompromised (64). This dual role as both a harmless commensal and a potential pathogen underscores the importance of understanding enterococci in both health and disease.

2.2 Virulence Factors of Enterococci

The pathogenic potential of *Enterococcus* species is also determined by a range of virulence factors. These virulence features, when coupled with their intrinsic resistance mechanisms, present a significant challenge to treatment efforts and contribute to their persistence in hospitals (65,66).

Some *E. faecalis* strains secrete an antimicrobial peptide known as cytolysin, which exhibits hemolytic activity and lyses both bacterial and eukaryotic cells (67,68). Cytolysin links to severe bloodstream infections and the potential to cause tissue damage. Additionally, pore-forming toxins belonging to the beta-barrel hemolysin family have recently been discovered in *E. faecalis* and *E. faecium.* These toxins serve to enhance their role in immune evasion and pathogenesis by binding to human leukocyte antigen I (HLA-I) and animal major histocompatibility complex I **(**MHC-I) molecules (69). The surface proteins of enterococci, including Esp (enterococcal surface protein), Agg (aggregation substance), Acm and Ace (collagen binding proteins), and SgrA (serine glutamate repeat containing protein A) are involved in the adhesion of bacteria to host tissues, facilitating the colonization and invasion of host systems (57,70-73). The gelatinase enzyme (GelE) is essential for forming biofilms, which play role in the establishment of chronic infections (74,75). The presence of a biofilm creates a physical barrier that protects these bacteria from environmental exposure by enabling them to adhere to both biotic and abiotic surfaces. This results in increased tolerance and makes them more resistant to antibiotic treatments (76,77).

These virulence factors coupled with vancomycin resistance, make VRE a significant challenge in healthcare environments.

2.3 Clinical Impact and Epidemiology of Enterococcal Infections

Enterococci have become one of the main causes of healthcare-associated infections, particularly in developed countries. Previously, considered to be harmless colonizers of the intestinal tract, these bacteria are now recognized for their ability to cause serious systemic infections (48). These bacteria are responsible for a variety of infections, including bacteremia, skin infections, intra-abdominal infections, central nervous infections and endocarditis (78,79). Enterococci are the third most common cause of hospital-acquired bloodstream infections and infective endocarditis worldwide (80). These infections account for approximately 10% of all cases of bacteremia and 30% of hospital-acquired endocarditis cases are caused by enterococci. Among the *Enterococcus* species, *E. faecalis* is more frequently associated with bloodstream and urinary tract infections, followed by *E. faecium* (81). In addition, enterococci have been isolated from various types of infections affecting the skin. In certain cases, they have been reported to cause septic arthritis, osteomyelitis, and central nervous system infections such as meningitis (82).

The emergence of multidrug-resistant strains limits the available range of treatment options. The mortality rate associated with *Enterococcus* species ranges between 14.3% and 32.3%, according to the latest data from the World Health Organization (WHO) (83). The rise of vancomycin-resistant enterococci has resulted in a notable increase in mortality rates associated with infections caused by these bacteria, ranging from 25% to 50%. VRE infections have a particularly severe impact on the prolonged hospitalized individuals and immunocompromised patients (82). Patients with hematological cancers, such as acute myeloid leukemia and lymphomas, are under particularly high risk of enterococcal infections due to their weakened immune system (83,84). This situation is further complicated by the widespread use of broad-spectrum antibiotics, which leads to increased colonization pressure and transmission of VRE (86,87).

As reported by the Centers for Disease Control and Prevention (CDC)'s National HealthCare Safety Network (NHSN), approximately 33% of all enterococci exhibited resistance to vancomycin during the 2006-2007 reporting period (88). A report of NHSN from 2014 data, indicates that, when examined within their genus group, *Enterococcus* species are the second-most prevalent pathogen group among all healthcare-associated infections, following *Streptococcus*. Additionally, they are the most common pathogens among central line-associated bloodstream infections (CLABSI) (89).

The prevalence of VRE exhibits considerable variation by region and hospital setting, with notable differences observed in European countries. A comparative study of hospitals in the Netherlands and Germany, revealed markedly higher VRE colonization rates in German hospitals, particularly among intensive care unit patients. Despite the similarities in cultural, social, antibiotic prescription habits and healthcare structure between the two countries, this contrast underscores the importance of local epidemiological surveillance in guiding infection control strategies (60).

From an epidemiological perspective, regionally, there has been a consistent level of severity and prevalence in VRE infections. Vancomycin resistance exhibits significant variations in European countries, according to a report of WHO in 2020, with seven countries, vancomycin resistance rates reported below 1% resistance rate, including Finland, France, Norway, Iceland, Netherlands, Ukraine and Sweden. In contrast, vancomycin resistance rates were equal to or greater than 25% in 13 countries while four countries, including Bosnia and Herzegovina, North Macedonia, Lithuania, and Serbia reported 50% or higher (90). A report from the European Antimicrobial Resistance Surveillance Network (EARS-Net) indicated an increase in the prevalence of vancomycin-resistant *E. faecalis* isolates from 10.4% to 17.3% over a four-year period, from 2014 to 2018 (91). The rate of VRE increased from less than 1% in 2001 to 14.5% in 2013 in Germany. A notable rise in the prevalence of VRE was highlighted by the German Antimicrobial Resistance Surveillance (ARS) system in hospital outpatient settings with an increase from 9.3% to 19.4% over the same period. (92). The meta-analysis of data from across Asian countries revealed a pooled prevalence

of VRE infections of 8.1%, with a significant regional variation. The highest prevalence was reported in Western Asia (11.4%), followed by South Asia (7.7%), East Asia (3.1%), and Southeast Asia (1.8%) (93). In Africa, a VRE prevalence of 34.6% was reported by Ashagrie et. al. in 2021 in Ethiopia (94). These statistical findings demonstrate the considerable variations in the prevalence of vancomycin resistance across the world.

In Türkiye, the first case of vancomycin-resistant *E. faecium* was reported from Akdeniz University Faculty of Medicine in 1998, after 12 years the first VRE was isolated in 1986 in Europe (95). This was followed by strains reported from Istanbul Medical Faculty and Ankara Gülhane Medical Academy in 1999 (96), and the number of centers where VRE was reported has gradually increased (97).

2.4 Antimicrobial Resistance in Enterococci

The emergence of antimicrobial resistance in enterococci, particularly in *E. faecalis* and *E. faecium* possess serious challenges to treatment of infections. Resistance against the two main antimicrobial agents, β-lactam antibiotics and vancomycin, is alarming among the available treatment options (2,11,56).

2.4.1 β-lactam resistance

Enterococci have intrinsic resistance or low-level resistance to β-lactams (98-100) but show susceptibility only to a few groups of penicillins like penicillin, ampicillin and piperacillin (2,101). β-lactam antibiotics, including cephalosporins, carbapenems, and penicillin, target PBPs involved in the bacterial cell wall biosynthesis. The PBPs catalyze the cross-linking of peptidoglycan pentapeptide side chains following a series of enzymatic reactions during the maturation of peptidoglycan layers (102). β-lactams are structural analogues of pentapeptide precursors of peptidoglycan chains (103). They bind covalently to form acyl-enzyme complexes in PBPs and inhibit their function, disrupting regular cell wall growth. Once PBPs are inhibited by β-lactams, they are inactivated, and peptidoglycan synthesis can no longer continue (102,104). In

the absence of functional PBPs, the substrate dipeptides accumulate on the outer surface of the cytoplasmic membrane (105). The production of β-lactamase is triggered by the accumulation of these dipeptides, which inactivate the repressor protein BlaI and enable the transcription of *blaZ*, beta-lactamase encoding gene (106,107).

The primary mechanism of resistance to β-lactam in Gram-positive cocci is the alteration of PBPs, resulting in PBP4 in *E. faecalis* (108), PBP5 in *E. faecium* (109), PBP2a in *Staphylococcus aureus* (110,111) and PBP2x in *Streptococcus pneumoniae* (112).

The accumulation of point mutations in the enzyme active site of PBP4 in *E. faecalis* has been linked to a reduction in affinity for β-lactams (100, 11-116). In a recent study, Lazarro and colleagues presented evidence indicating that amino acid alterations in the catalytic site of the class B PBP4 in *E. faecalis* resulted in changes to the expression level of the *pbp4* gene, and an increased affinity of PBP4, leading to elevated minimum inhibitory concentration (MIC) values against ceftobiprole, a new generation cephalosporin (117). A further study examined the crystal structure of PBP5 of *E. faecium* and demonstrated that particular amino acid changes may be associated with resistance by altering the configuration of the active site or β-lactam binding (118). Enterococci are less sensitive to β-lactams than streptococci, 10 to 1000 times more drugs are needed to inhibit. *E. faecalis* is more sensitive than the other dominant enterococcal species, *E. faecium* (51,54,119).

The production of β-lactamases is a common mechanism of resistance observed in other organisms of Gram-positive family, such as streptococci. However, it occurs rarely in enterococci (120-122). When the β-lactamase production does occur, it is usually at a low level and constant (104).

Enterococci can also acquire resistance to various antibiotics by acquiring new DNA through plasmids, transposons or mutations. In addition to intrinsic and acquired

resistance to various antibiotics, enterococci show tolerance to all cell wall-acting antibiotics and this tolerance is clinically important (123).

2.4.2 Vancomycin mechanism of action and resistance

Glycopeptide antibiotics represent a group of glycosylated cyclic or polycyclic non-ribosomal peptides produced by actinomycetes, which inhibit the synthesis of cell walls of Gram-positive bacteria (124). These groups of antibiotics act as a substrate binder for cell wall precursors, as opposed to the other antimicrobial agents that function as active-site blockers (125-128). Vancomycin is a member of the glycopeptide antibiotic class. In contrast to β-lactams, vancomycin prevents cross-linking of peptidoglycan layers by binding to the limited number of D-alanyl-D-alanine terminus on the lipid II ((undecaprenyl)-linked *N*-acetylglucosamine-*N*-muramyl pentapeptide) monomers on the outer surface of cytoplasmic membrane and inactivates the transpeptidase activity of PBPs (124,129). Although the lipid II substrate is present in most bacterial species, the antibacterial spectrum of vancomycin is limited to Gram-positive bacteria. In Gram-negative bacteria, the physicochemical properties of the glycopeptide structure prevent access to the lipid II target by blocking passage through the outer membrane (128). Vancomycin-resistant bacteria avoid antimicrobial action by replacing the glycopeptide binding target, the D-Ala-D-Ala peptidoglycan stem pentapeptide C-terminus with D-Ala-Lac or D-Ala-D-Ser (130-132). As a result of this replacement, the binding affinity for vancomycin reduces 1000-fold for D-Ala-D-Lac (134) and 6-fold for D-Ala-D-Ser (22) (**Figure 1**).

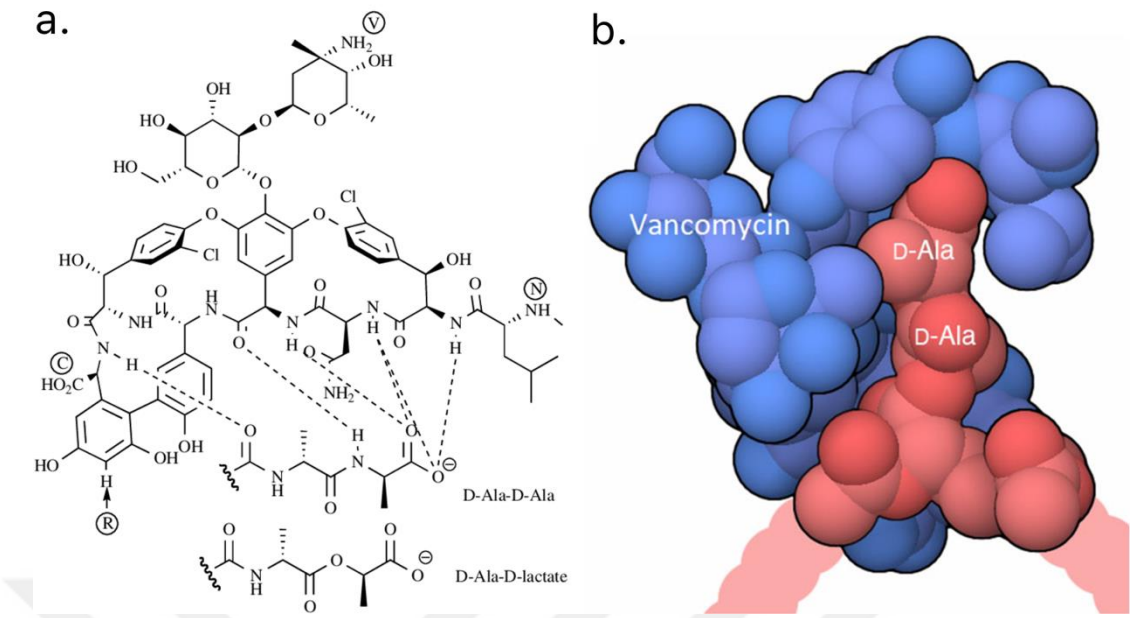

Figure 1. Structure of vancomycin with D-Ala-D-Ala in uncross linked peptidoglycan. a. The vancomycin dimers are tethered via the C, N, V, and R sites shown. b. Surface representation of vancomycin-D-Ala-D-Ala complex (135).

Unfortunately, over the last three decades, the rise of vancomycin resistance in enterococci has become a concerning challenge for clinical practice. It is mediated by the acquisition of a gene cluster, located in a mobile genetic element called a transposon, which allows for horizontal transfer of genetic material between bacteria. The genes in these clusters encode enzymes that are involved in the synthesis of low-affinity precursors and in the elimination of high-affinity precursors (131). At present, eleven distinct gene clusters have been identified as conferring vancomycin resistance. Among these clusters vanA, vanB, vanD, vanE, vanI and vanM encode the precursor peptide that terminates in D-Ala-D-Lac, whereas the vanC, vanE, vanG, vanL and vanN clusters encode the precursor peptide that terminates in D-Ala-D-Ser (136-140). For enterococci, vanA, vanB, vanC, vanD, vanE, vanG, vanL, vanM, vanN clusters have been identified as responsible for vancomycin resistance (141). Furthermore, vanF has been identified and observed only in *Paenibacillus popilliae*, and this variant exhibit high similarity to the vanA cluster (142).

The most prevalent resistance types observed globally are vanA and vanB, which are carried by transposons Tn1546 (143) and Tn1549 (144), respectively. These resistance types are predominantly observed in *E. faecium* and *E. faecalis* (54).

VanA type vancomycin resistance is an inducible, high-level resistance against vancomycin (MIC ≥ 64 μg/ml) and teicoplanin (MIC ≥ 16 μg/ml) (143). The expression of the genes in the vanA gene cluster results in products of the low-affinity D-Ala-D-Lac peptidoglycan precursors instead of high-affinity D-Ala-D-Ala (145). This operon contains genes encoding various proteins required for expression and regulation of vancomycin resistance, including *vanA, vanH, vanX*, *vanR* and *vanS*. VanA protein functions as a ligase to produce D-Ala-D-Lac peptides (21), whereas VanH protein functions as a D-hydroxyacid dehydrogenase for the generation of D-Lactate pool (146). VanX, D, D-dipeptidase, selectively hydrolases D-Ala-D-Ala, while exhibiting no activity against D-Ala-D-Lac (147). The regulation of these proteins is regulated by VanR and VanS proteins. VanS protein is a sensor histidine kinase, that self-phosphorylated upon detecting the presence of the vancomycin. This phosphorylated group then transferred to transcriptional activator protein, response regulator VanR which activates VanAHX system (10,54,141,143,144). VanY is a D, D-carboxypeptidase which cleaves the terminal D-Ala from native pentapeptides (148), and VanZ protein increases the teicoplanin MICs, but does not work for vancomycin. The genes of *vanY* and *vanZ* have no known requirement for vancomycin resistance (54).

VanB type vancomycin resistance is also inducible by vancomycin but in contrast to the vanA type, vanB type is not inducible by teicoplanin (56,149). Expression levels of vancomycin resistance are generally higher in vanA than in vanB strains (149). Also, the distribution of vanA and vanB types of resistance is uneven. VanA is more widely distributed and is the predominant type in Europe (150) while vanB is common in the United States (151). The genetic composition of vanB clusters is very similar to vanA. In the operon, *vanB* gene encodes D-Ala-D-Ala ligase and $vanY_B$ and $vanH_B$ encode peptidases, while $vanR_B$ and $vanS_B$ are two components of the signaling system. A gene named *vanW,* whose function in resistance is not fully understood, is present instead of the *vanZ* gene of the VanA cluster (101,141).

The vanC cluster is genetically distinct from vanA and vanB, and typically exhibits less virulence compared to previous clusters carrying enterococci

(22,152,153). The VanC type resistance is an intrinsic characteristic of *E. casseliflavus, E. gallinarum,* and *E. flavescens* (54). The VanC cluster ligase produces D-Ala-D-Ser instead of D-Ala-D-Lac, similar to the VanE phenotype. This results in a reduction in affinity to vancomycin (54,104,154). However, both retain the capacity to produce D-Ala-D-Ala, providing a low level of resistance to both vancomycin and teicoplanin (102). Unlike the other D-Ala-D-Ser producing operons (vanC, vanE, vanL and vanN), vanN can transfer by a conjugative plasmid to *E. faecium* (141). The vanD cluster is only chromosomal and displays similarities to both vanA and vanB. The vanD was isolated from VRE. *faecium* (155) and has been observed in only a small number of *Enterococcus* species (156). The *vanL*, *vanM* and vanN clusters were recently discovered. While vanM is genetically similar to vanA, vanB and vanD, both vanN and vanL are similar to vanC (141). Vancomycin resistance clusters are shown in **Figure 2.**

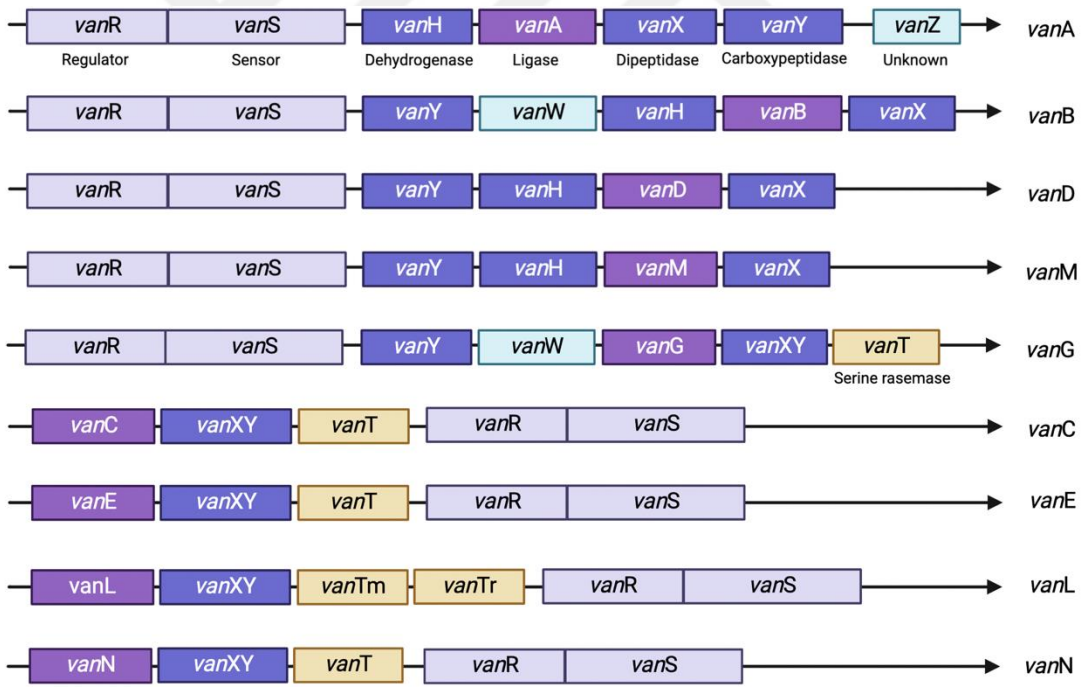

Figure 2. The schema of the vancomycin resistance clusters. vanTr: racemase vanTm: membrane-binding (Adapted from 141,149).

2.5 Cell Wall Biosynthesis

The cell membrane and the other structures that surround and protect the cytoplasm are just a basic membrane. In contrast to the cells of more advanced, higher organisms, the bacterium is exposed to an unpredictable and often challenging environment. A complex cell wall has evolved to protect this basic cell membrane while facilitating the selective passage of nutrients from outside and the removal of waste products from the inside, thereby allowing the bacterium to survive (157). The bacterial cell wall is a very important structural component that maintains the shape and integrity of cells. Along with providing mechanical strength, the cell wall plays an important role in protecting bacteria against environmental stress and antibiotics (158,159).

The biosynthesis of a bacterial cell wall is a highly complex process that involves several steps and requires the participation of many different enzymes and precursor molecules (159,160). The entire process, from the initial formation of UDP-GlcNAc (uridine diphosphate *N*-acetylglucosamine) to the ultimate cross-linking of peptidoglycan strands to create the peptidoglycan layers, also named sacculus, is crucial to maintaining the structural integrity of bacterial cells. Regulation of the pathway ensures that cell wall synthesis is synchronized with the growth and division of bacteria. The key distinctions between Gram-positive and Gram-negative bacteria lie in the composition and organization of their cell walls. In Gram-positive bacteria, the cell wall is characterized by a thick layer composed of multiple layers of peptidoglycan (158-161). These layers contain a mixture of long anionic polymers called teichoic acids which covalently attached to peptidoglycan layers (162). The other class of the wall teichoic acids, lipoteichoic acids serve to anchor the wall to the cytoplasmic membrane lipids, traversing the peptidoglycan layer (163). In contrast, Gram-negative bacteria have a thin layer of peptidoglycan, which is located between the inner cytoplasmic membrane and the lipid bilayer outer membrane containing lipopolysaccharide (157,164). These two concentric membrane layers define an aqueous cellular space, termed as periplasm (165,166) (**Figure 3**). Although the periplasm is known to be a structure unique to Gram-negative bacteria, a study

reported by Matias and Beveridge revealed the presence of a periplasmic space in *B. subtilis* cell envelope using cryo-electron microscopy (167). This provides a new insight into the structure of cell wall in Gram-positive bacteria and seems logical given that secreted proteins and penicillin binding proteins require a space in which to fulfill their function (168).

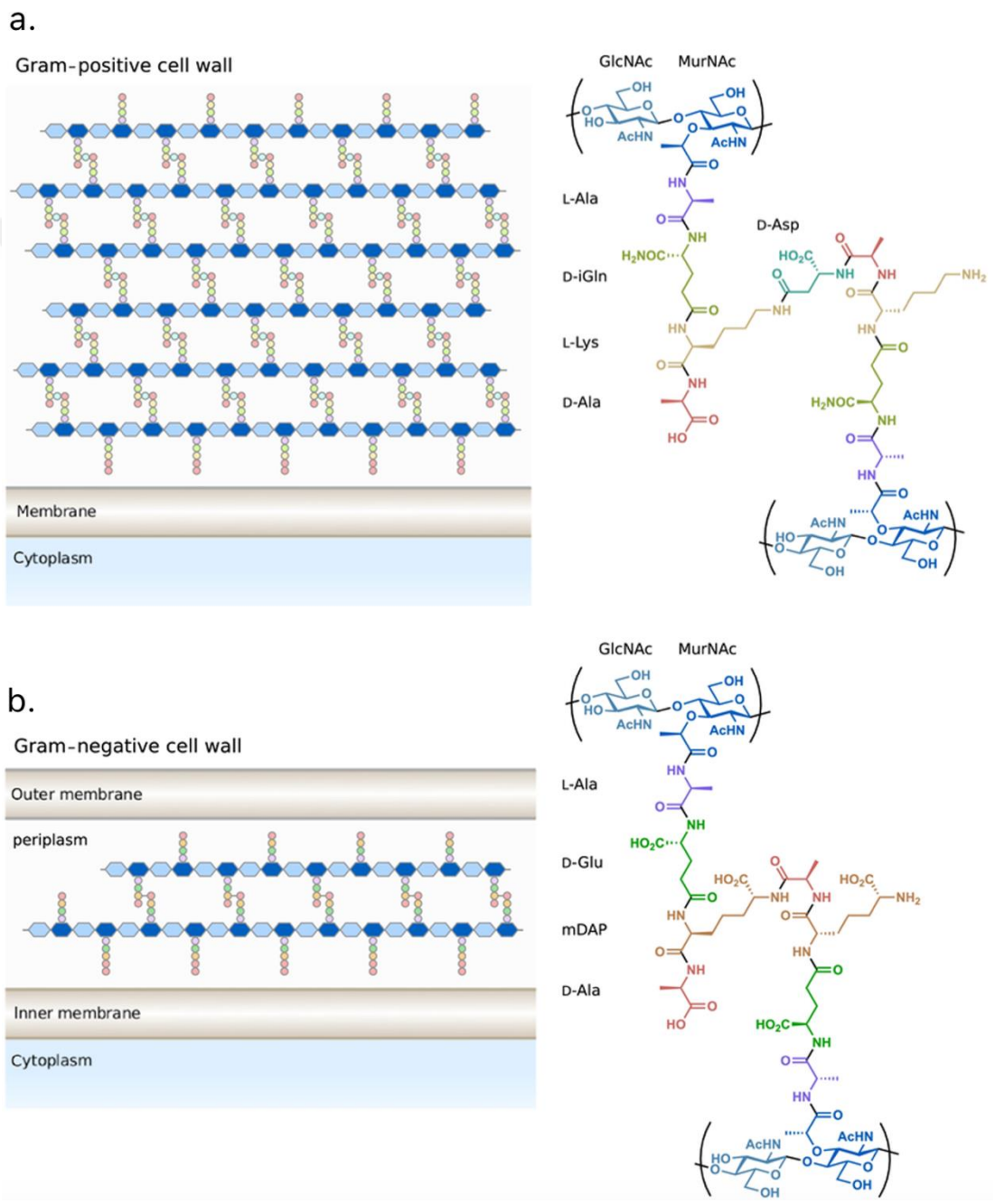

Figure 3. Structure of Gram-positive and Gram-negative peptidoglycan (166).

2.5.1 Peptidoglycan layer

Bacteria have a rigid exoskeleton that protects them from lysing in aqueous media. This exoskeleton is composed of peptidoglycan layers (157). Peptidoglycan (also known as murein) is a polymer consisting of glycan strands that can be described as hoop-like structures encircling the cell, and closely related to the processes of cell growth and cell division. They are forming the essential structural component, providing rigidity and shape to the cell (169,170).

Gram-positive bacteria are surrounded by multiple layers of peptidoglycan, forming a thick, protective shell-like barrier (157). They lack the outer membrane envelope. The peptidoglycan layers also serve as a scaffold for a group of molecules called teichoic acids that run perpendicular to the peptidoglycan layers (157,169,171). These molecules are unique to the Gram-positive cell wall and play roles in pathogenesis (172-174). Some of the other wall-associated proteins also function in cell envelope reconstruction during growth and division, in the uptake of nutrients and metals from the surrounding environment, and as adhesins that facilitate surface attachment and colonization (175).

