Investigation of host-microbe interactions in the probiotic bacteria *Escherichia coli* Nissle 1917

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Abstract

Probiotics are generally live preparations of bacteria that exert beneficial effects on host health when ingested in sufficient quantities. The novel probiotic *Escherichia coli* strain Nissle 1917 (EcN) has been shown to have a number of beneficial effects in this context, including protection against food-borne pathogens and infectious diarrheal diseases, and maintaining remission of inflammatory bowel diseases by virtue of its anti-inflammatory properties. However, little is known regarding the mechanisms underlying the beneficial effects of this organism. The overall aim of this work was to provide a greater understanding of the mechanisms through which EcN interacts with the mammalian gastro-intestinal epithelium to benefit human health.

In order to identify genes involved in host-microbe interaction in EcN, a random transposon mutagenesis based approach was adopted. A large bank of mini-Tn*5* mutants was constructed using the pRL27 suicide delivery system, and subsequently subjected to high throughput screening to identify mutants deficient in traits relevant to host-microbe interaction, and colonisation of the intestinal tract. These included screens for mutants with alterations in biofilm formation using a crystal violet microtitre plate assay, which we hypothesised would permit recovery of mutants with deficiencies in surface structures relevant to host-microbe interaction.

From a bank of 4,116 mutants screened, 21 with alterations in biofilm formation were recovered and a sub-set were characterised in greater detail. Identities of genes disrupted in these mutants were mainly predicted to encode proteins associated with the bacterial membrane, or to be involved in generation of cell surface structures. The interactions of selected mutants with intestinal epithelial cells were subsequently investigated using an *in vitro* co-culture model with the human intestinal Caco-2 cell line. This system was optimised for EcN and used to compare EcN wild-type, selected mutants, and the *E. coli* K12 intestinal commensal strain MG1655 in terms of adhesion, internalisation, effects on cell health and apoptosis.

One mutant was demonstrated to show significantly enhanced adherence to Caco-2 cells and elevated induction of apoptosis, compared to the wild- type and MG1655. This mutant was disrupted in the *kfiB* gene, part of the K5 capsule biosynthesis gene cluster in EcN but with no confirmed function. The *kfiB* gene was confirmed as responsible for the observed phenotype using RT-PCR to rule out polar effects from the mini-Tn*5* insert, on expression of downstream genes. To further investigate the function of the *kfiB* gene in host-microbe interaction, mutants with deletions in either *kfiB* or the associated *kfiC* gene (previously shown to be essential for K5 capsule biosynthesis), were generated by homologous recombination. Attenuation in K5 capsule synthesis was confirmed in both mutants (EcNΔ*kfiB* and EcNΔ*kfiC*) using the ΦK5 bacteriophage sensitivity assay, which attaches to the K5 capsule and only infects strains producing this surface structure. However, EcNΔ*kfiC* lacked the adherent and apoptosis-inducing properties on Caco-2 cells demonstrated by EcNΔ*kfiB* strains. These observations reinforce the importance of K5 capsule in mediating bacterial interactions with intestinal epithelial cells, and indicate that genes associated with capsule biosynthesis in EcN may mediate distinct aspects of host-microbe interaction; most likely through impact on capsule biosynthesis, but the possibility of independent effects on host cells cannot be excluded.

To gain an overview of other mechanisms through which EcN may interact with the human host, and contribute to the regulation of intestinal processes, the impact of EcN on neurotransmitter release was compared with non-pathogenic gut commensal *E. coli* strain MG1655. Exposure of sections of mouse ileum to EcN cells was found to significantly increase serotonin (5-HT) production in the enterochromaffin cells in a dose dependent manner, in contrast to MG1655. The produced 5-HT was not metabolised into 5-hydroxy-3-indoleacetic acid (5-HIAA) by EcN as observed in controls, suggesting that EcN was blocking the serotonin transporter (SERT) system which could result into an increase in host's gastro-intestinal motility.

In conclusion, this study elucidates novel mechanisms by which EcN interacts with the host intestinal epithelium and may regulate neurotransmitter mediated signalling the host. These results reveal pathways toward the development of more effective, rationally designed probiotic interventions for the prophylaxis of intestinal disorder, or the enhancement of human health.

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This thesis is lovingly dedicated to our precious daughter Joanna, born on 26th March 2014, a few days prior to submitting this report.

Author's Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not yet been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed

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Abbreviations and definitions

5-HIAA	5-hydroxyindole acetic acid
5-HT	5-hydroxytryptamine or serotonin
5-HTP	5-hydroxytryptophan
DAP	diaminopimelic acid
EcN WT	*Escherichia coli* Nissle 1917 wild-type
EcN	*Escherichia coli* Nissle 1917
GI	gastro-intestinal
GIT	gastro-intestinal tract
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
MG1655	*Escherichia coli* K12 MG1655
SCFA	short-chain fatty acid
SDW	sterile deionised water
Tn	transposon

Chapter 1: General introduction

1.1 Human 'super-organism'

The human body is a highly complex organism both in functions and composition, and plays host to a diversity of microbial communities including bacteria, fungi, viruses and archea. Human beings are thought to be germ-free *in utero* but pick up their first microbes as they pass through the birth canal, and then through the immediate exposure to the environment. From that oint on in a e rson's lifetime, normal and harmless microbial communities will establish themselves in various anatomical locations such as skin, conjunctiva, oral cavity, respiratory tract, gastro-intestinal tract (GIT) and urogenital tract. These microbial communities, also termed the human microbiota, are thought to have co-evolved with the human host for mutual benefits, and play critical roles in maintaining human health (Dethlefsen *et al*. 2007, Ley *et al*. 2008, Wallace *et al*. 2011).

The impact of the human microbiota on host-health is especially evident in the GIT, in which the greatest microbial diversity and abundance is found. Approximately, 100 trillion microbial cells, comprising an estimated 1,000 bacterial species inhabit the GIT of a healthy adult individual (Qin *et al*. 2010, Zhu *et al.* 2010). The total number of bacterial cells in and on humans is about 10 times more than "human" cells, whereas their collective genomes comprise more than 100 times the number of genes as the human genome (Ley *et al*. 2008, Qin *et al*. 2010). It is mainly on the basis of hosting such a dense complex and co-evolved microbiota, that the human body can be viewed as a 'su e r-organism' (Goodacre 2007, Zhu *et al.* 2010). Functionally, the gut microbiota is a 'microbial organ' performing a wide range of beneficial tasks for the host. These include those relating to metabolism (Martin *et al*. 2009), development and homeostasis of the immune system (Tlaskalova-Hogenova *et al*. 2004, Turner 2009), stimulation of gut motility (Samuel *et al.*

2008, Quigley 2011), and protection from enteropathogenic infections (Lu and Walker 2001, Tlaskalová-Hogenová *et al*. 2011).

1.2 Disturbance of the gut microbiota

The overall balance in the composition of the gut microbiota is important in order to exert the mentioned benefits on the host. Disturbance of the gut microbiota balance can be induced by various exogenous and endogenous factors. These include antibiotic treatment, dietary changes, and invasive pathogens that can lead to a shift in species composition and activities of the gut microbiota. In turn, the disruption of the gut microbiota can favour the outgrowth of potentially enteropathogenic bacteria. Alternatively, disruption of the gut microbiome can result in damage to the physiological barrier of the intestinal mucosa, epithelial irritation and inflammation or increased intestinal permeability, which may predispose the host to a higher risk of developing a variety of gastro-intestinal (GI) diseases (McCracken and Lorenz 2001, Guarner and Malagelada 2003, Chichlowiski and Hale 2008, Mai and Draganov 2009).

Table 1.1 illustrates GI diseases and dysfunctions that are associated with disturbances of the gut microbiota. These include infectious, functional, inflammatory, iatrogenic, and immunological-related diseases. These diseases account for considerable morbidity, mortality, and cost worldwide, with an estimate of 6-60 billion cases of GI illnesses and millions of deaths occurring every year (Payment and Riley 2002). For example, gastroenteritis is estimated as the third and second most common cause of deaths in adults and children less than 5 years old, respectively (Amar 2010). Other common gut disorders, which also remain prevalent in industrialised nations, include inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), as well as colorectal cancer. In the UK alone the prevalence of UC is ~240 per 100,000 people, with ~ 115,000 CD sufferers (NICE 2014). Colorectal cancer remains the third most common

Table 1.1: Gastro-intestinal diseases associated with disturbance of the gut microbiota

Gastro-intestinal diseases	Example
Infections	Shigellosis, salmonellosis, cholera, infections by *E. coli* (i.e. EPEC, ETEC, EHEC)*
Inflammatory bowel diseases (IBD)	Ulcerative colitis, Crohn's diseases
Diseases associated with anatomical and morphological changes	Colorectal cancer, diverticulosis, strictures, bling loops
Diseases associated with immunological disturbances	Food allergy, celiac disease
Iatrogenic diseases	Diarrhoea/ functional diseases after treatment with antibiotics, chemotherapeutic agents or radiation
Functional diseases	Irritable bowel syndrome (IBS), chronic constipation, non-ulcer dyspepsia

*EPEC, enteropathogenic *E. coli*; EHEC, enterohemorrhagic *E. coli*; ETEC, enterotoxigenic *E. coli.*

This Table was adapted from Sonnenborn and Schulze (2009).

malignancy worldwide, with over 100 new cases diagnosed daily in the UK alone (IARC/GLOBOCAN 2002, Cancer Research UK 2014, Office for National Statistics UK 2014). Considering these observations, it is possible that restoring balance to the disturbed gut microbiota can help to manage gastro-intestinal diseases to a certain extent.

1.3 Probiotics

The term "r obiotic" was first utilised in 1965 by Lilly and Stillwell, to designate substances secreted by one microorganism to kindle the growth of another (Lilly and Stillwell 1965). This definition was later im r oved by Parker (1974) as "substances and organisms which contribute to intestinal microbial balance." Nevertheless this definition became much debated as its meaning could include the antibiotics. It was subsequently revised by Fuller (1989) emphasizing the importance of live microbial cells. He then redefined r obiotics as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance." However, since some probiotics might not be administered orally or through food r e a rations, Fuller's definition was readjusted by the FAO/WHO expert committee as: non-pathogenic living microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO 2001).

Many probiotic bacteria are culturable members (able to grow in laboratory) of the human microbiota and have been used by humans for many centuries, before the term " r obiotic" was introduced. The most common probiotics are from the lactic acid bacteria (LAB) group that have been applied in fermentation of food products for many centuries. Moreover, *Streptococcus and Lactobacillus* members of the LAB are also important inhabitants of the human intestinal tract (Hayashi *et al*. 2005). In general, most probiotics are Gram-positive, mainly strains from *Lactobacillus* and *Bifidobactetrium* species. Also, some species from the genera *Enterococcus*, *Streptococcus*, and *Bacillus* have been

recognised and used as probiotics (Boyle *et al.* 2006). Gram-negative are rarely selected as probiotics and the best known of this group is probiotic *E*. coli strain Nissle 1917 (EcN).

1.4 *Escherichia coli* Nissle 1917

E. coli Nissle 1917 (EcN) is a Gram-negative rod-shaped bacterium, facultative anaerobe member of the family of *Enterobacteriaceae*. Serologically, *E. coli* Nissle 1917 belongs to the *E. coli* O6 group, serotype O6:K5:H1 and exhibits a semi-rough colony morphology on agar plate, and sensitivity to normal human serum (Grozadanov *et al*. 2002). This strain was first isolated by the army surgeon Alfred Nissle from the faeces of a World War I soldier who, in contrast to his comrades, was not affected by an outbreak of diarrhoea (Sonnenborn and Schulze 2009). Nissle hypothesised that this soldier harboured a special bacterial strain with antagonistic properties against infectious pathogens. He then performed a laboratory test screening stool samples from the healthy soldier, resulting in the isolation, and preliminary characterisation of EcN. This gastroprotective bacterium became the active component of the microbial drug Mutaflor® (Ardeypharm GmbH, Herdecke, Germany) which is currently marketed and used in several European countries (Sonnenborn and Schulze 2009).

1.5 Health-benefits of *E. coli* Nissle 1917

Amongst the different health benefits claimed for EcN, controlling inflammatory bowel diseases (IBDs) and inhibition of enteropathogens are the best studied. The exact causes of IBDs have yet to be clearly elucidated, but the current conventional therapy mainly consists of immunosuppressive agents. Alternatives to this include the use of anti-TNF-α antibodies and the probiotics as an emerging therapy. In particular, clinical trials have shown EcN to be therapeutically effective in IBDs. For example, in three randomised clinical trials involving more than 500 patients (Burke and Axon 1988, Malchow1997, Rambacken *et al*. 1999), EcN has been shown to maintain remission in patients of

ulcerative colitis (UC) at efficacies comparable to those of the standard mesalazine (an anti-inflamatory drug commonly used for treatment of IBD patients). Moreover, EcN has been shown to be effective for treatment of acute diarrhoea in infants (Henker *et al*. 2007, Henker *et al*. 2008) and constipation in adults (Möllenbrink and Bruckschen 1994, Sonnenborn and Schulze 2009).

Protective effects of EcN against enteropathogens have been studied using intestinal cells and animal models. For example, co-culturing EcN with invasive *E. coli* using intestinal epithelial cell (IEC) model was found to reduce the adhesion of the invasive *E. coli* by 97.2-99.9 % (Boudeau *et al*. 2003). In an other study, simultaneous administration of EcN and *Salmonella* to piglets resulted in up to 70 % decrease of *Salmonella* invasion (Altenhoefer *et al*. 2004). Although the antagonistic mechanisms of EcN were not clearly defined in these studies, it was suggested that this probiotic was a better coloniser of the gut that most of the enteropathogens.

Besides therapeutic and protective effects, EcN was found to demonstrate good safety aspects as a suitable candidate carrier for bioactive molecules into the human body (Westendorf *et al.* 2005). As a result EcN recombinants carrying anti-HIV microbicides (Rao *et al*. 2005), insulinotropic proteins (Duan *et al.* 2008), human α-defensin (Seo *et al*. 2012), and epidermal growth factors (Choi *et al*. 2012) have been constructed with the intention to improve human health.

1.6 Characteristics of *E. coli* Nissle 1917 with relevance to probiotic effects

As mentioned earlier in this chapter, EcN was discovered almost a century ago and has been in use for decades. However, it was not until recently that some of genetic and phenotypic characteristics of EcN were determined with respect to probiotic properties. EcN has now been thoroughly characterised using biochemical, microbiological, and

molecular genetic approaches (Blum *et al*. 1995, Blum-Oehler *et al.* 2003, Grozdanov *et al*. 2002, Grozdanov *et al*. 2004, Sun *et al*. 2005, Hancock *et al*. 2010b), and has demonstrated the absence of virulence factors (Grozdanov *et al* 2004, Sun *et al*. 2005). Moreover, a range of strain-specific attributes contributing to EcN survival and colonisation of the gut have been determined. These include production of microcins (Patzer *et al*. 2003), and various siderophore-mediated iron uptake systems (Grosse *et al.* 2006, Vassiliadis *et al*. 2010). Furthermore, EcN was found to express a special lipopolysaccharide (LPS) structure that is distinct from all other known LPS in *E. coli* (Grozadanov *et al*. 2002). The LPS of EcN is unique due to its O6 polysaccharide side-chain that was found to be very short, consisting of only one 're eating unit' of the oligosaccharide of the O6 antigen (Grozadanov *et al*. 2002). This unique LPS has been found to be res onsi ble for this strain's serum sensitivity, and has been suggested to contribute to its immunomodulating properties (Sonnenborn and Schulze 2009, Güttsches *et al*. 2012).

Despite growing evidence of clinical efficacy of EcN, little is known regarding the mechanisms underlying the proposed effects of this probiotic species or its interactions with the human host. Elucidation of these mechanisms would provide a greater understanding of the impact and functioning of probiotic bacteria, as well as the human gut microbiota in general. Such information would facilitate the rational design of the efficient probiotic interventions to promote life-long health and wellbeing.

1.7 Aims and objectives

The overall aim of this study was to elucidate the mechanisms underlying interaction of probiotic *E. coli* strain Nissle 1917 with the host intestinal cells and its probiotic effect.

Specific objectives were to:

- Evaluate and optimise transposon-based mutagenesis systems for *E. coli* Nissle 1917 and generate mutants deficient in traits relevant to host-microbe interaction, and colonisation of the intestinal tract.

- Optimise *in vitro* co-culture model of the human intestinal Caco-2 cell and use this to investigate the effect of *E. coli* Nissle 1917cell surface structures on adherence, internalisation and cell health.

- Investigate the effect of *E. coli* Nissle 1917 on neurotransmitter synthesis and metabolism by mouse intestinal mucosal cells.

Chapter 2: General materials and methods

2.1 General microbiological methods

2.1.1 Culture and manipulation of bacterial strains

Bacterial strains (Table 2.1) were typically grown in 5-15 mL Luria-Bertani (LB) broth (Oxoid, Basingstoke, UK) composed of tryptone (pancreatic digest of casein) 10 g/L, yeast extract 5 g/L and sodium chloride 10 g/L (Miller 1987) with shaking (150 rpm) at 37°C unless otherwise stated. Technical grade agar (Oxoid, Basingstoke, UK) was added (1.5 % w/v) when solid media were required. Sterile 50 mL polypropylene tubes (Alpha Laboratories, Eastleigh, UK) and 90 mm Petri-dishes (Scientific Laboratory Supplies, Hessle, UK) were used for the growth of liquid and colony cultures, respectively. The isolation of single colonies was performed by streaking bacterial cultures on LB agar and these were used to produce liquid cultures. Bacterial strains harbouring plasmids, or transposon insertions with selectable markers, were cultured on media supplemented with appropriate antibiotic selection unless otherwise stated. The antibiotic selection used for each plasmid and transposon are summarised in Table 2.2. Other general reagents were obtained from Fisher Scientific (Loughborough, UK).

