Dual fluorescence system for analysis of transcriptional response in multi-species planktonic and biofilm context

Miao, Huang

2009

Miao, H. (2009). Dual fluorescence system for analysis of transcriptional response in multi-species planktonic and biofilm context. Doctoral thesis, Nanyang Technological University, Singapore.

https://hdl.handle.net/10356/14964

https://doi.org/10.32657/10356/14964

NANYANG TECHNOLOGICAL UNIVERSITY

DUAL FLUORESCENCE SYSTEM FOR ANALYSIS OF TRANSCRIPTIONAL RESPONSE IN MULTI-SPECIES PLANKTONIC AND BIOFILM CONTEXT

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A thesis submitted to the Nanyang Technological University in fulfillment of the requirement for the degree of Doctor of Philosophy

2009

Acknowledgements

Doctoral study is like a long distance running and it would be impossible for me to reach here without the support of many people, who have never left me alone on the track.

My upmost gratitude goes to my supervisor, Dr. Sze Chun Chau, for leading me into the fascinating research of bacteriology, for the personalized and valuable teachings. Her scientific and cheerful attitude is influential to me. Every time when I was totally lost, her insight can always point me a direction where I can have less stumbles. Apart from research, I also thank her for caring my life over these years.

I want to extend my thanks to the members in Dr. Sze's lab, past and present. Thanks go to Shalini Ratnasingam, Malan Manish Desai and Tiong Chi Tze who contributed in the construction of promoter fusion plasmids; thanks go to Pu Chui San who contributed in the co-culture promoter assay; thanks also go to Chew Ley Byan and Chong Li-en who contributed in the *AsRed2* mutant library. I also thank them, as well as Chin Hooi Ming, Chan Sock Hoai, Yang Nan, Dr. Bhalla Ritu, for constant encouragement and warm friendships.

I thank Lee Cailing for assistance with flow cytometry. I thank my friends in School: Huang Yafen, Huang Dandan, Yang Ye, Hu Guangan for all the happy moments spent together. I also thank my boyfriend Lan Li for constant care and efforts to make me laugh.

I'm deeply indebted to my parents: Wang Jianhua and Miao Xinmin for giving me freedom to pursue my dream, for having belief in me and unconditional love.

Lastly, I thank Nanyang Technological University for research scholarship, and A*STAR Biomedical Research Council for the support of this project under Grant No. 04/1/22/19/357.

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Journal Paper

1. **H. Miao**, S. Ratnasingam, C. S. Pu, M. M. Desai and C. C. Sze (2009) Dual fluorescence system for flow cytometric analysis of *Escherichia coli* transcriptional response in multi-species context. *Journal of Microbiological Methods, 76(2): p.109-119*

Meeting proceedings

1. **H. Miao**, M. M. Desai, S. Ratnasingam, C. S. Pu, and C. C. Sze (2006) System for analyzing transcriptional responses of *Escherichia coli* in a multi-species context. *1st Korea-Singapore Conference on Bioscience & Biotechnology*, December 2006, Singapore. Poster Presentation

2. **H. Miao**, M. M. Desai, S. Ratnasingam, C. S. Pu, and C. C. Sze (2007) An in vitro system for analysis of transcriptional response of *Escherichia coli* in a multi-species biofilm context. *4th ASM Conference on Biofilms*, March 2007, Canada. Poster Presentation

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Summary

Bacteria found in various ecosystems are often multi-species in context and the interspecies interactions are likely to lead to complex intracellular changes not observed in single-species cultures. Here we describe a dual fluorescence system that allows analysis of transcriptional responses of *Escherichia coli* as influenced by other species. We used two other species reported to be members of the intestinal microbiota of neonates in addition to *E. coli*: *Klebsiella pneumoniae* and *Enterococcus faecalis*. Multi-species co-culture condition was established. *E. coli* strain MG1655 was genetically manipulated to generate strain SCC1, which constitutively expresses green fluorescent protein. Plasmids carrying promoters of interest, fused to a red fluorescent protein gene (*AsRed2*), were introduced into strain SCC1. When a co-culture of *E. coli* strain SCC1 carrying promoter-*AsRed2* fusion and a non-*E. coli* strain was analyzed by FACS, it enabled (i) distinction of *E. coli* SCC1 from the non-*E. coli* strain, (ii) analysis of the *E. coli* promoter activity via AsRed2 expression and (iii) identification of transcriptional heterogeneity within the *E. coli* population. Spatial distribution of promoter activities in biofilm co-cultures can also be visualized via CLSM. This system has revealed that *E. coli fadB* and *rpoE* transcription were differentially influenced by the partner species under various growth conditions. Exploration to develop this established system further is presented and discussed. This is the first analysis system reported to date to allow transcriptional response due to bacterial interspecies interactions to be studied, even when the species to be analyzed is a severe minority.

Abbreviations

A	adenine
AHL	acyl-homoserine lactone
Ap^r	ampicillin resistance
ATCC	American Type Culture Collection
BHI	Brain-Heart Infusion
bp	base pair
C	cytosine
CFU	colony forming unit
cm	centimeter
Cm^r	chloramphenicol resistance
dd H_2O	deionized distilled water
EB	ethidium bromide
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
E. faecalis	*Enterococcus faecalis*
EP-PCR	error-prone PCR
FACS	fluorescence-activated cell sorter
FP	fluorescent protein
G	guanine
Gfp	Green fluorescent protein mutant No.3*
gfp	gene of green fluorescent protein mutant No.3*
h	hour
K. pneumoniae	*Klebsiella pneumoniae*
kb	kilobase(s)
LA	Luria-Bertani agar
LB	Luria-Bertani
mRNA	messenger RNA
µg	microgram
µl	microliter
min	minute
ml	milliliter

MOPS	3-(N-Morpholino)propanesulfonic acid
MRS	deMan, Rogosa and Sharpe
NB	Nutrient Broth
ng	nanogram
nt	nucleotide(s)
OD_{600}	optical density at 600 nm
PCR	polymerase chain reaction
QS	quorum sensing
RBS	ribosomal binding site
rcf	relative centrifugal force
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	room temperature
s	second
SD	standard deviation
T	thymine
UTI agar	HiCrome UTI agar plate
v/v	volume per volume
w/v	weight per volume

Chapter 1 Introduction

1.1 Modes of bacterial growth

1.1.1 The planktonic mode

The planktonic form of bacteria is characterized by being unicellular, homogeneously suspended and free-living in a liquid medium. The development of the planktonic single-species laboratory culture by Koch in the mid 1800s [1] has been instrumental in winning the war against epidemic bacterial diseases. Since then, the majority of the knowledge about bacterial physiology such as bacterial structure, growth, metabolism and genetics has been accumulated through the studies of planktonic cultures.

There are two common techniques of culturing planktonic bacterial cells. In a batch culture, a small inoculum of bacteria in a fixed volume of fresh growth medium is cultured with shaking to provide the proper level of aeration and at temperatures as required by the specific species. The population of bacteria in a batch culture would go through three distinct phases: (i) lag phase, when bacteria are adapting from its previous state to the new environment, with no or little cell division; (ii) exponential phase, when bacteria undergo binary fission at a steady state and the generation (or doubling) time can be calculated from this phase; (iii) stationary phase, when the population enters a plateau because of nutrient depletion and metabolic waste accumulation. Another technique is the chemostat culture (also known as continuous culture). Bacterial cells are grown in a bioreactor in which fresh medium is continuously added while culture liquid is continuously removed at the same rate [2].

1.1.2 The biofilm mode

Most of the research has been solely focused on planktonic bacteria for over a hundred years before the early 1980s, and only since then that due attention was given to biofilm [3-5], another mode of bacterial growth. Biofilm is defined as "microbially derived sessile communities characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit altered phenotypes with respect to growth rate and gene transcription" [6]. Compared to the planktonic counterpart, which is the mode mainly cultured in laboratory, biofilm is the more prevalent form of bacteria existing in the environment. Growing into a biofilm confers to the bacteria some advantages, such as protection from the environment, metabolic cooperation and acquisition of new genetic traits [7]. Apart from the phenotypic disparities, gene expression is also very different between biofilm and planktonic cultures [8-10]. It has been speculated that the planktonic mode of growth that is found in nature corresponds to one specific stage in the biofilm life cycle (Fig 1.1.), i.e. when the bacterial cells detach and disperse from the matured biofilm and swim to colonize new sites [11].

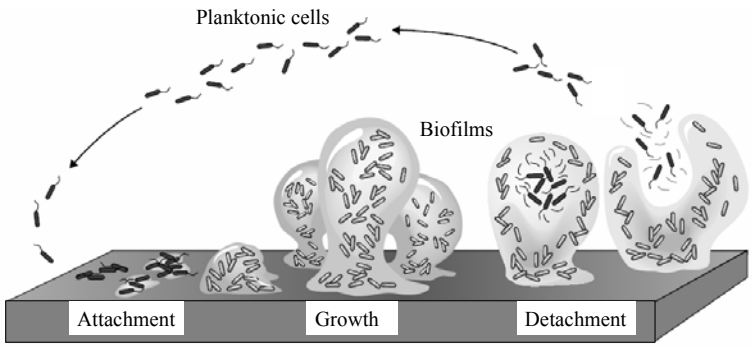

Fig 1.1. Schematics of the biofilm life cycle. Planktonic cells first attach to a surface, followed by cell division and maturation of biofilm architecture and finally dispersion of single cells from the biofilm. Adapted from [11] with minor modifications.

1.1.3 Single-species versus multi-species systems

Both planktonic and biofilm modes of growth have been studied extensively for a long time in single-species cultures. However, bacteria outside of laboratory rarely live a solitary life. Instead, they coexist with other species to form microbial communities, such as those within animal guts, oral cavities, soils, natural and artificial water systems and other environments [12]. In industrial applications such as biodegradation, for example, existence of multiple species is unavoidable, and even welcomed from the point of view of process efficiency [13]. Hence, from both the basic and applied perspective, studies on multi-species systems are greatly needed to answer important biological questions. Imperative as it is, research in this field has been limited to only a few approaches, largely due to the complexity associated with system set up and analysis. Interspecies interaction in multi-species system is especially interesting, because it is the interaction between the component species that is critical to determining the property of the multi-species system [14, 15].

1.2 Approaches to studying interspecies interactions

1.2.1 In situ studies

In order to obtain information on interspecies interactions within the natural environment, one approach is to characterize the existing species within various natural communities. Another approach is to study the metabolic dynamics of the bacteria in the consortia.

Cultivation efficiency of bacteria from natural communities are notoriously low [16], so in order to study the biodiversity in multi-species natural communities,

methods based on cultivation-independent analyses have to be employed. For example, polymerase chain reaction (PCR) combined denaturing gradient gel electrophoresis (DGGE) can perform community fingerprinting. PCR/DGGE separate PCR-amplified DNA fragments (i.e. 16S rDNA) based on the melting behavior of DNA [17], and it is used to analyze and compare biodiversities from different samples [18]. 16S rRNA phylogenetic analysis is utilized to characterize the environmental isolates by sequencing the PCR-amplified 16S rDNA [19]. Fluorescence in situ hybridization (FISH) is another common approach to visualize the biodiversity within a community [20]. The knowledge of bacterial rRNA based phylogeny allows designing of fluorescence labeled oligonucleotide probe to identify and quantify key players in complex communities. It has been successfully applied to individually detect microbial cells in situ in multi-species consortia [21]. Global approaches, such as metagenomics and metaproteomics are other emerging alternatives to reconstruct species diversity in microbial consortia [22, 23]. These approaches, in addition to providing a catalogue of the biodiversity, could also reveal interaction-related information, e.g. that certain bacterial species can alter its genome make up via DNA recombination with closely associated species as one of the survival strategies in extreme environments [24-26].

Metabolic dynamics studies have led to the understanding of syntrophic relationships within the natural consortia. For example, by measuring the dissolved H_2 concentration in sewage sludge and lake sediment, it was found that most H_2-dependent methanogenesis in those ecosystems occurs as a consequence of direct interspecies H_2 transfer made possible by juxtapositioned

microbial associations between H_2 consumers (e.g., methanogens) and H_2 producers (e.g., acetogens) [27]. FISH and microelectrodes have been utilized to study microbial nitrification (oxidation of ammonia to nitrate via nitrite) in domestic wastewater biofilms and they are capable of showing the clustering of nitrite-oxidizing bacteria around ammonia-oxidizing bacteria and in situ nitrification activity [28]. It has also been revealed through a metagenomic approach, that multiple bacterial partners behave cooperatively during syntrophic cycling of oxidized and reduced compounds to provide their eukaryotic host with an optimal energy supply and waste management [22].

1.2.2 In vitro studies

Although in situ studies are valuable because they are able to show bacteria behaviors in natural communities, it is limited in the types of biological questions that can be answered with respect to interspecies interactions. The difficulty is mainly due to the complex influences that are expected to exist when such a large number of species interact in the natural environment. In vitro studies can analyze interspecies interactions in a more dissective manner, thus bring broad knowledge to complement what we have learnt from in situ studies. To date, there are mainly three in vitro approaches in the studies of interspecies interaction: analyses of growth dynamics, metabolic interaction and cell-cell communication.

1.2.2.1 Growth dynamics

The techniques adopted for the study of growth dynamics are primarily for the purpose of growth evaluation within the co-cultures. For qualitative analyses, direct visual observation is possible if the growth dynamics is reflected by overall biomass at the macro level [29, 30]. With the aid of microscopy, the species composition and spatial distribution can be observed in details [31]. For quantitative analyses of bacterial growth, enumeration of colony forming unit (CFU) was often performed for viability count [30, 32, 33]. Optical density at 600 nm (OD_{600}) of suspension sample [29] and wet weight of biofilm sample can be used to quantify biomass [30]. FISH has also been applied in combination with microscopy to enumerate bacteria in biofilm chambers [34]. These methods are utilized to generate information about growth dynamics in co-cultures. There are two approaches in those studies. One is to monitor the establishment process of multi-species systems and another one is to examine the growth relationships of various species within the established system.

The establishment process of multi-species system is well studied in oral biofilms, because coaggregation (the recognition between genetically distinct cells in suspension and the resultant clumping) is fundamental for dental plaque formation [35, 36]. Using a flowcell with saliva-coated surfaces, coaggregation sequences of the oral bacteria have been studied extensively [37, 38]. Apart from oral biofilm researches, there were also reports on two-species systems in which the colonization properties of incoming planktonic cells of one species with pre-established biofilm of another species have been investigated [32, 39].

A number of researches concentrate on the growth interaction within the co-cultures after the establishment of its population equilibria. Competition is one common outcome of such interaction, as bacteria have to compete with other community members for the nutrient for their own fitness. Growth inhibitions, which operates in effective competition, can be specific in some cases, for example, bacterocin produced by some enteric bacteria are narrow-spectrum to closely related bacteriocin-sensitive bacteria [40]. In other cases, the inhibition can be broad-spectrum, such as the antibacterial compound AlpP from *Pseudoalteromonas tunicata* [31], group II capsular polysaccharide secreted by *E. coli* uropathogenic strain CFT073 [29] and the various antibiotics produced by soil bacteria [41].

Interactions other than those that result in competition have also been documented. Commensalism is one of them, in which the growth of one species benefits from the presence of another, while the "benefactor" remains unaffected. During the biodegradation of phenol by planktonic co-culture, growth of *Pseudomonas putida* F1 is enhanced by *Burkholderia* sp. JS150 [34]. Biofilm growth of *Lactobacillus* species is promoted by *Actinomyces* species and *Streptococcus mutans* [30]. Synergistic interactions, in which the benefit is mutual to both parties were also reported. For example, two early colonizer strains of tooth surface – *Streptococcus oralis* 34 and *Actinomyces naeslundii* T14V – allow each to grow in conditions where neither can survive alone [33].

1.2.2.2 Metabolic interaction

Focusing on metabolic interactions in multi-species systems is another approach to study interspecies interaction. Techniques utilized in this approach of study mainly have the purpose of visualizing the spatial distribution of the component species in the system, analyzing the metabolites and studying the underlying gene expressions. Hence, in addition to the techniques mentioned in section 1.2.2.1 (on pages 6~7), other methods have been included, such as confocal laser scanning microscopy (CLSM) [42-44], high-pressure liquid chromatography (HPLC) [42, 44] and usage of promoter fusion reporter genes [44, 45].

In a system containing two or more species, food chain could develop with the end-product of one organism serving as an energy source for the other. Such direct metabolic interactions have been documented in *Pseudomonas putida*–*Acinetobacter* sp. co-cultures. *P. putida* was shown to dominate over *Acinetobacter* in a biofilm by growing underneath *Acinetobacter* to utilize the leaked intermediate products from above. However, it would lose the competition if both were cultured in homogenous chemostat culture [42]. This two-species biofilm is interesting because it evolves to be more productive in the sense of nutrient utilization, but this is at the same time balanced by the resulting closer proximity of *P. putida* bringing detrimental effect to *Acinetobacter* due to intensified competition for oxygen [43]. Similar metabolic interaction is also reported in other two-species systems. In *Burkholderia* sp. LB400-*Pseudomonas* sp. B13(FR1) co-culture, structured biofilm is not formed until the system is fed with carbon source usable to *Burkholderia* sp. LB400 but

not to *Pseudomonas* sp. B13, which then moves from the unassociated form to the associated form with *Burkholderia* sp. LB400 to utilize its metabolic products [44].

Apart from the exploitative nature of the above examples, metabolic interaction can also be beneficial to both parties. In *Streptococcus gordonii–Veillonella atypica* co culture, *V. atypica* is unable to ferment sugars, but uses lactic acid, which is a major end product of *S. gordonii. V. atypica* induces expression of *S. gordonii* α-amylase-encoding gene *amyB* for more efficient sugar fermentation [45]. In methane-utilizing culture, the existence of "contaminants" that cannot utilize methane is found to be vital for the system stability in that they can utilize the inhibitory by-products in the methane-utilization pathway [13].

1.2.2.3 Cell-cell communication

Another major approach in the in vitro studies of interspecies interaction is through the analysis of cell-cell communication. In addition to the techniques mentioned in the previous sections, mutant strains were often utilized for functional analysis related to this aspect of interspecies interaction [46-48].

In the studies of cell-cell communication, quorum sensing (QS) is an area most thoroughly studied. QS mediates coordinated behaviors of bacteria at the population level in response to cell density, such as virulence, increased competence or biofilm formation [49, 50]. One of the classes of QS signals, the acyl-homoserine lactone (AHL) found in many Gram-negative bacteria is the

most well-characterized [51, 52]. It is reported that the competition ability of AHL-deficient mutant strain is impaired in biofilm co-cultures [46]. The ability of the bacteria to counteract the QS signaling of their competitors can boost their advantages in ecosystems, and AHL-degradation eyzymes have been identified in some bacteria species [53].

Apart from AHL which is used by bacteria as self-perception tool to synchronize group behaviour, another QS signal AI-2 [54, 55] produced by a wide variety of Gram-negative and Gram-positive bacteria has also been reported. Bacteria can detect the presence of other species via AI-2 (synthesized by the gene product of *luxS*). It is speculated that AI-2 may enhance cooperation and therefore the survival of most of the bacterial species in a mixed community [56, 57]. For example, *luxS* mutants of both *S. gordonii* and *Porphyromonas gingivalis* are incapable of forming mixed-species biofilms, and restoration of *luxS* (thus AI-2 production) in either strain reverses the defect [47]. Similar result was reported in *Streptococcus oralis* 34–*Actinomyces naeslundii* T14V co-culture [48].

There are evidences that other cell-cell communications are likely to exist in multi-species communities. A diffusible un-identified signaling molecule, that can induce specific gene expression in *S. gordonii* for symbiotic interaction is found in *S. gordonii*-*Veillonella atypica* co-culture [45]. Virulence genes of *Pseudomonas aeroginosa* are upregulated by host indigenous microflora, not purely by quorum sensing but also through some unknown interspecies communication mode(s) [58].

1.2.3 In silico modeling

The approach of mathematical modeling is used to simulate interaction-driven phenomena, which provides a tool for understanding the nature of these interspecies interactions by comparing the experimental results to those predicted with various interaction scenarios. One of the early models is pure-and-simple competition models, which assume that the two microbial populations do not interact in any way except to compete for one growth-limiting substrate [59-61] The fact that biomass calculated from this model is not consistent with experimental data facilitates the discovery of commensal interactions in a binary culture for phenol biodegradation [34]. Actually, situations that fail to be predicted by simple competition models are not rare, because other interactions are likely to exist between the microbial species and more than one substrate should be expected to influence the growth of the microorganisms.

More complicated mathematical models taking into considerations of several levels of interactions, multiple species and multiple substrates, have led us to broader and deeper understandings [62, 63]. For example, a model describing interactions between nitrifiers, heterotrophs and predators in activated sludge is validated by experiment and served as a helpful tool to process optimization [64]. Although those models have taken various factors into account, it is still challenging to model most of the multi-species systems, especially the complex systems like natural ecosystems or industrial bioreactor communities. More knowledge is needed for in silico modeling to help elucidate those situations.

1.3 Differential gene expression

To date, the in vitro studies on multi-species systems have been focused mostly on phenotypic properties, such as growth dynamics, metabolic interaction, and cell-cell communication. The basis of the interactions mentioned above can ultimately be traced to the bacteria's gene expression profiles [65]. Differential gene expression of a species gives rise to its unique metabolic capacity and output [14, 65], which in turn, results in influences on another species' physiology and responses, manifested by alteration of gene expression of the affected species [66-68]. Only a few researches have been done to study differential gene expression as a consequence of interspecies interactions. One such study showed that a specific promoter related to aromatic compound degradation is activated in *P. putida* with the presence of *Acinetobacter* in biofilm cultures [69]. Another study demonstrated that a promoter related to sugar fermentation in *S. gordonii* is induced by *V. atypica* [45]. Both of these studies utilized promoters fused to green fluorescent protein reporter gene to monitor the promoter activities.

Gene expression in prokaryotes has its own set of features that are distinct from eukaryotes. Genes of related functions in prokaryotes are usually organized into a cluster as a single transcriptional unit, also known as an operon [70]. To begin transcription at a particular promoter (usually upstream of the first gene in an operon), RNA polymerase must first interact with a σ factor, which ensures the recognition of a specific promoter sequence [71]. Once started, transcription and translation are coupled to encode protein efficiently. Codon usage in bacteria is different from that of eukaryote and it also varies among bacterial

species [72]. Individual messenger RNA (mRNA) molecules function for only a few minutes before being destroyed by RNaseE [73, 74] and replaced by newer, thus more timely, information [75, 76]. It is not an easy job for bacteria to make the right product in the right amount at the right time, and for reasons of economy, the key step to regulate gene expression is often transcription initiation [77]. As a result, much researches on gene regulation have been focused on transcriptional regulation. The methods introduced below have been used commonly to study differential gene expression in single-species bacterial cultures.

1.3.1 Methods for studying differential gene expression at the transcriptional level

1.3.1.1 Microarray

Microarray is a powerful tool to allow quantitative comparison of the transcriptional activity of entire bacterial genome (via global mRNA expression) under different conditions [78, 79]. The technique has been used to search for differentially expressed genes in biofilm mode compared to planktonic mode [8-10]. However, the harvest of cellular material will result in averaged data of the population, thus this method is unable to yield heterogeity information within the sample.

1.3.1.2 Differential fluorescence induction (DFI)

Differential fluorescence induction (DFI) relies on the use of a reporter gene, e.g. gene of green fluorescent protein (GFP), to monitor promoter activity. Bacteria library with random transcriptional fusions to *GFP* are screened on the

basis of stimulus dependent synthesis of GFP [80]. DFI has been used to identify genes involved in pathogenesis [81-84]. For example, in vitro conditions were chosen to mimic in vivo environment encountered by *Streptococcus pneumoniae* when it is in the host. The genes whose promoters are induced under these conditions are likely to have virulence related functions [83].

1.3.1.3 In vivo expression technology (IVET)

In vivo expression technology (IVET) shares similarity with DFI in that it also utilizes random transcriptional fusions. However, in contrast to DFI, the random promoter elements are fused with a selectable marker gene which is required for survival in the host animal. As a result, infection-specific promoter elements can be identified in the strains recovered from the animal, and subsequently screened for lack of in vitro expression [85]. This method is more relevant to pathogenesis studies (host-microbe interactions) and less so for interspecies interaction studies.

1.3.2 Other methods for studying differential gene expression

1.3.2.1 Signature-tagged mutagenesis (STM)

Signature-tagged mutagenesis (STM) uses transposons with unique DNA tags to mutagenize bacteria to generate a mutant library [86-88]. The genomic DNA is extracted from mutant libraries cultured under different conditions (input pool and output pool). The DNA tags from the two DNA pools can be used for hybridization to identify the differentially expressed bacterial genes. The tag that appears in the input pool but not in the output pool indicates that the gene

mutagenized by the transposon is essential for survival in the condition of the output pool.

1.3.2.2 Proteomic methods

Another method used to analyze differences in gene expression is proteomics. Conventional proteomics utilized two-dimensional gel electrophoresis based on molecular weight and isoelectric point, to separate individual proteins in total protein extract. In this way, the differentially expressed proteins between the two samples are revealed, followed by mass spectrometry to identify the protein(s) of interest. Via this technique, differentially expressed genes between planktonic and biofilm modes of growth, and among different developmental stages of biofilm growth have been identified [89, 90].

1.4 Statement of problems

There is no dispute that it is important to study multi-species systems, but obstacles exist at different levels. In situ studies are able to unravel the species diversity in natural consortia and probe the interspecies interactions within the ecosystem. But this approach has to face the problem of daunting complexity. It is challenging to dissect the multi-factorial influences conferred by the environment and other species to the one species of interest. In comparison, in vitro studies can be better controlled and used to answer specific biological questions. However, the majority of in vitro studies are restricted to two-species cultures due to technical difficulties. In particular, knowledge about differential gene expression as consequence of interspecies interaction is limited.

Diverse and useful as the methods described in section 1.3 (on pages 12~15) may be in the study of differential gene expression, it is challenging to utilize them for studying interspecies interactions in multi-species systems. The majority of the work using these strategies has been focused on single-species cultures, conveniently without contribution of contaminating materials from the other species in multi-species backgrounds. In the few studies that did look into the multi-species context, the overwhelming cellular materials from the other species compromised the detection sensitivity for the minority species [23].

Another problem is the loss of information about gene expression heterogeneity from those methods, as the principles of their analyses are based on the averaged data obtained from the wholesale harvest of cellular materials. The perception about bacteria genetics that gene expression is homogenous within the whole population has been long-existing and dominating. However, in recent years, it has become evident that a single bacterial population can manifest two or more different behavioral or developmental patterns, even though all members are, in principle, exposed to the same set of environmental conditions [91]. This fact has been particularly well established in biofilms [92-95], but also demonstrated in planktonic cultures [91, 96].

1.5 Aim of the study

We aim to establish an analytical system that is able to complement the methods used to date, allowing bacterial interspecies interactions to be studied from the perspective of the transcriptional response of one particular species.

1.5.1 Conceptual design of the strain for analysis

To tackle the problem of contamination from other species during analysis of multi-species system, the species of interest would be genetically manipulated to have self-expressed green fluorescent protein. This "labeled" species can be distinguished from other species via emission of green fluorescence. A second fluorescence component, a red fluorescent protein reporter gene fused to the promoter of interest, would be introduced into the green-labeled species. This red fluorescent component will therefore be able to reflect the promoter activity as influenced by the other species in the multi-species system. The species being analyzed is conceptually referred to as the "reporter strain" because this strain "reports" the influence exerted by the other species (Fig 1.2.).

Fluorescence-activated cell sorter (FACS) would be used to analyze the transcriptional response in planktonic co-cultures. In FACS, bacterial cells can be analyzed one by one to quantify the fluorescence intensity and the analysis is high-throughput (thousands of events per second). The single cell resolution of FACS can reveal different subpopulations in the culture. With this advantage, the transcriptional response (red fluorescence) of the reporter species (green fluorescence) can be analyzed within the green population, without contaminating information from other species. The heterogeneous nature of

promoter activities, if any, can also be discerned by analyzing the red positive and red negative subpopulation within the green population. The high-throughput property of FACS also implies that even if the reporter species develops into a minority in the consortium, we should, in theory, still be able to analyze the reporter strain by running sufficient volume of samples through to collect statistically significant number of data of the reporter species.

In addition, confocal laser scanning microscope (CLSM) can reveal the heterogeneous spatial architecture of a biofilm in its naturally hydrated state, by taking scans at different focal planes throughout the sample [97-99]. Hence, it would be utilized in this study to visualize the promoter activities in biofilm co-cultures.

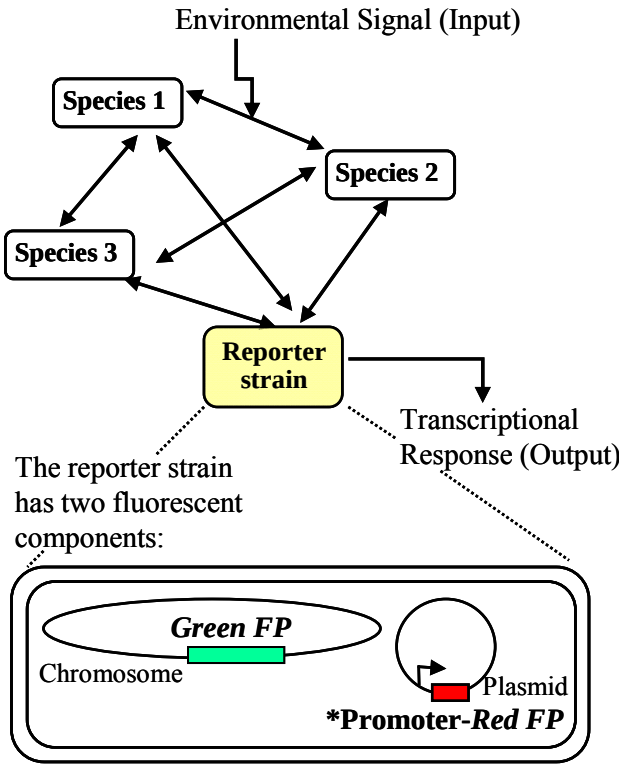

Fig 1.2. Schematics of the "reporter strain" with dual fluorescence components. The reporter strain has been designed to capture the transcriptional response that arises as a result of the interaction of several species. *Green FP* refers to constitutively expressed green fluorescent protein gene. *Promoter refers to the promoter of interest, which is fused upstream of a red fluorescent protein gene (*red FP*).

1.5.2 Biological systems used

1.5.2.1 Intestinal microbiota

Many multi-species systems are of interest to investigate, and in this study we focus on one of them: the intestinal microbiota. Intestinal microbiota refers to the community of bacteria in terminal ileum and the large bowel (cecum and colon) of gastrointestinal tract [100]. The gastrointestinal tract is sterile at birth but becomes colonized within hours. The microbiota in neonates is composed mainly of aerobic bacteria and facultative anaerobes, which quickly establish the low redox potential required to sustain anaerobic organisms. Upon weaning, a complex adult type microbiota, predominated by obligate anaerobes, becomes established [101]. The presence of the mucosal layer in the gastrointestinal tract serves as habitat and source of nutrients to the bacteria [102]. The commensal bacteria are reported to be beneficial to host [103, 104].

Majority of the studies for interspecies interactions in intestinal microbiota have been focused on those between pathogenic and commensal bacteria, which is clinically important for the battles against intestinal pathogens [105, 106]. It is reported that the virulence gene *aggR* of enteroaggregative *Escherichia coli* is varyingly regulated with the presence of different commensal species [107]. Interactions among the intestine commensal bacteria are also reported. For example, the presence of *Bifidobacterium longum* can elicit an expansion in the diversity of polysaccharides utilized by *Bacteroides thetaiotaomicron* [108]. Although reports with commensal bacteria have been limited compared to pathogenic bacteria, research in this field is equally valuable.

1.5.2.2 *E. coli, K. pneumoniae* and *E. faecalis*

From the myriad of organisms within the intestine microbiota, we chose three of them, *E. coli*, *Klebsiella pneumoniae* and *Enterococcus faecalis*. They are chosen for study because they are common commensals of the microbiota in neonates [109] and can be cultured aerobically thus easier to work with.