2.5.1.1 Structure of peptidoglycan

The basic structure of peptidoglycan is formed by linear chains of repeated units of disaccharide cross-linked through peptide side chains. This disaccharide units are highly conserved among bacterial species, is composed of *N*-acetylglucosamine (GlcNAc) linked to *N*-acetylmuramic acid (MurNAc) through a β-1,4-glycosidic bond (169,176). MurNAc, a disaccharide unique to bacteria, includes a C3 lactate group. In uncross-linked peptidoglycan of Gram-positive bacteria, this lactate group forms a bond with the N-terminus of a pentapeptide chain that consists of five amino acids. The first amino acid L-Alanine followed by D-isoglutamine, and the chain terminates in a D-Ala-D-Ala dipeptide. At the position three of the pentapeptide chain, either L-Lysine or meso-2,6-diaminopimelic acid (m-DAP) is found, depending on species. While L-Lysine is present in many Gram-positive bacteria, including *E. faecalis*, *E.*

faecium, S. aureus, Streptococcus pneumonia, Gram-negative bacteria such as *Escherichia coli, B. subtilis* utilize mDAP instead (176,177).

The cross-linking of glycan strands initiated by the forming of an amide bond. This bond forms between the side chain of L-Lys_3 of one stem pentapeptide and the carbonyl group of D-Ala_4 another pentapeptide, after the removal of the terminal D-Ala_5 (176,178).

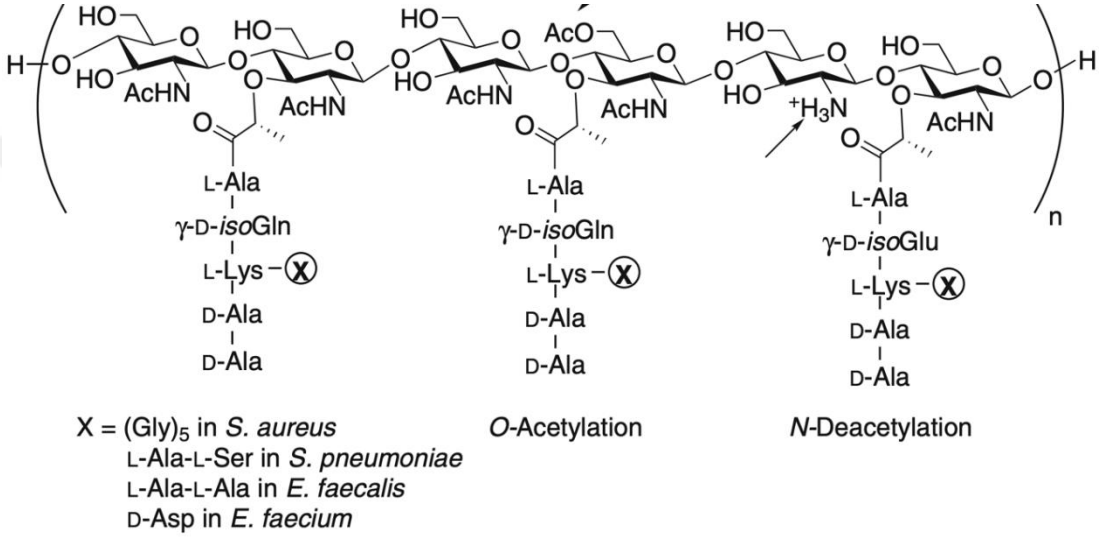

Figure 4. Peptidoglycan structure and types in common species (178).

The lengths of peptidoglycan strands show considerable variation between bacterial species. In the instance of *S. aureus*, the relatively short glycan strands typically consist of 6-18 disaccharide units (179). In contrast, the strands in *Bacillus subtilis* are much longer with 54-96 disaccharide units. But recent experiments using atomic force microscopes have suggested that these chains in length can extend up to 5,000 disaccharide units. These differences in glycan strand length may be related to cell shape, as cylindrical cells such as *B. subtilis* are subjected to more mechanical stress in their walls in comparison to polar ones (180).

2.5.1.2 Biosynthesis of peptidoglycan

Peptidoglycan biosynthesis is a highly coordinated and essential multi-step process that occurs in different stages. The process is divided into three stages: cytoplasmic stage, membrane-associated stage and extracellular stage (178,181).

Cytoplasmic stage: The initial stage takes place in the cytoplasm and involves the assembly of a UDP-MurNAc pentapeptide. The stage starts with the enzyme MurA facilitating the transfer of enol pyruvate from phospho-enol pyruvate to the C3 hydroxyl group of UDP-N-acetylglucosamine (UDP-GlcNAc), forming a key intermediate. Following this step, the enzyme MurB reduces the enolate group to form UDP-N-acetylmuramic acid (UDP-MurNAc), which becomes the foundation for subsequent peptide attachment (182). The pentapeptide chain is assembled in a stepwise manner by the Mur ligases (MurC, MurD, MurE) which sequentially add L-alanine, D-glutamic acid, and either L-lysine or mDAP respectively, depending on the bacterial species. At this stage, the UDP-MurNAc-tripeptide is formed. D-Alanine is converted from its L-form by the alanine racemase enzyme (Alr), and D-Ala-D-Ala dipeptide is produced by D-Ala-D-Ala ligase (Ddl). This dipeptide is then added to the UDP-MurNAc-tripeptide chain by MurF transferase (183-185). In this process, ligases use adenosine triphosphate (ATP)-activated amino acids to facilitate binding, due to the thermodynamically unfavorable nature of the peptide bond formation (186-188).

In Gram-negative bacteria, peptide cross-links occur directly between D-Ala_4 and $mDAP_3$ or two $mDAP_3$ of neighboring peptide chains from adjacent glycan chains. In Gram-positive bacteria, cross-linking can be either directly or mediated by an additional peptide bridge, which varies in amino acid composition and length (189). The general differences in the peptidoglycan structure of Gram-positive bacteria are due to variations in the amino acid sequence, the cross-bridge forming peptides. In most species in the *Enterococcus* genus, these cross-linking bridges are attached to the third position of amino acid L-Lys. The lysine residue is acetylated with D-Asp or D-Asn in *E. faecium*, while *E. faecalis* adds L-Ala-L-Ala (**Figure 5**), and *S. aureus* adds up to five glycine residues to the third L-Lys (176,190,191).

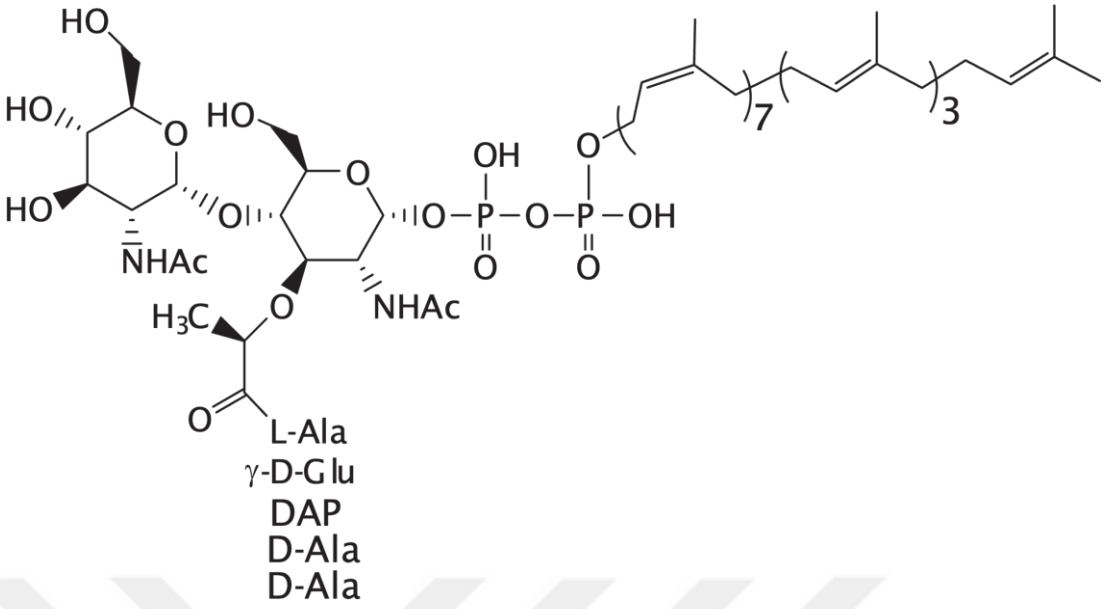

Figure 5. Lipid II with the L-Ala[2] peptide bridge (192).

Membrane-associated stage: Once synthesized in the cytoplasm, the precursor UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala (UDP-MurNAc-pentapeptide) is transferred to membrane-embedded lipid carrier molecule, undecaprenyl-phosphate (Und-P) by MraY enzyme, resulting the formation of lipid I, undecaprenyl-PP-MurNAc-pentapeptide (193,194). MurG enzyme catalyzes the assembly of GlcNAc to lipid I to create lipid II, undecaprenyl-PP-MurNAc(pentapeptide)-GlcNAc (195,196). Once lipid II is formed, additional peptide branches or amino acids are added, and these vary between different bacterial species. In *E. faecalis*, the L-Ala-L-Ala bridge is synthesized by the Fem-family of transferases and attached to lipid II-pentapeptide L-Lys_3 (191,193,197). The molecule is then transported to the external surface of cell membrane by MurJ, lipid II flippase (197). In *E. coli*, each MurJ protein is expected to potentially translocate approximately 700 lipid II molecules per minute, given the number of gene copies present in each bacterium (198-200). The identity of the flippase(s) that translocate lipid II is still a matter of debate. Since most of the research on lipid transfer enzyme, MurJ flippases has been done on *E. coli* and a limited number of Gram-positive *B. subtilis*, it is considered that this mechanism may not be the same in other Gram-positive bacteria, particularly enterococci, or that homologous or additional proteins may be used (201-204).

Extracellular stage: The subsequent stage of peptidoglycan biosynthesis is the polymerization of lipid II molecule into long glycan chains and cross-linking, which is synthesized in the cytoplasm and transported to the external surface of the membrane. Lipid II serves as a monomer for peptidoglycan (181). In the final stages of peptidoglycan biosynthesis, PBPs play a central role through their transpeptidase and glycosyltransferase activities. Following transport of lipid II into the outer membrane, PBPs with glycosyltransferase activity (also known as Peptidoglycan glycosyltransferase) polymerize lipid II subunits into glycan chains, while the peptide moiety of lipid II is cross-linked by PBPs with transpeptidase activity (205,206). These activities are frequently observed as domains in a single bifunctional protein, although monofunctional variates of both enzymes exist (178).

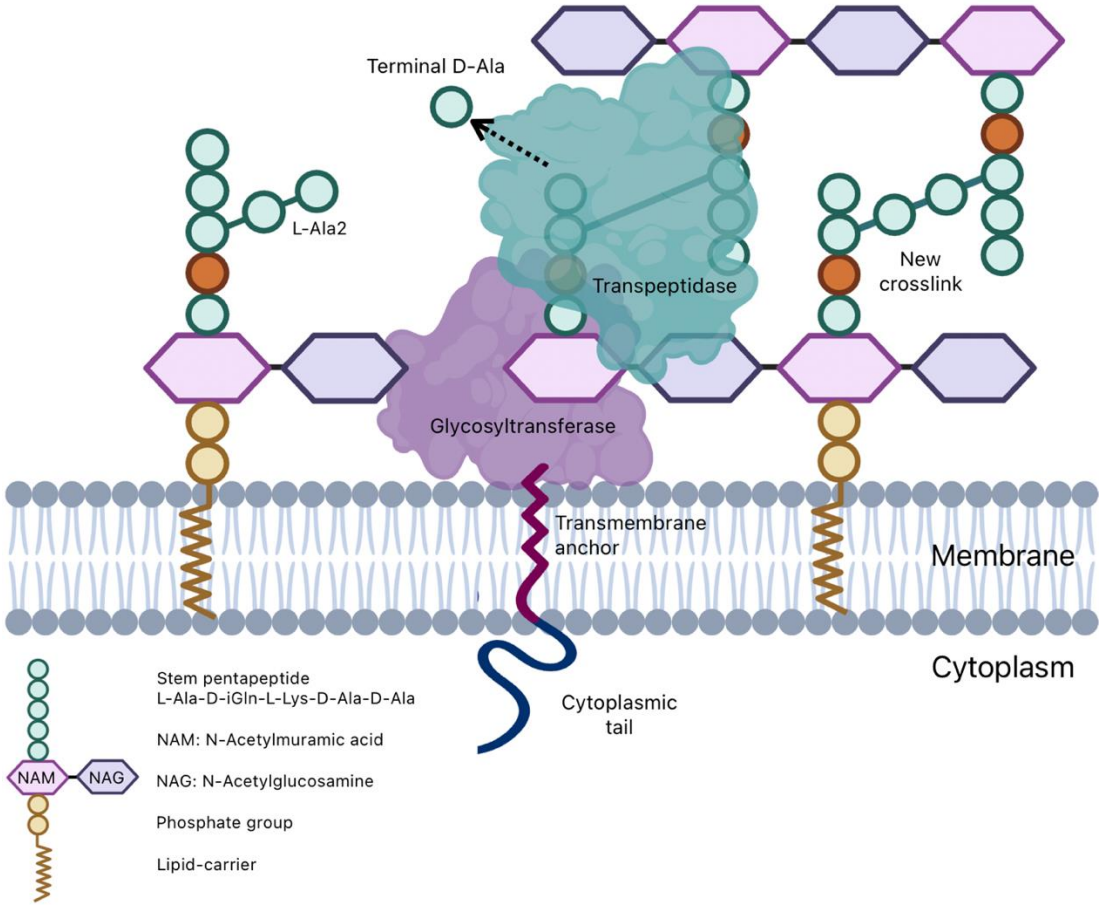

Figure 6. Schematic representation of the enzymatic activity of bifunctional PBPs in peptidoglycan synthesis at the extracellular stage of bacterial cell wall biosynthesis.

2.5.1.3 Degradation of peptidoglycan

The degradation of peptidoglycan is carried out by a diverse range of enzymes, which can be broadly categorized based on the bonds they break (**Figure 7**). Among these, glycosidic bonds of the glycan chains are cleaved by glycosidases, while amidases hydrolyze the amide bond between the first amino acid of the stem pentapeptide and the MurNAc. On the other hand, peptidases target peptide bonds between amino acids within the stem pentapeptides. Peptidoglycan peptidases can be divided into two categories: carboxypeptidases, which remove the C-terminal amino acid of the stem pentapeptide and endopeptidases, which cleave the cross-linking peptide bridges. Depending on the stereochemistry of the amino acids they target, these peptidases are referred as DD-, DL-, or LD-peptidases (169,207). While lysozymes and lytic transglycosylases both generate disaccharide-peptides with their hydrolytic activities. Lysozymes release a terminal reducing MurNAc, whereas lytic glycosyltransferases produce anhydro muropeptides characterized by the presence of 1,6-anhydro rings at MurNAc (anhNAM) (208,209).

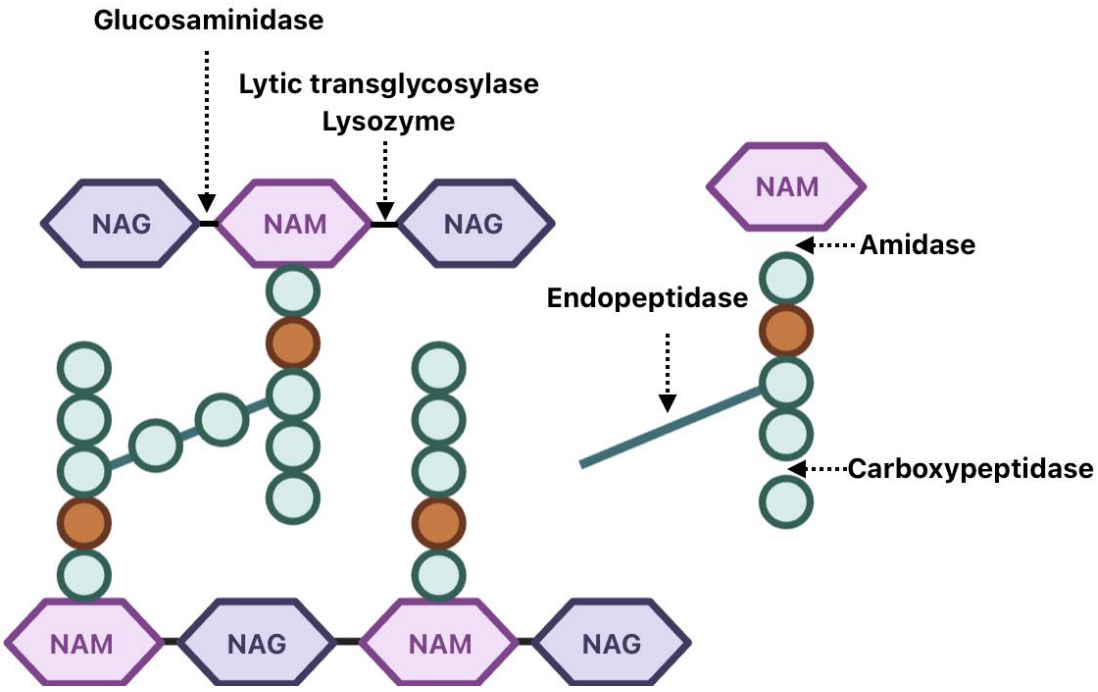

Figure 7. Peptidoglycan cleaving enzymes and their respective sites of action (Adapted from 207).

2.6 Penicillin Binding Proteins: The Builders of Bacterial Cell Wall

Penicillin binding proteins are key enzymes involved in the synthesis and maintenance of bacterial cell walls. They play a crucial role in the final steps of peptidoglycan biosynthesis, a vital process that shapes the structural integrity of bacterial cells (210,211). Their activities are particularly critical for Gram-positive bacteria, like enterococci where their involvement in the polymerization and cross-linking of peptidoglycan strands directly affects cell wall stability and bacterial survival (100), and their position makes them most appropriate target for antimicrobial agents. They are particularly notable due to their role in resistance to β-lactams and vancomycin in some significant pathogens, including *S. aureus* and enterococci.

2.6.1 Classification and function of PBPs

The number of PBPs present in each bacterial species is variable. Some are essential for the survival of the organism, while others are important for resilience in the face of stressful conditions. It is to be expected that rod-shaped bacteria like *B. subtilis* will have more PBPs than cocci, like *E. faecalis*, due to their occupancy (100). For instance, *B. subtilis* and *E. coli* have 16 and 12 PBPs, respectively, while *S. aureus* has 5, *E. faecium* and *E. faecalis* have 8 PBPs each, of which 6 are high molecular weight (212).

PBPs are divided into two categories according to their molecular mass: The high molecular weight (HMW) and the low molecular weight (LMW) PBPs (212). HMW PBPs contribute to the polymerization of peptidoglycan and its integration into the existing cell wall structure. LMW PBPs exhibit hydrolase activity, modulating the composition and structure of cell walls by cleaving peptide bonds during cell growth and division (205,213). Depending on the structure and function, HMW PBPs are further classified into class A or class B. Both class A and class B share a C-terminal domain responsible for transpeptidase activity, that cross-links adjacent glycan chains via peptide bonds. Class A PBPs are distinguished by their high molecular mass because of their double-domain structure. They are characterized by the presence of

an N-terminal glycosyltransferase domain and play a role in elongation of uncross-linked glycan chains. The other HMW class B PBPs are monofunctional, single-domain proteins that have only transpeptidase activity, taking over the transpeptidase function of class A PBPs inhibited by antibiotics. They lack glycosyltransferase activity (214,215) and they are thought to be associated with cell morphogenesis, through interactions with other proteins that regulate the bacterial cell cycle. (216,217) Furthermore, LMW PBPs are included in this classification as class C. The LMW Class C PBPs are monofunctional carboxypeptidases and endopeptidases that act as a "proofreader" in the cleavage and recross-linking of peptidoglycan in case of errors in the layering process (115,212).

The findings of deletion studies in *E. faecalis* suggest that at least one of the three class A PBPs is necessary for the proper synthesis of peptidoglycan layers by its glycosyltransferase activity (215). Class A PBPs are essential for *E. faecalis* but the absence of all three PBPs of class A is not lethal. A bacterium lacking all class A PBPs can still survive and produce peptidoglycan, but the growth rate is much lower than the wild-type bacteria and wall defects are frequently seen (215,218). This deficiency results in increased generation time and decreased peptidoglycan cross-linking. Since *E. faecalis and E. faecium* lack a monofunctional glycosyltransferase, glycan chain polymerization must be carried out by a new type of glycosyltransferase (212,215).

Furthermore, proteins that are involved in cell wall synthesis and exhibit similar functions to PBPs have been identified. A study in 2016, was reported a new PBP in the division site of *E. hirae*. A PBP5 synthesis repressor protein (PSR), renamed as LcpA (LytR-CpsA-Psr family A) has a role in cell wall metabolism, potentially functioning as a phosphotransferase that catalyze the attachment of secondary cell wall polymers like teichoic acids to the peptidoglycan (219). Additionally, some species including *S. aureus* and *E. coli*, contain a peptidoglycan glycosyltransferase without a transpeptidase domain unlike other bifunctional PBPs (220,221).

In *E. faecalis*, three class A and three class B PBPs have been identified (215), in addition to two low-affinity class C PBPs. The class A PBPs are PBP1A, PBP2A and

PBP1B, while the class B PBPs are PBP2B, PBPC (PBP3), and PBP4. Also, a D-alanyl-D-alanine carboxypeptidase and a serine hydrolase are present as class C PBPs (212,222,223).

2.6.2 Structure of PBPs

A typical PBP possess a cytoplasmic tail, a transmembrane anchor, and depending on the class of PBP, one or two enzymatically active penicillin-binding domains, connected by a flexible, β-rich, non-penicillin-binding linker (115,205,224) (**Figure 8a**). The penicillin-binding domain is formed of two sub-domains: a five-stranded β-sheet covered by three α-helices and a full helical domain (**Figure 8c**). The active site is located between these two sub-domains (**Figure 8b)** (115). The potential flexibility between these sub-domains may affect the ability of some PBPs to bind to various ligands (225). The active site contains three highly conserved motifs in PBPs, and these are sufficient to identify a PBP and the enzymatically active domain. The *motif I* contains the active serine (also known as the catalytic serine), followed by a lysine to form the *SxxK* motif. The active serine serves as a central component in the initiation of enzymatic reaction. The *motif II, (S/Y)xN*, is responsible for the protonation of leaving groups of β-lactams. And the final *motif III, KTG(T/S),* plays a role in substrate binding (115,212,226,227). The glycine and threonine or serine residues define the oxyanion hole, is a pocket that stabilizes the negative charge of the substrate's oxygen and is important for catalysis of enzymatic reactions (115,228). As a noteworthy detail, in the transpeptidase domain the residue that continues in *motif III* is typically a threonine or a serine in class A PBPs *(KTG(T/S)(T/S))*, while an alanine is present in class B PBPs *(KTG(T/S)A)* (227).

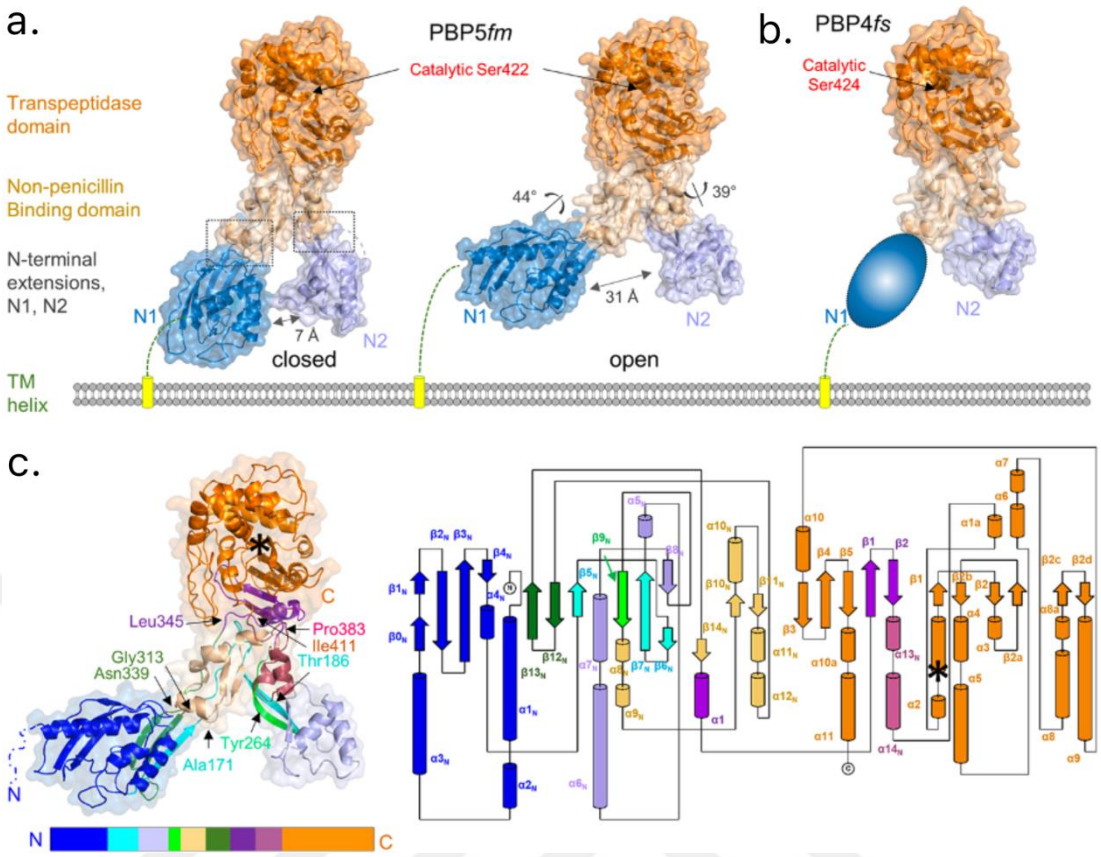

Figure 8. *E. faecium* PBP5 and *E. faecalis* PBP4 structures. a. Conformations of PBP5. b. Structure of PBP4. c. Secondary structure and topology map of PBP5. The asterix indicates the catalytic serine. The topology map is colored the same as the amino acid positions in the 3D conformations of the protein (115).

Historically, PBPs have been numbered according to their migration patterns in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which has caused some confusion. For instance, *S. aureus* PBP2 is functionally similar to *E. coli* PBP1A, while *S. aureus* PBP3 similar to *E. coli* PBP2, and *S. aureus* PBP1 similar to *E. coli* PBP3 (212). In other words, PBP names were assigned based on their molecular weight, rather than their structure or function. Recently, there has been a shift towards a more comprehensive approach to classification with the amino acid sequence alignments and the structural information derived from three-dimensional protein modeling studies with evolving bioinformatics approaches. This ongoing process aims to improve the reliability of the data.

2.7 PBPs as a Drug Target

PBPs were identified and named based on their affinity to penicillin, a β-lactam antibiotic (100,229). β-lactams are the mostly used class of antibiotics, due to their mechanism of action. Understanding the interaction between PBPs and β-lactams has formed the basis for many alternative antimicrobial therapies (181). β-lactam antibiotics act as a competitive inhibitor by mimicking the natural substrate of PBPs, D-Ala-D-Ala moiety of lipid II stem pentapeptide. They bind covalently to a highly conserved serine residue and acylate the active site of the transpeptidase domain of PBP, thereby inhibiting bacterial cell wall biosynthesis. This inhibition results in the prevention of proper cell wall synthesis, which leads to the death of the bacterial cell (230,231).

