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Generation of a Vancomycin-Resistant *Enterococcus faecium* Clinical Isolate Expressing a (FMN)-Based Fluorescent Protein

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ABSTRACT

Infections caused by multidrug-resistant (MDR) bacteria, including Vancomycin-Resistant Enterococcus (VRE), have become one of the greatest clinical challenges of the 21st century. Particularly, VRE strains of *E. faecium*, a natural member of the gastrointestinal consortia, have recently emerged as one of the most problematic cases of MDR nosocomial pathogens. It is widely known that the establishment of high-level intestinal colonization of this bacterium, triggered by antibiotic treatment of the host, potentially leads to bacteremia, endocarditis and surgical wound, urinary tract, and device-related infections in hospitalized patients. However, the unique determinants possessed by these strains, which enable them to benefit from the antibiotic-induced perturbations of the gut microbiota and host intestinal immune defenses, remain to be identified.

Essentially, what needs to be revealed is the differential gene expression of *E. faecium* in its niche at different time points; before and during antibiotic treatment of the host, and very interestingly, in the presence of probiotic bacteria. Differentially expressed genes will highlight how and why *E. faecium* is able to dominate the gut, as well as how it interacts with its surroundings, possibly pinpointing its potential weaknesses, as well as provide hints on what specific properties should be prerequisites for bacteria to be used as probiotics.

Since the global expression intended to be analyzed is that of *E. faecium* alone, a method to separate this bacteria from the rest of the gut microbiota is required. Failure to do so would result in the study of the gut microbiota's transcriptome as a whole: metatranscriptomics. In practice, the separation *VRE. faecium* from the rest of the commensal microbiota could be carried out by means of flow cytometry if the generation of a fluorescent *E. faecium* was achieved. Additionally, it would serve as a great tool to study a series of facets of *VRE faecium* colonization which remain virtually unknown. It would enable the monitorization of VRE infection through whole-body imaging of infected mice, fluorescence microscopy analysis of histological cuts to determine its most predominant locations, the use of FbFP as a translational or transcriptional reporter, etc.

Consequently**,** the main objective of this Final Degree Project has been to attempt the generation of a Vancomycin-Resistant *Enterococcus faecium* clinical isolate (C68) expressing a fluorescent protein whose chromophore can form under the anaerobic conditions found in the guts of animals: (FMN)-based fluorescent protein (FbFP).

In order to fulfill the main objective of this work, first, the FbFP gene was synthesized with the appropriate features for its expression *in E. faecium* and for its cloning. Next, the plasmid construction aimed to confer *E. faecium* fluorescent properties (pBT2-FbFP), was generated. This was done by inserting the FbFP gene into the pBT2 plasmid. As a previous step to the transformation of *E. faecium*, pBT2-FbFP was transformed into *E.coli DH5α* in order more efficiently validate the integrity of the plasmid construction. After validation, *E faecium* was finally transformed and the corresponding fluorescence analyses were performed.

Key words:
Vancomycin-Resistant Enterococcus, (FMN)-based fluorescent protein, *Enterococcus faecium,* Antibiotic resistance.

Generación de un aislado clínico de *Enterococcus faecium* resistente a la vancomicína mediante la expresión de una proteína fluorescente basada en FMN.

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RESUMEN:

Las infecciones causadas por bacterias resistentes a múltiples fármacos (MDR), incluyendo el Enterococo resistente a la vancomicina (VRE), se han convertido en uno de los mayores retos clínicos del siglo 21. En particular, las cepas de *E. faecium*, miembro natural de la microbiota gastrointestinal, suponen actualmente uno de los casos más problemáticos de MDR patógenos nosocomiales. La colonización intestinal con altos niveles de esta bacteria es provocada por el tratamiento con antibióticos y puede producir bacteriemia, endocarditis, infecciones del tracto urinario y de heridas quirúrgicas o relacionadas con dispositivos médicos, en pacientes hospitalizados. Sin embargo, aún no se han identificado los determinantes específicos que les permiten a estas cepas beneficiarse de las alteraciones inducidas por los antibióticos sobre la microbiota y las defensas del sistema inmune intestinal.

En esencia, se necesita conocer la expresión diferencial de los genes de *E. faecium* en su nicho y en diferentes momentos: antes y durante el tratamiento con antibióticos, así como en presencia de bacterias probióticas. Los genes expresados diferencialmente pondrían de relieve cómo y por qué *E. faecium* es capaz de dominar el intestino o la forma en que interactúa con su entorno, posibilitando la localización de sus debilidades potenciales y mostrando qué propiedades específicas deberían ser requisitos previos de una bacteria para ser utilizada como probiótico.

Dado que la expresión global a analizar es la de *E. faecium* solo, se necesita un método para separar esta bacteria del resto de la microbiota intestinal. No hacerlo, daría lugar al estudio del transcriptoma de la microbiota intestinal en su conjunto: metatranscriptómica. En la práctica, la separación de *VRE faecium* del resto de la microbiota comensal podría llevarse a cabo por medio de citometría de flujo una vez lograda la generación de un *E. faecium* fluorescente. Además, constituiría una gran herramienta para el estudio de una serie de facetas de la colonización de *VRE faecium* que siguen siendo prácticamente desconocidas. Por ejemplo, permitiría la monitorización de la infección por *VRE* a través de imágenes del cuerpo de ratones infectados, el análisis de microscopía de fluorescencia de cortes histológicos para determinar sus principales localizaciones, el uso de FbFP tanto como reportero transcripcional como de traducción, etc.

Por ello, el objetivo principal de este Trabajo de Fin de Grado la generación de un aislado clínico (C68) vancomicina resistente de *Enterococcus faecium* a través de la expresión de una proteína fluorescente cuyo cromóforo pueda formarse en las condiciones anaerobias de los intestinos de los animales: proteína fluorescente basada en FMN (FbFP).

Con el fin de cumplir con el objetivo principal de este trabajo, el gen FbFP fue sintetizado con las características apropiadas para su expresión en *E. faecium* así como para su clonación. A continuación, se generó la construcción del plásmido destinado a conferir propiedades fluorescentes a *E. faecium* (pBT2-FbFP). Esto se hizo mediante la inserción del gen FbFP en el plásmido pBT2. Como paso previo a la transformación de *E. faecium*, pBT2-FbFP se transformó en *E. coli DH5α* con el fin de validar de manera más eficiente la integridad de la construcción del plásmido. Después de su validación, *E. faecium* fue transformado y se realizaron los correspondientes análisis de fluorescencia.

Palabras clave:
Enterococo vancomicína resistente (VRE), proteína fluorescente basada en FMN, *Enterococcus faecium,* resistencia a antibióticos

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ABREVIATIONS:

Bp: Base Pairs

BLAST: Basic Local Alignment Search Tool

BHI: Brain Heart Infusion

BYGT: BHI Yeast extract, Glucose, Tris

CFP: Cyan Fluorescent Protein

C-Terminal: Carboxy-Terminal

GI: Gastro Intestinal

DIC: Differential Interference Contrast

DNA: Deoxyribonucleic acid

dNTPs: Deoxyribonucleotide triphosphates

dsDNA: Double stranded DNA

EcFbFPs: E. coli FbFPs

EDTA: Ethylenediaminetetraacetic acid

FACS: Fluorescence-Activated Cell Sorting

FbFPs: (FMN)-Based Fluorescent Proteins

FMN: Flavin Mononucleotide

GFP: Green Fluorescent Protein

LB: Luria-Beltani

LOV: Light Oxygen Voltage

MCS: Multiple Cloning Site

MDR: Multi-Drug Resistant

NCBI: National Center for Biotechnology Information

nm: Nanometers

N-Terminal: Amino-Terminal

OD: Optical Density

PBS: Phosphate Buffer Saline

PCR: Polymerase Chain Reaction

qPCR: Quantitative PCR

RNA: Deoxyribonucleic acid

RNA-Seq: RNA-Sequencing Technology

rpm: Revolutions per minute

RT: Reverse Transcription

TBE: Tris-Borate-EDTA

TE: Tris-EDTA

TRIS: Tris(hydroxymethyl)aminomethane

U.V.: Ultra Violet Light

VRE: Vancomycin-Resistant Enterococcus

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1. INTRODUCTION

1.1 Emergence of Enterococci as a challenging nosocomial problem

1.1.1 Enterococci

The *Enterococcus* genus forms part of the Enterococcaceae family, along with other bacterial genera (*Bavariicoccus*, *Catellicoccus*, *Melissococcus*, *Pilibacter*, *Tetragenococcus* and *Vagococcus)* (Lebreton *et al*., 2014). It consists of species that are normally commensal bacteria of human and animal gastro-intestinal (GI) tracts, as well as of the guts of insects. However, some are also adapted to survive and persist in fermented foods, dairy products, and different environments such as plants, soil and water.

These Gram-positive bacteria are characterized for being facultative anaerobic oval-shaped cocci, which are often arranged in pairs or small chains. Although unable to form spores, these organisms have been capable of surviving in such a broad spectrum of niches as a result of their ability to survive harsh conditions: high salt concentrations, and broad ranges of pH (4.5-10.0) and temperature (10 ºC to >45 ºC).

The taxonomy of enterococci has changed substantially over the past decade. At present, the genus includes more than forty distinct species adapted to various environments, with specific metabolic and phenotypic characteristics (Lebreton *et al*., 2014). It is of great importance to mention that *E. faecalis* and *E. faecium* are considered to be the most abundant enterococci species found in human fecal matter (Devriese *et al.,* 1994), and evidence reflects that they also account for the majority of enterococci-related diseases currently reported (Malani *et al.,* 2002)

1.1.2 The human gut microbiome

The human digestive system is home to trillions of microorganisms which play a key role in many different host-related processes and have a profound effect on human health and disease. This complex community of microorganisms is known as the gut microbiome and is often defined as “the vast collection of symbiotic microorganisms in the human gastrointestinal system and their collective interacting genomes” (Kimross *et al.,* 2011).

The gut flora comprises a highly organized ambiance, in which each species has a specific role, and where certain species balance other out, giving rise to a fluctuating, though relatively stable equilibrium. It has been described as an “extended genome” of the host, where millions of microbial genes are located in the GI tract (Lederberg, 2000). Therefore, it is not surprising that the human microbiome is bound to perform essential biochemical functions for the host, and that it plays an important role in our development, immunity, and nutrition (Kau *et al.,* 2012).

Alternatively, disorders of the microbiome are associated with various human disease processes (Kimross *et al.,* 2011), and consequently, in recent years, a great amount of effort has been made by scientists all around the world to study the microbiome and its activity. It is clear that a further understanding of the human gut microbiome is crucial for the development of future personalized medicine strategies, along with the potential identification of new therapy targets for drug development.

1.1.3 Antibiotic resistance: Rise of antibiotic resistant Enterococci

Antibiotic resistance is the ability of a microorganism to resist or evade the effects of a given antibiotic, and it occurs when the microorganism changes in some way which hampers the effectiveness of the drugs designed to cure or prevent its infections. As a result, these bacteria are capable of surviving and continue multiplying even under exposure to antibiotics. Multidrug resistant (MDR) bacteria or, more colloquially known as "superbugs", are terms which refer to bacteria that are resistant to multiple antibiotics. This phenomenon is currently a major threat to public health, and it represents one of the major clinical challenges and health concerns of the 21st century.

Resistance to antibiotics can be the result of spontaneous genetic mutations in genes involved in normal physiological processes and cell structures, and very importantly, additionally, through the acquisition of resistance genes from other bacteria by horizontal gene transfer, via conjugation, transduction or transformation (MICHIGAN STATE UNIVERSITY, 2011). Resistance genes are frequently carried by vectors (plasmids, transposons or integrons) that transfer these genes to other bacteria, though not necessarily pertaining to the same genus or specie.

Historically, Enterococci have been contemplated as second-rate pathogens due to the generally low virulence levels they display, evidenced by their ubiquitous presence in the human GI tract (Lebreton *et al*., 2014). However, factors such as the enormous plasticity of their genomes (acquired elements can comprise up to 25% of the genome), innate resistance to some antimicrobial agents (clindamycin, cephalosporins, aminoglycosides), ability to gain and spread antibiotic resistance genes, and their ability to survive long periods of time on environmental surfaces, contribute to the transformation of this second-rate pathogen into a first-class clinical challenge.

Although *E. faecalis* and *E. faecium* are currently the most frequent Enterococci fecal isolates and account for the majority of infections today, before mid-1990s *E. faecium* infections were rare, and *E. faecalis* accounted for 90–95% of clinical isolates (Lebreton *et al*., 2014; Lebreton *et al*., 2014). Nonetheless, ever since, *E. faecium* isolates have increased in proportion, a fact that hasn't passed unnoticed to many researchers in the field, and which most attribute to the dispersion of antibiotic resistance (vancomycin and ampicillin resistance in particular), a phenomenon highly associated with the increased use of vancomycin and other broad-spectrum antibiotics (Arias and Murray, 2012; Gilmore*et al*., 2013; Lebreton *et al*., 2014). It can be said that within the Enterococcus genus, *E. faecium* has emerged as the most therapeutically challenging organism (Arias and Murray, 2012).

Repercussions of this emerging problem are clinically dramatic. As mentioned earlier, some *E. faecium* strains possess unique traits which enable them to exploit their selective advantage over the rest of the gut microbiota under antibiotic exposure. The opportunistic facet of *E. faecium* is especially enhanced in the hospital setting, where antibiotic treatment is widespread. This environment, as described in detail in the next section, enables it to rise as a multidrug-resistant nosocomial pathogen, becoming a major clinical challenge in hospitals, and which potentially causes bacteremia and endocarditis, in addition to surgical wound, urinary tract and device-related infections in debilitated patients (Zang *et al*., 2013).

1.1.4 Gastro-intestinal tract colonization and the effect of antibiotic treatment

Despite the universal presence of Enterococci in the GI tracts of humans and animals, they habitually constitute a small percentage of the gut consortium, naturally comprising only

less than 1% of the adult microflora (Finegold *et al*, 1983; Sghir *et al,* 2000; Lebreton *et al 2014*). However, the use of antibiotics in hospitalized patients results in a substantial alteration of the gut microbiota, which in turn promotes the colonization by drug-resistant enterococci (Donskey *et al.,* 2000).

There is insufficient understanding of the mechanisms by which enterococci are able to conquer the GI tracts of either healthy individuals or hospitalized patients. Nonetheless, it is widely known that the settlement of a prominent intestinal colonization by drug-resistant Enterococci is a decisive step in a process that can culminate in serious nosocomial infections (Arias and Murray, 2012). Once *E. faecium* has become the predominant species in the GI tract, it may access the bloodstream by crossing the intestinal lining, causing bacteremia and infective endocarditis (Ubeda *et al*., 2010). Additionally, and taking into account the long survival period of Enterococci on environmental surfaces, this stage can also serve as a reservoir for fecal contamination of the skin and urinary tract, as well as for its dissemination in the hospital setting, altogether leading to catheter-related infections (Arias and Murray, 2012).

It is widely known that commensal microbiota normally aids in the protection against Gram-positive pathogens. Alterations in the susceptible microbiota triggered by antibiotics enhance the rise of drug-resistant enterococci as the dominant members of the GI tract, potentially leading to a severe clinical impact. The main reason for this phenomenon is the fact that commensal bacteria compete with Enterococci for the resources in the niche and produce substances which inhibit their growth and spread. Moreover, recent studies (Ubeda *et al*., 2010; Arias and Murray, 2012) have also shed light on the fact that antibiotic-depletion of the microbiota alters the immune defenses of the host (reduction of REGIII secretion), thereby indirectly facilitating colonization of the intestines by MDR Enterococci.

1.1.5 Commensal bacteria can prevent/combat Vancomycin-Resistant Enterococcus (VRE) infections (probiotics)

As explained in the previous section, the presence of a healthy gut microbiota is beneficial for the host, not only because it performs essential biochemical functions for the host, but also because it is key for keeping opportunistic bacteria (ei. *E. faecium*) in place. Notably, the beneficial effects of commensal bacteria have raised awareness on its potential use as probiotics. Basically, the idea behind the use of probiotics is the restoration of beneficial bacteria which can help the host fight infection by killing harmful bacteria, either directly (actively attacking other bacteria with bacteriocins and competing for the same resources in the niche), or indirectly (through the activation of the immune response). With the number of drug-resistant bacteria on the rise, and taking into account the time-consuming and tedious process antibiotic development entails, this alternative presents itself as ever more appealing.

Recent studies demonstrate that the transference of a healthy donor's microbiota into a sick recipient by means of bacteriotherapy (fecal transplantation), can prompt its recovery through the reestablishment of its normal microflora (Khoruts *et al*., 2010). Therefore, directed manipulation of the gut microbiome presents itself as clinically promising. In relation to VRE, *E. faecium* in particular, studies carried out by the co-supervisor of this Final Degree Project were able to identify bacterial populations which correlate with the elimination of this pathogen in the GI tracts of mice (Ubeda *et al*., 2013). So far, it appears that bacteria pertaining to Barnesiella genus protect the GI tract of its host from VRE domination. Withal, it is very likely that other bacterial genera may also contribute to VRE elimination, and that, rather than a complete recovery of the microbiota, the recovery of just a few key members could be sufficient for VRE clearance.