E. coli is a gram-negative, facultative anaerobic, rod shaped bacterium, first described by Theodor Escherich in the late 19th century in a series of pioneering studies of the intestinal bacterial of infants [110]. Since its discovery, it has been the best-studied prokaryotic model organism because of easy cultivation, simple genetics and easy manipulation. Despite the extensive quantity of studies done on *E. coli*, over 50% of the open reading frames (ORFs) in *E. coli* K12-MG1655 are still uncharacterized, according to the web database "Comprehensive Microbial Resource" (http://www.tigr.org/CMR) from The Institute for Genomic Research [111], so there is still huge knowledge gap to fill. Nevertheless, to construct a prototype of the proposed dual fluorescence system, we chose *E. coli* to be the reporter species. *E. coli* may exist as a harmless commensal or as an intra- or extraintestinal pathogen [112] and we opted to use the commensal wild type strain *E. coli* MG1655 in this study.

K. pneumoniae is a gram-negative, rod shaped bacterium and *E. faecalis* is a gram-positive, coccoid shaped bacterium. Both of them are facultative anaerobic [109].

Being commensal species in the same habitat, the three species (lineages shown in Table 1.) have been partners over an evolutionarily significant period of time, so they are likely to have established complex and stable interspecies interactions.

Table 1. Lineages of the three species.

Species	***E. coli***	***K. pneumoniae***	***E. faecalis***
Genus	*Escherichia*	*Klebsiella*	*Enterococcus*
Family	Enterobacteriaceae		Enterococcaceae
Order	Enterobacteriales		Lactobacillales
Class	Gamma Proteobacteria		Bacilli
Phylum	Proteobacteria		Firmicutes
Domain	Bacteria		

1.5.3 Project Overview

In this project, the proposed dual fluorescence system would be constructed, validated and assessed for its applicability, as shown below.

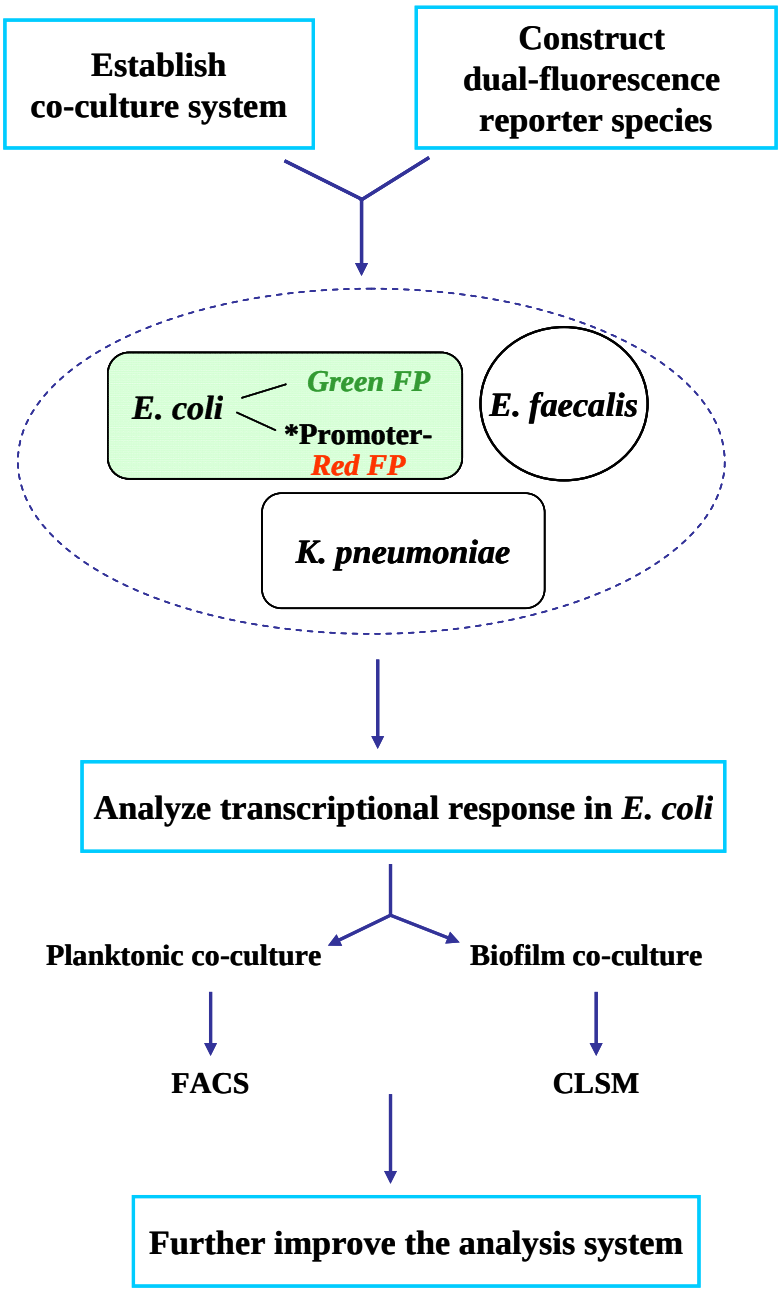

Fig 1.3. Project overview. The whole project includes four main parts (in blue boxes). After establishment of co-culture system and construction of dual-fluorescence reporter species, the reporter *E. coli* strain grows with the other two species: *K. pneumoniae* and *E. faecalis* into co-cultures. Promoter activity of *E. coli* in planktonic co-culture can be analyzed by FACS, and that of biofilm co-culture can be visualized by CLSM. The last part is the further development of the system.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 General reagents, kits and media

2.1.1.1 Reagents

10 mg/ml ethidium bromide	Stored at room temperature (RT)
10 mM each dNTPs mix	In 10 μl aliquot, stored at -20ºC

2.1.1.2 Laboratory stock solutions

10% (v/v) glycerol solution	Autoclaved and stored at RT
60% (v/v) glycerol solution	Autoclaved and stored at RT
20% L-(+)-arabinose	Stored at 4ºC
50% sucrose solution	Stored at 4ºC
Solution I (for preparation of chemically competent cell)	150 mM $MgCl_2$, 10 mM MOPS (pH 7.0), 10 mM RbCl, sterilized by filtration through 0.22 μm filter, and stored at 4ºC
Solution II (for preparation of chemically competent cell)	50 mM $CaCl_2$, 100 mM MOPS (pH 6.5), 10 mM RbCl, sterilized by filtration through 0.22 μm filter, and stored at 4ºC
10x TBE	0.9 M Tris, 0.9 M boric acid, 20 mM Na_2EDTA (pH 8.0)

TE	10 mM Tris/HCl (pH 8.0), 1 mM Na_2EDTA (pH 8.0)
Annealing buffer	100 mM Tris/HCl (pH7.4), 100 mM $MgCl_2$
6x loading dye	From Fermentas

2.1.1.3 Enzymes

Restriction enzymes	From Fermentas, New England Biolabs and Roche
Alkaline phosphatase (CIAP)	From Fermentas
Taq DNA polymerase	From Fermentas
PfuTurbo® Hotstart DNA Polymerase	From Stratagene

2.1.1.4 Commercially available kits

Rapid DNA Ligation Kit	From Roche
Nucleospin Plasmid	From Macherey-Nagel
NucleoBond PC100	From Macherey-Nagel
NucleoBond PC500	From Macherey-Nagel
Nucleospin® Extract II Kit	From Macherey-Nagel
In-Fusion™ 2.0 PCR Cloning Kit	From BD Clontech

2.1.1.5 Media for bacterial culture

All media were sterilized by autoclaving at 121ºC for 20 min.

Luria-Bertani (LB) medium	1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl

LB agar	LB media plus 1.5% (w/v) bacto-agar
SOC	2% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 10 mM KCl, 20 mM glucose
Nutrient Broth	From Pronadisa
Brain-Heart Infusion broth	From Becton Dickinson, Difco
Brain-Heart Infusion agar	From Becton Dickinson, Difco
Lactobacilli MRS agar	From Becton Dickinson, Difco
HiCrome UTI agar	From HiMedia™

2.1.1.6 Media supplements

100 mg/ml ampicillin	Prepared in ddH_2O and sterilized by filtration through 0.22 μm filter
30 mg/ml chloramphenicol	Prepared in ethanol
10% mucin	Prepared in Brain-Heart Infusion broth, and sterilized by autoclaving at 121ºC for 20 min
50% sucrose solution	Prepared in ddH_2O and sterilized by filtration through 0.22 μm filter

2.1.2 Bacterial strains and plasmids

2.1.2.1 Storage of bacterial strains in permanence

Single colony was inoculated into 3 ml medium with appropriate antibiotics (if any) and incubated at 37ºC with shaking for 12~16 h. The culture was stored in 20% glycerol at -80°C in a 2 ml screw-cap cryotube.

2.1.2.2 Bacterial strains (Table 2.1.)

Strain	Relevant description	Source
***E. coli* strains**		
E. coli MG1655	wild type, λ^-, F^-	ATCC 700926
E. coli TOP10	*araBAD* mutant, for arabinose-inducible P_{araB} expression	Invitrogen
E. coli SCC1	MG1655 with chromosomal insertion of $P_{A1/04/03}$-*gfp*mut3*	This study
E. coli S17 λ-*pir*	provides Pir protein, which is essential for replication of R6K-based plasmids	[113]
Non-*E. coli* strains		
Klebsiella pneumoniae NCTC 9633	enteric, Gram negative rod	ATCC 13883
Enterococcus faecalis OG1RF	enteric, Gram positive coccus	ATCC 47077
Lactobacillus casei	enteric, Gram positive rod	ATCC 11578
Serratia marcescens	enteric, Gram negative rod	clinical isolate
Bacillus cereus	environmental, Gram positive rod	environmental isolate
Micrococcus luteus	environmental, Gram positive coccus	environmental isolate
Acinetobacter baumanii	environmental, Gram negative rod	clinical isolate
Streptococcus pyogenes	skin, Gram positive coccus	clinical isolate
Staphylococcus aureus	skin, Gram positive coccus	clinical isolate

2.1.2.3 Plasmids

Table 2.2. Vector plasmids

Plasmid	Relevant description	Source
Vectors		
pBAD/*myc*-His B	Ap^r; ColE1 ori; low copy; 4.1 kb	Invitrogen
pUT-miniTn5-Km2	Ap^r; R6K ori; low copy; 6.0 kb	[114]
pDM4	Cm^r; R6K ori, low copy; 7.1 kb	[115]
pBluescript SK(+)	Ap^r; pUC ori, high copy; 3.0 kb	Stratagene
pBS516*	pBluescript SK(+) derivative	V. Shingler
pBS197*	pBluescript SK(+) derivative	V. Shingler
pBS-SacII*	pBluescript SK(+) derivative	V. Shingler
pBS-EcoRV/NotI*	pBluescript SK(+) derivative	V. Shingler

Multiple cloning sites of pBluescript SK(+) and its derived vectors which are marked with asterisks (*) in the above list:

pBluescript SK(+): *Sac*I-*Bst*XI-*Sac*II-*Not*I-*Eag*I-*Xba*I-*Spe*I-*Bam*HI-*Sma*I-*Pst*I-*Eco*RI-*Eco*RV-*Hin*dIII-*ClaI*-(*Hinc*II-*Acc*I-*Sal*I)-*XhoI*-(*Dra*II-*Apa*I)-*Kpn*I

pBS516: *Sac*I-*Bst*XI-*Sac*II-~~***NotI***~~-*Eag*I-*Xba*I-*Spe*I-*Bam*HI-*Sma*I-*Pst*I-*Eco*RI-*Eco*RV-*Hin*dIII-*ClaI*-(*Hinc*II-*Acc*I-*Sal*I)-*XhoI*-(*Dra*II-*Apa*I)-~~***KpnI***~~-***Nde*I**

pBS197: *Sac*I-*Bst*XI-*Sac*II-*Not*I-*Eag*I-*Xba*I-*Spe*I-*Bam*HI-*Sma*I-*Pst*I-*Eco*RI-*Eco*RV-*Hin*dIII-*ClaI*-(*Hinc*II-*Acc*I-*Sal*I)-~~***XhoI***~~-***Not*I**-(*Dra*II-*Apa*I)-*Kpn*I

pBS-SacII: *Sac*I-*Bst*XI-~~***SacII***~~-*Not*I-*Eag*I-*Xba*I-*Spe*I-*Bam*HI-*Sma*I-*Pst*I-*EcoRI*-*Eco*RV-*Hin*dIII-*ClaI*-(*Hinc*II-*Acc*I-*Sal*I)-*XhoI*-(*Dra*II-*Apa*I)-*Kpn*I

pBS-EcoRV/NotI: *Sac*I-*Bst*XI-*Sac*II-*Not*I-*Eag*I-*Xba*I-*Spe*I-*Bam*HI-*Sma*I-*Pst*I-*EcoRI*-~~***Eco*RV***~~-***Not*I**-*Hin*dIII-*ClaI*-(*Hinc*II-*Acc*I-*Sal*I)-*XhoI*-(*Dra*II-*Apa*I)-*Kpn*I

In bold are the restriction enzyme sites that are different from those of the parental pBluescript SK(+).

Table 2.3 Plasmids constructed by others

Plasmid	Relevant description	Source
(i) Plasmids constructed by other laboratories		
pJBA28	Ap^r; R6K ori, low copy, $P_{A1/04/03}$-RBS-*gfp*mut3*-T_0-T_1	[116]
(ii) Plasmids with fluorescence protein genes from commercial sources		
pFP series	Ap^r; pUC ori, high copy, the fluorescent protein (FP) genes are under the control of *lacZ* promoter (P_{lacZ})	BD Clontech

The fluorescence excitation/emission properties of the FPs are listed below:

pAmCyan	excitation=453 nm; emission =486 nm
pEGFP	excitation=488 nm; emission =507 nm
pd2EGFP	excitation=488 nm; emission =507 nm
pZsGreen	excitation=496 nm; emission =506 nm
pEYFP	excitation=513 nm; emission =527 nm
pZsYellow	excitation=531 nm; emission =540 nm
pDsRed-Express	excitation=557 nm; emission =579 nm
pDsRed-Monomer	excitation=557 nm; emission =585 nm
pAsRed2	excitation=576 nm; emission =592 nm
pHcRed1	excitation=588 nm; emission =618 nm

Table 2.4 Plasmids constructed in this study

Plasmid	Relevant description
(i) Plasmids with FP genes flanked by RBS and transcriptional terminators	
	Ap^r, pBAD/*myc*-His B derivative carrying the FP gene under the control of *araB* promoter
pCCS112	P_{araB}-RBS-*AmCyan*-T_1-T_2
pCCS103	P_{araB}-RBS-*EGFP*-T_1-T_2
pCCS113	P_{araB}-RBS-*d2EGFP*-T_1-T_2
pCCS132	P_{araB}-RBS-*ZsGreen*-T_1-T_2
pCCS111	P_{araB}-RBS-*EYFP*-T_1-T_2
pCCS133	P_{araB}-RBS-*ZsYellow*-T_1-T_2
pCCS102	P_{araB}-RBS-*DsRed-Express*-T_1-T_2
pCCS122	P_{araB}-RBS- *DsRed-Monomer*-T_1-T_2
pCCS126	P_{araB}-RBS-*AsRed2*-T_1-T_2
pCCS110	P_{araB}-RBS-*HcRed1*-T_1-T_2
(ii) Plasmids with promoters inserted upstream of *EGFP*	
	Ap^r; R6K ori; low copy, pUT-miniTn5-Km2 derivative
pCCS114	*rrnB* P1-*EGFP*
pCCS125	P_{lacZ}-*EGFP*

Table 2.4 (continued)

Plasmid	Relevant description
(iii) Plasmids with $P_{A1/04/03}$ -*gfp* flanked by Region A and Region B	
	sacB$^+$, Cm^r, pDM4 derivative, carrying $P_{A1/04/03}$ - *gfp*mut3* flanked by DNA fragments (Region A and Region B) recombination into *E. coli* chromosome
Coordinates shown below correspond to those from *E. coli* MG1655 genomic sequence	
pCCS167	Insertion region No. 7 Region A: 312037 bp-312754 bp Region B: 312771 bp-313495 bp
pCCS168	Insertion region No. 8 Region A: 4537564 bp-4538307 bp Region B: 4538348 bp-4538949 bp
pCCS169	Insertion region No. 9 Region A: 2902718 bp- 2902047 bp Region B: 2902026 bp- 2901544 bp
pCCS170	Insertion region No. 10 Region A: 3766819 bp-3767614 bp Region B: 3767641 bp-3768247 bp
pCCS171	Insertion region No. 11 Region A: 2065385 bp-2065992 bp Region B: 2066019 bp-2066642 bp
(iv) Plasmids with promoter-*AsRed2* fusions	
pCCS127	Ap^r, pBluescript SK(+) derivative with RBS-*AsRed2*- T_1-T_2
pCCS204	P_{fadB}-*AsRed2*, pCCS127 derivative
pCCS320	P_{sulA}-*AsRed2*, pCCS127 derivative
pCCS182	P_{acrA}-*AsRed2*, pCCS127 derivative
pCCS205	P_{rne}-*AsRed2*, pCCS127 derivative
pCCS319	P_{rpoE}-*AsRed2*, pCCS127 derivative
pCCS207	P_{recA}-*AsRed2*, pCCS127 derivative
pCCS206	P_{msrA}-*AsRed2*, pCCS127 derivative
pCCS175	P_{gadA}-*AsRed2*, pCCS127 derivative
pCCS184	P_{flgB}-*AsRed2*, pCCS127 derivative
pCCS177	P_{pspA}-*AsRed2*, pCCS127 derivative
pCCS318	P_{rbsD}-*AsRed2*, pCCS127 derivative

Table 2.4 (continued)

Plasmid	Relevant description
(v) Plasmid constructed for generating *AsRed2* mutant library	
pCCS325	Ap^r, pCCS127 derivative $P_{A1/04/03}$-*AsRed2* (parental plasmid for generating *AsRed2* mutant library)
pCCS326	$P_{A1/04/03}$-*AsRed2* mutant M1
pCCS339	$P_{A1/04/03}$-*AsRed2* mutant M2
pCCS340	$P_{A1/04/03}$-*AsRed2* mutant M3
pCCS341	$P_{A1/04/03}$-*AsRed2* mutant M4
pCCS342	$P_{A1/04/03}$-*AsRed2* mutant M5

Cm^r: chloramphenicol resistance

Ap^r: ampicillin resistance

sacB$^+$: *sacB* is a structural gene from *Bacillus subtilis*. It is used as a counter selection marker (refer to section 2.2.3.2 on page 55).

2.2 Methods

2.2.1 Molecular cloning

2.2.1.1 Agarose gel electrophoresis

2.2.1.1.1 Procedure overview

1.0% and 0.7% agarose gels were routinely used for analysis of 0.1~3 kb and > 3 kb DNA fragments respectively. Agarose was melted by boiling in 1x TBE, cooled for 30 min, followed by casting in gel apparatus (Bio-Rad). The gel was set at RT for at least 30 min. Electrophoresis was carried out in the same apparatus with the gel submerged 1~2 mm below the surface in 1x TBE. DNA samples were loaded into the wells with 1x loading dye. Electric field of about 10 volt/cm was usually applied for the separation of DNA fragments. After electrophoresis, the gel was transferred into 1 μg/ml ethidium bromide (EB) solution for staining. DNA fragments were then visualized by fluorescence over a UV light (302 nm, UV transluminator TM-20, UVP), under which DNA/EB complexes fluoresce, and the image was recorded with a Mitsubishi video copy processor.

2.2.1.1.2 DNA semi-quantification and purification

DNA concentration was routinely gauged using agarose gel electrophoresis, by comparing the intensity of samples with that of known quantity of 100 bp or 1 kb DNA ladder (GeneRuler™, Fermentas). Agarose gel electrophoresis was also used to isolate a particular DNA fragment in a mixture of fragments, for ligation or as a PCR template. For this purpose, the gel was run with 8 volt/cm for optimal separation. Gel containing DNA fragments of interest was excised under 70% power of UV light (Vilber Lourmat) and DNA was extracted using

Nucleospin® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

2.2.1.2 Polymerase chain reaction (PCR)

Amplification of DNA fragments was carried out using PCR through repeated cycles of DNA template denaturation, primer annealing and new strand synthesis by thermo-stable DNA polymerase. Thermocycling was performed in a Mastercycler® ep gradient thermal cycler (Eppendorf). All PCR experiments included a sample without DNA template, to serve as negative control, for checking the presence of contamination in PCR mixtures. PCR primers were designed with the aid of Vector NTI Suite 9 (Invitrogen) to ensure the absence of significant secondary structure, and to maintain %GC between 40~60%. If restriction enzyme site was added at 5' end of the primer, GC clamps of 2~6 bp were added to increase the efficiency of restriction digestion.

2.2.1.2.1 Standard PCR protocol

PCR was routinely performed in 100 µl or 20 µl reaction volumes for cloning or screening purposes, respectively. A typical PCR reaction contained less than 5 ng/µl template, 200 µM of each oligonucleotide primer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 1x buffer (provided in 10x concentration by the manufacturers) and 1~1.5 U DNA polymerase. *Taq* DNA polymerase (Fermentas) was used for colony PCR screening. *PfuTurbo*® Hotstart DNA Polymerase (Stratagene), with 3'-5' exonuclease (proofreading) activity to improve DNA synthesis fidelity and hot start activity to increase PCR specificity, was used to amplify DNA fragments for cloning. For colony PCR,

colonies were picked with 1µl sterilized pipette tips and partially deposited on the surface of a grid master plate, before being dipped into the PCR mixture to serve as the template.

Standard thermocycling reactions involved the initial denaturation at 94ºC for 3 min followed by 30 cycles of 94°C for 30 s (denaturation), T_a°C for 30 s (annealing), and 70°C for 1 min (extension). The reaction was completed by a further 7 min at 70°C. Annealing temperatures (T_a) was set at 5ºC lower than the higher T_m of the two primers. T_m refers to the melting temperature of the primer, at which 50% of DNA duplexes become single-stranded.

2.2.1.2.2 Site-directed PCR mutagenesis

The site-directed PCR mutagenesis carried out in this study followed closely to the standard PCR protocol as described above, except that the primers were designed to have several mismatched bases to introduce the intended mutations into the PCR products.

2.2.1.2.3 Error-prone PCR (EP-PCR)

EP-PCR was performed as documented by Cadwell et al [117]. The 20 µl PCR reaction contained: 2 ng DNA fragment (about 800 bp, 20 fmols), 300 µM of each oligonucleotide primer, 7 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1x buffer and 1 U *Taq* DNA polymerase. EP-PCR mixtures were incubated for 30 cycles of 94ºC for 1 min, 45ºC for 1 min and 72ºC for 1min, with no prolonged denaturation step in the beginning and no prolonged extension step after the last cycle.

2.2.1.3 Restriction endonuclease digestion

Restriction endonuclease digestions were usually carried out in 100 µl reaction volume with 20 U of enzyme for 2 µg DNA and incubated at 37ºC for 12~16 h, or in 20 µl reaction volume with 5 U of enzyme for 500 ng DNA for 2~5 h. Reactions also contained 100 µg/ml bovine serum albumin (New England Biolabs) to achieve higher digestion efficiency. Digestion with two enzymes was carried out simultaneously in a suitable buffer according to the manufacturer's instruction. All PCR-amplified fragments for cloning was digested with the corresponding endonuclease for which the sites were designed into the primers, unless otherwise stated. Both vectors and inserts for cloning were purified with Nucleospin® ExtracII Kit (Macherey-Nagel) to remove the restriction enzymes and buffers prior to ligation.

2.2.1.4 DNA ligation

2.2.1.4.1 Conventional ligation

DNA vectors with compatible ends were prepared by restriction enzyme digestion and followed by treatment with alkaline phosphatase, unless otherwise stated. Vector DNA (about 200 ng) and insert DNA (in 3:1 molar ratio to vector) were ligated using the Rapid DNA Ligation Kit (Roche). A reaction without insert DNA was included as negative control.

2.2.1.4.2 In-Fusion™ 2.0 PCR Cloning Kit

For the ligation using In-Fusion™ 2.0 PCR Cloning Kit (Clontech), the DNA insert was PCR-amplified with the primers containing at least 15 bases of homology with the sequence that flank the site of insertion in the linearized

vector. The PCR-amplified DNA insert was then fused with the linearized vector via single stranded regions generated by In-Fusion enzyme. PCR fragment and vector at a molar ratio of 2:1 was mixed in deionized H_2O in a total volume of 10 µl and added into the tube provided, mixed by pipetting up and down. The tube was incubated at 37ºC for 15 min, then at 50ºC for 15 min, and then transfered on ice. Mastercycler® ep gradient thermal cycler (Eppendorf) was used for the required incubations.

2.2.1.5 *E. coli* competent cells preparation

2.2.1.5.1 Chemically competent cells

The *E. coli* strain TOP10, stored at -80ºC was used to prepare chemically competent cells. Fresh colonies were prepared by streaking out cells from the -80°C frozen stock onto LB agar plate and incubating them overnight at 37°C. On the second day, a single colony of *E. coli* TOP10 was inoculated into 3 ml LB medium and grown overnight at 37°C. On the third day, 2 ml of the overnight *E. coli* culture was transferred into flask containing 100 ml pre-warmed LB mudium and incubated at 250 rpm at 37°C until the OD_{600} reached 0.4~0.6. This should take approximately 1.5~2 h. The flask was put on ice for 5 min to cool the cells. The cells were then transferred to 250 ml centrifuge bottles and centrifuged at 4ºC for 10 min at 2700 *g* with Avanti™ J-25 centrifuge (Beckman Coulter™). The pellet was resuspended in 20 ml ice-cold Solution I. After centrifugation as before, the cells were resuspended in 20 ml Solution II and the cells were incubated on ice for half an hour. Cells were collected by centrifugation as before and the pellet was resuspended in 10 ml

ice-cold solution II. Following the addition of 5 ml 60% glycerol, the cells were aliquoted into 600 µl/tube and stored at -80°C.

2.2.1.5.2 Electrocompetent cells

The *E. coli* strain MG1655, stored at -80ºC was used to prepare electrocompetent cells. 1 ml of overnight bacterial culture (similarly prepared as described in section 2.2.1.5.1 on page 36) was transferred into a flask with 100 ml pre-warmed LB medium and were incubated with shaking at 250 rpm at 37ºC until the OD_{600} reached 0.7~0.9. The cells were harvested by centrifugation at 4355 *g* for 10 min at 4°C. The pellet was rinsed in sterile ice-cold ddH_2O twice (100 ml and 50 ml respectively) and finally resuspended in 5 ml sterile ice-cold 10% glycerol solution. The cells were then aliquoted into 100 µl/tube and stored at -80°C.

2.2.1.6 Transformation of plasmid DNA

2.2.1.6.1 Into chemically competent cells

For each transformation, 200 µl *E. coli* TOP10 cells were thawed quickly and stored on ice immediately after thawing. After the addition of DNA (200 ng~300 ng), the mixture was mixed by tapping gently and then incubating on ice for 30 min, followed by heat shock at 42ºC for 90 s. 800 µl SOC medium was added, followed by incubation at 37ºC for 30 min to 1 h. This was spread onto agar plates containing the appropriate antibiotics.

2.2.1.6.2 Into electrocompetent cells

For each electroporation, 45 µl of *E. coli* MG1655 electrocompetent cells were thawed and transferred to ice-cold 0.2 cm electropration cuvette (Bio-Rad) and approximately 1 µg DNA (maximum volume 10 µl) was added. The cuvettes were gently tapped to mix and to ensure that there were no air bubbles. Gene Pulser (Bio-Rad) apparatus was set at 25 µF, 200 ohm and a voltage of 2.5 kV. Time constant would be displayed as > 4.5 for high electroporation efficiency. Immediately after electroporation, 1 ml of SOC medium was added into the cuvette and transferred to a sterile tube, and shaken at 37ºC for 1 h. The mixture was spread onto plates containing the appropriate antibiotics.

2.2.1.6.3 Into One Shot® TOP10 competent cell

For construction of the *AsRed2* mutant library, plasmids were transformed into One Shot® TOP10 chemically competent *E. coli* cells (Invitrogen), according to the manufacturer's instructions. Approximately 104 ng of DNA was transformed into 50 µl of One Shot® TOP10 competent cells. We added 300 µl of SOC medium to the tube before spreading on LB agar supplemented with 200 µg/ml ampicillin (LA-Ap).

2.2.1.7 Identification of colonies with recombinant plasmid

After transformation, colonies carrying recombinant plasmids were identified following two steps: (i) PCR screening (if suitable PCR primers were available) and (ii) restriction digestion. Colony PCR was carried out if the genomic DNA of *E. coli* would not interfere as a contaminating source of template. Alternatively, plasmid DNA was extracted and used as template for the PCR

screening. A negative control and if possible, a positive control were included in the experiment. Only colonies with positive PCR results were inoculated for small scale plasmid extraction and the insertion was further confirmed by restriction digestion. If the insert DNA was derived from PCR amplification, the absence of PCR-generated mutation would be verified by DNA sequencing.

2.2.1.8 Extraction and purification of DNA

For small scale purification of plasmid DNA, 3 ml (for high copy plasmids) or 5ml (for low copy plasmids) LB medium with appropriate antibiotics was inoculated with a single colony of the required strain and incubated at 37ºC, for 12~16 h, with shaking at 250 rpm. A Nucleospin Plasmid Kit (Macherey-Nagel) was used to extract DNA. For large scale purification of plasmid DNA, a NucleoBond PC500 Kit (Macherey-Nagel) was used for high copy plasmids and NucleoBond PC100 (Macherey-Nagel) was used for low copy plasmids. Genomic DNA of *E. coli* MG1655 was extracted using Nucleospin® Tissue (Macherey-Nagel). All kits were used according to the manufacturer's instructions.

2.2.2 Plasmid Construction

2.2.2.1 Construction of fluorescent protein (FP) cassettes

pBAD/*myc*-His B (Invitrogen) was used in this series of construction for the experiments discussed in section 3.2.2.1 (on pages 77~78) because it was desirable to flank the FP genes with the ribosomal binding site (RBS) and transcriptional terminators that are present on this plasmid. The resulting "RBS-FP-Terminators" will be referred to as FP cassette.

2.2.2.1.1 Strategy 1 of cloning FP gene into pBAD/*myc*-His B – pCCS126

(Construction of pCCS126 is described as representative of all FP constructs in Table 2.4-i on page 29, except pCCS112 and pCCS113)

The *AsRed2* fragment was digested from pAsRed2 (BD Clontech) using *Nco*I and *Eco*RI. After purification (Fig 2.1.A), it was ligated into the corresponding sites on pBAD/*myc*-His B (Invitrogen). With this fusion, RBS would be located 7 bp upstream of the start codon of *AsRed2*.

2.2.2.1.2 Strategy 2 of cloning FP gene into pBAD/*myc*-His B – pCCS112

(Construction of pCCS112 is described as representative of the set pCCS112 and pCCS113)

An alternative strategy (Fig 2.1.B) from the one described above had to be used for pCCS112 and pCCS113 because *Nco*I site in pAmCyan (for pCCS112) and pd2EGFP (for pCCS113) is not unique. We designed the forward primer so as to include a *Bam*HI site and to create an RBS at 6 bp upstream of the AmCyan start codons. The *Bam*HI site in the primer has compatible sticky overhangs with the *Bgl*II site in the vector. For PCR mutagenesis, PR32 is a reverse primer used in both constructions; PR53 and PR54 were forward primers for AmCyan and d2EGFP genes respectively.

The primer sequences are (mutated bases are in bold):

PR32: 5′-GCATCCGCTTACAGACAACTGTGACCG-3′;

PR53: 5′-TAGAGGATCC(*Bam*HI)CCGGGTAC**AGG****AG**G(RBS)CCACCA-3′;

PR54: 5′-TAGAGGATCC(*Bam*HI)AC**AGG****A**GG(RBS)CCACCATGGTGA-3′.