Vancomycin-resistant bacteria can avoid the inhibitory effect of vancomycin by altering the D-Ala-D-Ala terminus of stem pentapeptide to D-Ala-D-Lac. Although there are still conventional antibiotic treatment options for vancomycin-resistant bacteria, such as linezolid and oxazolidinone (232,233), the potential for resistance remains a concern. Considering the increase of vancomycin-resistant pathogens like *S. aureus, E. faecium and E. faecalis*, research into the development of novel biotechnological strategies became urgent and important. Given their important role in bacterial cell wall biosynthesis, PBPs could potentially be a highly effective drug target. The key to developing new therapeutics lies in understanding the structure and modifications of PBPs. Recent studies have modeled the crystal structures of various PBPs, revealing important aspects of their active sites (115). In particular, the flexibility of the binding pocket has been identified as an important factor in conferring resistance (227). This information has led to the idea of developing drugs that can effectively bind to these modified PBPs even when mutations or structural changes occur.

2.7.1 Modified antibiotics and allosteric inhibition

Ceftaroline has been highlighted as a novel cephalosporin capable of binding to mutated PBP2A of methicillin-resistant *S. aureus* (MRSA), which traditional b-lactams cannot efficiently target. Modifying the acyl side chain allows these drugs to better interact with active sites of modified PBPs, thereby enhancing their efficacy against resistant strains. Binding to the allosteric site detected in PBP2A enables conformational opening of the active site (234,235). A similar allosteric binding relationship was also reported between *S. pneumonia* PBP2X and cefuroxime. The cefuroxime molecule binds to the active site of PBP2X, while the other binds non-covalently to a distal area to the active site, called the serine-threonine kinase-associated (PASTA) domain (236). Although neither the function of this domain nor the structural basis for its recognition of β-lactams is entirely certain, the structural mobility of the PASTA domain can be governed by the presence of newly formed peptidoglycan or β-lactam antibiotics (237). Not all PBPs are capable of exhibiting allostery, the allostery observed in *S. aureus* PBP2A, *S. pneumonia* PBP2X may also be present in *E. faecium* PBP5 (118). However, it is not known how these PBPs acquire the allostery, but it may occur randomly in the organism (234,238).

The use of dimeric vancomycin derivatives that bind to lipid II precursors in peptidoglycan synthesis may be effective in strains carrying PBP mutations. The mechanism of action of these dimers is thought to be related to the inhibition of glycosyltransferase activity of PBP instead of binding to the D-Ala-D-Lac precursors (239,240).

The dual-action glycopeptide derivative oritavancin, which is an analog of vancomycin, has been shown to be effective against both VanA and VanB types of vancomycin-resistant strains and capable of binding the D-Ala-D-Lac moiety. It has three different mechanisms of action that limit the emergence of resistance, as follows: membrane depolarization, transpeptidation and glycosyltransferase inhibition (241,242).

2.7.2 PBP inhibitors with novel mechanisms

Allosteric sites can act by increasing the activity of modified β-lactams, as well as non- β-lactam PBP inhibitor compounds can directly inhibit the activity of PBPs. These inhibitors may act as the allosteric sites of PBPs, preventing the enzyme from functioning properly (234). In 2019, Levy et. al. reported the generation of a potential PBP2-specific inhibitor, the diazabicyclooctane compound CPD4, which demonstrated efficacy against *E. coli* and *Pseudomonas aeruginosa* strains. Their study showed that the ability of CPD4 to form a hydrophobic stacking interaction in the active site of PBP2 of *E. coli* could be a start for the development of broad-spectrum PBP inhibitors (**Figure 9**) (243).

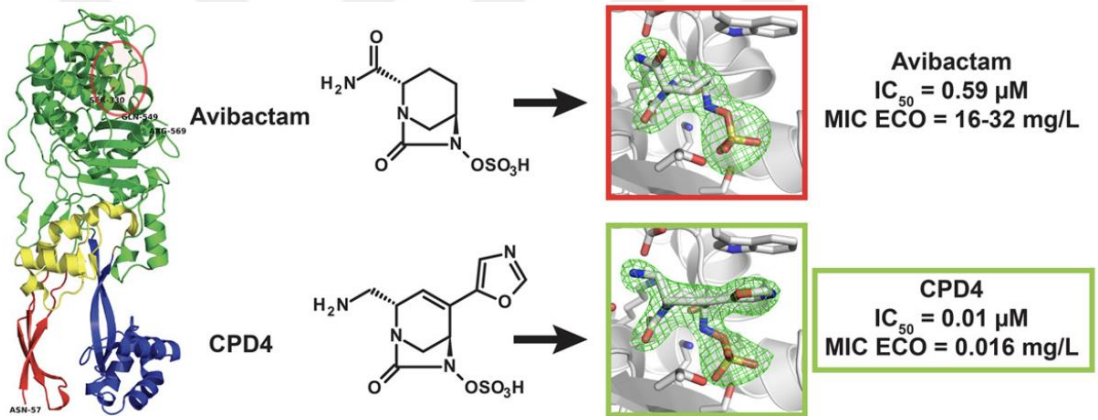

Figure 9. Comparative demonstration of the PBP-inhibitor CPD4 and avibactam (243). IC50: Half maximal inhibitory concentration.

2.7.3 Antimicrobial peptides

Liu et. al. in 2019, investigated the antimicrobial potential of the surface localized antimicrobial display (SLAY)-derived peptides (244) against vanA-mediated VRE (**Figure 10**). Their findings revealed that SLAY-peptides exhibited a significant synergistic activity when combined with vancomycin against VRE with the mechanism of action specifically inhibiting the transcription of regulatory *vanR* and sensor *vanS* genes in vanA cluster (245).

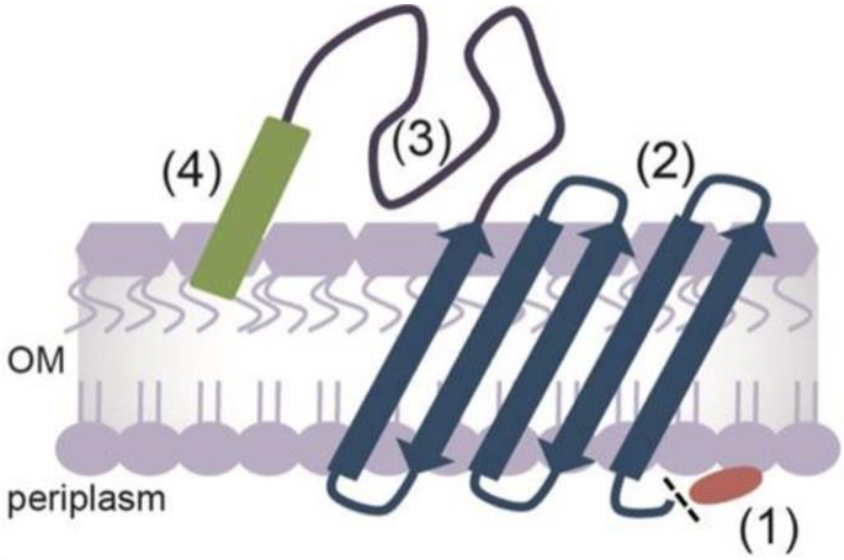

Figure 10. SLAY-peptide system. The system included a murein lipoprotein signal sequence for protein transport (1), a transmembrane protein (2), a flexible linker (3) and a C-terminal, hydrophilic, arginine-rich, cationic peptide (4) (244).

In conclusion, the challenge of combating PBP-mediated resistance in vancomycin-resistant bacteria is multifaceted, requiring a combination of novel drug design, strategic use of existing antibiotics, and innovative therapeutic approaches. The continued study of PBP structures and mechanisms remains critical to developing effective treatments that can stay ahead of bacterial evolution. The limited information available on the structure and mechanisms of action of PBPs, particularly in enterococci, has made research on this subject very important. The available information was based on inferences derived from the studies on rod-shaped bacteria *E. coli* and *B. subtilis*. However, this information may not be sufficient for cocci given the activity of PBPs on bacterial cell wall synthesis and, consequently, on cell shape. Recent research has focused on understanding the structural biology of PBPs and developing novel drugs that can overcome resistance mechanisms.

3 MATERIALS AND METHODS

3.1 Chemicals

The chemicals used in this study, along with commercial information are presented in **Table 1.** All chemicals were prepared according to the manufacturer's instructions before use.

Table 1. Chemicals.

Chemical	**Brand**	**Catalog number**
Mueller-Hinton Broth (MHB)	Oxoid, US	CM0405B
Luria Bertani Broth (LB)	Oxoid, US	CM0996
Tryptone Soya Broth (TSB)	Oxoid, US	CM0129
Agar	Sigma-Aldrich, US	A1296
Glycerol	Merck, Germany	104092
Skim milk powder	Merck, Germany	115363
MyTaq Hot Start DNA Polymerase	Meridian Bioscience, US	BIO-21111
E.Z.N.A Bacterial DNA Kit	Omega Bio-tek, US	D3350
E.Z.N.A Gel Extraction Kit(V-spin)	Omega Bio-tek, US	D2500
Ethidium bromide	Sigma-Aldrich, US	E1510
Orange G	Sigma-Aldrich, US	O3756
SYBR Gold Nucleic acid gel stain	Invitrogen, US	S11494
Ampicillin disk	Oxoid, US	CT0003B
Penicillin disk	Oxoid, US	CT0152B
Sodium D-lactate	Sigma-Aldrich, US	71716
Boc-D-Ala-OH	Sigma-Aldrich, US	853087
Sodium chloride (NaCl)	Merck, Germany	106406
TLC silica gel 60 F_{254}	Merck, Germany	105554
Magnesium sulfate heptahydrate ($MgSO_4$)	Merck, Germany	105882
Sodium sulfate anhydrous (Na_2SO_4)	Merck, Germany	106649
Potassium carbonate (K_2CO_3)	Sigma-Aldrich, US	209619
4-Dimethylamino pyridine (DMAP)	Sigma-Aldrich, US	107700
N,N-Dimethylformamide (DMF)	Merck, Germany	103053
Dichloromethane (DCM)	Sigma-Aldrich, US	34856
Dicyclohexylcarbodiimide (DCC)	Sigma-Aldrich, US	D80002
Diethyl ether (Et_2O)	Merck, Germany	100923
Ethyl acetate (EtOAc)	Isolab, Germany	920018
Sodium bicarbonate ($NaHCO_3$)	Merck, Germany	106329
Ethanol (EtOH)	Merck, Germany	100983
Methanol (MeOH)	Merck, Germany	822283

Table 1. Chemicals (continue).

Hydrochloric acid (HCl)	Merck, Germany	109060
Acetic acid (AcOH)	Sigma-Aldrich, US	695092
Acetyl chloride (AcCl)	Sigma-Aldrich, US	114189
Tert-Butanol (t-BuOH)	Merck, Germany	822264
N-Butanol (n-BuOH)	Merck, Germany	101990
Chloroform	Merck, Germany	102445
Dimethyl sulfoxide (DMSO)	Merck, Germany	102952
Toluene	Tekkim, Turkey	170590
Trifluoroacetic acid (TFA)	Merck, Germany	108262
Acetonitrile	Merck, Germany	100030
Celite 545 (0.02-01 mm)	Merck, Germany	102693
Trizma Base	Sigma-Aldrich, US	T1503
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, US	E9884
Sodium hydroxide (NaOH)	Merck, Germany	106462
Boric acid	Merck, Germany	100165
Na-lauryl-Sarcosine	Sigma-Aldrich, US	L9150
Na-deoxycholate	Sigma-Aldrich, US	30970
Proteinase K	Omega Biotek, US	AC116
RNase	Omega Biotek, US	AC117
Lysozyme	Thermo Scientific, US	90082
Lysostaphin	ProSpec, Israel	enz-269
Restriction enzyme (SmaI)	New England Biolabs, UK	R0141
Low melt agarose	Gold Biotechnology, US	A204
Pulsed Field Certified agarose	Bio-Rad, US	1620137

3.2 Devices and Instruments

The devices and instruments utilized in this study in addition to the software, programs, and online servers applied in bioinformatics analysis, are presented in **Table 2** below**.**

Table 2. Devices and instruments.

Herasafe biological safety cabinet	Thermo Scientific, US	51033761
Heratherm incubator	Thermo Scientific, US	IMH180
Environmental shaker-incubator	BioSan, Latvia	ES-20/60
Microcentrifuge	Thermo Scientific, US	MicroCL 21R
Heat block	BioSan, Latvia	Bio TDB-100
Nanodrop	Thermo Scientific, US	200c
Thermal cycler	Bio-Rad, US	T100
ORTE	TiBO, Turkey	ORT01
ChemiDoc MP Basic Imaging System	Bio-Rad, US	
CHEF-DR II PFGE System: CHEF Electrophoresis Cell CHEF Cooling Module, 120 V CHEF Variable-Speed Pump CHEF Cooling Module, 120 V	Bio-Rad, US	 1703649 1703654 1703644 1703654
Savant SpeedVac	Thermo Scientific, US	SPD300
ABI 3730XL DNA Analyzer	Applied Biosystems, US	A41046
BioEdit	Informer Technologies, US	
EXPASY Translate	SIB Swiss Institute of Bioinformatics, Switzerland	
Chimera	University of California, San Francisco, US	
AutoDock Vina	Molecular Graphics Laboratory, US	
Rotary evaporator Rotavapor R-300	BUCHI Corporation, US	
UV Cabinet 4	CAMAG, Switzerland	
Freeze dryer	Labconco, US	
1200 HPLC system	Agilent, US	
AdvanceBio Peptide Plus column	Agilent, US	695775-949
Avance IVDr NMR spectroscopy	Bruker, US	
Mass Spectroscopy LC/MS	Agilent, US	

3.3 Microbial Studies

3.3.1 Strains

Vancomycin-susceptible and vancomycin-resistant strains of *Enterococcus faecalis* and *Enterococcus faecium*, members of the *Enterococcaceae* family, were initially included in this study. All *E. faecalis* strains, including both VRE and VSE, were provided from Acibadem Labmed Clinical Laboratories, Istanbul. Similarly, *E. faecium* strains were provided from Hacettepe University, Ankara. The study initially

included three vancomycin-susceptible and two vancomycin-resistant *E. faecalis*, for a total of five strains. Following the initial analysis, an additional three vancomycin-resistant strains for PBP1B were included in the study. All the strains were frozen stocks of clinical isolates. The bacterial strains were identified using Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Microflex LT; Bruker Daltonik GmbH, Bremen, Germany), with further confirmed by polymerase chain reaction (PCR) with species-specific PBP primers.

The vancomycin susceptibility was determined for each strain by the Kirby–Bauer disk diffusion method. All VRE strains were identified to possess vanA-type resistance based on the detection of *vanA* gene using specific PCR primers: 5′AATACTGTTTGGGGGTTGCT3′ and 5′GCTTGACTAACTGGCGAACT3′ (246).

3.3.2 Cultivation of bacterial cells

The frozen stocks of clinical isolates were inoculated on Mueller–Hinton agar (MHA) (Dehydrated infusion from Beef 300.0 g/L; Casein hydrolysate 17.5 g/L; Starch 1.5 g/L., Agar 17 g/L) plates by single colony inoculation method and kept in an incubator at 37°C for approximately 24 hours. Single colonies were selected from the petri dishes and inoculated into Mueller–Hinton broth (MHB) (5 ml) (Dehydrated infusion from Beef 300.0 g/L; Casein hydrolysate 17.5 g/L; Starch 1.5 g/L., pH: 7.3 ± 0.1) medium in 50 ml of tubes and then incubated in a shaking incubator at 37°C with a speed of 180 rpm for approximately 16 hours.

Long-term bacterial stocks were prepared from the growth liquid medium in a 20% glycerol solution (liquid cell culture (800 µl) in sterile glycerol (200 µl) and a 10% skim milk solution (dry skim milk, 10 gr) in sterile double distilled water (ddH_2O, 100 ml) and the resulting solutions were stored at -80°C until required.

3.4 Molecular Studies

3.4.1 Extraction of bacterial genomic materials

Genomic materials of *E. faecalis* and *E. faecium* strains were extracted for the investigation of PBP genes. For this purpose, bacterial cell cultures were initially prepared as described in Section 3.3.2. Bacterial DNA was isolated using E.Z.N.A.® Bacterial DNA Kit (Omega Bio-tek, US) according to manufacturer's instructions. In order to isolate DNA from Gram-positive *Enterococcus* strains, an additional cell wall digestion step is added due to the thicker peptidoglycan layer that is typical of Gram-positive bacteria.

A maximum of 3 ml of culture (approximately $1x10^9$ cells) was harvested by centrifuging using MicroCL 21R centrifuge device (Thermo Scientific, US) at 4000 x g for 10 minutes at room temperature. The supernatant was discarded, and the cell pellet was retained. The bacterial cell pellet was resuspended thoroughly in TE Buffer (100 µl) (10 mM Tris-HCl, 1mM EDTA, pH 8.0) by vortexing to ensure complete dispersion. Lysozyme (10 µl, 20 mg/ml) and the same amount of lysostaphin were added to the resuspended cells and incubated at 37°C for a minimum of 10 minutes to facilitate the disruption of the cell wall. Upon completing incubation, TL Buffer (100 µl) (a lysis buffer containing Tris-HCl, EDTA, SDS) and Proteinase K solution (20 µl, 20 mg/ml) were added to the mixture, mixed briefly, and then incubated at 55°C in a heat block for a minimum of one hour, by vortexing the samples every 20 minutes. This step ensures protein digestion and cell lysis. Following lysis incubation, RNase (5 µl, 20 mg/ml) were added and incubated at room temperature for 5 minutes to degrade RNA. Following the RNase treatment, BL Buffer (200 µl) (a lysis buffer containing detergent) was added, and the mixed lysate was incubated at 65°C for 10 minutes, denaturing proteins and facilitating DNA binding to the column. pure ethanol (200 µl) was added and vortexed for 20 seconds at high speed and transferred the mixture including any precipitate formed into a HiBind DNA Mini Column placed in a collection tube. The tubes were centrifuged at 10000 x g for a minute and discarded the collected liquid. HBC Buffer (500 µl, high salt containing wash buffer) prediluted

with isopropanol was added to the column and centrifuged again under the same conditions. After discarding the collection tube, DNA Wash Buffer (700 µl, 70% ethanol) was added and centrifuged again under the same conditions. This washing process was repeated once more. For removing any residual ethanol, the DNA binding column was centrifuged at highest speed as empty for 2 minutes. Dried column was placed into a sterile 1.5 ml microcentrifuge tube and added ddH_2O (50 µl), preheated to 65°C, carefully directly onto the center of the silica matrix of the binding column. After waiting the tube for 5 minutes at room temperature, centrifuged at 10000 x g for a minute to collect the eluted DNA. The concentration of the eluted DNA was measured on a NanoDrop One spectrophotometer (Thermo Scientific, US) and stored at -20°C for long-term storage.

3.4.2 Design of primers

Primers were designed to produce multi copies of PBP genes in the PCR process. Primers are single-stranded, short DNA fragments of approximately 20 nucleotides long, which provide a starting point for DNA synthesis. In each PCR reaction, a primer pair is used to amplify a gene. The forward primer and reverse primer bind to the template DNA at the 5' and 3' ends, reciprocally, and define the boundaries of the region to be amplified.

In this study, the vancomycin-resistant *Enterococcus* strain *E. faecalis* V583, which was the first VRE strain to complete a whole genome sequencing, was selected as the reference for the design of the primers (222). The complete genome sequence of *E. faecalis* V583 is available in National Center for Biotechnology Information (NCBI) database (Reference sequence: NC_004668.1 and GenBank accession: AE016830.1) (223,247). Primers were designed using Primer BLAST (The Basic Local Alignment Search Tool) online bioinformatics tool (248).

The optimal operational range of the DNA polymerase enzyme utilized in the PCR process is up to 1000 bp per minute. Similarly, as the Sanger sequencing method also

works within this efficiency range, a complete PBP gene was amplified by dividing it into three parts, with each part overlapping, in order to maintain reading sensitivity.

Furthermore, a primer pair specific for *vanA* gene, which is responsible for vancomycin-resistance and indicated vanA-type resistance, was designed to confirm vancomycin-resistance and its type. The primer lengths to be used in this process are detailed in **Table 3** and **Table 4**.

Table 3. The PCR product lengths of *E. faecalis* V583 strain.

Gene product	**Base pair (bp) lengths**	**Fragment base lengths (bp)**		
		1.	**2.**	**3.**
PBP1A	2337	801	801	817
PBP1B	2412	821	852	824
PBP2A	2187	796	752	729
PBP2B	2136	790	753	694
PBP3	2229	727	782	835
PBP4	2048	748	714	698
vanA	989	-	-	-

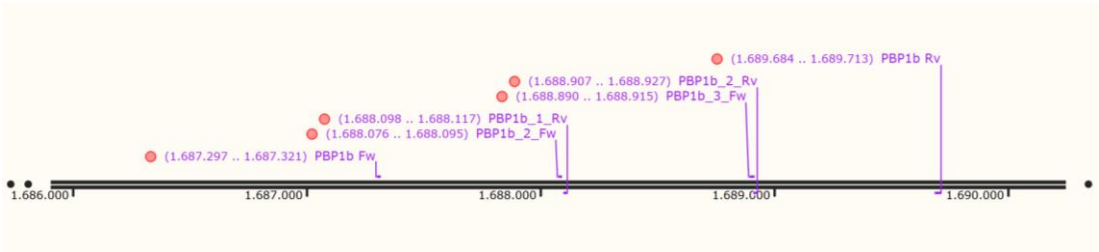

Figure 11. Diagram displaying the placement of overlapping primers on the PBP1B gene region.

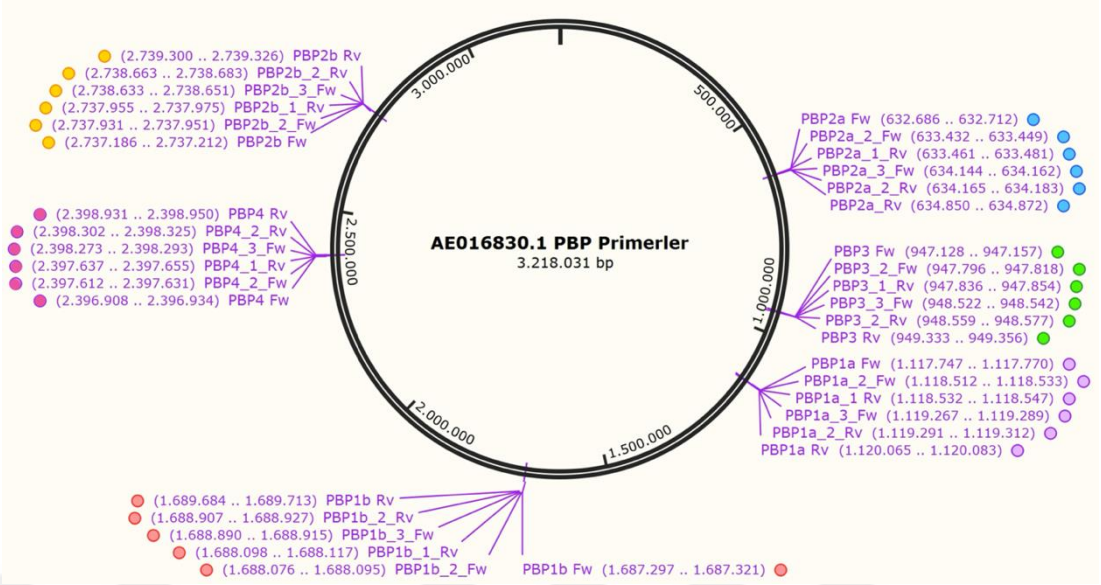

Figure 12. Diagram displays the locations of all PBP primers on the VRE *E. faecalis* V583.

Table 4. The primer sequences and properties of VRE *E. faecalis* V583.

Primer Tag	**Sequence**	**Primer Length (bp)**	**T_m (□C)**	**Product Length (bp)**
PBP1a Fw	TTATGCTGCTTTATTTTCATCTGG	24	54	2337
PBP1a Rv	ATGCCAACCGCAAATTCAG	19	56	2337
PBP1a_1 Rv	GCTTATGCGGCTTTCG	16	54	801
PBP1a_2_Fw	GTTATAAATCCCGTTATTGGCG	22	55	801
PBP1a_2_Rv	GTTGTACTTTACACAATGTACG	22	52	801
PBP1a_3_Fw	CGGCTTTCGAGATTTTTTTATTG	23	55	817
PBP1b Fw	ATGTATCATTTTATTGAGGTGAAGC	25	54	2412
PBP1b Rv	GCGAGTTAATTATTATTCTTTTTATTATCG	30	53	2412
PBP1b_1_Rv	GACAAATCGTCTTTCAAGGC	20	54	821
PBP1b_2_Fw	CACGCCCTATACTAACACTG	20	54	852
PBP1b_2_Rv	GCATCTGAAGCTGGATAGTTC	21	55	852
PBP1b_3_Fw	CACTACTTAAGTAAAATGAACTATCC	26	52	824
PBP2a Fw	ATGGACAATCTTAAACAATTTTTTAGT	27	53	2187
PBP2a_Rv	CTAATTTCCTAATAAGCCTCCGA	23	53	2187
PBP2a_1_Rv	CGACCATTAGTTGTAAAACGG	21	55	796
PBP2a_2_Fw	CATTGACAATGCCACAGC	18	53	752
PBP2a_2_Rv	CACCCGTTTGTAATCCTCC	19	54	752
PBP2a_3_Fw	CGGTACTATGGTTTAGCCC	19	55	729
PBP2b Fw	TTATTTTTTGTACATTTCCATATACGC	27	53	2136
PBP2b Rv	GAGGTATGAAGAAAAACTCATTTATAG	27	52	2136
PBP2b_1_Rv	CGGAACAACCATGCAATTAAC	21	52	790
PBP2b_2_Fw	GAATATTCTAACGCTTGCTCC	21	55	753
PBP2b_2_Rv	GAATGAAGGGGTAACAGAAGG	21	53	753
PBP2b_3_Fw	GCTGTGTGTTCACCAATTG	19	54	694
PBP3 Fw	TGAGTAAAAGACATAAATTTAAACAATTC	30	52	2229
PBP3 Rv	TTATTCTGTGCCTTCTAAAGTCAC	24	53	2229
PBP3_1_Rv	CAAAATACACACGGCCATC	19	55	727
PBP3_2_Fw	GGGACTAGAACAGACCTATAATG	23	54	782
PBP3_2_Rv	GAGGTTGCAACATTGAACC	19	54	782
PBP3_3_Fw	CCAAATGATGAAAGGTTTCTC	21	53	835
PBP4 Fw	TTATTTAATGGTTGCTTCTAAGTAATC	27	52	2048
PBP4 Rv	TTTATTACGATTGCTTCGTTCCAT	24	55	2048
PBP4_1_Rv	CATTGGTTTAGATGCAGGG	19	53	748
PBP4_2_Fw	CTAGCTCTTCATCTGGCTTC	20	55	714
PBP4_2_Rv	CAGCCAATATCAAAGCTTTTAGTG	24	54	714
PBP4_3_Fw	CCTTGGCTTAACTTTTGATTG	21	53	698

T_m: Melting temperature, Fw: Forward primer, Rv: Reverse primer

3.4.3 Acquisition of PBP genes

3.4.3.1 Conventional PCR

The PBP genes were amplified by PCR with genomic DNA extracted from *E. faecalis* and *E. faecium* strains using MyTaq Hot Start DNA Polymerase (Bioline, US) enzyme. The PCR mixture was prepared separately with primer pairs that were specially designed for each PBP encoding gene. The PCR conditions were adjusted in accordance with the enzyme manufacturer's instructions and the optimal melting temperatures of the primers (**Table 5**). The PCR reaction was performed on a T100 Thermal Cycler (Bio-Rad, US) device.

Table 5. The general PCR protocol for amplification of PBP genes.