In the foreseeable future, MDR enterococci are expected to continue posing a huge public health challenge. The trends in the epidemiology of enterococcal infections suggest that VR*E faecium* may become the most common species isolated from hospitalized patients soon (Kristich *et al*., 2014). As a result, novel therapeutic strategies focused on treating the infections provoked by these organisms are urgently needed. The probiotic approach is the one being taken by the lab department in which this "Final Degree Project" was carried out.

1.2 Importance of a complete Transcriptome study

The specific genetic determinants which promote the GI tract colonization of nosocomial *E. faecium* strains during antibiotic treatment remain to be identified (Zang *et al*., 2013). Until light is shed on the unique traits that enable this pathogen to benefit from the antibiotic-induced perturbations of the gut, it will continue to deceive our clinical barriers, hence threatening the lives of countless hospitalized patients. Studies focusing on revealing the key differences between the community-associated (commensal) and the nosocomial *E. faecium* are those of most urgent need. Essentially, what needs to be revealed is the differential gene expression of *E. faecium* in its niche at different time points; before and during antibiotic treatment of the host, and very interestingly, in the presence of probiotic bacteria. Differentially expressed genes will highlight how and why *E. faecium* is able to dominate the gut, as well as how it interacts with its surroundings, possibly pinpointing its potential weaknesses. This will provide hints on what specific properties should be prerequisites for bacteria to be used as probiotics.

To study this, transcriptomic tools are required. Transcriptomics is defined as "the study of the complete set of RNAs (transcriptome) encoded by the genome of a specific cell or organism at a specific time or under a specific set of conditions". Fundamentally, there are three techniques for tackling the transcriptome: Real-time quantitative PCR (qPCR), microarray-based expression profiling and Next-Generation sequencing technologies (specifically a technology known as "RNA-Seq").

Although the qPCR technique is remarkably quantitative and sensitive, it would not be suitable for analyzing the differential expression of *E. faecium*, for it is designed to interrogate a relatively small number of transcripts, hence defeating the purpose of a global expression analysis. However, both microarrays and RNA-Seq could offer global surveys of the transcriptome, and could thereupon be used for this purpose (Perkel, 2013). On one hand, microarrays are biased, only capable of detecting homologous sequences to those on the array, but, the technology is widely available, simple and economical. On the other hand, RNA-Seq is unbiased, though it remains expensive and computationally challenging.

Since a global expression analysis is required, both the microarray and RNA-Seq technologies could be used, though RNA-Seq seems more suitable as a result of its unbiased output. However, certain aspects must be taken into account. Since the global expression analysis intended to be analyzed is that of *E. faecium* alone, a method to separate this bacteria from the rest of the gut microbiota is required. Failure to do so would result in the study of the gut microbiota's transcriptome as a whole: metatranscriptomics. Furthermore, taking into account that under certain conditions VRE only represents a minor population of the microbiota, conducting RNA-Seq without previous separation of this bacterium would ultimately result in an output highly enriched with non-VRE sequences. Likewise, Microarray analysis of gene expression without taking the effort to separate the bacteria of interest would result in an outcome enriched with false positives, since homologous sequences from other commensal bacteria would bind to the probes on the chip.

1.3 (FMN)-based fluorescent proteins (FBFP's)

In practice, *E. faecium* could be separated from the rest of the commensal microbiota using flow cytometry, a technique that is widely used for counting and sorting microscopic particles, by suspending them in a stream of fluid and passing them through an electronic detection apparatus (UNIVERSITÉ DE PICARDIE JULES VERNE, 2014). This technique can be coupled to automated cell sorting devices which can sort successive droplets of the stream into different fractions by reason of the fluorescence emitted by each droplet (FACS: Fluorescence-activated cell sorting). Therefore, construction of a fluorescent VRE *faecium* clone would be of great interest for its in vivo separation from the rest of the microbiota, my means of FACS.

It is important to highlight that there are a series of fluorescent reporter proteins which are currently used as noninvasive molecular tools for in vivo real-time imaging, like for example, GFP-like proteins (green fluorescent protein) (Drepper *et al*., 2007). However, a considerable drawback of the members of the GFP family is that their utility is restricted to aerobic systems, since molecular oxygen is strictly required for the correct formation of their chromophores. This handicap motivated some researchers to generate Flavin mononucleotide (FMN)-based fluorescent proteins (FbFPs), which can be used as fluorescent reporters under both aerobic and anaerobic conditions. It is important to highlight the importance of the development of these proteins for studies involving fluorescence in the guts of animals, since these are anaerobic environments. These proteins were derived from the bacterial blue-light photoreceptors YtvA (from *Bacillus subtilis*), and SB2 (from *Pseudomonas putida*) that contain the light oxygen voltage (LOV) domains. In the engineered protein, the absorption peak is found at 449 nm, and its emission apex is 495 nm upon excitation with blue light (450 nm).

In this way, the generation of a VRE *faecium* clone expressing FbFP could be used to infect mice and subsequently recover it from feces at different time points and under different conditions (antibiotic treatment, presence of other bacteria, etc.), in order to separate it by means of FACS and study its transcriptome. This is one of the main objectives of the department in which this project was carried out.

1.4 Further applications of a fluorescent VRE *faecium* clone

In addition to its usefulness for global expression analyses, a fluorescent VRE *faecium* clone could be a great tool to study a series of facets of VRE *faecium* colonization which remain virtually unknown. For instance, whole-body imaging of VRE infected mice could be used for imaging the course of infection and its response to antibiotics or probiotics. Similar approaches have only been attempted through high expression levels of GFP-like proteins (Hoffman and Zhao, 2007), though FbFP appears more promising due to its fluorescent properties under anaerobic condition. This technique presents itself as non-invasive, for it enables imaging of FbFP-expressing bacteria from outside intact infected mice. Consequently, the temporal and spatial behavior of the infectious process could be monitored in great detail, making possible the determination of the spatial migration, tissue-specificity and response of VRE to chemical or biological agents.

Furthermore, little is known about the in vivo interaction of these microorganisms with the GI epithelium of colonized mice. Fluorescence microscopy could be used to study histological cuts of the GI tracts of infected mice in order to determine whether VRE is predominantly located on the epithelial surface and embedded in the mucus, inside intestinal lining crevices, etc. This information could give us insight on what specific niches VRE occupies, thus facilitating the study and development of strategies against it.

As an alternative to immunofluorescence microscopy, GFP-like proteins are commonly used as transcriptional or translational fusion reporters. As a transcriptional fusion reporter, the promoterless gene which codes for the fluorescent protein is fused to the promoter of the gene of interest. Consequently, the transcriptional activity of the promoter can be measured through the analysis of fluorescence, since the fluorescent protein is synthesized whenever the target gene is being transcribed (Roberts and Pautsian, 2002). This allows for the monitorization of the transcriptional regulation of the target gene. However, it ignores any possible posttranscriptional effects. In contrast, in translational fusions, the fluorescent protein is made whenever the target gene is being transcribed and translated. In this method, the fluorescent gene is inserted between the C-terminal and N-terminal of the protein of interest, in-frame and preferably at a site that does not disrupt the proteins function or topology. The resultant protein is basically the target protein bearing a fluorescent tag. As GFP-like proteins, FbFP should also be suitable for this purpose and its great potential lies in its application for the analysis of gene expression in anaerobic environments, like the human GI tract (Lobo *et al*., 2011). The use of these reporter proteins presents itself as an elegant alternative to immunofluorescence, for it not only allows in vivo real-time gene-expression monitorization, but can also be used for the determination of the localization, movement and interaction of gene products.

2. OBJECTIVES

The main objective of this work is the generation of a Vancomycin-Resistant *Enterococcus faecium* clinical isolate (C68) expressing a (FMN)-based fluorescent protein (FbFP) which could serve as a practical tool for the study of key aspects of VRE's colonization and persistence in the gut. These aspects remain practically unknown today but require special attention now that *E. faecium* has escalated to become a major public health concern.

In order to accomplish the main objective, a series of partial objectives were proposed:

1. The design and synthesis of a FbFP gene containing the necessary features for its adequate expression in *E. faecium* (Enterococcus codon-usage biased gene sequence, enterococcus constitutive promoter, and stop codon), as well as for its appropriate insertion into the pBT2 plasmid (BamHI and SalI restriction sites).

2. The generation of the pBT2-FbFP plasmid through the cloning of the fluorescent protein gene (FbFP) into pBT2 (in *E. coli DH5a)* and the subsequent verification of the integrity of the plasmid construction.

3. The optimization of the transformation protocol for *E. faecium* through the adjustment of the transformation protocol used for *E. faecalis*.

4. The successful transformation of *E. faecium* with the validated pBT2-FbFP plasmid construction.

5. The use of fluorescence microscopy techniques to analyze the putative fluorescent properties of the transformed *E. faecium* cultured at different conditions.

3. MATERIALS AND METHODS

3.1 Bacterial strains

3.1.1 *Escherichia coli DH5α*

E. coli DH5α strain (F– Φ80*lac*ZΔM15 Δ (*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1) was used for the molecular cloning procedures of the EcFbFP gene. This strain is particularly useful for recombinant DNA methods and provides the means for high-efficiency transformations.

3.1.2 *Enterococcus faecium C68*

Enterococcus faecium C68 is a clinical isolate that represented the most prevalent VRE clone in 11 Cleveland hospitals in 1996 (Lam *et al*., 2012). At present, within the *Enterococcus* genus, *E. faecium* has emerged as the most therapeutically challenging organism. *C68* readily colonizes the mouse gastrointestinal tract under antimicrobial selective pressure (Rice *et al*., 2009). Its genome has been completely sequenced (Lam *et al*., 2012). In this work, this strain was transformed with the pBT2 plasmid containing the EcFbFP insert, with the purpose of conferring it fluorescent properties.

3.2 Plasmids

3.2.1 pBT2

pBT2 is a temperature-sensitive, 6.97 Kb shuttle vector (**Fig. 1**) constructed from fragments of the pBR322 and pTV1Ts plasmids (Brükner, 1997). Some important features of the resulting plasmid include: origins of replication for *E. coli* and for Gram positive bacteria derived from the pBR322 and thermosensitive (does not replicate at 42 ºC) pTV1Ts plasmids respectively, ampicillin resistance to *E. coli* and chloramphenicol resistance to *Staphylococcus, E. coli* and *Enterococcus*, in addition to a multiple cloning site derived from the pUC18 plasmid. It is important to highlight that chloramphenicol resistance is not used as the selective marker of choice for *E. coli* throughout the experiments due to the relatively low expression levels of the chloramphenicol acetyltransferase gene in this bacteria; ampicillin resistance is used for this purpose instead.

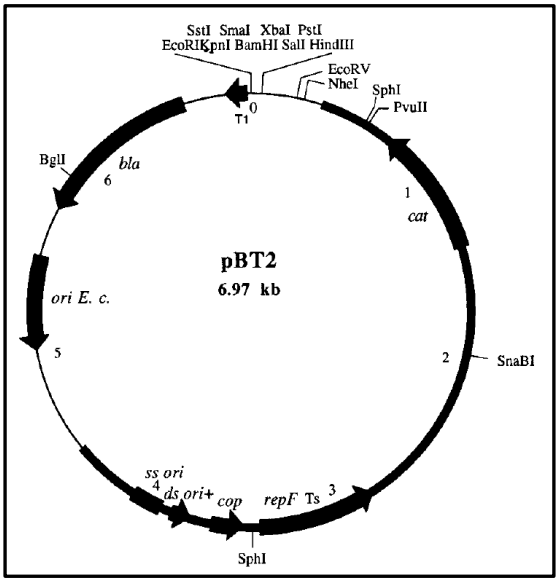

Figure 1. Restriction map of the shuttle plasmid pBT2. Thin lines represent DNA derived from pBR322, and thick lines DNA derived from pTV1Ts. The location and orientation of resistance genes, origins of replication and the T1 transcriptional terminator are marked by arrows. Some restriction sites are shown. Restriction sites indicated in the multiple cloning site are unique. In addition, EcoRV, NheI, and PvuII may be used for cloning in pBT2 (Brückner, 1997).

This plasmid was used to clone the FbFP gene into the BamHI, SalI sites, thereby generating the pBT2-FbFP plasmid. The plasmid was chosen to be temperature-sensitive as a requirement for steps that are to be carried out after the works described in this Final Degree Project. Basically, once the desired functional plasmid construction conferring *E. faecium* fluorescent properties is generated, there is intention to integrate the FbFP gene into the genome through recombination, and for this purpose, in order to select bacteria which have undergone recombination, temperature will be raised to provoke the plasmid loss. The bacteria which are able to grow in the selective media will prove to be recombinant.

3.2.2 pUC57

This plasmid was purchased from GenScript USA Inc. with the *Enterococcus* codon optimized cyan-green FbFP protein insert (441 bp). The gene was cloned into the EcoRV located in the multiple cloning site (MCS), as seen on (**Fig. 2)**.It is important to highlight that the replication origin is only compatible with *E.coli* and that the gene is inserted promoterless.

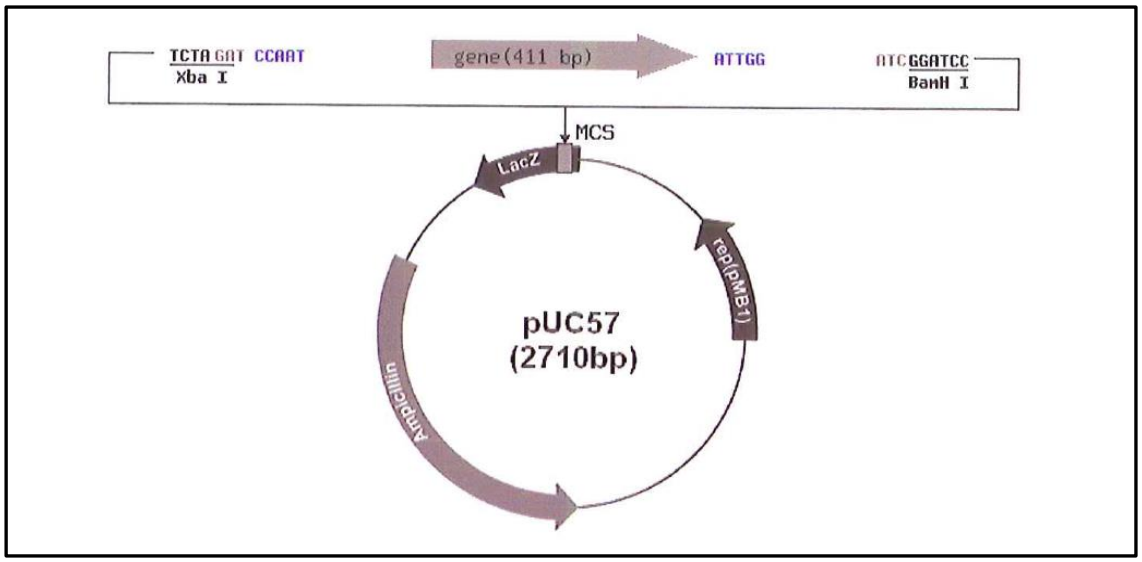

Figure 2. Plasmid construct map of the pUC57 vector and multiple cloning site detail. The location and orientation of resistance genes and origin of replication are marked by arrows. The multiple cloning site (MCS) has been enlarged to show the exact location and the orientation of the FbFP gene (411 bp).

3.2.3 pGLOW-KXN-Bs2

The pGLOW-KXN-Bs2 is a 6957 bp bacterial expression vector that was purchased from Evoglow (**Fig. 3**). It is designed for the expression of an *E. coli* codon optimized FbFP. It contains a series of features like: both a constitutive promoter (aphII) for continuous expression and an IPTG-inducible T7 promoter (for a stronger induction of fluorescence), chloramphenicol and kanamycin antibiotic resistance genes, a gene for plasmid replication (rep) in a broad range of gram-negative bacteria, and a gene for plasmid mobilization (mob). This plasmid was aimed for its use as a positive control for fluorescence. This plasmid cannot be used directly in *E. faecium*, for the gene promoter and replication origins are not compatible.

