Comparative *In Silico* Analysis of Serine/Threonine Protein Kinase in *Enterococcus faecium*

Naqib bin Ahmad Ghazali

(72453)

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Naqib bin Ahmad Ghazali

A thesis submitted in partial fulfilment of the Requirement of The Degree Bachelor of Science with Honours
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SUPERVISOR: DR HASHIMATUL FATMA BINTI HASHIM

Programme of Resource Biotechnology
Faculty of Resource Science and Technology
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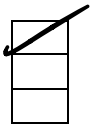
Final Year Project Report 
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Naqib bin Ahmad Ghazali

Programme of Resource Biotechnology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

Abstract

Enterococcus faecium is well distinguished for its ability to cause detrimental health issues including death among humans. Serine/threonine protein kinase gene, *stk* is a virulent gene present in the genome of *E. faecium* and the gene is believed to be the regulating factor which causes this condition, by possessing the ability to activate antibiotic resistance, virulence, and biofilm formation of *E. faecium*. Several genomic comparative analysis studies were conducted by various researchers to understand this detrimental mechanism. However, none of the studies are focussing on how serine/threonine protein kinase gene in the *enterococci* is regulating its antibiotic resistance, virulence, and biofilm formation property. This study aims to generate thorough understanding of the role of *stk* gene in the activation of those properties through the analysis of phylogeny, domain, and virulence genes in the bacterium. Advanced software and bioinformatic tools including MEGA11.0, Virulence Finder Database, ResFinder, CARD, and tools available from NCBI were utilized to achieve this aim. This study has provided evidence for the significant use of 16s rRNA in conducting phylogenetic analysis of *E. faecium* as clustering of the strains according to their isolates can be observed more clearly in the phylogenetic tree constructed using this gene compared to using *stk* gene. The domains of Stk in *E. faecium* are found to be very conserved based on the highly consistent E-values obtained, and insights of virulence genes present in the bacterium are exhibited as the essential outcome of virulence gene mining as the virulence factors regulated by *stk gene* were identified. This study enables *stk* to be made as target gene in an effective approach to control enterococcal infections in the future.

Key words: serine/threonine protein kinase, *Enterococcus faecium*, virulence

Abstrak

Enterococcus faecium amat dikenali dengan kebolehannya untuk menyebabkan masalah kesihatan yang memudaratkan termasuk kematian. Gen serine/threonine protein kinase, stk adalah suatu gen virulens yang terdapat di dalam genom E. faecium dan gen ini dipercayai menjadi faktor pengawal yang menyebabkan keadaan ini, dengan memiliki kebolehan untuk mengaktifkan sifat rintangan terhadap antibiotik, virulens, dan pembentukan biofilm dalam E. faecium. Sebilangan analisis perbandingan genomik telah pun dilaksanakan oleh para penyelidik untuk memahami mekanisme yang memudaratkan ini. Walau bagaimanapun, tiada penyelidikan yang memfokuskan bagaimana gen serine/threonine protein kinase di dalam enterococcus tersebut mengawal sifat rintangan terhadap antibiotik, virulens, dan pembentukan biofilm yang terdapat pada E. faecium ini. Kajian ini mensasarkan untuk menjana kefahaman yang menyeluruh terhadap sifat ini melalui analisis filogeni, domain, dan gen virulens milik bakterium ini. Perisian dan peralatan bioinformatik yang canggih termasuk MEGA11.0, Virulence Finder Database, ResFinder, CARD, dan peralatan yang terdapat pada NCBI digunakan untuk mencapai sasaran ini. Kajian ini telah membuktikan penggunaan 16s rRNA dalam melaksanakan analisis filogeni E. faecium adalah amat signifikan kerana pengelompokan strain E. faecium mengikut sumber perolehannya dapat dilihat dengan lebih jelas dalam pokok filogenetik yang dijana menggunakan 16s rRNA berbanding menggunakan gen stk. domain Stk dalam E. faecium didapati sangat terpelihara berdasarkan perolehan nilai E yang sangat konsisten, dan pengertian gen virulens yang terdapat pada bakterium ini dipaparkan sebagai hasil perlombongan gen virulens yang bernilai ekoran pengenalpastian faktor virulens yang dikawal oleh gen stk. Kajian ini membolehkan gen stk menjadi gen sasaran dalam kawalan infeksi enterococcal di masa hadapan.