Table 2.1: List of bacterial strains used in this study

Bacterial strains	Comments/use	Source
E. coli Nissle 1917	Wild-type probiotic strain used as an active component of the microbial drug Mutaflor®	Ardeypharm GmbH, Herdecke, Germany
E. coli K12 MG1655	Wild-type, commensal non-pathogenic *E. coli* of the human intestinal tract	Blattner *et al*. (1997)
E. coli EPI300 ™	Standard strain for cloning and propagation of plasmid vectors	Epicentre Technologies Corp., Madison, USA
E. coli JM109	Host for transformation of pGEM® vectors and for production of single-stranded DNA from M13 or phagemid vectors	Promega Ltd., Southampton, UK
E. coli DH5α	Standard strain for gene cloning and protein production	Stratagene Ltd., Cambridge, UK
E. coli S17.1	Standard strain for cloning and propagation of plasmid vectors. Also a λpir lysogen, producing π protein, which allows replication of plasmids with R6K origin of replication	Microscience Ltd., Warrington, UK
E. coli BW29427	Standard strain for cloning and propagation of plasmid vectors. It carries :: *pir* and is a diaminopimelic acid (DAP) auxotroph - The *pir* gene provides π protein that allows replication of plasmids with R6K origin of replication - Auxotrophy to DAP allows counter selection when the strain is used in conjugation	Datsenko, K.A. and Wanner, B. L., Purdue University

Table 2.2: Antibiotic selection used for cultivation of *E. coli* strains carrying plasmid vectors or transposons

Plasmid vector	Antibiotic selection [*]	Main features / uses	Source
pUC19	Am 100 µ g/mL	Commonly used plasmid cloning vector in *E. coli*	Yanisch-Perron *et al.* (1985)
pUTMini-Tn*5*Km2	[¥]Km 30 µg/mL, [≠]Am 100 µ g/mL	Suicide delivery vector pUT, carrying mini-Tn*5*Km2, *ori*R6K, *oriT*. Only replicates in *pir*+ host. Maintained in *E. coli* S17.1λ*pir*	de Lorenzo *et al*. (1990)
pLR27	[§]Km 30 µg/mL	Suicide delivery vector carrying mini-Tn*5,* hyperactive transposase*, ori*R6K, *oriT*. Only replicates in *pir*+ host. Maintained in *E. coli* BW29427, adiaminopimelicacid (dap) auxotroph	Larsen *et al*. (2002)
pLGBE	Tc 12 µg/mL	Carries bacterio hag e red λ recombination functions (*bet*, *exo, gam*) for gene deletion/replacement	Bloor and Cranenburgh (2006)
pTOPO-DifCAT	Cm 20 µg/mL	Provides target specific cassette (*dif-cat-dif*) for gene deletion. The cassette containing *dif* sites, substrates for native Xer recombinases that excise the antibiotic resistance gene (cat) resulting in markeless mutation	Bloor and Cranenburgh (2006)

[*] The antibiotics used were obtained from Sigma, UK. Km: kanamycin, Cm: chloramphenicol, Amp: ampicillin, Tc: tetracycline.

[¥] selection for mini-Tn*5*Km2 transposon, [≠] selection for vector pUT, [§] selection for both plasmid vector and mini-Tn*5* in pRL27.

2.1.2 Storage of bacterial strains

Fresh single colonies from bacteria growing on agar plates were used to establish stock cultures. The colonies were harvested with a sterile cotton swab and resuspended in LB medium supplemented with dimethyl sulfoxide (DMSO) at a final concentration of 8 %, in sterile 2 mL cryogenic tubes (Fisher Scientific, UK). Fresh colonies of transposon mutants were stored directly in 96-well microtiter polystyrene plates (Genetix, New Milton, UK) in 100 µL L broth e r well. The plates were sealed loosely in plastic bags then incubated at 37°C overnight. Cultures were then su l emented with an additional 50 µL of sterile 30 % glycerol making a final volume of 150 µL with a final concentration of 10 % glycerol. The plates were then stored at - 80°C.

2.1.3 Enumeration of bacterial cells

Bacterial cultures were grown in liquid media then serially diluted in phosphate buffered saline (PBS, Sigma) composed of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4 ; pH 7.4. Dilutions of the original culture ranging from 10^0 to 10^{-7} were established in 1.5 mL sterile Eppendorf tubes (Fisher Scientific) and aliquots (100 µL) of these were spread on LB agar plates supplemented with the appropriate antibiotic when required. Plating was carried out in triplicate for each dilution. Agar plates were then incubated at 37°C overnight to allow bacterial growth and colony formation. Colony counts were performed on all replicates from a dilution yielding between 30 and 100 colonies. The mean of the three replicate values was used to calculate the colony forming units per 1 mL (Cfu/mL) present in the original culture.

Cfu/mL = (number of colonies x dilution factor) / volume of culture per plate

2.1.4 Spectrophotometric determination of bacterial growth

Growth of bacterial strains in liquid media was indirectly measured or monitored over a period of time using a spectrophotometer at a wavelength of 600 nm (OD_{600}). Cultures

grown in volumes of 5 mL and above, were diluted (if required) at 1:10 to 1:5 in fresh growth media, then 1mL of each culture was transferred in polystyrene micro cuvette (1.5 mL capacity, 10 mm light path; Fisher Scientific). The OD_{600} then measured using a Jenway 6300 model spectrophotometer (Jenway Ltd., Dunmow, UK).The spectrophotometer was blanked using uninoculated growth medium. The total OD_{600} per culture was calculated as OD_{600} readings per sample x dilution factor. Growth of cultures incubated in 96-well plates was monitored using a Biochrom Asys UVM340 microplate reader (Biochrom Ltd., Cambridge, UK).

2.1.5 Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of various antimicrobial compounds that were tested against strains of EcN was assessed using a modified protocol of the standard method for determining MICs as described by Andrews (2001).

2.1.5.1 Determination of MIC in liquid media

Doubling dilutions of compounds being tested were prepared in LB broth in a 96-well plate. The wells were inoculated with an overnight starter culture of the test strain at a ratio of 1:1,000. After inoculation, plates were incubated between 12 and 24 hours at 37°C without shaking, and bacterial growth was determined using a spectrophotometer at 600 nm. Controls were included in all the experiments. These comprised: (i) uninoculated media without the compound being tested, (ii) uninoculated media with the compound for each concentration tested, (iii) inoculated media without the compound. The MIC-value was taken as the lowest concentration of the antimicrobial compound that inhibited detectable growth. For each compound tested, the detectable growth was defined based on OD_{600} readings from uninoculated media (but containing the compound being tested).

2.1.5.2 Determination of MIC on solid media

Doubling dilutions of compounds being tested were prepared in LB agar (cooled to 50°C). The medium was mixed gently by swirling then poured into plates and allowed to set at room temperature for 20 minutes. An aliquot of ~ 2µL of the overnight starter culture was spotted on each of the agar plates, containing various concentrations of the compound being tested. The inoculated plates were then incubated between 12 and 24 hours at 37°C, and the bacterial growth was detected by visualisation of colonies on the agar. Controls were included in all the experiments as stated in Section 2.1 5.1. The MIC-value was taken as the lowest concentration of the antimicrobial compound that inhibited the visible growth of the test strain (colony formation on agar).

2.1.6 Statistical analysis and reproducibility

Statistical analysis was performed using Microsoft Office Excel 2010 and Minitab 16 (Minitab Inc., PA, USA). All experiments were performed at least in triplicate, and the data were presented as the mean values from independent experiments. Where appropriate the standard error of the mean was also given. Significant differences between means were assessed using two- sample t-tests, or ANOVA when comparing three or more groups of samples, at the 95 % confidence interval where appropriate. The null hypothesis (*Ho*) for t-tests was of no significant difference between means tested. For ANOVA, *Ho* was that all group means being compared are equal.

2.2 General molecular biology methods

2.2.1 Separation of DNA fragments by gel electrophoresis

Fragments of DNA were analysed using agarose gel electrophoresis according to standard protocols (Sambrook and Russell 2001). Separation of fragments was conducted using the Fisher VG-SYS Vari-gel MAXI (Fisher Scientific) or the Biorad Mini-Sub® Cell GT (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) electrophoresis cells, which were run by the PowerPack 300 power supply (Bio-Rad). Tris-acetate-EDTA (TAE) buffer (Fisher Scientific) was used both as a running buffer and in the agarose (Fisher Scientific) gel at 1 x TAE working concentration (40 mM Tris-acetate, 1 mM EDTA; pH 8.3). The agarose gel was prepared according to expected sizes of the fragments being separated. Fragments > ~ 7 kb were separated using 1 % (w/v) agarose, those < ~ 7 kb, 1.5 % (w/v) agarose and 2 % (w/v) agarose for those < ~ 0.5 kb. A voltage of 50-100 V was applied. The gel was subsequently stained with ethidium bromide (EtBr) (Sigma-Aldrich) in TAE buffer at a final concentration of 0.5 µg/mL Et r for 15-20 minutes at room temperature (RT). DNA fragments were then visualised and photographed over UV light, using an Ingenious Gel Documentation System (Syngene, Cambridge, UK). A 1 kb molecular size standard (Promega, Southampton, UK) was included in all gels.

2.2.2 Measurement of DNA concentration

The concentration of DNA r e a rations was routinely measured using a NanoDro ™ spectrophotometer (Thermo Fisher Scientific). The spectrophotometer was first set against a standard blank consisting of the vehicle solution with no DNA. A 1.2 -µL aliquot of the appropriate DNA solution was pipetted onto the bottom pedestal of the spectrophotometer, and the DNA concentration was measured based on absorbance at 260 nm. The ratio of absorbance at 260 and 280 nm was automatically generated and used to assess the purity of the DNA. A ratio of 1.8-2.1 was generally acce t ed as " u re".

2.2.3 Standard protocol for the polymerase chain reaction (PCR)

Reaction mixes were transferred to thin walled, flat capped 0.2 mL PCR tubes (VWR International Ltd., Lutterworth, UK) and DNA amplification was carried out in a PTC-200 thermal cycler (MJ Research Inc, Watertown, Massachusetts, USA) or *i*Cycler 1x96 Well Reaction model (Bio-Rad). *Taq* polymerase enzyme, PCR buffers and solutions from the Qiagen *Taq* PCR Core Kit (Qiagen Ltd., Manchester, UK) were used. Primers were obtained from Eurofins MWG/Operon (London, UK). Forward and reverse sets of primers were combined and diluted to create stock solutions at a concentration of 10 pmol per primer; 1:1 forward: reverse ratio.

Each 25 µL PCR mix typically contained 5 µL 5 x Q solution, 2.5 µL 10 x buffer (with 15 mM $MgCl_2$, giving 1.5 mM $MgCl_2$ in the PCR reaction), 1.5 µL r imer mix (10 mo l of each r imer), 0.5 µL of a 1 mM deoxynucleoside triphosphates (dNTPs), and 0.2 µL Taq (5 U/µL, 0.04 U e r reaction). If required the PC was o ti mised by adding more $MgCl_2$ to the mixture or the substitution of the Q solution by nuclease free, sterile deionised water (SDW) before reactions were brought to a final volume of 24 µL with SDW. One µL of appropriately diluted template DNA (1-50 ng) was added to the final PCR mix. All PCR experiments included a negative control reaction (containing all other ingredients except the template DNA, substituted by SDW) in order to confirm purity of the reagents. Where feasible, a positive control reaction was added. This contained template DNA known to produce a specific product with the set of primers being used.

PCRs were routinely run using the following cycling conditions:

Initial denaturation, 95°C for 5 minutes
30 cycles of:
Primer specific anneal temperature for 45 seconds
72°C for 1 minute
94°C for 30 seconds
Final extension, 72°C for 5 minutes
Final hold, 4°C.

For longer PCR products the extension time was increased by 1 minute per 1 kb DNA fragment. The primer specific annealing temperature was empirically determined in a gradient PCR using the Biorad *i*Cycler 1 x 96 Well Reaction model thermocycler.

2.2.4 Restriction digest of DNA

DNA samples were digested with restriction endonucleases obtained from Promega (Southampton, UK) according to the manufacturer's instructions. If required, the suitable enzymes providing the desired digest fragments were initially selected using virtual digestion of the DNA sequence using pDRAW32 software (http://www.acaclone.com). A ty ica l restriction digest reaction consisted of 2 µL of 10 x enzyme restriction buffer, 0.2 µL of acetylated bovine serum albumin (BSA) 10 mg/mL, 0.5 µL of restriction endonuclease (5 units of restriction enzyme per reaction), and 0.2– 2.5 µg of substrate DNA in a volume ≤ 10 µL. SDW was added to give a final volume of 20 µL. Reagents were mixed thoroughly, and digests incubated for 16 hours at 37°C. Products of digestion were visualised by gel electrophoresis.

2.2.5 Purification of DNA products

Purification of DNA fragments (under 10 kb) from PCR products, restriction digests and vectors was carried out using the QIAquick gel extraction kit (Qiagen). The purification procedure was carried out according to the instructions provided by the manufacturer, using the Heraeus Pico17 microcentrifuge (Thermo Fisher Scientific) at 17,000 *g*. The purified DNA products were eluted in 30-50 µL of nuclease free SDW and were subsequently stored at -20°C.

2.2.6 Isolation of plasmids from bacterial cultures

Plasmid preparation from *E. coli* strains was carried out using the Qiagen QIAprep Spin Miniprep kit (Qiagen). Cells from 5 mL of overnight cultures were harvested by

centrifugation using the Heraeus Labofuge® 400 R model centrifuge (Thermo Fisher Scientific) (1,500 *g*) for 10 minutes RT. The supernatant was removed and the pellet resuspended in 250 µL buffer P1 (suspension buffer provided with the Qiagen Miniprep Kit) then transferred to a 1.5 mL sterile Eppendorf tube. Further steps of plasmid preparation were then carried out according to the manufacturer's instructions. The centrifugation steps performed during the protocol were carried out at 17,000 *g* using a Heraeus Pico17 microcentrifuge. All plasmids were eluted in 30-50 µL of nuclease free SDW and were stored at -20°C.

2.2.7 Isolation of genomic DNA from bacterial cultures

Genomic DNA was isolated from *E. coli* cultures by a mini-prep protocol based on the chloroform extraction method (Sambrook and Russell 2001). Cells from overnight LB broth cultures (1.5 mL) were transferred to 2 mL sterile Eppendorf tubes and harvested by centrifugation (17,000 *g* for 1 minute). The supernatant was discarded and pellet resus ende d in 200 µL of $T_{10}E_{10}$ buffer (10 mM Tris-Cl, 10 mM EDTA; pH 8) then mixed with 900 µL lysis buffer (1 % w/v sodium dodecyl sulfate, 50 mM Tris-Cl, 50 mM EDTA; pH 8) and incubated at RT with gentle rocking overnight.

After lysis, cellular debris and r oteins were reci i tated by the addition of 200 µL saturated ammonium acetate (Sigma-Aldrich) and vigorous shaking. The DNA was then extracted by adding 500 µL of chloroform (Thermo Fisher Scientific), vigorous shaking of the tubes and 2 minutes centrifugation at 17,000 *g*. The upper aqueous layer containing the DNA was transferred to a high yield 1.5 mL sterile tube and DNA precipitation was carried out by adding 3 volumes of ice-cold 100 % ethanol (Thermo Fisher Scientific). The tubes were inverted gently then incubated at -20°C for at least 1 hour to allow further precipitation of the DNA.

The DNA was subsequently harvested by centrifugation at 17,000 *g* for 25 minutes RT and was washed twice in 500 µL 70 % ethanol, and dried for 15 minutes at RT. The dried pellet was then resus ended in 50 µL of nuclease free SDW and stored at -20°C until required.

2.2.8 Extraction of total DNA from bacterial cultures by colony boiling

Crude extracts of total DNA (genomic and plasmid) for use in PCRs were prepared from *E. coli* fresh cultures by the colony boiling method as described by Moore *et al*. (1999). Freshly grown single colonies were picked from an agar plate using a sterile pipette tip toothpick or inoculating loop then transferred to sterile 1.5 mL Eppendorf tube containing 50 µL sterile T_{10} E_{10} buffer. The cell suspension was placed in a water bath at 95°C for 20 minutes. The obtained cell lysate was allowed to cool for 15 minutes at RT. The supernatant containing the DNA was then collected by centrifugation (17,000 *g*, 1 minute) and transferred to a fresh sterile tube, and subsequently used.

2.2.9 Generation of chemically competent *E. coli* cells

Generation of chemically competent cells (CCCs) from *E. coli* strains was conducted using the conventional calcium chloride method (Sambrook and Russell 2001). Cultures were grown in 5 mL LB broth in 50 mL polypropylene tubes overnight at 37°C, with shaking. Cultures were then cooled by placing the tubes on ice for 30 minutes. The cells were harvested by centrifugation at 4°C (1,500 *g*) for 10 minutes. The pellets were resuspended by swirling in 2 mL of ice-cold sterile 0.1M $CaCl_2$ solution (Sigma-Aldrich) then chilled on ice for 5 minutes and pelleted again at 1,500 *g* for 5 minutes at 4°C. The $CaCl_2$ treatment was repeated twice and the resulting CCCs were resuspended in 100 µL of the $CaCl_2$ solution. The 100 µL individual aliquot was used directly, or supplemented with 10 % glycerol then stored at -80°C until required.

2.2.10 Generation of electro - competent *E. coli* cells

Electro-competent *E. coli* were generated as described in Section 2.2.9**.** However, cells were treated with an ice-cold 10 % (v/v) glycerol solution instead of 0.1 M $CaCl_2$ solution (Sambrook and Russell 2001). Aliquots of electro-competent cells were directly used or snap frozen in liquid nitrogen then kept at -80°C until required.

2.2.11 Transfer of plasmid DNA to *E. coli* strains by chemical transformation

Plasmid DNA was transferred to chemically competent cells (CCCs) of *E. coli* by standard heat-shock transformation method (Sambrook and Russell 2001). An aliquot of CCC suspension (100 µL) was transferred into a pre-chilled sterile 1.5 mL Eppendorf tube containing 50 ng of the appropriate plasmid DNA, in a volume of 5 µL, and mixed gently by tapping 5 times then incubated on ice for 30 minutes. The tubes were transferred to a preheated water bath at 42°C and the cells heat-shocked for 50 seconds, then incubated on ice for 2 minutes before the addition of 900 µL of super optimal broth with catabolic repressor (SOC) medium (20 mM glucose, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 2.5 mM KCl, 10 mM NaCl, 20 g/L tryptone and 5g/L yeast extract). The tube contents were then transferred to a sterile 15 mL polypropylene tube (Alpha Laboratories, Eastleigh, UK) and incubated at 37°C for 1 hour, with shaking to allow cell recovery and expression of selectable markers. The transformants were identified by spreading the cultures over LB agar plates containing the required antibiotic selection (Table 2.2).