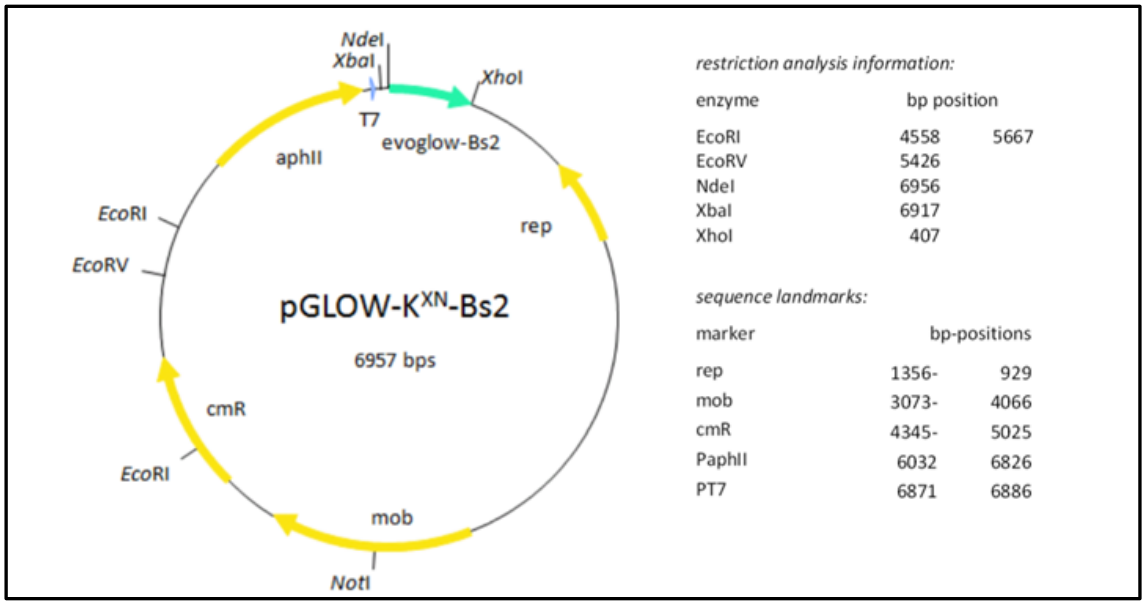

Figure 3. Restriction map of the pGLOW-KXN-Bs2 plasmid. The location and orientation of resistance genes (cmR), origin of replication (rep), EcFbFP (blue), constitutive promoter (aphII), inducible promoter (T7) and the mobilization gene (mob) are marked by arrows. A series of restriction enzymes are also displayed, in addition to their bp positions.

3.5 Primers

The oligonucleotide primers used in this work have been designed with vector NTI software. Primers EcFbFP-F-B, EcFbFP-R-S-2, T1-1R and Pbr322-1R were purchased from Isogen Life Sciences and FbFP-F3, FbFP-R4, EcFbFP-F5 and EcFbFP-R6 were purchased from Integrated DNA Technologies.

Table 1. List of oligonucleotide primers used for PCR Amplification in this work.

Primer	Sequence	Description	Reference
EcFbFP-F-B	GTCGGATCCGCTTGCATCAAAATAA ACTACATGGGTATAATAGCAATGAA ATGCATTTCAAAAATATTTTGAGGA GAATTTAGTATGGCTTCATTCCAATC ATTCGG	"Insert-specific" forward primer used for PCR amplification of the FbFP from pCU57, and for the introduction of a promoter and a BamHI restriction site for cloning.	This work
EcFbFP-R-S-2	ACTGTCGACTTATTCTAATAATTTTT CGTATTCTTTTTGTTTTGTG	"Insert-specific" reverse primer used for PCR amplification of the FbFP insert from pCU57, and for the introduction of a stop codon and the SalI restriction site for cloning.	This work
T1-1R	CGTTCACCGACAAACAACAG	Forward primer flanking the multicloning site of pBT2.	This work
Pbr322-1R	ATTGCATCAACGCATATAGC	Reverse primer flanking the multicloning site of pBT2.	This work
FbFP-F3	AGATCACGTTCGTGTTGGTG	Forward primer designed to amplify an internal region of the FbFP insert of pBT2-FbFP. Originally designed for Q-PCR.	This work

FbFP-R4	TCAACTTCAGCTGGATCTGTGT	Reverse primer designed to amplify an internal region of the FbFP insert of pBT2-FbFP. Originally designed for Q-PCR.	This work
EcFbFP-F5	CACCGCGCTGCAAAATAAAG	Forward primer designed to amplify an internal region of the EcFbFP insert of pGLOW-KXN-Bs2. Originally designed for Q-PCR.	This work
EcFbFP-R6	CCTTCTGCTTGGTGATGTCG	Reverse primer designed to amplify an internal region of the EcFbFP insert of pGLOW-KXN-Bs2. Originally designed for Q-PCR.	This work

3.6 Culture and growth conditions

Both the solid and liquid media detailed below (sections 3.6.1 and 3.62) were sterilized using an autoclave: 20 minutes at 1 atmosphere of pressure and 120 ºC. Temperature-sensitive compounds, such as antibiotics, were added after autoclave sterilization, and only once the media was at a temperature below 50 ºC. Solid media was stored at 4 ºC; while liquid media was stored at room temperature (liquid media was not stored at 4 ºC, as antibiotics were added right before its use). Liquid cultures were grown in sterile flasks or falcon tubes on an agitated incubator tray to ensure an adequate aeration. However, it is important to highlight that when anaerobic conditions were required, cultures were grown in the anaerobic chamber. Solid cultures were grown in petri dishes stored in an inverted position, inside the incubator.

3.6.1 *Escherichia coli*

E. coli was grown at 37 ºC with agitation (220 rpm) in LB (Luria-Bertani) medium, composed of (1%(m/v) tryptone, 0.5% (m/v) yeast extract y 1% (m/v) NaCl). When required, ampicillin (reason discussed in section 3.2.1) was used for selection at a concentration of 100 µg/mL. In order to prepare solid LB media, liquid LB was supplemented with agar (15 g/L).

3.6.2 *Enterococcus faecium*

E. faecium was grown at 37 ºC, or at 30 ºC (when carrying the temperature-sensitive pBT2 plasmid) with an agitation of 220 rpm in BHI medium, composed of: brain heart infusion from 250g (5 g/L), calf brains infusion from 200 g (12.5 g/L), disodium hydrogen phosphate (2.5 g/L), D(+)-glucose (2 g/L), peptone (10 g/L), sodium chloride (5 g/L) at pH 7.4±0.2 (25 °C). When needed, Chloramphenicol was used at a concentration of 12.5 µg/mL for selection purposes. When BHI agar medium was required, BHI broth was supplemented with agar (15 g/L).

The media for electroporation was prepared as described by (Li *et al.*, 1995; Arias and Murray, 2012):

-BYGT (BHI, yeast extract, glucose, Tris)
Composition: 19 g/L BHI, 5 g/L yeast extract, 2 g/L dextrose, 12.1 g/L Tris; (pH=8).
-BYGT Glycine (6%)

Composition: 19 g/L BHI, 5 g/L yeast extract, 2 g/L dextrose, 60 g/L glycine, 12,1 g/L Tris; (pH=8).
-BYGT-Sucrose
Composition: 19 g/L BHI , 5 g/L yeast extract, 2 g/L dextrose, 85.5 g/L sucrose, 12.1 g/L Tris; (pH=8).
- Sucrose-MgCl2
Composition: 213 g/L sucrose, 0,2 g/L Magnesium Chloride hexahydrate; (pH=4).

3.7 DNA manipulations

3.7.1 Plasmid extractions

Plasmid DNA extractions were carried out using the "QIAprep Spin Miniprep Kit (Qiagen)", following the "QIAprep Spin Miniprep Kit Using a Microcentrifuge" protocol on page 22 of the QIAprep Miniprep Handbook. This procedure is based on the alkaline lysis of bacterial cells, followed by adsorption of DNA onto a silica-gel membrane in the presence of high salt concentrations, finally leading to the washing and elution of the plasmid DNA.

It is important to highlight that plasmid extractions of *E. faecium* required an additional step: resuspension of the overnight 5 mL culture cell pellet in 250 µL of the Enterococci lysis solution (9 g/L glucose, 3.46 g/L EDTA, 3.03 g/L TRIS and 5 mg/mL of lysozyme (pH= 8)) and incubation for 1 hour on ice. The following steps are to be carried out as starting on the second step of the "QIAprep Spin Miniprep Kit Using a Microcentrifuge" protocol.

3.7.2 Conditions for PCR amplification

PCR amplification reactions were routinely done in sterile microcentrifuge tubes, and all pre-PCR steps were carried out inside laminar flow cabinets. A PCR negative control (no DNA template) was always used to allow detection of contamination of the PCR reagents and positive controls were used when possible.

Table 2. PCR reagents and concentrations.

Reagent/Component	Volume for one reaction (µL)
H2O milli Q	19.375
DNA Polymerase Buffer (10X)	2.5
dNTPs (10mM)	0.625
Taq Polymerase Enzyme (1U/ µL)	0.5
Forward primer (10 mM)	0.5
Reverse primer (10 mM)	0.5
DNA template (∓ 10-20 ng)	1
FINAL VOLUME	25*

Note* Some PCR reactions were carried out for a final reaction volume of 50µL, thus all reagent volumes were doubled.

All PCR amplification reactions were carried out in a thermal cycler (Techne TC-4000).

Table 3. PCR program.

Step	Temperature (ºC)	Time (min)	Number of cycles
Initial denaturation	94	5	1
Denaturation	94	0.5	35
Annealing	*	0.5	
Elongation	68	0.5	
Final elongation	*	5	1
Hold	4		

* Annealing temperatures depend on the different sets of primers used for each PCR reaction. See **Table 4** for the temperatures used for each pair of primers.

Table 4. Annealing temperatures used for each primer pair.

Primer pairs	Brief Description	Temperature (ºC)
EcFbFP-F-B and EcFbFP-R-S-2	Insert-specific	58
T1-1R and Pbr322-1R	vector –based/insert-flanking	56
FbFP-F3 and FbFP-R4	E. faecium qPCR	56
EcFbFP-F5 and EcFbFP-R6	E. coli qPCR	56

3.7. 3 Colony PCR

Colony PCR was a quick and economical technique used to rapidly screen transformation products for positive clones after a ligation reaction, before double checking with double restriction enzyme digestions. Putative transformant colonies were resuspended in 15 µL of PBS in a 96-well plate. 3 µL of each colony solution were plated on selective media plates in order to have access to each colony in case the PCR indicated a positive transformation. 1 µL of the solution was used for PCR. Initial denaturation at 94 ºC lyses the cells, hence allowing PCR amplification of the gene, if present. It is important to highlight that the insert-flanking, vector-based primers were used (reasons discussed in section 4.5).

3.7.4 Gel electrophoresis

Agarose gel electrophoresis were performed with 1.4% agarose gels and using TBE (Tris/Borate/EDTA) 1X buffer solution (89 mM Tris (pH 7.6), 89 mM boric acid, 2 mM EDTA). The molecular weight marker used was "GeneRuler ™ , ready-to-use, 100-10000 bp DNA ladder Mix; 0.1µg/µL" (Thermo Scientific) and DNA samples were mixed with Loading MIX (50 µL loading buffer 3X, 0.2 µL Gel Red) before loading them on the gel wells (Sample-Loading MIX ratio = 1/4). All gels were run during a period of 25 min and a 20 volt electric field was applied. Gel results were visualized under U.V. light, taking the proper precautions, and photographs of the results were taken using a polaroid photo documentation camera.

3.7.5 DNA quantification

DNA quantification was required for many experiments in this work, such as quantification of plasmid extractions, quantifications of samples before digestions and ligations, to find out if large quantities of sample were lost during purifications, etc. For all DNA quantifications, the picogreen method was used. Picogreen is a very sensitive fluorescent nucleic acid stain used for the quantitation of double-stranded DNA. Quant-iT™ PicoGreen® dsDNA Assay Kit From Molecular Probes (Invitrogen) was used for this purpose. The reaction

itself consisted on mixing 1 µL of the sample with 24 µL of TE buffer and 25 µL of PicoGreen® dye (1:200; dye/TE ratio) in microcentrifuge tubes. The reaction was then incubated in the dark for 5 minutes to permit the dye to bind to double stranded DNA. The fluorescence of the samples was then measured with "QuantiFluor® dsDNA System (Promega)" at excitation/emission of 435/535 nm.

3.7.6 Restriction enzyme digestion of DNA

All restriction enzymes and restriction buffers were purchased from New England BioLabs and were kept in ice at all times. Digestion reactions took place in 1.5 mL eppendorf tubes placed in a tube incubator for a period of 2 hours, at a temperature of 37 ºC.

Table 5. Reagents and volumes used for the restriction enzyme digestions of DNA.

Reagent	Volume (µL)
H20 milli Q	0**
Buffer *	3
Enzyme 1	0.5
Enzyme 2	0.5
DNA (0.1-0.5 µg)	26**
Final Volume	30

Note*: Buffer 3.1 was used for digestions with SalI and BamHI. In both cases, the selected buffer conferred 100% activity to both enzymes.
Note**: The volume of water and DNA will vary in relation to the concentration of the DNA sample to be digested.

3.7.7 DNA ligations

The DNA concentrations of the insert and vector (quantified as indicated above), as well as their size ratio, were used to calculate the volume of each required for an optimal ligation reaction, using a 1:4 (vector/insert ratio). Calculations were made aiming for the use 100 ng of pBT2 vector for the ligation reaction. The following formula was used:

$$\frac{\text{ng of vector X bp size of insert}}{\text{bp size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}}$$

Once the nanograms of vector and insert required for the reaction were calculated, and having quantified the DNA concentrations previously, the volumes required for the ligation reaction were calculated by multiplying the ng of (insert or vector required) by their respective concentrations.

Table 6. Reagents and volumes used for the DNA ligation reaction.

	Ligation reaction	Ligation Control (without insert)
Reagents	**Volume (µL)**	**Volume (µL)**
H20 milli Q	*	*
pBT2 plasmid (100 ng)	*	*
EcFbFP gene insert	*	0
Buffer (10x)	2.5	2.5
Ligase enzyme	1	1
Final Volume	25	25

Note *: These volumes depend on the calculations explained above.

Ligation took place overnight in a thermocycler running a program which consisted in maintaining a steady temperature of 16 ºC.

3.7.8 DNA purifications

All DNA purifications which did not required extraction of DNA from a gel slice, such as the purification of DNA after digestion, were done using "Excela Pure 96-well plates" (EDGE BIO). Using this method, contaminants are filtered to waste under vacuum pressure and the purified DNA products are retained on the well membrane. Purified products are recovered after a quick elution and ready for immediate use. In these cases, deionized water was added until the volume was 100µL, and then they were left to rest 10 minutes inside a well. After this, vacuum was applied to the plate until the wells were dry (10 min).Subsequently, 100µL of deionized water were used to resuspend the DNA sample. Again, the plate was left to rest 10 minutes, followed by the same vacuum operation. Finally, DNA had been purified, and after resuspending it in 30-50 µL of water it was stored for future use.

When Purifications required extraction of the DNA contained in a gel slice, (for example when the FbFP gene was amplified for its subsequent ligation with the pBT2 vector), the "High Pure PCR Product Purification Kit" was used in order to purify the amplicon from the agarose gel. Purification was therefore carried out once the desired DNA band was cut from the gel using an ethanol-cleaned scalpel on a UV light box, taking all the necessary precautions.

3.7.9 RNA purifications

RNA was extracted and purified with the "UltraClean® Microbial RNA Isolation Kit" (MO-BIO). This Kit is designed to obtain RNA from pure cultures of microorganisms. Through a bead beating technology, cells are lysed. Then, RNA is captured on a silica spin filter, washed and eluted in an RNase-free elution buffer. Finally, it is ready for reverse transcription and qPCR. When working with RNA, all the special necessary precautions were taken.

3.7.10 Sample storage

All DNA samples, reagents and enzymes were stored in freezers at -20 ºC. RNA was stored at -80 ºC and bacterial strains were stored as frozen stocks, at -80 ºC, containing 15% glycerol.

3.8 Transformation

3.8.1 *E. coli*

E. coli DH5α cells were transformed using the heat-shock transformation method. This technique is performed once cells are chemically made competent (permeable to foreign DNA), by keeping the cells and plasmids on ice for a period of time, and briefly heating the mixture. The rapid change of temperatures causes the disruption of the membrane, hence allowing the entrance of the exogenous plasmids into the bacterial cells (Panja *et al*., 2008).

3.8.1.1 Competent cell preparation

For the preparation of competent cells, a colony of *DH5α* cells was inoculated in a 50 mL falcon tube containing approximately 5 mL of LB broth for overnight culture (negative control for LB contamination was incubated parallelly). 24 hours later, 0.5 mL of overnight culture was inoculated in 12.25 mL of fresh LB broth. Cells were grown at 37 ºC, shaking at 220 rpm until the OD600 (absorbance measured in a spectrophotometer, at a wavelength of 600nm) was between 0.4-0.5 (generally, it took around 2 hours). It is important to mention that, from now on, all steps were carried on ice. Cells were centrifuged at 3750 rpm for 10 min at 4 ºC, and the pellet was resuspended in 6.25 mL ice-cold 0.1 M Calcium chloride. After a 30 min incubation on ice, cells were centrifuged again (3750 rpm, 10 min at 4 ºC) and the pellet was resuspended gently in 200 µL of 0.1 M Calcium Chloride.