Kata kunci: *serine/threonine protein kinase, Enterococcus faecium, virulens*

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List of Abbreviations

CARD	Comprehensive Antibiotic Resistance Database
DAP	Daptomycin
DDH	DNA-DNA hybridization
eDNA	Extracellular DNA
FASTA	Fast alignment
GGDC	Genome-to-Genome Distance Calculator
Mb	Mega base pair
MEGA	Molecular evolutionary genetics analysis
MGEs	Mobile genetic elements
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
NCBI	National Centre for Biotechnology Information
Pbp	Penicillin-binding protein
RAST	Rapid Annotation of Microbial Genomes using Subsystem Technology
SMART	Simple Modular Architecture Research Tool
Stk	Serine/threonine protein kinase
VRE	Vancomycin-resistant enterococci
VREfm	Vancomycin-resistant *Enterococcus faecium*

1.0 Introduction

Enterococcus faecium, a Gram-positive cocci species, is a worldwide important opportunistic pathogen capable of producing a wide spectrum of human infections with high fatality rates, especially in hospitalised patients (Gorrie et al., 2019). It is a commensal bacterium of the human gastrointestinal tract that is also a significant nosocomial pathogen, owing to its ability to develop resistance to a variety of medications, including vancomycin. In 1899, *Enterococci* were identified in human faeces for the first time.

E. faecium and *E. faecalis* can be considered as the major agents which cause infections in humans. *Enterococci* became a prominent cause of hospital-acquired infections in the 1970s (Gilmore et al., 2013). In addition, *E. faecium* is the species which is causing the most problems. This is due to the species having more than 80% strains showing resistance towards vancomycin and 90% strains showing resistance towards ampicillin (Morrisette et al., 2020). Glycopeptide resistance genes are grouped in van operons, which are found on mobile genetic elements (MGEs) (Zhou et al., 2020). *E. faecium* which are resistant to vancomycin are usually treated using daptomycin (DAP) (Tran et al., 2015).

1.1 Problem Statement

There have been a wide range of study encompassing the comparative analysis of *Enterococcus faecium* genome from various sources. Nevertheless, the study of comparative analysis of the gene that encodes for protein serine/threonine kinase in *E. faecium* and their interactions with other virulence gene is still lacking. This leads to the need of conducting this study.

1.2 Objectives

The detailed objective of this study is classified as follows:

1. To evaluate the phylogeny of *E. faecium* strains from different isolates using 16s rRNA and serine/threonine protein kinase.
2. To determine the domain of serine/threonine protein kinase, Stk of *E. faecium* strains from different origin.
3. To evaluate virulence profiles of *E. faecium* strains from different origin.

2.0 Literature Review

2.1 *Enterococcus faecium*

Enterococcus faecium is a commensal bacterium which is found in the human gastrointestinal tract and is a significant nosocomial pathogen. The first cases of vancomycin-resistant enterococci (VRE) were discovered in hospitals in the 1980s, and they have subsequently been found in health-care settings all over the world (Lam et al., 2012). Vancomycin-resistant *Enterococcus faecium* (VREfm) has been the substantial cause of nosocomial infections and therefore is classified as high priority in the worldwide priority list published by the World Health Organization involving antibiotic-resistant bacteria (Gouliouris et al., 2018).

2.2 Serine/Threonine Protein Kinases

A eukaryotic-type serine/threonine protein kinase known as Stk is a major determinant of cephalosporin resistance in *E. faecium* (Labbe & Kristich, 2017). The intrinsic resistance of *E. faecium* towards cephalosporin antibiotics and its lowered susceptibility to penicillin is accredited to the expression of Pbp5 which is a low-affinity class B penicillin-binding protein (Pbp) (Desbonnet et al., 2016). Pbp5-deficient *E. faecium* strains are vulnerable to β-lactam antibiotics, such as cephalosporins. In bacteria, the widely spread eukaryote-like serine threonine kinase/phosphatase systems are incriminated in a variety of cellular functions, which includes cell wall formation, cell propagation, and exposure to cell wall-active antibiotics (Pereira et al., 2011).