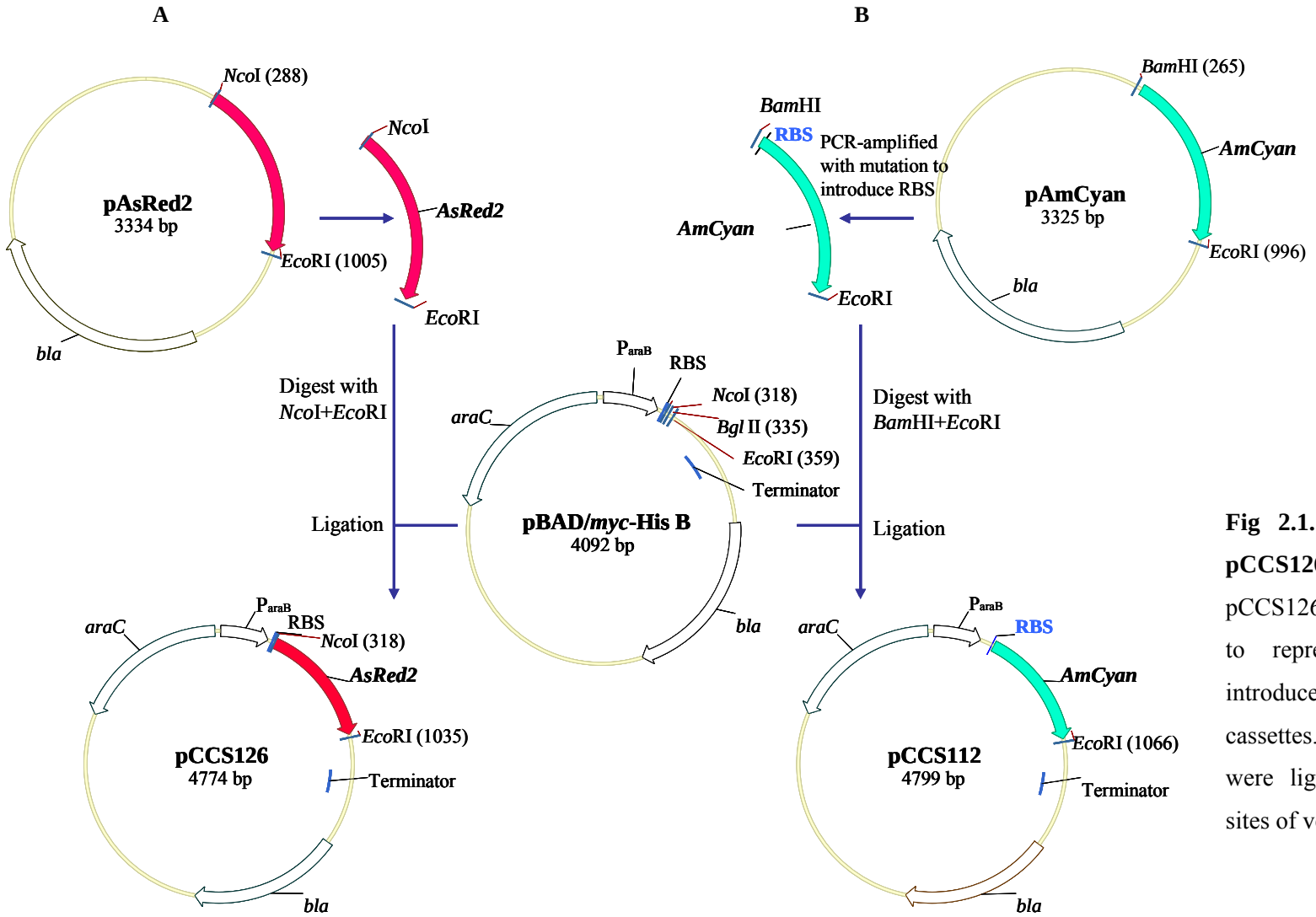

Fig 2.1. Construction maps of pCCS126 and pCCS112.

pCCS126 and pCCS112 are shown to represent the modifications introduced to create RBS-FP-T_1-T_2 cassettes. Insert DNA fragments were ligated into the compatible sites of vectors.

2.2.2.2 Insertion of promoters upstream of *EGFP*

2.2.2.2.1 Insertion of *rrnB* P1 upstream of *EGFP*–pCCS114

Promoter *rrnB* P1 was first PCR-amplified from *E. coli* genomic DNA using primers PR05 and PR06. Amplified PCR fragment was digested with *Hin*dIII and *Eco*RI and inserted into the corresponding sites on pBS516 to derive the intermediate plasmid pCCS99. RBS-*EGFP*-Terminator cassette was then PCR-amplified from pCCS103, using primers PR29 and PR30, and inserted into *Pst*I and *Bam*HI sites of pCCS99 to generate pCCS107. *Not*I fragment containing *rrnB* P1-*EGFP* cassette was PCR-amplified with PR51, PR52 and inserted into *Not*I site of miniTn5-Km2 to generate pCCS114.

The primer sequences are:

PR05: 5′-CGGCAAGCTT(*Hin*dIII)TGCGAATATTGCCTTTTGTA-3′

PR06: 5′-CGGCGAATTC(*Eco*RI)TGGTGGCGCATTATAGGGAG-3′

PR29: 5′-GCCCTGCAG(*Pst*I)TTTCTCCATACCCGTTTTTTGGG-3′

PR30: 5′-GCGGGATCC(*Bam*HI)ACCGACAAACAACAGATAAAACG-3′

PR51: 5′-GTGAGCGGCCGC(*Not*I)AATACGACTCACTATA-3′

PR52: 5′-GGCGCTATTCAGATCCTCTTCTGAGATGA-3′

2.2.2.2.2 Insertion of P_{lacZ} upstream of *EGFP*–pCCS135

pCCS134 was constructed by inserting P_{lacZ}-*EGFP* fragment from *Pvu*II and *Eco*RI sites of pEGFP into *Sma*I and *Eco*RI sites of pBS197. The *Not*I fragment from pCCS134 containing P_{lacZ}-*EGFP* was then inserted into *Not*I site of miniTn5-Km2 to generate pCCS135.

2.2.2.3 Plasmids constructed for the purpose of *gfp*mut3* (*gfp*) chromosomal integration

The initial constructions were on pBluescript-derived vectors, to exploit their ample restriction sites and high copy number, for cloning convenience (section 2.2.2.3.1). The constructions were then continued on pDM4-derived plasmids for chromosomal integration (section 2.2.2.3.2).

2.2.2.3.1 Plasmids constructed on pBluescript-derived vectors – pCCS115 and pCCS124

(Constructions of pCCS115 and pCCS124 are described as representatives of the Stage I and II plasmids, respectively, shown in Table 2.5.)

The two fragments (refer to Fig 3.9. on page 86, Region A and Region B) spanning Insertion region No.7 were PCR-amplified from *E. coli* genomic DNA (Fig 2.2.). The *Xho*I-RegionA-*Cla*I fragment corresponding to 312037-312754 bp (Region A) of *E. coli* MG1655 genome (GenBank accession no.U00096) was first inserted into the correspondingly double-digested vector pBS-EcoRV/NotI to generate pCCS115. Next, the *Bam*HI-RegionB-*Spe*I fragment corresponding to 312771-313495 bp (Region B) was ligated into pCCS115 to yeild pCCS124.

2.2.2.3.2 Plasmid constructed on pDM4-derived vectors – pCCS143 and pCCS167

(Construction of pCCS143 and pCCS167 are described as representatives of the Stage III and IV plasmids, respectively, shown in Table 2.5.)

The entire *Xho*I-*Spe*I (Region A + Region B) fragment from pCCS124 was subsequently inserted into the corresponding sites in the R6K-suicide plasmid pDM4 [118] to construct pCCS143 (Fig 2.2.). This is followed by insertion of the $P_{A1/04/03}$-*gfp*mut3* cassette, excised as a *Not*I fragment from pJBA28 [116], into the *Not*I site, to generate pCCS167.

Table 2.5. Constructed plasmids for *gfp*mut3* chromosomal integration.

Insertion region	in pBluescript vector		in pDM4 vector	
	Stage I	Stage II	Stage III	Stage IV
No.7	pCCS115	pCCS124	pCCS143	pCCS167
No.8	pCCS116	pCCS125	pCCS144	pCCS168
No.9	pCCS117	pCCS139	pCCS145	pCCS169
No.10	pCCS118	pCCS141	pCCS146	pCCS170
No.11	pCCS119	pCCS142	pCCS166	pCCS171

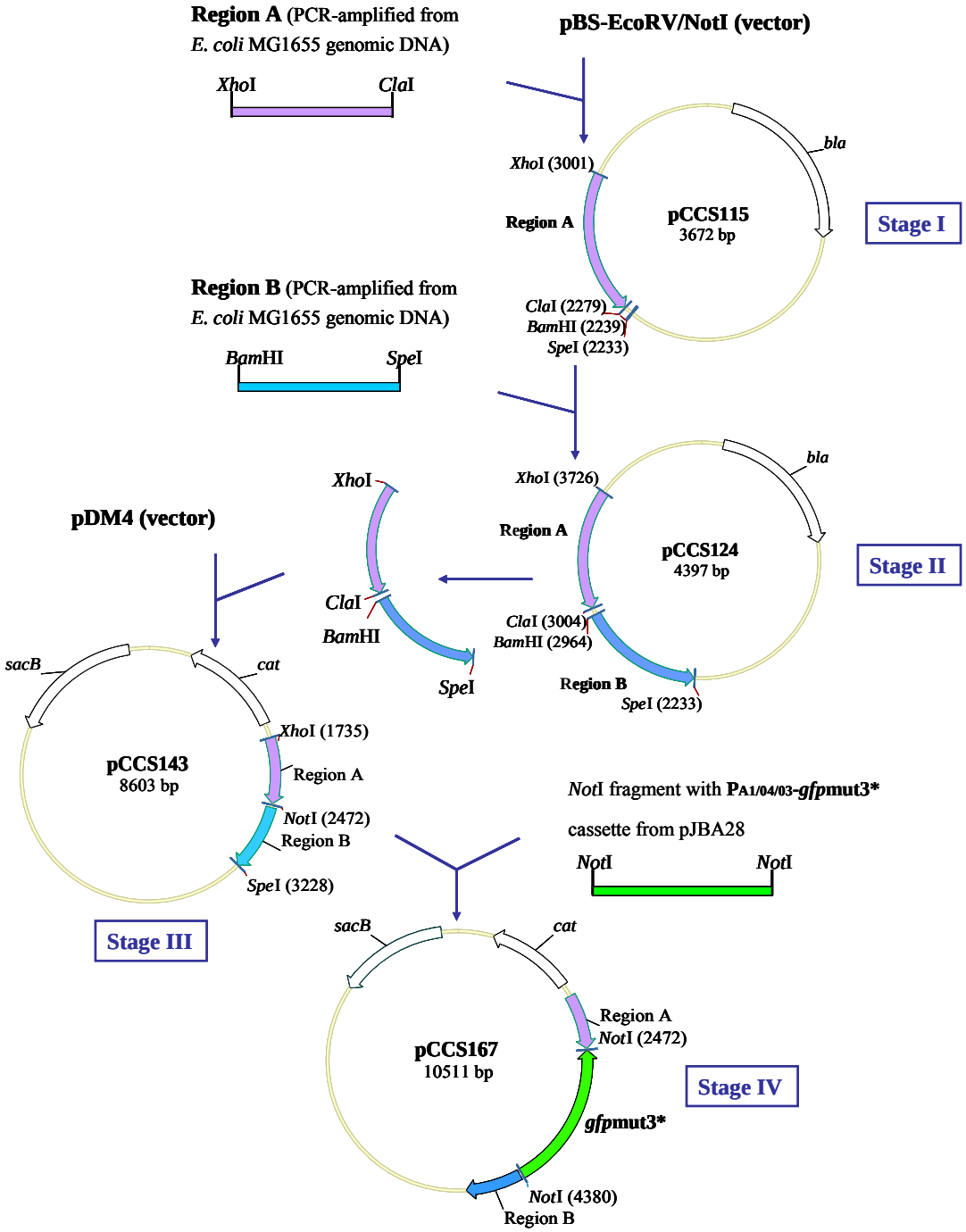

Fig 2.2. Construction maps of pCCS115, pCCS124, pCCS143 and pCCS167.

Table 2.6. Primers used for plasmid constructed in Table 2.5.

No.[a]	R[b]	PR[c]	Sequence (5'->3')
7	A	138	CCGCCGCTCGAG(*Xho*I)GATACATGCTCATCCGGATC
		139	CGCCCATCGAT(*Cla*I)CCAACAACGATATCTTGTATAACACCCCA
	B	140	CCGCGGATCC(*Bam*HI)TGAGTTCTATTAAACCGTCAACTA
		141	CGGGGGACTAGT(*Spe*I)CGATTTCCTTTTCCGCAGAAA
8	A	142	CCGCCGCTCGAG(*Xho*I)GCCCGCATAAACAATTAGCA
		143	CCATCGAT(*Cla*I)CACCAACATCAACAAGCCTCTCCAGAT
	B	144	CCGCGGATCC(*Bam*HI)CGGCGAATGGCTGTGA
		145	GCGGGGGACTAGT(*Spe*I)AGCTGTGCTGGGTTAGCAATAA
9	A	149	CCGCCGCTCGAG(*Xho*I)TCATTTATATTGTTTGTCGCC
		148	CCATCGAT(*Cla*I)GCTGGCGCGGGGAACTCGACA
	B	147	CCGCGGATCC(*Bam*HI)CGTTATCTCTTTCTCAAGTT
		146[d]	GGTTTATCCCCGCTGATGCAGGGAACA
10	A	150	CCGCCGCTCGAG(*Xho*I)ATTAGCCACCTGTTTATACA
		151	CCATCGAT(*Cla*I)GGTCTTATGATCTTTCCCTTACTTAATAAATAGC
	B	152	CCGCGGATCC(*Bam*HI)TTGCCGCTGATTGCATTGAA
		153	GCGGGGGACTAGT(*Spe*I)TGGAACTTTGCTCCTTGTAAGGA
11	A	154	CCGCCGCTCGAG(*Xho*I)ACCACAATTTCGCTCTCTC
		155	CGCCCATCGAT(*Cla*I)GAATATATCTACTGATGTACAAAACACAACA
	B	156	CCGCGGATCC(*Bam*HI)TTTCACCTGCTTGCAAAGCTTC
		157	CGCGGGGGACTAGT(*Spe*I)ACAAAGTACAAATGATTACTGTAATGATA

[a] No. refers to Insertion region No.

[b] R refers to Region A or B.

[c] PR refers to the primers used to PCR-amplify the Region A or B.

[d] Region B in Insertion region No.9 contains inherent *Spe*I site. Hence primer-directed mutagenesis (to add restriction enzyme site) was not necessary.

2.2.2.4 Insertion of promoters upstream of *AsRed2*

2.2.2.4.1 Promoterless *AsRed2* in pBluescript – pCCS127

A synthetic DNA linker that contained restriction sites *Sph*I, *Nar*I and *Bgl*II were made by annealing two oligonucleotides (PR79 and PR80). This was inserted into *Hin*dIII site of pBS-SacII to construct pCCS120. Annealing reaction was performed in 14 µl reaction, containing 6 µl of each oligonucleotide (10 mM) and 2 µl of annealing buffer, with one cycle of 70ºC for 15 min, 37ºC for 20 min, and RT for 10 min. 1~3 µl of the mixture was used for ligation. Since synthesized oligonucleotides do not have phosphate groups at both the 5' and 3' ends, the vector was not treated with alkaline phosphatase so that ligation would be possible. Screening was carried out based on the criterion that positive recombinant plasmids would be cut by *Bgl*II but not by *Hin*dIII. Orientation of the insertion was determined by sequencing. Fragment of RBS-*AsRed2*-Terminator cassette was PCR-amplified from pCCS126 using PR29 and PR30 (refer to section 2.2.2.2.1 on page 42), and inserted into *Pst*I and *Bam*HI sites of pCCS120 to generate pCCS127.

Primer sequences are:

	(*Hin*dIII)	(*Bgl*II)	(*Nar*I)	(*Sph*I)	(*Hin*dIII)
PR79:	5′ AGCT **AG**	AGATCT	TC GGCGCC	CAT GCATGC	**T** 3′
PR80:	3′ **TC**	TCTAGA	AG CCGCGG	GTA CGTACG	**A**TCGA 5′

Note: The base-pairs in bold were designed so that the original *Hin*dIII site:
5′...A▼AGCTT...3′
3′...TTCGA▲A...5′ would be destroyed after ligation.

2.2.2.4.2 Promoters of interest fused upstream of *AsRed2* – pCCS127-derived plasmids

Eleven different promoters were PCR-amplified from *E. coli* MG1655 genomic DNA and inserted into *Sph*I and *Bgl*II sites in pCCS127. Extents of promoters

were decided based on the information on the intergenic regions from Kyoto Encyclopedia of Genes and Genomes database (KEGG) [119] and with reference to the promoter regions documented ("Ref" column in Table 2.7.). Construction of pCCS204 with the promoter of *fadB* was shown in Fig 2.3., which is representative of other plasmids listed in Table 2.7.

Table 2.7. Primers used for construction of pCCS127-derived plasmids with promoter-*AsRed2* fusions.

Promoter[a]	Plasmid[b]	PR[c]	Sequence (5'->3')	Limit[d]	Ref[e]
P_{fadB}	pCCS204	118	GCCGCATGC(*Sph*I)TCCATTTTTTACCCTTCT	-194	[120]
		119	GGAAGATCT(*Bgl*II)GTCAGTCTCCTGAATCCG	-1	
P_{sulA}	pCCS320	240	GGGGCATGC(*Sph*I)CATAAAATTCCTTTTAAAAT	-221	[121]
		242	GGAAGATCT(*Bgl*II)TGTGAGTTACTGTATGGATG	-16	
P_{acrA}	pCCS182	221	CGGGCATGC(*Sph*I)ATGTTCGTGAATTTACAGGC	-141	[122]
		222	GGAAGATCT(*Bgl*II)TATGTAAACCTCGAGTGTCCGATTTC	+1	
P_{rne}	pCCS205	205	CCGCATGC(*Sph*I)CAGCAAGAAGTGAAAAAAC	-452	[123]
		117	CGTAGATCT(*Bgl*II)CTCATTATTCTTACATTGACG	-10	
P_{rpoE}	pCCS319	247	GGGGCATGC(*Sph*I)TGGTCAGCCAGGCGTAG	-500	[124]
		248	GGAAGATCT(*Bgl*II)CCCCAAACCAAATTTCCACGCG	-12	
P_{recA}	pCCS207	121	CGTGCATGC(*Sph*I)TCGTCAGGCTACTGCG	-140	[121]
		115	CGTAGATCT(*Bgl*II)TTTTACTCCTGTCATGCCG	+1	
P_{msrA}	pCCS206	120	CGTGCATGC(*Sph*I)CATTTTCTCCTGAATATC	-206	[125]
		113	CGAAGATCT(*Bgl*II)TGGTGTCGCTCTCCCGA	+1	

Promoter[a]	Plasmid[b]	PR[c]	Sequence	Limit[d]	Ref[e]
P_{gadA}	pCCS175	226	CGGGCATGC(*Sph*I)AACTCAGTATTTAACGTTGAGC	-888	[126]
		227	GGAAGATCT(*Bgl*II)CGAACTCCTTAAATTTATTTGAAGGCA	-2	
P_{flgB}	pCCS184	225	CGGGCATGC(*Sph*I)GTTTTATTATCAGCATTTTC	-171	[127]
		224	GGAAGATCT(*Bgl*II)CTCCTCCGCAGGTATCAAAA	-2	
P_{pspA}	pCCS177	230	CGGGCATGC(*Sph*I)GATGCGAAACCTGTTC	-239	[128]
		229[f]	CCGCGGATCC(*Bam*HI)TTGTCCTCTTGATTTCTGCG	-5	
P_{rbsD}	pCCS318	243	GGGGCATGC(*Sph*I)CGAAATCTAACCCAGACGC	-176	[121]
		245	GGAAGATCT(*Bgl*II)TCACAGCATCACACACCACCAGC	+88	

[a] Promoter refers to promoters of interest.

[b] Plasmid refers to constructed plasmid (pCCS127-derived plasmids).

[c] PR refers to primers used to PCR-ampilfy each promoter of interest.

[d] Limit refers to limits of promoters cloned upstream of *AsRed2* gene as defined by the primers. The numbers shown are with respect to the start codon of each gene.

[e] Ref refers to the references that documented the transcriptional start site of each promoter.

[f] The promoter region of P_{pspA} has inherent *Bgl*II,site, hence *Bam*HI site was incorporated into the primer instead.

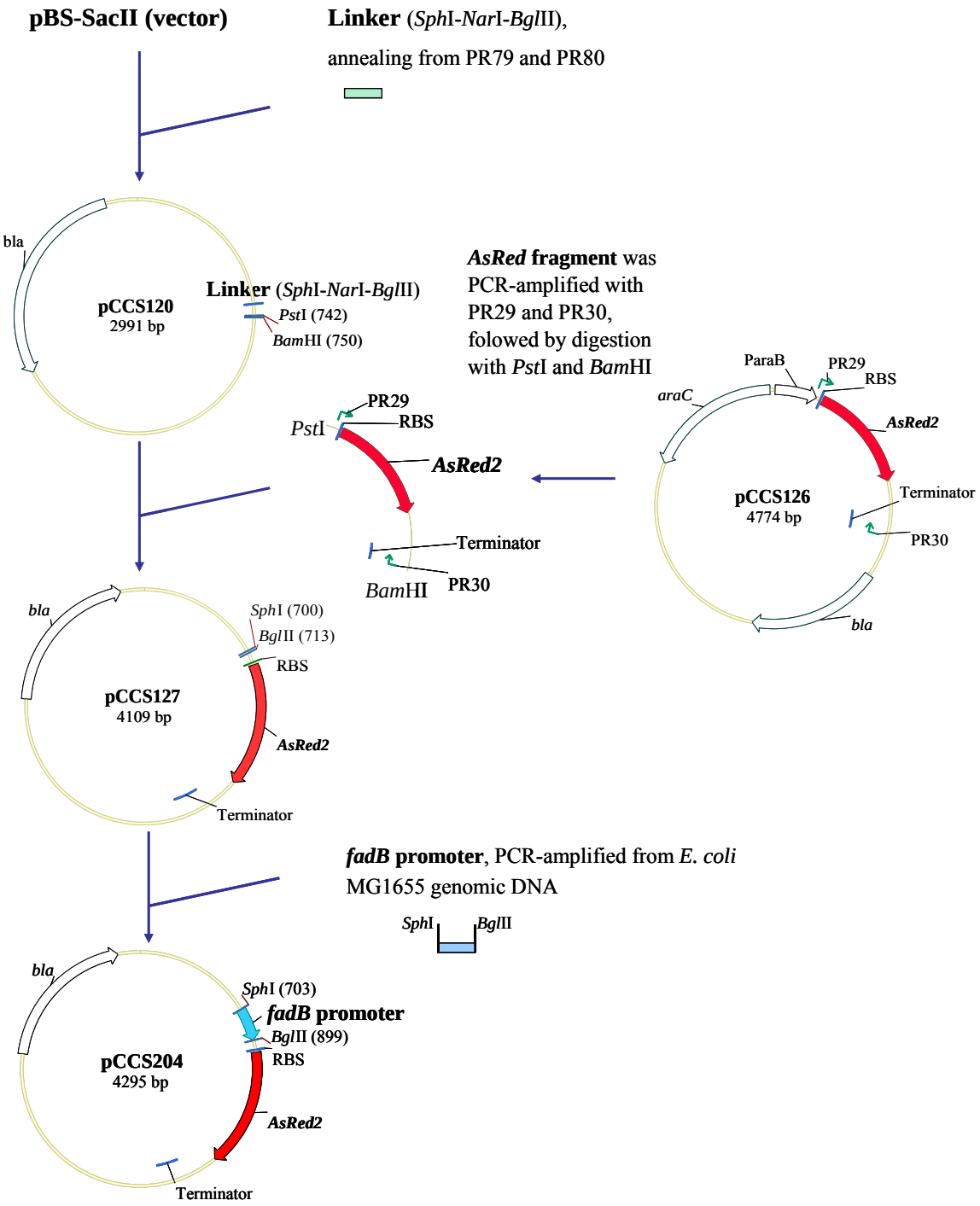

Fig 2.3. Construction maps of pCCS120, pCCS127 and pCCS204. The Linker *Sph*I-*Nar*I-*Bgl*II was inserted into pBS-SacII to generate pCCS120. RBS-*AsRed2*-Terminator cassette was PCR-amplified from pCCS126 and inserted into pCCS120 to derive pCCS127. Promoter of *fadB* was PCR-amplified from *E. coli* genomic DNA and inserted upstream of *AsRed2* to derive pCCS204. Insert DNA fragments were ligated into compatible sites on the vectors.

2.2.2.4.3 Plasmid constructed as parental plasmid for the generation of *AsRed2* mutant library – pCCS325

Fragment containing promoter $P_{A1/04/03}$ was PCR-amplified from pJBA28 [116] using primers PR102 and PR109, and inserted into *Cla*I and *Pst*I sites (upstream of the promoterless *AsRed2* gene) of pCCS127, to generate pCCS325, which would be used as the parental plasmid for *AsRed2* mutant library construction (refer to section 2.2.2.5 on pages 52~54).

Primer sequences are:

PR102: 5′-GGATCGAT(*Cla*I)ATTTATCAGGGTTATTGTCT-3′

PR109: 5′-GGCTGCAG(*Pst*I)TGTGTGAAATTGTTATCCG-3′

2.2.2.5 *AsRed2* mutant library construction

2.2.2.5.1 Preparation of vector and insert

Linearized vector was prepared by restriction digestion of pCCS325 with *Pst*I and *Hin*dIII (Fig 2.4.), followed by purification with Nucleospin ExtractII kit (Macherey-Nagel). The usual procedure of purification through agarose gel electrophoresis for mixed DNA sample was not necessary here because the later cloning strategy used a different mechanism from that of conventional ligation. DNA fragment (approximately 1.5 kb) containing *AsRed2* gene was excised from pCCS325 with the restriction enzymes *Kpn*I and *Sac*I, then purified through agarose gel electrophoresis to serve as the template for error-prone PCR. The primers were designed to contain more than 15 bp of sequence that is homologous to the ends of the linearized vector. This will ensure the *AsRed2* mutants generated would contain the homologous arms to allow fusion with the linearized vector via In-Fusion enzyme.

Primer sequences are:

PR304:5′-**GCGGATAACAATTTCACACAC**TGCAG(*Pst*I)-3′

PR307:5′-**CTGAGATGAGTTTTTGTTCTAGA**AAGCT(*Hin*dIII partial)-3′

The base pairs that are homologous to the ends of vector are shown in bold.
(Clontech online tool was used for primer design:
http://bioinfo.clontech.com/infusion/convertPcrPrimersInit.do)

2.2.2.5.2 Cloning using In-Fusion™ 2.0 PCR Cloning Kit and One Shot® TOP10 competent cells

Approximately 71.3 ng linearized vector and 32.8 ng insert DNA (amounts as derived from the online tool:http://bioinfo.clontech.com/infusion/molarRatio.do) were used to perform cloning using In-Fusion™ 2.0 PCR cloning kit. The product was then 5-fold diluted and 2.5 µl of the diluted sample was used for transformation into One Shot® TOP10 competent cells (refer to section 2.2.1.6.3 on page 38).

2.2.2.5.3 Harvesting and storage of mutant library

The LA-Ap plates with library transformants were incubated at 37°C for 16 h, followed by storage at 4°C for another 24 h. We observed that some colonies on the plates were visibly redder than others. Hence we took half of each of the observed red colonies and streaked it onto LA-Ap plates. The streaked LA-Ap plates were incubated at 37°C for 16 h and used for further analysis. We scraped off all the remaining colonies and transferred them to LB-Ap with an approximate cell density of 4000 colonies/ml. Glycerol was added to the culture to a final concentration of 20% and the library was aliquoted to be stored as frozen stock vials at -80°C, for further screening in the future.

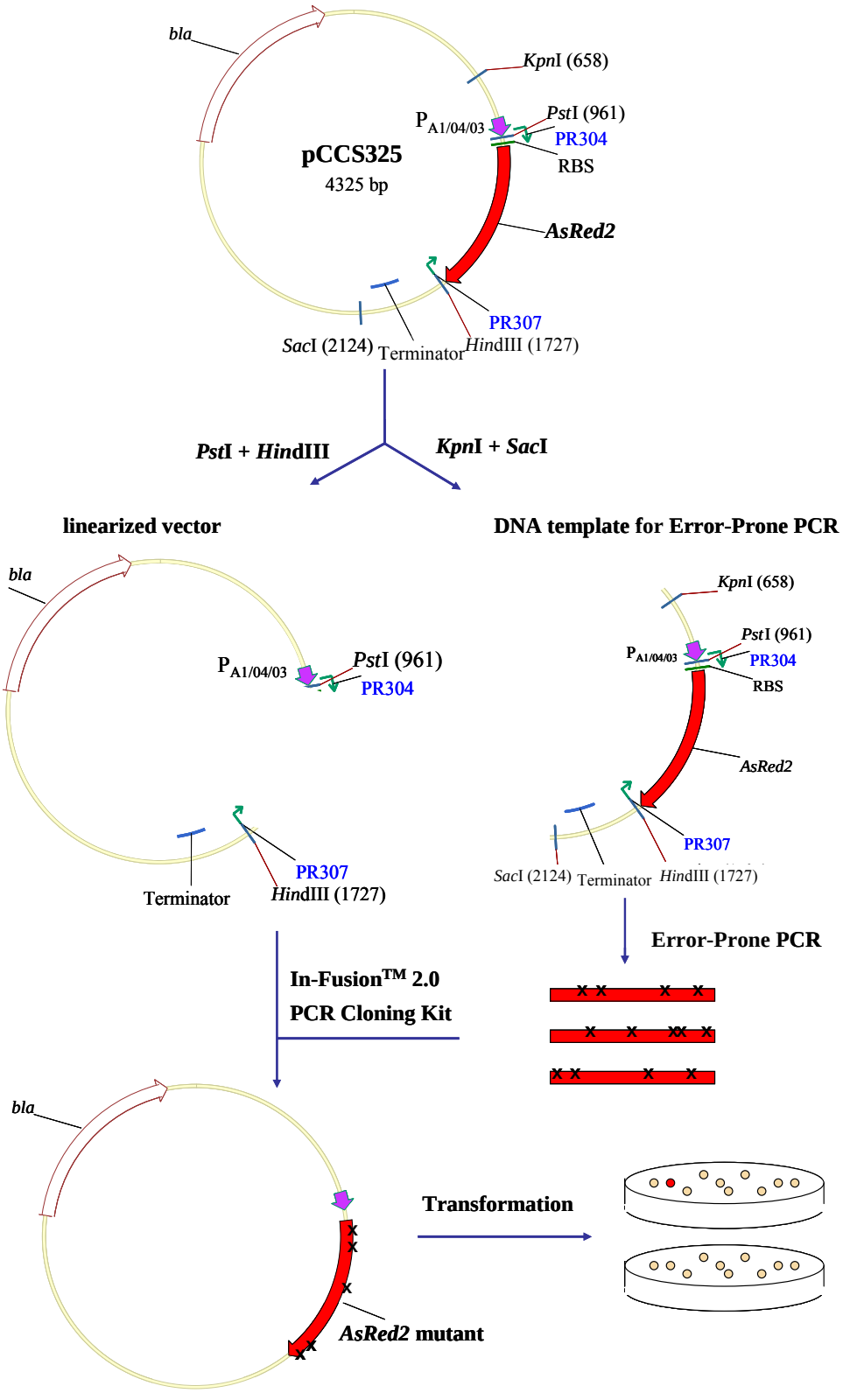

Fig 2.4. Construction of *AsRed2* mutant library. pCCS325 was digested to provide linearized vector and template for EP-PCR. Products of EP-PCR ("x" denotes point mutation) were fused into linearized vector using In-Fusion™ 2.0 PCR Cloning Kit, followed by transformation into One Shot® TOP10 competent cells.

