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Optimization of bacterial growth conditions for using fluorescent bioreporters in cell culture infection assays

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Abstract:

While fluorescent bioreporters are no longer a new thing, they are under constant improvement for application in different fields, and one of them is in medical research. They can be of a great importance for monitoring bacterial infection diseases and give us information about pathogen-drug interaction within the host. In this study, two bioreporters containing the *gfp* gene (IPTG inducible and constitutively expressing reporter gene) were used in uropathogenic *E. coli*. We tested different bacterial growth conditions and found the optimal one that is not affecting GFP fluorescence and also allow process of bacterial internalization in bladder cells.

Keywords:

Fluorescence, bioreporter, urinary tract infection, E. coli, cell culture infection

CERCS:

B230 Microbiology, bacteriology, virology, mycology; T490 Biotechnology

Bakterite kasvu tingimuste optimeerimine fluorestseeruvate bioreporterite kasutamiseks rakukultuuri nakatumise katsetes

Lühikokkuvõte:

Kuigi fluorestseeruvaid bioreportereid on kasutatud juba mõnda aega, siis jätkuvalt on vaja neid erinevates valdkondades, näiteks meditsiiniuuringutes, rakendamiseks pidevalt täiustatud. Bioreporteritel võib olla suur tähtsus bakteriaalsete nakkushaiguste jälgimisel ja nad saavad meile teavet patogeeni ja ravimi koostoime kohta peremeesorganismis. Käesolevas töös kasutati uropatogeenses E. coli tüves kahte kahte biorporterit, mis sisaldasid gfp geeni (IPTG indutseeritav ja konstitutiivselt ekspresseeriv reportergeen). Katsetasime erinevaid bakterite kasvutingimusi ja leidsime optimaalse tingimuse, mis ei mõjuta GFP fluorestsentsi ning võimaldavad ka bakterite sisenemist põie-epiteeli rakkudes.

Võtmesõnad:

Fluorestsents, bioreporter, kuseteede nakkus, E. coli, rakukultuuri nakkus

CERCS:

B230 Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia; T490 Biotehnoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

2x YT – 2x yeast tryptone

AMP – ampicillin

CFU – colony forming units

CFU/ml- colony formed units per millilitre

Em – emission

Ex – excitation

FACS – fluorescence-activated single cell sorting

FBS – fetal bovine serum

GFP - green fluorescent protein

IPTG - isopropyl β-D-1-thiogalactopyranoside

KAN – kanamycin

LB - lysogeny broth

LPS – lipopolysaccharide

MHB – mueller-hinton broth

MM 4-64 – membrane marker 4-64

OMPs - outer membrane proteins

SOB – super optimal broth

SOY – trypticase soy broth

UTIs – urinary tract infections

UT- urinary tract

UPEC – uropathogenic Escherichia coli

INTRODUCTION

UTIs are one of the most infectious diseases (bacterial) worldwide, and they incur one of the highest prescriptions for antibiotics. The high occurrence and recurrence of these infections lead to extreme repercussions ranging from high medical costs, antibiotic resistance, to death. The inappropriate ways of treating UTIs, which mostly focuses on symptom removal (due to the consideration that they are extracellular infections), but not eradication of intracellular bacterial reservoirs, is one of the reasons for recurrences and increasing antibiotic resistance. The utmost solution of this worldwide problem would be in a discovery and reasonable use of a drug, that can be as effective against extracellular as against intracellular bacteria.

The aim of this study was to test and optimize fluorescence of bioreporters within uropathogenic *Escherichia coli* (UPEC), to find the most suitable one for usage in subsequent *in vitro*- cell culture infection models. By establishing the infection model using human bladder cell line and fluorescent UPEC, we were able to get more information about bacterial localization and pathogenesis process. Aside from this, the model is relevant for testing efficiency of both commonly prescribed antibiotics in treatment of UTIs, and new drugs that are still in clinical trials.

In this study, we used UPEC, transformed with plasmids carrying fluorescent proteins. Our aim was to define the pre-growth condition which provides the highest and the most stable expression of fluorescent protein, but that at the same time do not affect bacterial internalization within bladder cells. For that purpose, we have used flowcytometry (FACS). Afterwards, we infected the bladder cells with the tested bacteria, and checked the internalization rate, followed with cytotoxicity assays and confocal microscopy. Through this study, we were able to successfully optimize bacterial bioreporters for better cell infection internalization, thus, improving the study of UTIs.

1 LITERATURE REVIEW

1.1 Urinary Tract Infections

Urinary tract infection (UTI) is defined as the presence of microbial pathogens in the urinary tract, usually at very high concentration reaching $\geq 10^5$ colony forming units per ml (CFU/ml). However, some pathogens can cause symptoms at much lower concentrations starting from 10^3 CFU/ml. UTIs can affect both men and women, but it is mostly considered a women's disease, which is predominantly due to the high rate at which it affects women. The major reason UTIs are more prevalent in women is due to their shorter urethra compared to men, and this makes it easier for bacteria to travel up the urinary tract to the bladder. About 150 million people around the world suffer from UTIs each year, with annual increase in occurrence of about 12.6% in women, and 3% in men. It has been found that at least, about 50% of women and 12% of men will suffer from a UTI in their lifetime (Jhang & Kuo, 2017; McLellan & Hunstad, 2016).

By the site of infection, UTIs can be classified as upper (kidneys) or lower (bladder, urethra) UTIs (see Figure.1). The second classification is based on the infection complexity which consists of uncomplicated and complicated UTIs. Uncomplicated UTIs occur in individuals with a non-complex urinary system, i.e., not involving instrumentation. On the other hand, complicated UTIs are diagnosed in individuals with complex urinary systems involving both structural and functional problems e.g., urethral catheter (Schmiemann & Hummers-Pradier, 2007). Thirdly, UTIs can also be classified based on how they were acquired on community acquired or hospital acquired (mostly involving catheterization). And finally, UTIs can be asymptomatic and symptomatic, depending on presence or absence of symptoms. The symptoms of UTIs range from mild to severe, including painful, recurrent, and pressing need to urinate, pain of the lower back, pelvic region pain, nausea, vomiting, fever, pyuria (cloudy or smelly) and haematuria (blood in urine) (Anderson et al., 2004; Terlizzi et al., 2017). The transition from asymptomatic to symptomatic infection depends on both human and bacterial features, e.g. host age, previous UT infections, gender, or from bacterial side production of bacteriocins, toxins, and siderophores. The most frustrating part of these infections is the high rate of recurrence. Having >3 UTIs within 12 months as well as >2 within 6 months defines a recurrent infection (Terlizzi et al., 2017). Various research has shown that approximately 26% of women with severe UTIs will have a recurrence in the space of six months, and many others will have multiple recurrences (Foxman, 2003; Foxman et al., 2000). The new episode of UTIs could be a result of a reinfection with the same or different bacterial

species, while a relapse is an infection that is usually caused by the same clone that caused the initial infection, and it usually happens within 1-2 week after the end of treatment (Geerlings, 2016). Recurrent UTIs being a predominant problem on its own can also lead to problems like renal scarring, antibiotic resistance, and bladder cancer (Anderson et al., 2004; Eto et al., 2006).

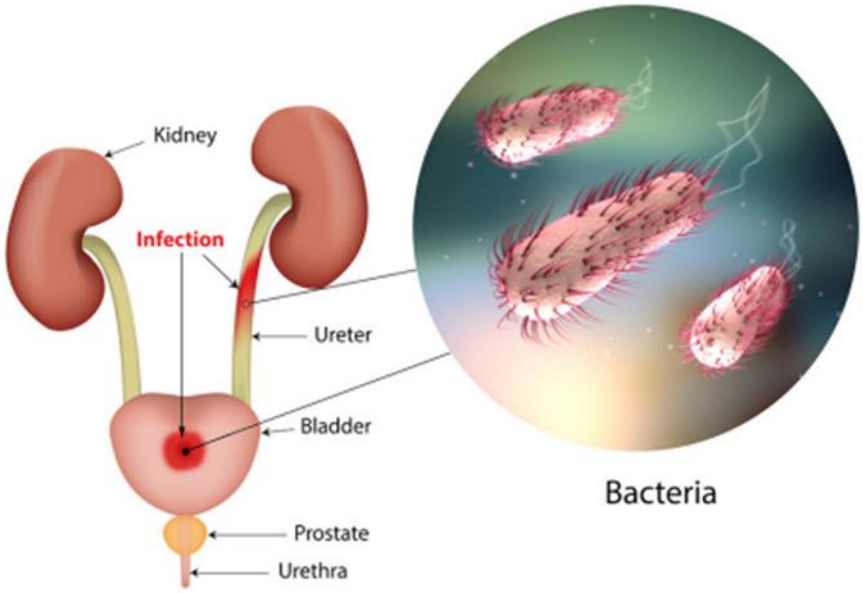

Figure 1. An illustration showing the urinary tract, and sites of infection. *The left part of the illustration shows upper urinary tract (kidney, ureters) and lower urinary tract (bladder, prostrate, urethra). Right side shows moving bacteria with extended pili and flagella, as the most important virulence factors in the first phases of establishing of infection.* ***Adapted from Terlizzi et al., 2017.***

UTIs are associated with different types of bacteria. These can include uropathogenic *Escherichia coli* (UPEC), *Staphylococcus saprophyticus, Klebsiella proteus, Pseudomonas aeruginosa,* and *Enterococcus faecalis. Enterobacter*, *E. coli*, and *S. saprophyticus* are usually linked with population acquired uncomplicated infections, while the rest are associated with irregular uncomplicated cystitis (bladder infection) and pyelonephritis (kidney infection). Rarely, one might acquire a UTI involving *Staphylococcus aureus.* Among the previously mentioned UTIs-associated bacterial species, the most common causative agent of UTIs is UPEC, which is found in approximately 90% of all infections (Anderson et al., 2004; McLellan & Hunstad, 2016; Vasudevan, 2014).