3.8.1.2 Heat-shock transformation

100 µL of the competent *DH5α* cell solution were mixed with 5µL of plasmid (2-10 ng/µL) and incubated in ice for 30 min. The other 100 µL were used as a negative control (no plasmidic DNA was added). The heat shock was carried out by incubating the tubes at 42 ºC for 90 seconds. After this, very quickly, 1 mL of fresh LB broth was added to both tubes, and then they were left shaking at 220 rpm for 40 min (37 ºC). Finally, transformed cells (and the negative control: transformation carried out without plasmid) were plated for overnight culture in LB plates with ampicillin (100 µg/mL) or kanamycin (50 µg/mL), used for selection purposes. The plated volume was 100 µL and glass beads were used for a more uniform spreading of the bacteria throughout the plate. Generally, the rest of tube's volume was centrifuged; the pellet was resuspended in 100 µL of fresh media, and finally plated in order to increase the probability of obtaining transformant colonies, just in case the transformation was not very efficient.

3.8.2 *E. faecium*

E. faecium cells were transformed using the electroporation transformation method. This technique also requires the bacterial cells to be made competent beforehand. The basic principle of this method is the brief exposure of the plasmid-cell mixture to a large electric pulse, which temporarily disturbs the cell membrane so that plasmid DNA molecules can pass into the cell. The transformation of *E. faecium* was achieved by following the protocol described previously by (Li *et* al. 1995, Arias and Murray, 2012), with a slight modification.

3.8.2.1 Competent cell preparation

For the preparation of the competent cells, *E. faecium* had to be grown in BYGT medium supplemented with a glycine concentration which led to optimal growth inhibition, for it has been found to help weaken the cell wall, hence enabling higher efficiency

transformations. *E. faecium* was grown overnight in BYGT supplemented with 4.8 % glycine. The overnight culture was diluted 10 fold in fresh BYGT with the same concentration of glycine (30 mL total) and incubated at 37 ºC for 1 hour. Afterwards, cells were chilled on ice, harvested by centrifugation (3750 rpm, 15 min at 4 ºC), and washed twice with chilled electroporation solution (sucrose-MgCl2), using ⅓ of the original volume (10 mL)). Finally, washed cells were resuspended in 500 µL of the sucrose-MgCl2 solution and then incubated on ice for 45 min.

3.8.2.2 Electroporation transformation

50 µL of the competent cells were mixed with 2 µg of purified plasmid DNA. The rest of the volume, up to 100 µL was filled up with more sucrose-MgCl2. Immediately after mixing, the solution was introduced in a clean chilled 0.2-cm cuvette and electroporated immediately with a Bio-Rad "Gene Pulser" apparatus at a capacitance of 25 mF, resistance of 200 V, and peak voltage of 2.5 kV (field strength of 8,750 to 10,000 V/cm). Cells were then incubated in 1 mL of BYGT-Sucrose solution for 2 hours, at 30 ºC (instead of 37 ºC due to the presence of the temperature sensitive plasmid inside the cells). Finally, tubes were centrifuged; the pellet was resuspended in 100 µL of BYGT-Sucrose solution, and plated with the aid of glass beads on BHI (containing chloramphenicol µg/mL) for overnight culture (30 ºC). Note that the electroporation process was also carried out without plasmidic DNA in order to have a negative control of the transformation process.

3.9 Sequencing and sequence analysis

Sequencing to verify the integrity of the FbFP gene insert cloned into the pBT2 plasmid was a necessary step. The Sanger Sequencing method was chosen for this purpose since it is the primarily used technology for small-scale sequencing operations and it allows especially long sequence reads (larger than 500 bp), an important characteristic taking into account the length of the FbFP insert (489 bp).The sequencing reaction was carried out with the BigDye Terminator V3.1 kit (Applied Biosystems) and resolved with a capillary sequencer from Sequencing Department of the University of Valencia.

The BigDye Terminator V3.1 Cycle Sequencing Kit provides the required reagent components for the sequencing reaction in a ready reaction, pre-mixed format. Reagents and volumes used to carry out the sequencing reaction can be seen on **Table 7**.

Table 7. Reagents and volumes used for Sanger sequencing reactions.

Reagents	Volume (µL)
Deionized water	3
BigDye® Terminator 5X Sequencing Buffer	1.6
Primer (0.5mM)*	1
BigDye® Terminator	0.4
DNA sample	2
Final reaction volume	8

Note*: Each reaction was performed either with the forward or reverse primer, separately.

A thermal cycler (Techne TC-4000) was used in order to carry out the sequencing reaction. The program used can be seen on the following table (**Table 8**).

Table 8. Sanger sequencing thermal cycler program.

STEP	Temperature (ºC)	Time	Cycles
Denaturation	95	30 sec	99
Annealing	56	5 sec	99
Final elongation	60	4 min	99
Hold	8		

Chromatograms were analyzed with "4peaks" (program that enables visualization and editing of sequence trace files). Poor-quality sequenced regions were eliminated with this program and sequence integrity was confirmed by performing a BLAST on NCBI with the original FbFP gene sequence.

3.10 Fluorescence microscopy

For the microscopy slides, bacterial colonies grown under the different conditions required (30ºC and 37 ºC, both under the presence and absence of molecular oxygen) were resuspended in 10 µL phosphate buffer saline (PBS) [4.3 mM dibasic sodium phosphate, 1.47 mM monobasic potassium phosphate, 137 mM sodium chloride, 2.7 mM potassium chloride, pH 7.4]. A drop of this suspension was added to each slide, spread by dragging another slide along the first, and subsequently allowed to air-dry. The coverslips were mounted with 25 µL of glycerol (87%) once the slides were dry.

All slides were analyzed with a Leica DM RXA2 Microscope and photographs were taken at 1000x magnification. CFP (Cyan Fluorescent Protein) filter was employed for fluorescent microscopy analysis: laser illumination aimed for maximal light absorption spectrum of FbFP (449 nm) and fluorescent emission was detected at 450 nm. Pictures of the same field views were also taken using "Nomarski" or Differential Interference Contrast (DIC) microscopy. In fluorescent microscopy images the background illumination was corrected by setting a common detection threshold with "ImageJ", a public domain, Java-based program for scientific image processing.

4. RESULTS AND DISCUSSION

4.1 Overview of the strategy used for the generation of a *VRE faecium C68* expressing FbFP

In order to fulfill the main objective of this work, a general workflow was designed (**Fig. 4**). The first step consisted in synthesizing the fluorescent gene (FbFP), followed by a gene-amplification step, which preceded the subsequent cloning steps into the pBT2 plasmid. The next step consisted in the transformation of *E. coli DH5α* with the resultant plasmid in order to obtain a large number of copies, as well as for confirming the presence and integrity of the plasmid construction. Finally, transformation of E. *faecium C68* with the validated construction was carried out, with the objective of performing a fluorescence analysis, as well as for attempting an in vivo FbFP gene-expression assay.

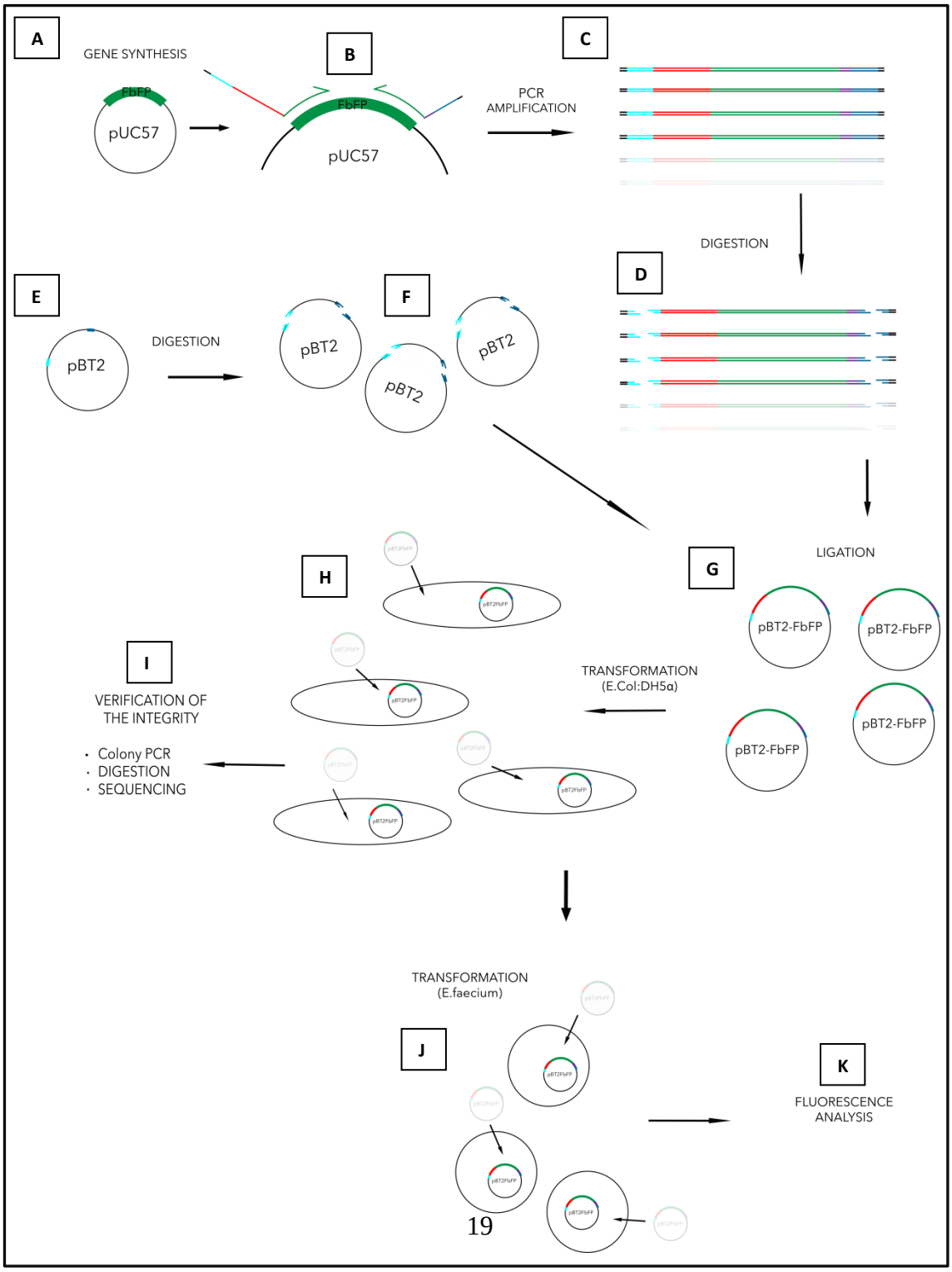

Figure 4. General workflow of the main experimental process followed in this work. (A) Gene synthesis (pUC57 plasmid). (B) Design of the insert-specific primers (C) PCR amplification of FbFP and addition of the required features for correct expression in *E .faecium* and for its appropriate integration in the pBT2 plasmid.(D) Double digestion (BamHI/SalI) of the PCR products. (E) pBT2 plasmid extraction. (F) pBT2 double-digestion (BamHI/SalI)(G)Ligation of the digested FbFP and pBT2. (H) Transformation of the pBT2-FbFP plasmid construction in *DH5α*. (I) Verification of the integrity of the plasmid construction through colony PCR, double digestion (BamHI/SalI), and insert sequencing. (J) Transformation of the validated pBT2-FbFP in *E. faecium*. (K) Fluorescent analysis of the *E. faecium* transformants.

4.2 FbFP Gene synthesis

4.2.1 pUC57 plasmid

The 411 bp gene sequence was obtained from the EcFbFP sequence that had been previously designed by (Drepper *et al.,* 2007). However, the DNA sequence used in the article was adjusted to the *E. coli* codon usage bias: phenomenon where specific codons are used more often than other synonymous codons during gene translation (Behura and Severson, 2013). It is thought that optimal codons help to achieve faster translation rates and higher accuracy, so the adjustment to Enterococci codon usage bias was carried out. This was achieved by using the codon-adaptation tool provided by (http://www.jcat.de/). Alignment of these two differently codon-biased sequences shows that their sequence differs significantly (**Fig. Annex 1**). However, although they are different nucleotide sequences, they code for the exact same amino acid sequence (**Fig. Annex 2**).

The modified sequence was sent to Genscript USA Inc. which synthesized the gene and cloned it into the 2710 bp pUC57 vector (**Fig. 3**). Upon arrival, pUC57 plasmid was transformed into *E. coli DH5α* cells. Transformed cells were of great interest at this point in time to generate large copy numbers for the extraction of as much insert-bearing plasmids as possible in order to carry out the following steps, and to simply act as containers of the plasmid for stock purposes. It is important to highlight that pUC57 plasmid only contains an origin of replication compatible with *E. coli,* hence the gene had to be cloned into a plasmid which could replicate in both *E. coli a*nd *Enterococcus*: pBT2.

Now, the synthesis of a functional FbFP gene was not yet complete, two more factors had to be taken into account: the addition of a stop codon (nucleotide triplet at the end of the sequence to signal the termination of translation) and the addition of an appropriate promoter (DNA sequence that defines where transcription of a gene by the RNA polymerase starts) at the beginning of the sequence. Both features were designed to be introduced to the original sequence upon PCR amplification of the gene, by designing adequate primers bearing the promoter and stop codon.

4.2.2 Oligonucleotide primer design and PCR amplification of the FbFP

As mentioned in the previous section, the promoter of the gene and the stop codon were designed to be introduced upon amplification of the FbFP gene. To make this possible, the primers for PCR amplification were designed to include these sequences, in addition to the appropriate restriction enzyme recognition sites which would allow its cloning into the pBT2 plasmid (**Fig. 9**). The stop codon (TAA) was designed to be located adjacent to the sequence of the reverse primer which is complementary to the final base pairs of the gene sequence. The promoter was designed to be located adjacent to the sequence of the forward primer which is complementary to the first base pairs of the gene sequence. The promoter sequence is a constitutive promoter of the bacA gene of *E. faecalis* which had been used previously to

overexpress Enterococcal proteins (Toledo-Arana *et al.,* 2001). It is of great importance to also mention that BamHI and SalI restriction restriction sites were included in the forward and reverse primers respectively. These sites were adjacent to the beginning of the promoter (BamHI) and behind the stop codon (SalI), and were designed to clone the FbFP gene into the pBT2 plasmid. Note that the 411 bp FbFP gene, upon amplification with these primers becomes 507 pb and when digested with BamHI and SalI: 501bp.

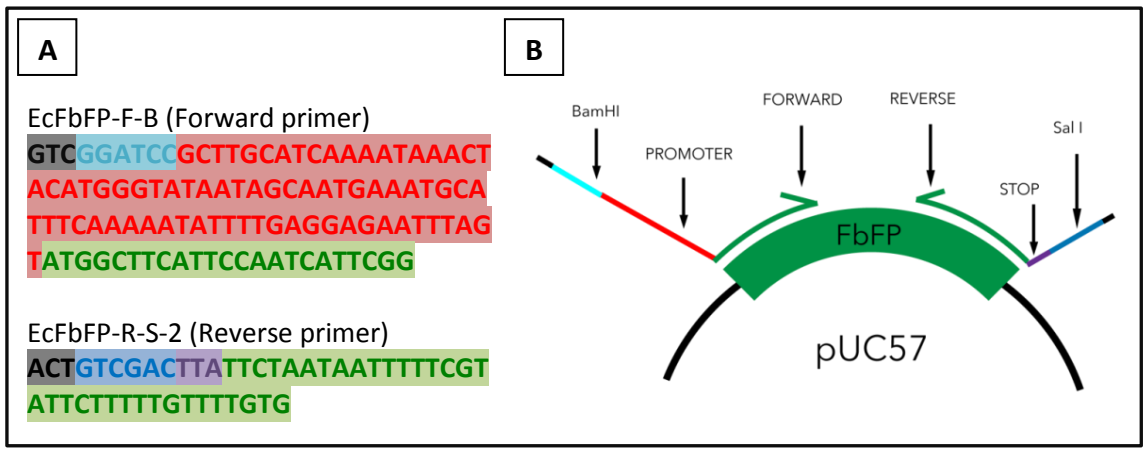

Figure 5. "Insert-specific" oligonucleotide primers designed for PCR amplification of the FbFP insert, which parallely introduce a stop codon, a constitutive promoter and two restriction sites (BamHI and SalI) upon PCR amplification. (A) Color-coded sequences of the forward and reverse primers. The promoter sequence, originally from *E. faecalis bacA* gene, is represented in red. Restriction sites are shown in blue (gtcgac for SalI and ggatcc for BamHI). Stop codon is labeled in purple (note that it is the reverse complementary of TAA). The sequences labeled in green represent the regions of the primers which bind to the extremes of the FbFP gene. It is important to mention that the bases labeled in black (right before the restriction sites) represent extra bases New England Biolabs recommends for optimal restriction enzyme cleavage. **(B) Schematic representation of how the insert-specific primers bind to the pUC57 plasmid PCR amplification.**

A PCR amplification of the FbFP gene was carried out with the primers described above, and using the pUC57 plasmid extracted from one transformed *DH5α* colony as the DNA template. Two 50µL PCR reactions were carried out. A small amount was used to check for amplification through gel electrophoresis (**Fig. 6A**). A band of the expected size (507 bp) was observed, while no band of this size was observed in the negative control. The rest of the reaction volume was run in an agarose gel (**Fig. 6B**), with the purpose of cutting the band and purifying the amplicon. Finally, the purified amplicon was quantified and its concentration was found to be 4.68 ng/µL.