2.2.3 Screening of *E. coli* MG1655 recombinants with $P_{A1/04/03}$-*gfp*mut3* chromosomal integration

2.2.3.1 First recombination event

The chloramphenicol resistance gene on pDM4-derived suicide plasmids was utilized for the first round of screening for plasmid integration. pCCS167~pCCS171 containing insertion regions No.7~11 were electroporated into *E. coli* MG1655 strain following the procedures described in section 2.2.1.6.2 on page 38. After electroporation, 1 ml SOC was added into each tube, and the mixture was statically incubated at 37ºC for 3.5 h. Then 500 µl of the mixture was diluted with 500 µl SOC and the static incubation was continued for another 14 h, before spreading the mixture onto LB agar supplemented with 15 µg/ml chloramphenicol (LA-Cm). Colony PCR using PR102 and PR109 was used to confirm the first recombination events, which amplified the promoter region of the *gfp*mut3* cassette.

2.2.3.2 Second recombination event

The *sacB* gene on the suicide plasmid served as a counter selective marker for the screening of those strains that had recombined a second time, and excised the plasmid backbone out of the chromosome, leaving only *gfp*mut3* cassette remaining on the chromosome. With the induction by sucrose, the *sacB* gene encodes an enzyme (levansucrase) that is lethal to Gram negative bacteria in the presence of 5% sucrose in the agar medium [129]. Only strains that did not carry the *sacB* gene could survive on 5% sucrose plate. Due to the delay of this lethal effect, strains with *sacB* gene could, in reality, form colonies on the sucrose-containing agar. However, they would lyse upon longer incubation, and

this would be manifested by "sticky" colonies when picked by toothpicks. To screen for second recombination events, the strains screened (refer to section 2.2.3.1 on page 55) were inoculated into 3 ml LB-Cm medium and shaken for 12 h at 37ºC. 30 µl of the LB-Cm culture was subcultured in 3 ml SOC and shaken for another 12 h to allow sufficient time for second recombination to occur. 30 µl of the SOC culture was then transferred into 3 ml LB medium supplemented with 5% sucrose (LB-5% sucrose) and shaken for 12 h to enrich for strains that had undergone second recombination. Cell density was adjusted to ensure good colony separation on the agar plates, before spreading the culture onto LA plate supplemented with 5% sucrose (LA-5% sucrose). The LA-5% sucrose plate was incubated for 24 h for the "sticky" phenotype to be apparent. Non-sticky colonies were picked onto a master plate of LA-5% sucrose, followed by screening of colonies with intact *gfp*mut3* cassette via PCR (refer to section 2.2.1.2.1 on page 33).

2.2.4 Culture conditions

2.2.4.1 Single- species cultures

2.2.4.1.1 Planktonic cultures

Single-species planktonic cultures were routinely incubated in 15 ml snap-cap tubes at 37°C with shaking at 250 rpm in a LM-570 incubator (MRC), unless otherwise stated. *E. coli* strains were either grown in LB medium for general propagation and cloning purposes, or Brain-Heart Infusion (BHI, Difco™, Becton Dickinson) medium for experiments involving co-cultures. Where appropriate, media were supplemented with 200 µg/ml ampicillin (USBio) or 15 µg/ml chloramphenicol (USBio). Four-round sub-culturing experiment was

performed by four subsequent rounds of 100-fold dilution of 24 h culture into 3 ml of fresh medium. For experiments that involved promoter activity assays, pure cultures were inoculated from -80ºC frozen stock vials and grown under inducing or non-inducing conditions. For P_{araB} induction, L-arabinose (Sigma Aldrich) was added to the fresh medium to derive at the indicated final concentrations, and the cultures were shaken at 37°C for 24 h. To assay for activities of P_{lacZ}, P_{fadB}, P_{sulA}, P_{acrA}, cultures were shaken at 37°C for 6 h (OD_{600} of 0.8 ~1.5) for late exponential phase, and 24 h (OD_{600} of 8 ~ 10) for late stationary phase. For P_{rpoE}, cultures were also shaken at 30°C and 42°C (in addition to 37°C), and assayed at 24 h. Non-*E. coli* strains were grown in BHI medium without antibiotics.

2.2.4.1.2 Biofilm static tank

Biofilms were cultured either on glass slides (26x76 mm, CellPath) for CFU count and FACS analysis, or on glass coverslips (24x50 mm, CellPath) for CLSM analysis (CellPath), both of which were slanted at a slight angle in a glass tank (Fig 2.5.) [130]. Biofilm cultures use BHI supplemented with 2% mucin (Sigma Aldrich) as the medium, and was inoculated with a starting cell density of $6x10^6$ cells/ml, and incubated statically at 37°C. Cell densities were adjusted according to the correlation of CFU/ml against OD_{600} as shown below (Chew et al, unpublished data):

Strains	**CFU/ml at OD_{600}=1**
E. coli MG1655	$3.858x10^8$
E. coli SCC1	$3.447x10^8$
K. pneumoniae	$1.127x10^9$
E. faecalis	$1.149x10^9$

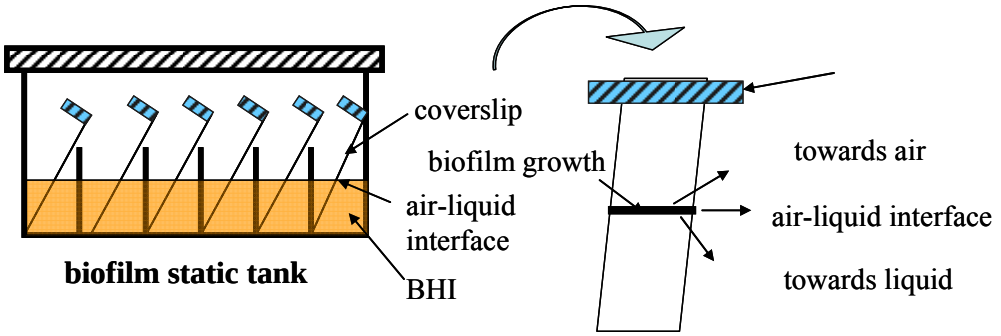

Fig 2.5. Schematics of a biofilm static tank. Biofilms are formed at the air-liquid interface. They can be removed from glass slide/coverslip and sonicated for CFU count, or directly analyzed by confocal laser scanning microscope (CLSM). An autoclaved tape was adhered on and folded over the top of coverslip to stabilize its position in the tank.

2.2.4.2 Co-cultures

2.2.4.2.1 Population dynamics

For experiments involving co-cultures for population dynamics, individual strains were first shaken in 3 ml BHI media for 12~16 h and their cell densities checked by measuring OD_{600}. These cultures were then mixed 1:1 to achieve a final density of $6x10^6$ cells/ml for each species, and then cultured planktonically (3.6 ml cultures in 15 ml snap-cap tubes) or as biofilms (25 ml cultures in static tanks) for 24 h or 48 h as described in previous sections.

2.2.4.2.2 Promoter activity assay

For experiments involving co-cultures for promoter activities, individual strains were first shaken in 3 ml BHI media for 12~16 h and their cell densities checked by measuring OD_{600}. *E. coli* strains carrying plasmids had been cultured with ampicillin to ensure the maintenance of plasmids during this stage. Subsequently, all old culture media were removed by centrifugation at 2540 *g* at RT for 10 min, followed by resuspension in 3 ml fresh BHI media. After

another round of centrifugation, both *E. coli* and non-*E. coli* strains were resuspended in fresh BHI media. Cultures of the strains were mixed 1:1 to achieve a final density of $6x10^6$ cells/ml for each species, and cultured planktonically or as biofilms for 24 h or 48 h, before being assayed.

2.2.5 Viability counts and growth monitoring

2.2.5.1 Colony Forming Unit (CFU) enumeration

Viable cell numbers were determined by CFU counts. A series of 10-fold dilutions were performed by transferring 200 µl cultures to 1800 µl sterile ddH_2O in 5 ml snap-cap tubes and colony forming units per ml (CFU/ml) were determined by plating 100 µl of suitably diluted cultures on HiCrome UTI agar plate (HiMedia™) (hereafter referred to as UTI agar) in triplicates, unless otherwise stated. Cultures with *E. faecalis* were also spread onto deMan, Rogosa and Sharpe (MRS) agar plates for selective enumeration of *E. faecalis*. UTI agar plates were routinely incubated for 12~16 h at 37°C, while MRS agar plates were incubated for 24 h under the same conditions. Only plates with the number of colonies in the range of 30~300 were counted.

2.2.5.2 Growth monitoring

2.2.5.2.1 Planktonic cultures

Growth of planktonic culture was monitored via OD_{600} on a BioSpec-mini spectrophotometer (Shimadzu). Raw measurements of OD_{600} were maintained within the linear range of 0.3~0.5 to minimize variability.

To obtain growth curves in planktonic cultures, OD_{600} of overnight cultures were first measured and subsequently diluted to an OD_{600} of 0.05 in 100 ml of corresponding medium in 500 ml baffled culture flasks and shaken at 37°C, 250 rpm in an incubator (LB-570, MRC). OD_{600} was taken at 30 min interval for the first 4 h and 1 h interval for the later time points. Mean data of the duplicate cultures in exponential phase were used to calculate generation time (G) using the formula (iii): $G = t / [3.3 \times \lg(b / B)]$.

This is derived from:
(i): G (generation time) = t/n;
(ii): $b = B \times 2^n$;
where t: time interval in minutes; b: OD_{600} of culture at the end of the time interval; B: OD_{600} of culture at the beginning of the time interval; n: number of generations (cell population doubling) during the time interval.

From (ii), solve for n:

$\lg b = \lg B + n\lg 2$;

$n = (\lg b - \lg B)/\lg 2$;

$n = 3.3 \lg(b/B)$,

Since (i) $G = t/n$

therefore (iii) $G = t / [3.3 \times \lg(b / B)]$.

2.2.5.2.2 Biofilm cultures

For biofilm cultures, slides were first gently rinsed in sterile ddH_2O to remove non-adherent cells. Stably attached biofilms from two slides were then scraped using 1 ml pipette tips into 1 ml of sterile ddH_2O, vigorously vortexed for 20 pulses, followed by sonication at 50% power on an ultrasonic bath (Tru-Sweep™ Ultrasonic Cleaners 575, Crest Ultrasonics) for 2 cycles of 15 min,

and vigorously vortexted for 20 pulses after each round of sonication, before OD_{600} measurement.

2.2.6 Equipments settings

2.2.6.1 Fluorometric microplate reader (fluorometer)

2.2.6.1.1 Parameter settings

Fluorescence levels of bacterial cultures were measured using an Infinite™ M200 multi-mode monochromator-based microplate reader (Tecan). The excitation/emission wavelength settings were 488 nm/530 nm for samples with Gfpmut3* expression, and 570 nm/600 nm for samples with AsRed2 expression. Duplicate aliquots of 200 μl of each appropriately diluted culture were dispensed into a 96-well black-wall clear-bottom microplate (Greiner). The green and red fluorescence levels (in Fluorescence Unit) and spectrophotometric absorbance at 600 nm (in Absorbance Unit) were sequentially measured and corrected by the medium control (blank).

2.2.6.1.2 Technical validation

The detection power for fluorescence was maximized (gain setting was at 95), with reference to two fluorescent *E. coli* strains: SCC1 (a green fluorescent strain, refer to section 3.2.3.3 on pages 90~92) and MG1655(pAsRed2) (strain MG1655 harboring pAsRed2, a red fluorescent strain). The Absorbance Unit has been shown to be accurate within the range of 0.02~0.35 (Chew et al, unpublished data). To examine the fluorescence quantification competency of the fluorometer, cultures of *E. coli* strains SCC1, MG1655 (non-fluorescent negative control) and MG1655(pAsRed2) were each diluted to generate a series

of increasing cell density. The fluorescence intensity and absorbance at 600 nm of these samples were measured subsequently (Fig 2.6.). Both the green Fluorescence Unit of SCC1 (green symbols in Fig 2.6.A) and the red Fluorescence Unit of MG1655(pAsRed2) (red symbols in Fig 2.6.B) had linear correlation with Absorbance Unit. This indicates that at this setting: (i) the fluorometer could quantify samples of a wide range of fluorescence intensity exemplified by the series of cell cultures used, with proper sensitivity and absence of signal saturation; and (ii) the ratio of Fluorescence Unit over Absorbance Unit is nearly constant for each fluorescent strain. Furthermore, the fact that MG1655(pAsRed2) in Fig 2.6.A and SCC1 in Fig 2.6.B shared similar profile with MG1655 suggested that there is very little signal spillage between the two fluorescence with respect to detection.

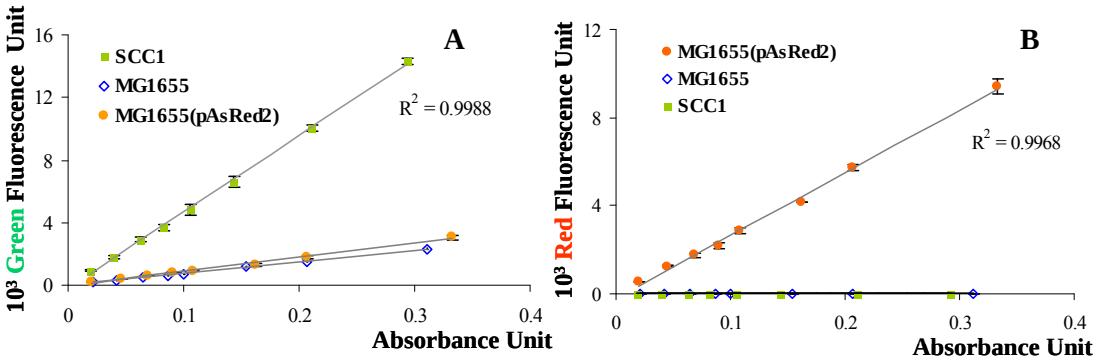

Fig 2.6. Linear relationship of fluorescence and absorbance signals detected by the fluorometer. Fluorescent positive or negative *E. coli* strains overnight cultures were diluted and their fluorescence intensity and absorbance were plotted. Green fluorescence (**A**) and red fluorescence (**B**) are plotted against absorbance for strains SCC1 (green), MG1655 (non-fluorescent) and MG1655(pAsRed2) (red). Linear correlation (R^2) are shown, which are both close to 1.

For fluorescence quantification, the fluorescence levels of the samples were normalized against the cell density to derive at Relative Fluorescence Unit (RFU): RFU = (Fluorescence Unit x 10^{-3}) / (Absorbance Unit at 600 nm).

2.2.6.2 Fluorescence activated cell sorter (FACS)

2.2.6.2.1 Parameter settings

Flow cytometric analyses were performed on FACSCalibur™ and/or FACSAria™ Cell Sorter Systems (both Becton Dickinson), while subpopulation sortings were performed only on FACSAria™. Both are equipped with air-cooled argon lasers at 488 nm. Filtered phosphate buffered saline was used as the sheath fluid, while BHI medium was used both as the diluent for samples and the collection fluid for sorted fractions. Detection of fluorescence on FACSCalibur™ was through FL1 filter (530/30 nm) for GFP and FL2 filter (585/42 nm) for AsRed2, while detection on FACSAria™ was through FITC filter (530/30 nm) and PE filter (585/42 nm) respectively. Compensation has been applied for FACSAria to ensure that the red fluorescence detected has minimal contribution from the green fluorescence of GFP, as recommended by the manufacturer. For FACSCalibur™, the following settings were used: the 90° side-scatter (SSC) signals at the level of 38 were set as the threshold to define an event to be counted, and the voltage settings for the parameters were: FSC E02, SSC 412, FL1 520, FL2 566, at flow rate "Lo". For FACSAria™, SSC signals at the level of 200 were set as the threshold, and the voltage settings for the parameters were: FSC 300, SSC 300, FITC 500, PE 650, at a flow rate of 1 or 2. All raw data were processed via the software FlowJo (Tree Star). Gates of G+/G- or R+/R- were defined by using green negative control (*E. coli* MG1655) and red negative controls (*E. coli* MG1655 and SCC1) Levels higher than those shown by the negative controls were defined as green or red positive gates. Fluorescence intensity of both FACSCalibur™ and

FACSAria™ was quantified by processing the geometric mean fluorescence intensity (GMFI) of the relevant sample sets.

2.2.6.2.2 Measurement procedures

To achieve analysis and sorting resolution approximating 1 event = 1 cell, the samples were diluted to a cell density of 10^7 cells/ml, and kept on ice prior to analysis. In general, 10,000 events of each sample were analyzed in triplicates unless otherwise stated. If the number of *E. coli* SCC1 events (defined as G+) was found to be very low in proportion within the co-culture, the sample was run until at least 2000 events of green positive cells had been detected and analyzed.

2.2.6.2.3 Checking the purity of sorted fractions

To check the purity of the sorted fractions from a mixture of *E. coli* and non-*E. coli* strains, 10,000 events each of green positive and green negative subpopulations (according to the gates shown in Fig 3.12.) were sorted into separate fractions in triplicates and plated on UTI agar for CFU counts.

2.2.6.3 Confocal Laser Scanning Microscope (CLSM)

Biofilms formed on the coverslips were placed on a flow chamber (Bjarke Bak Christensen, Technical University of Denmark) and kept hydrated with water in the flow chamber channels. This was then observed under an Eclipse 90i CLSM (Nikon) equipped with 488 nm and 543 nm lasers. To observe green fluorescence, the channel was set at 488 nm excitation and 515/30 nm emission; for red fluorescence, 543 nm excitation and 605/25 nm emission. The confocal

images of the two fluorescences were taken sequentially to minimize the crosstalk between them. Controls of the microscope were manipulated by iControl software (Nikon). Images were processed by EZ-C1 3.20 FreeViewer software (Nikon).

Chapter 3 Results

3.1 Growth relationship of *E. coli, K. pneumoniae and E. faecalis* in co-culture

The aim of the project is to build a prototype of an analytical system that allows the transcriptional responses due to interspecies interaction be studied (as described in section 1.5 on pages 17~22). Three species (*E. coli, K. pneumoniae, E. faecalis*) were chosen as components in our in vitro system. They are representative commensal organisms in the microbiota of neonatal intestines [109] and all can be readily cultured aerobically. We started first with the attempt to estabilish the co-cultures and monitor the growth relationship of the species in the co-cultures.

3.1.1 Choice of media

There are a few basic technical issues that need to be resolved to make the study of multi-species system possible. These are presented in this section.

3.1.1.1 Brain-Heart Infusion (BHI) medium as co-culture broth

Each component species has been recommended to grow in specific media by American Type Culture Collection (ATCC) − Luria-Bertani (LB) for *E. coli*, Nutrient Broth (NB) for *K. pneumoniae* and Brain-Heart Infusion (BHI) for *E. faecalis*. However, all three species would need to be grown in a single medium for co-culture experiments, so a suitable broth medium for planktonic and biofilm co-cultures needs to be selected.

3.1.1.1.1 Planktonic cultures

To test which broth media can best support all the three species in planktonic cultures, they were first inoculated in their ATCC-recommended media to obtain the seed cultures, which were then diluted into LB, NB and BHI media. The growth curves of the three species as monitored by measurement of OD_{600} (refer to section 2.2.5.2.1 on pages 59~60) are shown in Fig 3.1.A~C on page 68. All the three species were found to grow best in BHI, with the shortest generation time (*E. coli*: 24.2 ± 0.3 min; *K. pneumoniae*: 24.5 ± 0.9 min and *E. faecalis*: 31.3 ± 0.4 min) and the highest saturating cell density at stationary phase (reflected by OD_{600}). In comparison, the proliferation of the three species in LB and NB were slower (reflected by the longer generation times) and they ceased growth at lower saturating cell density.

3.1.1.1.2 Biofilm cultures

The biofilm cultures of the three species were grown in static tanks in various broth media. For each species, the largest biofilm biomass was observed with BHI medium (Fig 3.1.D~F). Biofilm biomass of *E. coli* and *K. pneumoniae* were observed to drop dramatically at 72 h data point. This is an indication of biofilm detachment, hence for later experiments, it was decided that biofilm cultures need only be grown until 48 h.

Both planktonic and biofilm data have shown that all three species could accumulate higher biomass in BHI than the other two media. Hence we deem BHI as a suitable co-culture broth media in this study, for ease of analysis derived from the adequate biomass.

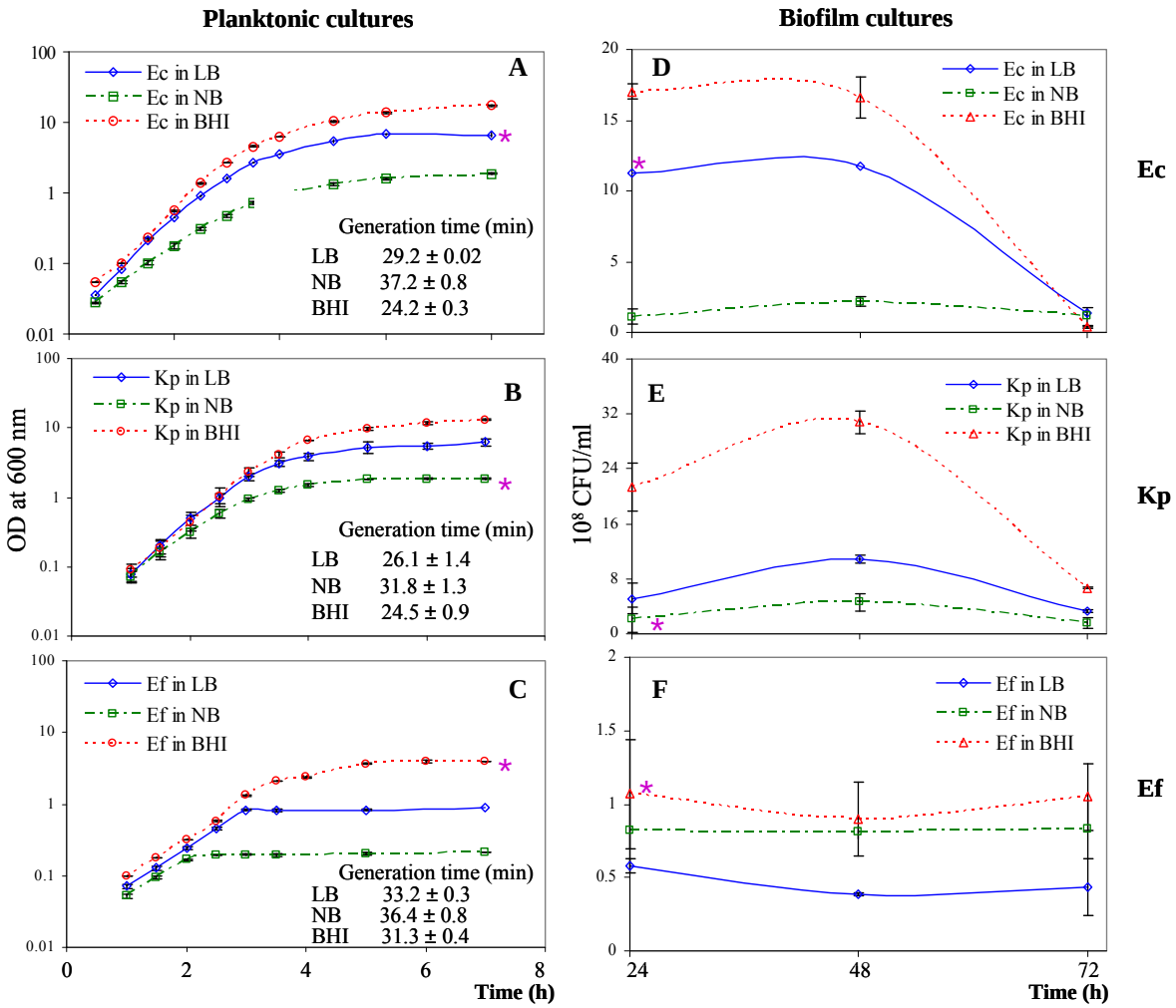

Fig 3.1. Growth of the three species in different media in planktonic and biofilm cultures. (A)~(C): Growth curves of the planktonic cultures in various media, as monitored by OD_{600}. Inset: generation time of each species in different media in minutes. Growth monitoring procedures and calculation of generation time were as described in section 2.2.5.2.1 on pages 59~60. **(D)~(F)**: Viability count of biofilms adhered on glass slides immersed in different media supplemented with 2% mucin, statically incubated at 37ºC. Ec: *E. coli*; Kp: *K. pneumoniae*; Ef: *E. faecalis*. Asterisk (*) denotes ATCC-recommended medium for each species. Data shown are the mean ± SD of duplicate samples.

3.1.1.2 Chromogenic UTI agar for CFU count of co-cultures

3.1.1.2.1 Qualitative differentiation

For the purpose of studying species composition within the co-culture, an agar medium that allows CFU count for the three species is desirable. Chromogenic UTI agar (hereafter referred to UTI agar) came under our consideration because according to the manufacturer's information, the three species would appear on this agar with different colony morphologies. As shown in Fig 3.2.A (on page 70), they have indeed unique colony colors on UTI agar. *E. coli* is purple, *K. pneumoniae* is dark blue and *E. faecalis* is light blue. Fig 3.2.B shows that they can be differentiated on UTI agar when they were mixed and spread. It was observed that the colony size and textures were also quite distinct. *E. coli* is big and flat, *K. pneumoniae* is big and mucoid, and *E. faecalis* is small and flat, with a white speck in the center. These unique colony morphologies make it possible to perform CFU counts for the three component species in the co-culture.

3.1.1.2.2 Quantitative consistency of CFU count derived from UTI agar compared to ATCC-recommended medium

We proceeded to examine whether CFU count derived from UTI agar can be consistent with that from ATCC-recommended media plate. To compare this, the same amount of bacterial culture was spread on both UTI and ATCC-recommended media plate for CFU count. Taking the CFU count from ATCC-recommended medium agar as 100% (indicated by the red dotted line in Fig 3.2.C), the percentage of CFU count on UTI agar compared to that of ATCC-recommended medium agar was presented. Fig.3.2.C shows that CFU counts

were consistent between UTI agar and ATCC-recommended medium agar for each species, indicating the viability of the species was not significantly affected on the UTI agar. Hence UTI agar is suitable to be used for studying species composition in later co-cultures experiments.

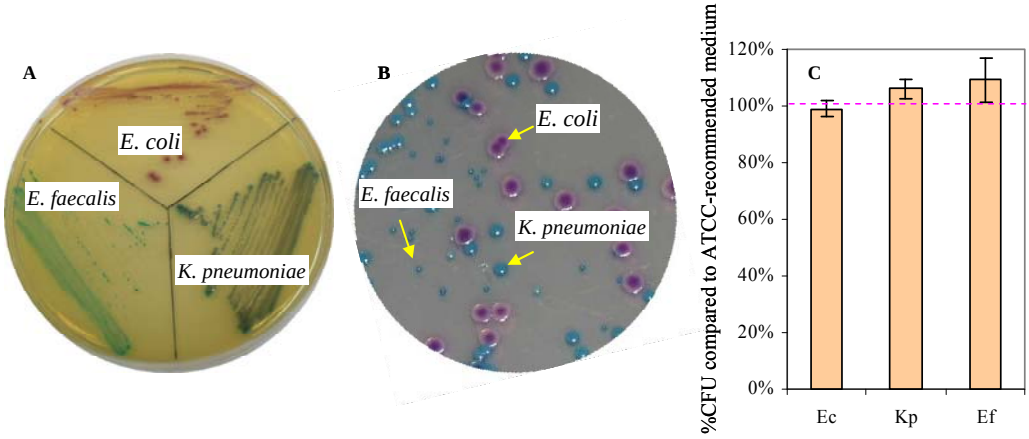

Fig 3.2. Differentiation and quantification of the three bacterial species on UTI agar. (A) The three species were streaked out on UTI agar. **(B)** The three species in mixed culture can be differentiated from each other by colony size, color and texture. **(C)** CFU counts derived from UTI agar and ATCC-recommended media were consistent. Ec: *E. coli*; Kp: *K. pneumoniae*; Ef: *E. faecalis*. Culture of each species was spread onto both UTI and ATCC-recommended medium agar, and the CFU count derived from the latter was deemed as 100% (indicated by the red dotted line). The percentage of CFU count on UTI agar as compared to ATCC-recommended medium agar was presented for each species. Data shown is mean ± SD of triplicate plates, and representative of three independent experiments.

3.1.1.2.3 Masking problem of *E. faecalis* by the other two species on UTI agar

Since CFU counts of the three species in co-cultures were to be carried out concurrently on UTI agar, the CFU count of each species may be adversely interfered by the existence of colonies from other species due to the crowdness on the agar surface, especially when colonies of the other species are greater in

number. We therefore mixed each species in 1:1 ratio and in 1:10 ratio with the other species, and compared its CFU counts in mixtures with that of its pure culture. The CFU count of the pure culture was represented by 100%, and those of mixtures were plotted proportionally in Fig 3.3.A (on page 72). All of the *E. coli* and *K. pneumoniae* samples (blue and red bars respectively) had percentages around 100% (marked by the red dotted line), suggesting their CFU counts were not affected in both 1:1 and 1:10 mixtures. However, *E. faecalis*'s CFU counts were affected by colonies from other species when it was in minority (yellow bars with asterisk, Fig 3.3.A). This could be attributed to the small colony size of *E. faecalis*, which added the difficulty to be counted in the background of big colonies from the other two species (Fig.3.2.B). To alleviate this masking problem of *E. faecalis*, selective media that could inhibit growth of *E. coli* and *K. pneumoniae*, but not inhibit growth of *E. faecalis*, was desirable.

3.1.1.2.4 Selective agar plates for *E. faecalis*

To explore the choice of selective medium for specific CFU count for *E. faecalis*, we started by supplementing compounds into UTI medium. Sodium azide was tried because it can inhibit Gram negative strains. Although the growth inhibition conferred by sodium azide to *E. coli* and *K. pneumoniae* was confirmed, the CFU count of *E. faecalis* dropped as well (data not shown). Alternatively, we looked for other types of medium, and deMan, Rogosa and Sharpe (MRS) agar plate [131] seems to be useful because other researchers had used it for selective enumeration of *Lactobacillus* from fecal samples [132]. Considering the large number of Gram negative strains in fecal samples, this

medium may be useful to eliminate *E. coli* and *K. pneumoniae* from the agar plate. As *E. faecalis* belongs to the family of lactic acid bacteria [133], it should be able to grow on MRS agar. When the three single-species cultures were spread on MRS agar, colonies of both *E. coli* and *K. pneumoniae* were undetectable after incubation for 24 h, while those of *E. faecalis* were visible and countable (data not shown). Viability quantification of *E. faecalis* on MRS plate was consistent when compared with UTI agar (Fig 3.3.B). MRS agar was therefore chosen to complement UTI agar to provide *E. faecalis*'s CFU data in co-culture samples later on.