2.2.12 Transfer of plasmid DNA to *E. coli* strains by electroporation

Electro-competent cells of *E. coli* strains were electroporated using a Gene Pulser Xcell Electroporation System (Bio-Rad). An aliquot of electro-competent cells (100 µL) was mixed with ~ 500 ng of the appropriate plasmid DNA (in a volume of < 5 µL) then transferred into a prechilled electroporation cuvette (0.1 cm gap, Bio-Rad). The contents of the cuvettes were electroporated at 1.8 kV, 200 Ω resistance, and 25 µF capacitance. Sterile SOC broth (900 µL) was immediately added to the cuvette contents, which were then transferred to a sterile 15 mL tube, and incubated at 37°C for 1 hour with shaking. The transformants were recovered by spreading the cultures over LB agar plates containing the required antibiotic selection (Table 2.2).

2.2.13 Transfer of plasmids to *E. coli* by conjugal transfer

Plasmid vector transfer to *E. coli* strains by conjugation depended upon the availability of counter-selection strategies allowing the elimination of the donor strain. Biparental matings were set up between, the recipient strain *E. coli* Nissle 1917 and donor *E. coli* BW29427, a diaminopimelic acid (DAP) auxotrophic strain carrying the plasmid to be transferred. Agar surface matings were performed according to a modified protocol of the method used by Lewenza *et al*. (1999). Cultures of the recipient and donor strains were grown separately in LB broth overnight. The growth medium of donor BW29427 was supplemented with 0.1 mM DAP and 30 µg/mL kanamycin.

An aliquot (1mL) of each bacterial strain's overnight culture was transferred to a sterile 1.5 mL Eppendorf tube and the cells were recovered by centrifugation (10,000 *g*, 1 minute). The supernatants were discarded and the pellets were resuspended in sterile 1 mL PBS. The donor cell suspension was washed twice with PBS to remove the antibiotic then concentrated into a final volume of 100 µL in P S. An aliquot (100 µL) of the recipient strain (EcN) cell suspension was mixed with the concentrated donor suspension and

centrifuged at 10,000 *g* for 1 minute. The supernatant was discarded, and the mixed cells were resuspended in 100 μL PBS. This final suspension was dropped on LB agar containing 10 mM $MgSO_4$ and 0.1 mM DAP. Drops were allowed to dry at RT and the agar plate was incubated overnight at 37°C.

After incubation, cells were then recovered from the agar surface using a sterile cotton swab, resuspended in 1mL sterile PBS, serially diluted in PBS, and spread over selective LB agar. This contained 50 μg/mL kanamycin but without DAP in order to eliminate the auxotrophic donor.

2.2.14 DNA Cloning

DNA fragments were cloned into a pGEM-T vector (pGEM®-T Easy Vector Systems, Promega) according to the manufacturer's instructions. The PC fragments being cloned were first purified using a QIAquick gel extraction kit (Qiagen) as described in Section 2.2.5, then dissolved in nuclease-free SDW. Ligation reaction consisted of: 1 μL T4 DNA ligase (3 units of the ligase), a linearized pGEM-T Easy fragment (50 ng, in 1 μL solution), and the appropriate amount of the target PCR product in a 3:1 (insert: vector) molar ratio, 5 μL of 2 X rapid ligation buffer, and nuclease-free water to a final volume of 10 μL. The reaction was performed in 0.2 mL PCR tubes, and was incubated at 4°C overnight. Ligations were transformed into *E. coli* JM109 electro-competent cells by electroporation (Section 2.2.12).

Successful pGEM-T clones containing the target insert (generally white colonies) were selected on LB agar supplemented with 100 μg/mL ampicillin, 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 80 μg/mL 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal). A sample of white colonies were picked at random and grown in liquid cultures. These were used to recover the plasmid. A DNA sample was taken from

the purified plasmid then digested with *EcoR*I, releasing the insert from the pGEM-T vector. Products of digestion were visualised by gel electrophoresis to confirm the presence of the insert before the clones were sequenced using T7 and SP6 vector primers.

2.3 Southern hybridisation

Southern hybridisation is a fundamental technique in molecular genetics. It is mainly used to detect and locate a specific nucleotide sequence in a large pool of DNA (Southern, 1975). The main steps involved in this technique include transfer of separated DNA fragments to a membrane and fragment detection by probe hybridisation as shown in Figure 2.1.

2.3.1 Transfer of DNA to positively charged membranes (squash blot)

Fragments obtained from genomic DNA digests were separated according to size by agarose gel electrophoresis (Section 2.2.1**)**. The squash blot was assembled and DNA fragments were transferred onto a positively charged nylon membrane (GE Healthcare, Little Chalfont, UK). Gels were first rinsed with T_1E_1 buffer (1 mM Tris-Cl, 1 mM EDTA; pH 8), and the unused portions were trimmed off, along with one corner to mark the orientation of the samples. Gels were subsequently incubated in 200 mL denaturing solution (0.5 M NaOH, 1.5 M NaCl) at room temperature with gentle rocking for 20 minutes.

Four pieces of quick draw paper (Sigma-Aldrich) were carefully cut to the same size as the gel then wetted by immersion in denaturing buffer. Three of these were placed in a suitable plastic tray that was subsequently used for the blot assembly. Gels were laid (face down) on top of the blotting paper inside the tray. A positively charged nylon membrane (GE Healthcare) fitting the size of the gel was labelled on one side (DNA side) and was subsequently placed on top of the gel. The fourth piece of the quick draw paper was laid on

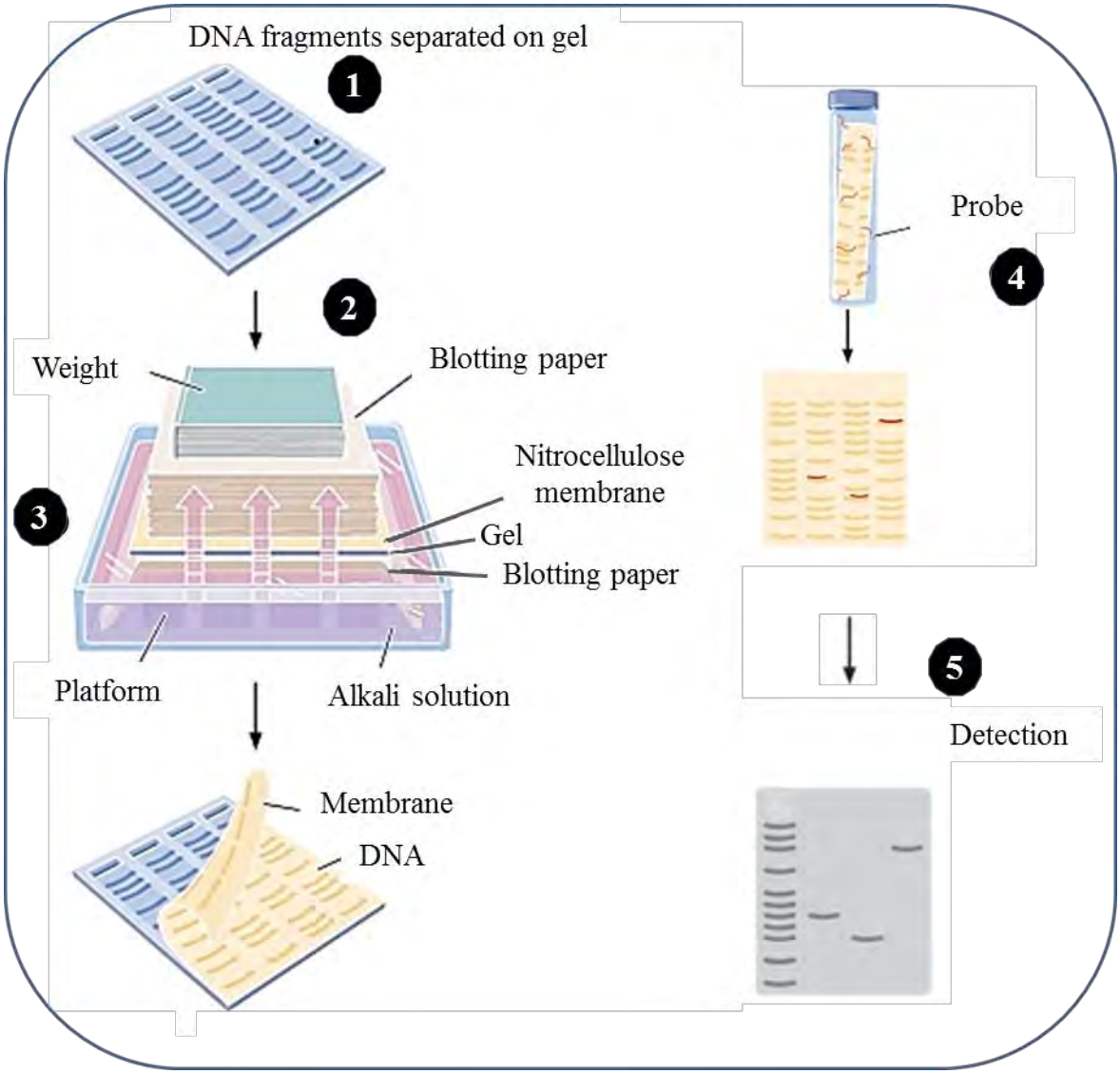


Figure 2.1: Southern blotting and hybridisation

Major steps include 1: DNA digest and separation on agarose gel, 2: Squash blot, 3: DNA transfer onto the membrane, 4: Hybridisation, 5: Detection of the bound probe.

The diagram was adapted from Pierce (2005).

top of the membrane, five additional layers (fitting the size of the gel) were positioned on top. The membrane and blotting papers were carefully handled using blunt-ended forceps in order to avoid contaminations and air bubbles between layers. More denaturing buffer was added into the bottom of the tray and a weight of about 2 kg was carefully positioned on top of the squash blot. The completed blot was incubated for 12 hours at room temperature, to allow the transfer of the DNA fragments onto the membrane. After the incubation, the nylon membrane was carefully removed from the gel then placed in equilibration buffer (0.5 M Tris-Cl, 1.5 M NaCl; pH8) for 5 minutes with gentle rocking, and rinsed with 2 x saline-sodium citrate (SSC) buffer (17.53 g/L NaCl, 8.82 g/L sodium citrate; pH 7.0). The membrane was then exposed to UV light (254 nm) for 3 minutes to allow fixation of the DNA.

2.3.2 Generation of digoxygenin labelled nucleic acid probes

Probes were labelled with digoxygenin (DIG; Roche, Burgess Hill, UK). Probes were generated by the standard PCR technique (Section 2.2.3), using primers specific to the DNA sequence of interest and DIG-labelled dNTPs (Roche) instead of the standard dNTPs. The PCR products obtained, the DIG-labelled probes were first visualised by gel electrophoresis to confirm that the PCR was successful and to verify the size of the DNA fragment. The PCR products were then purified using the QIAquick purification Kit (Section 2.2.5) and used directly or stored at -20°C, until required.

2.3.3 Hybridisation of digoxygenin-labelled probes

The DNA (fixed onto the nylon membrane) was probed using the generated DIG-labelled probes (specific to the sequence of interest). Membranes were immersed in T_1E_1 buffer then transferred to hybridisation tubes (Fisher Scientific), with the DNA side facing the interior of the tube. Hybridisation buffer, DIG Easy Hyb buffer (15 mL, Roche) was then added to the tubes. An aliquot (10 μL) of the DIG-labelled probe (described in Section

2.3.2) was mixed with 500 µL of the DIG Easy Hyb buffer, then transferred to the hybridisation tube. The tube was incubated overnight in a Robin Scientific Roller Oven, with continuous rotation at a probe-specific hybridisation temperature (HT). The HT was calculated according to the GC content and the percentage homology of probe to target using the following equation:

$$HT = Tm - 20\,^{O}C$$

$$Tm = 49.82 + 0.41\,(G+C\,\%) - (600/L)$$

$$HT = Tm - 20°C$$

$$Tm = 49.82 + (0.41 \times G+C\,\%) - (600/L)$$

Where Tm = Melting temperature, G+C% = G+C content of probe, and L = Probe length.

2.3.4 Chemiluminescent detection of digoxygenin labelled probes

The detection of the DIG-labelled probes, bound to homologous target sequences on nylon membranes, was carried out using a DIG chemiluminescent detection kit (Roche). The kit included anti-DIG antibodies conjugated to the enzyme alkaline phosphotase (anti-DIG-AP fragments), alkaline phosphatase substrate chloro-5-substituted adamantyl-1,2-dioxetane phosphate (CSPD) to produce a light signal, and membrane blocking reagent. Blocking solution, washing buffer and antibody solution were prepared using maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl; pH7.5).

After probe hybridisation (Section 2.3.3), the nylon membrane was kept in the hybridisation tube then washed at a temperature 10°C above the hybridisation temperature, to remove the unbound probes. The washing was first done twice for 15 minutes in 20 mL washing solution (17.53 g/L NaCl, 8.82 g/L sodium citrate, 0.1 % w/v SDS), then twice for

15 minutes in 20 mL of 0.1 x washing solution (0.877 g/L NaCl, 0.441 g/L sodium citrate, 0.1 % w/v SDS).

Membranes were subsequently rinsed in 100 mL washing buffer (0.1 M maleic acid, 0.15 M NaCl; pH7.5, 0.3 % w/v Tween 20) for 1 minute at RT, then incubated in 100 mL blocking solution (maleic acid buffer, 10 % w/v blocking reagent, Roche) for 30 minutes at RT with gentle rocking. After blocking, the membrane was incubated for 30 minutes in antibody solution (20 mL) at RT with gentle rocking. The antibody solution consisted of anti-DIG-AP fragments (Roche) diluted 1:10,000 in blocking solution. The membrane was then washed twice for 15 minutes in washing buffer and equilibrated by immersion in 20 mL of detection buffer (0.1 M Tris-HCl, 0.1 M NaCl; pH 9.5) for 5 minutes.

2.3. 5 Visualisation

The equilibrated wet membrane was transferred onto an acetate sheet and placed DNA side up. The CSPD substrate, 1 mL (diluted at 1:100 in detection buffer) was distributed over the membrane, which was immediately covered with the second acetate sheet. The membrane was then sealed using cling film and incubated for 1 hour at 37°C, in the dark to allow antibody-substrate reaction to take place. The membrane was finally visualised using the Xenogen *in vivo* imaging system 50 (IVIS 50) (Xenogen, Hopkinton, MA), with an integration time of 1 minute. Overlay images and luminescence measurements were made using Living Image software (version 2.50.1; Xenogen).

2.4 General tissue culture methods

2.4.1 Cell lines and culture conditions

The human adenocarcinoma (Caco-2) cells (Fogh *et al*. 1977, Pinto *et al.* 1983) were obtained from LGC standards (Middlesex, UK) and were used between passage number 55- 85. Cells were seeded in T75 flasks (75 cm^2 growth surface area) and grown at 37°C in a humidified atmosphere of 5 % CO_2 in Dulbecco's modified Eagle's medium-high glucose (DMEM, 4.5 g glucose /L), supplemented with 10 % fetal bovine serum (FBS) and 1x non-essential amino acids (NEAA). This formulation is referred to as "com l ete growth medium". Cell culture media and supplements were purchased from PAA Laboratories (Somerset, UK); flasks, plates, tubes and other reagents were obtained from Fisher Scientific, UK unless otherwise specified.

Cells were passaged upon reaching ~ 80 % confluence in T75 flask. The medium was removed from the flask and the monolayer was washed with sterile hos ha te-buffered saline (P S). The cells were then detached from the flask surface by the addition of 3 mL of try sin -EDTA solution (0.05 % w/v trypsin/0.5 mM EDTA), followed by incubation for 7 minutes at 37°C. After this incubation, the trypsin was neutralised by the addition of 10 mL complete growth medium. The detached cells were then transferred to a 20 mL sterile universal tube then centrifuged at 500 g for 5 minutes. The supernatant was discarded and the pellet was resuspended in 1 mL complete growth medium and subsequently distributed into fresh flasks. Caco-2 cells were routinely seeded into new flasks at a 1:8 ratio, which required 4 days to form a monolayer (~ 80 % confluence). The complete growth medium was replaced once every 2 days.

2.4.2 Storage and thawing of cells

Long-term storage of cells was achieved by cryogenic preservation in liquid nitrogen at - 130°C or lower. Caco-2 cells were grown in T75 flask to reach ~ 80 % confluence then trypsinised as previously described in Section 2.4.1. The cells from the entire flask were resuspended in 4 mL of complete growth medium containing 5 % (v/v) DMSO. Aliquots (1 mL) of the cell suspension were transferred in 2 mL sterile cryo-tubes. The cryo-tubes were put in a freezing pot, stored in polystyrene containers at -80°C for 48 hours then transferred to liquid nitrogen at -130°C until required.

To thaw the cells, the cryo-tubes were removed from storage and immediately placed in a water bath at 37°C. The thawed cell suspension (1 mL) was then transferred to a sterile 50 mL tube containing 15 mL of complete growth medium then to T75 flask and incubated at 37°C in a humidified atmosphere of 5 % CO_2.

2.4.3 Quantification of cell number by haemocytometer

The haemocytometer count was routinely used to determine the seeding density of cells before passaging. Suspensions of freshly trypsinized cultured cells were diluted with sterile PBS in 1.5 mL Eppendorf tubes and counted using a Neubauer haemocytometer. The haemocytometer and its cover slide were properly cleaned with 70 % ethanol then loaded with 10 µL (per chamber) of cell suspension using a pipette. Cells within the counting areas were manually counted using a microscope at 20-100X magnification. Cell counting was performed in at least in 4 counting squares (per sample) and the cell concentration was calculated using the following formula:

$$\textbf{Total cells/mL} = \frac{\text{Total cells counted x dilution factor x } 10^4 \text{ cells/mL}}{\text{\# of Squares}}$$

2.4.4 Assessment of cell viability by trypan blue exclusion

The number of viable cells was determined using the trypan blue exclusion method (Freshney 2000). Suspensions of freshly trypsinized cultured cells were diluted with sterile P S. An aliquot of r o e rly mixed cells (50 µL) was transferred to a sterile 1.5 mL Eppendorf tube and mixed with an equal volume of trypan blue solution (0.25 %, w/v in PBS). The resulting mixture was loaded onto a haemocytometer and the cells were observed using a microscope. The total number of cells was counted as well as the number of dead cells (stained blue). The number of viable cells was determined by calculating the difference between the total cell count and the number of dead cells.