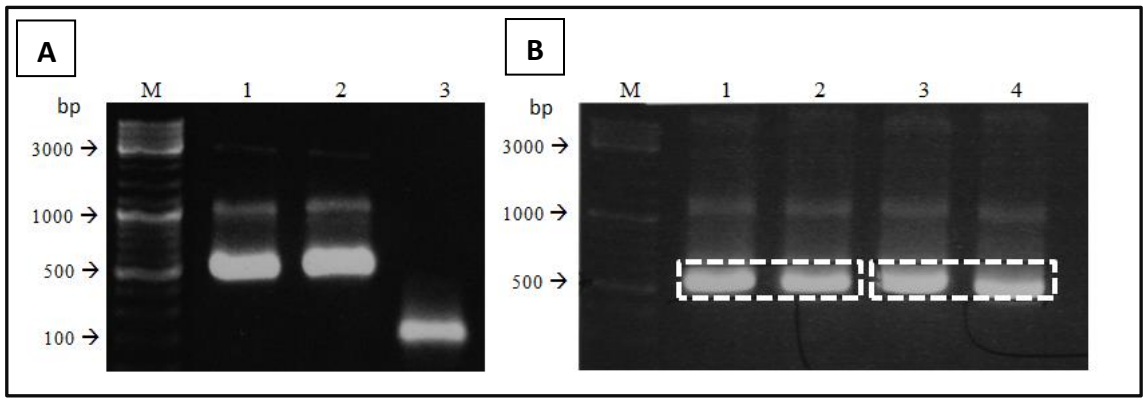

Figure 6. Gel electrophoresis of the PCR amplification (using the insert-specific primers) of the FbFP inserted in pUC57. (A) Gel run with 4 µL of PCR product to check for amplification. Lanes 1 and 2 correspond to two PCR amplifications of two plasmid extractions from one *DH5α* colony. The 3rd lane

corresponds to a PCR negative control (water). The band observed on this lane is probably due to the formation of primer dimers. **(B) Gel electrophoresis with the remaining PCR volume, aimed for band extraction.** The dotted boxes represent the cutting of the bands for subsequent purification. Lanes 1 and 2 correspond to one plasmid extraction and 3 and 4 the other.

4.3 FbFP gene cloning

4.3.1 Digestion and ligation of the pBT2 plasmid and the FbFP amplicon

The amplified and purified FbFP insert, as well as the pBT2 plasmid, had to be double digested with the proper restriction enzymes (SalI and BamHI) (**Fig. 4D, F**) before they could be ligated to yield the resultant pBT2-FbFP plasmid construction (**Fig. 4G**).

Since the pBT2 plasmid (recipient vector) had not yet been extracted, this was carried out from transformed *DH5α* cells, and quantification revealed it was at a concentration of 7.01 ng/µL. Taking into account that the FbFP amplicon was at a concentration of 4.68 ng/µL and pBT2 at a concentration of 7.01 ng/µL, digestion conditions were set to match those described in materials and methods. After digestion, pBT2 and FbFP were purified to get rid of the restriction enzymes and the remainder cut off fragments. Subsequent quantification revealed that the digested and purified pBT2 and FbFP were at a concentration of 10.78 ng/µL and 3.143 ng/µL respectively.

The DNA concentrations of the insert and vector, as well as their size ratio, were used to calculate the volume of each required for an optimal ligation reaction, using a 1:4 (vector/insert ratio).

Calculations:

DNA quantification results:
- pBT2: 10.78ng/µL
-FbFP: 3.143 ng/µL

Using the formula described in materials and methods, the ng of plasmid and insert required for the ligation reaction were calculated the following way:

$$\frac{100\ \text{ng} \times \mp 500\ \text{bp}}{\mp 7000\ \text{bp}} \times \text{molar ratio of } \frac{4}{1} = 34.1\ \text{ng FbFP}$$

Once the nanograms of vector and insert required for the reaction were calculated using the formula, and having quantified the DNA concentrations (of the purified digestions) previously, the volume required for the ligation reaction was calculated as follows:

-Vector: (100 ng) / (10.78 ng/µL) = 9.27 µL
-Insert: (34.1ng) /(3.143 ng/µL) = 10.84 µL

Table 9. Reagents and volume used for the DNA ligation reaction.

	Ligation reaction	Ligation Control (without insert)
Reagents	**Volume (µL)**	**Volume (µL)**
H20 milli Q	1.39	12.23
pBT2 plasmid (100 ng)	9.27	9.27
FbFP gene insert	10.84	0
Buffer (10x)	2.5	2.5
Ligase enzyme	1	1
Final Volume	25	25

4.3.2 Transformation of *DH5α* with the ligation product

The ligation product was transformed into *DH5α* cells to verify the presence and integrity of the desired plasmid construction and for its propagation (**Fig. 4H**). This was done as a previous step to the transformation of *E. faecium C68*. At first glance, it may seem as if validating the plasmid construction in *DH5α*, rather than directly doing it on the bacterial strain of interest (E. *faecium C68*) is a waste of time. However, the reason why *E. faecium C68* was not chosen is due to the fact that *E. coli DH5α* is an ideal strain for routine cloning procedures, specially designed to enable highly efficient transformations, a very convenient trait when trying to transform a ligation product, since these are commonly very inefficient. By using *DH5α,* several tedious and inefficient limiting steps can be bypassed.

4.3.3 Verification of the correct insertion of FbFP in pBT2

4.3.3.1 Colony PCR screening

After transformation of *DH5α* cells, the colony-PCR technique was utilized as a preliminary screening method for determining which transformed colonies indeed had incorporated the desired plasmid construction. In order to perform the colony PCR, a different pair of primers was used to amplify the FbFP gene. These primers were designed to bind to the flanking regions of the site where FbFP was supposedly cloned into (**Fig. 7**). These vector-based primers were used as a precaution method to avoid the obtention of false positive colony-PCR bands that could occur if the insert-specific primers (primers used for the FbFP amplification step) were to be used. This false positive phenomenon could happen, for example, if non-inserted FbFP molecules were carried along with the ligation volume used for the transformation, and were plated onto the culture media along with the transformant cells. As the number of FbFP molecules that could be present on the plate is very large, and only one molecule could give rise to a false positive, chances are that when picking a colony, non-inserted FbFP molecules could be accidentally picked up into the PCR mixture at the same time, potentially leading to the observation of false positives. Therefore, the best way of making sure FbFP had been inserted correctly into pBT2 was to use insert-flanking, vector-based primers.

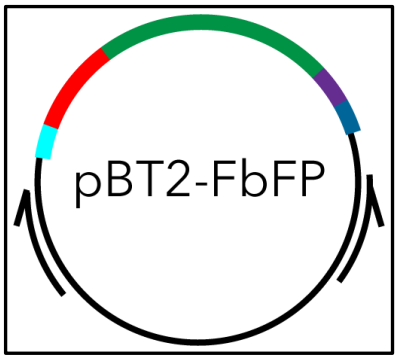

Figure 7. Schematic representation of the manner in which the "vector-based", "insert-flanking" primers bind to the pBT2-FbFP plasmid for PCR amplification.

To carry out the colony-PCR, 9 colonies that had grown overnight in the selective media were picked and amplified using the vector-based, insert-flanking primers. An agarose gel was run with the colony-PCR products (**Fig. 8**). The expected fragment size is about 1000 bp, since the insert is roughly 500 bp and the sum of the amplifiable parts of the plasmid is also about 500 bp).

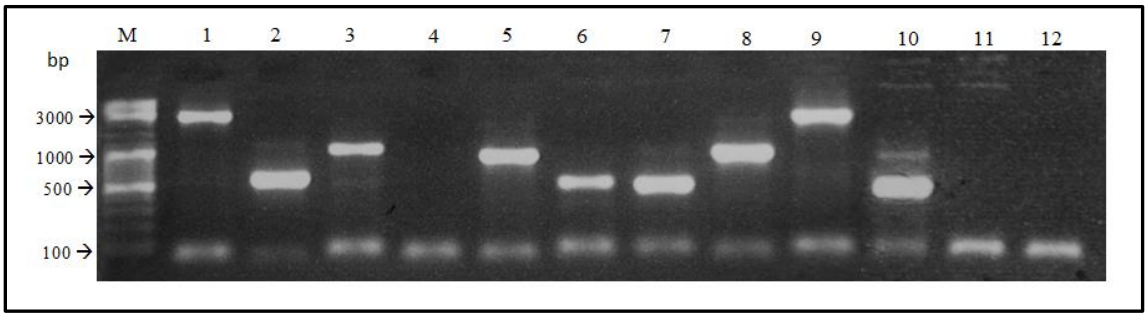

Figure 8. Colony PCR results. Lanes 1-9 show the colony PCR products for the 9 colonies that were picked. Lane 10 shows the PCR product of the pBT2 plasmid. Since it has no FbFP insert, the amplified region is the sum of the amplifiable flanking regions (roughly 500 bp); it acts as the positive control. Lane 11 shows the PCR product of the pUC57 plasmid in which primers cannot bind, hence acting as a negative control. A negative control for the PCR reaction is shown on lane 12 (water). The band triplets observed on the upper part of lanes 10 and 11 shows the 3 different plasmid migration types.

According to the molecular marker, the bands which correspond to this size are those pertaining to colonies 3, 5 and 8. Additionally, by analyzing the gel, one can easily see that colonies 2, 6 and 7 were transformed with a plasmid which did not contain the insert, for the amplified fragment corresponds to the sum of the vector's amplifiable flanking regions (about 500 bp), a band size also displayed by the negative control (pBT2 without insert, tenth lane). This could most probably be the result of a poor digestion: either, neither BamHI nor SalI digested pBT2, or alternatively, only one of them was able to digest the plasmid, which subsequently re-ligated (the double-digestion scenario followed by the plasmid's re-ligation is not contemplated, for BamHI and SalI produce distinct cohesive ends). Finally, colonies 1 and 9 show bands which correspond to excessively large sized fragments, most likely ligation artifacts. It is important to note that the bands observed at the lower part of the gel in all lanes are PCR by-products: primer dimers.

4.3.3.2 Restriction enzyme double-digestion

Once colony-PCR had pinpointed colonies 3, 5 and 8 as the major candidates for containing the desired plasmid construction, plasmid was extracted from each of these colonies with the intention of doing a restriction enzyme double-digestion with BamHI and SalI. Restriction enzyme digestions give more accurate results and serve as a double-check to faithfully determine if the colonies indeed carry the recombinant pBT2-FbFP plasmid. **Figure 9** very neatly displays how all three colonies (3, 5 and 8) undoubtedly possess the FbFP insert, since a band corresponding to its size (about 500 pb) is observed in all gel lanes except the negative control (pBT2 plasmid without insert). Additionally, an intense band corresponding to the plasmid's expected size can be seen in the upper part of all gel lanes (negative control included).

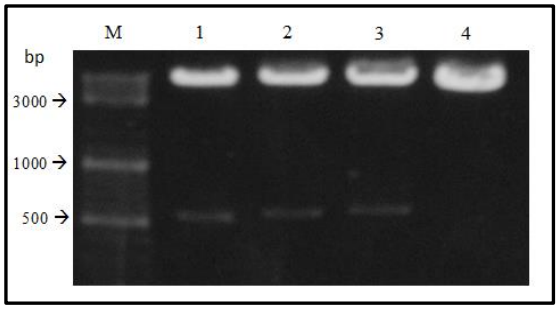

Figure 9. Gel electrophoresis displaying the digestion products of the pBT2-FbFP construct. Lanes 1, 2 and 3 correspond to digestions of the plasmids extracted from colonies 3, 5 and 8 (**Fig. 8**). Lane 4 is a negative control: pBT2 plasmid (without FbFP). The FbFP insert (about 500 bp) is observed in all three colonies.

4.3.3.3 pBT2-FbFP insert sequencing

An important step during the cloning of a gene into a plasmid is the verification of the inserted sequence through its DNA sequencing, a recommended step before advancing to any further operations. Since the FbFP sequence had undergone many steps of amplification and a great deal of handling, the possibility that it may have had acquired mutations or suffered any kind of modification throughout the process was not discarded. For this reason, pBT2-FbFP plasmid was extracted from colony 3 (see previous section) for sequencing analysis.

Sequencing was achieved by means of the Sanger Sequencing method (see materials and methods). Sanger sequencing was carried out with the reverse insert-specific primer: EcFbFP-R-S-2 (**Fig. 10A**), and with the forward vector-based primer: T1-1R (**Fig. 10B**) Surprisingly, Sanger sequencing performed with the reverse and forward primers both revealed a point mutation in the FbFP gene, where an Adenine nucleotide was exchanged for a Guanine (**Fig. 10A, B**). Alignment of the mutated sequence of FbFP and the original FbFP sequence is shown reveals a mismatch (**Fig. Annex 3**).These types of substitution mutations are known as transition mutations, and, in contrast to transversion mutations, are much more frequent and involve interchanges of similarly-shaped bases: interchanges of two-ringed purines (A / G) or of two-ringed pyrimidines (C /T). Fortunately, this was also a silent mutation, for it did not result in a change in the amino acid sequence of FbFP; the affected codon was changed to a synonymous one. Alignment of the mutated and original FbFP translated sequences demonstrates amino acid sequence identity (**Fig. Annex 4**).

A **Forward (T1-1R)**

TTTTATTTGATGCCTCAAGCTAGAGAGTCATTACCCCAGGCGTTTAAGGGCACCAATAACTGCCTTAAAAAAATTACGCCCCGCCCTGCCACTCAT
CGCAGTGCAGCGGAATTCGAGCTCGGTACCCGGGGATCCGCTTGCATCAAAATAAACTACATGGGTATAATAGCAATGAAATGCATTTCAAAAA
TATTTTGAGGAGAATTTAGTATGGCTTCATTCCAATCATTCGGTATCCCAGGTCAATTAGAAGTTATCAAAAAAGCTTTAGATCACGTTCGTGTTG
GTGTTGTTATCACAGATCCAGCTTTAGAAGATAACCCG* ATCGTTTACGTTAACCAAGGTTTCGTTCAAATGACAGGTTACGAAACAGAAGAAA
TCTTAGGTAAAAACGCTCGTTTCTTACAAGGTAAACACACAGATCCAGCTGAAGTTGATAACATCCGTACAGCTTTACAAAACAAAGAACCAGTT
ACAGTTCAAATCCAAAACTACAAAAAAGATGGTACAATGTTCTGGAACGAATTAAACATCGATCCAATGGAAATCGAAGATAAAACATACTTCGT
TGGTATCCAAAACGATATCACAAAACAAAAAGAATACGAAAAATTATTAGAATAAGTCGACCTGCAGGCATGCAAGCTTTAATGCGGTAGTTTAT
CACAGTTAAATTGCTAACGCAGTCAGGCACCGTGT

B **Reverse (EcFbFP-R-S-2) (reverse complementary)**

AAAATAAACTACATGGGTATAATAGCAATGAAATGCATTTCAAAAATATTTTGAGGAGAATTTAGTATGGCTTCATTCCAATCATTCGGTATCCCA
GGTCAATTAGAAGTTATCAAAAAAGCTTTAGATCACGTTCGTGTTGGTGTTGTTATCACAGATCCAGCTTTAGAAGATAACCCG* ATCGTTTACG
TTAACCAAGGTTTCGTTCAAATGACAGGTTACGAAACAGAAGAAATCTTAGGTAAAAACGCTCGTTTCTTACAAGGTAAACACACAGATCCAGCT
GAAGTTGATAACATCCGTACAGCTTTACAAAACAAAGAACCAGTTACAGTTCAAATCCAAAACTACAAAAAAGATGGTACAATGTTCTGGAACGA
ATTAAACATCGATCCAATGGAAATCGAAGATAAAACATACTTCGTGGATCCAAAA

Figure 10. Sanger sequencing reads for colony 3: Forward and reverse. (**A**) Sequence output obtained by sequencing the insert using the forward "vector-based" primer. (**B**) Reverse complementary sequence of the sequence output obtained by sequencing the insert using the reverse "insert-specific" primer. (**A,B**) Color code: Orange = pBT2 plasmid; Purple = stop codon; Green = Insert; Red = bacA promoter. The A→G mutation has been highlighted in yellow and marked with an asterisk.