1.1.1. UPEC

As stated earlier, the primary agent associated with UTIs is UPEC. It belongs to a group of extraintestinal pathogenic *E. coli* that might have originated from the gut (Kot, 2019; Schmiemann & Hummers-Pradier, 2007). However, unlike gut *E. coli*, UPEC contains mobile genetic elements known as pathogenicity islands (PAIs). These islands are genomic regions that aids in the expression of the virulence elements that makes UPEC highly specialized in surviving and persisting in the UT (Schmidt & Hensel, 2004).

There are many variants of UPEC, which can be classified into phylogenetic groups such as A, B1, B2, D and E, based on their genomic similarities (Terlizzi et al., 2017). Majority of the UPEC isolates belong to B2 group, e.g. (CFT073, UTI89, J96, NU14 and 536). On the other hand, UPEC strains from other phylogenetic groups have not been properly isolated and researched (Lloyd et al., 2007). However, in the research on UTIs, the most toxic internalizing UPEC studied in the mouse model is CFT073 (Chockalingam et al., 2019; Sivick et al., 2010). It was isolated from the blood of a patient with pyelonephritis (Mobley,' et al., 1990).

1.1.2. UPEC virulence factors

Virulence elements are essential for bacterial survival and multiplication in UT, and they play crucial role in the process of UTI pathogenesis. The most important elements include toxins and adhesins, type 1 pilus, curli, outer membrane proteins (OMPs), adhesins, siderophore receptors, lipopolysaccharide (LPS), and flagella (Figure 2) (Anderson et al., 2004; Blango & Mulvey, 2010; Eto et al., 2006; Terlizzi et al., 2017).

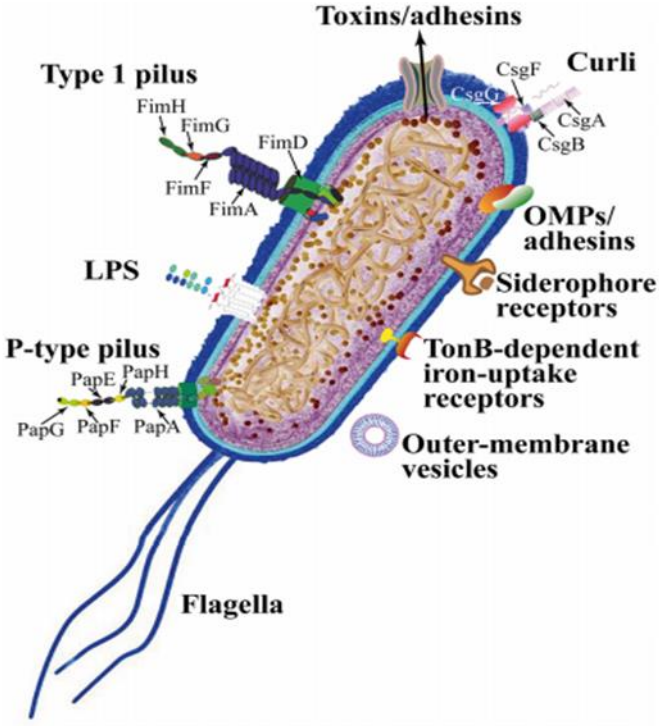

Figure 3. UPEC major virulence elements. *Virulence factors on the surface of UPEC responsible for establishing of UT infection. Adapted from Terlizzi et al., 2017.*

Starting with LPS, this molecule's morphological constituents help in multiple aspects of the UPEC life cycle, this stems from the fact that it has amphipathic (one part of the molecule is hydrophobic, and the other part is hydrophilic) properties. Colonizing the bladder, forming reservoir, evoking bladder's protective response to bacteria, combined with providing resistance against hydrophobic antibiotics, for which their amphipathic properties are very important. A decreased expression of LPSs on UPEC's surface increase susceptibility to hydrophobic molecules. Type 1 - and P type pili (associated with the kidney, with the P standing for pyelonephritis) are also very important molecules synthesized by the fim and pap operon, respectively (Anderson et al., 2004; Terlizzi et al., 2017). These pili provide important adhesins known as PapG and FimH. In the urinary tract, FimH binds mannosylated residues from uroplakin molecules on the surface of the urothelium. On the other hand, PapG binds to the globoside on the kidney epithelium. PapG is more associated with chronic and recurrent UTIs (Anderson et al., 2004; Flores-Mireles et al., 2015; Foxman et al., 2000; Terlizzi et al., 2017). Curli grow on the UPEC surface, and they secrete subunits from the cell as soluble monomeric proteins, which aids in the formation of biofilms and bacteria survival externally (Terlizzi et al., 2017). Curli moderates cellular interactions with other cells and surfaces to promote the adhesion of bacteria to other mammalian cells, plant cells, and other surfaces, thus it is also important for bacteria internalization, and resistance against

urine flow forces threatening to wash it out of the bladder (Allsopp et al., 2012; Cegelski et al., 2009; Floyd et al., 2015). To do this, curli can interact with host glycoprotein and cause the clustering of bacterial cells (Zalewska-Piatek & Piatek, 2019). Flagella also play important roles in biofilm formation. They give invasive properties, their roles are adherence, maturation, and dispersal (Cegelski et al., 2009; Floyd et al., 2015; Terlizzi et al., 2017).

Iron acquisition system is very important for UPEC survival in the urinary tract. It is composed of receptors, known as siderophores, which main role is in capturing of ferric iron. Siderophore receptors on the surface of the cell promote iron uptake into the cell with the aid of other factors (Terlizzi et al., 2017).

Finally, UPEC produces different toxins that aid in pathogenesis. One toxin worthy of noting is alpha hemolysin which plays a major role in renal scarring and damage and enhances the urinary tract colonization by interrupting the normal flow of urine (Allsopp et al., 2012; Emody et al., 2003; Schreiber Iv et al., n.d.; Terlizzi et al., 2017).

Of all these virulence factors, none is solely responsible for the UPEC pathogenesis, but all together, the ability to cause UTIs depends on these factors, and these factors have been found in most UPEC strains (Allsopp et al., 2012; Emody et al., 2003; Terlizzi et al., 2017).

1.1.3. UPEC pathogenicity

Until recently UPEC was considered only as an extracellular pathogen. Its intracellular lifestyle and the pathogenic pathway in bladder tissue were first discovered in mice (Barber et al., 2016; Carey et al., 2016; Sivick et al., 2010; Vasudevan, 2014). Due to structural similarities with human bladder, mice model has been extensively used in studying UTIs. The mice bladder epithelium is, same as human, transient and composed of multiple layers (the basal layer, the intermediate layer, and the superficial layer). The epithelium is transient in the sense that due to the superficial cells layer being removed every 3-6 months, the lower intermediate layer cells are differentiated into the superficial cells, and the basal cells are differentiated into the lower intermediate cells (Anderson et al., 2004; Lewis, 2000). The superficial layer makes the bladder impermeable. The superficial cells also express integral membrane proteins known as uroplakins (UPs). These uroplakins help the integrity of the bladder epithelium, provide water impassibility, but they can also contribute to UPEC pathogenicity, through UP subunits that allow bacterial attachment to it (Anderson et al., 2004; Blango & Mulvey, 2010; Terlizzi et al., 2017).

As stated earlier, UPEC expresses several virulence factors that have a role in colonization and invasion of the bladder epithelium. UPEC enters the bladder using actin and microtubule dependent processes mediated by type 1 pili, then FimH binds mannose containing glycoprotein receptors containing α3β1 integrin complexes and uroplakin that coat the entire internal surface of the bladder. The association of FimH with these uroplakin plaques mediates UPECs entry into the superficial bladder cells. Simultaenously, α3β1 integrin receptor complexes on the urothelium can also mediate UPEC invasion of the intermediate cells. Once internalized, UPEC can be ejected from the cells, or trafficked into the endosomes, where they can remain in a quiscent state, bound by the actin filaments. On the other hand, in the superficial cells where the actin filaments are rare, UPEC can invade adjacent superficial cells and multiply, forming biofilm like inclusions, called intracellular bacterial communities (IBCs) (Anderson et al., 2004; Justice et al., 2004; Mulvey et al., 2001; Terlizzi et al., 2017).

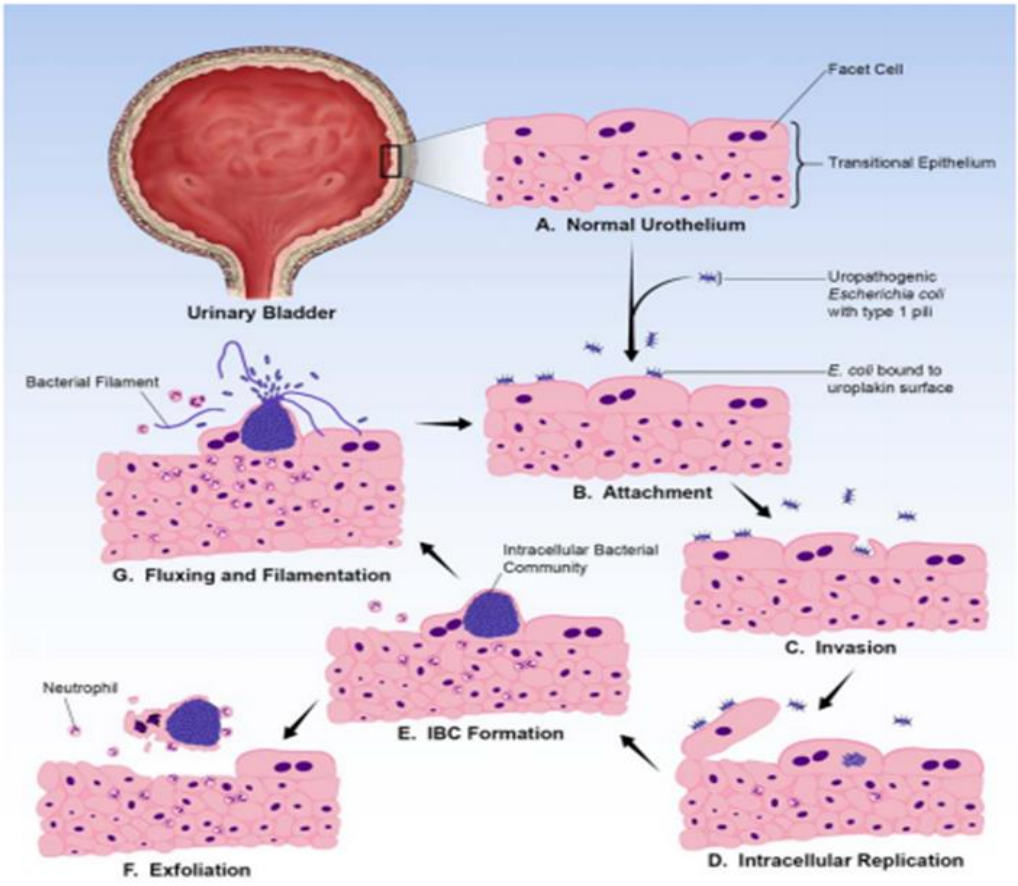

Figure 3. Intracellular lifestyle of UPEC as observed in the murine cystitis model. *(A) The normal full transitional multilayered urothelium without any affiliation. (B) With the aid of pili attachs to UPs on the surface of the bladder epithelium. (C) Through association with the UPs, the UPEC invades the cells. (D) While in the cell, they replicate intracellularly to*