2.4.5 Analysis of cell health by Hoechst propidium iodide (HPI) staining

Cell health (death and viability) of Caco-2 cells was investigated using the Hoechst 33342/ Propidium iodide (HPI) double fluorescence staining technique as described by Dive *et al*. (1992). Hoechst 33342 is a blue-fluorescence dye that binds the DNA of eukaryotic cells, staining the condensed chromatin of apoptotic cells more brightly than the chromatin of healthy normal cells (Elstein and Zucker 1994). The red-florescent propidium iodide (PI) dye only binds permanently to dead cells, staining the DNA of necrotic cells (Elstein and Zucker 1994, Darzynkiewicz *et al*. 1997).

The HPI staining solution was prepared complete growth medium containing 5 mg/mL Hoechst 33342 (Thermo Fisher) and 1 mg/mL propidium iodide (Sigma-Aldrich). Monolayers of Caco-2 cells were exposed to experimental conditions in 6-well plates. The medium was gently removed from wells which were then rinsed once with PBS. An aliquot (250 µL) of the HPI solution was distributed evenly over the cell monolayer in each well. Plates were immediately covered in foil then incubated for 5 minutes at room temperature in the dark. The stained cells were then visualised using the Zeiss AxioVert 25 inverted fluorescent microscope with imaging system.

2.4.6 Analysis of apoptosis by detection of caspase 3/7 activity

Activity of effector caspases, caspase-3 and -7 was measured in Caco-2 cells using the Caspase-Glo® 3/7 Assay (Promega Ltd., Southampton, UK) according to the manufacturer's instructions**.** The assay kit includes a luminogenic caspase-3/7 substrate containing the tetrapeptide sequence DEVD in a reagent which has been optimised for caspase activity, luciferase activity and cell lysis. Caco-2 cells were seeded in 96-well plates (5,000 cells/well in 100 µL complete growh medium) then grown at 37°C in 5% CO_2 for 60 hours to reach ~60 % confluence (~10,000 cells/well). For the first 48 hours, complete growth medium was used, and then replaced with serum–free growth medium for the rest of the incubation period. The medium was then removed from all the cells and wells were washed once with PBS followed by exposure to experimental conditions in serum-free serum–free growth medium (100 µL/well).

After the cells were exposed to experimental conditions, the Caspase-Glo® 3/7 reagent (100 µL) was added to each well representing experimental and control samples (each already containing 100 µL of medium). The contents of the plate were mixed gently using a microplate shaker at 300 rpm for 2 minutes then incubated in the dark for 1 hour at room temperature to allow the reactions to take place. Luminescence proportional to caspase 3/7 activity was measured as relative light units (RLUs) using a Synergy Multi-Mode Plate Reader (BioTek, Potton, UK) operated with BioTek Gen5.20 software. The results were plotted on a graph and caspase activities were compared between experimental and control samples.

Chapter 3: Development and optimisation of molecular genetic approaches to study host-microbe interactions in *E. coli* Nissle 1917

The use of genomic-based strategies and molecular tools in functional studies of bacteria has been proved to be efficient for the identification of mechanisms by which probiotics interact with the host (McAuliffe and Klaenhammer 2002, Callanan 2005, Klaenhammer *et al.* 2005, Salminen 2006, Amor *et al*. 2007, Lebeer *et al*. 2008). These tools have contributed towards the genetic dissection of the bacterial traits underlying virulence and beneficial effects on human health (Grozdanov *et al*. 2004, Sun *et al.* 2005, Vebø *et al*. 2010, Lukjancenko *et al.* 2012), and have also facilitated the identification of genes relevant to the probiotic effect. For instance, genes involved in survival of probiotic bacteria in the gut environment (Klaenhammer *et al*. 2005, Begley *al*. 2006, Whitehead *et al*. 2008); those essential for adherence to the host intestinal mucosa and its colonisation (Mack *et al* 2003, Pretzer *et al*. 2005, Lasaro *et al*. 2009, Troge *et al.* 2012); those enhancing human intestinal epithelial barrier function (Schlee *et al.* 2007, Schlee *et al.* 2008, Seth *et al*. 2008); and genes involved in modulation of host immune responses (Grangette *et al*. 2005, Iliev *et al*. 2005, Hafez *et al.* 2009), have all been explored using several molecular genetic approaches.

3.1 Genomic-based strategies to study the beneficial functions of *E. coli*

E. coli being the best studied and characterised microbial organism (Casali 2003, Hobman *et al*. 2007), offers many possibilities to analyse probiotic *E. coli* Nissle 1917 (EcN) at the genetic level using a broad range of molecular techniques. Suitable techniques for studying the beneficial properties of probiotics include comparative genomics, gene expression-based strategies, gene inactivation, and gene transfer strategies (McAuliffe and Klaenhammer 2002). These approaches are compared in Table 3.1 on the basis of their application in elucidating the genes underlying host-microbe interactions in EcN.

Table 3.1: Application of molecular genetic approaches to study probiotics properties relevant to the EcN interactions with host

Group	Advantages	Limitations	Reference
Comparative genomics	- No requirement for established molecular genetic systems - Applicable in preliminary studies predicting functions of genes from sequence data - Helpful for designing further functional studies	- Comparisons are limited by available sequence data and incomplete nature of public databases - Requires supplementary methods to verify functions of genes - Requires properly annotated genome of EcN which, is not yet available	McAuliffe and Klaenhammer (2002), Grozdanov *et al.* (2004), Sun *et al.* (2005)
Expression based methods (e.g. DNA microarray, quantitative RT-PCR, Western blotting)	- Real-time monitoring of gene expression which can help to determine functions of genes during host-probiotic interactions or when bacteria are exposed to various stimuli or conditions - Multiple genes expressed under a particular condition can be determined in single experiment - Can also detect genes downregulated in response to stimuli	- Requires supplementary methods to verify functions of genes - May require properly annotated genome of EcN, which is not yet available - Transcriptome data may not account for post-transcriptional regulation and modifications likely to affect rates of protein turnover - Lack of *in vitro* co-culture systems adapted for studies of host-probiotic interactions	Callanan (2005), Hancock *et al.* (2010b), Desmond *et al*. (2006)
Gene-transfer/genetic library-based approach (e.g. gene cloning)	- Potential discovery of genes underlying a given trait - Can facilitate confirmation of phenotype of interest and of gene identity by sequencing	- Laborious and reliant on availability of highly efficient cloning systems - Requires availability of suitable closely related, but innocuous secondary host strain to express genes encoded - Requires supplementary methods to verify functions of genes, including complementation approaches	Desmond *et al*. (2006), Sleator and Hill (2007)

Group	Advantages	Limitations	Reference
Directed mutation-based methods (e.g.. gene deletion, replacement)	- Can be used to confirm function of gene already identified	- Relies on availability of sequence data and clear knowledge of target gene - Many genes involved in probiotic interaction with hosts remain unknown	Ozimek *et al*. (2004), Primrose and Twyman (2006), Schlee *et al*. (2007)
Random mutation-based methods	**Use of mutagens** (e.g. chemical agent, UV, thermal stress) - Can be used to generate very large numbers of mutants quickly, without requiring a prerequisite knowledge of target genes	- Likely to cause multiple mutations within a single genome complicating determination of functions for individual genes - Identifying mutated gene difficult or impossible	Alberts *et al.* (2002), Lee and Salminen (2009)
	Transposons (Tn) (e.g. Tn*5*, Tn*10*) - Can reveal function of unexpected genes in phenotype related to probiotic effects - Does not require prerequisite knowledge of genes involved - Generally generate single and stable chromosomal mutation - Identification of gene disrupted is facilitated by transposon - Availability of Tn insertion systems adapted to *E. coli* strains hosts	- Preferential, non-random insertion may occur (hot-spots) - Requires supplementary methods to verify function of disrupted genes - Transfer of Tn system and isolation of mutants need optimisations which can be laborious or difficult	Lodge *et al*. (1988), Ito *et al.* (2010), Ruiz *et al.* (2013), Lasaro (2009), Lee and Salminen (2009)

Adapted from Hensel and Holden (1996)

Their advantages and limitations are also highlighted, considering that a complete and annotated genome sequence of this strain is not yet publicly available.

Gene inactivation strategies are quite useful for studying the genetic basis of particular probiotic effects as they can help to associate gene products with phenotypes. These strategies can be divided into two main categories, random mutagenesis and site-directed mutagenesis. Random mutagenesis holds an advantage over site-directed mutagenesis and the rest of the molecular techniques due to its ability to reveal novel genes associated with a trait of interest, without requiring prior information about these genes. Random mutagenesis approaches have been employed to discover novel genes involved in probiotic effects in *Lactobacillus* (Ito *et al.* 2010), *Bifidobacterium* (Ruiz *et al.* 2013), and in EcN (Lasaro *et al*. 2009). Conversely, methods of site-directed mutagenesis require prior knowledge of the target gene, and are highly reliant on the availability of sequence data. Consequently, they are typically used to prove roles of the genes for which the functions are already predicted, or those highly likely to be involved in a given trait. Site-directed mutants have been successfully generated to confirm gene functions in antipathogenic and immunomodulatotory effects of probiotic *Lactobacillus* strains (Grangette *et al*. 2005, Corr *et al*. 2007, Dobson *et al.* 2007, Duncker *et al*. 2008) and EcN (Hafez *et al*. 2009).

3.1.1 Random mutagenesis-based methods

Random mutagenesis causes the disruption of non-specific genes. Mutants showing alterations in the phenotype of interest can then be identified (Hayes 2003, Lee and Salminen 2009). This approach requires the use of techniques facilitating the generation of large-scale library of mutants in order to cover a high number of possible mutations in the genome of the target bacteria. The larger the population size of randomly generated mutants, the higher the probability of inactivating any gene that might be associated with the trait of interest. Particularly, generation of a large mutant library can increase the

chances of discovering novel genes in mutants showing alterations in traits relevant to host-microbe interactions in probiotic bacteria. The isolation of such mutants is possible when the desired phenotype can be easily screened. Figure 3.1 displays basic principles involved in random mutagenesis of probiotic bacteria and the isolation of mutants.

The choice of a suitable random mutagenesis strategy for use in a particular bacterial strain, is crucial for the quality and characterisation of the mutants. There are two main random mutagenesis-based strategies, the use of chemical mutagens and transposons (Tn) (de Lorenzo and Timmis 1994, Hensel and Holden 1996, Camilli *et al*. 2001). Chemicals such as *N*-methyl-*N*-nitroso-*N*-guanidine have been used to promote mutagenesis in Gram-positive probiotic strains of *Lactobacillus* (Arsenijevic *et al*. 2000, Ibrahim *et al*. 2000, Grill *et al*. 2000) but have often shown major disadvantages of multiple mutations within a single genome. This can complicate the identification of individual genes responsible for the phenotype of interest. Another disadvantage of using chemical mutagens is that the mutation sites are not marked. This in turn, makes the identification of mutated genes more difficult, even in the case of a single disruption. This approach can be more useful if used in conjunction with next-generation sequencing (NGS) technologies for deep sequencing of mutant genomes, which may potentially permit identification of mutated genes, but this is still not straightforward. In contrast, transposons are capable of generating stable, single-gene disruption mutants, which are much easier to characterise (Camilli *et al*. 2001, Snyder and Champness 2003).

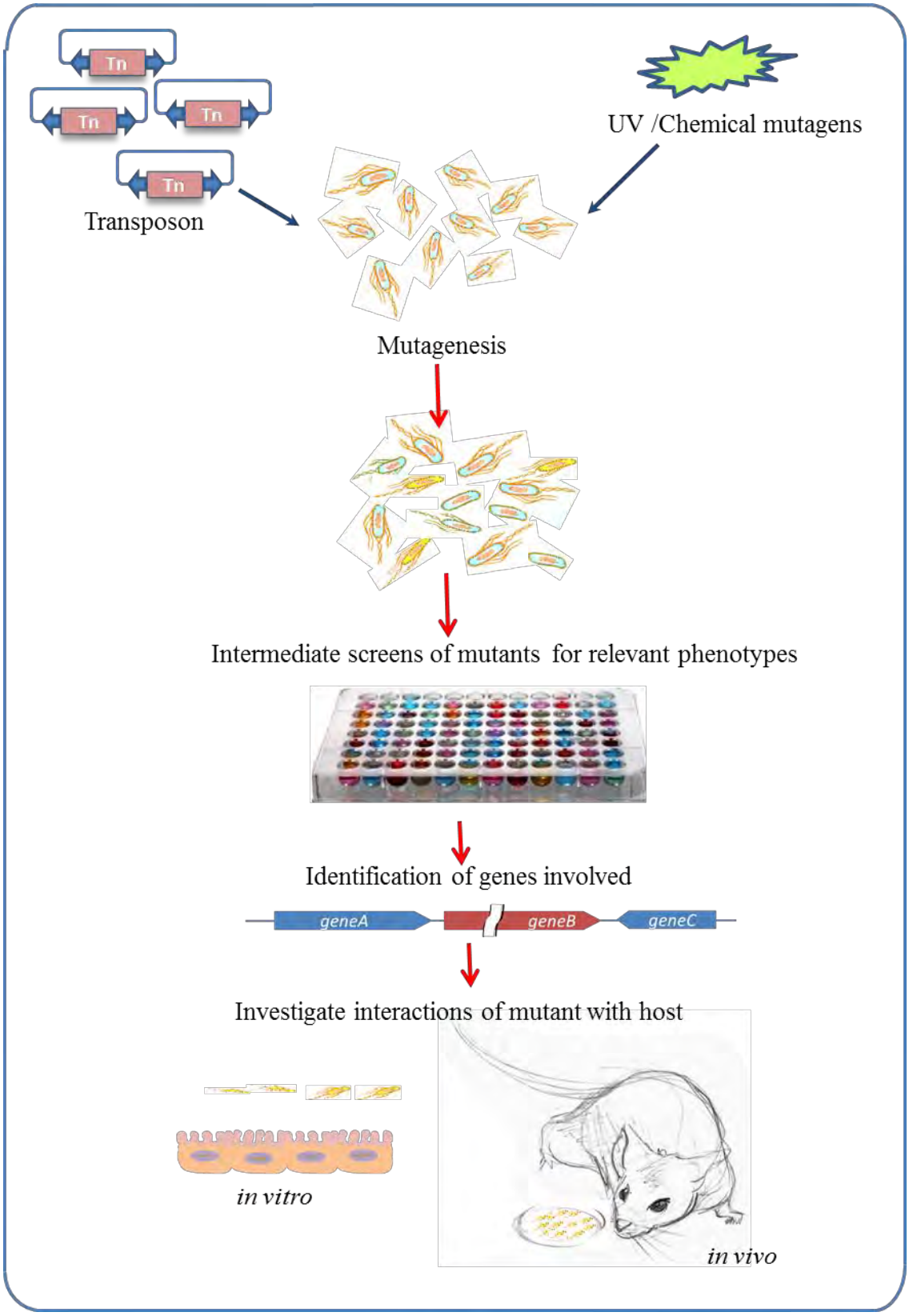


Figure 3.1: Basic strategies for the elucidation of genes involved in probiotic interactions with the host

3.1.2 Random transposon mutagenesis

Transposons are mobile genetic elements that naturally occur in bacteria and other living organisms. They have the ability to "ho " from one location to another within a genome, a movement known as transposition (Kazazian 2004). These elements produce transposase (Tnp), a multifunctional protein that catalyses all the steps of transposition (Berg and Berg, 1996, Boeke 2002). A successful insertion can interrupt an open reading frame (ORF), disrupting gene expression that in turn generates a mutant with a recognisable phenotype (Berg and Berg 1996). There are many varieties of wild-type bacterial transposons that fall under two main classes depending upon their structural properties, mode of transposition, and DNA sequence homology. These are Class I composite transposons, and Class II noncomposite transposons (Kleckner 1981, Moat *et al.* 2002).

Composite transposons such as Tn*5* and Tn*10*, typically consist of a central DNA region (often carrying one or more genes conferring resistance to antibiotics) flanked at both termini by two short insertion sequences (IS) of less than 2 kb in size, which encode the functions required for transposition (Mahillon and Chandler 1998). The IS are terminated by short inverted repeats (IRs) of about 10-40 bp that serve as recognition signals of the Tnp enzyme in order to initiate the process of transposition (Mahillon and Chandler 1998). The Class I transposons normally employ a conservative "cut-and- a ste" mechanism in which the transposable element is first excised from the donor replicon then integrated into the target DNA, leading to complete loss of the transposon from the donor replicon (Kleckner *et al*. 1996, Steiniger-White *et al.* 2004). In contrast, noncomposite transposons such as Tn*3* display a more complex structure and usually use a replicative "copy-and- a ste" mechanism whereby the transposable element is first replicated, releasing one of the copies to insert into the target genome (Bennett 1991, Snyder and Champness 2003). The remaining copy is kept intact within the donor replicon, thus a probability of further

transposition events, and multiple insertions exists. These properties make Class I transposons more suitable for use in mutagenesis where a single mutation per genome is desirable.

Besides the transposition functions, most of the wild-type transposons harbour antibiotic resistance genes which can confer an easily selectable phenotype on the host bacteria that is able to express the gene. The selectable marker can facilitate the identification and characterisation of transposon insertion in mutants of interest. The concept and properties of natural transposition have been exploited by molecular biologists to develop a broad range of random mutagenesis systems (Shaw and Clewell 1985, Kleckner *et al*. 1991, Berg and Berg 1996, de Lorenzo and Timmis 1994, Hensel and Holden 1996, Chalmers *et al.* 2000, Lawley *et al*. 2010). Among the most widely studied and used include Tn constructs derived from composite transposons Tn*10* and Tn*5* (Kleckner *et al*. 1991, de Lorenzo *et al*. 1998).

3.1.3 Tn*5*-based transposition mutagenesis of bacteria

Tn*5*- based transposition systems represent a useful tool for bacterial DNA manipulation. This is mainly because of the simplicity of the wild-type Tn*5*, its well-studied mechanism of transposition, its antibiotic resistance offering easily selectable phenotype, and transposability with random insertions in a wide range of bacteria, Gram-negative in particular (Berg *et al.* 1984, Reznikoff 1993, Zhou and Reznikoff 1997, de Lorenzo *et al*. 1998, Reznikoff 2002). The wild-type Tn*5* is a well-characterised 5,700 -bp composite element with a pair of nearly identical IS*50* insertion sequences (designated IS*50*R and IS*50*L), present in inverted orientations (Berg *et al*. 1984, Reznikoff 1993). These flank an internal segment comprising genes encoding antibiotic resistance to kanamycin/neomycin (Kan^R), bleomycin (Ble^R) and streptomycin (Str^R) (Berg *et al*. 1984).