4.3.4 Transformation of *E. faecium*

4.3.4.1 Optimization of the Enterococci transformation protocol

Transformations in *E. faecalis* have been well described in the past by (Li *et* al. 1995, Arias and Murray, 2012) in which growth media is supplemented with glycine (6%), for it has been observed that, although having an inhibitory effect on bacterial growth, it enables greater transformation efficiencies, probably through the debilitation of the cell wall. Even though previous studies with E. *faecalis* had shown a 70 to 90 % reduction in optical density at OD600 (the so called "optimal growth inhibition") when supplementing with 6 % glycine, *E. faecium* did not tolerate such high amounts. Consequently, experiments to test different glycine concentrations were carried out with the purpose of determining a suitable glycine concentration that would enable efficient *E. faecium* transformations.

First, an experiment testing a series of glycine concentrations was designed. In this experiment, *E. faecium* was grown overnight in an array of BYGT cultures, supplemented with different glycine percentages (0, 0.375, 0.75, 1.5, 3 and 6) (**Table 10**). Growth inhibition was assessed by comparing the absorbance of each overnight culture with the absorbance of a culture containing no glycine. It is important to mention that since the darkness of the media increased with glycine addition, absorbance blanks containing the same glycine percentage as the samples were performed before each sample measure. This precaution was aimed to avoid the bias the increase in darkness would have produced to the readings.

Table 10. Results of the 1st experiment designed to test the inhibitory effects of an array of glycine %.

Glycine %	Absorbance (OD600)	Growth inhibition (%)
0	1.741	0
0.375	1.732	0.5
0.75	1.556	10.6
1.5	1.389	20.2
3	1.257	27.8
6	0.001	99.94

A sudden drop in the growth was observed between cultures containing 6% and 3% glycine (**Table 11**) Inhibition was still too far away from the optimum growth inhibition described by (Arias and Murray, 2012), so a second experiment was carried out, though this time, testing intermediate glycine percentages: 3, 3.6, 4.2, 4.8, 5.4, and 6%.

Table 11. Results of the 2nd experiment designed to test the inhibitory effects of an array of glycine %.

Glycine %	Absorbance (OD600)	Growth inhibition
0	1.577	0
3	1.504	2.3
3.6	1.557	1.3
4.2	1.508	4.4
4.8	0.575	63.5
5.4	0.021	98.7
6	0.008	99.5

Again, a sudden growth drop was observed, this time between the cultures supplemented with 4.8 and 5.4 % glycine. As the growth inhibition optimum described previously was close to the inhibition achieved with 4.8% glycine, transformation of *E. faecium* with this concentration was attempted.

4.3.4.2 Transformation of *E. faecium C68* with pBT2-FbFP

Transformation of *E. faecium* was performed as described in materials and methods (**Fig. 4J**). Two putative transformants (colonies grown on the selective media plates) were grown for plasmid extraction. A gel was run with extracted plasmid from two colonies (**Fig. 11**). As it can be observed, the DNA appears degraded, as a light smear. This is not suitable for a restriction enzyme digestion analysis, for it would not yield results. It could be possible that genomic DNA was sheared, and thus could not be isolated well from the plasmidic DNA. Parallelly, nucleases from Enterococci could have partially degraded the plasmid, and so an indirect method to find out if *E. faecium* is really a transformant would be to re-transform *DH5α* with the poorly-extracted pBT2-FbFP plasmid. This would be an interesting technique since *DH5α* is an endonuclease free strain: mutation "endA" in the Endonuclease I eliminates the non-specific endonuclease activity, allowing for more efficient plasmid extractions. Obtention of *DH5α* transformants would enable the verification of the integrity of the plasmid since the strain has proven to yield highly enriched plasmid extractions.

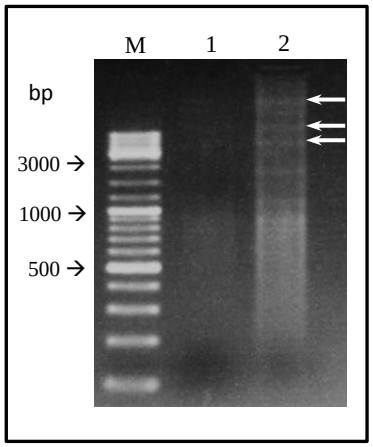

Figure 11. Agarose gel run with the plasmid extractions of two putative *E. faecium* transformants. Lanes 1 and 2 correspond to extractions from two different colonies. Lane 1 shows a very unsuccessful extraction, a very light smear is observed. Lane 2 is similar, but much more intense. It is possible that in this lane, the different conformation migrations of the plasmid can be observed (Arrows).

4.3.4.3 Indirect confirmation of the transformation of *E. faecium* with pBT2-FbFP

As discussed in the previous section, transformation of *E. coli DH5α* with the presumptive pBT2-FbFP plasmid (extracted from hypothetical *E. faecium* transformants) was carried out in order to indirectly determine whether *E. faecium* had successfully undergone transformation or not. Transformation procedure was executed as described in materials and methods.

After transformation, PCR amplification of the FbFP insert was performed with the vector-based, insert-flanking primers, using extracted plasmid as the PCR template. Gel electrophoresis for the PCR products (**Fig. 12A**) revealed bands which correspond to the expected amplicon size (roughly 1000 bp); thereby indirectly demonstrating that *E. faecium* had indeed been successfully transformed with the pBT2-FbFP vector.

An additional experiment was carried out in order to double check the results exposed by the previous gel. The experiment consisted in a double digestion with BamHI and SalI (restriction enzymes used for cloning FbFP into pBT2) of the extracted plasmid. The results

from this experiment confirmed the previous findings, for the gel that was run with the digestion products (**Fig. 12B**) displayed bands which correspond to the FbFP expected insert size (around 500 bp) and no band on the negative control. Note that the upper band seen for all lanes corresponds to the plasmid. As a result, it can be said that DH5a cells were transformed with the pBT2-FbFP plasmid which was extracted from the E. faecium, and therefore, although obtained indirectly, there is sufficient evidence as to confirm the successful transformation of the latter.

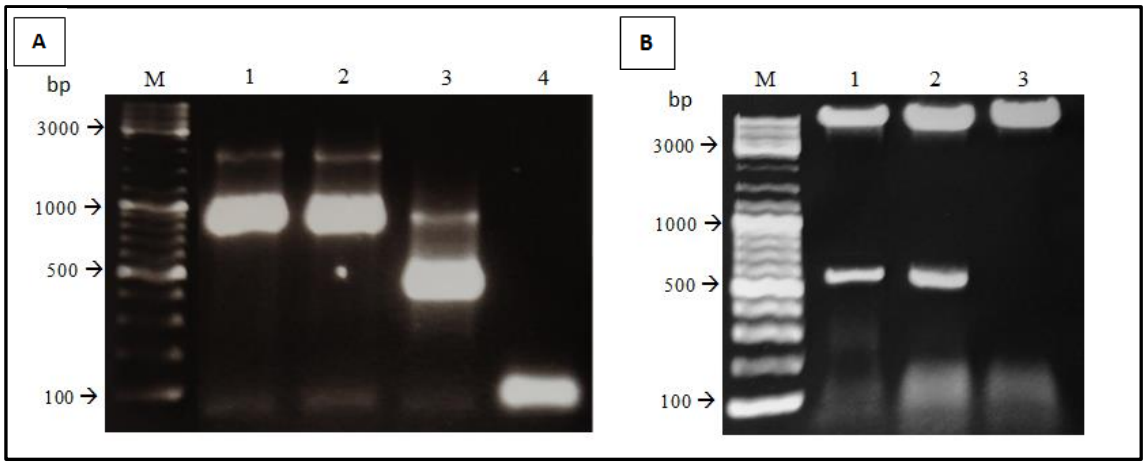

Figure 12. (A) Gel results for the PCR amplification of the FbFP insert performed with the vector-based, insert-flanking primers, using extracted plasmid (from *DH5a*) as the PCR template. Lanes 1 and 2 corresponf to two extractions (2 colonies) of pBT2-FbFP. Lane 3 represents a positive control for the amplification (pBT2 without FbFP insert) and lane 4 probably shows the formation of primer dimers on the negative control for the PCR reaction(water). (**B**) **Agarose gel showcasing the results of double-digestion reaction (BamHI and SalI) of the extracted pBT2-FbFP.** Lanes 1 and two represent the two extractions (FbFP insert is present) and lane 3 a digestion of the pBT2 plasmid without insert (negative control). Upper bands reflect the presence of the plasmids.

4.5 Analysis of fluorescence

Once the transformation of *E. faecium* with pBT2-FbFP was confirmed, visualization of the bacteria through a fluorescent microscope was of great importance, as this technique had the potential to confirm whether or not the main objective of this work had been achieved (**Fig. 4K**). In order to carry out this task, an experiment was carefully designed to mimic the in-vivo niche conditions of this bacterium and meet all other experimentally relevant demands.

In the first place, since the use of the intended fluorescent *E. faecium C68* is principally aimed for its use in anaerobic environments (such as the GI tracts of animals), the experiment was designed in such a way as to, not only study the fluorescent properties of FbFP under aerobic conditions, but very importantly, also under anaerobic conditions. This experimental design parallelly allowed the confirmation of previous studies which claimed that FbFP, in contrast to GFP-like proteins, is equally fluorescent in the presence and absence of molecular oxygen (Drepper *et* al. 2007).

Secondly, it is important to mention that up until this point in time, all culturing steps which involved the transformed *E. faecium* had been carried out at 30 ºC as a precautionary measure, since pBT2 is a temperature-sensitive plasmid and temperatures close to 42 ºC would prompt its loss. However, since the temperatures of the GI tracts of the animal hosts of this bacterium are usually around 37 ºC, avoiding to carry out the experiments at this temperature, whilst continuing to perform them at 30 ºC, would constitute a failure in the

experimental design. Therefore, both aerobic and anaerobic cultures were cultured at both 30 ºC and 37 ºC, as it enabled to determine if FbFP is expressed similarly and can be folded equally well at both temperatures. Moreover, this design also provides the means to experimentally determine whether the plasmid is lost at 37 ºC or not.

As part of a proper fluorescence experimental design, a positive control which is fluorescent under the same conditions as those to which the samples are subjected is always required. To serve this purpose, *E. coli DH5a* was transformed with a plasmid containing the EcFbFP gene which would confer it with florescent properties: pGLOW-KXN-Bs2 plasmid (look at materials and methods for more information).

The presence of the plasmid containing the gene was confirmed by performing a PCR amplification of an internal region of the EcFbFP gene, using extracted plasmid as the PCR template (**Fig. 13**) It is important to draw attention to the fact that the positive control was cultured under all the different conditions *E. faecium* was subjected to (37 ºC and 30ºC, both under anaerobic and aerobic conditions). Negative controls for both *E. coli* and *E. faecium* transformants were also designed. These consisted in both of the bacterial species without undergoing transformation. However, these were only tested under one condition (37 ºC; aerobic), since they did not contain a plasmid with the FbFP gene and were merely used to check that the strains did not have already fluorescent properties, as well as for comparison with fluorescence intensities obtained by the transformants.

As an additional control which would indicate the presence of bacteria (even if they did not display fluorescence), microscopic photographs of all conditions were taken using DIC (Differential Interference Contrast; Nomarski) microscopy, in addition to the pictures (of the same field of view) taken through fluorescent microscopy. Fluorescence and DIC microscopy analysis was carried out as described in materials and methods.

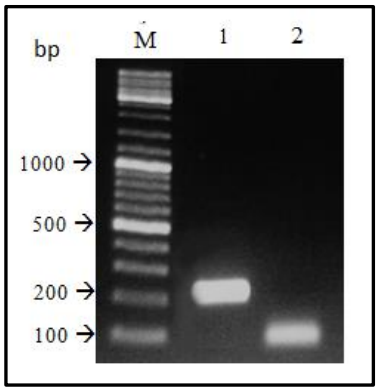

Figure 13. Confirmation of the Transformation of *DH5a* cells with the commercial plasmid. Lane 1 corresponds to the PCR product of the amplification of an internal region of the EcFbFP insert using extracted plasmid as template. An amplicon corresponding to the expected size (about 200 bp) confirms the presence of the gene. Lane 2 represents the negative control for the PCR (water)

Since no significantly relevant differences were observed when comparing the fluorescent results of the different sets of conditions (temperature and oxygen presence), only the results which correspond to the anaerobe experiment carried out at 37 ºC (closest in-vitro conditions to the in-vivo gut environment) are displayed below (**Fig. 14**). However, the rest of the images exhibiting the remaining conditions tested can be observed on **Fig. Annex 5.** The fact that all conditions display the same general result indicates that conclusions obtained from just analyzing the condition shown in the figure below can be extrapolated to the rest of conditions, since neither the temperature range used or the presence/absence of oxygen appear to have a differential effect on the expression and function of the fluorescent protein.

It is important to mention that as the signals of interest appeared very dim, long camera exposures were applied to enhance image background contrast. However, this was problematic, as it gave rise to the detection of certain autofluorescence which interfered with

the detection of the bacterial fluorescence of interest. Although very dull, some degree of autofluorescence signals can be observed on the negative controls (**Fig. 14 B, D**).

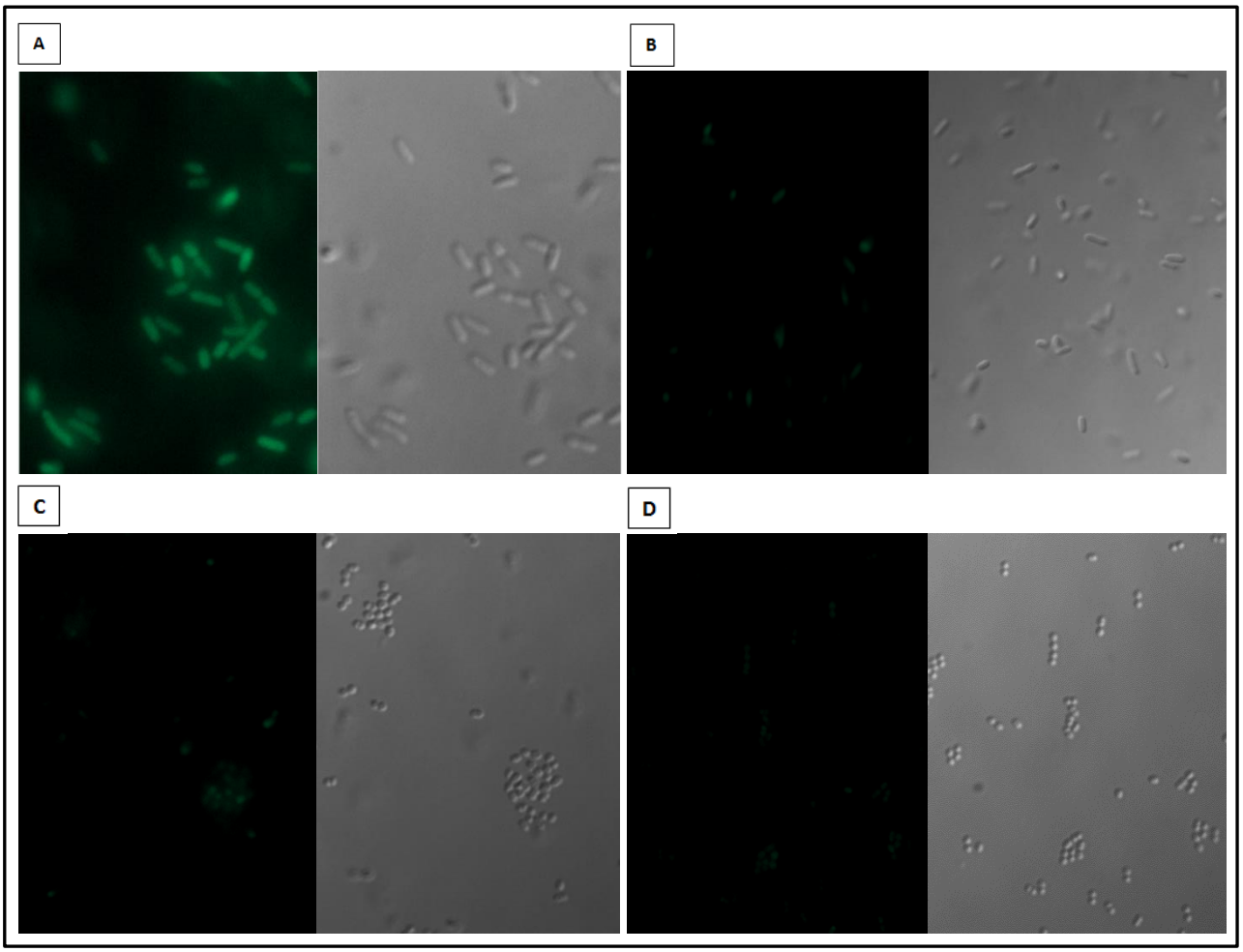

Figure 14. Fluorescence CFP (Cyan Fluorescen Protein) filter microscopy and Differential Interference Contrast; Nomarski) microscopy photographs (1000x) taken with the Leica DM RXA2 Microscope. In each panel (**A,B,C,D**), pictures on the left were taken with the CFP filter and the pictures on the right with DIC. (**A**) ***DH5α* cultured at 37ºC under anaerobic conditions, transformed with the pGLOW-KXN-Bs2 (positive control).** Bacteria show fluorescence when compared to its negative control (**B**)**: *DH5α* cells without the plasmid**. (**C**) ***E. faecium* cultured at 37ºC under anaerobic conditions, transformed with the pBT2-FbFP plasmid**. Although the intensity of signal is slightly higher that the negative control (**D**), suggesting that the FbFP protein is expressed, though at very low levels compared to the positive control (**A**), the results are not conclusive as to faithfully determine that FbFP is responsible for the signal observed.