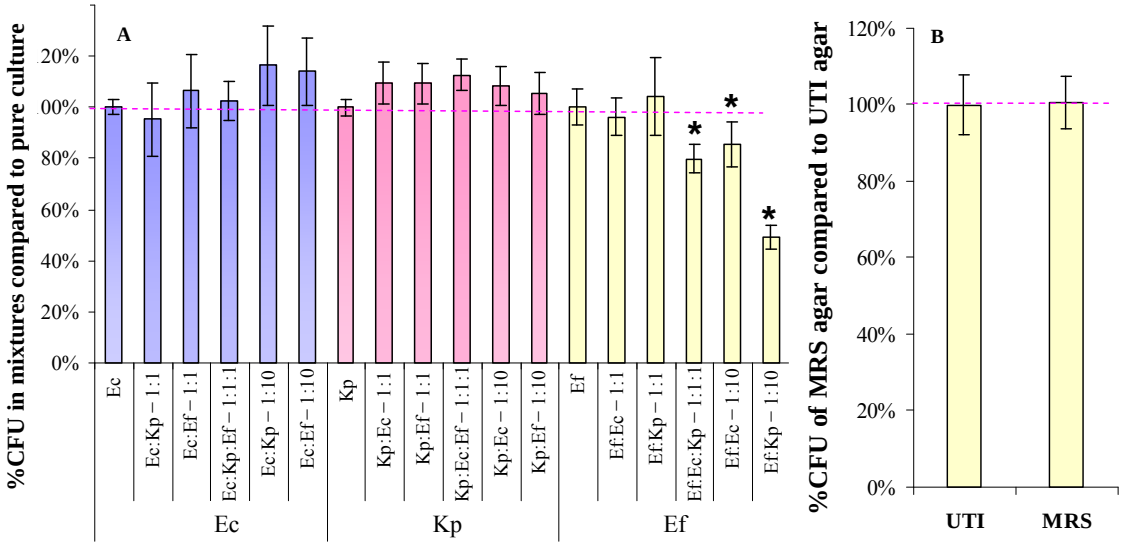

Fig 3.3. Masking problem of *E. faecalis* and selective medium. (A) Comparison of CFU counts of each species in mixtures and in pure culture. Ec: *E. coli*; Kp: *K. pneumoniae*; Ef: *E. faecalis*. Culture of each species was first diluted to have cell density of approximately 150 colonies per plate before mixing in 1:1 and 1:10 ratio with other species. The CFU count derived from the pure culture sample was represented by 100% (indicated by red dotted line), and those of the mixtures were plotted proportionally. Data shown is mean ± SD of triplicate plates, and representative of three independent experiments. **(B)** Comparison of CFU counts between UTI and MRS agar plates. *E. faecalis* culture was spread on both UTI and MRS agar. The CFU count on UTI plates was represented as 100% (indicated by red dotted line), and that of MRS agar was plotted proportionally. Data shown is mean ± SD of two independent experiments.

3.1.2 Population relationship in dual/multi-species cultures

Population relationship among the three species in co-cultures was then investigated via CFU count to monitor the growth properties of the three species when they were grown with other species. Co-cultures were set up with initial ratio of 1:1 for each species (refer to section 2.2.4.2.1 on page 58) and CFU count was performed at 24 h to study the species composition in both planktonic and biofilm co-cultures.

3.1.2.1 Planktonic co-cultures

The three species were grown in planktonic co-cultures (Fig 3.4.A on page 75). All species in co-cultures dropped in CFU/ml compared to their pure cultures, albeit to different extents. In *E. coli–K. pneumoniae* (Ec–Kp) co-cultures, both species had about 2.5 times reduction in cell density compared to their pure cultures, with a total cell density (horizontally shaded bar) similar to that of the *E. coli* pure culture. *E. coli* was a minority in the Ec–Kp co-culture. In *E. coli–E. faecalis* (Ec–Ef) co-cultures *E. coli* was also reduced to about 1.8 times compared to its pure culture. However it was dominating over *E. faecalis*, which may be due to the faster growth rate of *E. coli* (generation time is 24.2 min for *E. coli* and 31.3 min for *E. faecalis*, refer to Fig 3.1.A and C on page 68). Drop in cell density was most dramatic in *E. coli–K. pneumoniae–E. faecalis* (Ec-Kp-Ef) co-cultures. Unlike Ec-Kp co-cultures, here *E. coli* had a similar cell density with *K. pneumoniae*, and the co-culture had a total cell density less than the *E. coli* pure culture. The population relationships in planktonic co-cultures indicated that there is sufficient interactions present to be reflected by differences in CFU count.

3.1.2.2 Biofilm co-cultures

The three species were also grown in biofilm co-cultures (Fig 3.4.B). Similar to the planktonic counterparts, all species in biofilm co-cultures dropped in CFU/ml compared to their pure cultures. In Ec-Kp co-culture, *E. coli* cell density dropped almost 10 times, and became a more severe minority in the co-culture than the planktonic co-culture. In Ec-Ef co-culture, *E. coli* biomass also dropped considerably (around 6 times reduction compared to *E. coli* pure culture), but the dominance of it over *E. faecalis* was even more overwhelming (*E. coli* was about 28 folds over *E. faecalis*) than the planktonic co-culture. Lowest cell density of *E. coli* was observed in Ec-Kp-Ef co-cultures. The different population relationship profiles imply the interspecies interactions in biofilms may be very different from that within the planktonic co-cultures.

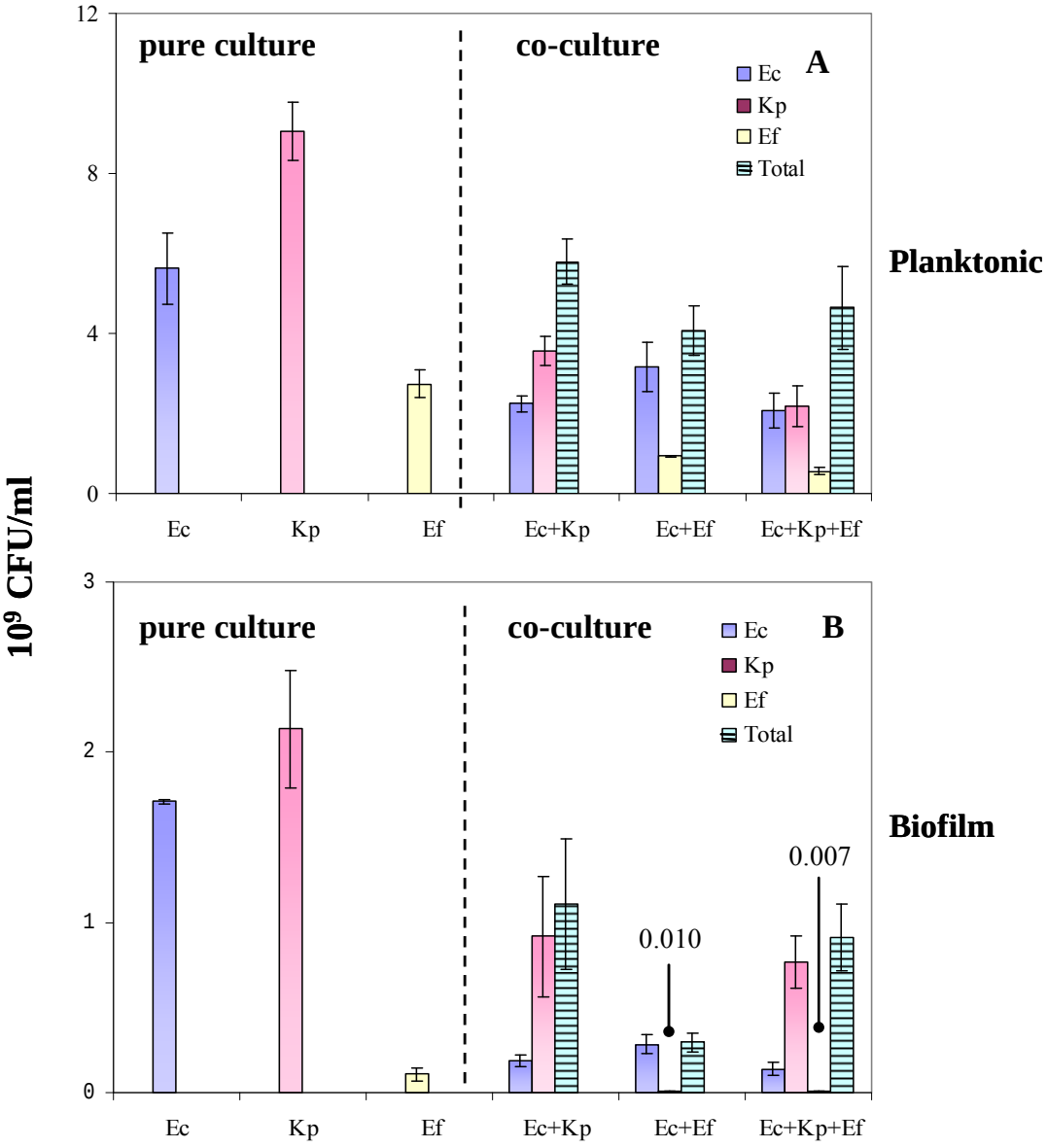

Fig 3.4. Population relationship in planktonic and biofilm co-cultures as assessed via CFU counts. (A) planktonic co-cultures and **(B)** biofilm co-cultures were grown under conditions described in section 2.2.4.2.1 on page 58. Harvest of biofilm cultures was described in section 2.2.5.2.2 on page 60. Numbers shown are CFU data of *E. faecalis* as the values are too small to be visible on graph. Data shown are the mean ± SD of triplicate samples.

3.2 Dual fluorescence system for the analysis of transcriptional response in multi-species contexts

3.2.1 The proposed dual fluorescence system

In order to study the interspecies interactions from the angle of transcriptional responses in one of the component species of our system (refer to section 1.5 on pages 17~22): *E. coli*, the "reporter *E. coli* strain" needs two fluorescence components (Fig 3.5. on page 77): (i) a constitutively-expressed green fluorescent protein (Green FP) to be distinguished from non-*E. coli* species, and (ii) a red fluorescent reporter (Red FP) gene, fused to the *E. coli* promoter of interest, to reflect its transcriptional activity.

The reporter *E. coli* would be labeled via integration of the Green FP gene into chromosome to ensure the consistent maintenance of the gene in *E. coli*. On the one hand, all *E. coli* cells should carry this gene to be fluorescent, and on the other hand, non-*E. coli* species should remain non-fluorescent during the process of experiments. Plasmid-borne FP gene need antibiotics in the growth media to maintain the selective pressure, which is not desirable in co-culture experiments with other susceptible component species. Furthermore, horizontal gene transfer in multi-species co-cultures may occur due to the plasmid, (although this is likely to occur at a very low frequency) to non-*E. coli* species.

The second fluorescence component is the promoter-fusion Red FP gene. Ideally the same criterion mentioned above should be applied, but unlike the Green FP gene, which can be regulated under a sufficiently strong promoter to

allow detection at single copy, Red FP gene would be fused to various promoters with a range of strengths. As a prototype to begin with, we decided to use plasmid-borne *Red FP* to acquire as much information as possible.

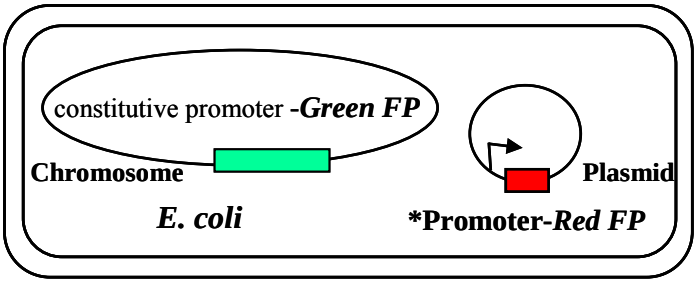

Fig 3.5. A schematic representation of the proposed dual fluorescence system in *E. coli* reporter strain. Green FP gene will be inserted into chromosome of *E. coli*. Red FP gene is plasmid-borne. *Promoter refers to the promoter of interest.

3.2.2 Choice of suitable Green FP and Red FP

Before searching for two suitable fluorescence proteins to label the *E. coli* reporter strain, the autofluorescence of all the three component species were examined by FACSCalibur™ (Fig 3.7.C on page 81 and Fig 3.8.A on page 83). The low autofluorescence ensured a low noise level for fluorescence detection in the proposed dual fluorescence system.

3.2.2.1 The FP gene cassette

The ten commercially available FP genes (BD Clontech) in our laboratory (Table 2.3-ii on page 28) can be grouped into two categories (as indicated by the yellow dotted line in Fig 3.6.A on page 78): green FPs and red FPs, according to the compatability of their emission wavelengths with detection filters of FACS. Before screening for the suitable FPs, Ribosomal binding site (RBS)-FP gene-Terminator cassettes were constructed in pBAD/*myc*-His B

derived plasmids (refer to Table 2.4-i on page 29 and section 2.2.2.1 on pages 39~41). Three benefits can be gained with the constructed FP cassette: (i) the RBS upstream of the initiation codon of FP gene ensures translational efficiency in prokaryotic cells; (ii) *rrnB* T_1 and T_2 transcriptional terminators (Terminator) suppresses readthrough transcription of downstream genes [134]; (iii) the FP was fused downstream of a tightly regulated promoter P_{araB} (or P_{BAD}) in the intermediate plasmids (pCCS126 as representative in Fig 3.6.B), which is useful for the optimizations of the dual-fluorescence system later.

A

Fluorescent Protein (FP)	Excitation (nm)	Emission (nm)
AmCyan	453	486
EGFP	488	507
d2EGFP	488	507
ZsGreen	496	506
EYFP	513	527
ZsYellow	531	540
DsRed-Express	557	579
DsRed-Monomer	557	585
AsRed2	576	592
HcRed1	588	618

B

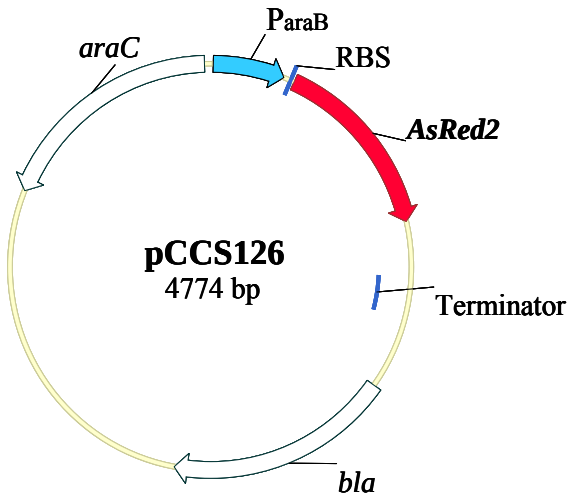

Fig 3.6. Fluorescent proteins properties and a schematic representation of the constructed FP gene cassette. (A): Excitation and emission wavelengths of the ten commercial fluorescent proteins. FPs above the yellow dotted line were categoried as green FPs and FPs bellow the same line were categoried as red FPs in this study. **(B)**: pCCS126 is an intermediate plasmid carrying RBS-*AsRed2*-Terminator cassette. The complete FP cassette can be PCR-amplified for usage in later applications. pCCS126 is shown to represent other similarly constructed plasmids with FP gene cassettes (Table 2.4-i on page 29).

3.2.2.2 Choice of the Green FP gene cassette

As described previously, a suitable Green FP need to be selected to "label" the *E. coli* strain so that it can be distinguishable from non-*E. coli* species.

3.2.2.2.1 Expression stability of EGFP and Gfpmut3*

This Green FP should be stably expressed in *E. coli*, to allow the reporter strain carrying the Green FP gene to authentically represent all *E. coli* cells. Expression stability is related to the entire gene expression process, from transcription, translation, protein folding to protein degradation. We examined the final outcome of the FP gene expression: fluorescence intensity, as an indicator of expression stability in this study.

Four-round sub-culturing is a stringent condition to monitor expression stability, which involves continuous propagation of cultures through daily transfer of a sample of population into fresh broth media with 100-fold dilution over four rounds (refer to section 2.2.4.1.1 on pages 56~57). If the FP causes any unfavorable consequences to cells, sub-culturing would select for spontaneous mutations that could silence its expression. The existence of spontaneous mutation can be reflected by a non-fluorescent subpopulation. For FPs listed in Fig 3.6.A (on page 78), sub-culturing experiment was done with *E. coli* strains carrying the pBAD/*myc*-His B derived plasmids (refer to section 3.2.2.1 on pages 77~78). P_{araB} upstream of FP genes was induced with the addition of L-arabinose in the media to the final concentration of 0.1%, a concentration that can fully activate P_{araB} (data not shown).

The green FPs in Fig 3.6.A (above the yellow dotted line, on page 78) and another FP available in our laboratory: Gfpmut3* [116], underwent the four-round sub-culturing experiment, and green fluorescence intensity was monitored by FACSCalibur^{TM}. EGFP and Gfpmut3* (hereafter referred to as Gfp) were more stably expressed than the others. The profile of EGFP (Fig 3.7.A on page 81) represented green FPs with stable expression over the four sub-culturings. ZsGreen (Fig 3.7.B) was shown to represent green FPs with poor expression stability, which had unpredictable expression patterns over sub-culturings. The non-fluorescent subpopulation suggested spontaneous mutation in this strain. From this experiment, EGFP and Gfpmut3* were selected for further study.

3.2.2.2.2 Intensity level comparison of the green FP gene cassettes

Since the green FP gene cassette would be expressed at single gene copy after being inserted into chromosome, ideally the product of the gene cassette should have high level of fluorescence intensity to ensure adequate detection sensitivity. *rrnB* P1 and P_{lacZ} promoters were inserted upstream of EGFP (refer to section 2.2.2.2.1 and 2.2.2.2.2, respectively, on page 42) to compare with the $P_{A1/04/03}$-*gfp* cassette in pJBA28 [116]. All the three gene cassettes were carried in plasmids derived from the same vector, thus with same copy number, so their expressions are comparable. *rrnB* P1 is a promoter responsible for synthesizing ribosomal RNA, hense constitutively expressed as long as the cell is actively growing. P_{lacZ} is the promoter of *E. coli*'s *lac* operon, generally known to be inducible by IPTG, but the "leakiness" of the promoter gives considerable basal expression [135]. $P_{A1/04/03}$ is a derivative of P_{lacZ} that has been mutated to

become strongly constitutive [136]. The fluorescence intensities of all three cassettes were compared and that of $P_{A1/04/03}$-*gfp* was observed to be the highest (Fig 3.7.D).

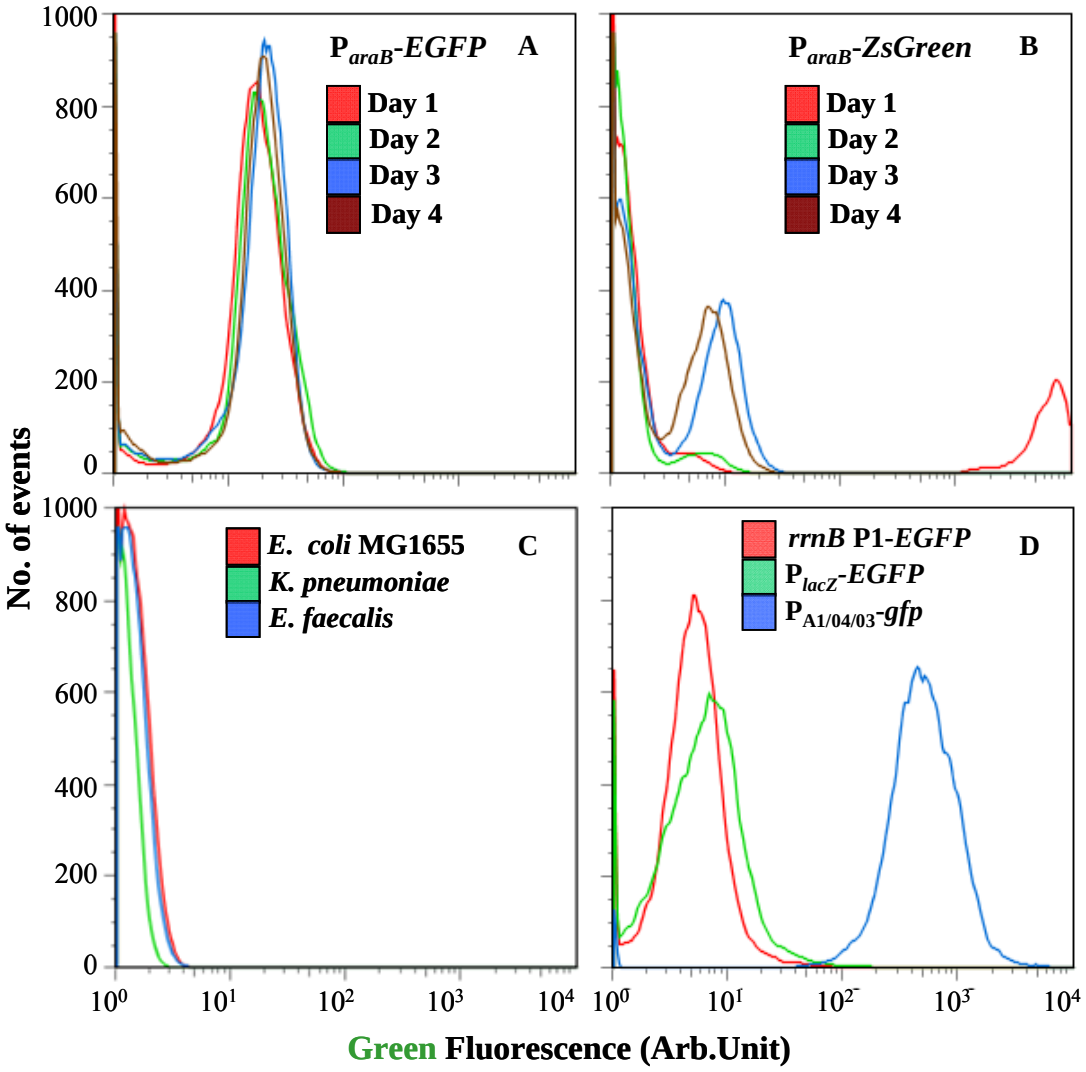

Fig 3.7. Histogram overlay of green fluorescence profiles via FACS analysis. (A) and **(B)**: Green fluorescence profiles of EGFP and ZsGreen, respectively, in four-round sub-culturing experiments. **(C)**: Green autofluorescence profiles of *E. coli* MG1655, *K. pneumoniae* and *E. faecalis*. **(D)**: Comparison of three green FP gene cassettes. P_{lacZ}-*EGFP*, *rrnB* P1-*EGFP* and $P_{A1/04/03}$-*gfp*mut3* were all in pUT-miniTn5 derived plasmids. 30,000 events were analyzed by FACSCalibur™ for each sample and data shown are representative of triplicate samples.

3.2.2.3 Choice of Red FP

A suitable red FP was needed to report the promoter activity in *E. coli*. The ideal Red FP should meet these criteria: (i) high intensity level, (ii) ability to report promoter activity fused upstream and (iii) stable expression for data reproducibiilty. The fluorescence levels of the four red FPs (below the yellow dotted line in Fig 3.6.A on page 78) were examined in *E. coli* strains carrying P_{lacZ}-*red FP* cassettes, which were expected to have high basal expression (for plasmids refer to Table 2.3-ii on page 28). Fig 3.8.B (on page 83) has shown that DsRed-Express had the highest intensity level, AsRed2 had the second highest, while those of DsRed-Monomer and HcRed1 had intensity level similar with autofluorescence background of the basic strains (the red plot in Fig 3.8.A). *E. coli* strains carrying four P_{araB}-*red FP* cassette (for plasmids refer to Table 2.4-i on page 29) were next examined with the inducing condition described in section 3.2.2.2.1 on page 79. Fig 3.8.C shows that expression of AsRed2 was as expected, but DsRed-Express was not induced. Expression of DsRed2-Monomer and HcRed1 were low. Since AsRed2 had reasonable intensity level when compared with other red FPs under the same promoter (P_{lacZ}) and can readily report promoter activiy (of P_{araB}), it was considered to be a more suitable red FP compared to others. We went on to monitor the expression stability of AsRed2 via four-round subculturing experiment (refer to section 3.2.2.2.1 on page 79). Fig 3.8.D has shown that AsRed2 expression was consistent over the four sub-culturings. Hence AsRed2 was selected to be the Red FP to report promoter activity in the proposed dual fluorescence system (Fig 3.5. on page 77).

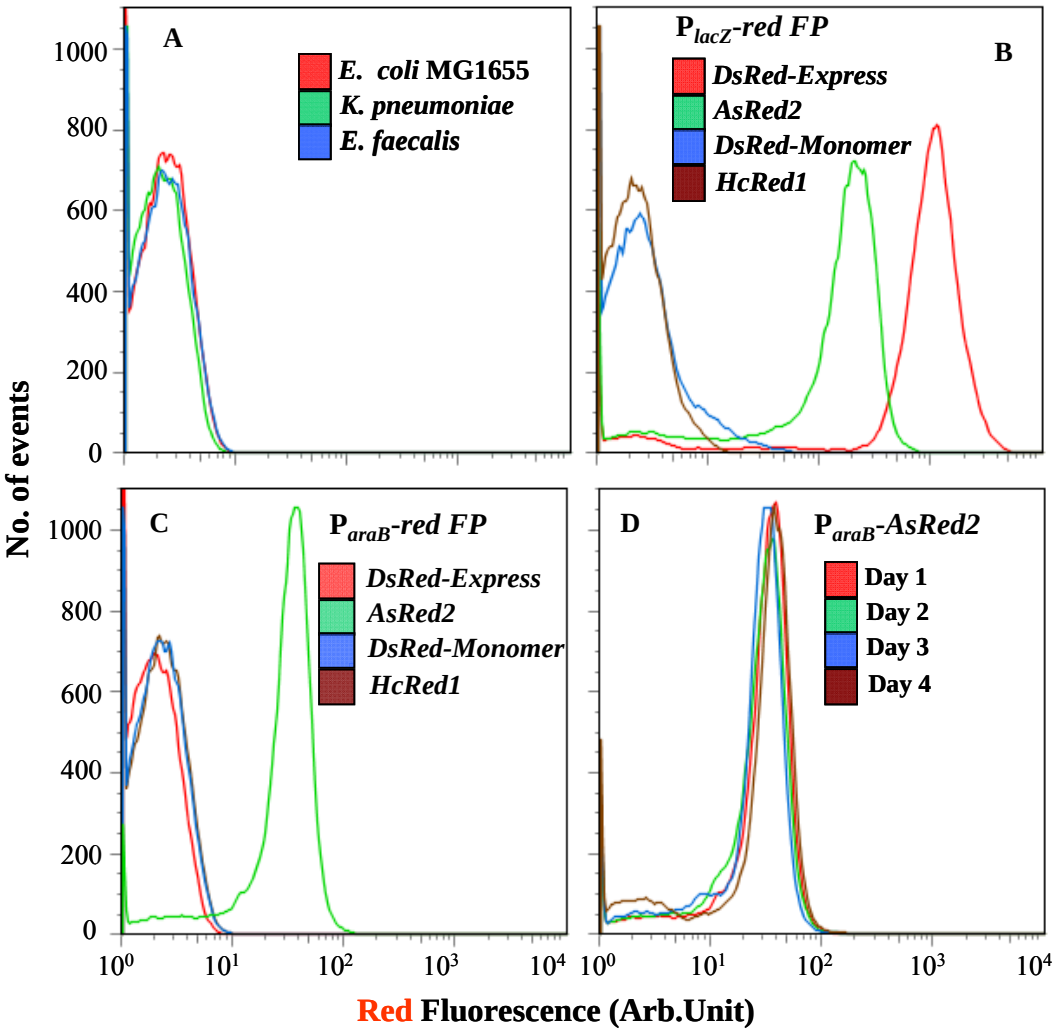

Fig 3.8. Histogram overlay of red fluorescence profiles via FACS analysis. (A): Red autofluorescence profiles of *E. coli* MG1655, *K. pneumoniae* and *E. faecalis*. **(B)**: Comparison of red FPs expression under the same promoter P_{lacZ}. The red FPs are DsRed-Express, AsRed2, DsRed-Monomer and HcRed1. **(C)**: Comparison of the ability to report P_{araB} activity of the four red FPs. **(D)**: Expression of P_{araB}-*AsRed2* in four-round sub-culturing experiment. 30,000 events were analyzed by FACSCalibur™ for each sample and data shown are representative of triplicate samples.

3.2.3 Construction of Gfp-expressing *E. coli* reporter strain

3.2.3.1 Chromosomal insertion of $P_{A1/04/03}$-*gfp*mut3* into *E. coli*

As was mentioned previously (refer to section 3.2.1 on pages 76~77), a green FP gene would be inserted into chromosome of *E. coli*. Some strategies exist for this task. λ phage-based tools could deliver the FP gene into specific attachment sites in *E. coli* chromosome. However, there is a remote possibility for the FP gene to be transferred to the other species in multi-species system despite of host specificity, due to the mobile nature of the λ phage element. Transposon (e.g. by pUT-miniTn5 system) has been engineered to be defective of horizontal gene transfer, but the insertion site is random. The random insertion of FP gene may disrupt certain essential gene of *E. coli* thus affecting *E. coli*'s natural physiology and consequently distort the transcriptional responses. Homologous recombination into the chromosome overcomes these problems, because the FP can be precisely inserted into non-coding region, by flanking it with two DNA fragments that are homologous to the chromosomal DNA (Region A and Region B in Fig 3.9. on page 86), and the possibility of horizontal gene transfer is also reduced due to this strategy.

The potential insertion regions for homologous recombination were chosen based on the criteria: (i) known to be non-coding; (ii) around 1 kb to facilitate two rounds of recombination events to occur; and (ii) sufficient distance (at least 200 bp [137]) from the neighboring genes, hence unlikely to have any regulatory functions. Five such regions in *E. coli* MG1655 genome (GenBank accession no.U00096) were selected and their coordinates are indicated in Table 3.1 on page 86.

The $P_{A1/04/03}$-*gfp* cassette was cloned onto an R6K-based suicide plasmid pDM4 [115] (Cm resistance, refer to Fig 3.9.), that allows replication of the plasmid only in a host that synthesizes Pir protein, e.g. *E. coli* S17-λ*pir*. In a non-permissive host, such as *E. coli* MG1655, no replication of plasmids occurs; hence Cm^r clones can only arise as a result of insertion of the plasmid into the chromosome. There are two rounds of screenings for the insertion of *gfp* cassette (Fig.3.9.). The first round of screening made use of the Cm^r gene on the pDM4-derived suicide vector, to get recombinants in which a first cross-over event had occurred (refer to section 2.2.3.1 on page 55). The second round of screening used the counter selection marker *sacB* on the suicide vector, by growing on media containing sucrose, which is lethal to *sacB*-expressing cells (refer to section 2.2.3.2 on pages 55~56). Only the recombinants that had lost the *sacB* gene by a second cross-over event survived. Four recombinants, each with the *gfp* inserted in a different non-coding region (Insertion region No.7, 8, 9, 11 respectively) were generated.

Table 3.1.: Coordinates of the five selected insertion regions on the *E. coli* MG1655 genome (GenBank accession no.U00096)

No.	Total Length (bp)	Region A (bp)			Region B (bp)		
		Start	End	Length	Start	End	Length
7	1458	312037	312754	717	312771	313495	724
8	1385	4537564	4538307	743	4538348	4538949	601
9[a]	1174	2902718	2902047	671	2902026	2901544	482
10	1428	3766819	3767614	795	3767641	3768247	606
11	1257	2065385	2065992	607	2066019	2066642	623

[a] Seqeunces of the Insertion region No. 9 are complemental.

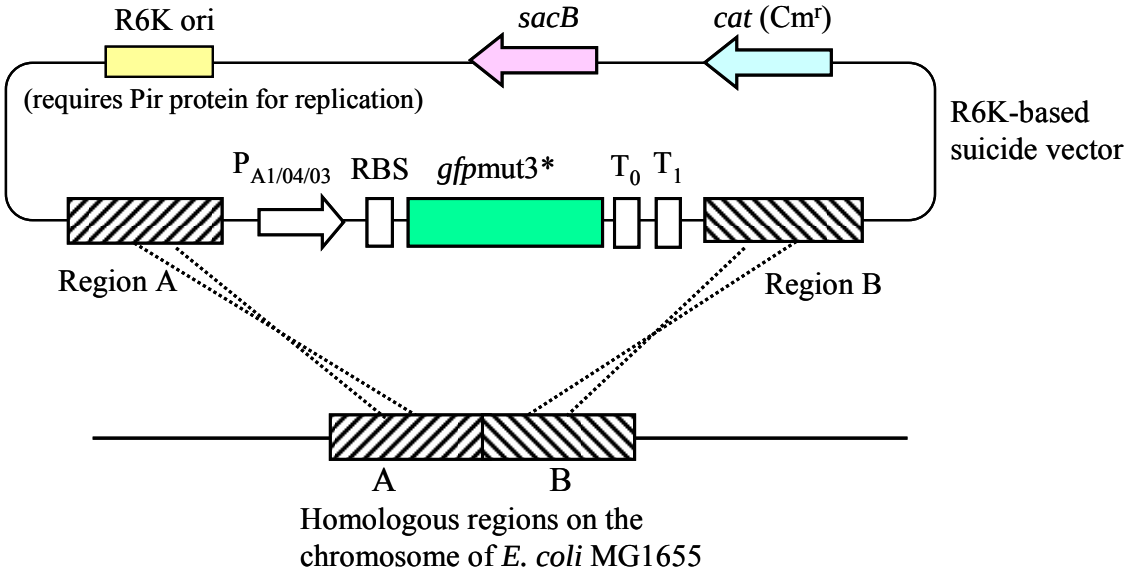

Fig 3.9. pDM4 (R6K-based)-derived suicide plasmid for chromosomal insertion of $P_{A1/04/03}$-*gfp* in *E. coli*. RBS: Ribosomal binding site; T: transcriptional terminator; *cat*: Cm resistance gene; *sacB*: a structural gene from *Bacillus subtilis*. It is used as a counter selection marker (refer to section 2.2.3.2 on pages 55~56).