Ingredients		**Conditions**			
	Volume	**Step**	**Temp.**	**Time**	**Repeat**
5x MyTaq Buffer (5 mM dNTPs, 15 mM $MgCl_2$)	5 µl	Initial denaturation	95^0 C	3 min	
MyTaq HS DNA Polymerase	0.5 µl	Denaturation	95^0 C	15 sec	34 cycles
Primers (20 µM each)	0.5 µl	Annealing	58^0 C	15 sec	
Template DNA (50 ng/µl)	as required	Extension	72^0 C	15 sec	
Water (ddH_2O)	up to 25 µl	Final extension	72^0 C	5 min	

3.4.3.2 Agarose gel electrophoresis (AGE)

AGE is used to separate DNA molecules based on their size. The negatively charged backbone of DNA allows for migration of the molecule towards the positively charged electrode in an electric field applied in a solution. Agarose gel provides a porous matrix that creates a resistance to larger molecules which slows their passage relative to smaller molecules.

In this study, agarose gel was prepared at a concentration of 1% (1 g agarose powder in 100 ml of 1X TAE buffer) in 1X TAE conductive buffer (40mM Tris, 20mM Acetate, 1mM EDTA, pH 8.6). The mixture was heated in a microwave oven for 1-3 minutes until the agarose was fully dissolved, being careful to avoid boiling. The

melted agarose solution was then poured carefully on to a horizontal tray, with a plastic comb placed on top to create wells for the loading of DNA.

Once the agarose gel had solidified, it was transferred to a tank containing 1X TAE buffer and the PCR product DNA, which had been mixed with an equal volume of Orange G loading dye and Sybr Gold fluorescent dye solution (Thermo Scientific, US). A 1 kb DNA ladder (ZG100, ZGeneBio, China) was also loaded into a well as a marker. The gel was run at between 100 and 120 volts (V) for 30-40 minutes. The ORTE (Observable Real Time Electrophoresis, TiBO, Türkiye) device was used for both running and imaging procedures. In addition to the ORTE, the gel was run on an EasyCast B2 Mini Gel Electrophoresis System (Thermo Scientific, US), stained in 0.004% (v/v) ethidium bromide (EtBr) solution for 20 minutes, and imaged using ChemiDoc MP Basic Imaging System (Bio-Rad, US).

3.4.3.3 DNA purification

After a DNA fragment of the gene of interest was identified in the agarose gel, the DNA fragment was excised along with the gel using a clean scalpel. DNA purification from agarose gel was performed using the E.Z.N.A Gel Extraction Kit (V-spin) (Omega BioTek, US) according to the manufacturer's instructions. The DNA containing agarose gel piece was transferred into a sterile 1.5 ml microcentrifuge tube. An equal amount of XP2 Binding Buffer was added and melted in a heat block at 60 ^{0} C for 10 minutes. The DNA-agarose solution was transferred into a HiBind DNA Mini Column placed in a collection tube and centrifuged at 10000 x g for a minute at room temperature. The collection tube was discarded and reused for the washing step with pure ethanol added SPW Buffer (700 µl) under the same conditions. The binding column was transferred into a sterile 1.5 ml microcentrifuge tube and added apyrogenic sterile water (50 µl), preheated to 65°C, carefully directly onto the center of the silica matrix of the binding column. After waiting the tube for 5 minutes at room temperature, centrifuged at 10000 x g for a minute to collect the eluted DNA.

3.4.3.4 Sanger sequencing

Sanger sequencing method is a highly accurate classical chain-termination method for DNA sequencing. This method requires a single-stranded DNA, a primer, a DNA polymerase enzyme, normal and fluorescently labeled dNTPs (249). These labeled nucleotides lack a 3'-OH group which terminates the strand elongation and creates various lengths of fragments. These fragments are separated by their size using capillary gel electrophoresis and fluorescent labels are read to determine the sequence (250).

In this study, the nucleotide sequence analysis of PBP genes was conducted using the Sanger sequencing method on an ABI 3730XL DNA Analyzer (Applied Biosystems, US). The sequencing services were purchased from GATC Biotech AG, Germany. The amplified and purified PBP genes of *E. faecalis* strains were prepared and dispatched to the company for sequence analysis at a DNA concentration of 10-30 ng/µl each with specific primers.

3.4.4 DNA fingerprinting

The term "DNA fingerprinting" refers to a technique in which large-scale genomic DNA is digested with restriction enzymes, producing multi-band maps of DNA fragments of varying sizes. DNA fingerprints have high variability, high individual specificity and stable heritability (251). PFGE is used to detect large genomic polymorphisms and rearrangements in all types of organisms, as well as a strain-typing method for pathogens (252).

3.4.4.1 Preparation of DNA agarose discs

To investigate the genetic diversity of the *E. faecalis* strains included in this study, a DNA fingerprinting analysis was conducted using the PFGE method. Bacterial cells cultured in MH broth medium (as described in section 3.3.2) were harvested in a 1.5 ml microcentrifuge tube and centrifuged at 10000 x g for 2 minutes to remove medium

residue. The collected pellet was resuspended in Tris-saline solution (500 µl, 10 mM Tris pH 8.0, 1 M NaCl) and centrifuged under the same conditions. This washing process was repeated twice. The final pellet was suspended in Tris-saline solution (200 µl). The concentration was then measured and adjusted to OD_{620}= 5.0 with the same solution according to the formula below:

$$V_{add}\ (\mu l) = (OD \times 40 \times 210) - 210$$

Bacterial cells were embedded in agarose discs in order to extract intact genomic DNA. An agarose solution was prepared at concentration of 0.15% (w/v) with low-melting agarose powder in Tris-saline solution and melted in a microwave. The melted agarose solution and the bacterial suspension were then placed in a water bath and their temperatures were equalized to 42°C at least 10 minutes before use.

To prepare the discs, a glass plate was coated with parafilm to create a hydrophobic surface. The microscope slides were positioned on this plate in two rows with the lower row positioned slightly above the upper row. The distance between the rows was less than one slide length, allowing the discs to form in the space between the slides. All parts were cleaned with 70% ethanol. The agarose solution and the bacterial suspension were mixed in a 1:1 ratio (100 µl each) in a new microcentrifuge tube and immediately dropped 20 µl each onto the prepared plate. The drops were covered with another clean slide to form a disc shape, which is essential for increasing the surface area and facilitating the enzymes' passage through the agarose matrix and penetration of the cells and DNA in subsequent steps. The glass plate with the droplets was kept at -20°C for 5 minutes and after transferred to room temperature for at least 10 minutes. The agarose discs were carefully collected into a 15 ml capped centrifuge tube using a disposable loop.

The cell membrane of the cells embedded in agarose discs were lysed in lysis solution (0.5 ml) (6 mM Tris pH 8.0, 1 M NaCl, 100 mM EDTA pH 8.0, 0.5% Na-lauryl sarcosine, 0.2% Na-deoxycholate, supplemented with RNase A (5 µl/ml, 10 mg/ml), lysozyme (5 µl/ml, 20 mg/ml) (Omega Bio-Tek, US) and lysostaphin (5 µl/ml,

10 mg/ml) (ProSpec, Israel) enzymes) to facilitate the release of bacterial genomic DNA. The incubation was set at 37°C in a shaking incubator for approximately 20 hours or more, until the discs became completely transparent.

The lysis solution was removed by sterile gauze and proteinase-K solution (0.5 ml, 0.5 M EDTA pH 9.0, 1% sarkosyl containing 1 mg/ml proteinase-K powder) was added. The mixture was incubated in a shaking incubator at 65°C for approximately 20 hours to digest the remaining protein residues.

At the end of the incubation, the solution was removed with sterile gauze. The residual materials were then removed in the TE buffer (13 ml, 10 mM Tris pH 8.0, 1 mM EDTA pH 8.0). The discs were cleaned in a rocker at room temperature. The TE buffer was renewed every 30 minutes for 5 times. Following cleansing, the agarose discs were transferred into a sterile 2 ml round-bottom microcentrifuge tube, containing the TE buffer (1 ml) and stored at 4°C for long-term preservation.

The DNA-embedded agarose discs were cut with *SmaI* restriction enzyme (New England Biolabs, UK) to create DNA fragment patterns unique to each strain. The restriction mixture was prepared by adding rCutSmart Buffer (4 µl, 50 mM Potassium acetate, 20 mM Tris-acetate, 10 mM Magnesium acetate, 100 µg/ml Recombinant albumin, pH 7.9), *SmaI* enzyme (1 µl, 20 U/ml) and ddH_2O (35 µl), and a disc was combined with this mixture. The incubation was held at 37°C for at least one hour. Once the incubation was completed, the restriction solution was carefully removed using a micropipette and the disc was melted at 70°C for five minutes for facilitating the loading of the running gel for further processes.

3.4.4.2 Pulsed-field gel electrophoresis

The genomic DNA of *E. faecalis* comprises of more than 3.2 megabases (Mb, million base pairs). In order to facilitate the separation of the DNA fragments, which are large even after restriction enzyme digestion, it is required to apply a prolonged, variable and pulsed-field electric current. For this purpose, restricted DNA was

separated by PFGE. The PFGE running gel was prepared as described in section 3.4.5.1 in concentration of 1% with pulsed-field certified agarose powder (Bio-Rad, US). The sample was mixed with Orange G loading dye (2 µl), loaded into wells and the molten disc was allowed to solidify again. The PFGE procedure was performed on the CHEF DRII System (Bio-Rad, US). After the PFGE running gel solidified, it was placed in the tank, previously filled with 0.5X TBE (20 mM EDTA pH 8.0, 108g/L Tris, 55g/L Boric Acid) and cooled to 14°C.

The PFGE running protocol was configured to the optimal settings, which were identified through a series of trials. These settings were as follows: in B1 mode, the voltage was set to 6.0 V/cm, with pulse durations of 5 seconds for initial switch and 35 seconds for final switch, and the protocol was run for 18 hours. At the end of the running, the PFGE gel was stained in a 0.004% EtBr solution and visualized using the ChemiDoc MP Basic Imaging System (Bio-Rad, US).

3.5 Bioinformatics Studies

The bioinformatics studies encompassed within this study include the search and analysis of genomic data of enterococci, the identification of PBP encoding gene regions, and a comparative and comprehensive analysis of the similarities of these genes in individuals belonging to the same species, other enterococcus species and other bacterial species both Gram-positive and Gram-negative.

3.5.1 Data collection and analysis of PBP genes in various bacterial species

For data collection and analysis of PBP genes, a number of key databases that are accessible via the NCBI platform were utilized. The Nucleotide database was the primary repository, comprising sequences sourced including GenBank (GenBank Sequence Database) (247), RefSeq (Reference Sequence Database) (253) and PDB (Protein Data Bank)(254). This comprehensive sequence source formed the basis for research into genetic structures. Additionally, BLAST (The Basic Local Alignment Search Tool) (248,255) was utilized to identify regions of similarity, which allows

comparisons between nucleotide or protein sequences. BLAST, which calculates sequence match statistics, enables the search for potential links between sequences, as well as the identification of related gene family members.

The complete genome sequences of VRE *E. faecalis* V583 (Reference sequence: NC_004668.1, GenBank accession: AE016830.1) and VRE *E. faecium* Aus0004 (Reference sequence: NC_017022.1) are available in the NCBI database. PBPs of vancomycin resistant and susceptible strains whose complete genome data was obtained from the GenBank database were analyzed comparatively (**Table 7**).

E. faecalis V583, the first VRE was isolated from a blood culture in the US in 1987 and was the first VRE completed genome. It has *van*B-type resistance (222,256). *E. faecalis* ATCC 51299 is also VRE and used as a reference strain for vancomycin resistance studies like V583. It has both *van*A and *van*B-type vancomycin resistance (257,258). *E. faecalis* ATCC 29212 is a vancomycin-susceptible reference strain, which is commonly used in laboratory studies. It was isolated from a human urine sample in the US (257,259). *E. faecalis* D32, vancomycin-susceptible strain, was isolated from fig feces in Denmark in 2001. It represents the first complete genome sequence of an animal isolate (260,261). *E. faecalis* OGRF1 is a laboratory-produced rifampicin and fusidic acid resistant derivative of non-antibiotic-resistant human oral isolate OG1. It is otherwise susceptible to commonly used antibiotics (262,263). *E. faecalis* 62, vancomycin-susceptible strain, was isolated from a healthy infant in Norway. It is the third *E. faecalis* strain to sequenced complete genome after V583 and OGRF1 (264,265). *E. faecium* Aus0004 is a *van*B-type VRE. It was first isolated from blood culture in Australia in 1998. It is the first strain of *E. faecium* to have its complete genome sequenced (266,267).

The PBP encoding open reading frames (ORFs) were initially identified following a preliminary search and evaluation with the Protein BLAST tool, by detecting motif sequences consisting of a few highly conserved marker amino acids, which are detailed in section 1.5.1. For the analysis of multiple bacterial strains (**Table 8**), the PBPs were aligned using the Clustal Omega multiple sequence alignment algorithm (268) in

standard settings and the presence of the motifs was determined using the MEGA X program (269).

3.5.2 Alignment and comparative sequence analysis

The PBP genes of *E. faecalis* clinical strains were amplified by PCR and the resulting DNA fragments were sequenced by Sanger sequencing method. The sequence data were processed using BioEdit software (version 7.0.5.5.3). The ClustalW algorithm was applied with default settings, utilizing the BLOSUM62 matrix. Comparative analysis of both VRE. *faecalis* and VSE. *faecalis* PBPs was performed by incorporating the sequences of a positive control strain, VRE *E. faecalis* V583. Mutation points were determined in the alignment studies and these nucleotide sequences were translated into amino acid sequences in the reading frame using the EXPASY Translate (Swiss Institute of Bioinformatics, Switzerland). Further studies were then conducted on these amino acid sequences at the protein level.

3.5.3 Protein modeling and docking studies

In this study, homology modeling was applied to predict the 3D structures of wild-type and mutant PBPs from VRE.fs and VSE.fs strains. The SWISS-MODEL homology modeling server (270), accessible online from Swiss Institute of Bioinformatics, was utilized to generate the models by aligning amino acid sequences of target PBPs to reference structures available in the Protein Data Bank. This approach aimed to create reliable structural representations of PBPs to enable further functional and interaction analysis.

In construction of the homology models, it was ensured that the level of coverage was as high as possible. The reference protein exhibiting the highest degree of identity (≥30%) and root-mean-square deviation (RMSD) of less than 4 Angstroms (Å), and as close as possible to 1 Å, was selected as the protein with the highest resolution. In accordance with the specific criteria, the properties of the templates are as follows. For PBP1B, penicillin-binding protein 1B from *Streptococcus pneumoniae* R6 (PDB ID.

2JE5) (271), the sequence identity is 50.71%, the coverage is 93-780th amino acid, and the X-ray diffraction resolution is 2.40 Å. For PBP2A, penicillin-binding protein 4 from *Listeria monocytogenes* (PDB ID. 3ZG7) (272), the sequence identity is 48.77%, the coverage is 190-646th amino acid, and the X-ray diffraction resolution is 1.99 Å. For PBP3, penicillin-binding protein 2X from *Streptococcus thermophilus* LMG 18311 (PDB ID. 5U47) (273), the sequence identity is 39.64%, the coverage is 68-738th amino acid, and the X-ray diffraction resolution is 1.95 Å.

Following the modeling, ligand binding dynamics were examined using the University of California, San Francisco (UCSF) Chimera (version 1.16) (274) program and AutoDock Vina (version 1.1.2) software. These tools facilitate the docking of specific ligands, natural L-Lys-D-Ala-D-Ala and modified L-Lys-D-Ala-D-Lac, into the active sites of each PBP model, allowing for the calculation of binding affinities and visualization of interaction patterns. Chimera X (version 1.3) (275) was employed to visually inspect and analyze the resulting 3D-structures and docking results.

The ligands were initially designed in ChemDraw (CambridgeSoft, UK) and then converted into 3D structure formats proper to process in Chimera, for compatibility with docking procedures. Comparing the binding interactions of each ligand on VRE and VSE PBPs enabled the identification of potential differences in ligand affinity and binding configuration, which may contribute to understanding resistance mechanisms.

3.5.4 Phylogenetic analysis of PBPs

In order to gain insights into the evolutionary pattern of PBPs, a total of 16 bacterial strains were analyzed, comprising nine Gram-positive and seven Gram-negative strains (**Table 8**). The strains were selected based on the availability and accuracy of their complete genome sequences.

A comprehensive analysis was performed on a set of 124 PBP genes from these well-documented bacterial species. The amino acid sequences were obtained from the GenBank database and identified according to their protein accession IDs and gene

annotations. Domains were analyzed on the Conserved Domain Database (CDD), to confirm the presence of penicillin-binding domains (276). Protein properties, including molecular-weight and isoelectric point (pl) were predicted using the ProtParam tool (277).

For the purpose of evolutionary analysis, the sequences were aligned using the MUSCLE (Multiple Sequence Comparison by Log-Expectation) algorithm with standard settings to generate a highly accurate alignment (278). A phylogenetic tree was constructed using the Maximum Likelihood (ML) method in MEGAX. The analysis employed the JTT (Jonas-Taylor-Thorton) model (279) and with 300 bootstraps to assess tree reliability (280).

3.6 Development of Peptide Inhibitors Studies

The modified D-Ala-D-Lac stem pentapeptide of lipid II monomer enables the bacterium to continue synthesizing the cell wall without being affected by the presence of vancomycin. In this study, the increased affinity of PBPs for this modified substrate inspired the question of whether the synthesis of a form of D-Ala-D-Lac that binds irreversibly to PBPs could potentially inactivate or inhibit these proteins. To verify this hypothesis, free peptide traps of D-Ala-D-Lac were synthesized for testing their antimicrobial activities.

3.6.1 Peptide synthesis

D-Ala-D-Lac were synthesized using the Solution-Phase Peptide Synthesis method. This method is basically based on protecting functional groups and forming the ester bond between the lactic acid and alanine in a solution medium. The synthesis was conducted in four steps: (1) acetylation of alcohol, (2) tert-butylation of the carboxyl group, (3) Boc-D-Ala-OH binding to D-Lac, and (4) removal of the protecting groups (**Figure 13**).

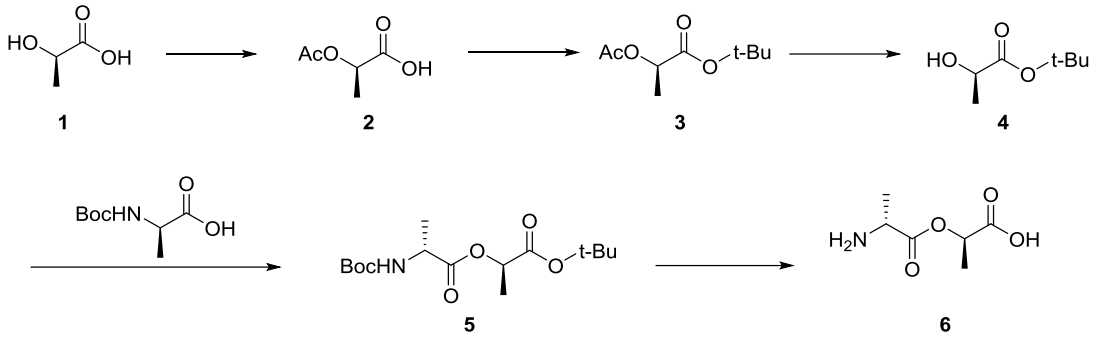

Figure 13. D-Ala-D-Lac synthesis reaction pathway.

The initial step of the peptide synthesis involved the protection of the alcohol group of D-lactic acid through the addition of an acetyl (Ac-) group. Sodium D-Lactate (8.92 mmol, 1000mg) (Thermo, US) was dissolved in acetic acid (6 ml) in a glass flask. Acetyl chloride (1.9 ml) was added dropwise to the reaction mixture at 0ºC with continuous stirring on a magnetic stirrer. The reaction was allowed to proceed overnight at room temperature. The acetic acid was subsequently removed from the reaction medium via rotary evaporator (BUCHI Corporation, US). The substance, formed in gel form, was dissolved in a solution of five times the volume of dH_2O and dried in a freeze dryer (Labconco, US). The formation of acetylated D-Lac (**Figure 13.2**) was confirmed by nuclear magnetic resonance spectroscopy (NMR) with an Avance IVDr System (Bruker, US). The NMR spectroscopy service was bought from the Acibadem Chromatography and Spectroscopy Laboratory, Istanbul.

The acylated D-Lac (**Figure 13.2**) (2.75 mmol, 363.74 mg), was weighed and dissolved in dichloromethane (DCM, 2 ml). 2.2-fold amount of tert-butanol (580 µl) was added, and dry 4-dimethylaminopyridine (DMAP, 100.8 mg, 0.825 mmol) was dissolved in the reaction mixture on a magnetic stirrer. The reaction temperature was reduced to 0 ºC, and dicyclohexylcarbodiimide (DCC, 3.575 mmol, 737.6 mg) dissolved in DCM (1 ml) was added dropwise. The reaction was carried out overnight in a nitrogen-filled environment at room temperature with continuous stirring. The solid particles formed during the reaction were removed by filter paper filtration. The liquid phase was washed once in a separatory funnel with an equal volume of distilled water. An additional washing procedure was applied to eliminate any residual DCC and DMAP, a solution of 10% acetic acid and 10% sodium bicarbonate was added to

the liquid phase, and washed in a separatory funnel, twice and one, respectively. The resulting organic phase in DCM was evaporated in a fume hood. The expected reaction product, acetylated D-Lac-(O-tBu) (**Figure 13.3**) was analyzed using NMR.

In the third step of the synthesis, the protecting acetyl group was removed. The 3-fold amount of potassium carbonate (K_2CO_3, 3.3 mmol, 456 mg) of substance (1.11 mmol, 209 mg) was initially dissolved in distilled water (3 ml) in a round bottom glass flask and then added methanol (1 ml). The methanol: water ratio was equalized by adding an additional methanol (2 ml) dropwise to the reaction mixture on ice on a magnetic stirrer. The reaction was allowed to proceed for a minimum of 3 hours in a nitrogen-filled environment at room temperature with continuous stirring. At the end of the reaction period, the substance (**Figure 13.4**) (21.5 mg) was immediately reacted with Boc-D-Ala-OH (0.16 mmol, 30.5 mg) (Thermo, US) in DCM (2 ml). After the addition of solid DMAP (0.16 mmol, 19.5 mg) to the mixture, DCC (0.16 mmol, 33 mg) dissolved in DCM (1 ml) was added dropwise on ice. The reaction was allowed to proceed overnight in a nitrogen-filled environment at room temperature on a magnetic stirrer. At the end of the reaction period, the material (**Figure 13.5**) was washed with equal volumes of 10% acetic acid, 10% sodium bicarbonate and distilled water using a separatory funnel, respectively. The organic phase in DCM was filtered through celite (Merck, Germany), and the DCM was evaporated in a fume hood.

In order to remove the Boc- and -tBu protecting groups on the synthesized dipeptide, a solution (2 ml) containing 95% trifluoroacetic acid (TFA), 3% triisopropyl silane (TIPS) (Sigma- Aldrich, US) and 2% distilled water was added and the reacted for 3 hours at room temperature on a magnetic stirrer. Once the reaction had completed, an equal volume of synthesis material and pure diethyl ether, cooled to -20 ºC, were mixed and then diethyl ether was removed by centrifugation at 14000 x g for 10 minutes. This process was repeated three times, and freeze-dried. The final product (**Figure 13.6**) was analyzed by 1H-NMR spectroscopy.

3.6.2 Analysis and purification of D-Ala-D-Lac

The products derived from each step of the synthesis were analyzed using Thin-layer Chromatography (TLC), whereby the products were absorbed onto a silica-covered layer and run in an appropriate solvent solution, according to its polarity. The solvent solution used was typically DCM and methanol mixture with the addition 0.5% acetic acid, based on the solubility characteristics of the product. TLC silica layers were visualized at short-wavelength UV light (254 nm) using UV Cabinet 4 (CAMAG, Switzerland).

The synthesized substance was analyzed and purified using a semi-prep column (Advance Bio Peptide Plus colon (2.1x150 mm, 2.7 micron)) in Agilent 1200 Infinity System (Agilent, US), a high-performance liquid chromatography (HPLC) device. The substance was dissolved in distilled water (1 mg/ml) and introduced into the system. It was run in an acetonitrile and water mixture gradient elution ranging from 0% to 100% over a period of 5-35 minutes. The elution profile was monitored at 214, 254 and 280 nm wavelengths.

3.6.3 Antimicrobial susceptibility tests

The disk diffusion method was used to test the susceptibility of VRE strains to the synthetic D-Ala-D-Lac dipeptide. This method is a quantitative technique that is based on the diffusion of antimicrobial agents in agar. The diffusion extent is determined by measuring the diameter of the inhibition zone (281).

The disk diffusion test was performed using VRE *E. faecalis* (VRE 6) strain as the test organism. The bacteria was cultivated in an MHA plate at 37ºC for approximately 18 hours. A small bacterial colony was resuspended in pure water (5 ml) containing a sterile glass tube with a swab, and the turbidity was adjusted to McFarland 0.5 (108 microorganisms/ml). The inoculum was subsequently plated on MHA plates using the spread plate method. A vancomycin disk was placed on the plate for the control of vancomycin resistance, and 10% isopropanol, peptide solvent

solution was used as a negative control. Synthetic peptides were dropped onto the plates in series of dilutions, with concentrations of 16, 8, 4, and 2 μg/ml. Additionally, dehydrated peptides were tested on a separate MHA plate prepared according to the same method. The plates were incubated in an incubator at 37ºC for 18 hours.

4 RESULTS

4.1 Information Retrieval on PBPs from Open Access Databases

The data regarding PBP genes was collected from complete genome sequences accessible via open access databases. At the beginning of the study, the retrieved information was largely in the form of raw nucleotide sequences or particularly for PBPs, hypothetical protein ORFs lacking gene annotations. During the study, the sequences were updated several times. Through local alignment search and motif analysis of raw data, we were able to successfully identify PBPs and confirm their true identities. Our findings were subsequently validated by updates to the original data set.

In line with our research objective to examine their role in the mechanism of vancomycin resistance, we investigated HMW Class A and Class B PBPs that process D-Ala-D-Lac stem peptides and exhibit transpeptidase activity. The six genes encoding PBP1A, PBP1B, PBP2A, PBP2B, PBP3, and PBP4 of *E. faecalis* were the focus of the study.

The first vancomycin-resistant enterococcal strain to undergo complete genome analysis, *E. faecalis* V583, was isolated from a blood culture in the US in 1987 (256), exhibiting vanB-type vancomycin resistance. The most significant result of the V583 genome analysis is estimated 25% of its genomic content that comprises mobile DNA. The locus tag codes, and protein IDs of these genes are presented in **Table 6**.

Table 6. Classification of PBPs of VRE *E. faecalis* V583.

Class	**Protein**	**Locus tag**	**Protein ID**
Class A	PBP1A	EF_1148	AAO80948.1
	PBP2A	EF_0680	AAO80501.1
	PBP1B	EF_1740	AAO81514.1
Class B	PBP4	EF_2476	AAO82193.1
	PBPC/3	EF_0991	AAO80797.1
	PBP2B	EF_2857	AAO82549.1
Class C	D-alanyl-D-alanine carboxypeptidase	EF_3129	AAO82807.1
	PBP/Serine hydrolase	EF_0746	AAO80564.1

Vancomycin-resistant *E. faecium* Au0004 was included in the PBP analysis of the same genus *E. faecalis*. The nucleotide sequence alignment of PBP1A revealed no significant similarity except the motif regions (**Figure 14**). However, an amino acid sequence alignment by *E. faecalis* V583 (Protein ID: AAO80948.1) and *E. faecium* Aus0004 (Protein ID: AFC63082.1) showed 65.60% identity on the protein BLAST tool (**Figure 15**).