Without doubt, the *DH5α* positive control displays fluorescence in comparison to the negative control (**Fig. 14A, B**). However, fluorescence emissions originated by *E. faecium* are not so clear. It is hard to determine if the signals obtained are really the product of FbFP expression or simply very dim false positives resulting from autofluorescent signals. At first glance, signals appear to be more intense than those observed on the negative control, suggesting that the fluorescent protein may be expressed, though at very low levels (**Fig. 14C, D**). Nevertheless, it could also be argued that autofluorescence intensity could appear higher than on the negative control as a consequence of a higher aggregation of bacteria.

It is clear that further studies are required to complement fluorescence microscopy in shedding light upon this ambiguous issue. For instance, Real-Time quantitative PCR analysis of

the expression of FbFP in *E. faecium* would be an interesting approach to find out if the gene is expressed, and if so, how much. Additionally, if the gene was shown to be expressed, a western blot could be carried out to determine if the gene is translated, and if it is the case, at what levels is the protein found inside the cells.

4.6 RNA extractions for q-PCR

As mentioned in the previous section, measurement of FbFP gene transcription would be an interesting approach to complement and possibly clarify the results obtained through fluorescence microscopy, as it would allow to determine that FbFP is expressed. This technique is known as Real Time/Quantitative-PCR (q-PCR), and it requires the previous extraction of the RNA from the cells, as well as its retro-transcription into double stranded cDNA.

It is important to highlight that a certain volume of cells (cultured under the different conditions used for the microscopy experiment explained in the previous section) was used to extract RNA, both from the transformed *E. faecium* and the positive *DH5α* control. RNA extractions were carried out as explained in materials and methods. A gel was run with the extraction products with the purpose of assessing the extraction results (**Fig. 15**). As it can be seen, the bacterial rRNA bands corresponding to 23S (~2,900 bases) and 16S (~1,500 bases) are visible. The blurred appearance of these bands, along with the presence of smears indicates that the RNA may be partly degraded, phenomenon which is especially pronounced for RNA extractions from *DH5α* cells.

As surprising as it may seem at first glance, taking into account that the same procedure was carried out for both bacterial samples, the fact that *DH5α* RNA extractions present a poorer quality may be explained by a simple detail: the type of bacterial cell wall. Since *DH5α* is a Gram-negative bacteria (containing a thin peptidoglycan layer), in contrast to *E. faecium,* which is Gram-positive (thicker peptidoglycan layer), it is quite possible that during the mechanical lysis step of the RNA extraction, the higher rigidity of the Enterococcus cell wall attributed this bacteria a higher resistance to disruption. In turn, *DH5α* may have lysed very early in this mechanical process, hence provoking a premature shearing of its RNA. Adding to this theory, the spherical coco shape of *E. faecium*, in comparison to the rod-like shape of *DH5α* may confer the first an even greater resistance to disruption.

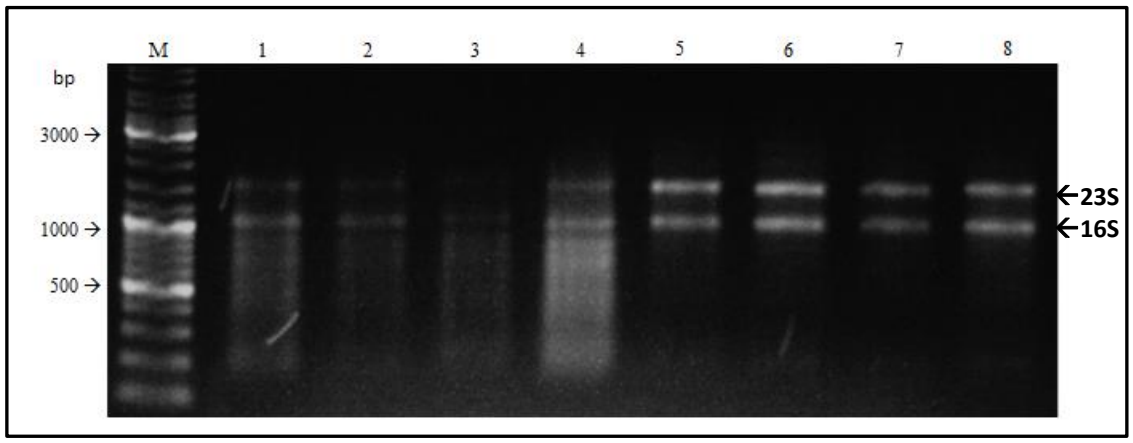

Figure 15. Gel electrophoresis of the total RNA extracted from transformed *DH5α* and *E. faecium* cells. Lanes 1-4 show RNA extracted from Dh5a and lanes 5-8 show RNA extracted from E. faecium. Bands for the 16s rRNA and 23S rRNA are indicated.

To make sure the RNA extraction had successfully isolated RNA from any DNA trace of the FbFP gene (even though the extraction protocol contained a step designed to eliminate DNA traces through DNAse 1 digestion), PCR amplification of an internal region of this gene was performed, using the primers originally designed for the q-PCR. Surprisingly, as displayed by the gel electrophoresis carried out with the PCR products (**Fig. 16**), all sample lanes showed a band corresponding to the amplicon's expected size (roughly 200 bp). This clearly indicated that there were still traces of the FbFP gene, since the Taq polymerase cannot use RNA as a template.

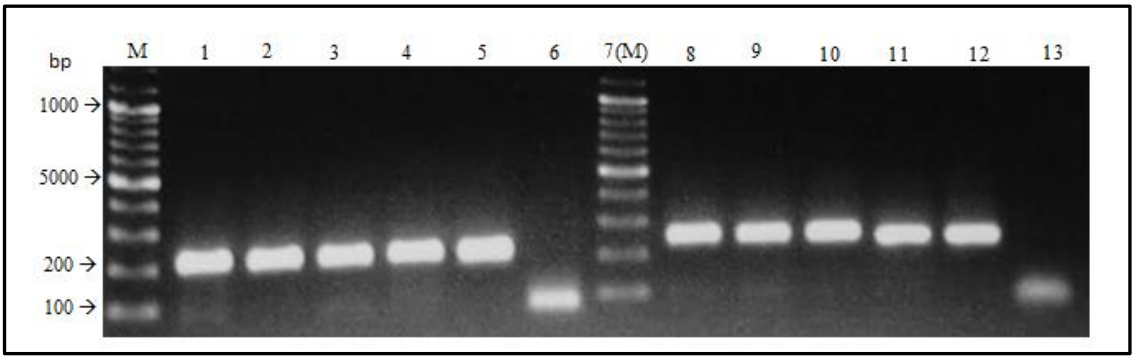

Figure 16. PCR amplification of the total RNA extractions from transformed *DH5α* and *E. faecium*, using primers for the amplification of an internal region of EcFbFP and FbFP respectively. Lanes 1-4 correspond to *DH5α* samples and lanes 8-11 correspond to *E. faecium* samples. Lanes 5 and 12 show the positive controls for PCR amplification, pGLOW-KXN-Bs2 and pBT2-FbFP plasmids respectively. Lanes 6 and 13 show bands corresponding to the primer dimers formed in the negative controls (water). An amplicon corresponding to the expected amplicon size (about 200 bp) is observed for all samples and the positive controls, indicating the contamination of all RNA samples

The quality and purity of RNA samples are critical parameters for reliably obtaining accurate and reproducible gene expression analyses. Performing a q-PCR without eliminating FbFP gene traces would result in false positives, since amplification would be observed for all samples, though it would be impossible to determine whether this was due to the presence of the desired retro-transcribed RNA or the original FbFP gene. For this reason, an additional 30 minute DNAse 1 digestion step was carried out (see materials and methods). However, PCR continued to show amplification, though at a lesser degree (**Fig. 17**).

An ultimate attempt was made to eliminate all traces of DNA. Since the last digestion had significantly lowered the amount of contaminating DNA it was thought that a greater digestion time would solve the problem. Two additional digestions were designed: one lasting 45 minutes and the other 60 minutes. Gel results for the PCR amplification are shown on (**Fig. 17**). It can be seen that the results for both digestions are very similar: practically no amplification is observed for *DH5α* samples and some amplification can be observed for *E. faecium* samples, specially the last two lanes.

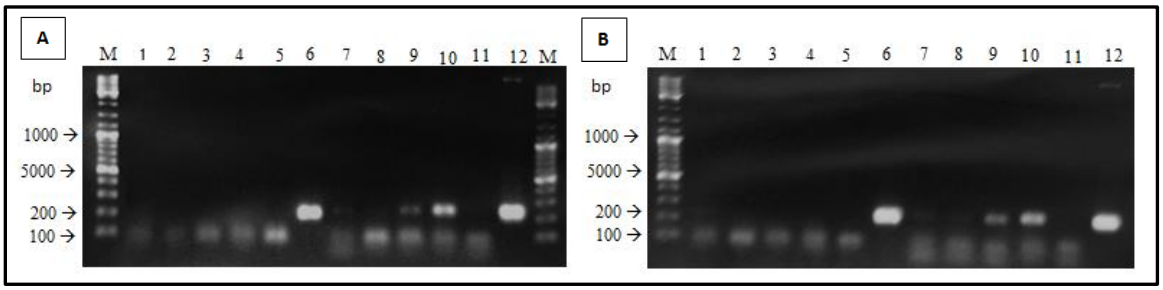

Figure 17. Gel electrophoresis of the PCR products obtained from the amplification of the total RNA extracted from *DH5a* and *E. faecium* and after 45 (A) and 60 (B) minute digestions with DNase1. For both figures (**A,B**), lanes 1-4 represent DH5a samples and lanes 7-8 represent E. faecium samples. Lanes 5 and 11 represent the negative controls for the PCR reaction (water), and lanes 6 and 12 show the amplicons obtained when amplifying pGLOW-KXN-Bs2 and pBT2-FbFP plasmids respectively (positive controls). FbFP gene contamination is still observed after 45 and 60 minute digestions, especially in *E. faecium* samples.

Although there were still DNA remnants in some samples, and thus not suitable for pPCR, it was interesting to run a gel with the digested RNA (**Fig. 18**). This allowed to determine if the incubation temperatures used in the digestion had a negative effect on its quality, since it is widely known that RNA is very delicate, and the temperatures used for the digestion were considerably high (37 ºC for incubation and 65 ºC for DNAse1 inactivation). As expected, although the presence of DNA contamination was shown to be negatively correlated to the time of DNAse1 incubation, apparently so did the quality of RNA (**Fig. 15** and **18**).

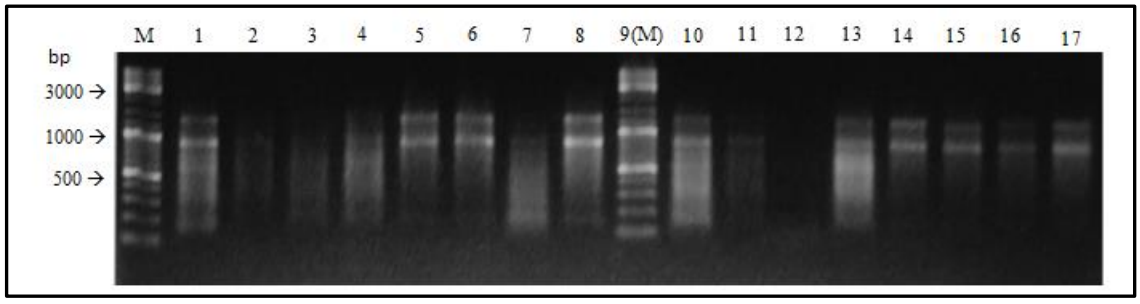

Figure 18. Agarose gel run with the *DH5a* and *E. faecium* RNA extractions after 45 and 60 minute digestions with DNase1. Lanes 1-8 correspond to RNA samples after a 45 minute digestion (lanes 1-4 show *DH5a* samples, and 5-8 show *E. faecium* samples). Lanes 10-17 correspond to RNA samples after a 60 minute digestion (lanes 10-13 show *DH5a* samples, and 14-17 show *E. faecium* samples. It can be seen that RNA sample quality decreases after the addition of DNAse1, compared to (**Fig. 15**).

These results suggest that a different approach, other than augmenting the digestion time, is required. Perhaps, increasing the enzyme concentration and decreasing the RNA amount rather than the digestion time, or simply by purchasing a commercial kit designed for this purpose, such as the Agilent Total RNA Isolation Mini Kit, specially devised for efficient removal of transfected plasmid DNA from total cellular RNA.

4.7 Additional Experiments

As a result of time frame restrictions, no additional experiments were able to be performed. However, it is of great importance for the understanding of this work as a whole to highlight the experiments which need to be carried out.

Firstly, once a method for extracting and purifying RNA which yields suitable RNA for pPCR analysis is developed, FbFP gene-expression analyses should be performed. qPCR will complement the results obtained through fluorescence microscopy analysis in this work, indicating whether no expression is achieved with the plasmid construct or if the expression levels are very low. In either case, the substitution of the promoter bacA used would be a good idea, since this promoter is originally from *E. faecalis*, not *E. faecium*. Therefore, it would be of great interest to try a strong promoter pertaining to an *E. faecium* gene. However, this task would be difficult, as there is a great lack of information in relation to strong constitutive promoters in *E. faecium*.

Once suitable intensity levels of fluorescence are achieved, the integration of the fluorescent gene into the genome should be carried out in order to avoid the loss of fluorescence, as plasmids can be lost through successive bacterial divisions. For this purpose, sites for recombination would need to be cloned flanking the inserted FbFP gene. These sites would be identical to regions in the genome to enhance recombination. Additionally, a gene terminator should be cloned after the FbFP gene insert to avoid the continuation of gene transcription when integrated. As explained in materials and methods, recombinant bacteria would be selected by growing them at 42 ºC, hence enhancing the loss of the temperature sensitive plasmid. Upon plasmid loss, bacteria that continue displaying fluorescent properties, but have become sensitive to chloramphenicol (due to plasmid loss) would therefore be products of recombination.

5. CONCLUSIONS

- The design and synthesis of a (FMN)-based fluorescent protein gene containing the necessary features for its expression in *E. faecium* and for its appropriate insertion into the pBT2 plasmid has successfully been achieved.

- Different analyses focused on the validation of the integrity of the pBT2-FbFP plasmid construction in transformed *E. coli DH5a* cells have evidenced the correct insertion of the FbFP gene into the pBT2 plasmid.

- The application of the transformation protocol used by (Arias and Murray, 2012) for *E. faecalis* has shown to be inadequate for the transformation of *E. faecium*. Adjustment of the glycine concentration used for the preparation of competent cells, from 6% to 4.8% glycine, although not attaining the optimum growth inhibition (70-90% reduction), has proven to yield efficient transformations in this bacterium.

- pBT2-FbFP plasmid extractions from transformed *E. faecium* cells did not allow for down-stream validation analysis of the integrity of the transformed plasmid. However, transformation of *E. coli DH5a* cells with the poorly extracted plasmid from *E. faecium* has demonstrated to be an indirect, yet very practical operation for this purpose.

- Fluorescence analysis of FbFP-expressing bacteria grown under different culturing conditions has revealed that, at least in *E. coli DH5a*, FbFP shows no signs of differential expression under aerobic and anaerobic conditions, thereby confirming the results obtained by other researchers in the field (Drepper *et al*, 2007).

- Fluorescence analysis of putative FbFP-expressing *E. faecium* transformants was not enough for definitely concluding that this bacterium had acquired fluorescent properties. The fact that the images taken with the fluorescent microscope show mildly enhanced intensity differences with the negative control suggest this bacterium might express the FbFP, although at very low levels, hence indicating that the design novel strategies to achieve an adequate expression of FbFP in *E. faecium are required*.

- Total RNA was successfully extracted from both *E. coli* and *E. faecium* with the purpose of conducting qPCR analyses which would complement and possibly clarify the results obtained through fluorescence microscopy. However, DNA containing the FbFP gene was also extracted with the RNA, contaminating all samples, and defeating the purpose of performing qPCR.

- The experiments designed to eliminate the DNA contamination from the RNA extractions through the augmentation of digestion's incubation times with DNase1 did not result in RNA outputs suitable for qPCR analysis. FbFP gene DNA traces remained in some samples and RNA quality declined considerably with incubation time. Alternative methods for the elimination of contaminating DNA need to be studied.

- It is clear that additional work will be required in order to complement the results obtained in this Final Degree Project. qPCR and western blot analysis present themselves as the most suitable techniques with the potential of ultimately clarifying whether FbFP expression in *E. faecium* has legitimately been achieved, and to design novel strategies to achieve and adequate expression of FbFP in *E. faecium*.