2.3 16s rRNA

For decades, sequence-based bacterial analysis has relied on the 16S rRNA gene (Johnson et al., 2019). 16s rRNA is the gene which is responsible in encoding small subunit ribosomal RNA molecules present in ribosomes which is important for significant conversion processes from genetic messages to cell components with their functions through the successful mRNA translation to proteins (Byrne et al., 2018). Analysis of the sequence and structural modelling of the 16S rRNA gene has proven that multiple conserved and variable segments are present in the gene's sequence.

In environmental microbiology and molecular evolution, prokaryotic 16S ribosomal RNA (rRNA) sequences are commonly utilised as trustworthy markers for phylogentic analysis of bacteria and its bacterial taxonomic clustering (Yang et al., 2016). Hence, several bioinformatics techniques were combined in this study to create an *in-silico* analysis to assess the phylogenetic responsiveness of hypervariable areas in comparison to full sequences.

2.4 Virulence Genes

Bacterial pathogens which exist either in human or animal host, regardless of by manner of natural or accidental, interpret and therefore become adapted to its environment through a global scale gene expression modification (Malachowa et al., 2011; Mandlik et al., 2011). Some of these genes are significant in the disease-causing ability of the bacterium. Hence, the outcome of these genes is referred to as the determinant of virulence or pathogenicity due to their providence of aid for colonisation, survival, and harmful ability of the bacteria against the host (Thomas & Wigneshweraraj, 2014). Tetracycline resistance in *E. faecium* was linked to the presence of *tet M* and *tet L* genes, while some other virulence genes of *E. faecium* includes the *efaAfm, fms8, pilA, pilB, sgrA,* and *hyl*.

2.4.1 *pilA & pilB*

There is a high presence of *pilB* in different isolates of *E. faecium* based on a study conducted by Soheili et al., (2014). *PilA* is also present but in a relatively lower frequency compared to *pilB* with a percentage difference of about 80%. Pili or commonly referred to as fimbriae are found in Gram positive bacteria. It is an organelle found on the surface which plays a role in occurrence of endocarditis and formation of biofilm in Gram positive bacteria. It also acts as a mediator for the attachment of bacteria to skin and epithelium of humans while enabling resistance in defiance of macrophages.

2.4.2 *acm*

acm gene codes for a collagen-binding protein that serves the function to bind type I and type IV collagens (Soheili et al., 2014). These collagens are vital as they act as antigen in humans that encounter endocarditis. The pathogenicity of bacteria is known to also be affected through cell adhesion by the involvement of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs).

2.4.3 *efaAfm*

efaAfm is another virulence gene commonly found in different isolates of *E. faecium.* Although its specific function has yet to be known, the scientific community trusted that the gene plays a role in adherence of the *enterococci* against cell wall, and therefore involved in the antibiotic resistance of *E. faecium.*

2.4.4 *sgrA*

E. faecium with pathogenic ability has a high content of *orf2351* that codes for *sgrA* which is involved in surface adhesion (Soheili et al., 2014). Hence, it can bind to nidogen 1 and 2 extracellular matrix molecules. This virulence gene also plays a role in biofilm formation.

2.4.5 *hyl*

As another virulence gene present in several *E. faecium* strains from different isolates, *hyl* is known for its ability to code for putative glycosyl hydrolase (Soheili et al., 2014). This hydrolase is deemed as the gene that harbours plasmids involved in colonizing mice gastrointestinal tracts, which consequently has resulted in a rise in pathogenic ability of *E. faecium.*

2.5 Multiple Antibiotic Resistance

Enterococci are abundant in nature and are resistant to a variety of circumstances (Tremblay et al., 2011). *Enterococci* antimicrobial resistance is not limited to nosocomial human settings. Resistance genes can be found on plasmids, transposons, or integrons, resulting in a wide range of multi-resistance phenotypes and co-selection mechanisms (Tremblay et al., 2011). A recent study by Bouymajane et al., (2018) reported that every *Enterococcus* involved in the study is resistant to ampicillin. Several *E. faecium* samples were also found to be resistant to streptomycin and tetracycline, which shows an elevating multiple antibiotic resistance index for *Enterococcus* species (Bouymajane et al., 2018).