form (E) IBC. (G) These IBCs can eventually cause a rupture and flux out of the cell. These cells might be released into the bladder and cause reinfection of the neighbouring cells, additionally, some cells become filamentous and can't be easily digested by neutrophils. (F) These IBCs can also be evicted from the bladder, by the host cell exfoliation. Adapted from David A. Rosen et al., 2007.

As a host defence mechanism, disruption of the urothelium can occur due to the UPEC induced exfoliation and influx of neutrophils, plus other inflammatory responses, e.g., mucus production, secretion of antimicrobial peptides and proteins, proinflammatory chemokines etc. (Chromek & Brauner, 2008; Vasudevan, 2014). When the superficial cells are overwhelmed with bacterial cells, then they initiate apoptosis. The apoptosis leads to the cell's exfoliation their removal from the UT with normal flow of urine. This is very efficient way of eradicating most of infected cells, however, bacteria can get easier access and infect underlaying and immature urothelial cells. This can be combated by the superficial cell's rapid regeneration, but it is not enough. In this moment of the bladder's vulnerability, the bacterial cells have an opportunity to go into the basal cells and form quiescent intracellular bacterial reservoirs (QIRs) (Chromek & Brauner, 2008; Lewis et al., 2016; Mulvey et al., 2000; Terlizzi et al., 2017). Therefore, these defence mechanisms aimed at protecting the host ends up enhancing UPEC persistence in the host, alongside IBCs and extracellular biofilms. All in all, UPEC have developed mechanisms providing persistence and protection mechanisms. The QIRs in the intermediate/basal cells in conjunction with the actin filaments may conceal UPEC from many of the host surveillance mechanisms. IBCs and extracellular biofilms protect UPEC from antimicrobial activities of neutrophils and other host defences (Anderson et al., 2003, 2004; David A. Rosen et al., 2007; Eto et al., 2006; Terlizzi et al., 2017).

1.1.4. Infection models

There have been various infection models carried out concerning UTIs. These model systems are made as an approximation of natural human infections and allow aspects of the infections to be controlled (Barber et al., 2016). Pairing known genetic and molecular techniques with model systems allow researchers to find key bacterial and host factors involved in UTIs pathobiology and identify new therapeutic targets (Barber et al., 2016). There are two common infection models, the cell culture infection model which we used in this research, and the animal infection model (Barber et al., 2016; Carey et al., 2016).

a. Cell culture infection models

Cell culture-based assays have been used extensively to investigate the mechanisms and consequences of UPEC interactions with host cells (Blango & Mulvey, 2010; Carey et al., 2016). These infections are done in addition to primary and cancer cell lines derived from the bladder, kidney, or other host cell tissues. These host cells are usually grown as undifferentiated monolayers, but they can be influenced to stratify and partially differentiate when grown using appropriate media on membrane scaffold in dishes or appropriate conditions (Barber et al., 2016; Blango & Mulvey, 2010). Pharmacological reagents can also be used to interfere with host enzymes and signalling cascades, but dosages should be closely monitored. The ease of use, low-cost amenability to high through-put assays, genetic manipulation, and biochemical analysis makes cell culture-based systems attractive alternative to animal infection models. Flow cell technology even makes it possible to mimic urinary tract conditions and examine UPEC-host interactions using bladder cells monolayers in the presence of urine flow (Barber et al., 2016).

b. Animal infection models

In animal infection models, predominantly murine especially mice, they are used for deciphering most of the UPEC pathogenic pathway. In mice, which is the predominant animal model for the study of UTIs (Barber et al., 2016; Chockalingam et al., 2019; Justice et al., 2004; Mulvey et al., 2001), it usually entails the transurethral catheterization of rodents and ensuing instillation of bacteria directly into the bladder lumen. Murines share so much in common with humans, including conserved immunological factors and similar anatomical features within the urinary tract. This model system allows detailed investigation of ascending and colonizing UTIs as well as comparing fitness and virulence potentials of wild type and mutant UPEC as well as examining host responses to UTI (Barber et al., 2016; Carey et al., 2016; Chockalingam et al., 2019).

Other animal species can also be used to study UTI e.g. humans and other primates, through analyzing the urine, zebrafish, swine, avian, nematodes, and teleost (the last five through intentional infections) (Barber et al., 2016).

1.2 Bioreporters

1.2.1. General description of bioreporters

Bioreporters are important research tool that give us better understanding of the world of microorganisms. They are the result of genetic engineering by which a responsive promoter is fused with a suitable reporter gene and then incorporated into bacterial cell (Johan HJ

Leveau & Steven E Lindow, 2002). Responsive promoters can respond to changes in environmental or metabolic conditions and provide easily measurable signal in return (Kohlmeier et al., 2007; Van Der Meer et al., 2004; Yoo et al., 2007). Such properties of a whole-cell bioreporters make them applicable in different fields (e.g. detection of pollution in the environment, and agriculture, in food control, medical diagnostics and metal detection etc.) (Johan HJ Leveau & Steven E Lindow, 2002; van der Meer et al., 2004).

Bioreporters can be classified either by the ones that increase their output signal when they sense specific target compounds (e.g., antibiotics, heavy metals), the ones that increase their output signal due to stressful conditions (e.g. pH, oxidative stress), or the ones that increase their output signal in reaction to a wide range of conditions (Kohlmeier et al., 2007; Shemer et al., 2015; van der Meer et al., 2004).

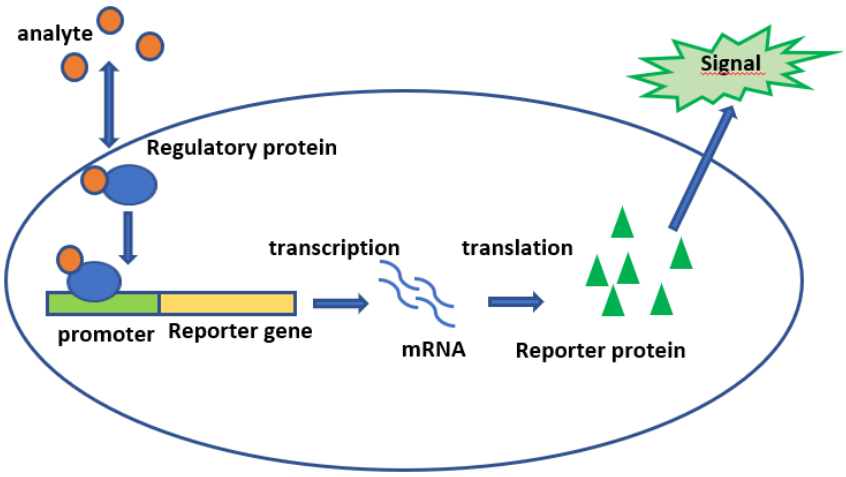

Figure 4. Schematic showing the whole-cell bioreporter. *The analyte responsible for inducing the expression of the bioreporter enters the cell. After entering the cell, a regulatory protein recognizes and binds to it. This binding leads to the expression of a reporter gene and synthesis of a reporter protein, which as a result creates an easily measurable signal. Adapted from Shemer et al., 2015.*

There are two types of bioreporters, the lights off, and lights on bioreporters. The lights off reporters' cells are designed to continuously emit light, and the lights on reporters, the principle is more complex as the cells are designed to emit light only when exposed to a specific substance (Figure 4) (Su et al., 2011; Xu et al., 2013). The lights on bioreporters are designed to contain a reporter gene that needs to use an analyte for inducing the expression of its signal. The analyte enters the cell, and a regulatory protein recognizes it and binds it to the

promoter of the reporter gene. This activates the expression of the mRNA that translates to the reporter protein and gives off an easily measurable signal. On the other hand, a lights-off bioreporter is designed with a reporter gene containing a constitutive promoter that always activates the expression of the protein responsible for giving of the signal. These signals can either be bioluminescence (e.g., coded by *lux* gene), florescence (e.g., coded by *gfp* gene), or colorimetric endpoints (e.g., β-galactosidase coded by *lacZ* gene) (Kohlmeier et al., 2007; Shemer et al., 2015; Su et al., 2011; van der Meer et al., 2004).

Bioluminescence is the light generation produced due to chemical reaction within a living organism and is used in biosensing through a reporter element (Cevenini et al., 2016; Xu et al., 2013). The chemical reaction causing bioluminescence happens when an enzyme reacts with a substrate to produce visible light photons (Cevenini et al., 2016; Xu et al., 2013).