In nucleotide location 1787, the matched region "*AAAACAGGGACT*" sequence shown with dots (**Figure 14**) encodes the KTGT amino acid sequence, which is one of the markers of PBP the motif III. The motif sequences demonstrate similarity, whereas in other regions, no significant similarity is observed. The nucleotide at position 1795 demonstrates codon usage pattern with a silence mutation in *E. faecium* Aus0004, diverging from that observed in *E. faecalis* strains. Specifically, it exhibits no change in amino acid sequence, with a transition from G to A.

The PBPs were found to be significantly divergent from one another, making amplifying them using PCR unsuccessful. To ascertain whether the mutations identified in the nucleotide sequences would result in changes to the protein structure, it was essential to make examinations on the level of amino acid sequences and construct 3D models of the folded functional proteins.

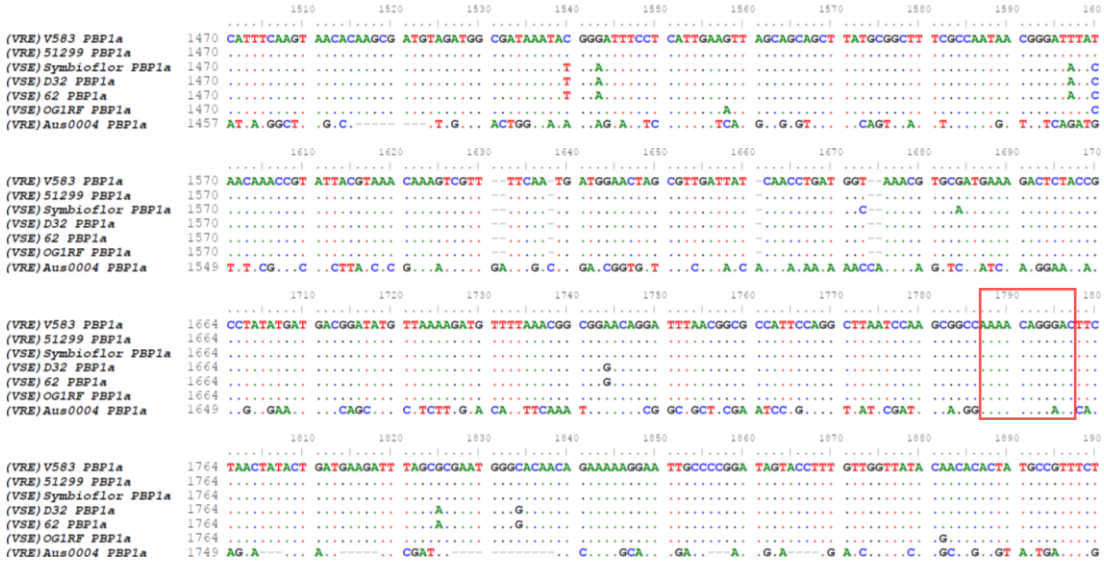

Figure 14. Nucleotide alignment of the PBP1A genes of VRE and VSE *E. faecalis* and VRE *E. faecium* Aus0004 strains. Red rectangle indicated the motif III.

penicillin-binding protein 1A [Enterococcus faecalis V583]

Sequence ID: AAO80948.1 Length: 778 Number of Matches: 1

Range 1: 36 to 692 GenPept Graphics ▼ Next Match ▲ P

Score	Expect	Method	Identities	Positives	Gaps
881 bits(2277)	0.0	Compositional matrix adjust.	431/657(66%)	517/657(78%)	1/657(0%)

```
V583     40   LFIKILLGILSFFCILFLAGVGLFWYYAKDAPELTDKKLDATVSSKLYTQDGELFEDLGA
Aus0004  36   .VL..F..L.IAGMVA.........F..RQ..K.E.D..N........DINN.I......

V583     100  EKREKISANELPKTLEDAIVSVEDRRFYKHIGVDPIRIIGSALSNFTSGGLQGGSTLTQQ
Aus0004  96   ....L.QP.DV.QL.K.............................VKN............

V583     160  LIKLSFFSTSAEDQTLKRKAQEAWMAVRLEQKKSKQEILTYYVNKVYMSNGLYGMETASE
Aus0004  156  .....Y...KES...................RE...E......I.....A..F......A.

V583     220  MYFGKKLSELSLPQTALLAGMPQAPSAYDPYVYPDQAKKRRDTVLYTMLQNEKISQTEYD
Aus0004  216  N.Y..H....D..............NS....TK..T..E...V.....YD.K...KA..E

V583     280  QAVNVPVTDGLQELTQSDDNTKIVDNYVKEVINEVQEKTDKNVYTDGLEIYTNLDLDAQK
Aus0004  276  K.KAT.IDE..VP.KA....R.V............KA..G........D......MN...

V583     340  KLYDIVNTDQYVSYPDDEMQVASTLIDTNTGKVKAQIGGRHIAEDVTLGNNLAVNTSRDF
Aus0004  336  Q......S....AF...K......V..VAS.Q.R........PD..Q.........Q..V

V583     400  GSTMKPVTDYGPAFEYLKYSTGKTITDAPYNYEGTSTPVGNWDNQYMGTITLRQALYLSR
Aus0004  396  ...V..IM.....I.N.N....RLMV.K.TK.P..DID.F.S.LT.Q.V..M.R.IMG..

V583     460  NVPAVKLFNEVGSDKVASFLKNLGIEYSTIHQSNAISSNTEEQDGTKYGASSLKMAAAYA
Aus0004  456  .TT..QT.D...KENIMP.I.G...D.KNLEA........SDV..D...I....L.....

V583     520  AFANGGTYYKPQYVNKIVFQDGTEETYEPDGKTAMSPETAYMITDILKDTITEGTGTNAQ
Aus0004  516  ....N.I.N..Y....V..N...SVD.Q....R..KDS....M..M...VLNG...F.GA

V583     580  IAGLYQAGKTGTSNYTDDEYAKLGIS-SGVYPDILFAGYTPNYSISVWTGYNKKMTPVTS
Aus0004  576  .P..I..A.........EDL.RM.TTEK.IA..ST.V...TH.AV.......DRN..IYQ

V583     639  ESSHVASDVYRELMQYVSANVTNTDWEMPSGLIRVGGELYYKDQYTARSNAITPSTT  695
Aus0004  636  .YYGI.......I.S.L.Q..S.D..VQ.DSVV...N...V..A.EVPNVQVL....  692
```

Figure 15. VRE *E. faecalis* V583 and VRE *E. faecium* Aus0004 PBP1A alignment.

The data regarding the *Enterococcus* strains whose complete genome sequences are available for comparison with the previously mentioned reference strain V583 are presented in **Table 7**. The PBPs of these bacteria were not correctly identified in their complete genome datasets. The ORFs were either not classified or named only as "penicillin-binding protein", with an emphasis on transpeptidase activity, or left unannotated as hypothetical protein. A small number of PBPs classified were incorrect, thus these ones were renamed according to the sequences and amino acid sizes of PBPs identified in the reference strain V583. The vancomycin-resistant strains V583 and ATCC 51299 were compared with the other vancomycin-susceptible strains, ATCC 29212, D32, 62 and OGRF1.

The findings of the alignment of PBPs in the NCBI database with the protein and genome accession numbers given in **Table 7**, are out of the six HMW PBPs, three exhibited amino acid substitutions, while the remaining three did not. While PBP1A, PBP3 and PBP4 remained unchanged, PBP1B exhibited three amino acid substitutions, PBP2A two and PBP2B 35. The changed PBPs were as follows: PBP1B (from threonine to asparagine at position 491(T491N), P554S, T671A) (**Figure 17**), PBP2A (E89K, K336G) (**Figure 18**), PBP2B (A151S, V381F, G396A, A419T, L422I, Q423K, T427V, L429T, P432A, I434L, T438S, S447A, G448S, A452T, Q453T, V473L, L477M, V487I, I492V, K496T, Y505V, I512T, L518I, S522T, P523T, Y525I, V526Q, P533S, A534S, K538Q, N552T, T554S, P555A, Q627P, V659N) (**Figure 19**).

Table 7. Information of comparatively analyzed *Enterococcus* strains.

Strain	***E. faecalis* V583**	***E. faecalis* ATCC 51299**	***E. faecalis* ATCC 29212**	***E. faecalis* D32**	***E. faecalis* 62**	***E. faecalis* OGRF1**	***E. faecium* Aus0004**
Vancomycin-resistance	+	+	-	-	-	-	+
Accession number	AE016830.1	JSES0000	CP008816.1	NC017316.1	CP003726.1	CP002491.1	NC017022.1
Protein ID. PBP1A	AAO80948.1	KGQ75598.1	AIL04424.1	AFO43846.1	ADX79825.1	WP_002360569.1	AFC63082.1
Protein ID. PBP1B	AAO81514.1	KGQ74720.1	AIL04223.1	AFO44358.1	ADX80336.1	WP_002414033.1	-
Protein ID. PBP2A	AAO80501.1	KGQ75158.1	AIL05477.1	AFO43389.1	ADX79303.1	WP_002355517.1	-
Protein ID. PBP2B	AAO82549.1	KGQ73632.1	AIL05666.1	AFO45341.1	ADX81159.1	WP_002359399.1	-
Protein ID. PBP3	AAO80797.1	KGQ73849.1	AIL03712.1	AFO43695.1	ADX79669.1	WP_002381310.1	-
Protein ID. PBP4	AAO82193.1	KGQ72602.1	AIL04845.1	AFO44975.1	ADX80903.1	WP_002356658.1	-

Figure 16. The changed amino acids of PBP1B of *E. faecalis* strains. Dots indicate the VRE strains.

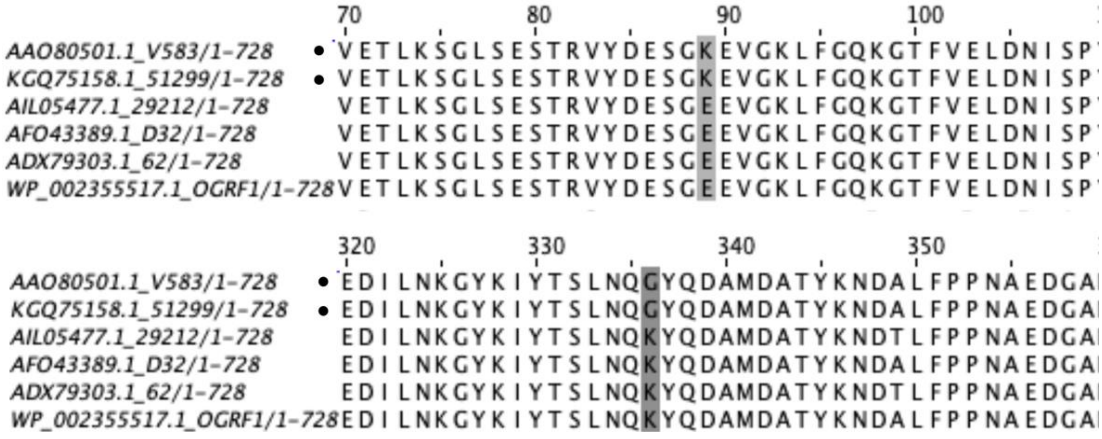

Figure 17. The changed amino acids of PBP2A of *E. faecalis* strains. Dots indicate the VRE strains.

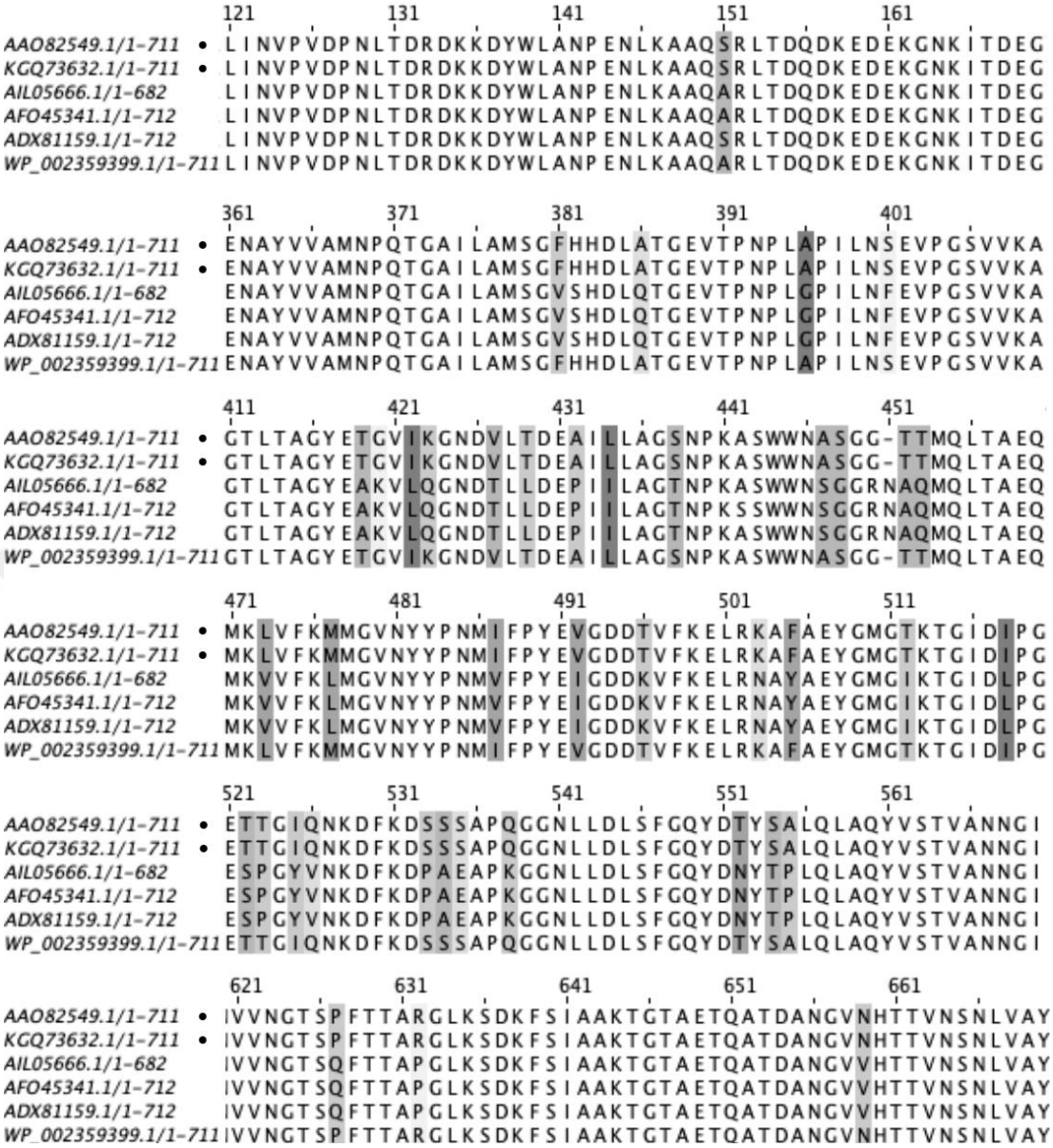

Figure 18. The changed amino acids of PBP2B of *E. faecalis* strains. Dots indicate the VRE strains.

4.2 Phylogenetic Relationships among PBPs

The phylogenetic analysis of PBPs across various bacterial species revealed distinct clustering patterns between Gram-positive and Gram-negative bacteria, confirming their revolutionary divergence (**Table 8**). Two PBPs were identified within the genomes of *E. faecalis* and *E. faecium* as belonging to the "PBP1A family penicillin-binding protein" category based on GenBank annotations. Further

investigation using BLAST and alignment confirmed that these PBPs corresponded to PBP1A and PBP2A, thus allowing for accurate classification within the PBP family.

Table 8. The complete genome information of widely investigated bacterial strains analyzed for PBPs.

Strain	Accession number	Reference
Gram-positive strains		
Bacillus subtilis 168	NC_000964.3	282
Enterococcus faecalis V583 (VRE)	NC_004668.1	222
Enterococcus faecalis ATCC 29212 (VSE)	NZ_CP008816.1	283
Enterococcus faecium XY2 (VRE)	NZ_CP039729.1	284
Enterococcus faecium DO (VSE)	NC_017960.1	285
Staphylococcus aureus MRSA252 (VSSA)	NC_002952.2	286
Staphylococcus aureus VRS5 (VRSA)	NZ_AHBO00000000.1	287
Streptococcus pneumoniae R6	NC_003098.1	288
Streptococcus pyogenes NGAS638	NZ_CP010450.1	289
Gram-negative strains		
Escherichia coli K-12	NC_000913.3	290
Klebsiella pneumoniae HS11286	NC_016845.1	291
Pseudomonas aeruginosa PAO1	NC_002516.2	292
Proteus mirabilis HI4320	NC_010554.1	293
Salmonella enterica LT2	NC_003197.2	294
Shigella dysenteriae ATCC 13313	NZ_CP026774.1	295
Haemophilus influenzae Hi375	NZ_CP009610.1	296

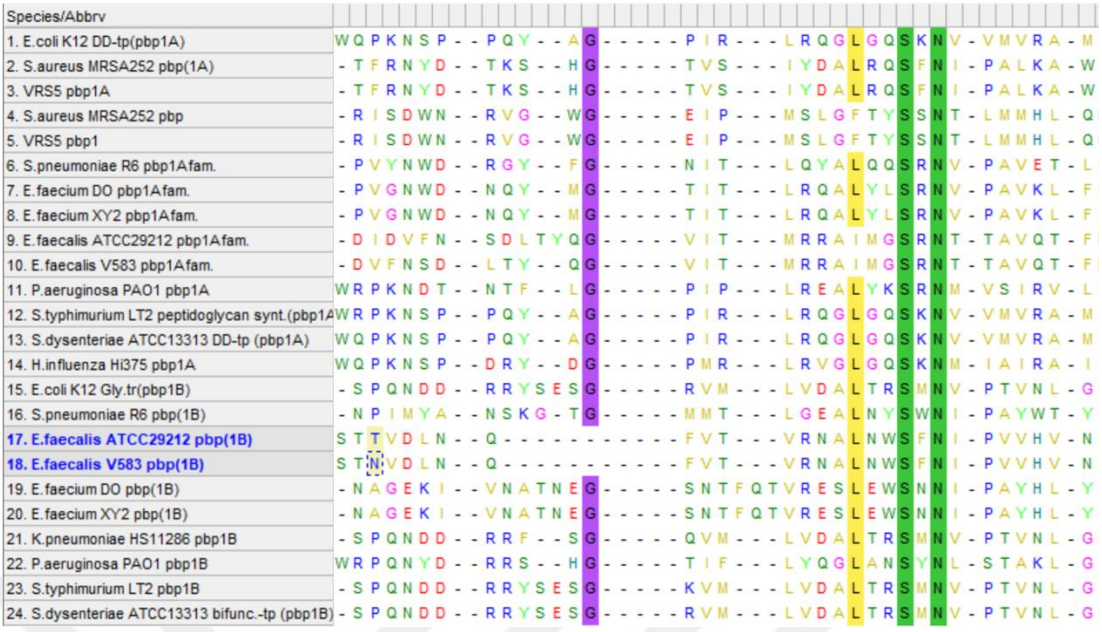

Figure 19. Determination of motif II ((S/Y)xN), in PBP alignment. The motif is indicated in the green colored column.

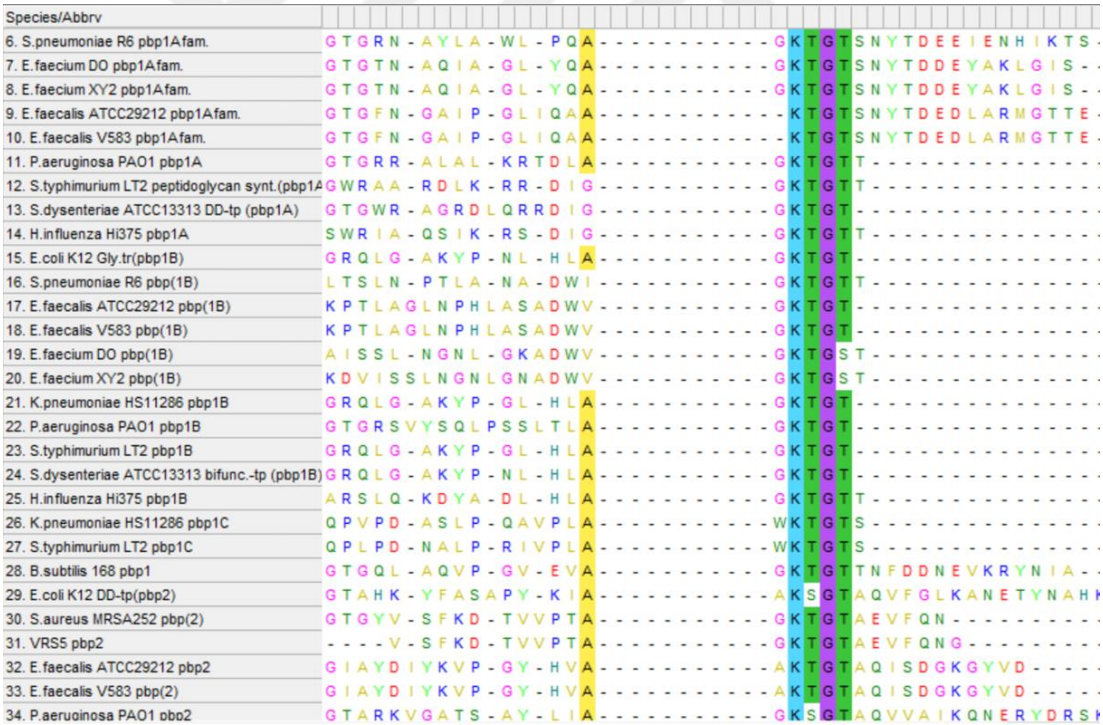

Figure 20. Determination of motif III (K(T/S)GT) in PBP alignment. The motif is indicated in blue-green-purple colors in a single column.

A noteworthy clustering pattern was identified among the proteins associated with vancomycin resistance. Specifically, the proteins *E. faecalis* PBP4, *E. faecium* PBP5, methicillin-resistant *Staphylococcus aureus* PBP2A, *Streptococcus pneumoniae*

PBP3, and *Bacillus subtilis* PBP3 were located in the same clade (**Figure 22.1**). Given that *Bacillus* species are known to facilitate horizontal gene transfer, the close phylogenetic proximity of these taxa suggests that PBPs associated with vancomycin resistance may be acquired through horizontal transfer, potentially contributing to the evolution of resistance mechanism.

Another distinctive observation within the phylogenetic tree is the positioning of *S. aureus* strain VRS5, a rare high-level vancomycin-resistant strain. Its distinctive PBP, referred to as PBPX, has an approximately 34.5 kDa molecular weight and is significantly smaller than other PBPs. This protein clusters closely with Enterococcus PBP1B (approximately 88 kDa) (**Figure 22.2**), indicating a potential functional relationship. This proximity suggests the possibility that the smaller PBPs in methicillin- and vancomycin-resistant *S. aureus* strains may be a part of glycosyltransferase and/or transpeptidase activity, similar to PBP1B, or may act as regulatory elements affecting the activity of other PBPs. Moreover, PBPX exhibited similarities to *S. pneumoniae* PBP2X, a known antibiotic resistance-related PBP, indicating a potential involvement in resistance pathways.

The two different types of cleaving peptidases were observed to be positioned in the same clade. The *Escherichia coli* K12 D-alanyl D-alanine endopeptidase PBP4, which specifically functions to cut cross-linkage between neighboring glycan chains, was identified in the same clade as D,D-carboxypeptidases, which functions to remove terminal amino acids or dipeptides. This revolutionary convergence suggests that *E. coli* K12 PBP4 may have undergone a functional change during the evolutionary process. Furthermore, the Gram-positive *B. subtilis* 168 PBPX, was found in the same clade with the Gram-negative bacteria.

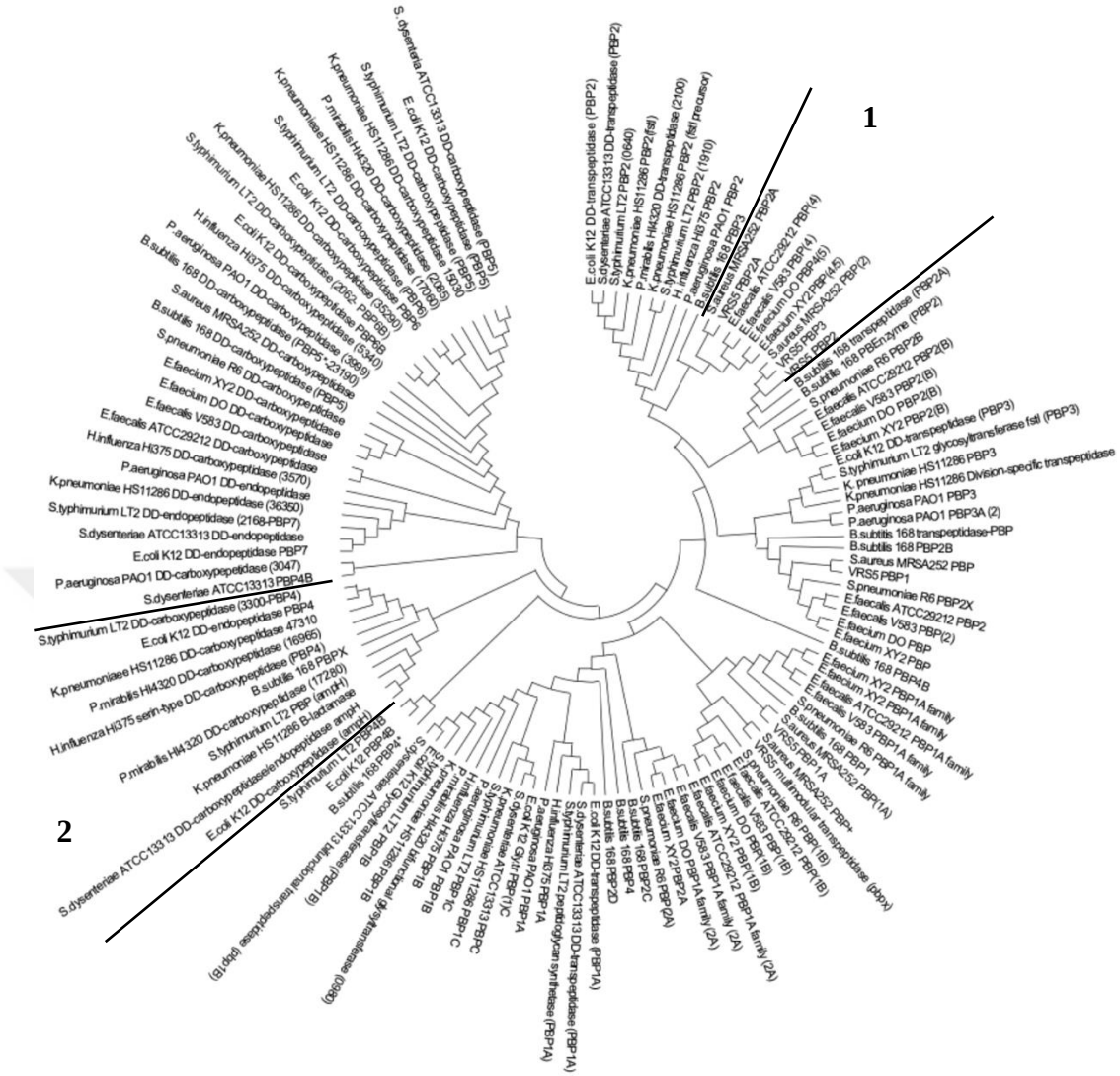

Figure 21. The phylogenetic tree of PBPs of multi-strain.