2.6 Biofilm formation

Understanding the pathogenesis of *E. faecium* biofilms is critical for developing innovative methods to prevent and treat infections caused by the enterococci. A significant autolysin is required for extracellular DNA (eDNA) release in the biofilm matrix in various bacteria, which contributes to biofilm adhesion and stability (Paganelli et al., 2013). *Enterococcus faecium*-related nosocomial infections are on the rise, and treatment approaches are becoming scarce due to the resistance to antibiotics that are significantly increasing and caused by biofilm-associated infections.

Multilayer biofilms formation is a complicated process which initiated from the binding of single cells to the formation of a three-dimensional bacterial population surrounded by an extracellular matrix. The matrix is a key component that helps biofilms to remain stable and defend themselves against antimicrobials and immune cells (Abee et al., 2011).

2.7 Previous studies

There were several comparative genome analysis and studies involving *E. faecium* that have been conducted by various researchers across the world. The examples of these studies include first, a study by Jahansepas et al., (2020) in the article entitled Comparative analysis of *Enterococcus faecalis* and *Enterococcus faecium* strains isolated from clinical samples and traditional cheese types in the Northwest of Iran: antimicrobial susceptibility and virulence traits. This study provides insights on the susceptibility of the 50 *enterococci* samples studied towards antibiotic. Second, in an article entitled Comparative genome analysis reveals key genetic factors associated with probiotic property in *Enterococcus faecium* strains by Ghattargi et al., (2018) which discusses on the effect of genomic variety of *E. faecium* towards its pathogenic, non-pathogenic, and probiotic property.

The third and final example is from an article by Zhong et al., (2019) entitled Comparative genomic analysis revealed great plasticity and environmental adaptation of the genomes of *Enterococcus faecium* which provided a thorough study of *E. faecium* being a vital nosocomial pathogen. Observing from these examples, it is very clear that although several genomic studies of *E. faecium* were carried out, none of it studied the bacterium by focussing specifically on the serine/threonine protein kinase gene, *stk*.

3.0 Materials and Methods

3.1 Strains used in the study

Data of *Enterococcus faecium* strains obtained from NCBI GenBank Database are shown in the following table.

Table 1 Genomic features of *Enterococcus faecium* strains used in the study

Strains	Source	Accession number	Size (Mb)	Reference
64/3	Human (hospitalized patient)	CP012522	2.57	Bender et al., 2015
Aus 0004	Human (blood)	CP003351	2.96	Lam et al., 2012
Aus 0085	Human (clinical isolate)	CP006620.1	2.99	Lam et al., 2013
DO	Human (blood)	CP003583.1	2.70	Qin et al., 2012
E1162	Human (hospitalized patient)	ABQJ01000104.1	2.71	van Schaik et al., 2010)
E1573	Animal (bison rumen)	AHWX01000013.1	2.81	Lebreton et al., 2013
E4452	Animal (dog feces)	AEOU01000170.1	2.77	(de Regt et al., 2012)
NRRL B-2354	Food (milk and dairy utensils)	CP004063.1	2.85	Kopit et al., 2014
QU 50	Encvironment (egyptian soil)	AP019394	2.54	Abe et al., 2019
VREA3	Environment (sewage)	NZ_JACYGU010000007.1	3.07	Klees et al., 2020

3.2 Evolutionary Analysis

The nucleotide sequences of *E. faecium* strains from table 1 is available in the GenBank Database of NCBI. The sequences were downloaded as FASTA format using the NCBI-genome-download programme with default options. FASTA is an analysis tool service provided by NCBI database which can be utilized to search for similarity in sequences (McWilliam et al., 2013). Genome sequences that were retrieved are only the ones that have been published and therefore available to be downloaded.