Figure 3.2a shows the functional organisation of Tn*5*. Of the two IS*50* elements, IS*50*R controls the movement the whole Tn segment by encoding two proteins, a *cis*-acting Tnp that catalyses transposition and a *trans* active Inh enzyme, which inhibits transposition (Berg *et al*. 1984, Reznikoff 2002). The latter also serves to provide the transposon integrant cells with "immunity" to further transposition events. The IS*50*L element contains a promoter, which programs the expression of the antibiotic resistance genes, and encodes two inactive truncated forms of those transposition proteins in IS*50*R (Reznikoff 1993, Zhou and Reznikoff 1997**)**. The inactivity of IS50L keeps the whole Tn unit structurally intact, increasing a possibility to achieve a stable recombination in proficient host cells **(**Reznikoff 1993, Zhou and Reznikoff 1997**)**. Both IS*50* elements are delineated by a 19-bp inverted repeat (IR) sequences, at both inside (IE) and outside (OE) ends (Johnson and Reznikoff 1983, Sasakawa 1983). The OE ends are sites at which the Tnp enzyme acts to initiate transposition in a “cut and a ste” mechanism as shown in Figure 3.2b.

Disadvantages of natural Tn*5* transposons for use in random mutagenesis include their large size, which may hinder transposition efficacy, their termini made up of repeated sequences that can promote DNA rearrangement hence causing unstable and unwanted mutations, and the Inh mediated transposition immunity, which inhibits subsequent transposon insertion in the same strain (de Lorenzo *et al*. 1998). To resolve these drawbacks, varieties of Tn*5* derivatives such as mini-Tn*5* have been developed to meet specific demands for bacterial genetic manipulations.

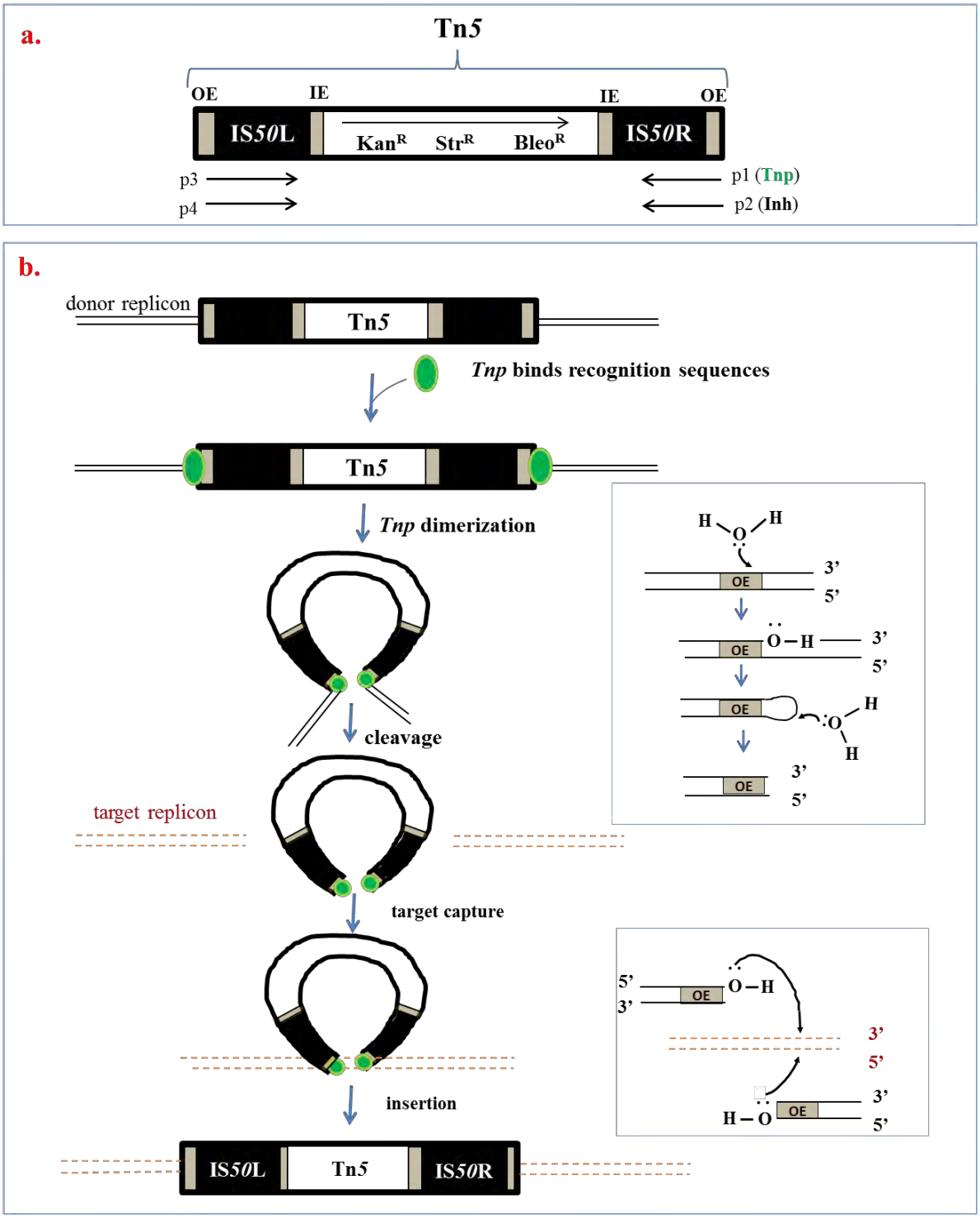


Figure 3.2: Functional organisation transposition mechanism of Tn*5*

Organisation of Tn*5* (a): Genes encoding for antibiotic resistance are bracketed by two IS*50* elements, each delineated by 19 bp IR sequences, the inside (IE) and the outside (OE) sequences. IS*50*R encodes for proteins Tnp and Inh, and IS*50*L regulate synthesis of p3 and p4.

Tn*5* cut-and-paste mechanism (b): involves i) binding of the Tnp (green sphere) monomers to the IRs, ii) oligomerization of the bound Tnp; iii) cleavage from donor replicon, and iv) target capture.

This diagram was adapted from Berg *et al*. (1984) and Steiniger-White *et al.* (2004).

3.1.4 Mini-Tn*5* delivery systems for *in vitro* transposition

Mini-Tn*5* is a generic name derived from wild-type Tn*5* in which functional DNA fragments have been genetically engineered to achieve shorter and more stable insertion elements, which are then cloned into a plasmid vector in order to optimize their transfer, and uses (de Lorenzo *et al*. 1998). The pUT mini-Tn*5* vectors developed by Herrero *et al*. (1990) and de Lorenzo *et al.* (1990) are the most popularly used systems for random Tn mutagenesis (de Lorenzo *et al*. 1998, Christie-Oleza *et al.* 2013). However, more versions of mini-Tn5 have been recently constructed to achieve more efficient transposition, and application in a broader range of microorganisms as compared to the pUT system (Larsen *et al.* 2002, Lyell *et al*. 2008).

Overall, the key steps towards successful development of mini-Tn*5* constructs have been: i) optimisation of the functionality of the mobile unit (shorter and optimised IR terminal sequences, and single selectable marker, often Kan^R gene) (de Lorenzo *et al*. 1990, Zhou et *al.* 1998, Lyell *et al*. 2008); ii) placement of the *tnp* gene outside of the mobile unit so that this is lost after transposition, resulting in stable transposon insertions and a possibility for subsequent transpositions if required (Way *et al*. 1984, Herrero *et al*.1990, de Lorenzo *et al.*1994); iii) improvement of the *tnp* functional characteristics to achieve hyperactive forms of transposase, thus a higher transposition efficiency (Dennis and Zylstra 1998; Goryshin and Reznikoff 1998, Larsen *et al.* 2002, Lyell *et al*. 2008); and iv) provision of a 'suicide' la smid vector (based on a conditional origin of replication allowing maintenance of the Tn element inside the plasmid replicable only in a permissive host strain but not the target recipient strain) resulting in subsequent delivery of the mini-Tn*5* (Stalker *et al.* 1982, Herrero *et al*.1990, Metcalf *et al*. 1996, Le Borgne *et al.* 1998).

It should be noted that any mini-Tn*5* system is most useful in elucidating functions of unknown genes in a particular bacterial strain when: i) a successful transfer of the plasmid

vector harbouring the Tn element into the target cell can be achieved; ii) high-throughput screenings of mutants for a desired phenotype can be established, and iii) strategies facilitating accurate genetic characterisation of those insertion mutants are available.

The transfer of the plasmid vector carrying the Tn into the target bacteria strain is often the limiting factor for construction of random transposon mutant libraries. This is because the number of mutants obtained reflects both the transposition frequency of the transposon system used and the efficiency of the plasmid vector transfer into cells (de Lorenzo *et al.* 1994). Given that the efficiency of plasmid transfer in bacterial cells can be highly dependent on the method used, it is important that an appropriate method for plasmid transfer is chosen and adapted, in order to optimise its functionality in the target strain.

3.1.5 Methods for transfer of plasmid vectors into bacterial cells

The ability to transfer plasmid DNA into bacteria cells is central to the successful application of mutagenesis systems, especially those delivered by plasmid vectors. Several methods for transfer of circular plasmids or linear DNA fragments into cells of different bacteria species have been developed (Yashida and Sato 2009). The most applicable to *E. coli* include chemical transformation, electroporation, and conjugal transfer (Woodall 2003, Sambrook and Russell 2006). Each method offers distinct advantages and disadvantages as presented in Table 3.2. Both chemical transformation and electroporation involve the use of chemical solutions to develop a state of cell competence, which is characterised by an increased cell membrane permeability facilitating the uptake of free DNA. Instead of using purified DNA, conjugal transfer requires direct physical contact between donor and recipient cells and requires at least a biparental mating, depending on how plasmid mobilisation functions are provided (Schweizer 2008). Biparental mating is applicable when the plasmid vector is self-transmissible. The triparental is often used when the plasmid of interest is mobilisable but not self-transmissible, requiring the use of helper

Table 3.2: Comparison of methods applied in the transfer of plasmid vectors into *E.coli*

Method and principle	Advantages	Disadvantages	Reference
Chemical transformation: chemical treatment to induce competence (e.g. with $CaCl_2$) and heat-shock at 42 °C	- Simple, accessible, and cost-effective - No need for specialised equipment	- Highly dependent on cell-wall structure and the low intrinsic permeability of cell membrane - Only works *for E. coli* - Can yield poor efficiency for transfer of large plasmids	Bergmans *et al*. 1981, Inoue *et al.* 1990, Chen, X. *et al*. 2001, Swords 2003
Electro–transformation: exposure of cells to electric pulse in presence of purified plasmid DNA	- Can yield higher transformation efficiency as compared to chemical method - Effective with small quantities of plasmid DNA	- Invasive and can cause DNA damage - High running cost and requires specialised equipment - Can yield poor efficiency for transfer of large plasmids	Calvin and Hanawalt 1988, Dower *et al*. 1988, Woodall 2003
Conjugal transfer: contact-dependent transfer of DNA plasmid from donor bacterium to a recipient cell	- Non-invasive and no plasmid purification required - Can easily transfer large plasmids	- Requirements for vector to be self-transmissible or at least mobilisable - Relies on availability of suitable counter-selection strategies to select against donor strain - May require vector to vector optimisation	Steele *et al*. 1994, Willetts and Wilkins 1984, Rainey 1997, Davidson 1999

plasmid that provides necessary mobilisation functions.

While conjugation is often considered the most efficient DNA transfer method in many bacterial species, it does require counter-selection strategies in order to isolate the resulting trans-conjugants from the donor (Szpirer *et al.* 1999, Schweizer 2008). Counter-selection can be difficult to establish especially when conjugation involves bacterial strains from the same group, sharing intrinsic properties. In such cases, adding a counter-selectable marker (i.e. antibiotic resistance gene) to the recipient strain might require the addition of foreign DNA that can confer unwanted properties to the microorganism.

Although electroporation can yield relatively higher transformation efficiencies compared to chemical transformation, its use can often be limited by a high running cost and requirement for specialised equipment (Dower *et al.* 1988). In contrast to electroporation, chemical transformation is a cost-effective, simple and highly reliable method for *E .coli*. Considering these differences, chemical transformation can offer a good balance between cost, efficiency and reliability, thus a potential choice for further optimisation in EcN transformation, and subsequent use for the transfer of mini-Tn*5* plasmid vectors.

3.1.6 Efficiency of chemical transformation in *E. coli*

Mandel and Higa (1970) first reported the chemical transformation of *E. coli* by demonstrating the effect of bacterial culture treatment with ice-cold $CaCl_2$ solution on the induction of cell competency. The mechanisms involved in the process are not very clear but the Ca^{2+} divalent cation was found to help permeabilisation of the cell outer membrane and heat-shock to facilitate vectorial transport of DNA (van Die *et al*. 1983, Hancock 1984). Subsequently, several modifications of this method have been established with the ultimate goal to improve the efficiency of DNA transformation in *E. coli* (Bergmans et *al*. 1981, Hanahan 1983, Huff *et al.* 1990, Nakata *et al.* 1997, Chen, X. *et al.* 2001, Tu *et al*.

2005, Singh *et al*. 2010). The standard protocol has been proposed by Sambrook and Russell (2006).

The most notable parameters of the optimised methods include the physiological status of bacterial cells, such as growth phase and cell density (Hanahan 1983, Huff *et al.* 1990, Tu *et al*. 2005); growth conditions, such as incubation temperature and media composition (Inoue *et al*. 1990,Chan *et al*. 2006, Brooke *et al*. 2009); type and concentrations of cations used in production of competent cells (Hanahan and Bloom 1996, Tu *et al*. 2005); DNA size and concentration (Hanahan 1983, Brooke *et al*. 2009); heat-shock temperature and duration (Van Die *et al*. 1983, Singh *et al*. 2010).

Besides these parameters, the genetic individuality of the strain has also been found to contribute to the efficiency of plasmid DNA transfer into cells (Tu *et al*. 2005). In their studies on the efficiency of plasmid transformation using different *E. coli* strains in standard chemical transformation, Tu and colleagues (2005) have reported variations in conditions leading to optimum transformation efficiencies in each one of the strains used. Their attempt to optimise the transformation protocol raised the efficiencies up to 100-300 fold as compared to the yield obtained using the standard transformation protocol. Considering these results, optimisation of chemical transformation in a particular bacteria strain might be required, especially when this is a candidate for genetic manipulation in random mutagenesis.

3.1.7 Establishment of high-throughput screening strategies

The successful generation of a transposon insertion mutant library is as important as the establishment of high-throughput (HTP) screening strategies, which permit the isolation of those mutants exhibiting traits of interest. The availability of HTP screenings of mutants phenotypes with relevance to host-microbe interactions, remains a limiting factor in

probiotic studies employing random mutagenesis approaches. This is mainly due to a shortage of *in vitro* and *in vivo* systems that can effectively mimic the natural microbial processes along the human gastro-intestinal tract (GIT), and which offer a scalable HTP assay for traits of interest.

An alternative to these systems can be the use of HTP screens of mutant libraries for intermediate phenotypes, those indirectly related to host-microbe interactions. This approach can help to isolate a reasonable number of mutants, which can be further studied using host cell, tissue or animal models. As an example, large-scale screening of mutants for biofilm formation, survival in bile and low pH media have been successfully performed to select those with deficiency in virulence factors among enteropathogenic bacterial strains (Begley *et al*. 2003, Dowd *et al*. 2007, Puttamreddy *et al*. 2010, Hadjifrangiskou *et al*. 2012). Therefore, this approach can be adapted to facilitate studies of EcN probiotic effects in random transposon mutants of this strain.

3.1.8 Characterisation of transposon insertion mutants

Genetic characterisation of transposon (Tn) insertion mutants with a recognisable phenotype, is often the ultimate goal of using random transposon mutagenesis approaches. The characterisation consists initially of revealing the identity and location of the disrupted gene in the genome sequence of the strain under investigation. There is a variety of techniques that facilitate the mapping of random Tn insertion sites by comparing sequences of the disrupted genes to the available genome sequences. These range from direct cloning of flanking DNA (Camilli *et al.* 1990, Woods *et al.* 1999), direct sequencing of genomic DNA with transposon-specific primers (Hoffman *et al*. 2000, Krin *et al*. 2001, Qimron *et al.* 2003) and various PCR methods to amplify DNA segments flanking the transposon

prior to sequencing (Prod'Hom *et al*. 1998, Karlyshev *et al*. 2000, Manoil 2000, Geoffroy *et al.* 2003, Jacobs *et al*. 2003, Das *et al.* 2005).

The direct cloning method consists of cloning restriction digests of chromosomal DNA extracted from the mutants, and subsequent sequencing of the clones (Camilli *et al.* 1990). This technique is long, laborious and involves the use of rare-cutting restriction enzymes, making its application in the analysis of large-scale mutant library unwieldy. Furthermore, it might be difficult or impossible to size select and clone large DNA fragments of interest.

An alternative to direct cloning is the direct sequencing of chromosomal DNA extracted from the mutant, and subsequent sequencing of the Tn-flanking fragments using a Tn-specific primer. This approach can be unreliable, mostly because of the inadequate concentration of the target template in a complex mixture of the untreated genomic DNA. Successful direct sequencing of the Tn-flanking sequences from a complete bacterial genomic DNA would require additional treatment of the DNA extract, as well as optimisation of the sequencing protocol, but this is still challenging to achieve (Qimron *et al.* 2003). In contrast, PCR-based methods that enrich the Tn-flanking sequences prior to sequencing are likely to increase the chances of identifying the genes disrupted in Tn insertion mutants. Thus this can be a good choice for large-scale characterisation of mutants.