4.3 Identification of PBP Sequences and Mutation Points in *E. faecalis*

The antibiogram data of clinical *E. faecalis* strains were obtained from routine tests performed at Acibadem Labmed Laboratories, the source of the strains. The data are presented in Table 9.

Table 9. Antimicrobial susceptibility test results of *E. faecalis* clinical isolates.

Strain	**VSE1**	**VSE2**	**VSE3**	**VRE4**	**VRE5**	**VRE6**	**VRE7**	**VRE8**
Sample	Urine	Urine	Urine	Skin fistula	Rectal swab	Urine	Rectal swab	Blood
Ciprofloxacin	S(<=0.5)	S(<=0.5)	R(>=8)	R(>=8)	S(>=8)	R(6mm)	ND	ND
Trimethoprim-sulfamethoxazole	IS	IS	IS	R(>=320)	R(>=320)	ND	ND	ND
Vancomycin	S (2mm)	S (1mm)	S (1mm)	R(6mm)	R(6mm)	R(6mm)	R(6mm)	R(6mm)
Tigecycline	S(<=0.12)	S(<=0.12)	S(<=0.12)	S(<=0.12)	S(<=0.12)	ND	S(25mm)	S(35mm)
Linezolid	S (2mm)	S (2mm)	S (2mm)	S(24mm)	S(25mm)	S(29mm)	S(24mm)	S(30mm)
Erythromycin	R	R	R	R	R	ND	ND	ND
Penicillin G	R	R	R	R	R	ND	ND	ND
Teicoplanin	S(<=0.5)	S(<=0.5)	S(<=0.5)	R(10mm)	R(11mm)	R(6mm)	R(6mm)	R(6mm)
Ampicillin	R(<=2)	R(<=2)	R(<=2)	R(<=2)	R(<=2)	R(10mm)	S(15mm)	S(25m)
Clindamycin	R	R	R	R	R	ND	ND	ND
Tetracycline	R	R	R	R	R	ND	ND	ND
Gentamycin (High-level)	S	S	R	R(6mm)	R(6mm)	R(6mm)	R(6mm)	R(6mm)

S: Sensitive, IS: Intermediate sensitive, R: Resistant, ND: No data.

PCR-amplified target genes were identified in 1% agarose gel by electrophoresis. The expected size of the amplified products was determined by the length of the primer pairs used in the reaction. The DNA fragments were then extracted from the gel.

The bioinformatic analysis revealed that the nucleotide sequences of the PBPs of *E. faecalis* and *E. faecium* were significantly divergent (**Figure 15**) to be amplified using PCR with the same primer pairs. PCR using several VRE and VSE *E. faecium* strains obtained from Hacettepe University and *E. faecalis* PBPs-specific primers did not yield the desired amplification of PBP genes. However, in a vancomycin-resistant *E. faecium* strain, named VRE12, PBP genes were succesfully amplified (**Figure 29**). Further Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF) examination of VRE12 confirmed that it was *E. faecium*.

The DNA fragments extracted from the gel were submitted to the service provider for nucleotide sequence determination via Sanger sequencing.

Figure 22. Electrophoresis of VanA-type vancomycin-resistance detection by PCR with 100bp DNA Ladder.

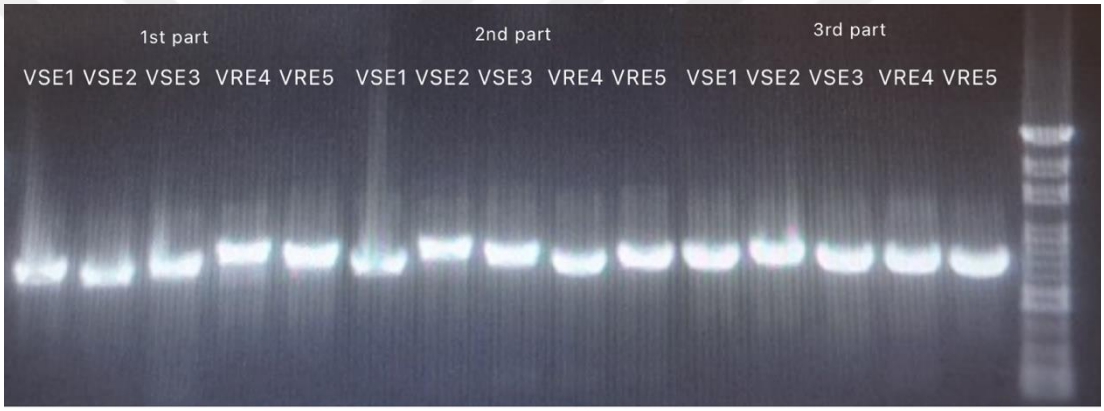

Figure 23. Electrophoresis of *E. faecalis* PBP1A genes in 1% agarose gel.

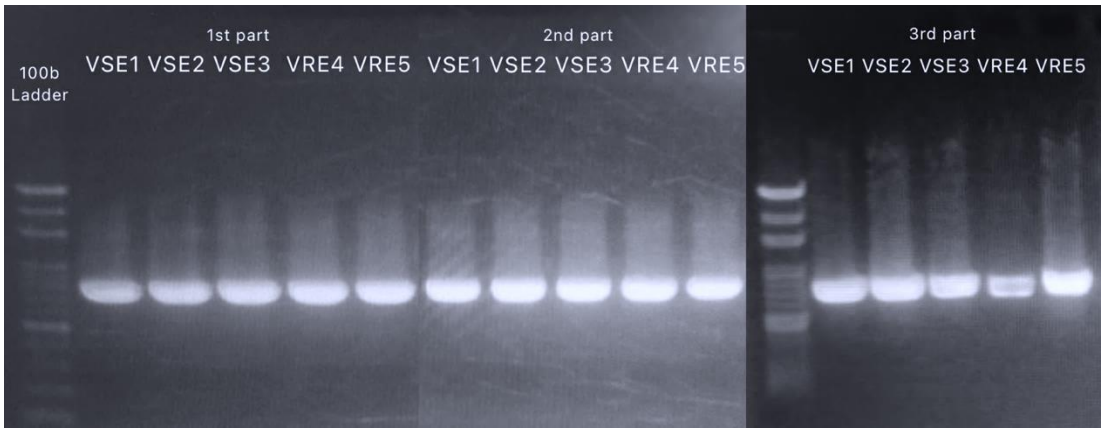

Figure 24. Electrophoresis of *E. faecalis* PBP1B genes in 1% agarose gel.

Figure 25. Electrophoresis of *E. faecalis* PBP2A genes in 1% agarose gel.

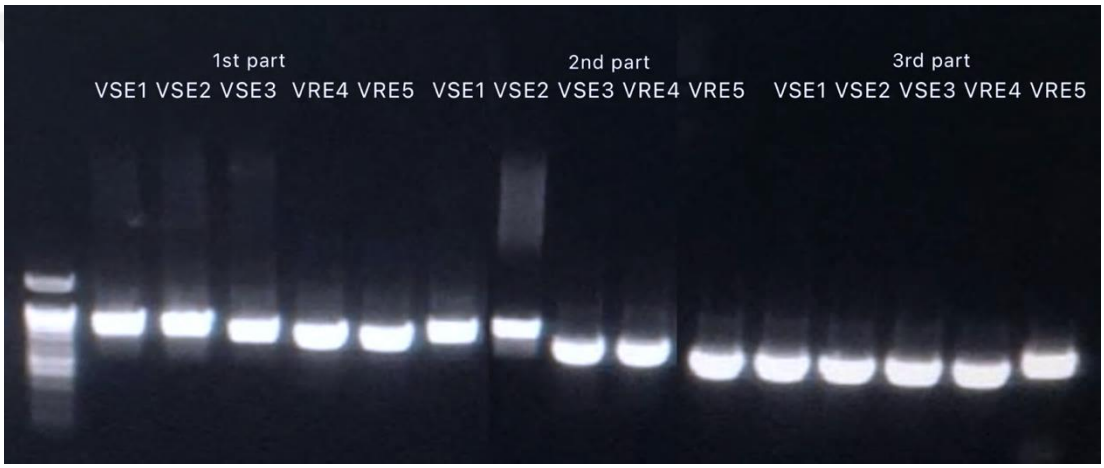

Figure 26. Electrophoresis of *E. faecalis* PBP2B genes in 1% agarose gel.

Figure 27. Electrophoresis of *E. faecalis* PBP3 genes in 1% agarose gel.

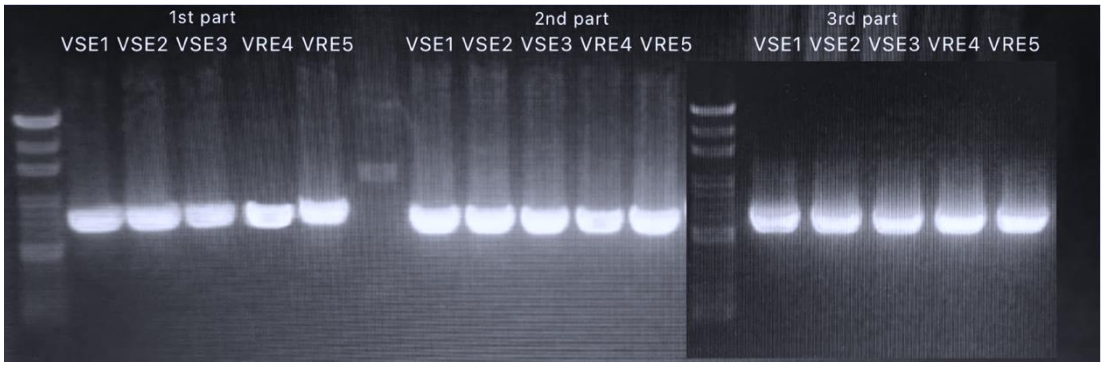

Figure 28. Electrophoresis of *E. faecalis* PBP4 genes in 1% agarose gel.

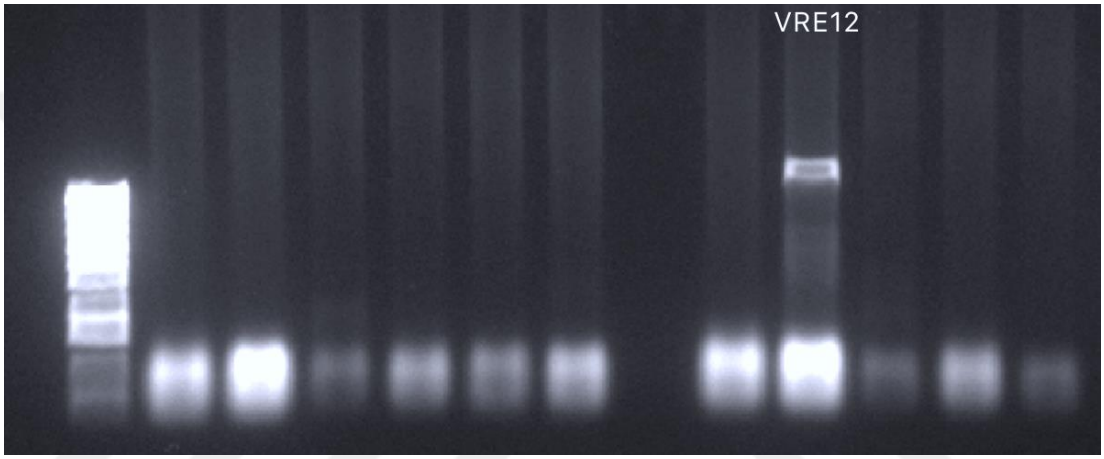

Figure 29. Electrophoresis of *E. faecium* PBP amplified with *E. faecalis* PBP primers in 1% agarose gel.

The DNA fingerprinting study provided information on the genetic diversity of the clinical strains studied. In addition, three VRE strains obtained from Acibadem Labmed Laboratories, Istanbul, that were included for PBP1B, were also included in the DNA fingerprinting study. All VSE strains exhibited distinct DNA patterns. Among the five strains, VRE4 and VRE5, VRE6 and VRE 7 showed similar patterns, while VRE8 showed a different pattern. The three VSE strains and the VRE strains, VRE4-5, VRE6-7 and VRE8, were found to have different genetic structures, indicating the presence of three distinct VRE strains (**Figure 30**).

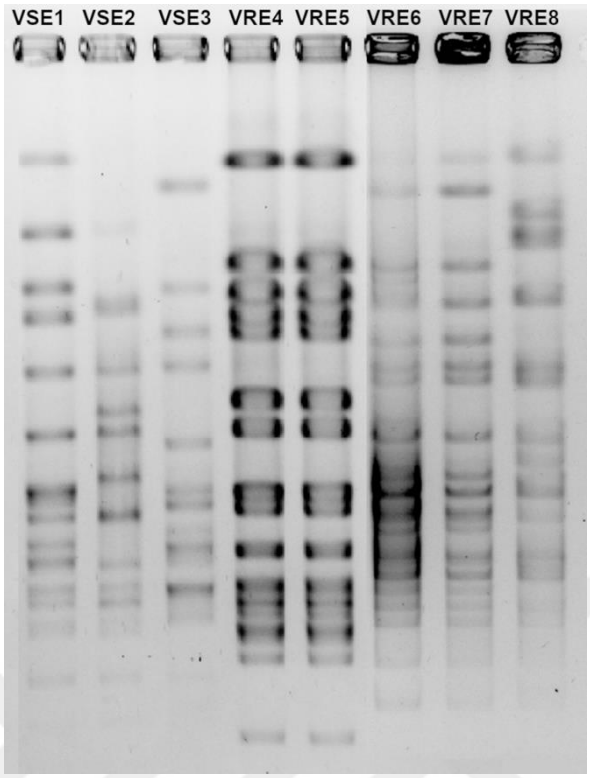

Figure 30. DNA fingerprinting of SmaI-digested, EtBr-stained *E. faecalis* genomic DNA in 1% PFGE gel.

The nucleotide sequences of PBPs determined via Sanger sequencing were subsequently analyzed using the BioEdit bioinformatics analysis program (ver. 7.0.5.3). Although a PBP gene was amplified in three parts, which were sufficient for Sanger sequencing, it was observed that the first and third parts of each PBP gene did not have sufficient reading accuracy. The second part that was read with the optimal level of accuracy also encodes the penicillin-binding domain of the protein, which contains the active site. Consequently, the investigation was concentrated on the enzyme active site and its surrounding area. In each PBP, the catalytic serine (S) amino acid, represents the active site, was determined and focused on the amino acid substitution in this region. The motif indicating catalytic serine, and the number of amino acid substitutions were presented in **Table 10**.

The amino acid substitutions identified in the PBPs of the clinical strains were consistent with our search in the PBPs from NCBI database. While substitutions were detected in PBP1B, PBP2A and PB2B in the sequences in the database, PBP2B was replaced by PBP3 in the clinical strains in this study and substitutions were detected

in PBP1B (T491N) (**Figure 31**), PBP2A (K336G) (**Figure 32**) and PBP3 (N326D, L345I, S350T, A353S, I354M, L359Q, S364L, I368V, T372Q, T376V, I378V, V383Y, G384T, N385R, K387N, D388G, T390E, S391T, T393N, N408K, R412K, G415D, D416E, V419M, S429T, T436S, S437G, H439S, T440A, D445G, N446T, T447N, I448F, N453M, G460A) (**Figure 33**).

The substitution from threonine (Thr or T) to asparagine (Asn or N) at location 491 in PBP1B in all VREs, the sequence data of which were collected from GenBank database. Threonine was located at this position, in all VSE except VSE3, which is identical with VRE strains at this region. This VSE3 strain was observed to exhibit resistance to both high-level gentamycin and ciprofloxacin in antibiotic susceptibility test (**Table 9**), a resistance profile that differs from that of other VSE strains and is consistent with that of VRE strains.

Table 1. Amino acid substitutions detected in *E. faecalis* PBPs.

Protein	**Substitution(s)**	**Active site indicating Motif I**	**Catalytic serine location**
PBP1A	Not Detected	STVK	417
PBP1B	T491N	STIK	450
PBP2A	K336G	SSLK	402
PBP2B	Not Detected	SVVK	406
PBP3	N326D, L345I, S350T, A353S, I354M, L359Q, S364L, I368V, T372Q, T376V, I378V, V383Y, G384T, N385R, K387N, D388G, T390E, S391T, T393N, N408K, R412K, G415D, D416E, V419M, S429T, T436S, S437G, H439S, T440A, D445G, N446T, T447N, I448F, N453M, G460A	STIK	343
PBP4	Not Detected	STFK	424

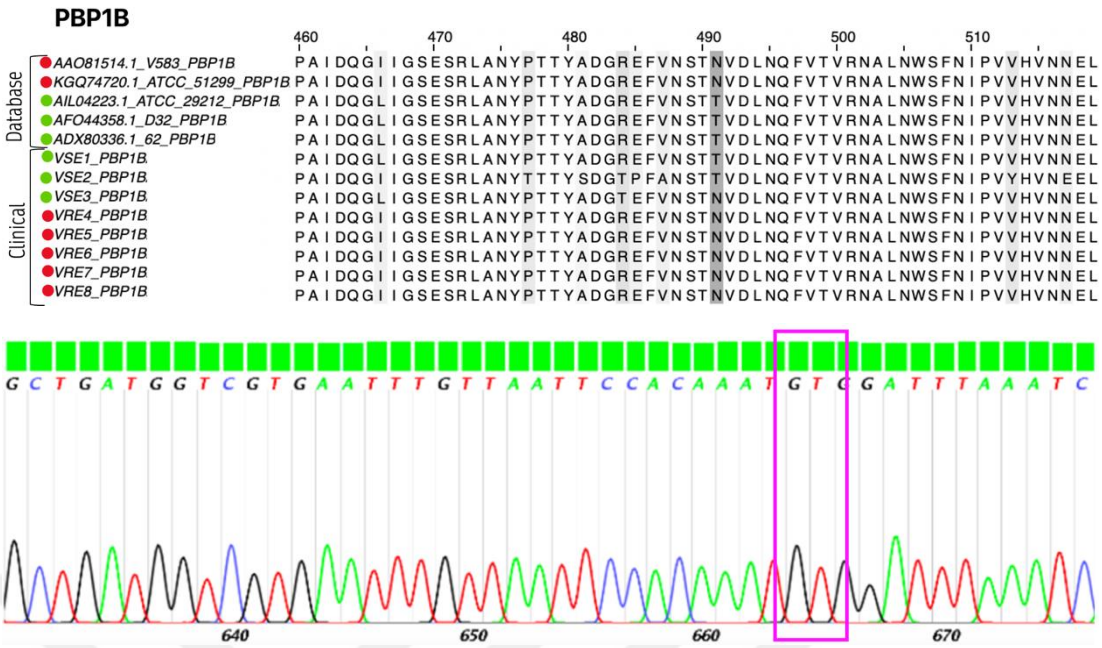

Figure 31. The substitution points for PBP1B and the Sanger sequencing chromatogram of the region of mutations.

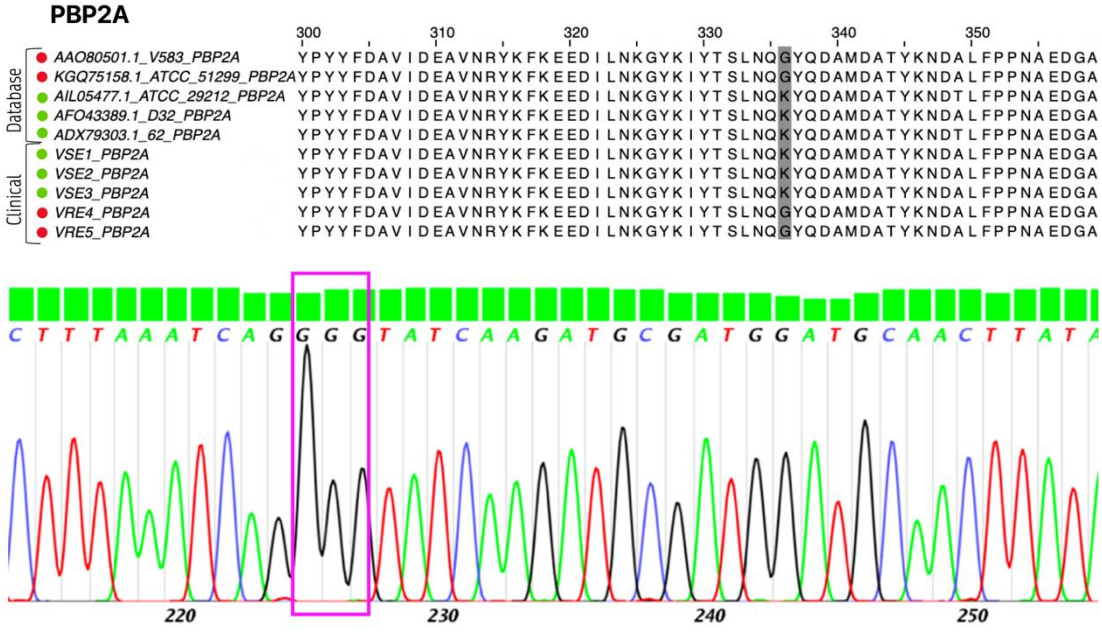

Figure 32. The substitution points for PBP2A and the Sanger sequencing chromatogram of the region of mutations.

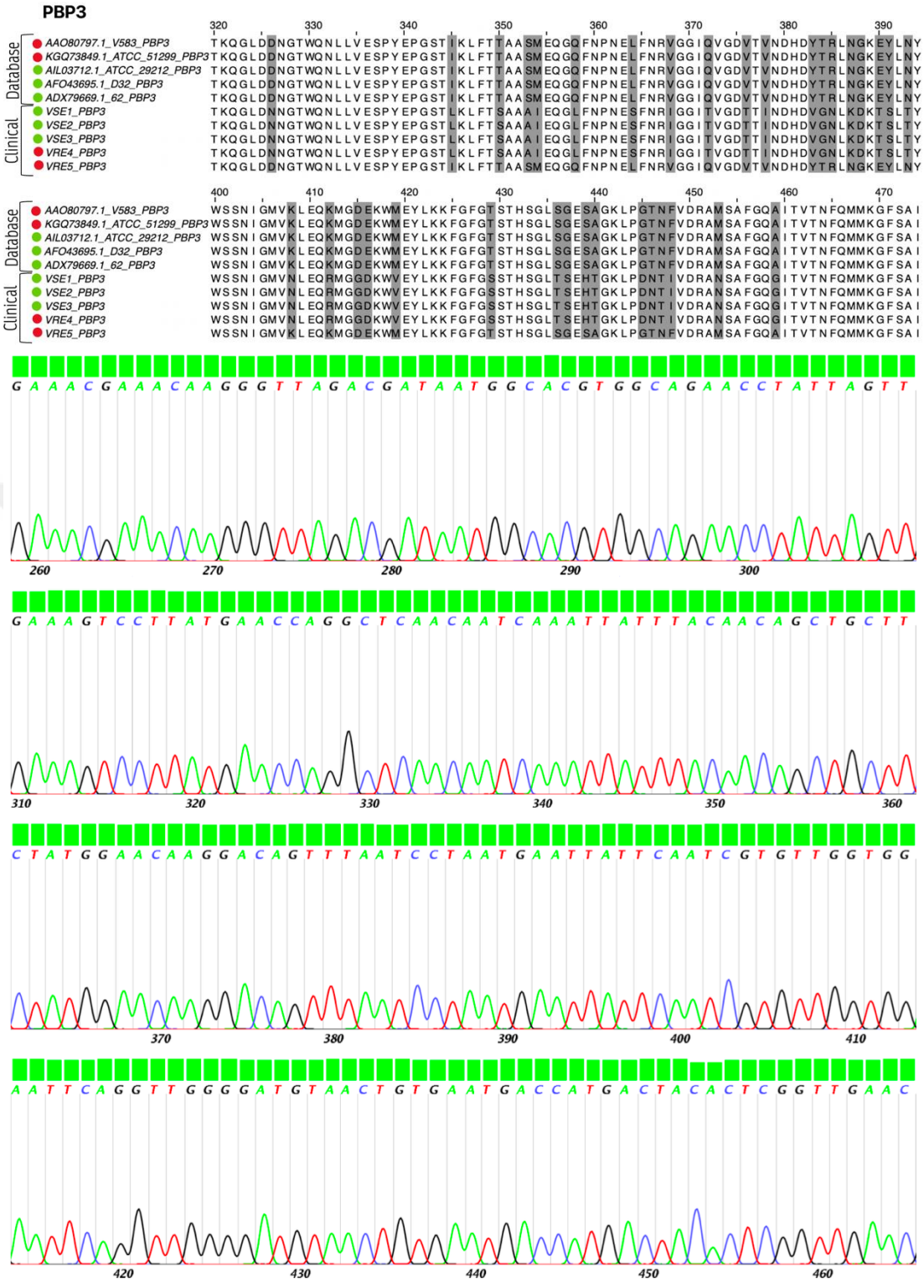

Figure 33. The substitution points for PBP3 and the Sanger sequencing chromatogram of the region of mutations.

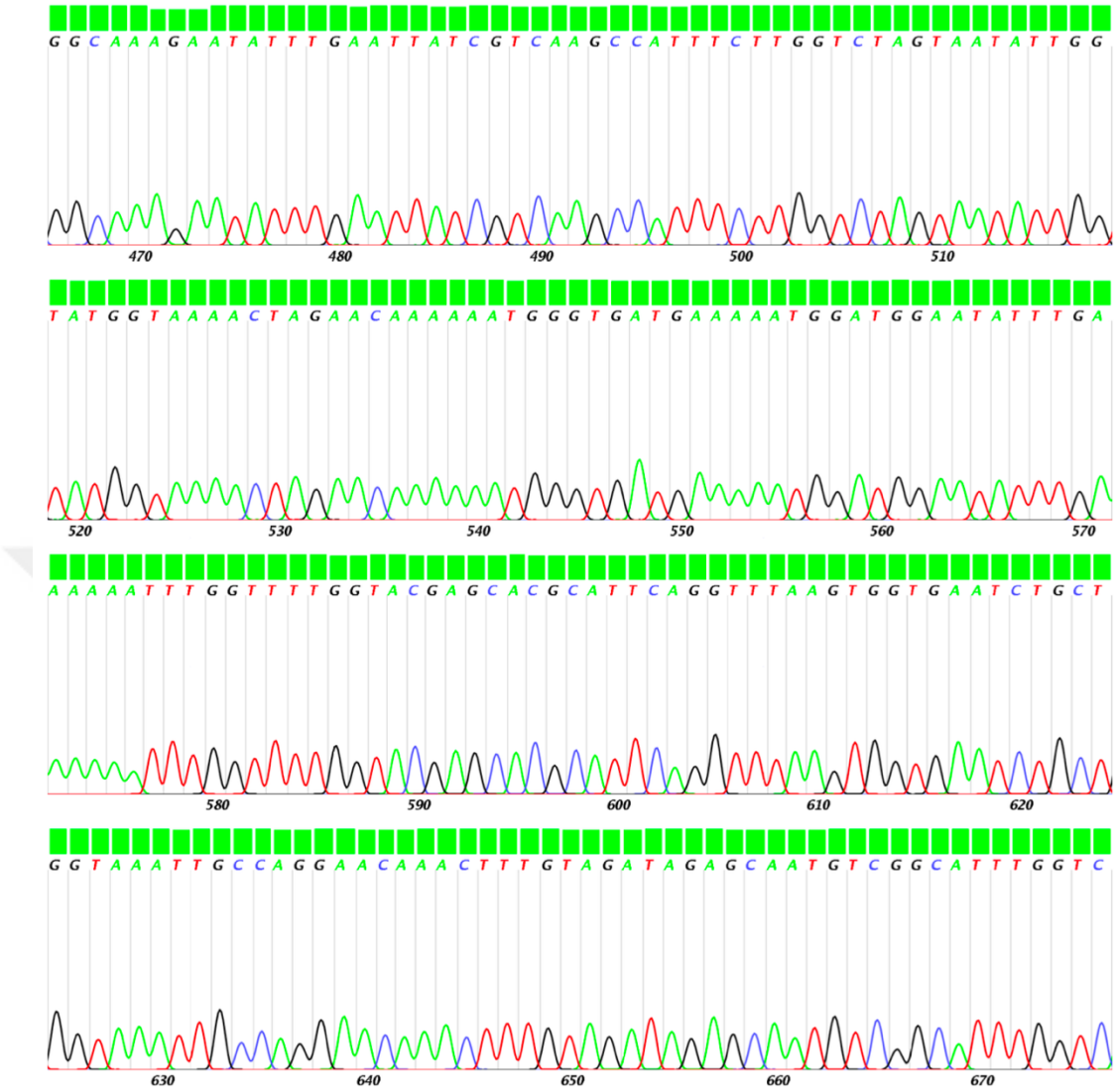

Figure 33. The substitution points for PBP3 and the Sanger sequencing chromatogram of the region of mutations (continue).