Fluorescence is basically light emission that occurs within nanoseconds after light absorption. Filtering out source of exciting light without blocking the resulting emission, makes it possible to see only fluorescent objects (Lichtman & Conchello, 2005).

Fluorophores/fluorochromes are molecules containing fluorescent properties (e.g., membrane marker MM 4-64). Absorption of light by a fluorophore leads the molecules to become excited to a maximal energy level, and the transition to this state is very swift. This excited state only lasts for some nanoseconds, before the molecules move back to the ground state, releasing the absorbed energy, this is the emission state of the molecule (Adan et al., 2017; Lichtman & Conchello, 2005).

Fluorescence and fluorochromes typically follows a principle known as Stokes Shift as illustrated in the Figure 5. It primarily explains that when light is absorbed and emmited, emmision energy contains lower energy than the absorbed energy, so, the wavelength of emmision will be longer than the wavelength of absorption, and will give a different colour of light (Adan et al., 2017; Lichtman & Conchello, 2005). The difference between excitation energy and emmision energy is known as Stokes Shift . Fluorescence studies uses this principle to determine good fluorophores, and when fluorescent molecules have a large stokes shift, detection of different molecules is easier using confocal microscopy or flow cytometry (Adan et al., 2017; Lichtman & Conchello, 2005; Rahman et al., 2006).

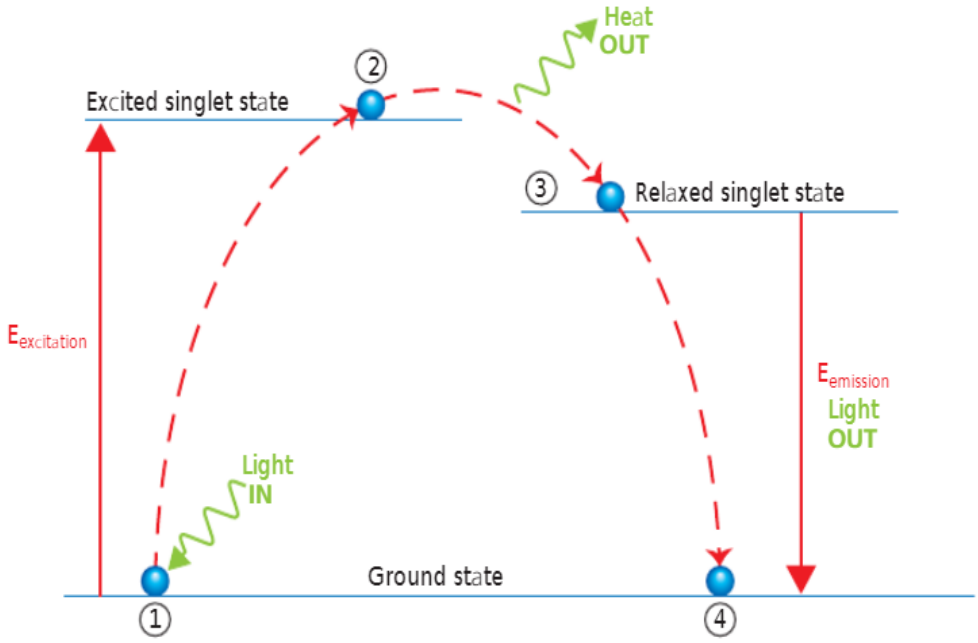

Figure 5. Stokes shift diagram. *This illustration explains the whole process of fluorescence production. (1) the electron is on the ground state, and absorbs reflected light (excitation), and transits to its maximum excited state. (2) the excited state lasts for a few nanoseconds, and releases some absorbed energy, giving off heat. (3) the electron falls to a stable energy state, the relaxed state. (4) the electron starts moving to the ground state, emitting the remaining absorbed energy as fluorescence (emission energy). Adapted from Rahman et al., 2006.*

In this study, GFP (excitation_Ex = 488nm, emmision_Em = 510nm) was used as a fluorescent marker, and it was introduced to the UPEC CFT073 using two different plasmids to make two different bacterial bioreporters, pET-GFP_mut2_KAN®, and pSC101-GFP_Δscarlet_AMP®. The first one needs IPTG molecule to induce its GFP expression, while the second one always expresses its GFP.

2 THE AIMS OF THE THESIS

The main aim of this work was to optimize bacterial growth conditions, so that eventually bacteria can be used in cell culture infection assays.

For that purpose, we tested:

- Bioreporter's fluorescence levels (GFP)
- Internalization rate and intracellular survival (in bladder cells)

The ideal bacterial growth conditions result in unhampered production of reporter proteins (high levels of green fluorescence) and do not affect expression of bacterial surface virulence factors, which are essential for infection process.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Bacterial strain and preparation of dimethyl sulfoxide (DMSO) stocks

CFT073 strain of *E. coli* (Mobley,' et al., 1990) was used in all experiments. Bacteria, from glycerol stock was streaked on LB agar plate, incubated over night at 37 °C and next day a single colony was taken for making a DMSO stock. With a plastic loop the colony was transferred into 10 ml of LB media and bacteria was grown over night, 37 °C, 120 rpm shaking conditions. The subsequent day, overnight culture was 100x diluted into fresh LB media and placed back into the shaker at 37 °C. When optical density (OD_{600}) of culture reached 0.8, DMSO was added to the final concentration of 8%, and cells were stored in 120 µl aliquots at -80 °C.

3.1.2 Making electrocompetent cells

To make the cells electrocompetent, overnight culture of CFT073 wild type (WT), that was cultured in LB, 37 °C, 120 rpm shaking conditions, was 100x diluted in 50 ml super optimal broth media (SOB) and put to grow in 37°C shaker incubator. Once OD_{600} reached 0,35 – 0,4 the cells were immediately put on ice for 20 – 30 minutes, swirling occasionally to allow even cooling. Afterwards, the entire 50 ml culture was poured into a cold 50 ml tube, and centrifuged at 3000 g for 10 minutes at 4°C. The supernatant was removed and pellet was resuspended in 10% glycerol. The process was repeated consecutively three more times. Eventually, the cells were resuspended in 400 µl of 10% glycerol, aliquoted, 50 µl each tube and used immediately for transformation. The remaining tubes with electrocompetent cells were stored at -80°C.

3.1.3 Bacterial transformation by electroporation

Bacterial transformation is the process where we take advantage of lateral gene transfer to insert foreign genetic information into a bacterial cell. Bacterial transformation by electroporation involves the use of an electroporator to intentionally create holes in the bacterial cells, so the bacteria can take up the genetic information in its environment.

All materials used, liquids, as well as bacterial cells were prechilled on ice for approx. 20-30 minutes before transformation. One aliquot of the electrocompetent cells (50 µl) was

mixed with the plasmid (100ng/µl) and then added into the electroporation cuvette (2mm). Electroporation was then done by applying 2500 voltage in electroporator, and afterwards cells were recovered by incubation in 1ml of warm nutrient rich media (LB). When cells were transformed with plasmid carrying kanamycin resistance gene (pET_GFP_mut2_KAN®), recovery- incubation time was for 1 hour, but when cells were transformed with plasmid carrying ampicillin resistance gene (pSC101_GFPΔscar-let_AMP®) recovery- incubation time was for half an hour.

After the period of incubation, the cells were centrifuged for 30 seconds at 15000 rpm, 900 µl of supernatant was removed, and the pellet was resuspended in remaining 100 µl of LB and plated on LB agar plates containing the appropriate antibiotic for selection (25 µg/ml of kanamycin or 100µg/ml of ampicillin).

Successfully transformed bacteria formed colonies on selective agar plates, and one was taken for making 8% DMSO stock (described above).

3.1.4 Optimization of bacterial growth

Bacterial growth was tested in both liquid and solid nutrient media (with addition of agar up to 1.5%).

In case of the liquid media, we tested the growth in LB at static (in incubator 37 °C) and shaking conditions (37 °C, 120 rpm). Bacteria from DMSO stock was 100x diluted into LB medium, with addition of selective antibiotic (kanamycin or ampicillin, 25 and 100 µg/ml respectively).

Bacteria transformed with pSC101_GFP_Δscarlet_AMP® plasmid can constitutively express green fluorescent protein (GFP), while bacteria transformed with pET_GFP_mut2_KAN®, need 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to be added into growth media to induce GFP expression.

For optimization of bacterial growth on solid nutrient media we had used different types of media: lysogeny broth (LB), super optimal broth (SOB), trypticase soy broth (SOY), mueller-hinton broth (MHB), and 2x yeast tryptone broth (2x YT). From the DMSO stock, 100 µl of bacteria was directly taken and used for spreading on the top of agar plates, using a glass spreader.

The next day, bacterial samples were prepared for flow-cytometry analysis (FACS) (described below in the text) and for determination of colony forming units (CFUs). Firstly, we measured an OD_{600} of liquid cultures, and adjusted it to be 1 in 1xPBS (corresponds to10^9

CFU/ml). For bacteria growing on top of solid media, we used plastic loops to collect and inoculate them into 1xPBS, and eventually OD_{600} was also adjusted to 1. Afterwards, serial dilutions were made to adjust bacterial concentration to 10^6 CFU/ml (optimal for FACS analysis). Samples were further prepared for flow cytometry analysis and appropriate dilutions plated on LB agar plates (with glass beads) for CFU/ml determination. All experiments were repeated three times.

3.1.5 Fluorescence-activated single cell sorting (FACS)

Flow cytometry is a method that enables detection and quantification of characteristics of cell populations or particles using a flow cytometer. A flow cytometer analyzes cell or particle populations by passing them singularly through lasers using hydrodynamic focusing. The particles are examined for visible light scatter and fluorescence parameters. The light scatter can be measured in two directions, at 90° (side scatter, SSC) which shows the cell or particle granularity, or at forward direction (forward scatter, FSC) which is used to show relative size of the cell or particle. Samples analyzed with a flow cytometer usually express fluorescent proteins or are stained with fluorophores(McKinnon, 2018; Rahman et al., 2006).