3.1.9 PCR-based methods of enriching the transposon-flanking sequences

Variations of standard PCR have been developed to allow amplification of the unknown DNA sequence flanking the site of transposon (Tn) insertion, using the genomic DNA extracted from mutants as a template. The main techniques that are used for this purpose are outlined in Figure 3.3. These include: i) the inverse PCR (iPCR) (Ochman *et al*. 1988,

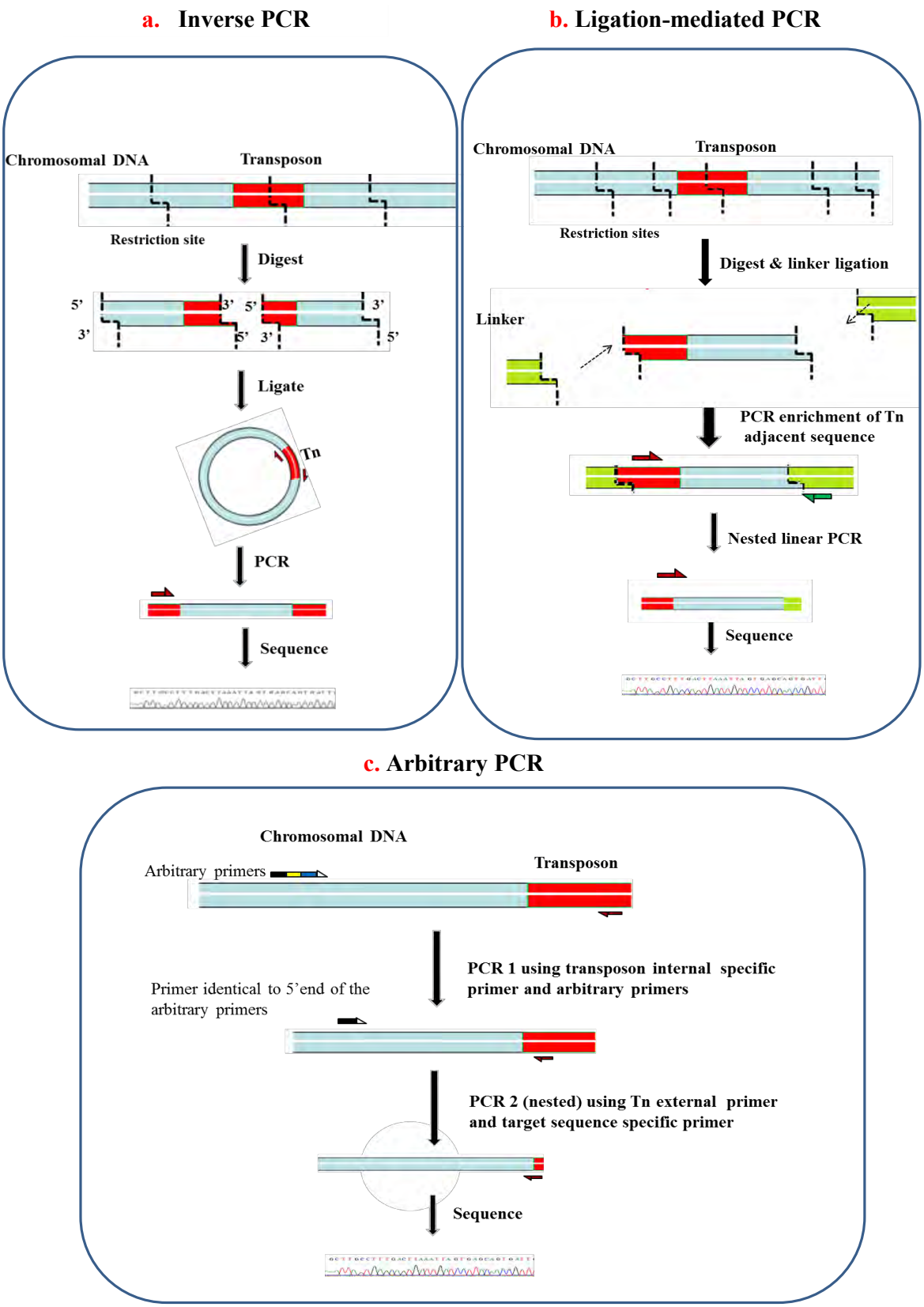


Figure 3.3: Schematic outline of PCR methods for mapping transposon insertion site

Rich and Willis 1990, Martin and Mohn 1999), ii) ligation-mediated PCR (LM-PCR) methods (Riley *et al*. 1990, Siebert *et al.* 1995, Prod'hom *et al*. 1998), and iii) arbitrary primed PCR (AP-PCR) (Parker *et al.* 1991, Caetano-Annoles 1993, O'Toole and Kolter 1998b, Manoil 2000, Mishra *et al*. 2002, Knobloch *et al*. 2003).

iPC involves restriction digestion of the mutant's genomic DNA and the trans oson at a single site, then ligation of the blunt-ended fragments under conditions that facilitate circularisation. The looped fragment is then amplified by PCR using primers facing outward from the transposon sequence, and the obtained fragments can then be sequenced. This method is primarily limited by uneven distribution of restriction sites across the bacterial genome, and unreliability of the circularisation step, especially when ligating large DNA fragments (Prod'hom *et al*. 1998, Krin *et al*. 2001, Yuanxin *et al*. 2003).

The LM-PCR method uses linkers, small DNA oligonucleotides that are first ligated to the random restriction fragments generated from chromosomal DNA of mutants, which are subsequently amplified with primer pairs recognizing the linker and transposon sequences. Non-specific primer annealing and amplification is a major drawback of this method as the primers designed to bind linkers can easily attach to random chromosomal regions (Prod'hom *et al*. 1998). Both the iPCR and LM-PCR methods require laborious steps, which include restriction digestion, tailing and ligation. The use of AP-PCR method can circumvent these issues and is thus a relatively better choice for characterising Tn insert sites.

The AP-PCR approach consists of two rounds of PCR. During these, sets of arbitrary degenerative primers that can prime to multiple regions in the genome and Tn specific r imers are normally used. This in turn facilitates random am l ification of mutant's genomic DNA fragments including those flanking the Tn, which can then be sequenced

(O'Toole and Kolter 1998b, Manoil 2000, Das *et al.* 2005). The first round is an arbitrary PCR, which is performed with Tn-specific primers paired with a single or a mixture of arbitrary primers, producing an amplicon between the transposon and one of the adjacent chromosomal regions (Das *et al.* 2005). The arbitrary primers are designed to include short sequence overhangs at their 5'- ends that can be easily primed in subsequent PCR enriching the Tn-flanking regions. Products from the first-round reactions are then used as templates for second-round PCR employing nested, Tn-specific primer paired with target sequence specific primer, which is identical to the 5' overhangs of the first-round arbitrary primers. The resulting amplicons are then subject to DNA sequence analysis using the Tn-specific primer used in the second-round PCR (O'Toole and Kolter 1998b, Manoil 2000).

Although the AP-PCR technique has been gaining popularity recently for the characterisation of transposon mutants (Bahrani-Mougeot *et al*. 2002, Knobloch *et al* 2003; Jones *et al*. 2004, Boddicker *et al*. 2006, Ge *et al*. 2008), failure to get appropriate PCR product capturing Tn- flanking segments have also been reported in some cases (Bahrani-Mougeot *et al*. 2002, Knobloch *et al*. 2003, Burall *et al.* 2004). In order to minimise such failures, Das *et al.* 2005 have suggested that a careful primer design and optimisation of quantity and quality of template DNA could increase the specificity and sensitivity of the PCR. Therefore, optimisation of the AP-PCR in EcN would be required in order to facilitate the genetic characterisation of mutants, once the transposon mutagenesis of this strain is established. The application of random transposon mini-Tn*5* systems and AP-PCR strategy in the current study will hopefully help elucidate the mechanisms involved in the colonisation and survival of EcN in the human gut, and could potentially identify novel genes involved in host-probiotic interactions.

3.2 Aims

The aims of the work presented in this chapter are to establish an optimised protocol for efficient plasmid transformation of probiotic *E. coli* Nissle 1917 based on standard chemical transformation, and test the functionality of mini-Tn*5* based systems for large-scale random transposon mutagenesis of this strain.

3.3 Material and methods

3.3.1 Optimisation of plasmid transformation in *E. coli* Nissle 1917

Introduction of plasmid pUC19 (Yanisch-Perron *et al*. 1985) DNA into bacterial cells was conducted using the standard chemical transformation method of *E. coli* by $CaCl_2$ treatment (Sambrook and Russell 2006) which is illustrated in Figure 3.4. Three parameters of this method, namely culture growth temperature, cell density, and heat-shock duration were optimised. *E. coli* strains Nissle, EPI300 and S17.1 were used in this study.

An overnight culture obtained from growth of a single colony was subcultured at 1:100 into 5 mL fresh LB in 50 mL polypropylene tubes and incubated at either room temperature (RT, 18-20°C) or 37°C with shaking at 150 rpm, to a range of cell population densities: 0.5, 1.0 and 5.0 (as determined by OD_{600}) at harvesting. A high-density culture (5.0 OD_{600}) was diluted to 2.5 OD_{600} before use. The tubes were transferred on ice and chilled for 30 minutes, then the cells were pelleted by centrifugation at 1,500 *g* for 10 minutes at 4°C. The pellets were resuspended by swirling in 2 mL of ice-cold sterile 0.1 M $CaCl_2$ solution then chilled on ice for 5 minutes and pelleted again for 5 minutes at 4°C. The $CaCl_2$ treatment was repeated twice and the resulting chemically competent cells (CCCs) were resuspended in 100 µL of the $CaCl_2$ solution.

The freshly prepared CCCs (100 µL aliquot) were transferred into a pre-chilled sterile 1.5 mL Eppendorf tube containing 50 ng pUC19 plasmid DNA, in a volume of 5 µL, and mixed gently by tapping 5 times, then incubated on ice for 30 minutes. A negative control containing cells with no added DNA was also included. The tubes were transferred to a preheated circulating water bath at 42°C and heat-shocked for 50, 90 or 180 seconds, then

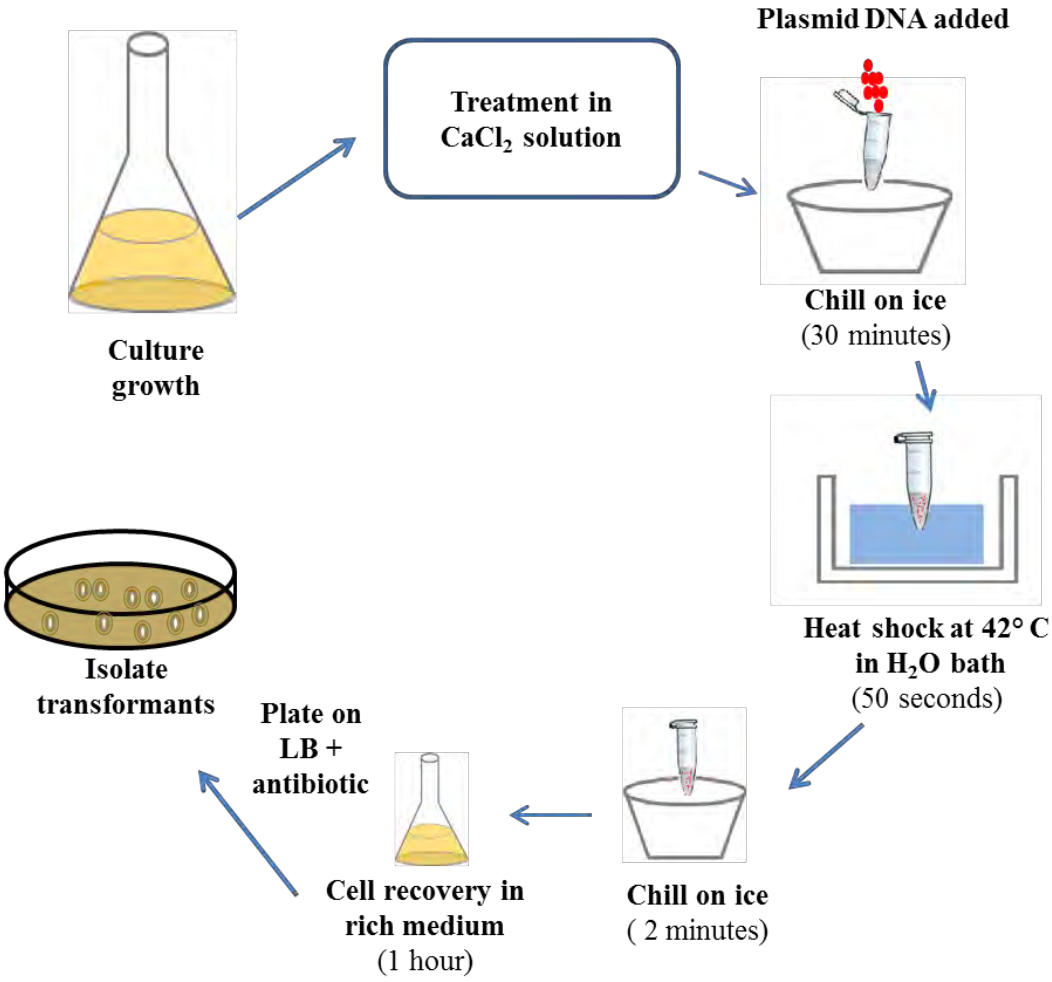


Figure 3.4: Standard chemical transformation of *E. coli*

Culture growth temperature, cell density, and heat-shock duration were optimised in *E. coli* Nissle transformation using pUC19 DNA.

incubated on ice for 2 minutes before adding 900 µL SOC broth (medium composition defined in Section 2.2.11). The tube's contents were then transferred into a sterile 20 mL universal tube and incubated at 37°C for 1 hour, with shaking to allow cell recovery, and the transformants to express the antibiotic resistance marker that is encoded by the pUC19 plasmid. The cultures were serially diluted in sterile PBS and spread on LB agar supplemented with ampicillin 100 µg/mL and grown overnight at 37°C. The efficiency of transformation was estimated by calculating the number of transformants per µg of pUC19 DNA.

3.3.2 Random transposon mutagenesis of *E. coli* Nissle 1917

3.3.2.1 Delivery of transposons into bacterial cells

Random transposon mutagenesis of *E. coli* Nissle 1917 (EcN) was performed using mini-Tn*5* systems, pUTKm2 (de Lorenzo *et al*. 1990) and pRL27 (Larsen *et al*. 2002); both represented in Figure 3.5. Plasmid DNA of these were purified from donor *E. coli* strains S17.1λ*pir* and BW29427- *dap* auxotroph*, ::pir*, respectively using the Qiagen mini prep kit (as described in Section 2.2.6) then transformed into CCCs by chemical transformation. The CCCs were prepared from EcN cultures grown at room temperature to an OD_{600} of 0.5 and the cell/plasmid DNA mixture was heat-shock treated for 180 seconds at 42°C. Transformants harbouring the transposons were selected on LB agar containing 50 µg/mL kanamycin. Alternatively, pRL27 was conjugally transferred into EcN using biparental mating (as described in Section 2.2.13) with donor *E. coli* BW29427- DAP auxotroph, :: *pir*. Mating plates consisted of LB agar supplemented with $MgSO_4$ 10 mM/mL and DAP 0.1 mM/ mL. Trans-conjugants harbouring the transposon were selected on LB agar containing 50 µg/ mL Km kanamycin without diaminopimelic acid (DAP) to eliminate the donor strain.

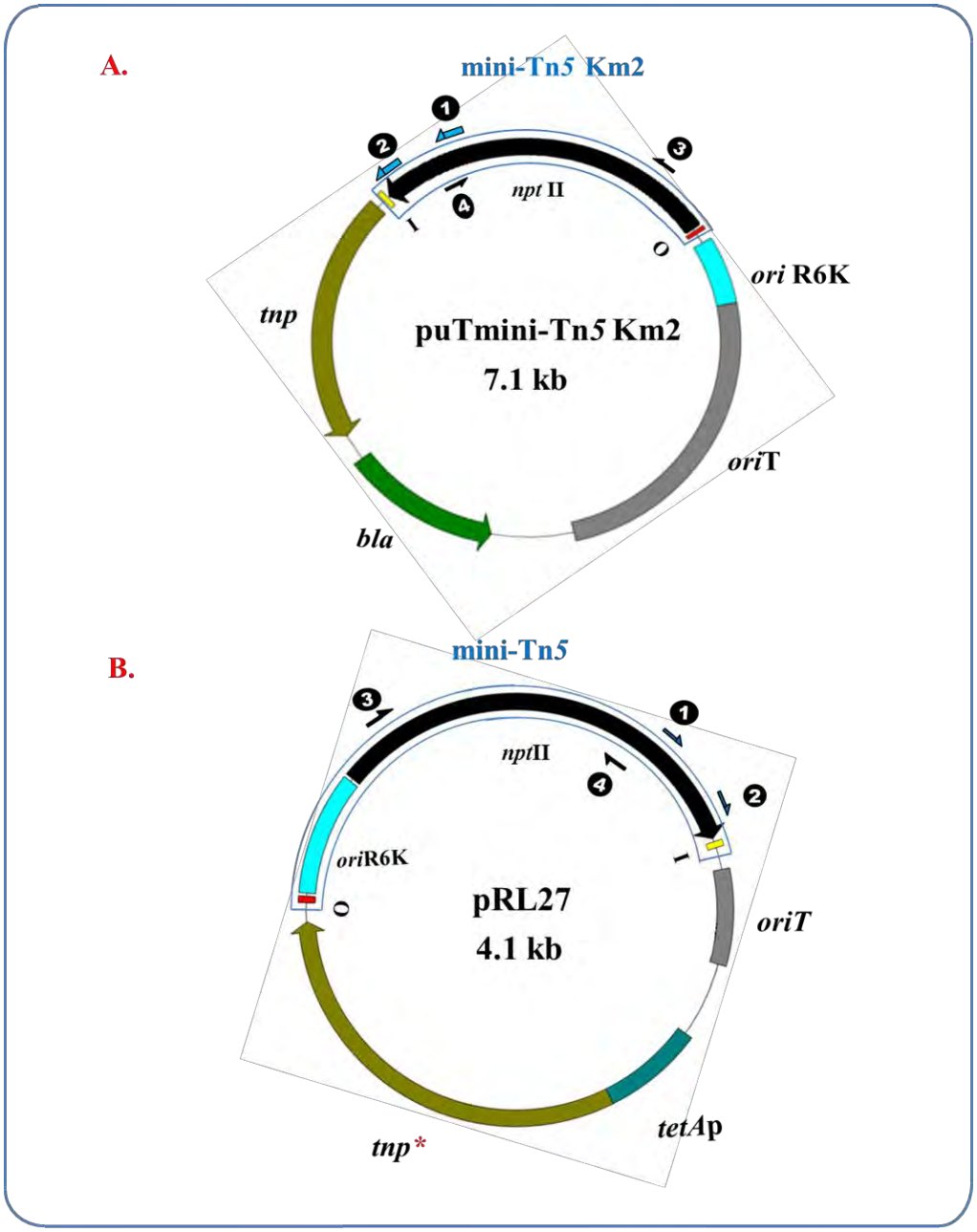


Figure 3.5: Structure of suicide delivery vectors harbouring mini-Tn*5* systems

The *npt*II gene: neomycin phosphotransferase II encoding for Km^R; O and I: Tn*5* inverted repeats; *tnp*: gene for Tn*5* transposase; *tnp* *: gene for hyperactive Tn*5* transposase*; ori*R6K: the origin of replication; *oriT*: the origin of transfer; *bla*: ampicillin resistance; *tetA*p: *tetA* promoter for *tnp*; 1 and 2: transposon specific primers, both useful in characterisation of insertion sites in genome ; 3 and 4: *npt*II forward and reverse primers, respectively.