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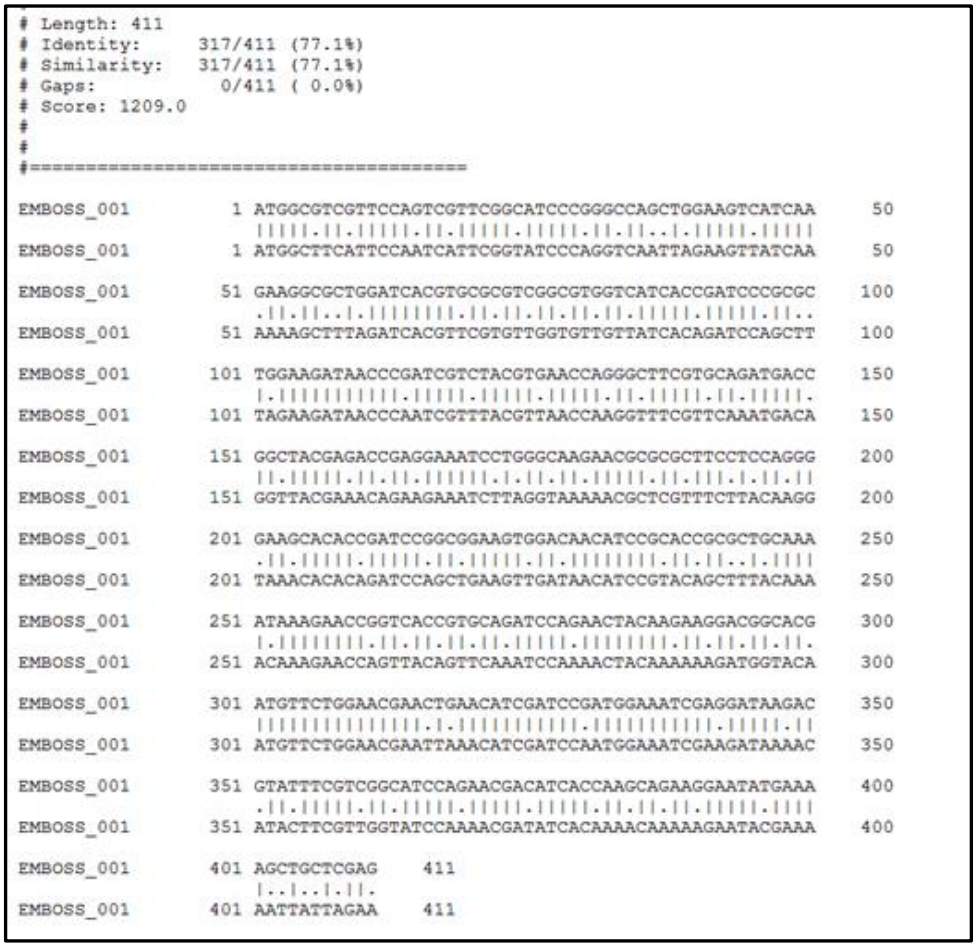
7. ANNEX



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EMBOSS_001        51 GAAGGCGCTGGATCACGTGCGCGTCGGCGTGGTCATCACCGATCCCGCGC    100
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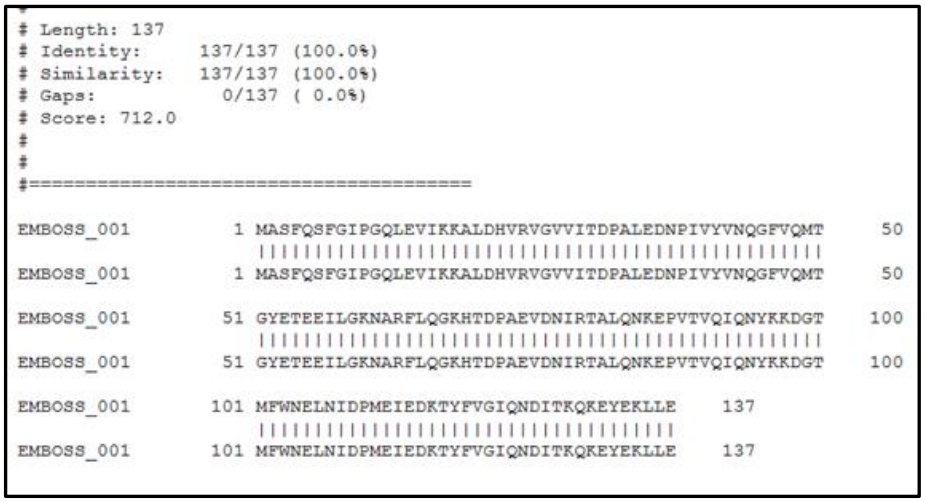
EMBOSS_001       251 ACAAAGAACCAGTTACAGTTCAAATCCAAAACTACAAAAAAGATGGTACA    300

EMBOSS_001       301 ATGTTCTGGAACGAACTGAACATCGATCCGATGGAAATCGAGGATAAGAC    350
                     ||||||||||||||.|.||||||||||.||||||||||.|||||.||
EMBOSS_001       301 ATGTTCTGGAACGAATTAAACATCGATCCAATGGAAATCGAAGATAAAAC    350

EMBOSS_001       351 GTATTTCGTCGGCATCCAGAACGACATCACCAAGCAGAAGGAATATGAAA    400
                     .||.||||||.||.||||||.||||||.||.||.||.||||||.||||
EMBOSS_001       351 ATACTTCGTTGGTATCCAAAACGATATCACAAAACAAAAAGAATACGAAA    400

EMBOSS_001       401 AGCTGCTCGAG    411
                     |..|..|.||.
EMBOSS_001       401 AATTATTAGAA    411
```

Figure Annex 1. Alignment of the original EcFbFP nucleotide sequence (*E. coli* codon-biased) and the modified FbFP sequence used in this work (Enterococcus codon-biased). Vertical lines (|) represent matched bases and dots (.) represent mismatches. Although they are different nucleotide sequences, they code for the exact same amino acid sequence (**Fig. A2**).



```
# Length: 137
# Identity:     137/137 (100.0%)
# Similarity:   137/137 (100.0%)
# Gaps:           0/137 ( 0.0%)
# Score: 712.0
#
#
#=======================================

EMBOSS_001         1 MASFQSFGIPGQLEVIKKALDHVRVGVVITDPALEDNPIVYVNQGFVQMT     50
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001         1 MASFQSFGIPGQLEVIKKALDHVRVGVVITDPALEDNPIVYVNQGFVQMT     50

EMBOSS_001        51 GYETEEILGKNARFLQGKHTDPAEVDNIRTALQNKEPVTVQIQNYKKDGT    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001        51 GYETEEILGKNARFLQGKHTDPAEVDNIRTALQNKEPVTVQIQNYKKDGT    100

EMBOSS_001       101 MFWNELNIDPMEIEDKTYFVGIQNDITKQKEYEKLLE    137
                     |||||||||||||||||||||||||||||||||||||
EMBOSS_001       101 MFWNELNIDPMEIEDKTYFVGIQNDITKQKEYEKLLE    137
```

Figure Annex 2. Alignment of the translated EcFbFP and FbFP (aligned on Fig. A1). Vertical lines (|) represent matched bases and dots (.) represent mismatches. No dots are observed, meaning that the translated sequences are identical. In a sense, although the nucleotide sequences are different, they code for the same amino acid sequence

```
# Length: 489
# Identity:     488/489 (99.8%)
# Similarity:   488/489 (99.8%)
# Gaps:           0/489 ( 0.0%)
# Score: 2436.0
#=======================================

EMBOSS_001         1 GCTTGCATCAAAATAAACTACATGGGTATAATAGCAATGAAATGCATTTC     50
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001         1 GCTTGCATCAAAATAAACTACATGGGTATAATAGCAATGAAATGCATTTC     50

EMBOSS_001        51 AAAAATATTTTGAGGAGAATTTAGTATGGCTTCATTCCAATCATTCGGTA    100
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001        51 AAAAATATTTTGAGGAGAATTTAGTATGGCTTCATTCCAATCATTCGGTA    100

EMBOSS_001       101 TCCCAGGTCAATTAGAAGTTATCAAAAAAGCTTTAGATCACGTTCGTGTT    150
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       101 TCCCAGGTCAATTAGAAGTTATCAAAAAAGCTTTAGATCACGTTCGTGTT    150

EMBOSS_001       151 GGTGTTGTTATCACAGATCCAGCTTTAGAAGATAACCCAATCGTTTACGT    200
                     |||||||||||||||||||||||||||||||||||.|||||||||||||
EMBOSS_001       151 GGTGTTGTTATCACAGATCCAGCTTTAGAAGATAACCCGATCGTTTACGT    200

EMBOSS_001       201 TAACCAAGGTTTCGTTCAAATGACAGGTTACGAAACAGAAGAAATCTTAG    250
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       201 TAACCAAGGTTTCGTTCAAATGACAGGTTACGAAACAGAAGAAATCTTAG    250

EMBOSS_001       251 GTAAAAACGCTCGTTTCTTACAAGGTAAACACACAGATCCAGCTGAAGTT    300
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       251 GTAAAAACGCTCGTTTCTTACAAGGTAAACACACAGATCCAGCTGAAGTT    300

EMBOSS_001       301 GATAACATCCGTACAGCTTTACAAAACAAAGAACCAGTTACAGTTCAAAT    350
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       301 GATAACATCCGTACAGCTTTACAAAACAAAGAACCAGTTACAGTTCAAAT    350

EMBOSS_001       351 CCAAAACTACAAAAAAGATGGTACAATGTTCTGGAACGAATTAAACATCG    400
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       351 CCAAAACTACAAAAAAGATGGTACAATGTTCTGGAACGAATTAAACATCG    400

EMBOSS_001       401 ATCCAATGGAAATCGAAGATAAAACATACTTCGTTGGTATCCAAAACGAT    450
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       401 ATCCAATGGAAATCGAAGATAAAACATACTTCGTTGGTATCCAAAACGAT    450

EMBOSS_001       451 ATCACAAAACAAAAAGAATACGAAAAATTATTAGAATAA              489
                     |||||||||||||||||||||||||||||||||||||||
EMBOSS_001       451 ATCACAAAACAAAAAGAATACGAAAAATTATTAGAATAA              489
```

Figure Annex 3. Alignment of the FbFP sequence obtained by Sanger sequencing the FbFP insert of pBT2-FbFP plasmids extracted from "colony 3" (see section 4.4.2.3) and the original FbFP sequence. Vertical lines (|) represent matched bases and dots (.) represent mismatches. A single mismatch is observed (highlighted in yellow), indicating that a mutation has occurred at some point during the handling process (transition from A→G). The affected codon is highlighted in green.

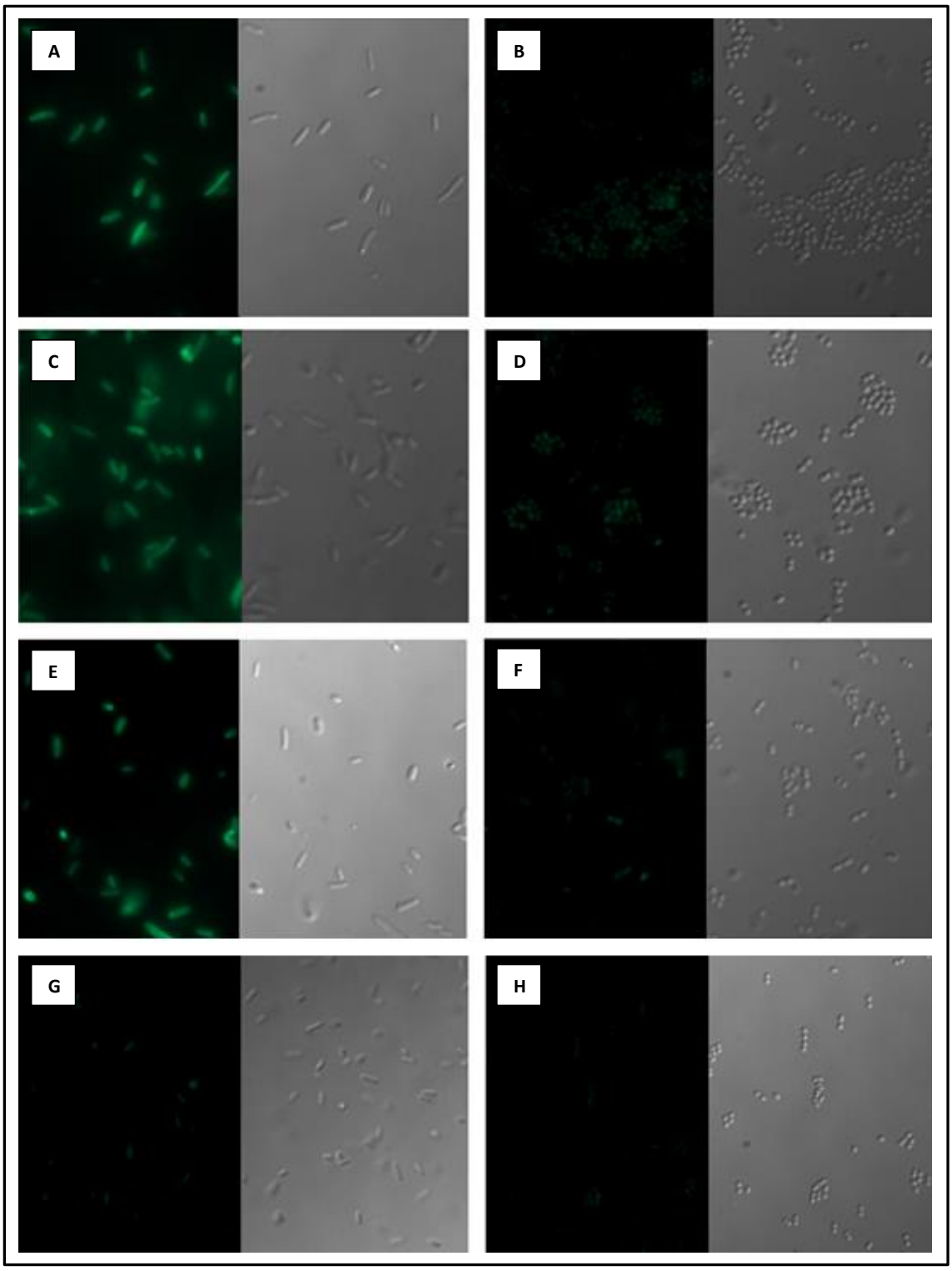
```
# Length: 138
# Identity:     138/138 (100.0%)
# Similarity:   138/138 (100.0%)
# Gaps:           0/138 ( 0.0%)
# Score: 713.0

EMBOSS_001         1 MASFQSFGIPGQLEVIKKALDHVRVGVVITDPALEDNPIVYVNQGFVQMT     50
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001         1 MASFQSFGIPGQLEVIKKALDHVRVGVVITDPALEDNPIVYVNQGFVQMT     50

EMBOSS_001        51 GYETEEILGKNARFLQGKHTDPAEVDNIRTALQNKEPVTVQIQNYKKDGT    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001        51 GYETEEILGKNARFLQGKHTDPAEVDNIRTALQNKEPVTVQIQNYKKDGT    100

101 MFWNELNIDPMEIEDKTYFVGIQNDITKQKEYEKLLE*    138
    |||||||||||||||||||||||||||||||||||||
101 MFWNELNIDPMEIEDKTYFVGIQNDITKQKEYEKLLE*    138
```

Figure Annex 4. Alignment of the translated original and mutated sequences of FbFP. Vertical lines (|) represent matched bases and dots (.) represent mismatches. As it can be seen, there are no mismatches. The amino acid sequences are identical since the (A→G) mutation produced a codon chage (CCA→CCG) which codes for the same amino acid (Proline). Therefore, it can be said that it is a silent mutation, having no effect on the final protein output.



<u>Figure Annex 5.</u> Fluorescence microscopy (CFP filter) and Differential Interference Contrast; Nomarski microscopy photographs (1000x) taken with the Leica DM RXA2 Microscope. See section 4.5 for a detailed explanation of the experiment. (A) *DH5α* cultured at 37ºC under aerobic conditions (B) *E. faecium* cultured at 37ºC under aerobic conditions (C)*DH5α* cultured at 30ºC under aerobic conditions (D) *E. faecium* cultured at 30ºC under aerobic conditions. (E) *DH5α* cultured at 30ºC under anaerobic conditions. (F) *E. faecium* cultured at 30ºC under anaerobic conditions. (G) Negative control: *DH5α* (without pGLOW-KXN-Bs2) (H) Negative control: *E. faecium* (without pBT2-FbFP). It can be seen how transformed *DH5α* cells indeed display fluorescent properties, in comparison to the negative control.

However, although the images suggest that transformed *E. faecium* cells are slightly more fluorescent than the negative control, the intensity is not conclusive. Changes in temperature or the presence/absence of molecular oxygen appear to have no effect on the fluorescence emission.