4.4 Structural Modeling and Ligand-Binding Dynamics of Changed PBPs

The structural changes induced by the identified amino acid substitutions in folded functional proteins were visualized using homology modeling to better understand the impact of these changes on the proteins' overall structure.

The T491N substitution identified in PBP1B was observed to be located at a critical position within the folded protein. The amino acid substitution is located in the active site of the transpeptidase domain, in a position opposite the catalytic serine (also

known as active serine), inside the active cleft (**Figure 35**). This position was also revealed to affect ligand-binding dynamics through affinity analysis. Asparagine in VRE PBP1B, contains an amide group and might create a different local hydrogen bonding opportunity. A negatively charged polar hydroxyl group (OH) of threonine replaces with an uncharged polar amide group (NH_2) on the side chain of asparagine resulted in a decrease the binding affinity to L-Lys-D-Ala-D-Ala and penicillin (**Table 11**).

Since D-Lac moiety contains a negatively charged group which is expected to be attracted to the positive polar group of asparagine, this may explain the affinity increase to D-Ala-D-Lac by this amino acid change. Furthermore, the analysis of structural models indicates that the Asn substitution induces minor conformational changes in the binding site, resulting in the formation of a more compacted region (**Figure 35b**) in comparison to the presence of Thr (**Figure 34b**). This change may optimize the active cleft for D-Ala-D-Lac binding. These results suggest a selective adaptation in which VRE develops mutations that provide a functional advantage in the presence of antibiotics targeting cell wall synthesis. This finding aligns with the observed tendency of PBPs in VRE to utilize D-Ala-D-Lac moieties instead of D-Ala-D-Ala.

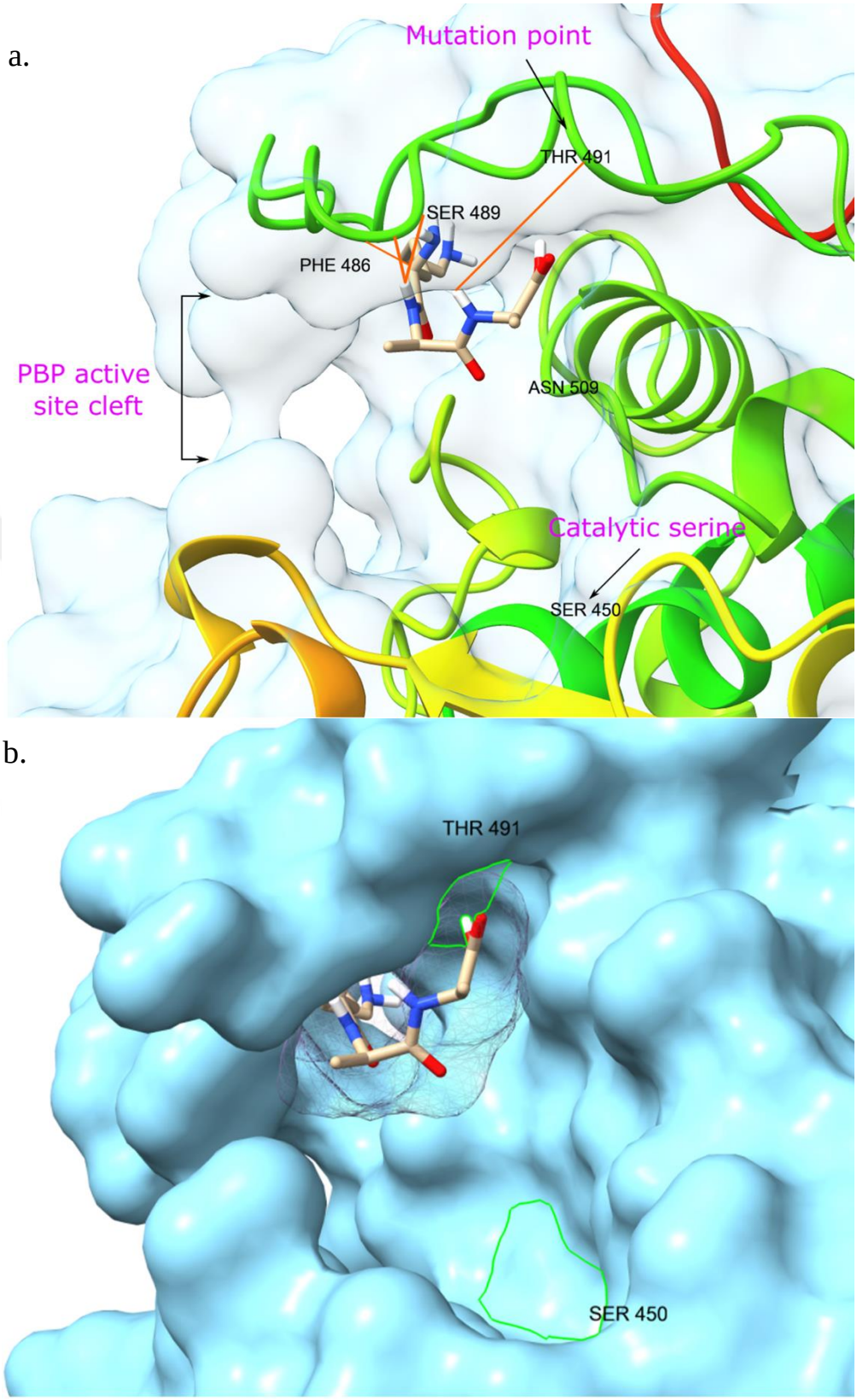

Figure 34. a. Representative model of VSE PBP1B and interactions with the native ligand L-Lys-D-Ala-D-Ala. b. The surface representation of VSE PBP1B and docked native ligand.

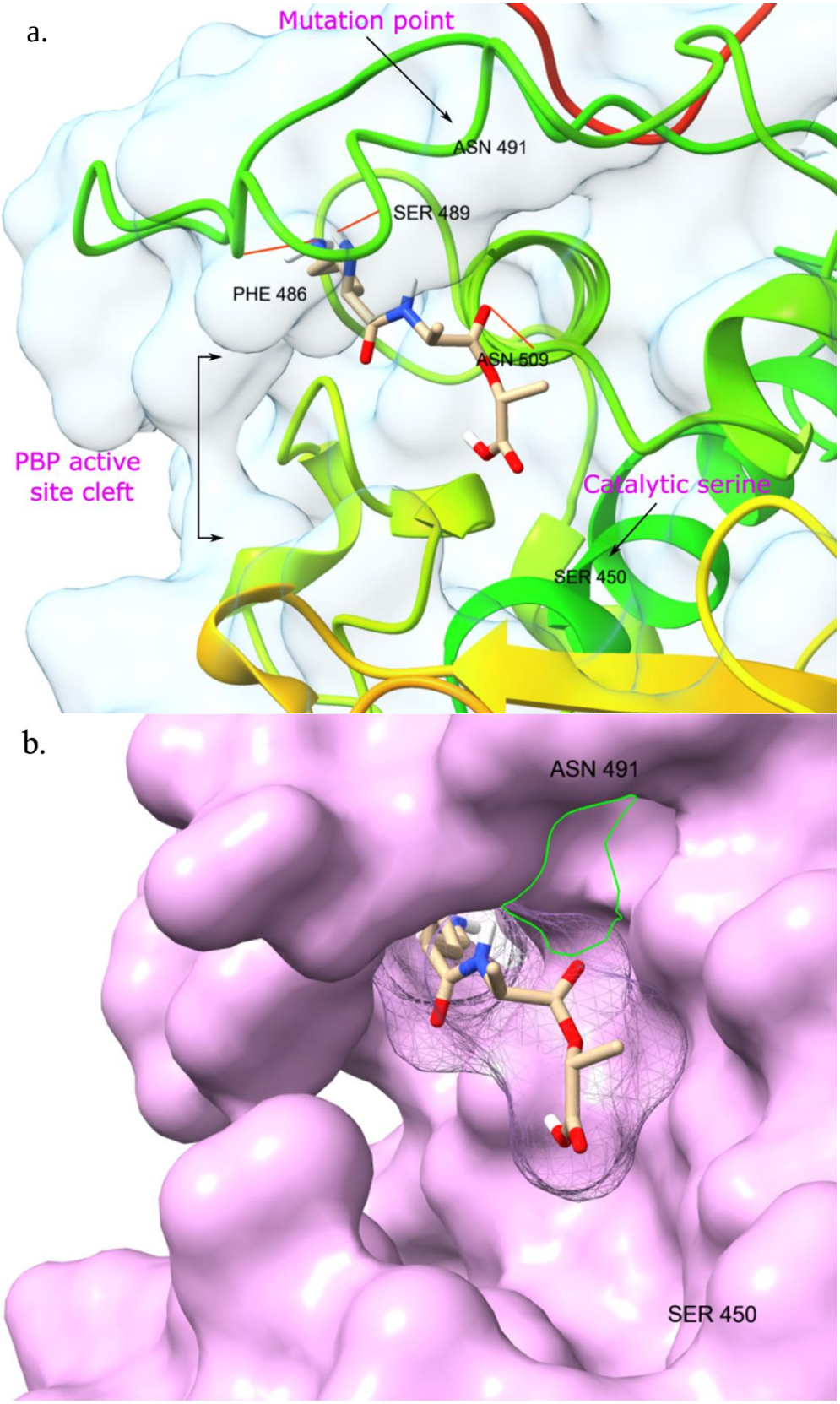

Figure 35. a. Representative model of Asn-modified VRE PBP1B and interactions with the altered ligand L-Lys-D-Ala-D-Lac. b. The surface representation of VRE PBP1B and docked altered ligand.

A substitution from lysine (Lys or K) to glycine (Gly or G) at position 336 (K336G) was identified in PBP2A. This substitution from a lysine, positively charged, polar amino acid with long side chain to an uncharged, non-polar amino acid glycine, lack of side chain, is expected to increase the flexibility of the protein in this region. This substitution was not located at the active site or near. It was observed to be located relatively distal to the active site, closer to the transmembrane domain (**Figure 36**). The substitution at such position may have effects on flexibility and stability of the protein in the proximity of the membrane interface, with the potential to mediate substrate accessibility.

Binding affinity scores showed similar values for D-Ala-D-Ala in both VRE and VSE PBP2A (-6.1 and -6.2 kcal/mol, respectively) . However, VSE PBP2A exhibited a reduced affinity for D-Ala-D-Lac (-5.5 kcal/mol) compared to VRE PBP2A (-6.1 kcal/mol) (**Table 11**).

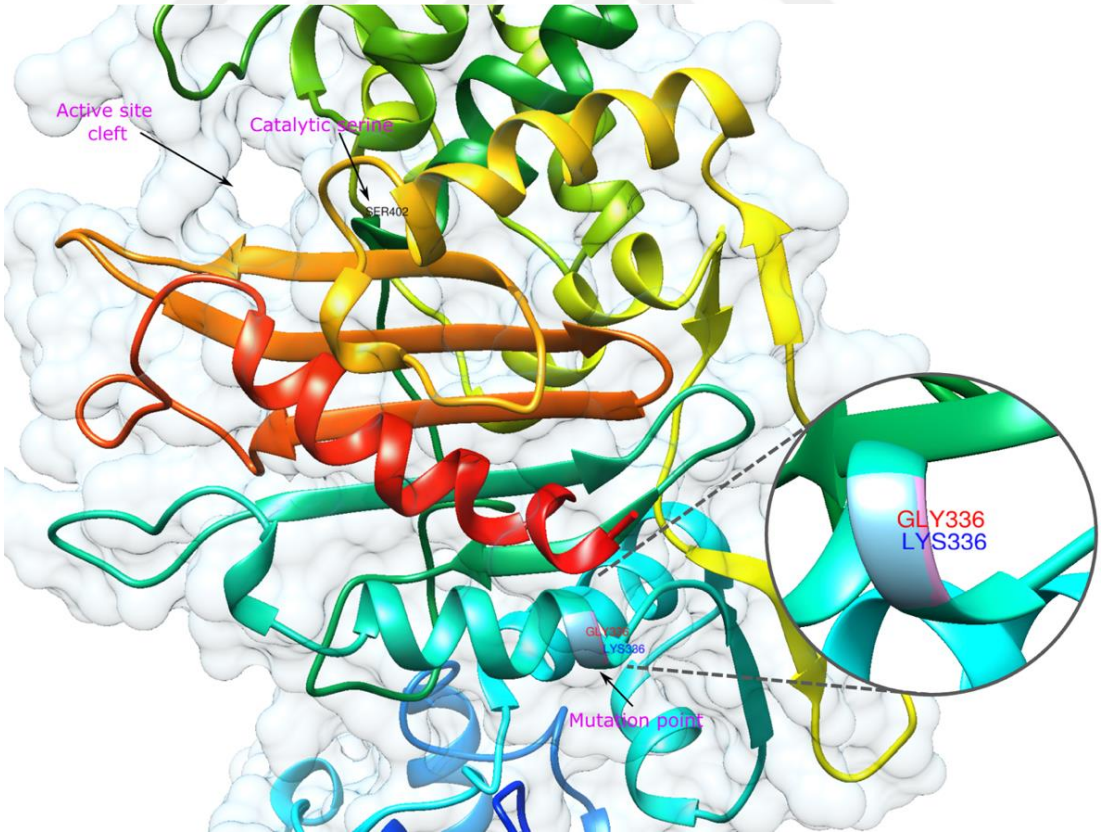

Figure 36. Representative model of PBP2A showing mutation point wherein VRE and VSE strains differ. The amino acids in the mutation point are shown in the zoomed-in area and are labelled in red and green for VRE and VSE, respectively.

PBP3, shared the same amino acid sequences in all VSE including VRE4. However, in VRE5 and VRE reference strain V583, a major change affecting 35 amino acids was observed located in the region forming the active site of the transpeptidase domain of PBP3. The most notable structural change in VRE PBP3 is the formation of a barrier-like structure due to the substitution of amino acid 385 from asparagine to arginine (N385R) (**Figure 38b**). This structure is situated at the entrance of the active cleft, leading to a narrowing of the cleft region.

A noteworthy detail was identified for Class B, monofunctional PBPs. The thirty-five amino acid substitutions were identified in PBP3 of the clinical strains and in PBP2B of the database strains. Due to the number of substitutions and their location, PBP3 in the strains in this study and PBP2B in the GenBank reference strains were exhibited a meaningful similarity. However, no substitution was observed in the clinical samples in PBP2B.

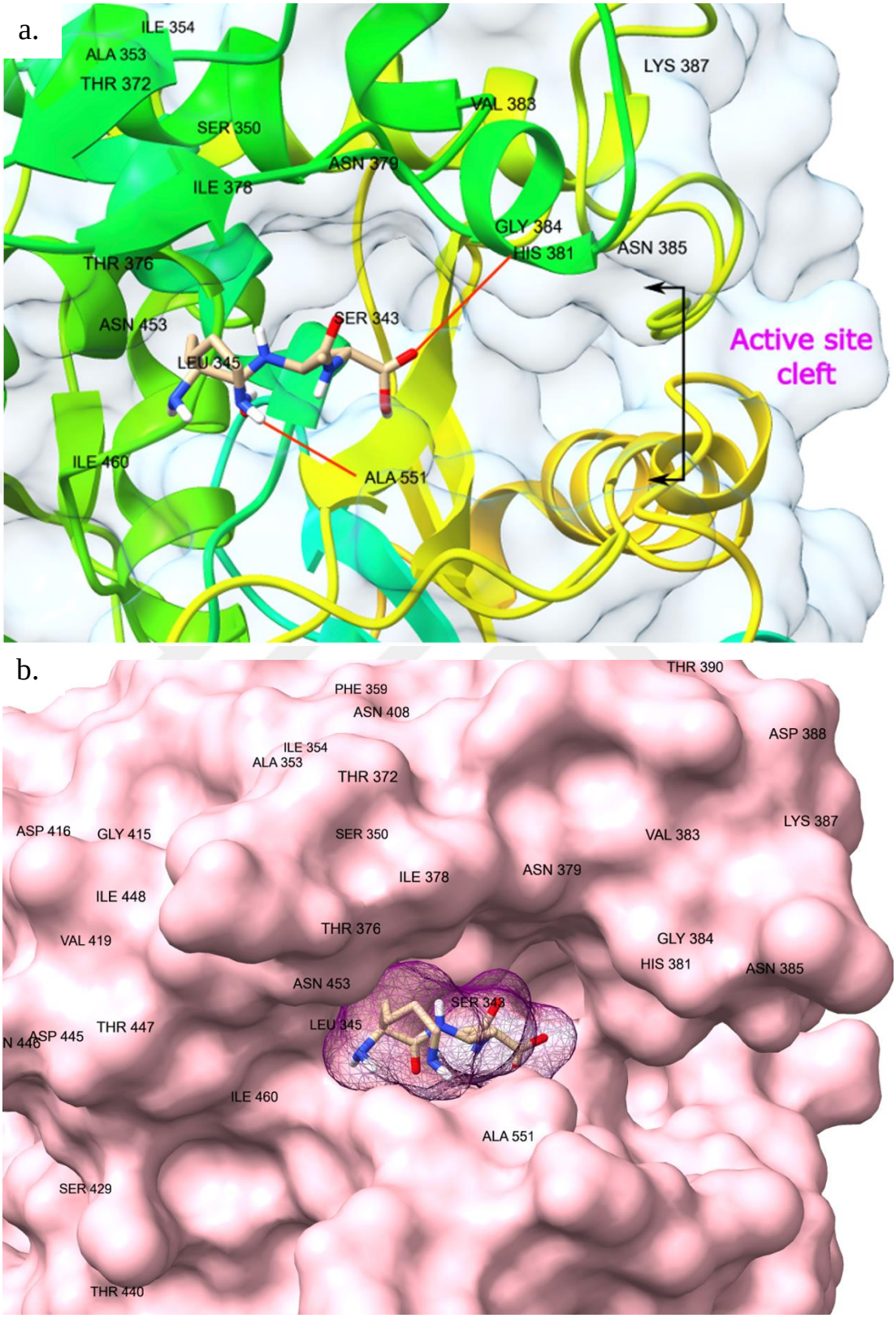

Figure 37. a. Representative model of unmodified VSE PBP1B and interactions with the native ligand L-Lys-D-Ala-D-Ala. b. The surface representation of VSE PBP1B and docked native ligand.

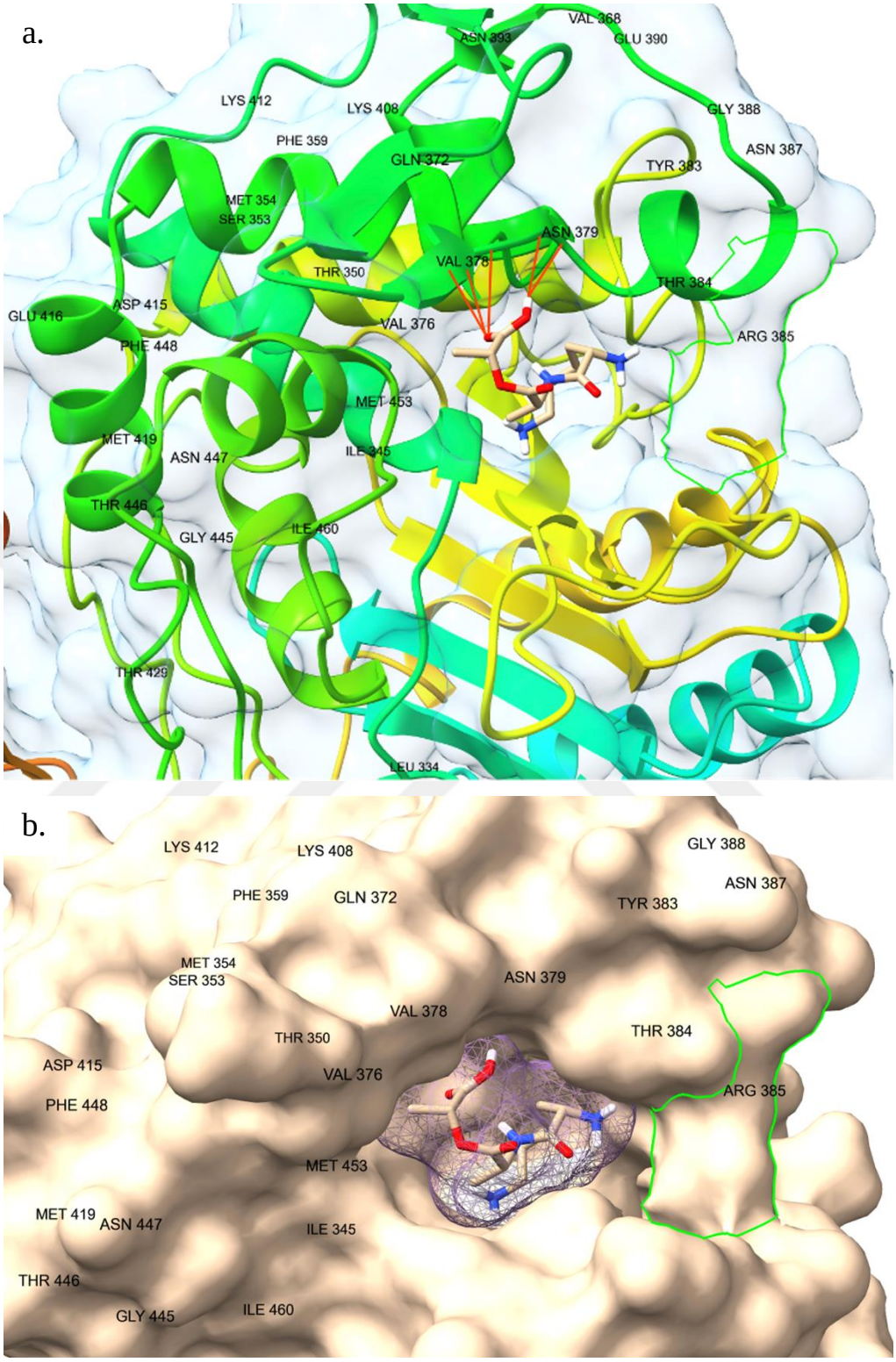

Figure 38. a. Representative model of VRE PBP3 indicates substitutions on around the transpeptidase domain and interactions with the altered ligand D-Ala-D-Lac. b. The surface representation of VRE PBP3 and the altered ligand complex shows a barrier-like structure framing the green line at the entrance of the active cleft. The structure is formed by the replacement of asparagine by arginine at residue 385.

Table 21. Receptor binding affinity results for both the native and altered ligands.

Receptor	**Ligand**	**Score* (kcal/mol)**
VSE PBP1B	L-Lys-D-Ala-D-Ala	−6.2
VRE PBP1B	L-Lys-D-Ala-D-Ala	−5.5
VSE PBP1B	L-Lys-D-Ala-D-Lac	−5.6
VRE PBP1B	L-Lys-D-Ala-D-Lac	−6.1
VSE PBP2A	L-Lys-D-Ala-D-Ala	−6.2
VRE PBP2A	L-Lys-D-Ala-D-Ala	−6.1
VSE PBP2A	L-Lys-D-Ala-D-Lac	−5.5
VRE PBP2A	L-Lys-D-Ala-D-Lac	−6.1
VSE PBP3	L-Lys-D-Ala-D-Ala	−5.4
VRE PBP3	L-Lys-D-Ala-D-Ala	−5.5
VSE PBP3	L-Lys-D-Ala-D-Lac	−5.7
VRE PBP3	L-Lys-D-Ala-D-Lac	−5.6

*Scores refer to binding free energy values, where a lower score indicates higher binding stability.

4.5 Synthesis and Antimicrobial Efficacy of Synthetic D-Ala-D-Lac

The synthesis of D-Ala-D-Lac dipeptide was conducted in five steps using the Solution-Phase Peptide Synthesis method. The process included the protection and deprotection of functional groups at intermediate stages, which was essential to ensure the structural integrity of D-Ala-D-Lac. The synthesis yielded a fibrous, hydrophobic, white precipitate in its dipeptide form. The insolubility of the intermediates in the most commonly used solvents was critical to develop a method for dissolving the intermediates and to identify a non-toxic solvent. The final product was found to be soluble in isopropanol, DCM, and methanol but exhibited limited solubility in water and chloroform. Despite its solubility limitations at higher concentrations, isopropanol was selected as the solvent.

TLC was used to analyze the intermediates, with the separating solvent varying in ratios of 0.5% acetic acid added DCM and methanol (98:2, 95:5 and 9:1). The substance was effectively visualized with the 95:5 and 9:1 solution (**Figure 39**). However, purification attempts using HPLC at wavelengths of 193 nm, 214 nm, 254 nm and 280 nm were unsuccessful. While products were isolated at three peaks observed at 214 nm, NMR analysis indicated that the desired dipeptide was not

obtained. Consequently, the synthesis steps were continued without further HPLC purification in order to minimize material loss. The product was synthesized in a yield of approximately 24 mg, which is relatively low.