3.2.3.2 Confirmation of *gfp* insertion status

To confirm the *gfp* insertion status in the generated four recombinants, we examined three aspects: (i) whether the *gfp* cassette has remained in the recombinant; (ii) whether the vector backbone was excised as desired; and (iii) the *gfp* insertion orientation was determined.

The second round of screening (refer to section 3.2.3.1 on page 85) might have selected for recombinants that had the second cross-over event occurring in the same homologous DNA fragment region as the first cross-over event, resulting in the loss of the *gfp* cassette. To confirm the existence of *gfp* cassette, *gfp* specific primers (PR158 and PR308) were used for PCR amplification. PCR products of the expected size (Lane 2~5 in Fig 3.10.A bottom panel on page 89) verified the presence of the *gfp* cassettes in all the four recombinants.

The second round of screening might also select for recombinants that underwent spontaneous mutation to survive on sucrose yet with the backbone region of the plasmid remaining in the chromosome. To exclude this possibility, primers that are specific to the vector backbone (PR193 anneals within *sacB* gene and PR185 anneals within *cat* gene) were designed. If the second recombination did occurred, the vector backbone (region below the red dotted line in Fig 3.10.A top panel) should have been excised out. As a result, there should be no PCR product, and this is shown by Lane 6~9 (Fig 3.10.A, bottom panel). The recombinants that underwent only one round of cross-over event and hence maintained the vector backbone, were included as positive control.

The PCR products of the expected size are shown in Lanes 10~13 (Fig 3.10.A bottom panel).

The third issure we examined is the insertion orientation of *gfp*. During the construction of pDM4-derived suicide plasmid for double recombination (refer to section 2.2.2.3.2 on pages 43~45), the *gfp* cassette was carried on a *Not*I fragment, inserted between Region A and Region B, so the insertion could be in either orientaton (Fig.3.10.B top panel). To determine the insertion orientations of each *gfp* tagged strain, PCR was performed. PR109, a reverse primer that annealed within $P_{A1/04/03}$, was used in every primer set. Each insertion region has its unique pair of primers: P_{up} and P_{down}, which are located at the start of Region A and at the end of Region B, respectively. If *gfp* was inserted in the (a) orientation, only P_{up}-PR109 would have the PCR product, and if *gfp* was inserted in the (b) orientation, only P_{down}-PR109 would have the PCR product. For all the four recombinants, P_{down}-PR109 showed PCR products of the expected size (Fig.3.10.B bottom panel), indicating that all recombinants had the *gfp* cassette inserted in the (b) orientation.

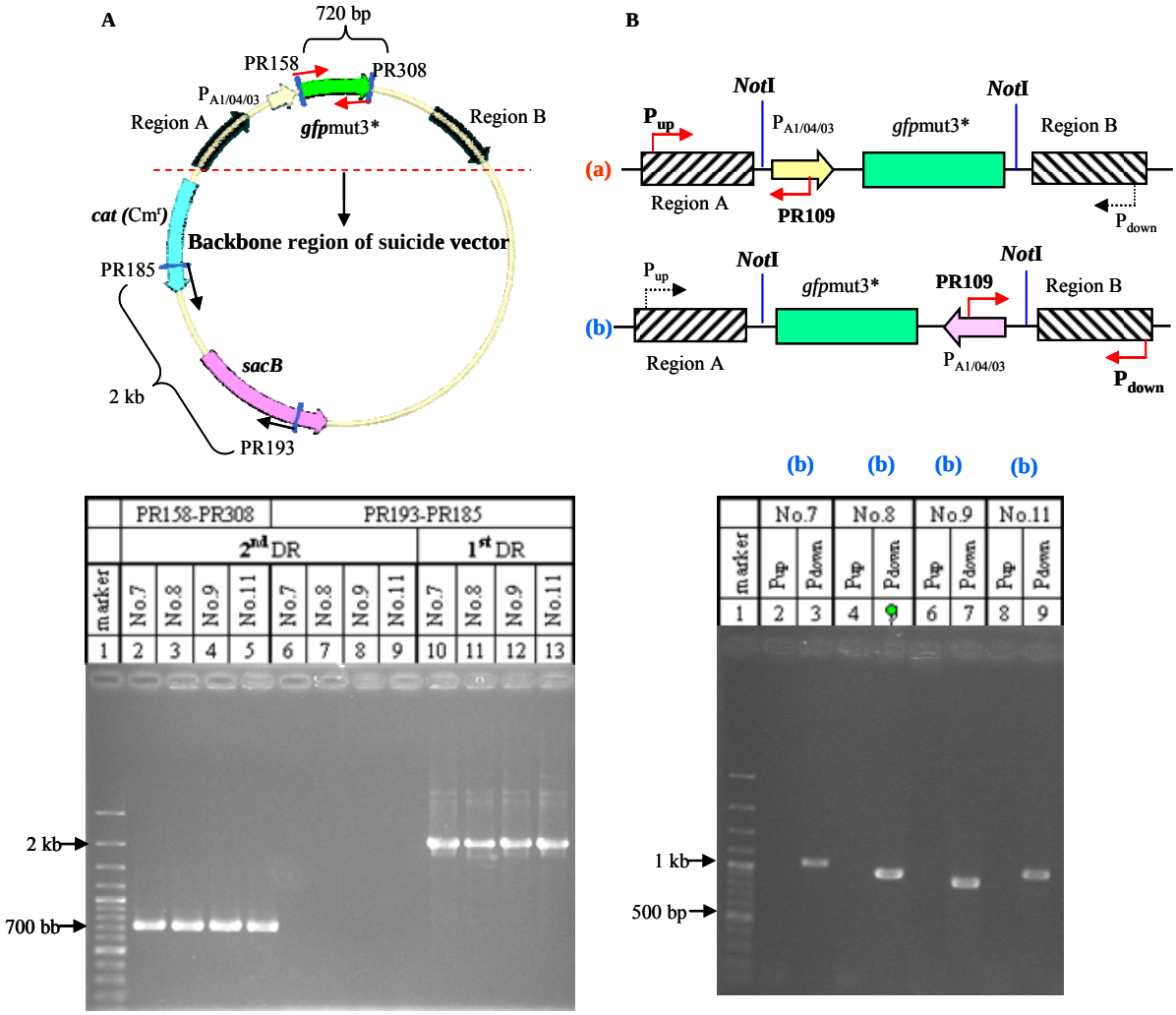

Fig 3.10. PCR to validate (A) the occurrance of second recombination and (B) insertion orientation of *gfp* cassette. (A) The occurrence of second recombination in all the four recombinants was validated. PR158 and PR308 are primers that flank the *gfp* gene; PR193 and PR185 are primers that anneal to the vector backbone. 2nd DR refers to PCR reactions which used the recombinants obtained from the second round of screening as templates; 1st DR refers to PCR reactions which used the recombinants with only one cross-over event as PCR templates. **(B)** Insertion orientation of the *gfp* cassette. (a) and (b) are the two possible insertion orientations. PR109: primer anneals within $P_{A1/04/03}$; P_{up} is located at the start of Region A and P_{down} is located at the end of Region B. Each insertion region has its unique pair of P_{up} and P_{down}. No.7, 8, 9, 11 refer to the four recombinants with the *gfp* cassette inserted into Insertion region No.7, 8, 9, 11, respectively.

3.2.3.3 *E. coli* strain-SCC1

From the four candidate strains with the *gfp* cassette inserted in the chromosome of *E. coli* MG1655, we need to select one to represent *E. coli* in the later co-culture experiments. Although all of them have *gfp* inserted in non-coding regions, the contexts (e.g. chromosome conformation, neighboring genes) may influence the *gfp* expression. Preliminary result revealed that they all have similar levels of fluorescence intensity (data not shown), so our criterion of selection was based on the stable expression of *gfp* in the strain. As the sorting functions and multi-parametric analysis of FACS would be required for later experiments, and FACSAria™ is more superior than FACSCalibur™ in those two aspects, subsequent analyses were carried out on FACSAria™ only.

We may need to monitor the co-cultures over a long period of time; hence the ideal reporter strain should not drop in fluorescence during this process, in order to consistently represent the *E. coli* population. The expression stabilities of the candidate strains were examined by FACS over as long as four days. All the candidate strains did not show obvious shift of fluorescence profiles over the four days. The fluorescence profiles of the strain with *gfp* inserted in Insertion region No.7 is shown in Fig 3.11.A (on page 92) as a representative of the other three strains.

As mentioned previously (refer to section 3.2.2.2.1 on page 79), four-round sub-culturing experiment is a stringent condition for detection of spontaneous mutation in the strain. We next examined the fluorescence profiles of the candidate strains in sub-culturing experiment via FACS. If the *gfp* had been

inserted into a region that causes unfavorable consequences to *E. coli* cells, a non-fluorescent subpopulation would appear in the profile. No such non-fluorescent subpopulation was observed for all four strains, but there was a slight shift of fluorescence profiles over sub-culturings. The strain with *gfp* inserted in Insertion region No.7 (Fig 3.11.B) was relatively more stable than the others, with an average shift in geometic mean fluorescence intensity (GMFI) of less than 10%. So this strain was selected to be the *E. coli* reporter strain for co-culture experiment and would be referred to as *E. coli* strain SCC1 from now on.

Fig 3.11.A~B have shown the homogeneous fluorescence intensity level of the strain SCC1 in culture under shaking condition, indicating that all the *E. coli* cells are green in planktonic mode of growth. To examine whether all the *E. coli* cells are green in biofim mode of growth, biofim sample was analyzed by FACS (Fig 3.11.C). *E. coli* SCC1 in biofilm was also homogenously green (black dotted line), albeit with a reduced intensity compared to the planktonic counterpart (green solid line). It is conceivable that the *gfp* expression is reduced due to the low metablic rate within biofilm cells [138]. However, biofilm image can still be captured with good resolution (Fig 3.11.D) using confocal laser scanning microscope (CLSM). CLSM is a suitable approach to analyze biofilm samples because it allows visualization of the spatial distribution of bacterial cells in situ. The drop in fluorescence intensity in biofilm culture may compromise the sorting and analysis accuracy of FACS, which depends on high fluorescence intensity of SCC1 to differentiate *E. coli*

from other species in multi-species system. Hence only planktonic cultures were analyzed by FACS in this study.

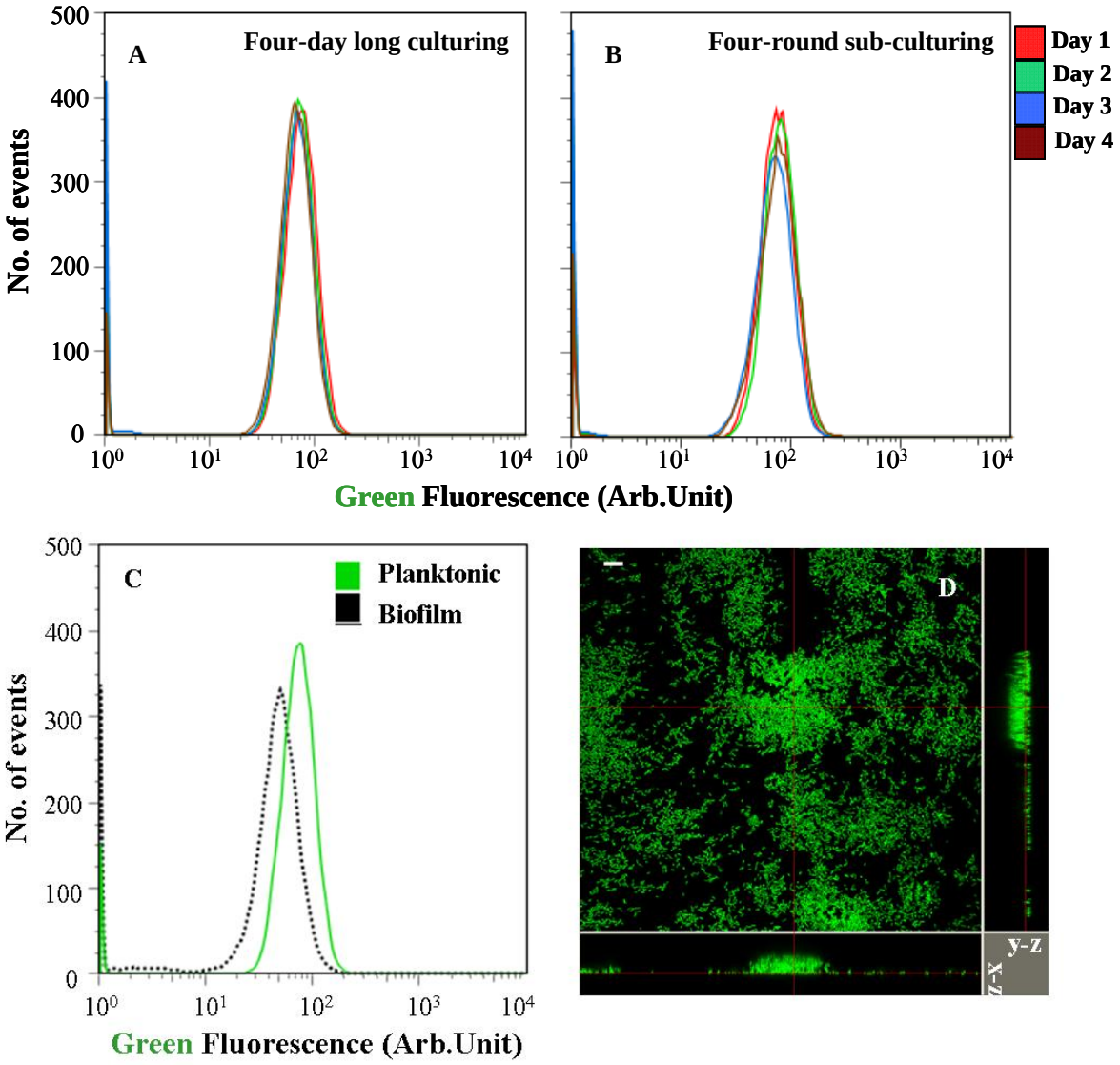

Fig 3.11. Green fluorescence of *E. coli* strain SCC1. (A) Fluorescence profiles of the strain with *gfp* inserted in Insertion region No. 7 (SCC1) in four-day long culture with continuous shaking. **(B)** Fluorescence profiles of the SCC1 strain in four-round sub-culturing experiment. **(C)** Fluorescence profile of *E. coli* strain SCC1 in bioflm and planktonic cultures at 24 h. Data shown in (A)~(C) were collected by FACSAria™. 10,000 events were analyzed for each sample and data shown are representative of triplicate analyses. **(D)** Confocal laser scanning micrographs of *E. coli* SCC1 biofilm at 24 h (size bar = 10 μm). Shown are x-y plane (horizontal section), x-z and y-z planes (vertical sections), corresponding to the red lines indicated in the respective perpendicular sections.

3.2.3.4 Separation of *E. coli* SCC1 from non-*E. coli* species

E. coli SCC1 has been satisfactory because of homogeneous *gfp* expression and high fluorescence level. Quantified by FACS, 98.7~99.6% of cells in SCC1 culture belong to G+ gate (defined as described in the legend of Fig 3.12. on page 94). The green fluorescence level of SCC1 is approximately two orders of magnitude higher than non-*E. coli* species (Fig 3.12.A~B). We next checked if *E. coli* SCC1 could be separated apart from non-fluorescent strains by FACS sorting.

To examine this, nine non-*E. coli* species were selected, with a spread of Gram positive versus Gram negative bacteria, and rods versus cocci (refer to Table 2.1. on page 26). We mixed planktonic cultures of *E. coli* SCC1 with a series of non-*E. coli* species, at a cellular ratio of 1:1, and sorted the mixtures using FACS. For illustrative purpose, we have shown the FACS-analyzed green fluorescence distribution of the *E. coli*–*E. faecalis* mixtures only (Fig 3.12.C), since the corresponding profiles of the other species are very similar. The purity of the green positive (G+) and green negative (G-) fractions from the *E. coli* SCC1 mixtures, sorted by the FACSAria^{TM}, were verified by CFU counts on the UTI agar (Table 3.2. on page 95). Most of the *E. coli* and non-*E. coli* populations could be sorted to purity as high as 99.0~100%, while a minority had purity that fell in the 97.5~98.9% range.

Since our FACS-based analytical system is intended for studying interactions that require co-cultures (in contrast to "mixtures" where single-strain cultures were mixed just prior to analysis), *E. coli* SCC1 was also co-cultured for 24 h

with single or dual partners (*K. pneumoniae* and/or *E. faecalis*) and then sorted by FACS. As shown in Table 3.2., the purity of the sorted fractions was in the range of 96.71~99.41%. The slightly lowered purity of the fractions sorted from co-cultures, compared to the fractions sorted from mixtures, is within our expectation as cell-cell contact during co-cultures, if sufficiently stable, may affect the resolution during flow cytometric sorting. Nevertheless, as the level of contamination is consistently less than 5%, we considered it to be of sufficient purity for significant analyzes.

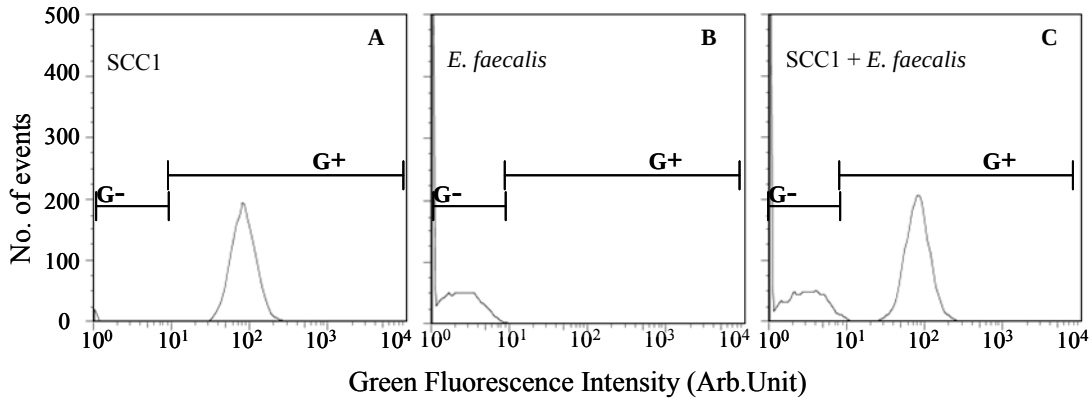

Fig 3.12. Histogram of green fluorescence intensity of cells in pure and mixed cultures analyzed by FACS. (A) pure culture of *E. coli* SCC1; **(B)** pure culture of *E. faecalis*; **(C)**: mixture of SCC1 and *E. faecalis.* G+: green positive gate; G-: green negative gate. The gates G+ and G- were set using the non-fluorescent *E. coli* MG1655 as the control, i.e. the fluorescence level within which the MG1655 population falls was defined as G-, and levels above, as G+. Data was collected by FACSAria™. 5000 events were analyzed for (A) and (B) and 10,000 events were analyzed for (C). Data shown are representative of triplicate analyses.

Table 3.2. Purity of fractions sorted by FACSAria™.

Samples	% purity of *E. coli* fraction[a]	% purity of non-*E. coli* fraction[b]
Mixture of *E. coli* SCC1 with[c]:		
K. pneumoniae	99.71 ± 0.11	99.01 ± 0.75
E. faecalis	99.41 ± 0.47	99.41 ± 0.43
L. casei	99.20 ± 0.45	98.62 ± 1.48
L. casei + *E. faecalis* + *K. pneumoniae*	98.71 ± 0.77	99.38 ± 0.44[d]
S. marcescens	97.59 ± 0.64	99.71 ± 0.96
B. cereus	99.09 ± 0.53	99.92 ± 0.24
M. luteus	99.89 ± 0.18	100.00 ± 0.00
A. baumanii	97.86 ± 2.35	99.93 ± 0.14
S. pyogenes	99.51 ± 0.40	100.00 ± 0.00
S. aureus	98.79 ± 0.84	100.00 ± 0.00
Co-culture of *E. coli* SCC1 with[e]:		
K. pneumoniae	97.34 ± 1.07	98.17 ± 0.61
E. faecalis	99.15 ± 0.51	99.41 ± 1.01
K. pneumoniae+*E. faecalis*	96.71 ± 2.18	99.24 ± 0.97[d]

[a] Fraction of 10000 events defined as green positive (G+) in Fig 3.12.

[b] Fraction of 10000 events defined as green negative (G-) in Fig 3.12.

[c] Preparation of the mixture: each strain was adjusted to a final cell density of 10^7 cells/ml, and mixed in equal volumes.

[d] Fraction contains more than one non-*E. coli* species.

[e] Co-cultures were analyzed at 24 h, as described in section 2.2.4.2.1 on page 58.

All data shown are expressed as mean ± SD of three independent sortings.

3.2.3.5 Comparison of physiology of SCC1 with wild type MG1655

Although strain SCC1 has been verified with respect to its competence to represent *E. coli* via *gfp* expression, whether the *gfp* expression would affect the basic physiology of SCC1 need to be investigated before it can be used in co-culture experiment.

We observed no significant difference between strains SCC1 and MG1655 with respect to cell morphology and biofilm structure (data not shown). The side-scatter (SSC, considered to be related to the internal granularity of the cell) and forward-scatter (FSC, often correlated to particle size) profiles of the two strains analyzed by FACS appeared highly similar (Fig 3.13.A on page 97). Growth profiles (Fig 3.13.B) and conversion relationship of cell density (CFU/ml) against OD_{600} (Fig 3.13.B inset) of strains SCC1 and MG1655 were also similar.

Furthermore, when strain SCC1 was co-cultured with other species in both planktonic and biofilm cultures, the percentage of each component species within the total population (green bars, Fig 3.13.C~D) were similar to those observed in MG1655 co-cultures (yellow bars, Fig 3.13.C~D), suggesting that Gfp expression does not affect population relationships. Based on these phenotypes, we considered strain SCC1 to be suitable for use in the development of the proposed dual fluorescence system.

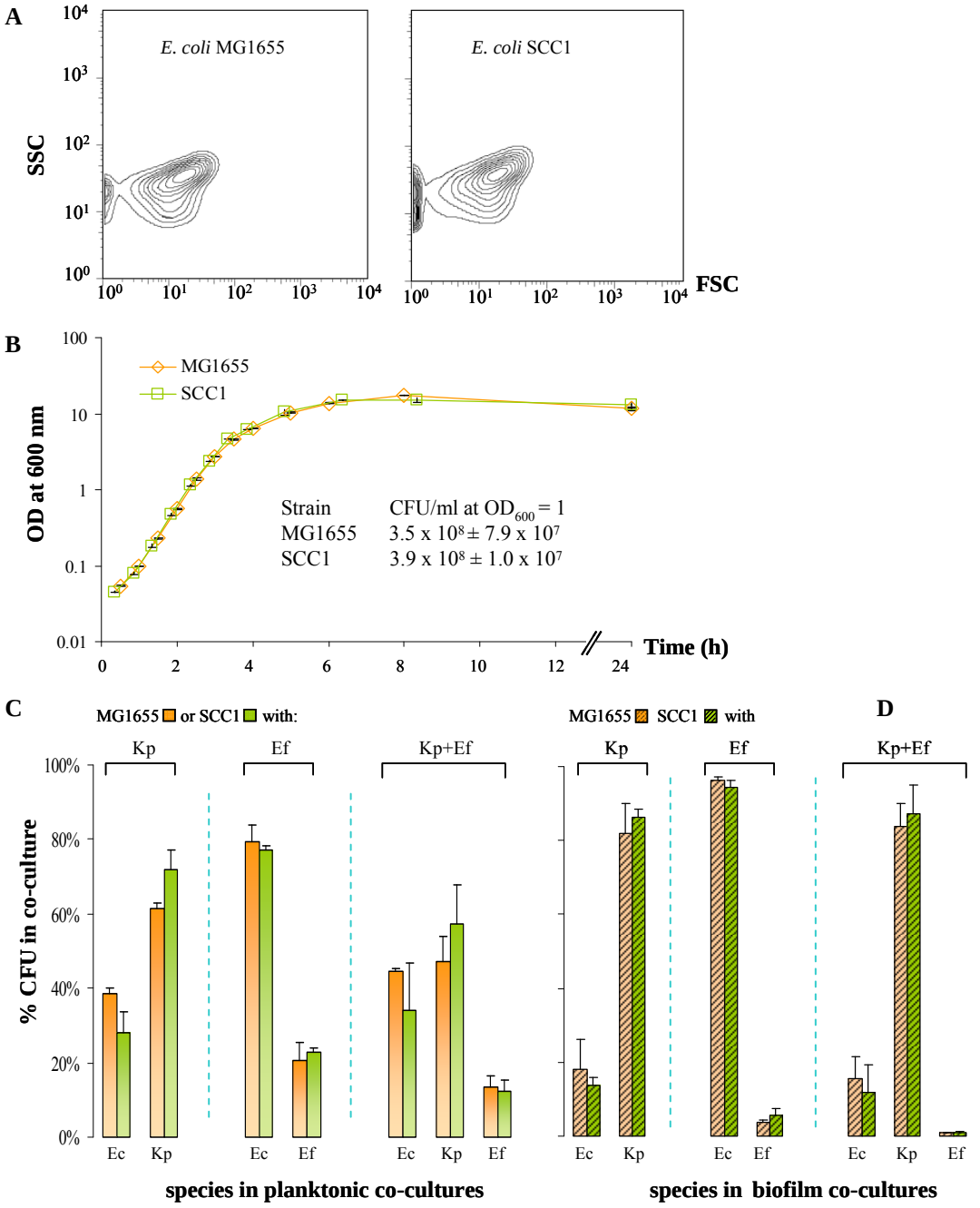

Fig 3.13. *E. coli* strain SCC1 mimics the basic physiology of MG1655. (A) Contour plots of side-scatter (SSC, y-axis) against forward-scatter (FSC, x-axis) of 12~16 h planktonic cultures, analyzed by FACSAria™. **(B)** Growth curve of planktonic cultures in BHI medium, shaken at 37ºC (refer to section 2.2.5.2.1 on pages 59~60). Data shown are the mean ± SD of duplicate samples. **(C)** and **(D)** Percentage contribution of each species to the total CFU, after 24 h planktonic (C) and biofilm (D) co-culture with *E. coli* MG1655 (yellow bars) or SCC1 (green bars) in BHI medium at 37ºC. Data shown are the mean ± SD of duplicate or triplicate samples.

3.2.4 Construction and validation of promoter fusion plasmids

In addition to the *gfp* insertion on the chromosome, the other component in the dual fluorescence system is the promoter-*AsRed2* fusion as a reporter for the transcriptional responses of *E. coli* (Fig 3.5. on page 77). *AsRed2* was first verified for its reporter capability and the plasmids with various promoter fusions were then constructed and validated for this task.

3.2.4.1 Reporter capability of *AsRed2*

AsRed2 was selected as a reporter for transcriptional responses (refer to section 3.2.2.3 on pages 82~83), and its capability to be the reporter need to be further verified in the context of our system. Fig 3.14.A (on page 99) shows that when the *AsRed2* reporter was under the control of the arabinose-inducible promoter P_{araB}, the increase and saturation of P_{araB}'s transcriptional activities in response to the increasing concentration of arabinose could be quantified via fluorometer. The P_{araB} activation profiles were also quantified by FACS (Fig 3.14.B). FACS analysis revealed that, with the increasing induction strength, a bigger subpopulation became promoter active, and not a homogenous shift of the whole population to higher GMFI. This was consistent with previous report that expression from P_{araB} as a function of arabinose concentration is proportional to the percentage of cells that are fully induced (versus uninduced) rather than the expression level in individual cells [139]. As can be observed from these data, although fluorometer is adequate for general quantification of promoter activity, FACS is capable of exploring the heterogeneous nature of a sample, which would be utilized in later promoter activity assays for co-cultures. Since *AsRed2*'s reporter capability had been verified to be satisfactory, we proceeded

to construct plasmids with various promoters fused upstream of the *AsRed2* gene.

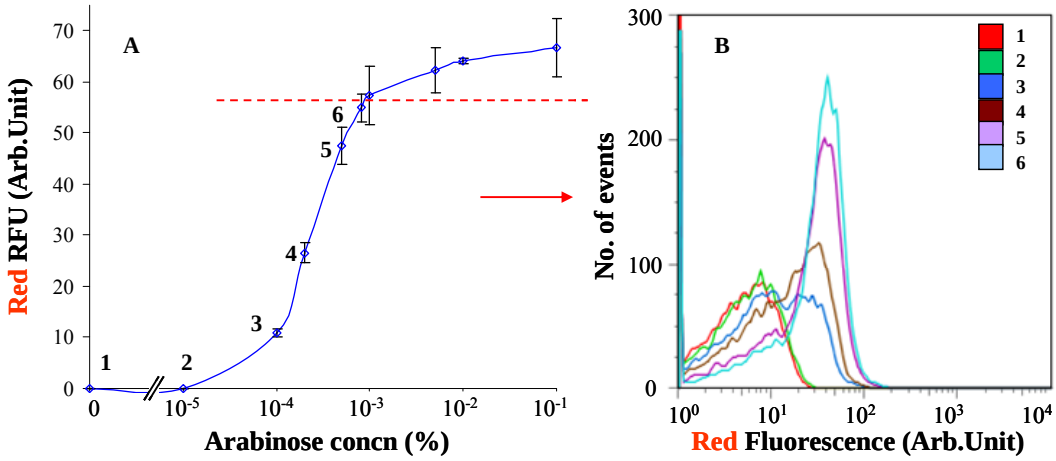

Fig 3.14. Promoter activities quantified via the red fluorescence of the AsRed2 reporter. (A) *E. coli* strain carrying pCCS126 [P_{araB}-*AsRed2*] was cultured in LB-Ap for 24 h, induced with the indicated concentration of L-arabinose. Data shown are the mean ± SD of duplicate samples analyzed by fluorometer. **(B)** Histogram overlay of the red fluorescence of the six samples (numbered 1~6) below the red dotted line in (A). For each sample, 10,000 events were analyzed by FACSAria™, and data shown are representative of duplicate analyses.

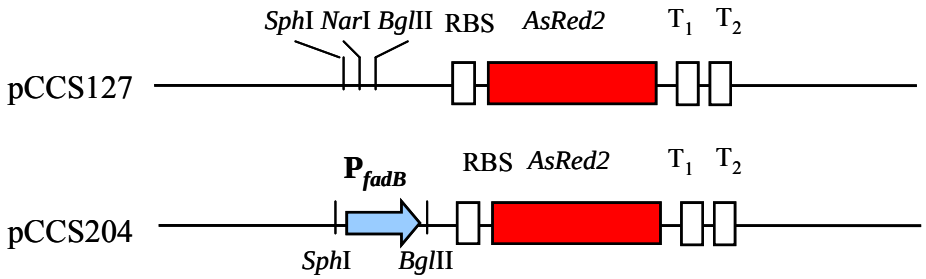

Fig 3.15. Schematics of pCCS127 and pCCS204. In pCCS127, a linker of *Sph*I-*Nar*I-*Bgl*II was inserted upstream of promoterless *AsRed2* for ligation of various promoters, and pCCS204 is one of the representative constructs that has P_{fadB} fused upstream of *AsRed2* [P_{fadB}-*AsRed2*].