3.3.2.2 Verification of suicide plasmid vector loss

To verify plasmid vector loss in EcN, a sample of ten trans-conjugants were picked at random from LB + Km 50 µg/mL agar plates and subcultured in LB broth containing the antibiotic, and subsequently used for plasmid DNA extraction using the Qiagen mini prep kit (as described in Section 2.3.2). EcN wild-type harbours two very stable cryptic plasmids of known sequence (Blum-Oehler *et al.* 2003) that can easily be compared to those of mini-Tn*5* plasmid vectors. Loss of the suicide plasmid vector was confirmed by *EcoR*I digest of 1 µg "plasmid DNA", from each of the selected trans-conjugants and controls. The banding patterns of the digests were visualised by gel electrophoresis and compared to control extractions obtained from "empty" unmated EcN wild-type and preparations of the mini-Tn*5* plasmid vector.

3.3.2.3 Confirmation of transposon presence in the genome of trans-conjugants

The randomly selected trans-conjugants, which had been used to confirm loss of the suicide plasmid vector, were also used to confirm presence of the transposon (Tn) in the genome by PCR using Tn-specific primers. Genomic DNA was purified from the transformants and control EcN wild-type using the chloroform extraction method (as described in section 2.2.7), then used as a DNA template in PCR amplification of the transposon-specific fragment (*npt*II gene), using primers NptIIF1, 5′-CTTGCTCGAGGCCGCGATTAAATT-3′ and NptII 1, 5′-TTCCATAGGATGGCAAGATCCTGG-3′ (Jones *et al*. 2004). The obtained PCR products were separated by agarose gel and compared to control reactions performed on EcN WT genomic DNA and purified DNA of the mini-Tn*5* plasmid.

3.3.2.4 Verification of single and random insertion

Single and random transposon insertions were verified by Southern blot analysis as described in Section 2.3. The verification was performed on a subset of randomly selected trans-conjugants which had also been used to confirm loss of the suicide plasmid vector. Genomic DNA was extracted from the trans-conjugants, and EcN wild-type (as a control) using the chloroform extraction method (as described in section 2.2.7); 2 µg genomic DNA from each sample was digested with the restriction enzyme *Pst*I, and the resulting fragments separated on a 1 % (w/v) agarose gel at 50 V, then analysed by Southern hybridisation using digoxigenin (DIG)- labelled transposon-specific probe. The probes were generated by PCR from plasmid pRL27::mini-Tn*5* template and primers NptIIF1& NptIIR1 (sequences in previous Section 3.2.2.3). Hybridised fragments bands and controls were visualised using the Xenogen *in vivo* imaging system 50 (*IVIS* 50).

3.3.2.5 Identification of genes disrupted by mini-Tn*5* inserts

Identification of the genes disrupted by the pRL27 mini-Tn*5* in EcN mutants was conducted using a "cloning free" AP-PCR based approach that was proposed by Manoil (2000). The approach consisted of two rounds of PCR, which were optimised using genomic DNA obtained from a subset of mutants which had been confirmed to have single and random Tn inserts. Negative and positive control reactions contained EcN wild-type genomic DNA and purified pRL27::mini-Tn*5* DNA, as template, respectively. The AP-PCR method and primers used are outlined in Figure 3.6.

In the first round PCR, 50 ng DNA template was amplified in a 25 µL reaction using the Qiagen Taq PCR Core Kit. Each reaction included primers (sequences listed in Figure 3.6), Tn-specific primer pRL27 Primer1 paired with a mixture of degenerative primers Primer 2a, 2b and 2c, at a ratio of 1:1:1:1, 0.5 pM each , 2 mM dNTPs, 0.04U *Taq* polymerase, and 1.5 mM $MgCl_2$.

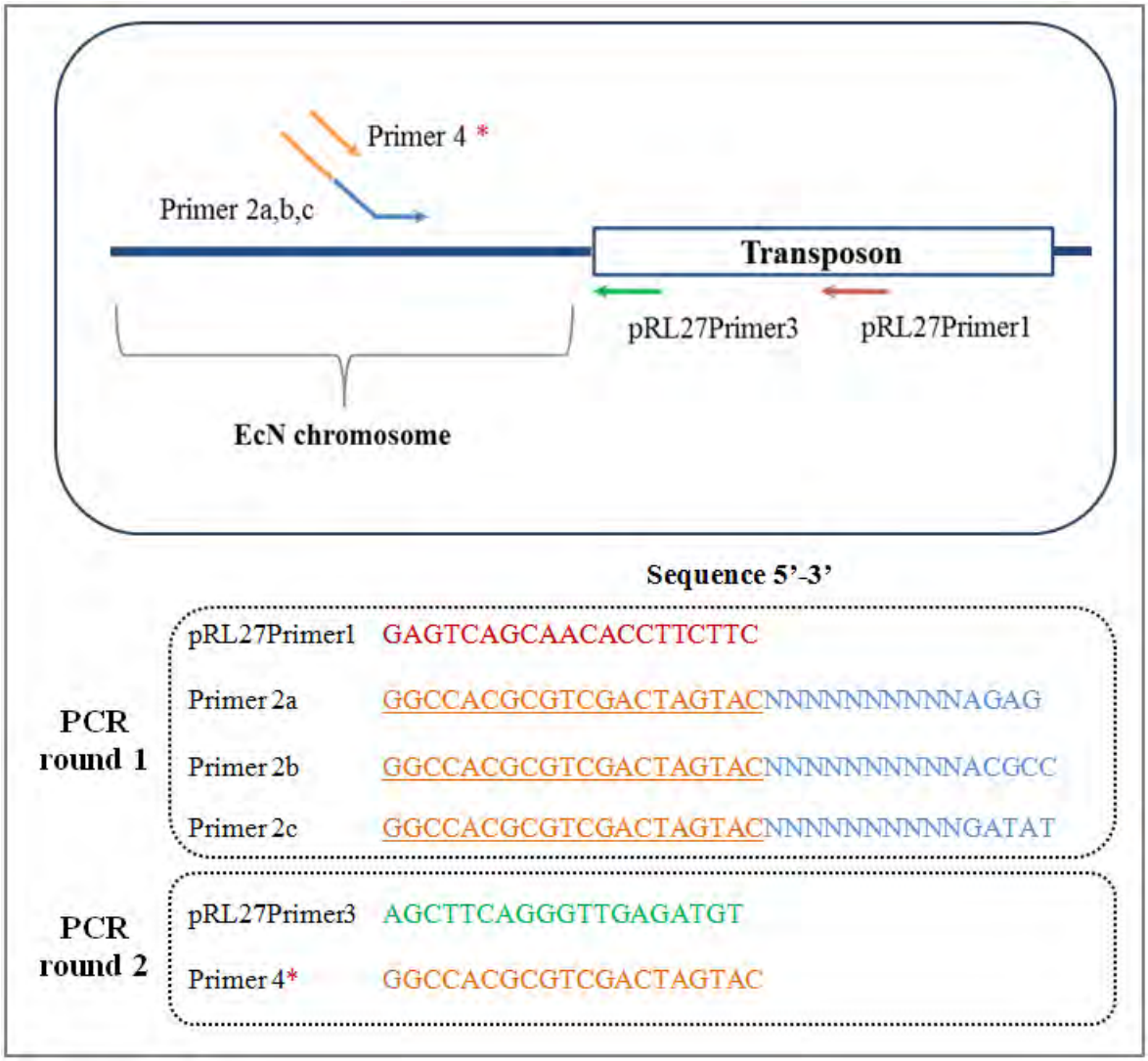


Figure 3.6: Cloning free PCR-based approach for amplification of transposon-genomic DNA junctions

The method consists of 2 rounds of PCR. First round AP- PCR uses primer pRL27Primer1 and a mixture of degenerate primers Primer 2a,b,c hybridising to transposon sequences and at many sites in the EcN genome, respectively. Degenerative primers contain overhangs (underlined) identical to Primer 4.

Second round nested-PCR uses DNA template from product of first PCR and employs nested primer, transposon –specific pRL27Primer3 paired with Primer4 (*: identical to the 5' sequence overhangs of degenerative primers used in the first-round PCR).

The following cycling conditions were used in first-round PCR:

Initial denaturation, 95^0C 5 minutes

8 cycles of

30^0C 35 seconds (primer annealing)

72^0C 45 seconds

94^0C 30 seconds

30 cycles of

43^0C 35 seconds (primer annealing)

72^0C 45 seconds

94^0C 30 seconds

Final hold, 4°C.

Products of the first PCR were purified using the Qiagen QIAquick gel extraction kit (Section 2.2.5), quantified using a NanoDrop 2000 spectrophotometer (Section 2.2.2) then used as a template, 100 ng DNA in a 50 μL PCR. For the second round PCR, nested primer, the Tn-specific primer pRL27 Primer3 was paired with Primer 4 that recognises the5'end of each of the degenerative primers, and were used at a ratio of 1:1, 0.5 pM of each , 1 mM dNTPs, 0.04U *Taq* polymerase, and 1.5 mM $MgCl_2$ of the Qiagen Taq PCR Core Kit. The following cycling conditions were used for second-round PCR:

Initial denaturation, 95^0C 5 minutes

30 cycles of

43^0C 35 seconds (primer annealing)

72^0C 45 seconds

94^0C 30 seconds

Final extension, 72°C for 5 minutes,

Final hold, 4°C

Final PCR products were purified and a sample was taken then visualised on a 1 % (w/v) agarose gel to detect bands of DNA fragments, which were an indicator of the PCR method working. Bands were not expected in the DNA template from EcN wild-type genomic (control). Purified DNA samples, 1µg, each were submitted to GATC Biotech Ltd. (London, UK) for sequencing of the mini-Tn*5* flanking regions using Tn-specific primer, pLR27Primer3. Sequences were first processed using BioEdit version 7.0.5.3 then analysed by Basic Local Alignment Search Tool (BLAST) at NCBI, to search for homologous DNA and protein sequences from bacterial strains, which the genome has already been sequenced.

3.4 Results

3.4.1 Optimisation of plasmid transformation in *E. coli* Nissle

Optimization of *E. coli* Nissle plasmid transformation was performed to improve the transfer of plasmid delivery vectors harbouring mini-Tn*5* elements, for the generation of mutants from this strain. Three parameters of the standard chemical transformation ($CaCl_2$/heat shock) method were optimised using plasmid DNA of the pUC19 cloning vector, 2.7 kb (Yanisch-Perron *et al*. 1985). These were culture growth temperature, cell density and heat-shock duration.

3.4.1.1 Effect of culture growth temperature on plasmid transformation

To evaluate the effect of culture growth temperature on *E. coli* Nissle transformation efficiency with pUC19, cells were grown at either room temperature (RT) or 37°C to a range of cell densities and used for preparation of CCCs. A standard 50 seconds heat-shock at 42°C was applied to the CCCs/plasmid DNA mix, and the resulting transformants were isolated on selective media for cells containing pUC19 (ampicillin 100 µg/mL) and compared in Figure 3.7. Overall, cultures grown at RT resulted in significantly higher numbers of tranformants as compared to those grown at 37°C, whereas an increase in cell density decreased the efficiency significantly ($P < 0.01$).

The highest transformation efficiency was 6.9×10^5 transformants/µg pUC19 DNA obtained from cells grown at RT up to an OD_{600} of 0.5, a 4.5-fold increase in the efficiency as compared to those grown at 37°C to the same density. The lowest numbers of transformants were observed in high-density cell cultures (OD_{600} of 2.5) which yielded 8.6×10^4 and 3.4×10^4 transformants/µg pUC19 DNA, from RT and 37°C cultures, respectively. Given the positive effect of RT on EcN transformability, this condition was ultimately selected for growth of cultures in further experiments of this study.

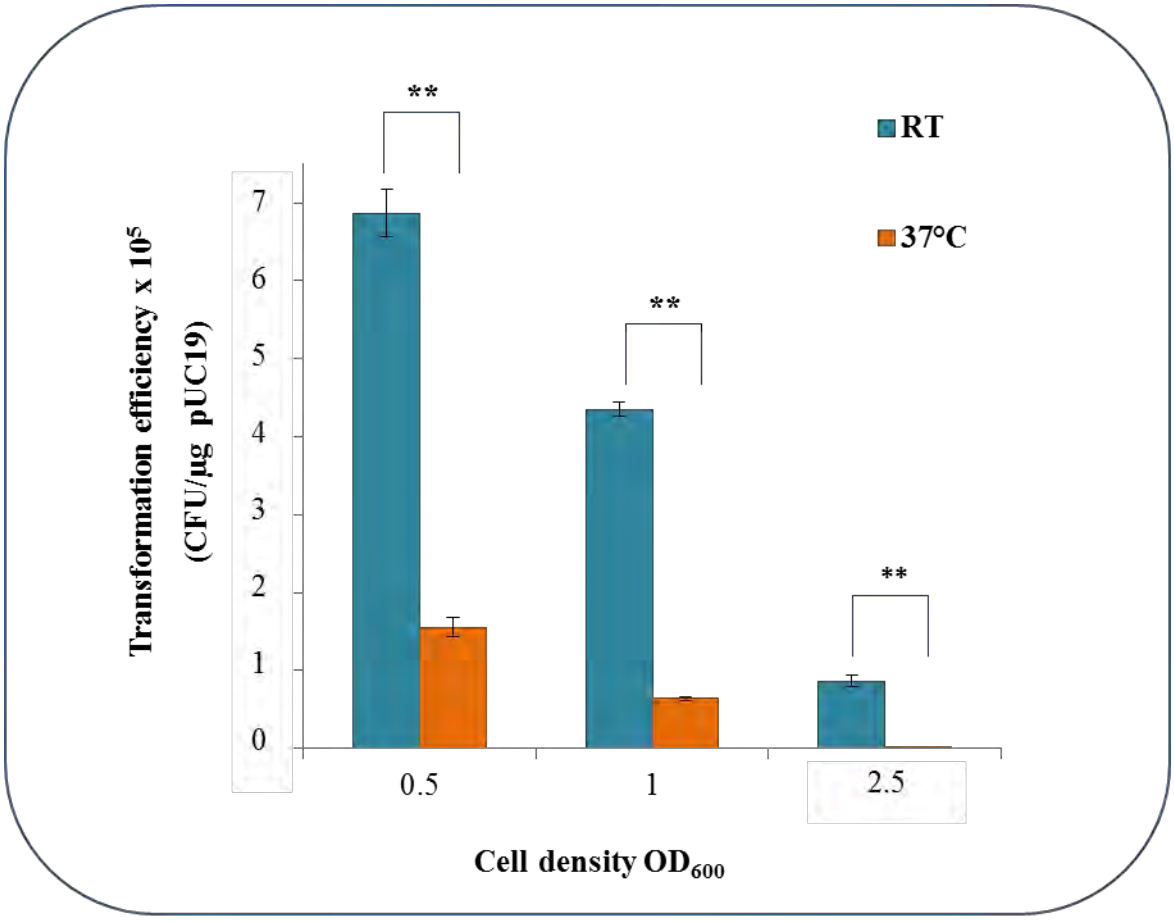


Figure 3.7: Optimisation of culture growth temperature

Transformation efficiencies of CCCs were compared using pUC19 DNA and a 50 seconds heat-shock treatment. Data are expressed as the mean of three independent experiments (n=3). Error bars show SE of the mean. **: significant difference as compared to cultures grown at 37°C to same density of cell populations, $P < 0.01$(t-test).

3.4.1.2 Effect of duration of heat-shock treatment on plasmid transformation

To determine the optimum duration of heat-shock treatment in EcN transformation, CCCs/pUC19 DNA mix were exposed to heat-shock at 42°C for time points, 50, 90 and 180 seconds, individually. The results are presented in Figure 3.8 and show an increase in transformation efficiency with an increase in heat-shock duration.

A prolonged heat-shock treatment of 180 seconds produced significantly higher transformation efficiency in low-density cell populations. The highest was ~20 $x10^5$ transformants/µg pUC19 DNA in cells grown at RT to an OD_{600} of 0.5 or 1.0, which is 3-5 times higher than when a 50 or 90 seconds heat-shock was used. In addition, this optimum yield represents a 23-fold increase in the transformation efficiency when compared to that of cells grown at 37°C, keeping other transformation factors constant. The heat-shock duration did not significantly affect the transformation efficiency in high-density cultures (2.5 OD_{600}), which also yielded the lowest number of transformants as compared to low-density cell populations.

While the prolonged heat-shock duration increased EcN transformation efficiency using pUC19 DNA, the converse was true for the *E. coli* cloning strains EPI300 and S17.1, as shown in Figure 3.9. Both EPI300 and S17.1 have shown optimum transformation when a 50 sec heat-shock treatment was used. At this minimum length of heat-shock exposure, transformation efficiency was 27.7 x 10^6 and 2.1 x 10^6 transformants/µg pUC19 DNA in EPI300 and S17.1, and then dropped to 16.8 x 10^6 and 1.7 x 10^6 transformants/µg pUC19 DNA, respectively when the heat-shock was increased to 180 seconds.