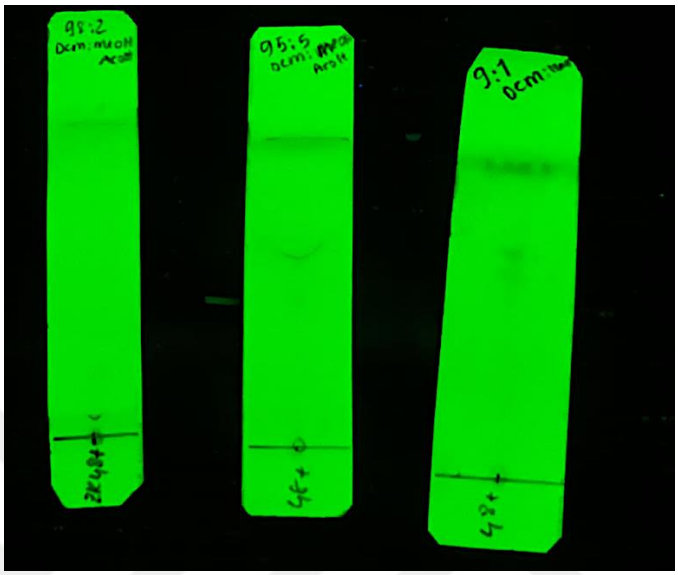

Figure 39. The visualization of the D-Ala-D-Lac dipeptide by TLC

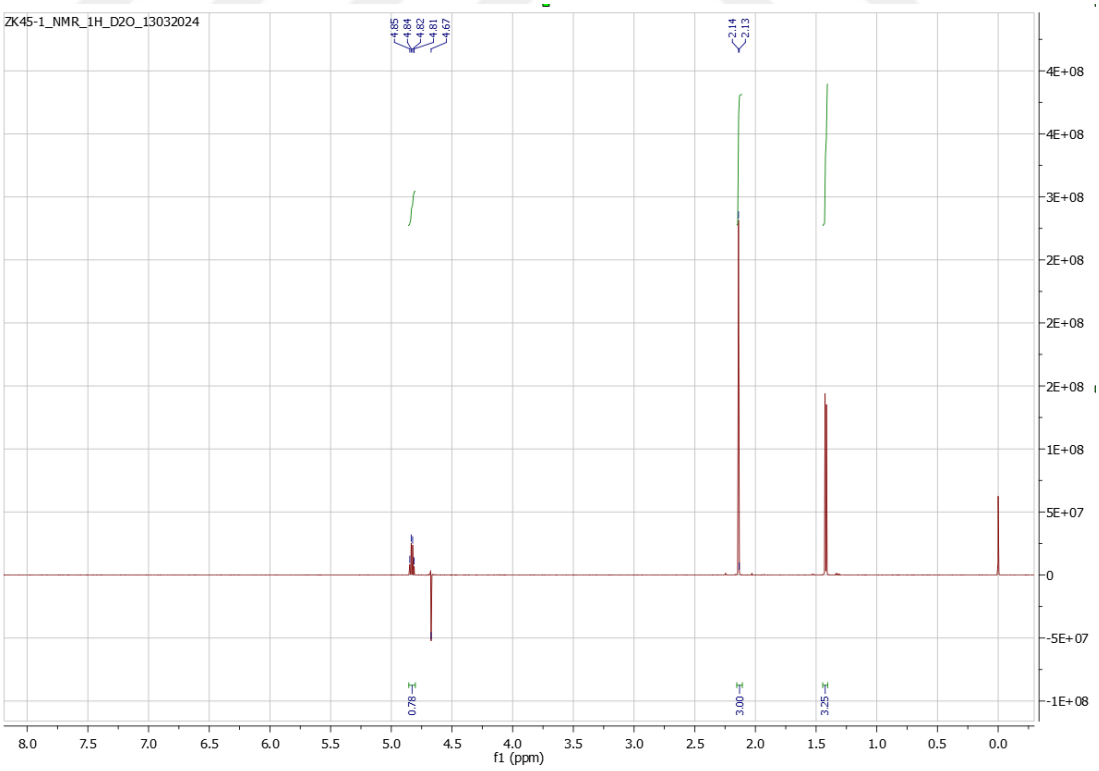

Figure 40. The 1H-NMR (in D2O) spectrum of the initial product.

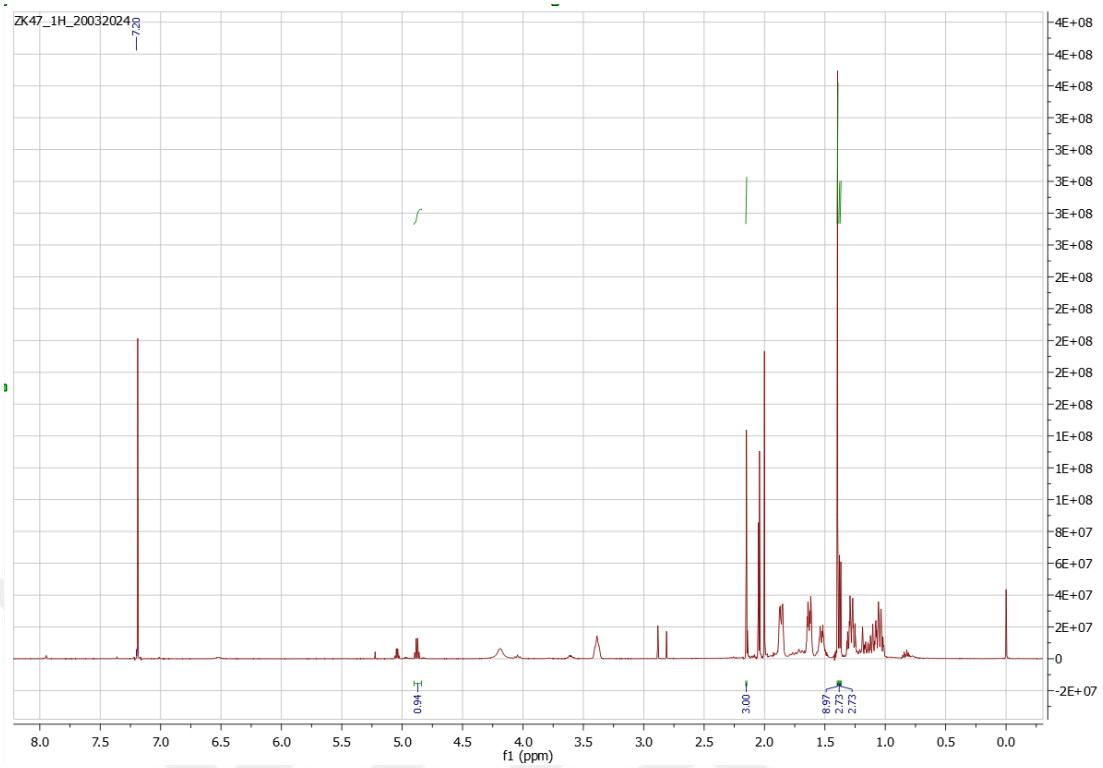

Figure 41. The 1H-NMR (in D2O) spectrum of the second step product.

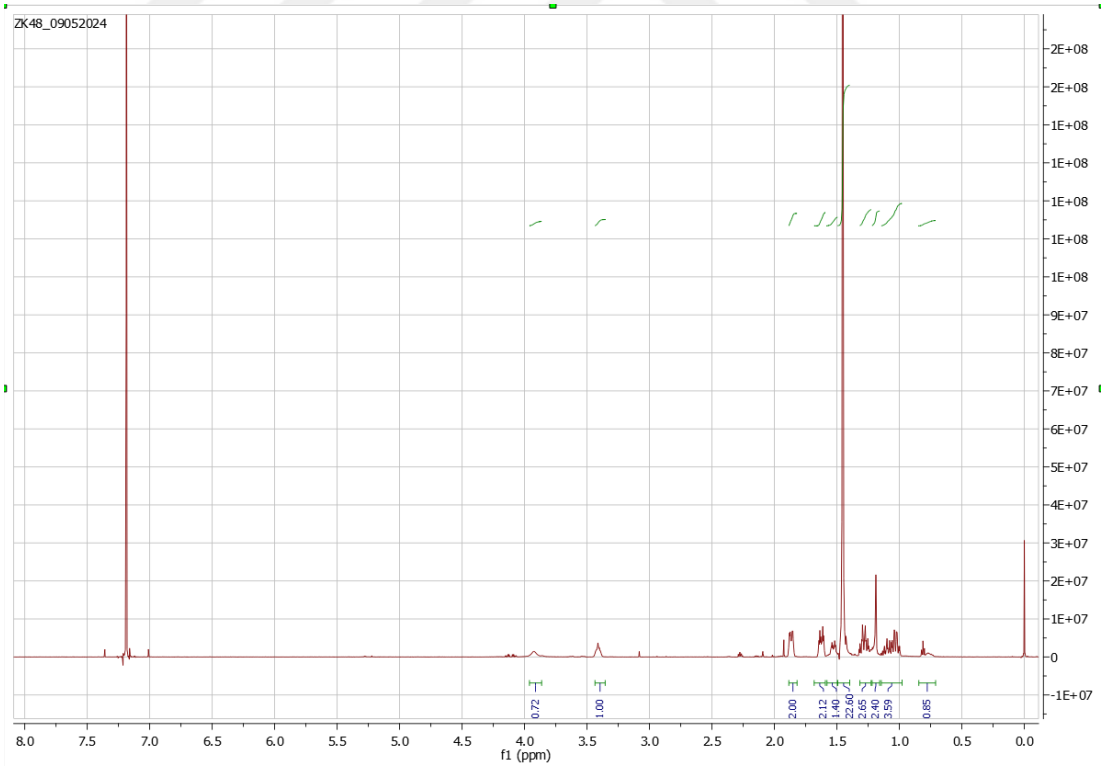

Figure 42. The 1H-NMR (in D2O) spectrum of the final product D-Ala-D-Lac.

The antimicrobial activity of the synthesized D-Ala-D-Lac dipeptide was evaluated using the disk-diffusion method. Initial trials with low concentrations (16, 8, 4, and 2 μg/ml in 10% isopropanol) of D-Ala-D-Lac on Mueller Hinton spread agar plates (VRE6) did not demonstrate any inhibitory effects on bacterial growth. Based on these results, high-concentration trials were performed to assess the activity of D-Ala-D-Lac in more concentrated forms.

In high-concentration trials, lyophilized pure peptide powder was put on the spread agar plate, but failed to exhibit any antimicrobial activity. However, the higher concentration in solution, the dipeptide showed limited but observable antimicrobial effect. Specifically, at a concentration of 8 mg/ml, a 2 mm inhibition zone was observed, while at 16 mg/ml, no distinct inhibition zone was present, but a reduction in bacterial growth within the dropping zone was noticeable.

These findings suggest that the D-Ala-D-Lac may have weak antimicrobial properties at higher concentrations, through further optimization and investigation with large-scale production are required to enhance its efficacy.

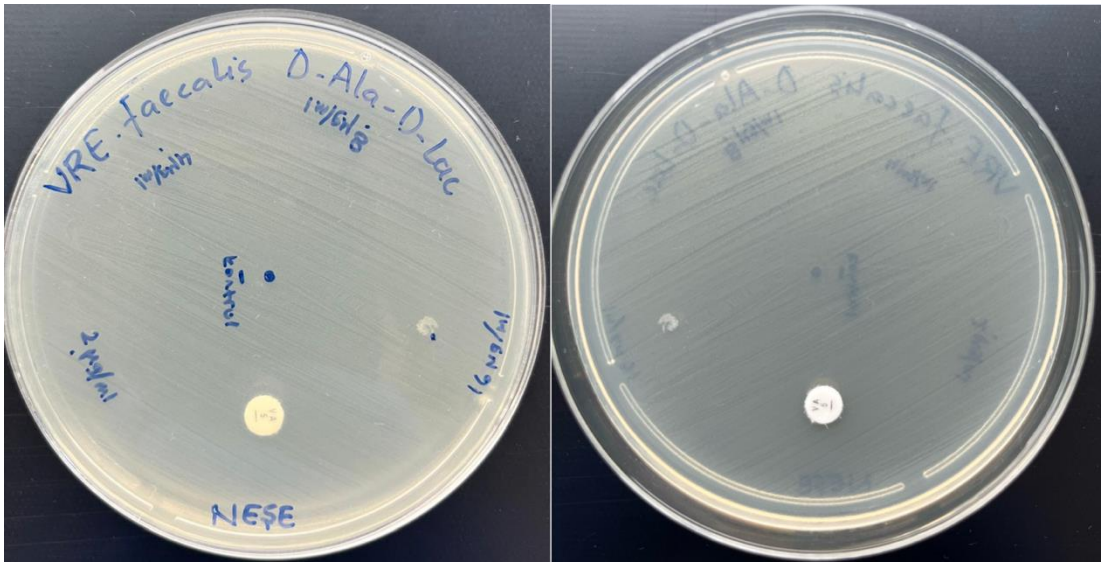

Figure 43. VRE6 disk-diffusion test with optimal concentration of synthetic D-Ala-D-Lac.

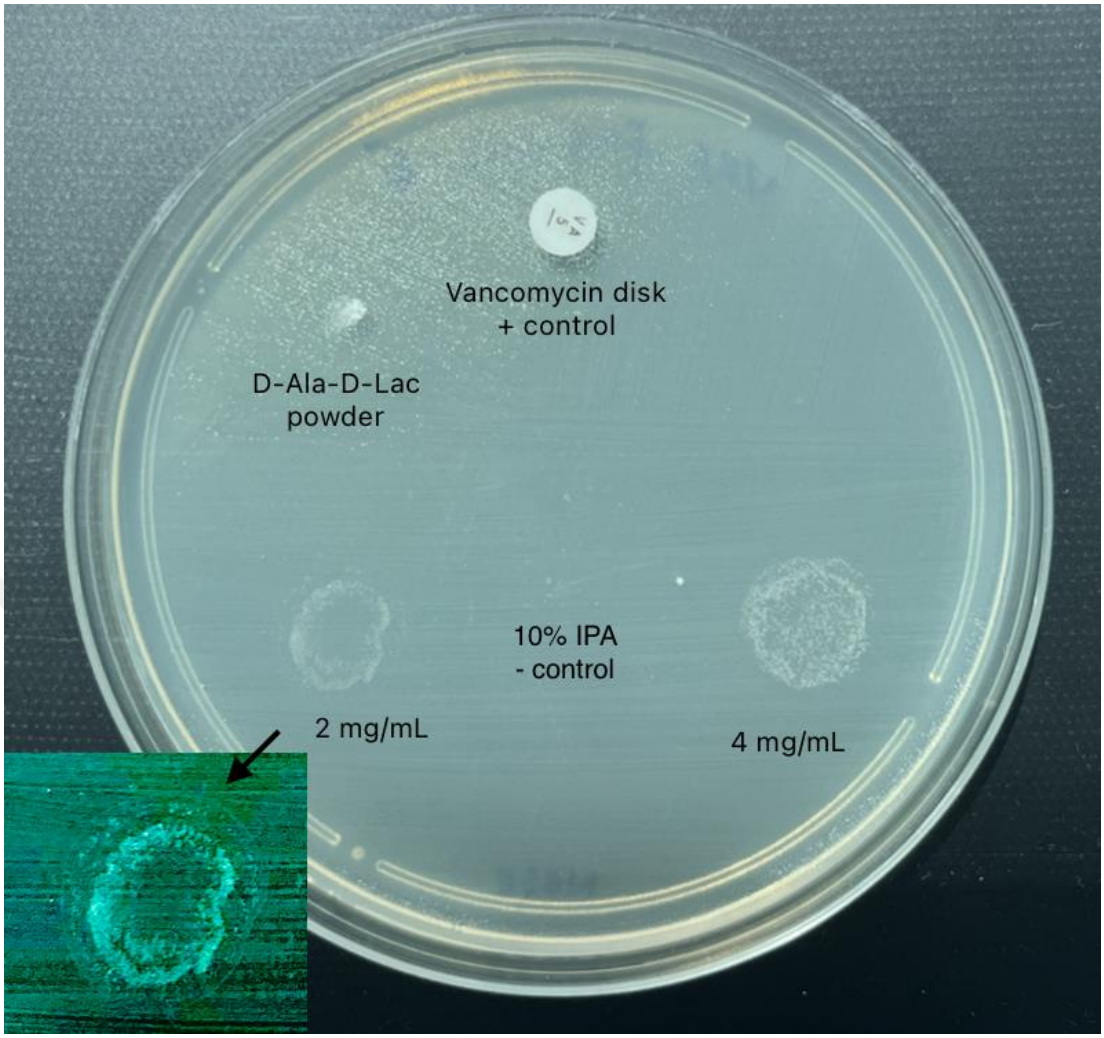

Figure 44. VRE6 disk-diffusion test with high concentration of synthetic D-Ala-D-Lac.

5 DISCUSSION

The data regarding PBP genes was collected from complete genome sequences accessible via open access databases, that continue to evolve, update and even change like a living organism. In recent years, the development of next-generation sequencing technologies and bioinformatics studies has led to creation and updating of these sources on a daily basis. The number of vancomycin-resistant stain sequences has increased as the emergence of new strains has been observed. Upon analysis of collected PBP sequences, it was observed that, contrary to our initial hypothesis that they would exhibit high levels of conservation, PBPs demonstrated a high degree of sequence divergence even among species belonging to the same genus. However, the 3D-structures of the functional proteins and their functions exhibited notable similarity. This suggests that, while the sequences may not be identical, proteins are conserved across genera and even taxonomic families.

Due to the large amount of genomic data in the NCBI database from individual isolates and closely related bacterial strains, the same proteins were represented using a single non-redundant protein accession number (prefixed with 'WP_') to reduce redundancy. The non-redundant data protein record was identified in strain OGRF1 and is thought to be the same sequence as V583, given the high level of sequence identity.

The study discovered various mutations in three of the six HMW PBPs present in VRE, leading to alterations in the structure and interaction capacity of these proteins. The changed PBPs of the clinical strains investigated in this study were PBP1B, PBP2A and PBP3, while the sequences of PBP1A, PBP2B and PBP4 remained identical to those found in VSE. Specifically, the mutation in PBP1B which is a bifunctional high molecular weight PBP, was remarkable due to its location and the effect to protein-substrate interaction. The substitution of threonine (Thr or T) to asparagine (Asp or N) at the active site of PBP1B, at a position that is critical for the active serine in substrate binding, results in functional changes to the enzyme. Threonine is a polar, commonly found in functional centers of proteins, possess a

reactive hydroxyl group (OH) capable to forming hydrogen bonds with various polar substrates, facilitating efficient binding to the unchanged native substrate D-Ala-D-Ala. On the other hand, asparagine has a polar side chain but a bulkier amide group (NH_2), typically located on the protein surface where it interacts with an aqueous environment and frequently involves in binding site of an enzyme. Its polar side chain is highly reactive, readily interacting with other polar or charged atoms, enhancing interactions with the altered substrate D-Ala-D-Lac, associated with vancomycin resistance. The 3D model of PBP1B revealed that this amino acid substitution occurs at the lid region of the active cleft which directly interfere with the substrate recognition (**Figure 35**). Molecular docking analysis further showed that the Asp-modified PBP1B had a higher affinity for D-Ala-D-Lac, whereas the wild-type Thr-containing PBP1B showed a preference for D-Ala-D-Ala (**Table 11**). The increased affinity of Asp-modified PBP1B for altered substrate is explained by negatively charged group of the D-Lac moiety being attracted to the positive charged polar group of asparagine. In addition, the analysis of structural models indicates that Asn substitution induces minor conformational changes in the binding site, resulting in the formation of a more compacted cleft (**Figure 35b**) in comparison to the presence of Thr (**Figure 34b**). This alteration may optimize the binding site for binding of D-Ala-D-Lac. The results obtained suggest a selective adaptation in which VRE develops mutations that provide a functional advantage in the presence of antibiotics targeting cell wall synthesis. This finding is in line with the previously mentioned tendency of PBPs in VRE to utilize D-Ala-D-Lac moieties instead of D-Ala-D-Ala. This substitution presents a potential target for inhibitor design, offering the possibility of modifying the enzyme's affinity for the altered substrate while maintaining its interaction with the native substrate.

Moreover, this critical mutation was observed in the VSE3 strain, which exhibits a phenotype similar with the VRE strains. Despite being susceptible to vancomycin, the VSE3 strain is resistant to ciprofloxacin and gentamycin (**Tablo 9**). The aminoglycoside antibiotic gentamycin disrupts protein synthesis by binding to the bacterial 30S ribosomal subunit (297). Ciprofloxacin, a fluoroquinolone, inhibits DNA replication by targeting DNA gyrase and topoisomerase IV enzymes (298). The

presence of a mutation at this critical location of PBP1B in VSE3, in association with resistance to these antibiotics with different mechanisms of action, may indicate that this substitution does not directly confer resistance to vancomycin, but rather represents an intermediate stage of adaptation. This mutation in PBP1B, could be a part of a broader genetic adaptation that also results in resistance to gentamycin and ciprofloxacin. The unique resistance profile of VSE3 strain offers a requirement for a deeper analysis of VSE3 to understanding its molecular background and evolutionary relationships.

A substitution from lysine to glycine at position 336 of PBP2A was identified in PBP2A (**Figure 36**). The substitution of amino acid 336 from lysine with its positively charged, long and flexible side chain to a small, lacks side chain glycine may disrupt the interactions provided by lysine and add flexibility to the protein. The position of this substitution, while relatively distal to the active site, is proximal to the transmembrane domain of the protein. Binding affinity scores showed similar values for D-Ala-D-Ala in both VRE and VSE PBP2A (-6.1 and -6.2 kcal/mol, respectively). Notably, VSE PBP2A demonstrated a reduced binding affinity for D-Ala-D-Lac (-5.5 kcal/mol) in comparison to VRE PBP2A (-6.1 kcal/mol) (**Table 11**). This substitution suggests a potential impact on the flexibility and stability of the protein, particularly in the vicinity of the membrane interface, which may consequently affect substrate accessibility.

A further considerable number of substitutions observed in PBP3. The thirty-five amino acids surrounding the enzyme's active site was identified in the transpeptidase domain of PBP3 which results in substantial structural changes in the protein. Such changes in polarity, charge, and hydrophobicity might disrupt the stability, binding properties or enzymatic function of the protein. The N326D, N385R, K387N and R412K substitutions involve charged residues and result in changes to the enzyme's electrostatic properties. The substitution of asparagine (N, neutral) with aspartate (D, negative) at position 326, and similarly, the positively charged arginine (R) and lysine residues at locations 385 and 412 affects the protein's electrostatic interactions. Additionally, hydrophobicity changes are observed in the substitutions L345I, I354M,

I368V, V383Y, V419M, and I448F. The substitutions S350T, A353S, S364L, T372Q, T376V, S437G, and T440A result in changes to the polarity and hydrogen bonding capabilities of the enzyme. The structural modifications resulting with the substitutions D388G, D45G, G460A, particularly that of glycine provide increased conformational flexibility due to the small size and lacks side chain of this amino acid. The substitutions are positioned close to the active site, with N326D, T372Q, T440A, and N453M, directly affecting substrate binding and catalytic efficiency.

A notable alteration observed at position 385 from asparagine (Asn or N) 274 to arginine (Arg or R), forming a barrier-like structure at the entrance of the active cleft. While this barrier-like structure appears to narrow the active cleft, which is hypothesized to have a negative impact on substrate binding, no significant difference in binding affinity values was observed for either ligand. This result suggests that the active cleft, even in its narrowed state, possesses sufficient opening to accommodate small molecules such as D-Ala-D-Ala or D-Ala-D-Lac.

These multiple substitutions were observed in the VRE5 clinical isolate, as well as in the V583 reference strain, and the other clinical isolate VRE4 were identical to the susceptible strains. Although there is no direct evidence linking these conformational changes to vancomycin resistance, the potential for affect protein function may have resulted in the formation of an allosteric site in the protein. It is also important to elucidate the impact of these changes on the protein's functionality through additional functional and structural investigations.

Furthermore, substitutions indicated exceptions between some VRE and VSE strains, with the VSE3 strain being identified as possessing the same mutation at position 491 in PBP1B as resistant strains, and a similar exception being observed for VRE4 in PBP3, in line with the susceptible strains. The presence of identical mutations between VRE and VSE strains may be attributed to horizontal gene transfer or spontaneous mutation.

Despite the observed similarity in DNA fingerprinting patterns between VRE4 and VRE5, a significant number of mutations were identified in VRE5 in PBP3. This can be explained by the absence of the SmaI restriction enzyme cutting site, was used in the DNA fingerprint analysis, in both the wild type and mutant PBPs.

The synthesis of D-Ala-D-Lac dipeptides was undertaken to ascertain the capacity of these compound to inhibit the modified PBPs. The antibacterial activity of the synthesized D-Ala-D-Lac dipeptide was found to be minimal in vancomycin-free environment. This finding supports our hypothesis that VRE conduct this change only in the presence of vancomycin. In the absence of vancomycin, they preserve energy by continuing cell wall production with conventional D-Ala-D-Ala ended pentapeptides rather than D-Lac. This approach is consistent with the idea that these bacteria have two separate sets of PBPs: one with unchanged PBPs that bind D-Ala-D-Ala, and the other with changed PBPs that have a high affinity for D-Ala-D-Lac. To further confirm this hypothesis, the synthesized peptide should be tested in vancomycin-containing agar dilutions to mimic the antibiotic's selective pressure.

The study focuses on answering the crucial question of how a changed PBP might be successfully inhibited. In addition, it aims to provide an alternative treatment option for infections caused by VRE, opening the door for more effective drug-resistant pathogens management. In antimicrobial susceptibility testing of the dipeptides synthesized for this purpose, a faint but distinct inhibition zone was observed. Although the zone was not obvious, this observation indicates a limited but potential antimicrobial effect of the D-Ala-D-Lac. This result suggests that the dipeptide may serve as an enzyme inhibitor or competitive substrate by disrupting bacterial metabolic processes or weakening the resistance to vancomycin.

6 CONCLUSION

In recent decades, VRE has emerged as a significant cause of nosocomial infections. In 2019, VRE accounted for 30% of hospital-acquired infections and related deaths in the USA (150). A study by the European Centre for Disease Prevention and Control reported an approximate 2-fold increase in VRE infections between 2007 and 2015, followed by a 2.5-fold increase from 2016 to 2020 (299). Moreover, the emergence of vancomycin resistance in methicillin-resistant staphylococci (MRSA) represents a significant cause for concern (300).

It is important to understand the mechanism of vancomycin resistance for the development of new therapeutic strategies to treat serious infections caused by VRE. The changes in glycan side chains which are responsible for vancomycin resistance are well established (144,301,302,303), the question of how PBPs can adapt to use D-Ala-D-Lac as a substrate during transpeptidation remains an area of research that requires further investigation so far. Earlier studies have shown that specific amino acid substitutions in PBPs can lead to resistance to β-lactam antibiotics (114,116,117, 304). In the present study, the PBPs of both vancomycin-resistant and vancomycin-susceptible enterococci were investigated, and amino acid changes in certain PBPs were detected that enable the adaptation of the modified D-Ala-D-Lac dipeptide of stem side chain.

The synthesis of D-Ala-D-Lac terminated monomers of peptidoglycan is strictly regulated by van clusters, which are responsible for vancomycin resistance. In the absence of the vancomycin, D-Ala-D-Ala terminals are not converted to D-Ala-D-Lac, thus allowing cell wall synthesis to proceed in a similar way to that of native PBPs, with only three changed PBPs of the six in VRE. The observation that one PBP in class A is changed while another remains in their original confirmations, and the similar pattern is observed in class B, suggests the presence of a coordinated compensatory mechanism among these varying numbers of the PBPs. These adaptive changes among PBPs provide new insights into the collaborative roles of different PBPs in cell wall

synthesis. In the absence of vancomycin, D-Ala-D-Ala may remain the preferred substrate, emphasizing the binding-dependent functionality of these proteins.

The findings of this study offer a further understanding of the molecular basis of D-Ala-D-Lac utilization in transpeptidation during peptidoglycan synthesis. A deeper understanding of the mechanism underlying the utilization of this altered peptide may provide a novel target for the development of new therapeutic agents to combat VRE. Given the fact that β-lactam antibiotics are analogues of D-Ala-D-Ala, this study demonstrated that their affinity for changed PBPs in VRE is notably reduced. Consequently, the designing of D-Ala-D-Lac analogues as the same strategy with β-lactams could potentially inhibit the activity of changed PBPs. In order to test this potential therapeutic effect, the D-Ala-D-Lac dipeptide synthesized in this study were observed to have promising effects on VRE at optimal concentrations and in combination therapy, although the limitations of producing pure and sufficient quantities.

Our results provide a novel insight into the molecular mechanism underlying D-Ala-D-Lac utilization during the transpeptidation of PBPs. This information may be useful in the development of alternative therapeutics to combat highly resistant bacteria, such as VRE, and may help to solve medical treatment issues.

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