Sample preparation*:* After adjusting concentration of bacteria to 10^6 CFU in 1ml of 1x PBS, 250µl of cell suspension was transferred into two Eppendorf tubes which were then centrifuged at 15000 rpm for 5 minutes. Pellet was dissolved in 1ml of 1xPBS. Eventually, in the one tube we added MM dye (Ex=558nm, Em=734nm) to final concentration of 2µg/ml. MM stains all the cell membranes red. In the second tube we did not add any dye. After 15 min of incubation at room temperature samples were analyzed using a flow cytometer (Attune NxT Flow Cytometer).

3.1.6 Bladder cell infection protocol

For establishing the model of *in vitro*-cell culture infection model we used bladder epithelial cancer cell line 5637 (ATCC® HTB-9™). Bladder cells were seeded in 12- well plates, in 2ml of RPMI-1640 (ThermoFisher) media, supplemented with 10% fetal bovine serum (FBS), at concentration of $4*10^4$ cells/ml. Plates were then incubated at 37°C in presence of 5% CO_2 for two days, so the confluency of cells on the day of infection was about 80-90% (coverage of the well bottom). On another hand, bacteria for infection (CFT073_pSC101-GFP_Δscarlet amp®) was grown at static conditions in LB broth for 24 hours, then 100x diluted into fresh LB medium and grown for additional 24 hours. We also tested in infection model bacteria that was grown on the surface of solid media (LB agar plate) for 48 hours.

Counting of bladder cells: On the day of infection, one well in 12- well plate was used for the determination of number of HTB9 cells. Cells were first detached from well bottom using trypsin 0.5 % with EDTA (PAN Biotech) and resuspended in total volume of 2ml in RPMI 10% FBS. 10 µl of cell suspension was further mixed with 10 µl of trypan blue dye, incubated for 5 minutes at room temperature. Automated cell counter (Invitrogen^{TM} Countess) was used to define the number of bladder cells.

Infection protocol: For infection of bladder cells, we used multiplicity of infection 50 (MOI50; 50 bacterial cells per one eukaryotic cell). 2 ml of RPMI media with 10% FBS, containing bacteria, was added into each well on top of the bladder cells. Plates were then centrifuged at 1000 rpm for 5 minutes to provide better attachment of bacteria to the cell surface. Followed by 2 hours incubation at 37°C in presence of 5% CO_2, allowing bacterial internalization. Non-phagocyted bacteria were killed by 2 hours incubation in presence of 100 µg/ml of gentamicin and washed with 1xPBS. After this step, initial infection rate (Time zero CFU/ml) was determined by lysing of bladder cells in 1ml of filtered (0,20 µm) distilled water. Half of the lysate was used for CFU determination and the other half for FACS analysis. The rest of infected wells were incubated for additional 24 hours at 37°C, 5% CO_2 incubator. To prevent extracellular contamination infected cells were incubated with 10 µg/ml of gentamicin. After the 24 hours incubation period, washing and lysing steps were repeated and samples were taken for CFU and FACS analysis.

3.2 RESULTS

3.2.1 OPTIMIZATION OF BACTERIAL GROWTH FOR CELL INFECTION ASSAY

As stated earlier, in this study, two bioreporters were used, all transformed in CFT073. The inducible bioreporter containing plasmid pET_GFP_mut2_KAN®, and the constitutively expressed bioreporter containing plasmid pSC101_GFPΔScarlet_AMP®.

To observe if it is possible to optimize bacterial growth without affecting the expression of the bacterial bioreporter, the bacteria was grown in different media and conditions. This is important in order to detect if bacterial growth conditions in general can affect bioreporter expression, detection and internalization. Afterwards, the growth and fluorescence levels were quantified and then compared.

3.2.1.1. pET_GFP_kan® IPTG inducible GFP

The bacteria transformed with plasmid pET_GFP_KAN® (IPTG inducible expression of GFP) was tested at first. This bacterial bioreporter is a lights-on-bioreporter (described under bioreporter section), and IPTG is its analyte.

In this part of the study, the bacteria containing the plasmid mentioned above were grown in LB agar, LB liquid shaking and LB liquid static conditions.

Flow cytometry (FACS) – single cell analysis

FACS analysis allowed us to analyze fluorescence and size properties at single cell level within bacterial populations, which were grown in different conditions.

Based on presence of green fluorescence, due to expression of GFP bioreporter, we could gate bacterial populations, like it is shown on the Figure 6A, B, C. Bacteria grown on LB agar surface and in LB liquid with shaking (6A, B) can be easily separated and distinguished from the noise ($<10^3$). In contrast, bacterial population grown in LB liquid static conditions (6C), which is commonly used in infection procedures, shows many events with very low GFP levels (close to the threshold). On histogram (6D) is presented overlay of these bacterial populations, and in case of static LB liquid conditions, there is clearly distinguishable sub-population (smaller peak) with low GFP signal.

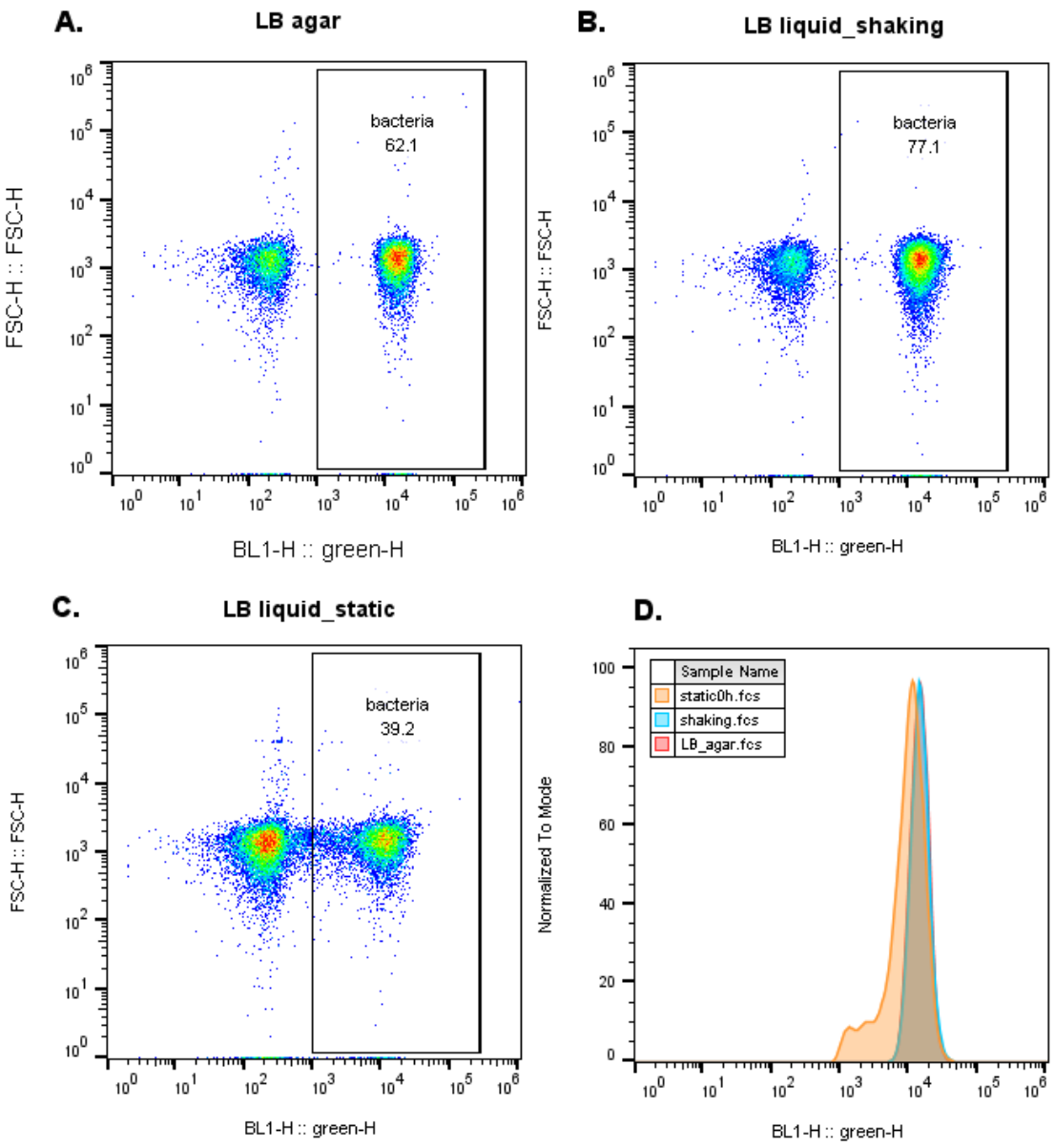

Figure 6. Flow cytometry analysis of bacterial populations expressing IPTG inducible GFP bioreporter. *Bacterial populations were gated based on presence of green fluorescence (above the threshold of 10^3). Gated bacterial populations, presented on panels A, B, and C were overlapped and presented in a histogram, shown on panel D.*

In order to find out if there are some bacteria that are not gated due to absence of green fluorescence (e.g., loss of plasmid) we used cell membrane dye MM 4-64. As explained in the materials and methods- flow cytometry section, MM dye stains cell membrane, hence, all the population.