3.2.4.2 Prototype promoter-*AsRed2* fusions

The plasmid pCCS127 was constructed for the convenience of generating promoter fusions. A linker with multiple cloning sites *Sph*I−*Nar*I−*Bgl*II had been designed and inserted into a pBluescript-derived intermediate plasmid carrying the *AsRed2* cassette to generate pCCS127 (Fig 3.15. on page 99). These three sites were chosen not only because they are common, but also because they have compatible sticky ends with 4-base cutting restriction enzymes, e.g. *Sau*3AI, *Alu*I which are useful for inserting random genomic fragments for the construction of promoter-capture library in the future. This plasmid was also used as the vector control in later experiments.

Eleven different promoters were inserted between *Sph*I and *Bgl*II sites upstream of *AsRed2* (pCCS204 [P_{fadB}] is shown in Fig 3.15 as a representative of the other constructed plasmids in Table 2.4-iv on page 30). These fusions served as prototypes to be tested in our system. The promoters were selected because they are known to belong to diverse functional categories (Table 3.3. on page 101), and have also been reported to be up-regulated in biofilms [10].

Table 3.3. Functions of the genes regulated by the promoters selected in this study

Gene	Definition[a]	Operon[b]	Functional category	Ref[c]
fadB	fatty acid oxidation complex	*fadBA*	Lipid transport and metabolism	[140]
sulA	SOS cell division inhibitor	*sulA*	SOS response	[141]
acrA	multidrug efflux system	*acrAB*	Antibiotic resistance	[142, 143]
rne	ribonuclease E	*rne*	Degrade mRNA, process rRNA	[144]
rpoE	RNA polymerase sigma E factor	*rpoE-rseABC*	Deal with unfoded periplasmic or membrane protein caused by heat shock	[124, 145]
recA[d]	DNA strand exchange and recombination protein	*recA*	SOS response	[146]
msrA[d]	methionine sulfoxide reductase A	*msrA*	Post-translational modifications, protein turnover and chaperones	[125]
gadA[d]	glutamate decarboxylase A	*gadA*	Tolerance to acidic condition	[147]
flgB[d]	flagellar basal-body rod protein	*flgBCEG HIJLM*	Flagella biosynthesis	[148]
pspA[d]	regulatory protein for phage-shock-protein operon	*pspABC DE*	Response to heat shock, hyperosmotic shock	[149, 150]
rbsD[d]	predicted cytoplasmic sugar-binding protein	*rbsDACB K*	high affinity membrane transport system for ribose	[151]

[a] Definition of the gene was taken from *Escherichia coli* K12-MG1655 sub-database in Encyclopedia of Genes and Genomes database (KEGG) [119]

[b] Operon refers to the operon that the gene belongs to.

[c] Ref refers to the related reference(s) for the genes of interest

[d] Promoter-*AsRed2* fusions of these genes were only used in the experiment shown in Fig 3.18.A.

3.2.4.3 Validation of promoter-*AsRed2* fusions in planktonic cultures

The competence of the promoter fusions to reflect transcriptional responses in *E. coli* MG1655 and SCC1 strains need to be validated first before they can be used to analyze *E. coli* transcriptional responses in co-culture conditions.

3.2.4.3.1 Promoters of *fadB, sulA, acrA* induced in stationary phase

It has been reported previously that the promoters of *fadB* [152], *sulA* [153] and *acrA* [154] were induced not during exponential phase but during stationary phase. To confirm this, the red fluorescence intensity in both *E. coli* strains MG1655 and SCC1, carrying plasmids with promoter-*AsRed2* fusions (pCCS204 [P_{fadB}], pCCS320 [P_{sulA}], pCCS182 [P_{acrA}]) were quantified using the fluorometer. *E. coli* strains with pAsRed2 [P_{lacZ}] were included as positive controls. As mentioned previously, although P_{lacZ} is generally known to be lactose-inducible, it is leaky at high copy and showed transcriptional activity over both exponential and stationary phases in rich media [135]. The expression of *AsRed2* from P_{lacZ} in *E. coli* was high at both phases (Fig 3.16. on page 104). The expression of *AsRed2* from the three promoters (P_{fadB}, P_{sulA}, P_{acrA}) was extremely low during exponential phase and much higher (i.e. induced) at stationary phase, as expected (Fig 3.16.).

3.2.4.3.2 Promoters of *rpoE* and *rne* induced by heat shock stress

It was reported that the promoters of *rpoE* [155] and *rne* [123] were induced by heat shock stress in planktonic cultures. To confirm this, red fluorescence intensity were monitored for both *E. coli* strains MG1655 and SCC1 carrying promoter fusion plasmids (pCCS319 [P_{rpoE}], pCCS209 [P_{rne}]) at 30ºC (basal

level) and 42ºC (inducing condition). Fluorescence levels at 42ºC were indeed higher than that of 30ºC for both promoters (Fig 3.16.).

3.2.4.3.3 Similar expression of promoter fusions in strains MG1655 and SCC1

Initially, it was a concern whether the expression of *gfp* in SCC1 would compete for the limited transcriptional/translational machineries in *E. coli* and consequently affect AsRed2 expression. The similar expression profiles of AsRed2 within SCC1 (green bars in Fig 3.16.) compared to its parental wildtype MG1655 (yellow bars in Fig 3.16.) under all the tested conditions validated that the AsRed2 expression was not affected by Gfp expression in SCC1. Hence the promoter fusions are capable of reflecting transcriptional responses in *E. coli* SCC1 strains.

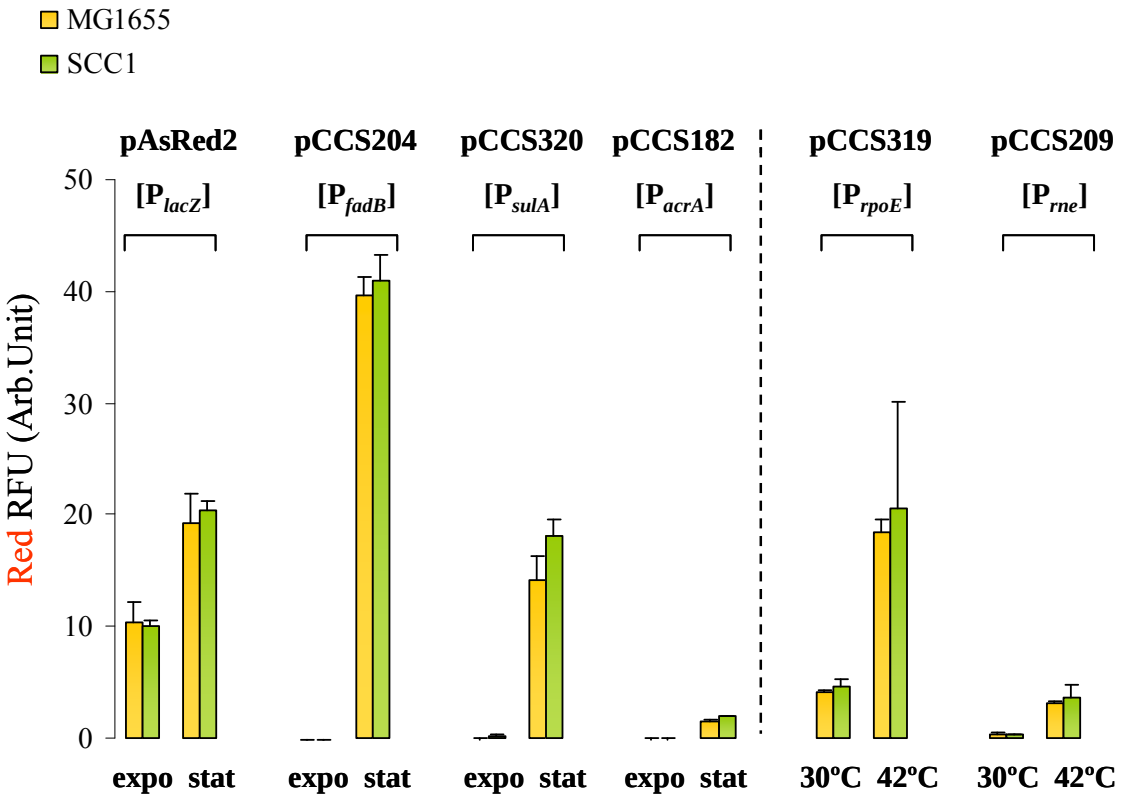

Fig 3.16. Promoter induction at stationary phase or due to heat shock stress assayed via AsRed2 fluorescence. *E. coli* strains MG1655 and SCC1 carrying promoter fusion plasmids were assayed for red fluorescence under the conditions shown (refer to section 2.2.4.1.1 on pages 56~57) by fluorometer. expo: late exponential phase; stat: late stationary phase. Data shown are the mean ± SD of triplicate samples.

3.2.4.4 Visualization of promoter activaties in biofilm cultures

The above tested promoter strains were also grown in biofim cultures, in which their promoters have been reported to be activated [10]. The heterogeneous nature of biofilm was reflected by the promoter activity of P_{fadB} towards the air region in the 24 h biofilms of both MG1655 and SCC1 strains, seen with high magnification under the CLSM (Fig 3.17.A on page 106). The activation profiles of all the promoters were captured with lower magnification, with DIC phase contrast image overlayed to show the boundaries of the biofilms, and the red fluorescence to show the promoter activity distribution (Fig 3.17.B). All the promoters were activated at 24 h and the profiles were similar between *E. coli* strains MG1655 and SCC1 (left and middle columns in Fig 3.17.B). Overall, the promoter activities were distributed fairly evenly through out the biofilms. Pockets of heterogeneity were more easily viewed in SCC1 due to the green fluorescence overlay (right column, Fig 3.17.A and B).

The results have shown that the promoter-reporter fusions were able to reflect the induction trends of the promoters in the biofilms of wild type *E. coli* MG1655, and similarly that in the reporter strain SCC1.

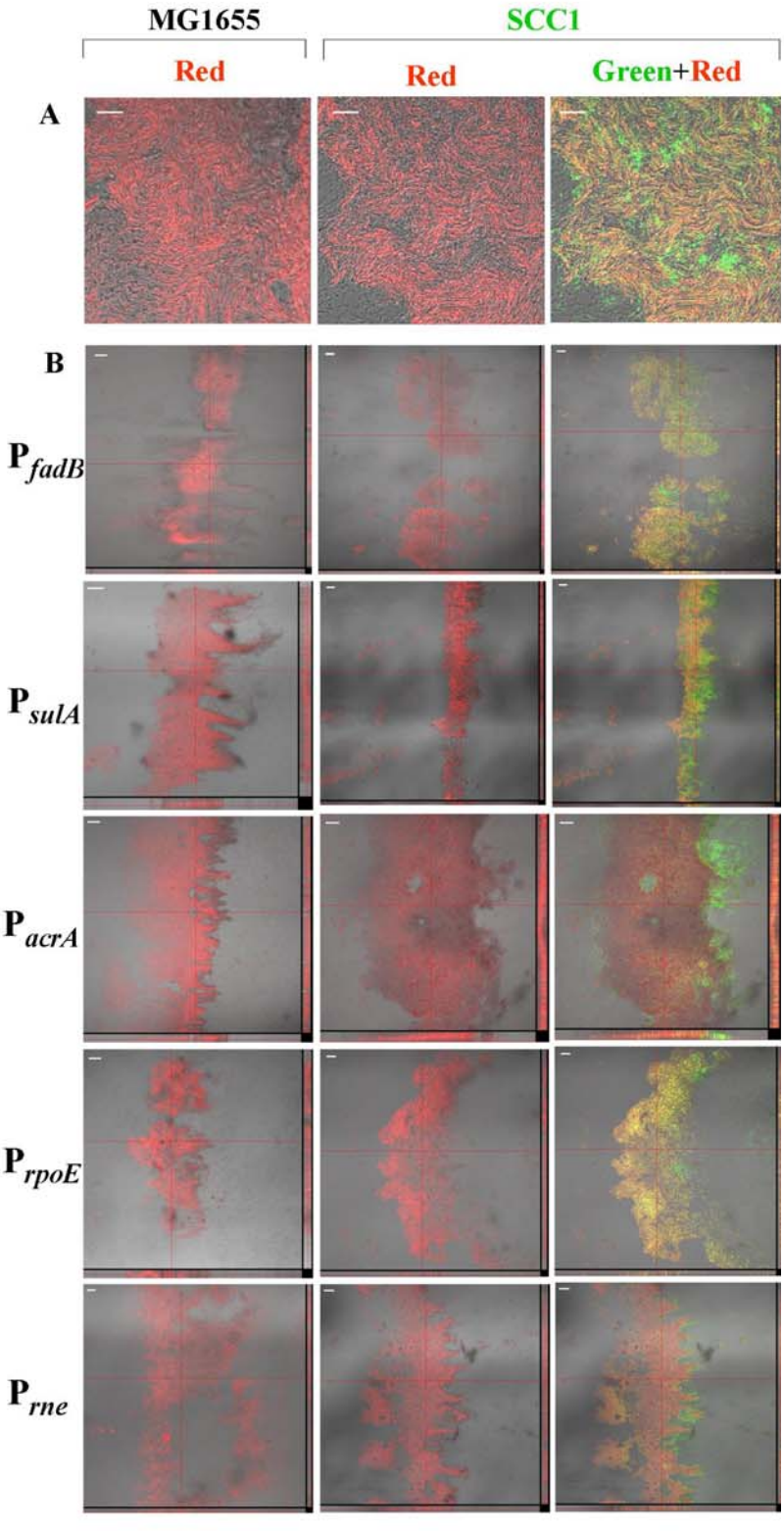

Fig 3.17. Promoter activities in the biofilm cultures. (A) Higher magnification of a single focal plane of the air-liquid interface region of the 24 h biofilm of *E. coli* MG1655 and SCC1 carrying (pCCS204)[P_{fadB}] (size bar = 10μm). **(B)** 24 h biofilms of *E. coli* MG1655 and SCC1 with various promoter-*AsRed2* fusions (size bar = 50 μm). Shown in each panel are the x-y plane (horizontal section), x-z and y-z planes (vertical sections), corresponding to the red lines indicated in the respective perpendicular sections. Left and middle columns are the overlays of red fluorescence and DIC; right column is the overlay of red, green fluorescence and DIC. All the images were taken with an orientation whereby the left side is towards the air, and the right side is towards the liquid medium on the slide, as shown in **(C)**.

3.2.4.5 Gfp expression of SCC1 carrying promoter-*AsRed2* fusions

Since the system depends on GFP expression to set apart *E. coli* from other species for analysis, we checked if the promoter-*AsRed2* component would affect GFP expression adversely in *E. coli* SCC1 (e.g. by competing for transcriptional/translational machineries). We surveyed the green and red fluorescence levels of SCC1 strains carrying various promoter-*AsRed2* fusions (refer to Table 2.4-iv on page 30, pCCS127-derivatives) under various inducing and non-inducing conditions (Fig 3.18.A on page 108). The levels of green fluorescence varied considerably but only within one order of magnitude, and no significant inverse correlation between AsRed2 expression and Gfp expression was observed.

We next checked if the variable Gfp expression level could fall to a value so low that *E. coli* SCC1 is no longer distinguishable from the non-fluorescent non-*E. coli* strains. *E. coli* SCC1 strains carrying promoterless *AsRed2*, P_{lacZ} -, P_{fadB} - or P_{rpoE}-*AsRed2* were selected for this purpose because by conventional fluorometry, the green fluorescence levels of these strains ranged from 19.4 to 108.1 RFU (Table 3.4. on page 109), spanning the range revealed in Fig 3.18.A. When analyzed by FACS, the green geometric mean fluorescence intensity (GMFI) ranges from 16.3 to 187.6 (Table 3.4.). The FACS histogram profiles for selected cultures (asterisks in Table 3.4.) are presented in Fig 3.18.B. With the exception of strain SCC1(pCCS319) [P_{rpoE}] grown at 42°C (Fig 3.18.B, arrow), all other SCC1 derived strains could be cleanly sorted within the green positive (G+) gate. The lowered green fluorescence level of SCC1(pCCS319) at 42°C (%G+ of 87.94% in Table 3.4.) could be due to the stress associated with

growth at this temperature, and/or the intrinsic thermosensitivity of Gfp [156] that remained even after it has been mutated for improved performance [157]. This information will be considered later to derive appropriate interpretation of data obtained from 42ºC cultures.

After validation of the strain SCC1, the promoter-fusion plasmids, and a combination of both, we considered the SCC1 strain carrying promoter fusion plasmid to be ready for use in the multi-species co-cultures.

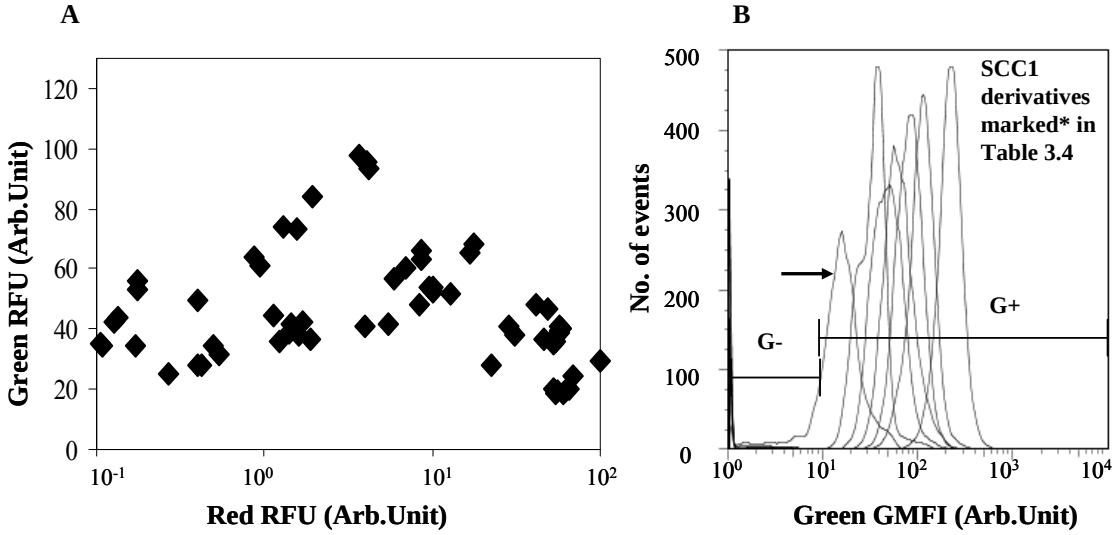

Fig 3.18. Gfp expression of *E. coli* SCC1 strains carrying various promoter fusions assayed by fluorometer and FACS. (A) Scatter plot of the green fluorescence level (y-axis) and red fluorescence level (x-axis) of *E. coli* SCC1 strains carrying various promoter-*AsRed2* fusions, grown under inducing and non-inducing conditions and assayed by fluorometer. **(B)** Histogram overlay of green fluorescence of SCC1 strains carrying derivatives of pCCS127 under various growth conditions, as described and marked with asterisks (*) in Table 3.4. 10,000 events were analyzed for each sample by FACSAria^{TM}. Data shown are representative of triplicate analyses. G+: green positive gate; G-: green negative gate.

Table 3.4. Green fluorescence level of *E. coli* SCC1 strains carrying promoter-*AsRed2* fusions

Plasmid [Promoter]	Condition	Fluorometer	FACS	
		Green RFU[a]	Green GMFI[b]	%G+[c]
pCCS127, vector	37°C, expo[d]	65.0 ± 6.1	67.7 ± 3.0	98.48 ±0.15
pCCS127, vector	37°C, stat[e]	62.7 ± 2.3	62.4 ± 0.6*	96.47 ± 1.78
pAsRed2 [P_{lacZ}]	37°C, expo	61.0 ± 0.8	80.5 ± 0.8	98.63 ± 0.08
pAsRed2 [P_{lacZ}]	37°C, stat	27.1 ± 2.5	26.3 ± 0.2*	97.20 ± 0.57
pCCS204 [P_{fadB}]	37°C, expo	48.8 ± 3.5	45.3 ± 1.6*	98.40 ± 0.15
pCCS204 [P_{fadB}]	37°C, stat	64.4 ± 0.9	51.1 ± 4.1*	96.27 ± 1.37
pCCS319 [P_{rpoE}]	30°C, stat	108.1 ± 1.9	187.6 ± 27.0*	96.00 ± 0.25
pCCS319 [P_{rpoE}]	37°C, stat	85.1 ± 2.0	94.9 ± 20.1*	95.88 ± 2.92
pCCS319 [P_{rpoE}]	42°C, stat	19.4 ± 0.0	16.3 ± 1.1*	87.94 ± 1.23

[a] Relative Fluorescence Unit, calculated as described in section 2.2.6.1.2 on pages 61~62.

[b] Geometric Mean Fluorescence Intensity analyzed by FACSAria™

[c] Percentage of cells falling within the green positive (G+) gate (refer Fig 3.18.B) from the total of 10,000 events analyzed.

[d] Late exponential phase, as described in section 2.2.4.1.1 on pages 56~57

[e] Late stationary phase, as described in section 2.2.4.1.1 on pages 56~57

* Distribution of the population is illustrated as a histogram in Fig 3.18.B

Data are expressed as mean ± SD of two to three independent experiments.

3.3 Study of interspecies interactions with the established dual fluorescence system

Using the established dual fluorescence system, we proceeded to investigate the transcriptional response of *fadB* and *rpoE* promoters in *E. coli* SCC1 when co-cultured with different partner species. As mentioned previously, the enteric species *K. pneumoniae* and *E. faecalis* were selected as they are likely to have evolved significant interactions with *E. coli* and it appeared that sufficient interactions had occurred in our co-cultures to be reflected by their population relationship (Fig 3.4 in section 3.1.2 on page 75).

3.3.1 Promoter activities of *E. coli* in planktonic co-cultures

E. coli SCC1 strains carrying either P_{fadB}- or P_{rpoE}-*AsRed2* were co-cultured at a starting cellular ratio of 1:1 with *K. pneumoniae* or *E. faecalis*. Three-species co-cultures at the starting ratio of 1:1:1 were also attempted. After 24 h of planktonic growth, the promoter activities of *E. coli* SCC1 strains in these co-cultures were analyzed by FACS. For the P_{fadB} set, co-cultures were grown at 37°C and assayed at the stationary phase where induction of P_{fadB} was expected to occur (refer to Fig 3.16. on page 104). For the P_{rpoE} set, co-cultures were grown at 30°C, 37°C and 42°C, to examine the induction profiles based on growth temperatures. The FACS data of all the samples were first screened to identify cases of clear transcriptional heterogeneity (distinct R+ and R- subpopulations in the red fluorescence histogram plots) within the G+ (*E. coli*) populations. An example of such heterogeneity is shown in Fig 3.19.C (on page 113), which illustrates the profile of *E. coli* SCC1(pCCS319) pure culture at

42°C. Among all cultures analyzed, only the pure and co-cultures of SCC1(pCCS319) showed such R+/R- heterogeneity in the G+ *E. coli* population. Hence, all data shown in Fig 3.19.A refer to the red GMFI of the whole populations of G+ *E. coli* cells analyzed from the cultures, except the cultures of *E. coli* SCC1(pCCS319) at 42°C for which the data for G+R+ subpopulation has been additionally presented (slant shaded bars for red GMFI in G+R+ subpopulation). To illustrate the levels of heterogeneity, the percentages of R+ in G+ subpopulation (%R+ in G+, checkered bars) are also shown.

3.3.1.1 Influence conferred by *K. pneumoniae* seem to override that of *E. faecalis*

From Fig 3.19.A, it can be observed that the activity of the *fadB* promoter in *E. coli* SCC1 was positively influenced by co-culture with *E. faecalis*, but negatively affected by *K. pneumoniae* (2.1~4.0 times reduction). This negative effect on the *fadB* promoter by *K. pneumoniae* was evident even in the three-species co-cultures (1.9~2.7 times reduction). In the case of the *rpoE* promoter, it was observed to remain uninduced at 30°C regardless of the co-culture partner(s). At 37°C, *K. pneumoniae* exerted an influence that was negative (3.1~4.0 times reduction), while *E. faecalis* was neutral or possibly slightly positive (1.0~1.4 times increase). Like the *fadB* promoter, the negative effect of *K. pneumoniae* on the *rpoE* promoter at 37°C was also evident in the three-species co-cultures (3.7~5.5 times reduction). At 42°C, the effect of *K. pneumoniae* (red bars) appears to be positive (1.9~2.3 times increase) to the *E. coli* G+ population (red bars), and the influence by *E. faecalis* (yellow bars)

is negative (9.7~12.2 times reduction). However, in the three-species co-culture, this dramatic negative effect by *E. faecalis* was not observed. Instead, the level of positive effect in the transcriptional response (1.8~2.5 times increase) is similar to that in the *K. pneumoniae* co-culture at 42°C.

Furthermore, due to the heterogeneous responses of P_{rpoE} in 42° C cultures as mentioned previously, the red GMFI in G+R+ subpopulation (slant shaded bars, Fig 3.19.A) and percentages of R+ in G+ subpopulations (%R+ in G+, checked bars, Fig 3.19.A) were also plotted. With reference to the information obtained from the fluorescence intensity (GMFI), the percentage of promoter activated cells reflected by %R+ serves as a useful parameter to describe heterogeneous promoter responses. It revealed that although the effect of *E. faecalis* has been shown to be negative to the overall G+ population, the R+ subpopulation of *E. coli* actually had even higher red GMFI than others. It is the %R+ that was extremely low (yellow checkered bar, Fig 3.19.A). Similar with observations derived in overall G+ population, in the three-species co-cultures, *K. pneumoniae* influences seem to override the *E. faecalis* influences in both red GMFI (in G+R+ subpopulation, slant shaded bars) and %R+ (in G+ subpopulation, checkered bars).

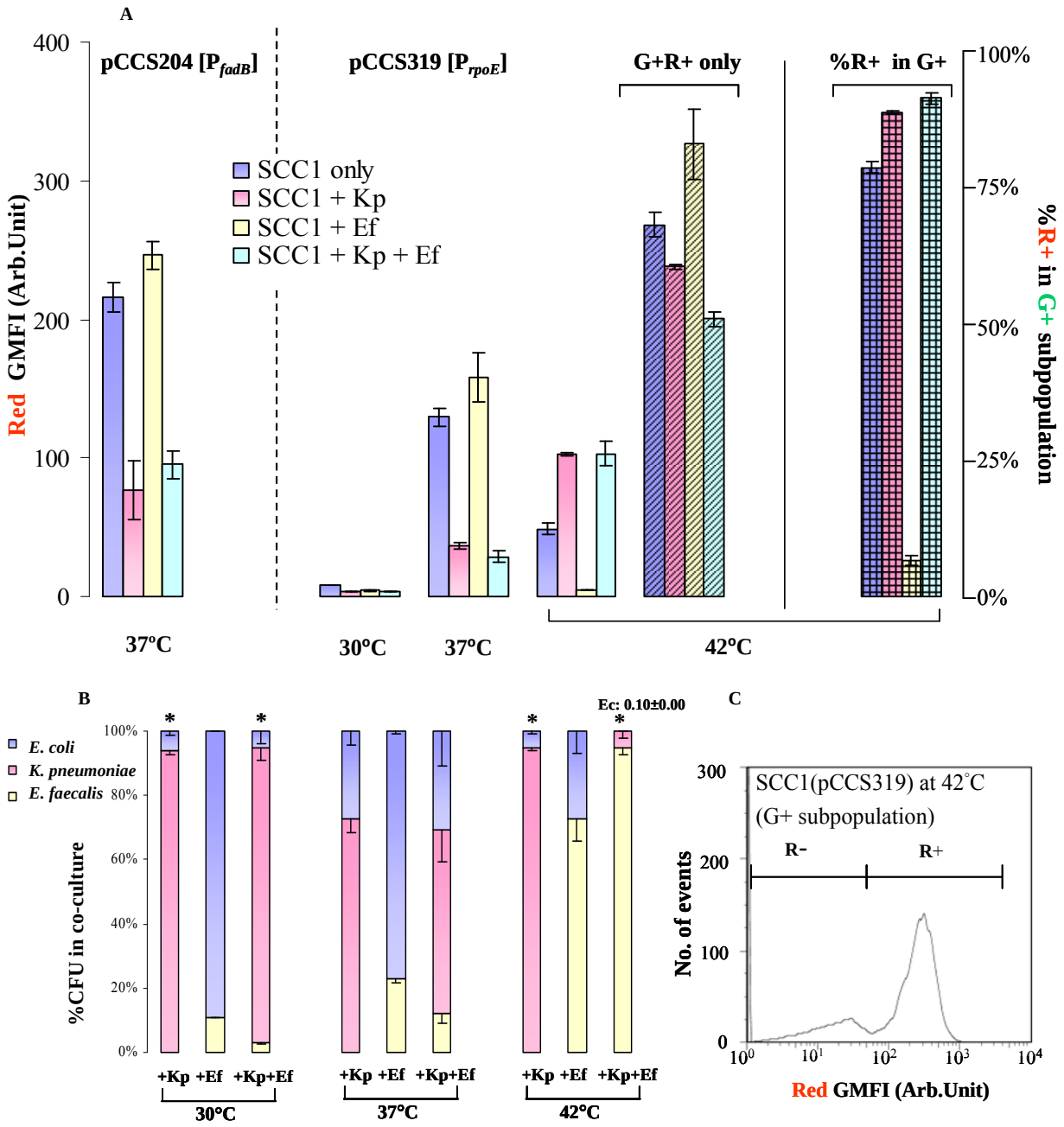

Fig 3.19. *E. coli* SCC1 was co-cultured planktonically with *K. pneumoniae* and/or *E. faecalis*. (A) Transcriptional response of P_{fadB} and P_{rpoE} in *E. coli* SCC1 included in G+ gate of the cultures quantified via AsRed2 fluorescence by FACSAria™. Slant shaded bars represent red GMFI in G+R+ subpopulation and checkered bars represent percentages of R+ in G+ subpopulation. **(B)** Percentage contribution of each species in 24 h co-cultures at the indicated growth temperatures. Asterisks (*) denote co-cultures in which *E. coli* was in <10% minority. *E. coli*'s percentage value was indicated above the last bar because the value is too small to be visible on the graph. Data shown in (A) and (B) are the mean ± SD of triplicate samples. **(C)** Heterogeneous promoter activities of P_{rpoE} in G+ gate of SCC1(pCCS319) pure culture grown at 42ºC.

3.3.1.2 Promoter activity reduction cannot be totally attributed to species dominance

We noted that *K. pneumoniae*, which exerted negative influences on the two promoters in both two- and three-species co-cultures at 37°C, was the dominant species in these co-cultures in the previous study (Fig 3.13.C on page 97). We proceeded to examine if the negative influences correlate with species dominance within the co-cultures. To investigate this, we performed viability counts of the respective co-cultures at all three temperatures, and analyzed the population ratios, as presented in Fig 3.19.B. At 42°C, *K. pneumoniae* was the dominant species in the two-species culture (94.6%) but was a minority (4.9%) in the three-species culture. However, its positive influence to *E. coli* was similar in both co-cultures, both in terms of red GMFI and %R+ in G+ subpopulation. Hence, changes in promoter activity due to partner species cannot be simply attributed to the effects of species dominance and competition.

3.3.2 Promoter activities of *E. coli* in biofilm co-cultures

The dual fluorescence nature of our reporter strain can also be utilized to visualize the distribution of promoter activity within the *E. coli* population, both in single-species and multi-species biofilms. Fig 3.20. (on page 116) shows the information we were able to obtain from such analysis. In the 48 h biofilm cultures (Fig 3.20.A~D), both *fadB* and *rpoE* promoter activities were observed to be localized towards air side (left side of the panel). Under this general trend, two activity distribution profiles existed. The promoter-active cells (cells in orange, Fig 3.20.B) were spatially distinct from promoter-basal cells (cells in green, Fig 3.20.B) in P_{fadB} sample, while a transition zone (cells in yellow, Fig 3.20.D) was observed between the cells of high and basal promoter activities in the P_{rpoE} sample.