The nucleotide sequences of the gene that encodes for serine/threonine protein kinase were utilized in the construction of phylogenetic tree that was done using MEGA software (Stecher et al., 2020). Generation of the nucleotide sequence set multiple alignment was performed using MUSCLE in the MEGA software. The alignments were then combined into a single alignment, before being used for the construction of phylogenetic tree using Maximum-Likelihood methods (Hall, 2013).

3.3 Domain Interaction Analysis

Functional Domain and virulence gene interaction in *E. faecium* from different sources was analysed by using the Simple Modular Architecture Research Tool (SMART) (Farmanullah et al., 2021). The SMART database combines a robust web-based interface with manually curated hidden Markov models for a variety of fields, as well as different analysis and visualisation capabilities (Letunic et al., 2021).

3.4 Virulence Gene Mining

The complete genome sequence of ten *E. faecium* strains of different isolates were obtained from the NCBI GenBank Database in FASTA format. To identify and analyse the virulence genes present in the strains, the whole genome sequence of each strain was submitted to the VFanalyzer tool, which is a virulence gene mining tool available at Virulence Factor Database (VFDB). Utilization of this tool at http://www.mgc.ac.cn/VFs/ enables the detection of putative virulence factors in all of the *E. faecium* strains isolated from different sources (Liu et al., 2012).

Identification of virulence gene in the ten *E. faecium* strains was also done through the utilization of VirulenceFinder website which can be reached at http://cge.cbs.dtu.dk/services/VirulenceFinder/. On the website, taxonomic group of the studied *enterococci* was selected before the whole genome sequence of each strain was submitted in FASTA format, allowing the program to run and provide results of virulence genes present in all of the strains (Kleinheinz et al., 2014). Similar procedure as that was done on the VirulenceFinder website was also carried out in Resfinder 4.0 which is a free to use website reachable at http://cge.cbs.dtu.dk/services/ResFinder/ for retrieval of more virulence gene (Bortolaia et al., 2020).

Retrieval of antibiotic resistant genes was carried out also by submitting the FASTA file of every *E. faecium* strains involved in this study to the Comprehensive Antibiotic Resistance Database (CARD) which is available at https://card.mcmaster.ca/home. This is to identify and further analyse the broad antibiotic resistance genes spectrum exhibited by *E. faecium* strains (Lal Gupta et al., 2020).

4.0 Results

4.1 Comparative Phylogenetics Analysis

The approach taken in conducting comparative phylogenetic analysis is by constructing a phylogenetic tree. The alignment of multiple serine/threonine protein kinase gene and 16s rRNA gene present in varying *E. faecium* sequences was done through the utilization of Multiple Sequence Comparison by Log- Expectation (MUSCLE) program which is an embedded program in the MEGA software (Stecher et al., 2020). The following phylogenetic trees was constructed by applying Maximum Likelihood methods in MEGA software (Hall, 2013).

These maximum likelihood trees were conducted with *Lactobacillus plantarum* WCFS1 and *Lactobacillus paracasei* ATCC 334 as the outgroup (Zhong et al., 2017). The MEGA 11.0 software was utilized in constructing this phylogenetic tree. The purpose for inclusion of outgroup in generation of the phylogenetic tree is to show large difference in distance and root when comparing the outgroup strains with *E. faecium* strains as suggested by its supposed function which is the providence of evolutionary knowledge and distinct features of the ingroup (Kinene et al., 2016).

The numbers visible at each node of the tree indicates the bootstrap confidence value with a basis of 1000 replicates, while substitution per site is indicated by the scale bar below the tree. The shown values only consist of the percentage of 50% or higher (de Moraes Russo & Selvatti, 2018). Bootstrapping analysis was done to enable the judgement of how strong the support of the branching tree is (Silakari & Singh, 2021). A scale bar is also visible in both phylogenetic trees which indicates 0.10 substitutions for one nucleotide position. This simply means that there is a nucleotide substitution that occurs in every 10 nucleotides.

4.1.1 Comparative Phylogenetics Analysis using serine/threonine protein kinase Nucleotide Sequence

From the phylogenetic tree, only two branches can be observed. As exhibited in **Figure 1**, all ten strains of *E. faecium* are clustered together in one branch while the strain of *L. paracasei* ATCC 334 and *L. plantarum* WCFS1 which were selected as the outgroup of the phylogenetic tree are clustered together in a separate branch.