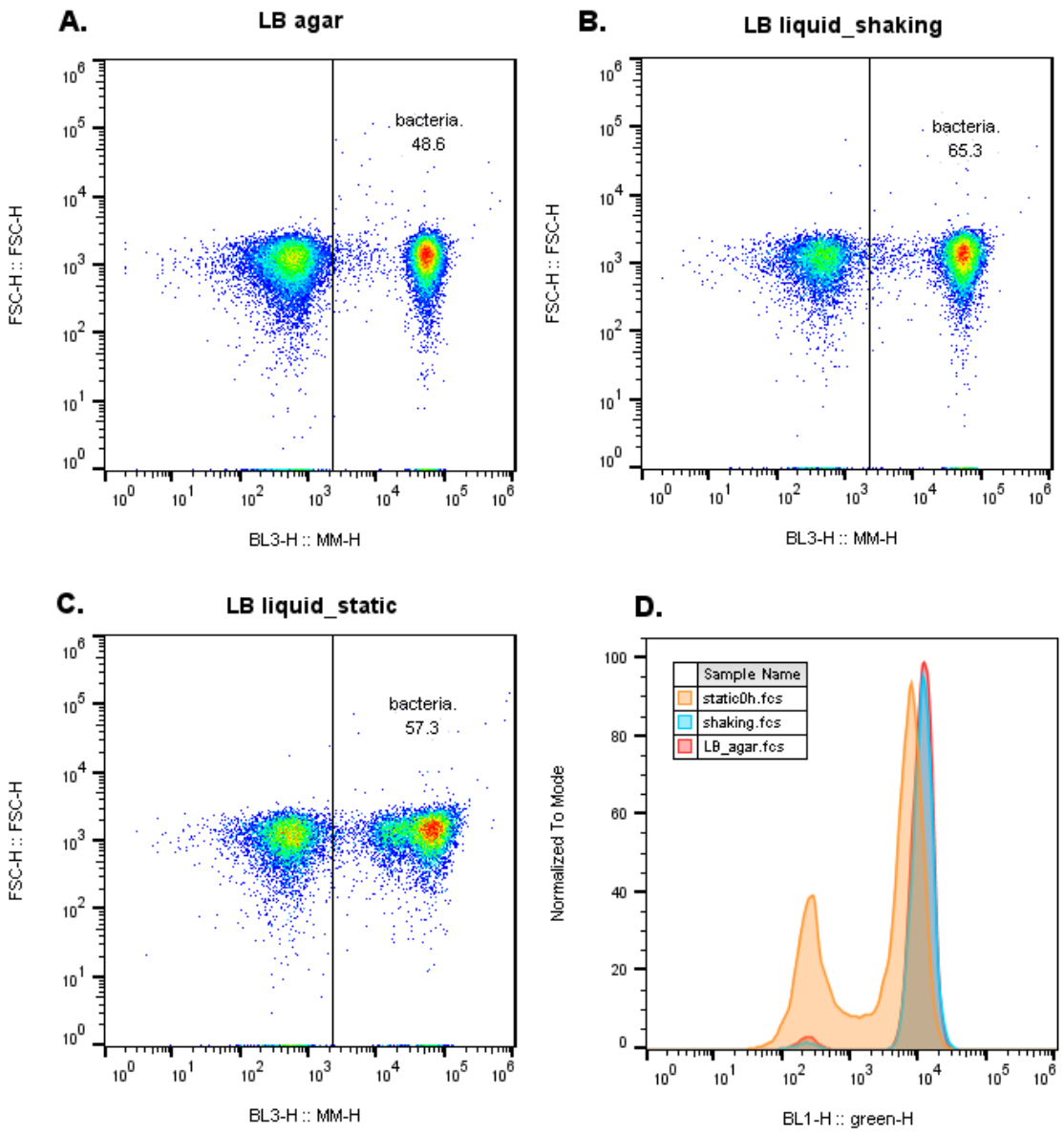

Figure 7. Flow cytometry analysis of bacterial populations using additional membrane dye MM4-64. *Bacterial populations were gated based on MM 4-64 staining of the cell's membrane above the threshold. On panel D, gated populations shown on panels A, B, and C were overlapped and checked using GFP parameters*.

Firstly, bacterial populations from three different pre-growth conditions, were gated based on presence of MM 4-64 dye -red fluorescence (Figure 7A, B, C). On their overlay, shown on Figure 7 D, bacteria grown in static liquid conditions is forming a huge subpopulation of cells that do not express green fluorescence, but have red fluorescence (membrane dye). Last but not least, we wanted test if cell viability is affected, by defining CFU/ml and comparing it with FACS events/ml.

Correlation between CFU/ml vs FACS events/ml

Subsequently, CFU- and FACS events per ml were defined and compared.

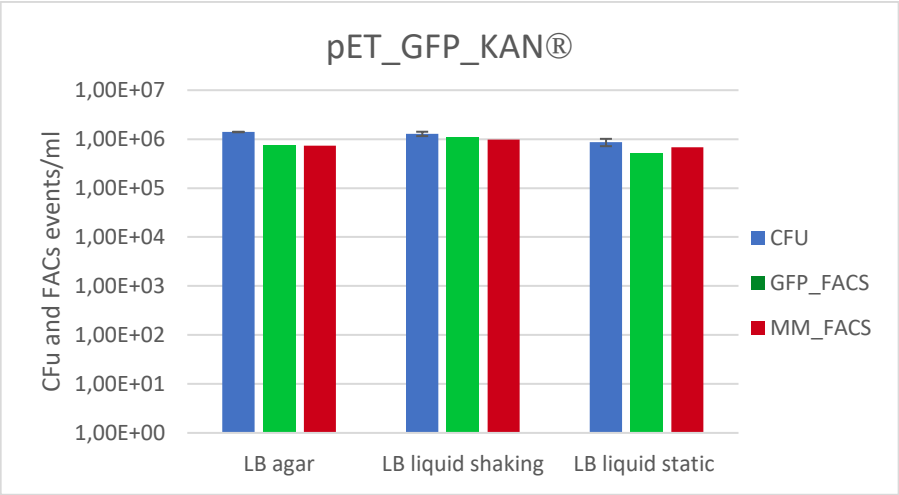

Figure 8. Comparison of CFU and FACS data of bacterial populations from three different pre-growth conditions. *CFU/ml is presented with blue bars. FACS events expressing GFP in green bars and events with red fluorescence (MM 4-64 dye) with red bars.*

In Figure 8, CFU count shows that all bacteria are viable, and the FACS events supports this data and correlates with it. However, in the case of LB liquid static, there is a slight decrease in GFP expressing populations.

3.2.1.2. pSC101_GFPΔscarlet AMP®

The next bioreporter that we have tested is a lights-off bioreporter (explained under bioreporter section), and it constitutively expresses it's GFP. Here, bacterial growth was examined on solid media (LB with and without AMP; SOY, SOB, MHB) and liquid LB media (static and shaking conditions).

Flow cytometry (FACS) – single cell analysis

FACS samples were analyzed in the same way as for previously examined bioreporter. On Figure 9 is presented gating of bacterial populations cultured in LB liquid (shaking, static) and on LB agar plates. However, gating of populations cultured in other types of media is presented in the appendix 1. In LB agar, LB liquid shaking, the bacteria expression of GFP is easily separated from the threshold ($<10^3$). On the other hand, bacteria grown in LB liquid static conditions (9C), show events with very low GFP levels (close to threshold). To see the shift in GFP expression intensity, we decided to make an overlapping histogram (figure 9D)

showing the three GFP expressing population that was gated. On the histogram, in the case of LB liquid static, there is a shift towards the left with low GFP signal (<10^3).

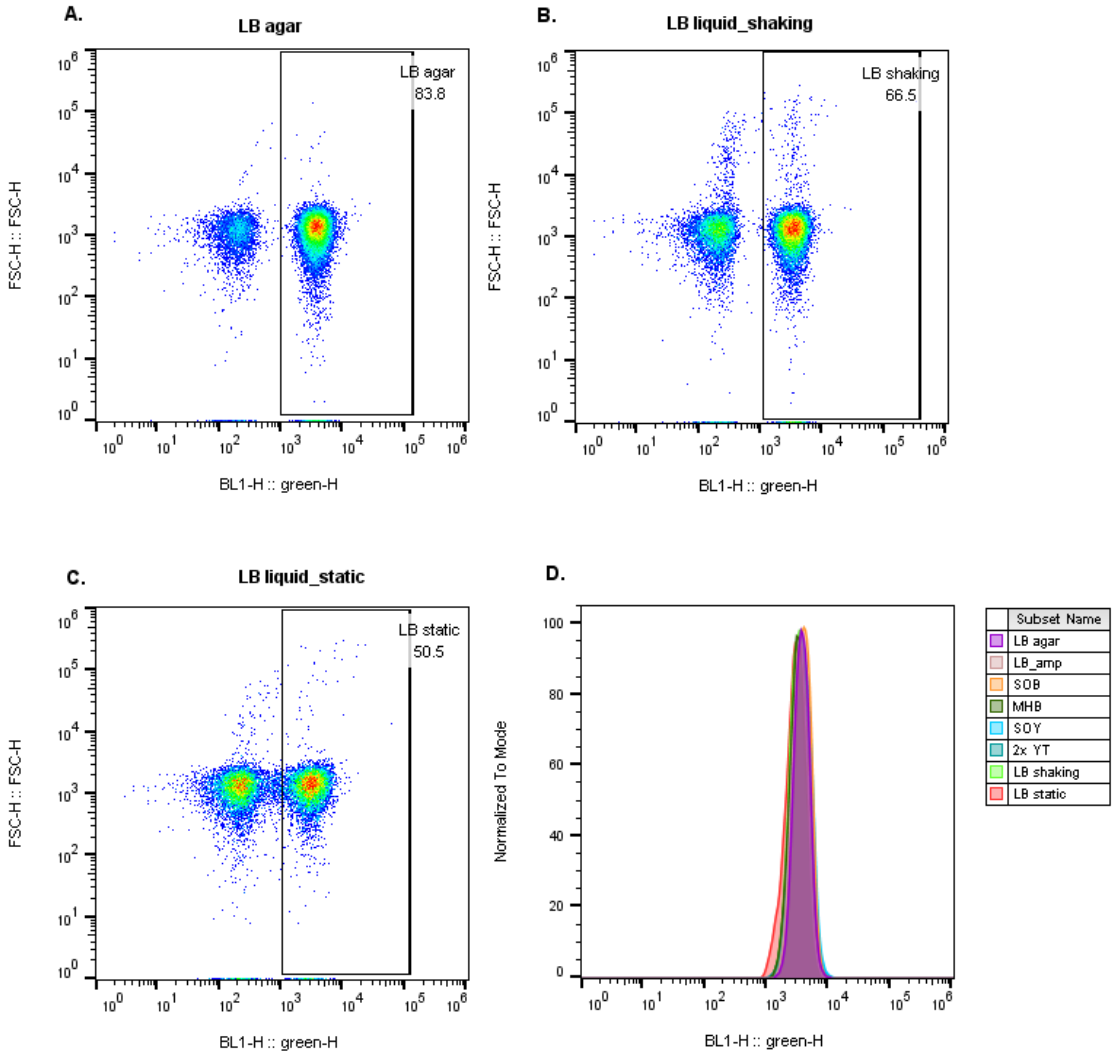

Figure 9. Flow cytometry analysis of bacterial populations of constitutively expressed GFP bioreporter. *Bacterial populations were gated based on presence of green fluorescence (above the threshold of 10^3). Gated bacterial populations, presented on panels A, B, and C were overlapped and presented in a histogram, shown on panel D. The rest of the tested media overlay is included, but gating data is in appendix 1.*

From this result, some bacteria grown in LB liquid static conditions are also below the threshold due to loss of GFP expression like in the first experiment. To see the population

of bacteria that were not gated due to absence of green fluorescence, we used MM 4-64 to stain the cell membranes.