Co-cultures with *E. faecalis* resulted in different profiles compared to the pure cultures for both promoter samples. As *E. faecalis* was very minor in composition in the biofilm co-culture as revealed in previous study (Fig 3.4.B on page 75 and Fig 3.13.D on page 97), and has no significant contribution to the biofilm boundaries, hence the green fluorescence shown in Fig 3.20.E~H is sufficient to define the biofilm in this case. It was observed that P_{fadB} was activated almost throughout the biofilm (Fig 3.20.E~F), with strong activation in some regions towards liquid side (righ side of the panel). P_{rpoE} was reduced throughout the *E. coli* population, as a result of co-culture with *E. faecalis*. This is in contrast to the situation observed in planktonic co-culture, where no negative effect by *E. faecalis* could be detected at 37°C (Fig 3.19.A).

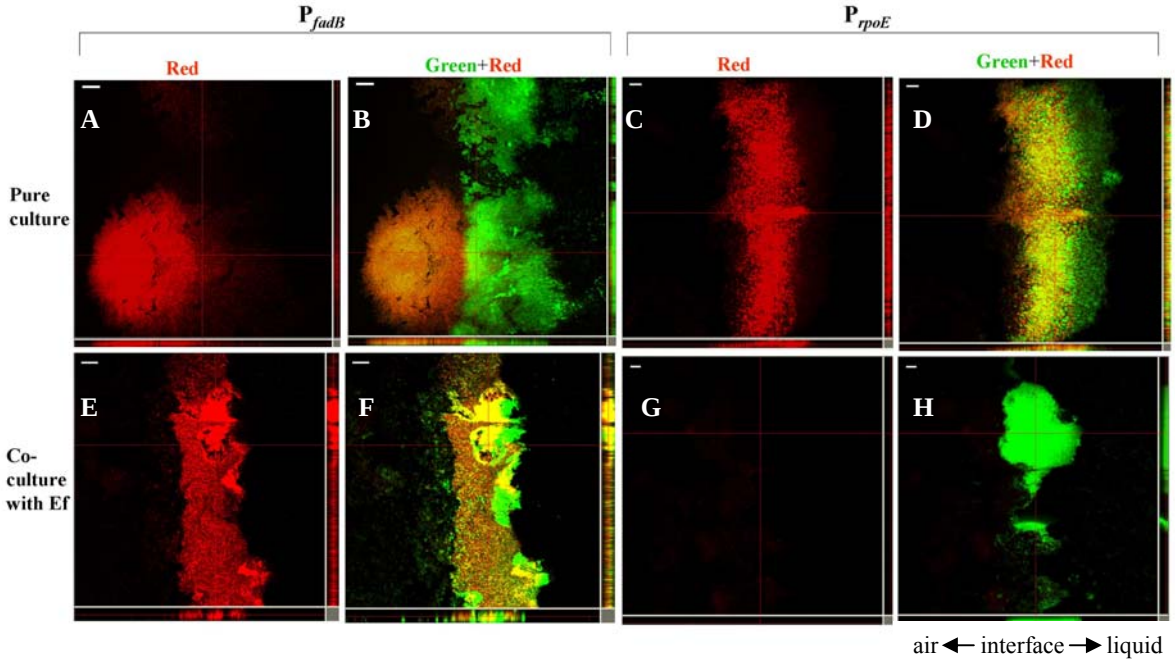

Fig 3.20. Promoter activities in biofilm co-cultures. Confocal laser scanning micrographs of biofilm cultures at 48 h. (size bar = 50 μm) Left half: *E. coli* SCC1(pCCS204) [P_{fadB}] pure culture (**A** and **B**) and co-culture with *E. faecalis* (**E** and **F**). Right half: *E. coli* SCC1(pCCS204)[P_{rpoE}] pure culture (**C** and **D**) and co-culture with *E. faecalis* (**G** and **H**). Shown in each panel are the x-y plane (horizontal section), x-z and y-z planes (vertical sections), corresponding to the red lines indicated in the respective perpendicular sections. All the images were taken with an orientation as indicated by the arrows below (**H**).

3.4 Exploring the possible limitations of the dual fluorescence system

In this section, we attempted to identify factors that may compromise the effectiveness of the established dual fluorescence system, and explored possible options to improve the system.

3.4.1 Influence of various factors on green fluorescence of *E. coli* SCC1

The system depends on the green fluorescence of *E. coli* SCC1 to distinguish *E. coli* from other species in the co-cultures. Hence factors that may influence the Gfp expression of SCC1 and affect this distinction need to be noted.

The green fluorescence intensity of SCC1 was obviously affected when cultured at 42ºC as described previously (Fig 3.18.B in section 3.2.4.6 on page 108). To explore this further, we examined the development of green fluorescence over time in cultures grown at the three temperatures: 30ºC, 37ºC and 42ºC. We also attempted two culture conditions with different culture volumes and vessels (tube versus flask). The fluorescence profiles between the two culture conditions were surprisingly different (compare Fig 3.21.A and B on page 119). The tube cultures at three temperatures reached distinctly different fluorescence levels at 12 h. The culture at 30ºC had the highest fluorescence intensity and the culture at 42ºC had the lowest. In contrast to the tube cultures, all the flask cultures reached similarly high fluorescence level at 12 h. We further examined the growth profiles of the cultures under the two

conditions and they were found to be similar (compare Fig 3.21.C and D). So the contrasting influences seen between Fig 3.21 A and B were unlikely to be due to growth differences. The reason behind this difference is as yet unidentified.

Apart from the temperatures and culture conditions, we noted that the green fluorescence level of SCC1 strain was slightly influenced by non-*E. coli* species in the co-cultures (Fig. 3.21.E). However, the fluorescence levels were still much higher than the lower limit of 26.3 GMFI (dotted line Fig 3.21.E), above which the entire *E. coli* population would fall into G+ gate as shown in Fig 3.18.B. The high sorting purity (within the range of 97.34~99.41%) in Table 3.2. on page 95 implied that FACS analysis was not affected by this variation of fluorescence levels. As a confirmation, we conducted an experiment to compare the CFU count from the co-cultures to the FACS %G+ data. The %G+ data was in good agreement with the CFU count data (Fig 3.21.F). This further confirmed that the influences from the partner species used in this study were not significant enough to compromise FACS analysis of *E. coli* SCC1.

As this experiment showed that temperatures do affect Gfp expression of *E. coli* SCC1, and the influences differed with specific culture conditions, it is advisable to make precautionary checks before making analysis under different co-culture conditions.

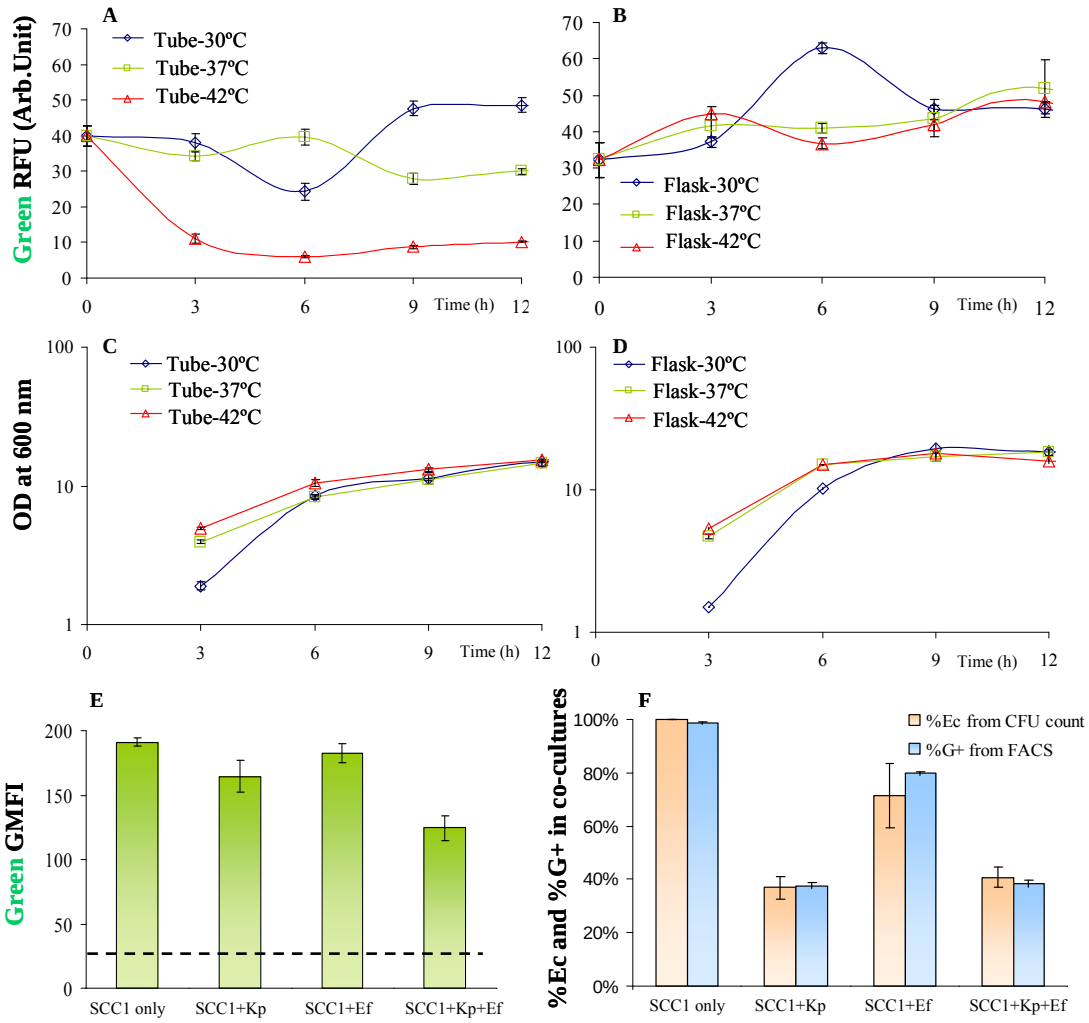

Fig 3.21. Green fluorescence intensity of *E. coli* SCC1 was influenced by various factors. (A) and **(B)**: Green fluorescence intensity of *E. coli* SCC1 strain over time, grown at three temperatures (30ºC, 37ºC and 42ºC), and in two culture conditions: tube (A) or flask (B). **(C)** and **(D)** Growth profile of *E. coli* SCC1 at the three temperatures in tube (C) and in flask (D) as monitored by OD_{600}. Data shown in (A)~(D) is mean ± SD of duplicate samples. **(E)**: Green GMFI of events fell into G+ gate in co-cultures as analyzed by FACS. Dotted line represent the lower GMFI limit above which the entire histogram profile of the sample would fall into G+ gate (refer to Fig 3.18.B on page 108 and Table 3.4. on page 109). **(F)**: Consistency between %Ec data obtained from CFU count and %G+ data obtained from FACS. Co-cultures at 24 h were analyzed both by CFU count of *E. coli* and FACS for G+%. Ec: *E. coli*; Kp: *K. pneumoniae*; Ef: *E. faecalis*. Data shown in (E)~(F) is the mean ± SD of triplicate samples.

3.4.2 Expanding the reporter capability of *AsRed2*

AsRed2 was selected as the reporter gene because it was better compared to the other red FP genes, with respect to reasonable fluorescence intensity and competence to report promoter activity (Fig 3.8 in section 3.2.2.3 on pages 82~83). Two promoter fusions (P_{fadB}-*AsRed2* and P_{rpoE}-*AsRed2*) have been shown to reflect promoter activities of varying strengths in *E. coli* as influenced by other partner species (Fig 3.19 on page 113). Some promoter fusions we have constructed, however, showed too low a fluorescence level to be detected, presumably because of the weak promoter strength. This means that the current *AsRed2* reporter is unable to reflect promoter activities in that low promoter strength range, and thus the repertoire of promoters that can be studied is reduced. Hence we attempted to generate an improved version of AsRed2 with higher fluorescence intensity, for expanding its reporter capability in the dual fluorescence system.

There is not clear strategy how we may improve the fluorescence intensity of AsRed2. One speculation is that its codon usage may have influenced the translation efficiency. The *AsRed2* coding sequence had been engineered via silent base pair changes so that the codons present would be favourable for efficient and optimal translation in mammalian cells, but perhaps these are not favorable in prokaryotic cells [158]. We decided not to employ a targeted engineering strategy to improve AsRed2 performance, but a random mutagenesis strategy instead.

3.4.2.1 *AsRed2* mutant DNA library

To obtain an improved version of the *AsRed2* reporter, we generated *AsRed2* mutant DNA using error prone PCR (EP-PCR) and obtained a random *AsRed2* mutant library via a non-conventional cloning method (In-Fusion^{TM} 2.0 PCR Cloning Kit). The constitutive promoter $P_{A1/04/03}$ was first fused upstream of the *AsRed2* gene in pCCS325, which served as the parental plasmid (wild type) for PCR mutagenesis. EP-PCR was conducted to yield *AsRed2* mutant DNA (refer to section 2.2.1.2.3 on page 34), which was then inserted downstream of the constitutive promoter $P_{A1/04/03}$. A library with 81,615 colonies was generated.

3.4.2.2 Analyses of clones of interest from *AsRed2* mutant library

53 colonies were visibly red at the time of harvesting the library, so they were isolated and screened by quantification of their fluorescence intensity. Profiles of the five clones with the highest fluorescence levels were shown in Fig 3.22. All the five *AsRed2* mutants had significantly higher fluorescence intensity compared to the wild type (asterisk, Fig 3.22.), and that of mutant M1, in particular, was almost 25 fold higher.

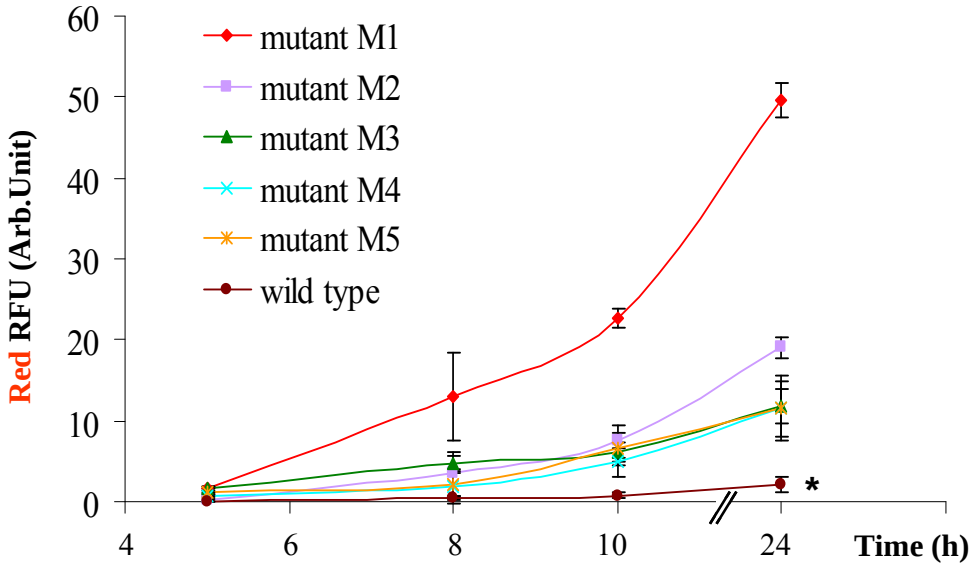

Fig 3.22. Red fluorescence intensity of the *AsRed2* mutants as assayed by fluorometer. Fluorescence profiles over time of *AsRed2* mutants M1~M5 and *AsRed2* wild type (indicated by asterisk) were quantified by fluorometer. Data shown are the mean ± SD of eight replicate samples.

The five *AsRed2* mutants were sent for sequencing, the results of which were aligned against wild type *AsRed2* using Vector-NTI software and confirmed with manual checking. Mutations are expected to exist between the two regions defined by the primers used in the PCR, i.e. -42 ~ +723 (numbered with respect to the start codon of *AsRed2*) as indicated in Fig 3.23. on page 124, in which +1 ~ + 699 is the coding region. A total of 32 point mutations were found in the

five mutants, i.e. averaging 6.4 mutations/mutant, which is fairly consistent with the theoretically calculated average of 5.4 mutations/mutant according to the erroneous base incorporation rate of $7x10^{-3}$ per nucleotide [117]. Mutations were all nucleotide substitutions. Table 3.5. shows that out of the total 32 mutations, 9 of them occurred in non-coding regions and 8 of them did not result in amino acid changes. Of the remaining 15 mutations that led to amino acid changes, 6 of them resulted in changes from one neutral aminal acid to another, and the remaining 9 resulted in changes to amino acids with different side-chain properties.

Among the five *AsRed2* mutants analyzed, we took the most interest in the sequence of mutant M1 as this mutant had the highest fluorescence intensity (Fig 3.22.). Out of the 8 mutations in mutant M1, only 1 led to an amino acid residue change (Tyr211 to Phe). What is also noteworthy is that there are 3 mutations occurring in the non-coding region slightly upstream of the start codon. Although they would not contribute to the property of AsRed2 protein, they may contribute to the secondary structure of the transcribed mRNA and presumably stabilize the *AsRed2* transcript.

This AsRed2 mutant M1 with improved fluorescence intensity may be used to analyze transcriptional activities of weaker promoters.

```
-150  TTTCGTCTTC ACCTCGAGAA AATTTATCAA AAAGAGTGTT GACTTGTGAG
-100  CGGATAACAA TGATACTTAG ATTCAATTGT GAGCGGATAA CAATTTCACA
-50   CACTGCAGTT TCTCCATACC CGTTTTTTGG GCTAACAGGA GGAATTAACC
            -42
+1    ATGGCCTCTT TGCTGAAGAA GACCATGCCC TTCAGGACCA CCATCGAGGG
+50   CACCGTGAAC GGCCACTACT TCAAGTGCAC CGGCAAGGGC GAGGGCAACC
+100  CCCTGGAGGG CACCCAGGAG ATGAAGATCG AGGTGATCGA GGGCGGCCCC
+150  CTGCCCTTCG CCTTCCACAT CCTGTCCACC TCCTGCATGT ACGGCTCCAA
+200  GGCCTTCATC AAGTACGTGT CCGGCATCCC CGACTACTTC AAGCAGTCCC
+250  TCCCCGAGGG CTTCACCTGG GAGCGCACCA CCACCTACGA GGACGGCGGC
+300  TTCCTGACCG CCCACCAGGA CACCTCCCTG GACGGCGACT GCCTGGTGTA
+350  CAAGGTGAAG ATCCTGGGCA ACAACTTCCC CGCCGACGGC CCCGTGATGC
+400  AGAACAAGGC CGGCCGCTGG GAGCCCTCCA CCGAGATCGT GTACGAGGTG
+450  GACGGCGTGC TGCGCGGCCA GTCCCTGATG GCCCTGGAGT GCCCCGGCGG
+500  TCGCCACCTG ACCTGCCACC TGCACACCAC CTACCGCTCC AAGAAGCCCG
+550  CCTCCGCCCT GAAGATGCCC GGCTTCCACT TCGAGGACCA CCGCATCGAG
+600  ATCCTGGAGG AGGTGGAGAA GGGCAAGTGC TACAAGCAGT ACGAGGCCGC
+650  CGTGGGCCGC TACTGCGACG CCGCCCCCTC CAAGCTGGGC CACAACTGAA
                          +723                          +699
+700  GCGGCCGCGA CTCTAGAATT CGAAGCTTTC TAGAACAAAA ACTCATCTCA
+750  GAAGAGGATC TGAATAGCGC
```

Fig 3.23. *AsRed2* coding region and flanking sequences. Sequences were numbered with respected to the start codon of *AsRed2*. Sequence corresponding to $P_{A1/04/03}$ is underlined (red "A" in bold denotes the transcriptional start site); *AsRed2* coding region is in bold (+1 ~ +699); and sequence corresponding to the primers used for EP-PCR are in blue. Point mutations are expected to be introduced between the two primer sites (-42 ~ +723) due to errors in base incorporation during PCR.

Table 3.5. Nucleotide and amino acid changes in *AsRed2* mutants.

AsRed2 mutant	Position[a]	Nucleotide change[b]	Amino acid (aa) change[c]	aa side chain property
M1	-26	T → G	non-coding region	
	-12	G → A	non-coding region	
	-3	A → C	non-coding region	
	+63	C → T	none	
	+285	C → T	none	
	+384	C → T	none	
	+621	G → A	none	
	+632	A → T	Tyr211 → Phe	neutral → neutral
M2	-20	G → T	non-coding region	
	-17	A → C	non-coding region	
	-1	C → A	non-coding region	
	+1	A → T	Met1 → Leu	neutral → neutral
	+45	C → T	none	
	+96	C → T	none	
	+449	T → A	Val150 → Glu	neutral → acidic
	+506	A → G	His169 → Arg	weak basic → strong basic
	+579	C → T	His193 → Arg	weak basic → strong basic
	+710	A → G	non-coding region	
M3	-40	T → A	non-coding region	
	+4	G → A	Ala2 → Thr	neutral → neutral
	+446	A → G	Glu149 → Gly	acidic → neutral
	+468	C → T	none	
	+612	G → A	none	
	+667	G → A	Asp223 → Asn	acidic → neutral
	+686	T → A	Leu229 → Gln	neutral → neutral
M4	-42	T → A	non-coding region	
	+401	A → G	Gln134 → Arg	neutral → basic
M5	+397	A → T	Met133 → Leu	neutral → neutral
	+494	C → A	Pro165 → His	neutral → weak basic
	+518	A → G	His173 → Arg	weak basic → strong basic
	+565	A → T	Met189 → Leu	neutral → neutral
	+626	A → T	Lys209 → Met	basic → neutral

[a] Position of the nucleotides is numbered with respect to the start codon of *AsRed2* as shown in Fig 3.23.

[b] A-Adenine; T-Thymine; C-Cytosine; G-Guanine

[c] Ala-Alanine; Arg-Arginine; Asn-Asparagine; Asp-Aspartic acid; Gln-Glutamine; Glu-Glutamic acid; Gly-Glycine; His-Histidine; Leu-Leucine; Lys-Lysine; Met-Methionine; Phe-Phenylalanine; Pro-Proline; Thr-Threonine; Tyr-Tyrosine; Val-Valine

Chapter 4 Discussions and future work

4.1 The strengths of the system

When interspecies interaction is the biological focus of an in vitro study, co-culture of different species becomes a necessary condition. However, although it is desirable to analyze the biological response of one specific member of the consortium in a dissective manner, it is technically challenging due to the following factors.

Firstly, contamination from partner species during data acquisition may undermine the confidence to derive meaningful interpretation from such data. Secondly, in accordance with the dynamics of the consortia under study, the population of species-of-interest may be vastly reduced to the point that the sensitivities of the analytical techniques are compromised. Thirdly, some assumptions made in analysis may not be correct as many basic effects of interspecies interaction are not yet known or completely established, e.g. it is often assumed that the percentage yield of cellular materials collected from different species using the same technique is the same, but that may not be the case. Finally, the multi-factorial nature of interspecies interactions may demand that more than one parameter be assessed simultaneously to make sense of an analysis, compounding the difficulty mentioned above.

In this study, we described the establishment of a dual fluorescence system that has the potential to address these challenges by exploiting the capacity of FACS.

The premise is that at the single-cell level, the species-of-interest, labeled with green fluorescence, can be distinguished from other species, and the promoter activity of this species can further be assessed via a red fluorescence reporter. Using this system, we were able to assess the transcriptional response of *fadB* and *rpoE* promoters in *E. coli* as influenced by co-culture partners *K. pneumoniae* and/or *E. faecalis*.

4.1.1 Suitability of *E. coli* SCC1 as reporter strain

Morphologically (data not shown) and physiologically (Fig 3.13.B on page 97), the Gfp-expressing *E. coli* strain SCC1 was found to mimic the wild type MG1655 strain closely. Importantly, its interactions with other species with respect to the population relationships were not affected by the Gfp expression (Fig 3.13.C~D), and its promoter activities–monitored through the *AsRed2* reporter–did not deviate significantly from that of the wild type (Fig 3.16. on page 104) as well. When mixtures or co-cultures of *E. coli* SCC1 with other species were sorted by FACS, 96.7~100.0% purity could be achieved for both the *E. coli* and the non-*E. coli* fractions (Table 3.2. on page 95). Using this reporter strain, we were able to examine the transcriptional response of *fadB* and *rpoE* promoters in *E. coli* as influenced by co-culture partners.

4.1.2 Detection capacity when species of interest is in minority

As the species-differentiation is being carried out by virtue of the green fluorescence, the promoter activity is simultaneously assessed at the single-cell level in our system. The advantage of species-differentiation and simultaneous transcriptional analysis has an impact particularly in a situation where the

species-of-interest has developed into a minority within the co-culture (asterisks, Fig 3.19.B on page 113). In more severe cases, the percentage of *E. coli* can drop to as low as 0.1%, as observed in the three-species co-culture at 42°C in this study (Fig 3.19.B). Despite *E. coli* being a severe minority, its transcriptional response could still be analyzed with confidence (Fig 3.19.A) by ensuring that a statistically significant number (2000 events) of data for *E. coli* cells were collected.

Although certain techniques such as quantitative reverse transcription-PCR (RT-PCR) can be applied to characterize specific gene expression in *E. coli* when in co-culture [68], if the other species overwhelm(s) *E. coli* in cell number (i.e. *E. coli* is a severe minority), *E. coli* mRNA will likewise be overwhelmed by the other species' mRNA with respect to copy number in the final preparation. It may be difficult to select primers with the appropriate level of specificity under such extreme bias. This problem is particularly prominent for genes from related species which are fairly homologous in sequence. Ironically, the biologically important question of interactions among closely related species will be most handicapped by this limitation. We have directly experienced this while attempting a parallel study using RT-PCR to look at *E. coli fadB* and *rpoE* expression in co-culture with *K. pneumoniae,* which carries these genes with 85% and 87% sequence similarity, respectively, to those of *E. coli*. Despite careful and rational designs of primers, a severe excess in copies of homologues from the dominant partner species has led to non-specific products, undermining the confidence in interpretation (data not shown). Hence, the FACS-based analysis system provides an alternative to

serve better under such circumstances, and increases the repertoire of gene expression versus community make-up that can be studied.

4.1.3 Ability to reveal heterogeneity in gene expression

In addition to the advantages described above, the single-cell analysis mode of FACS on the dual fluorescence system provides a handle to address the issue of heterogeneity in gene expression. Heterogeneity in genetically homogeneous bacteria has been reported in both planktonic and biofilm cultures [91, 159, 160]. It is an area in which analysis using DNA microarray or proteomics have not been sufficiently developed to tackle. An and Parsek [161] in a recent review warned of the perils of transcriptional profiling in biofilm communities, and suggested flow cytometric sorting of the metabolically active and inactive subpopulations prior to DNA microarray analysis, to obtain their profiles separately. The dual fluorescence system we have developed is capable of discerning the heterogeneous nature of the specific promoters under study (Fig 3.19.A and C on page 113).

4.1.4 Complementary data to the analysis of transcriptional response

Although FACS-based analysis of the transcriptional response is the main information we wish to derive from the dual fluorescence system, it is also able to provide other information to complement the main data. The dual fluorescence property makes biofilm visualization feasible (Fig 3.20. on page 116), adding other dimensions, such as spatial distribution of the promoter activities, to the analysis. Another potential derivation of information from FACS analysis is through the percentage of green positive events (%G+)

counted. In the current study, %G+ was able to reflect the population ratio of *E. coli* in the co-culture (Fig 3.21.F on page 119), although we have adhered to using the viable count (CFU) data as an indicator of population ratio for the relevant discussions (Fig 3.13.C~D on page 97 and Fig 3.19.B on page 113).

4.1.5 Application of the system at the genomic level

In addition to the capability of the system to study the specific genes of interest as described in this study, it can be applied to investigate the gene expression in multi-species context at the genomic level. By insertion of random DNA fragments upstream of the promoterless *AsRed2*, a promoter-capture library can be generated, and the clusters of genes that are differentially expressed in co-cultures could be identified.

4.2 Limitations and further improvement

4.2.1 Reporter strain *E. coli* SCC1

Reporter strain *E. coli* SCC1 has been capable of representing *E. coli* population in co-culture conditions in this study, but we cannot exclude the possible effect(s) on *E. coli* SCC1 with respect to its Gfp expression in other types of co-cultures. As mentioned previously green fluorescence intensity of *E. coli* strain SCC1 was influenced by various factors including temperatures, culture conditions, partner species (Fig 3.21. on page 119), etc. As a result, the level of Gfp expression in *E. coli* SCC1 needs to be checked under the specific culture conditions, with regards to growth temperature, culture volume, aeration condition, and culture vessels. The influences conferred from other partner

species to Gfp expression should also be checked before application in other co-culture systems.

4.2.2 Promoter reporter *AsRed2*

4.2.2.1 *AsRed2* cassette on high copy plasmid

Although the multiple copies of promoter-fusion plasmid can increase the system sensitivity, the plasmid-borne reporter has its limitations. The *AsRed2* reporter in high copy may lead to artifacts in representation of certain transcriptional activity, particularly those promoters that are sensitive to changes in regulator:promoter ratio.

With the improved fluorescence intensity of *AsRed2* mutant M1 (Fig 3.22. on page 122), the elevated sensitivity of the system may be utilized to circumvent the problems of high copy plasmid by having *AsRed2* mutant M1 on low copy plasmid or even as single copy insertion onto the chromosome of *E. coli* SCC1.

4.2.2.2 Stability of AsRed2 and the consequence

The AsRed2 protein is very stable, and able to maintain its fluorescence at least 12 h post-expression (data not shown). Hence its fluorescence reflects the accumulation of gene expression in the cell rather than expression at the time of observation. As a result, temporal changes such as transient reduction in promoter activity are unlikely to be registered. For such observations, an unstable version of AsRed2 will be useful. Similar approach as that of generating the unstable Gfp versions [116] can be adopted, whereby the

addition of specific C-terminal oligopeptide rendered the Gfp susceptible to degradation by indigenous housekeeping proteases.

4.3 Biological questions generated in this study

With the genetically manipulated *E. coli* strain SCC1 carrying promoter-fusion *AsRed2*, we were able to observe altered transcriptional responses of *E. coli* as influenced by other species in multi-species cultures. In the further exploration of the system, AsRed2 mutants with high fluorescence intensity were obtained. Two biological questions are generated from this study.

The promoters of *fadB* and *rpoE* in *E. coli* were found to be differentially influenced by the partner species in this study. It would be interesting to further investigate questions such as how and why the transcriptional responses of *E. coli* were altered in this way by the partner species in the co-cultures. The functional consequences of these transcriptional responses in *E. coli*, is a direction worth exploring.

Another intriguing biological question is how the mutations in *AsRed2* mutant M1 resulted in the improved fluorescence performance. Are there essential point mutations that could account for the better performance? Which aspect of the protein was "improved" – the translational efficiency, protein folding efficiency, or protein structure?

Conclusion

Knowledge of interspecies interaction among bacteria of different species is important as most bacteria in nature exist in a multi-species context. The project presented in this thesis had as a goal to construct a dual fluorescence system to analyze transcriptional response of the species of interest within a multi-species context. Co-cultures composed of *E. coli*, *K. pneumoniae* and *E. faecalis* were established. Transcriptional responses of *E. coli* within the co-cultures were analyzed via a genetically modified SCC1 strain, which has a constitutively expressed Gfp to distinguish from non-*E. coli* species and a promoter fusion *AsRed2* to report transcriptional activities. Promoters of *fadB* and *rpoE* in *E. coli* had shown altered expressions when co-cultured with the partner species. To increase the sensitivity of the system and thus broaden the repertoire of promoters that can be analyzed, *AsRed2* mutant library was generated, and clones with high fluorescence intensity in *E. coli* were obtained from the library. The analytical system described in this study is able to complement the current methods used for the studies with multi-species systems. This is the first system reported to date that allows transcriptional response due to bacterial interspecies interactions to be studied, even when the species to be analyzed is a minority.

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