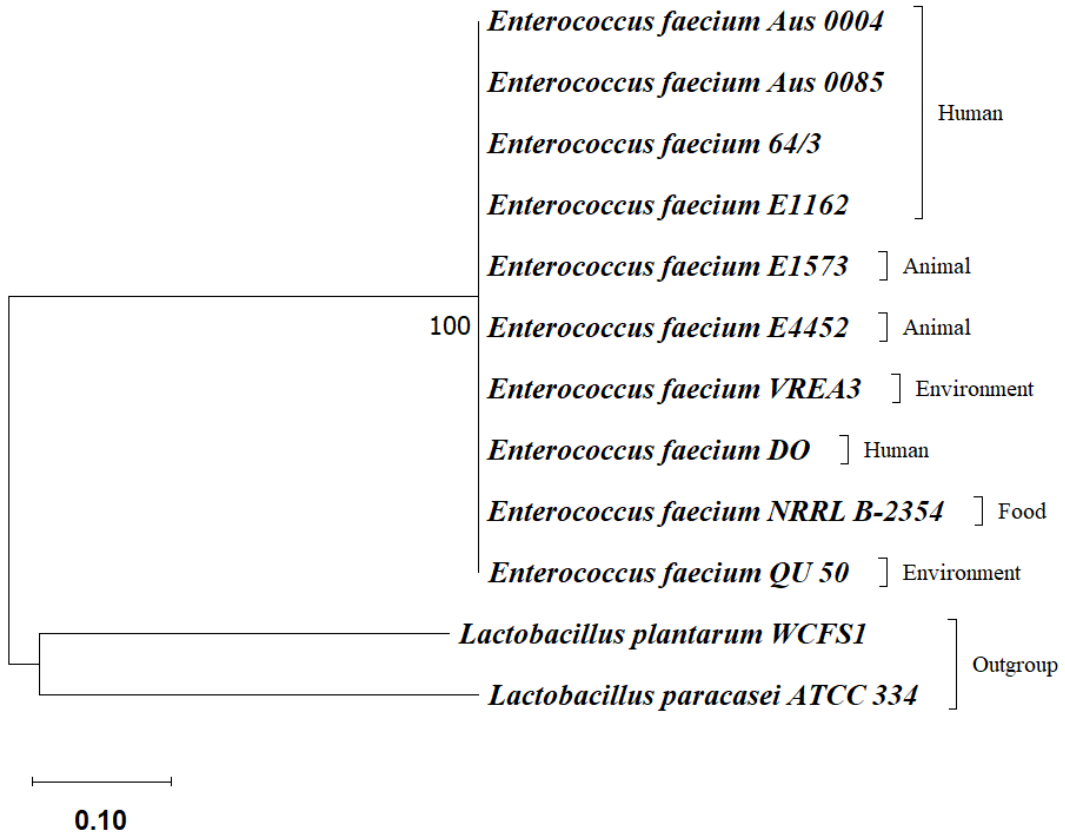

Figure 1 The phylogenetic tree of different *E. faecium* strains, constructed using the nucleotide sequences of their respective serine/threonine protein kinase genes.

4.1.2 Comparative Phylogenetics Analysis using 16s rRNA Nucleotide Sequence

Unlike what was observed in the phylogenetic tree constructed using the nucleotide sequence of serine/threonine protein kinase gene in **Figure 1**, the following phylogenetic tree, **Figure 2** exhibits three visible branches. The first branch is consisting of the clustering of *E. faecium* strains isolated from environment (*E. faecium* VREA3), human (*E. faecium* 64/3, *E. faecium* Aus 0004, *E. faecium* E1162) food (*E. faecium* NRRL B-2354), and animal (*E. faecium* E1573). The second branch consists of the clustering of *E. faecium* strains isolated from environment (*E. faecium* QU 50), human (*E. faecium* DO, *E. faecium* Aus 0085) and animal (*E. faecium* E4452). The third branch is where *L. paracasei* ATCC 334 and *L. plantarum* WCFS1 are clustered together as the outgroup of this phylogenetic tree.