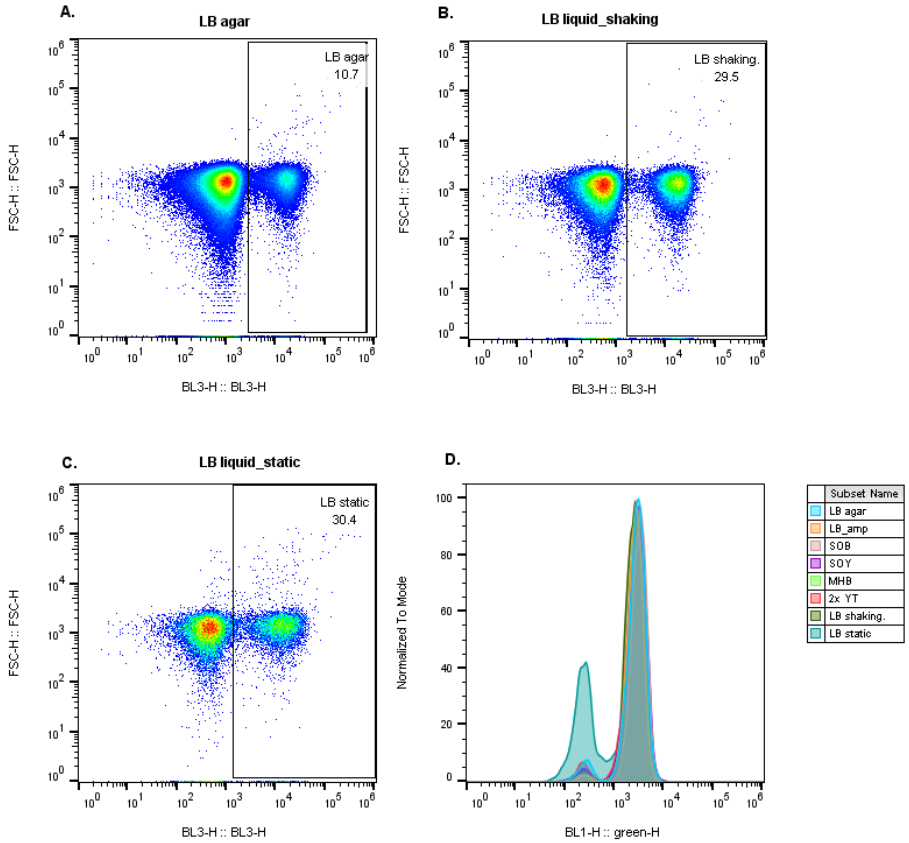

Figure 10. MM dyed populations. *Bacterial populations were gated based on MM 4-64 staining of the cell's membrane above the threshold. On panel D, gated populations shown on panels A, B, and C were overlapped and checked using GFP parameters. The rest of the tested media gating is in appendix 1, but the overlay is seen in Figure 10D.*

Firstly, bacterial populations from three different pre-growth conditions, were gated based on presence of MM 4-64 dye or red fluorescence (Figure 10A, B, C). On Figure 10 D, bacteria grown in static liquid conditions is forming a huge subpopulation of cells that do not express green fluorescence but have red fluorescence (membrane dye). Finally, we checked cell viability, by defining CFU/ml and comparing it with FACS events/ml.

CFU vs FACS

In the graph below, the CFU and FACS bacterial populations were compared.

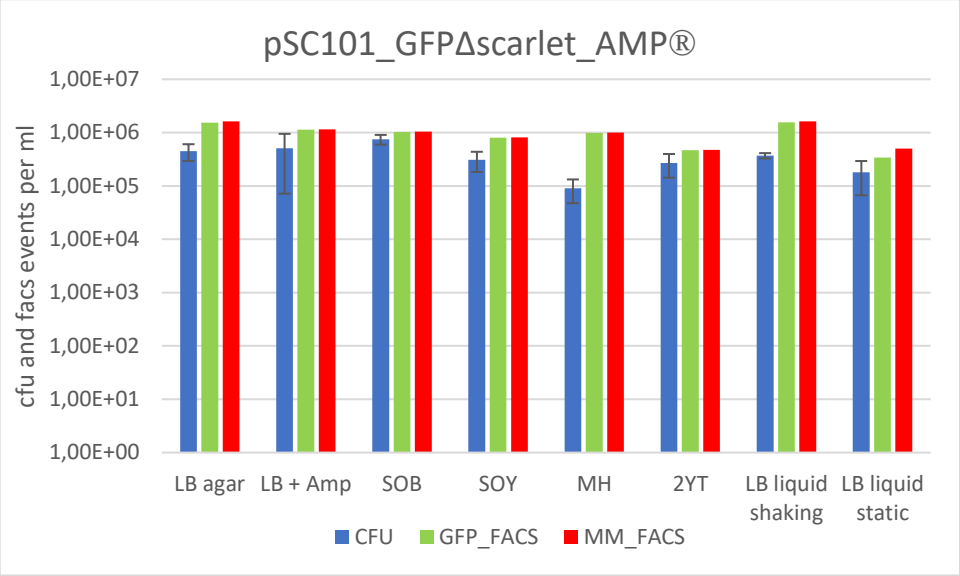

Figure 11. CFU vs FACS data comparison of bacterial populations from three different pre-growth conditions*. As the legend above describes, CFU/ml is shown with blue bars. For FACS events, green bars show GFP expressed events, and red bars show population expressing red fluorescence.*

Overall, high bacterial bioreporters growth and GFP expression are not mutually exclusive and is achievable as seen in bacterial bioreporters grown on LB agar. With this bioreporter, as noticed in the previously tested bioreporter, LB liquid static grown bacteria tends to lose more fluorescence expression compared to other conditions.

3.2.1.2 Cell culture infection assay

Apart from testing bioreporter's fluorescence levels under different growth conditions, my supervisor Ivana Kerkez tested internalization rate using CFT073 strain transformed with pSC101_GFP_Δscarlet_AMP® plasmid and bladder cells.

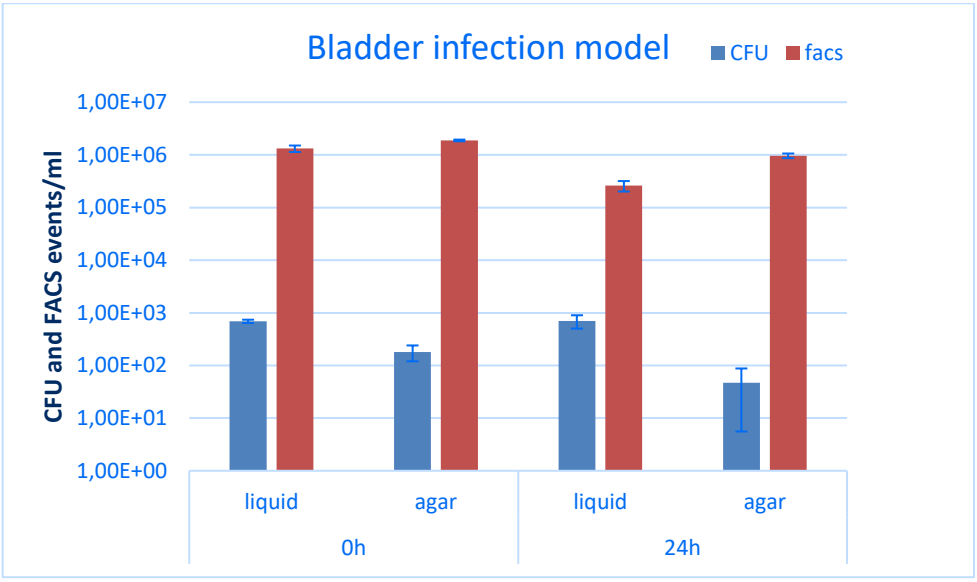

Figure 12. Effect of bacterial pre-growth conditions on internalization rate in bladder epithelial cells. *Samples for CFU determination (blue bars) and FACS analysis (red bars) were taken just after infection of cells (0h) and after 24 h of incubation with low concentrations of gentamicin (10 µg/ml).*

The internalization rate of the bacteria bioreporter mentioned above was tested. Bacteria grown on agar plates had a lower internalization than bacteria grown in LB liquid static conditions. Generally, there is a ≥3 log difference between CFU and FACS for both LB liquid static and LB agar grown bacteria. That might be because dead bacteria are firmly attached to the surface of the bladder cells and that's why they can be detected by FACS (because they were not lysed) but they do not form colonies.

3.3 DISCUSSION

Bacteria transformed with the two bioreporters (IPTG induced bioreporter, constitutively expressed bioreporter) exhibited unhampered growth in all examined growth. However, we noticed that some growth conditions might affect fluorescence expression.

According to the FACS analysis, both IPTG induced bioreporter and constitutively expressed bioreporter exhibited more loss of GFP fluorescence expression in LB liquid static growth compared to the other growth conditions. It was surprising to encounter these results because in most previous researches, all infections were always done with bacteria grown in LB liquid static conditions e.g., (Blango & Mulvey, 2010; Justice et al., 2004).

However, maybe we found a solution, our data suggests that for cell infections, bacteria grown on LB agar is more suitable, as fluorescence is not lost.

Furthermore, after infecting bladder cells with the bacteria, we found a compromising result. The LB agar cells had slightly lower internalization rate compared to LB liquid static grown bacteria. Our infection model showed big FACS – CFU difference suggesting that most were dead and attached to the surface of the bladder cells. To use this data in the future, we must find a better way to properly lyse the extracellular bacterial cells.

The entire results imply that even if we had great bacterial bioreporters growth or GFP expression during the initial optimization, the same cannot be said for its internalization, and reporter expression in the bladder cells. A classic example is seen in the lower internalization rate of bioreporters grown in LB agar conditions using constitutively expressed GFP bioreporters. Another example is seen in LB static grown bacteria bioreporter. One possible hypothesis is that some of the liquid static grown bacteria used their ATP to synthesize pili instead of expressing GFP, and might have lost the plasmid since it was of no use. Bacteria are highly adaptable organisms, and there is no surprise if this is the case. But these must be subject to further research for confirmation.

While there has been little documented study on the optimization of bioreporters for usage in cell culture infection models, getting these results means a lot for the further development of UTI studies. It means easier observation of the intracellular lifestyle of bacteria without having to inoculate for several days and harvest organs for inspection. If these bioreporters could be further developed for cell culture infections, it will create a great way to study and combat UTIs without animal cruelty, high expenses, or time consumption.

SUMMARY

Whole cell bioreporters are slowly gaining its importance in cell culture infection models. They can be used to detect different bacteria internalization rate and make the study of cell infections easier.

In this study, we used two bioreporters, one was an inducible bioreporter (IPTG inducible GFP bioreporter) transformed with plasmid pET_GFP_KAN®, and the other was a constitutively expressed bioreporter (constitutively expressed GFP) transformed with plasmid pSC101_GFPΔscarlet_AMP®. The bioreporters were selected with their antibiotic markers, and bacterial growth conditions were subsequently optimized.

The optimization experiment included the growing of bacteria overnight in different media and conditions – on lysogeny broth (LB), mueller-hinton broth, super optimal broth, trypticase soy broth, and in LB liquid static, LB liquid shaking conditions (200 rpm) all in 37°C. Afterwards, the bioreporter samples were analysed using CFU and FACS.

Both bioreporters carrying E. coli strains grow well in all tested condition. But one thing that was not expected was the lower fluorescence expression in LB liquid static grown bioreporter compared to the other growing style. This was not expected because this is the style normally used for growing bacteria in various research infection assays.

When the bladder cells were infected with these bioreporters grown on the LB agar had nice internalization and GFP expression in the bladder cells. Bacteria from LB static growth condition had slightly better internalization, but lower GFP fluorescence. One theory is that some of the liquid static grown bacteria used their ATP to synthesize pili in the liquid media instead of expressing GFP and might have lost the plasmid since it was of no use.

In conclusion, this study shows that LB agar grown bacterial bioreporter is the best for infection assay and can be used in cell culture infection experiments in the future.

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Appendix

I. GFP and MM 4-64 gating of SOB, SOY, MHB, LB agar (with and without AMP)

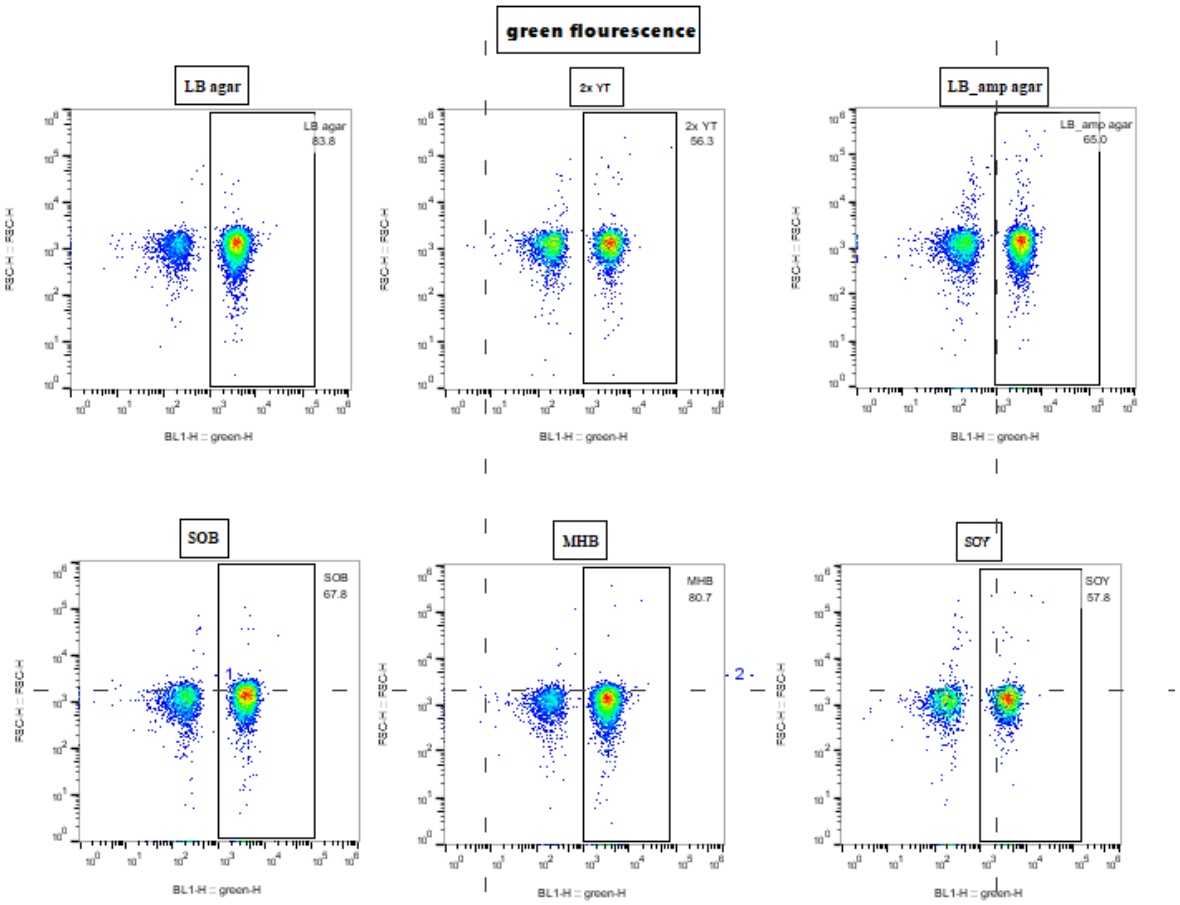

Figure 13. FACS analysis of bacterial population constitutively expressing GFP. *Bacterial populations were gated based on the expression of GFP. LB agar was added here to show similarity in GFP populations*

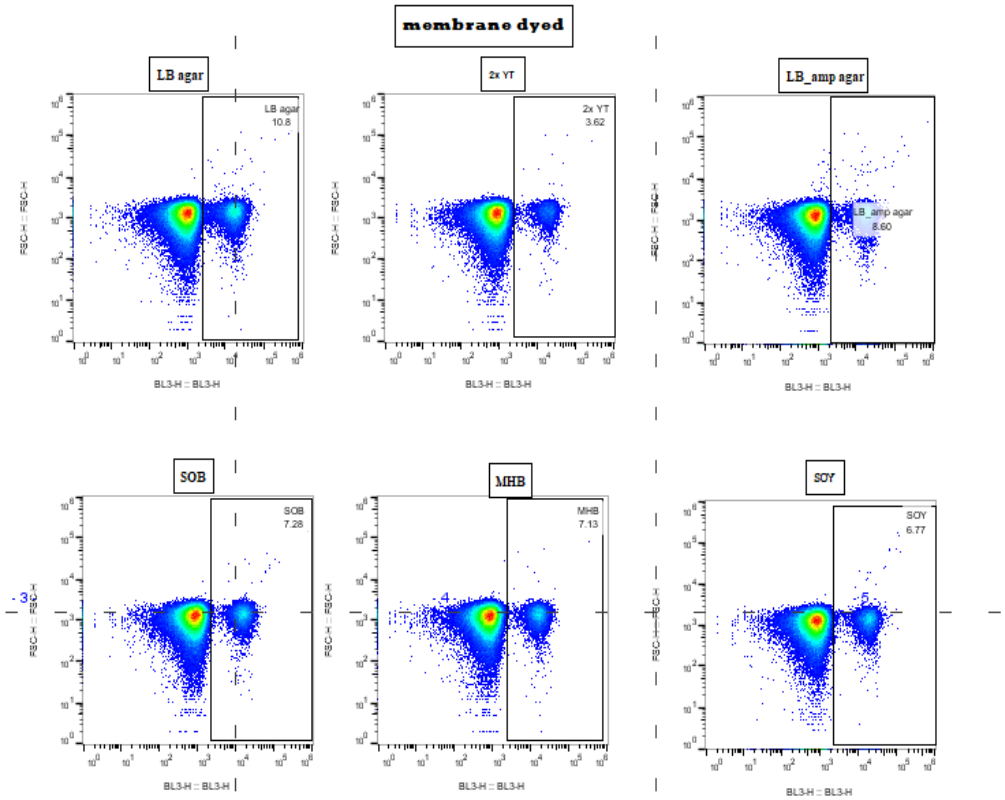

Figure 14. FACS analysis of bacterial population constitutively with MM stained membranes. *Bacterial populations were gated based on MM 4-64. LB agar was again added here to show similarity in MM populations*

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20/05/2021