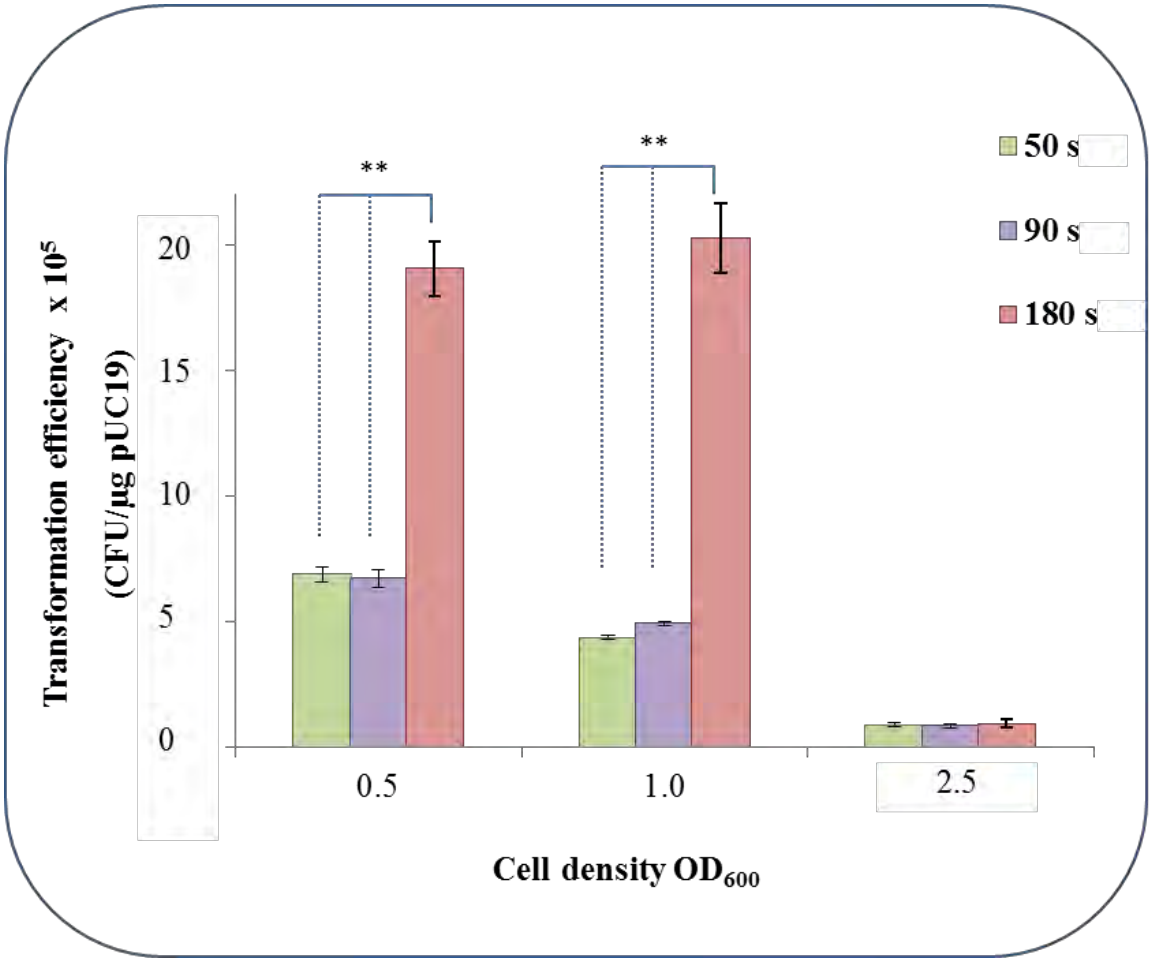


Figure 3.8: Optimisation of heat-shock treatment duration in *E. coli* Nissle

Competent cells were obtained from cultures grown at RT then transformed with pUC19 DNA and treated for various durations of heat-shock. Data are expressed as the mean of three independent experiments (n=3). Error bars show SE of the mean. **: significant difference in 180 seconds – treated cells as compared to other heat-shock durations at same density of cell populations, P< 0.01(t-test).

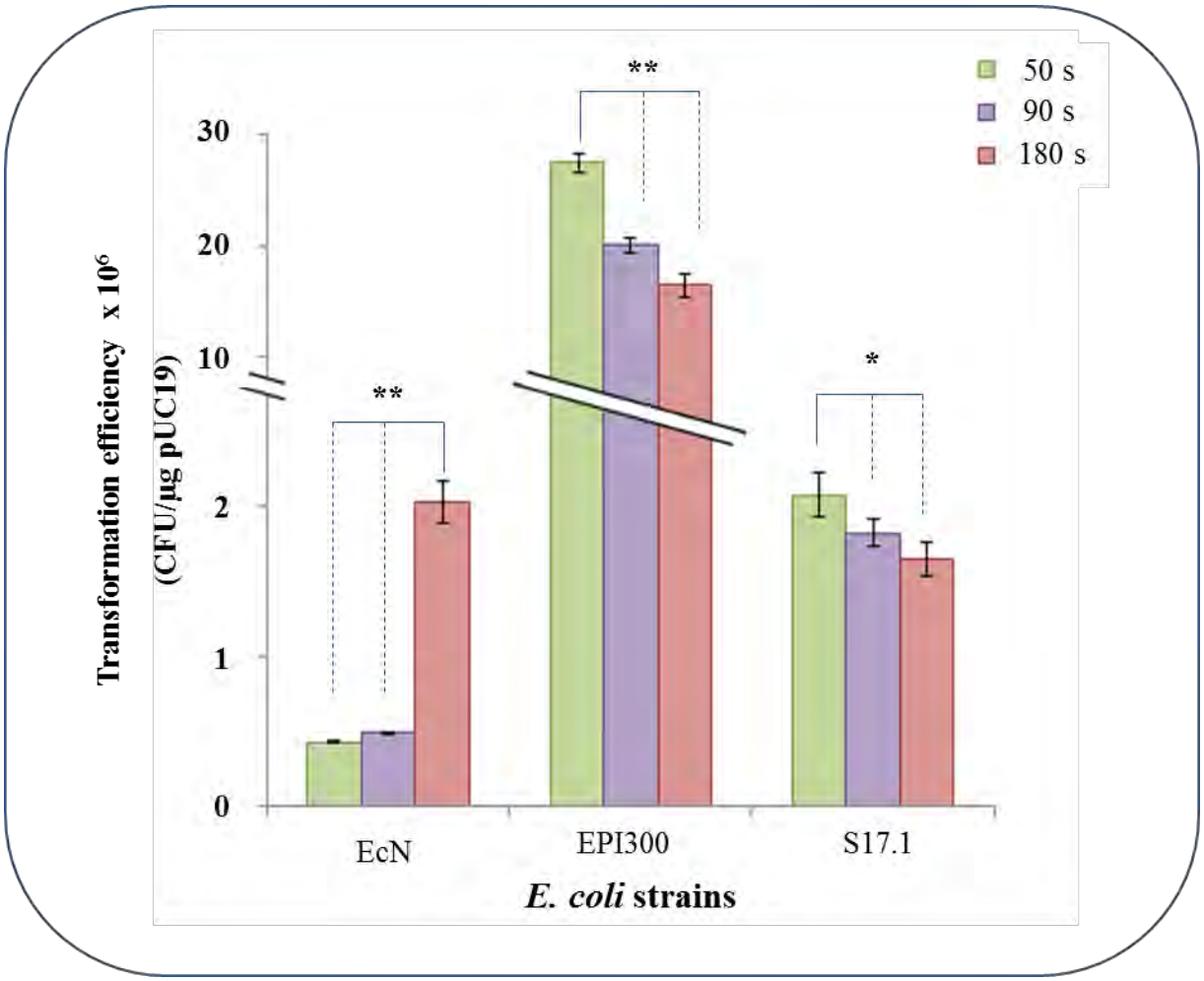


Figure 3.9: Effect of heat-shock treatment duration on other *E. coli* strains

Competent cells were obtained from cultures grown at RT to an OD_{600} of 1.0. Cells/pUC19 DNA mix treated for various heat-shock durations. Data are expressed as the mean of three independent experiments (n=3). Error bars show SE of the mean. **: significant difference in180 sec-treated cells as compared to other heat-shock durations at same density of cell populations with $P < 0.01$, *: with $P < 0.05$ (t-test).

All the optimised parameters combined together, applying a heat-shock of 180 seconds on chemically competent cells produced from low-density cultures grown at RT, was demonstrated to yield optimum transformation efficiency in *E. coli* Nissle. These conditions were subsequently chosen to establish EcN-specific transformation protocol for genetic manipulation using mini-Tn*5* systems.

3.4.2 Mutagenesis of *E. coli* Nissle 1917

Two mini-Tn*5* based systems for random transposon mutagenesis, pUTmini-Tn5Km2 (de Lorenzo *et al*. 1990) and pRL27::mini-Tn*5* (Larsen *et al*. 2002) were tested for the generation of mutants from *E. coli* Nissle 1917. Initially, plasmid delivery vectors pUT and pRL27 were introduced to chemically competent cells produced from EcN cultures using an optimized protocol of chemical transformation (Section 3.3.1.2). The pRL27 system generated transformant colonies on medium selective for the mini-Tn*5* (kanamycin 50µg/mL) with a yield of 624 transformants/µg pRL27::mini-Tn*5* DNA (also potential mutants), an improvement from only 21 transformants/µg pRL27::mini-Tn*5* DNA when the standard transformation protocol was used . However pUT failed to generate any kanamycin-resistant colony (transformant of pUT).

Plasmid pRL27::mini-Tn*5* was also transferred from donor *E coli* BW29427- *dap* auxotroph*, :: pir* to *E. coli* Nissle wild-type by conjugation, which was only possible with pRL27 but not pUT. The pRL27 system generated trans-conjugant colonies on medium selective for EcN harbouring mini-Tn*5* and counterselective for donor *E coli* BW29427 (with kanamycin 50 µg/ml, without diaminopimelic acid). The conjugal transfer resulted in ~2,400 trans-conjugants per plate-mating experiment, a yield that was relatively high as compared to the chemical transformation. This was therefore preferred for use in further steps testing the functionality of pRL27::mini-Tn*5* in EcN mutagenesis.

3.4.2.1 Verification of loss of mini-Tn*5* delivery vector pRL27

Ten trans-conjugants were randomly selected from L medium Km 50 (μg/mL) and used to confirm that the delivery vector pRL27 was not maintained after conjugal transfer to EcN. This was evaluated by restriction digest of plasmid extracts from trans-conjugants, for which the banding patterns were compared on agarose gel to those obtained from the digest of controls. The positive control was pRL27 vector alone (4.1 kb), whereas the negative consisted of extracts from "empty" unmated EcN which harbours two indigenous, cryptic plasmids pMUT1 and pMUT2, of 3.2 and 5.5 kb in size, respectively (Blum-Oehler *et al*. 2003).

The agarose gel results are presented in Figure 3.10 and demonstrate the absence of both the pRL27 and transposon fragment from *EcoR*I plasmid digests obtained from the trans-conjugants. Instead, the gel shows DNA banding patterns of the sample fragments to be identical (in size and number) to those of the negative control "empty" EcN wild-type, and not plasmid pRL27. This confirms that delivery pRL27 was not maintained in the trans-conjugants and was eventually lost. Moreover, the loss did not result in the Tn being inserted into the EcN cryptic plasmids, which would have altered the restriction profle of these plasmids. Therefore, pRL27::mini-Tn*5* could be used to successfully generate EcN transposon mutants.

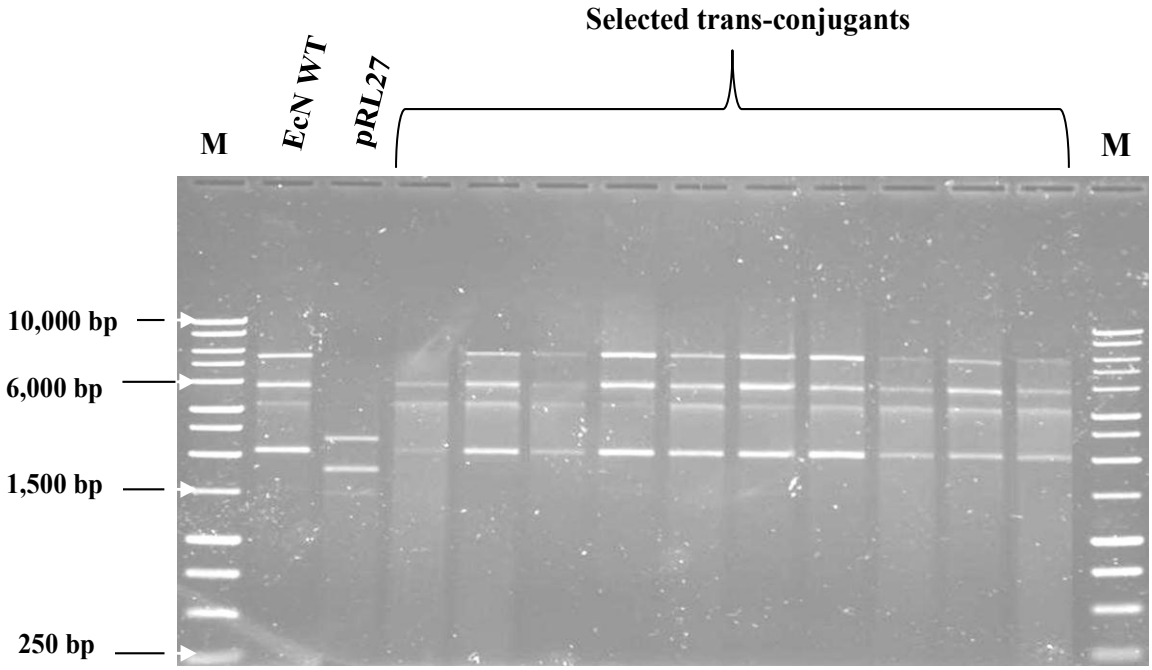


Figure 3.10: Verification for loss of pRL27 delivery vector in *E. coli* Nissle trans-conjugants

Replication of pRL27 delivery vector in EcN trans-conjugants (selected at random) and possible insertion of mini-Tn*5* into EcN cryptic plasmids were assessed by *Eco*RI enzyme restriction digests of plasmid DNA extracts from trans-conjugants (isolated from LB agar + Km 50 (μg/mL) and com ar ed to those from EcN and purified pLR27 DNA. Digests (250 ng DNA from each sample) were loaded and separated on a 1 % (w/v) agarose gel.

M=1kb Promeda DNA ladder.

EcN WT= plasmid from EcN (negative control)
pRL27 = purified plasmid pRL27 (positive control)
M=1kb Promeda DNA ladder

3.4.2.2 Verification of transposon presence in *E. coli* Nissle trans-conjugants

Trans-conjugants that had been used to confirm loss of the pRL27 vector were also used to verify the presence of mini-Tn*5*. Mini-Tn*5* was confirmed in all ten trans-conjugants by PCR analysis of Tn-specific sequence using primers NptIIF1 & NptIIR1 (Section 3.3.2.3) and chromosomal DNA extracts as template. The results obtained are presented in Figure 3.11 and indicate that the pRL27 delivery vector could have successfully introduced the mini-Tn*5* into *E. coli* Nissle by conjugation.

3.4.2.3 Verification of single and random insertion in *E. coli* Nissle genome

The trans-conjugants confirmed to have lost the pRL27 delivery vector and to be positive for mini-Tn5 based on *npt*II PCRs were also investigated for single, random insertion in the chromosome. *Pst*I digested genomic DNA extracted from trans-conjugants and EcN wild-type were separated by agarose gel electrophoresis (Figure 3.12a) and assessed for single and random insertion of Tn by Southern hybridisation with mini-Tn*5* specific probes (Figure 3.13). *Pst*I has no target site within the probe (*npt*II fragment of 676 bp), ensuring that a single transposition event could yield only one hybridisation fragment on the blot per genome. All the tested trans-conjugants showed hybridisation sized fragments of various sizes and indicated random Tn insertions, each as single copy per genome. Therefore, these results show that pRL27::mini-Tn*5* can be used to generate mutants with single transposon inserts in *E. coli* Nissle.

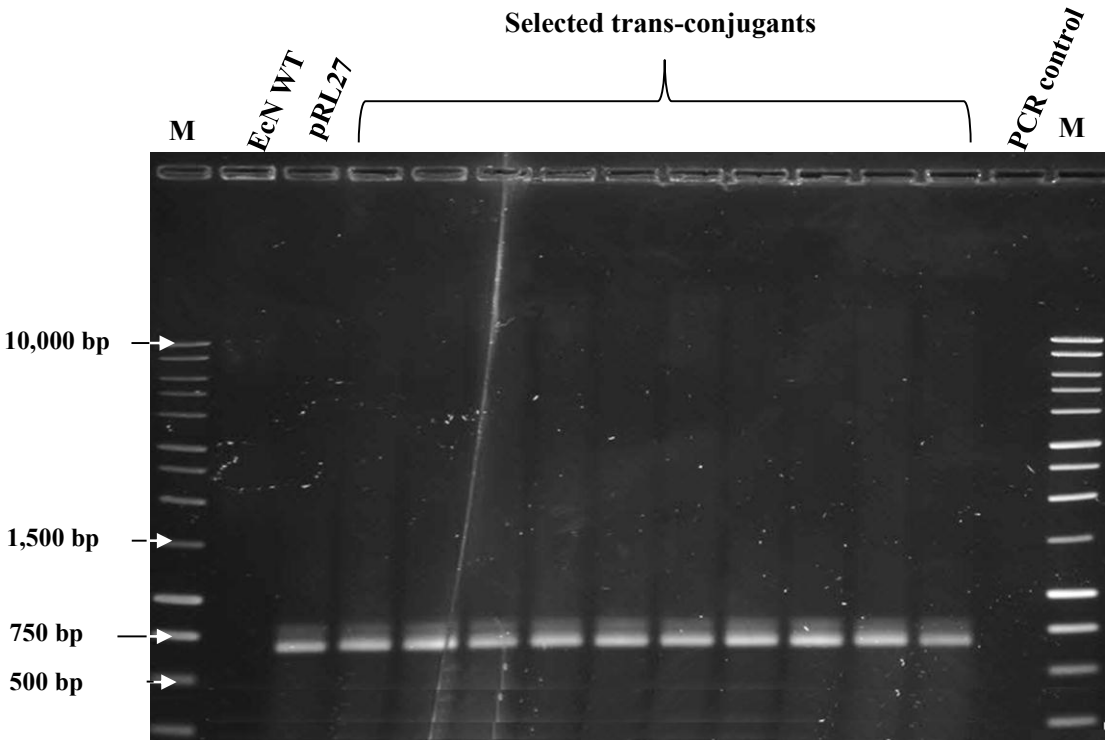


Figure 3.11: PCR confirmation of mini-Tn*5* presence in EcN trans-conjugants

Trans-conjugants (confirmed for loss of pRL27 vector) were used to verify presence of mini-Tn*5*Tn*5* intoEcN, by PCR with primers NptIIF1/R1using chromosomal DNA from trans-conjugants as templates. PCR products reveal ~ 670 bp, a segment of the mini-Tn*5* *npt*II gene in the positive control and transconjugants. PCR products were separated on a1 % (w/v) agarose gel.

EcN WT= genomic DNA extracted from EcN wild-type (negative control)
pRL27 = purified plasmid pRL27 (positive control)
PCR control =sterile deionised water
M=1kb Promeda DNA ladder

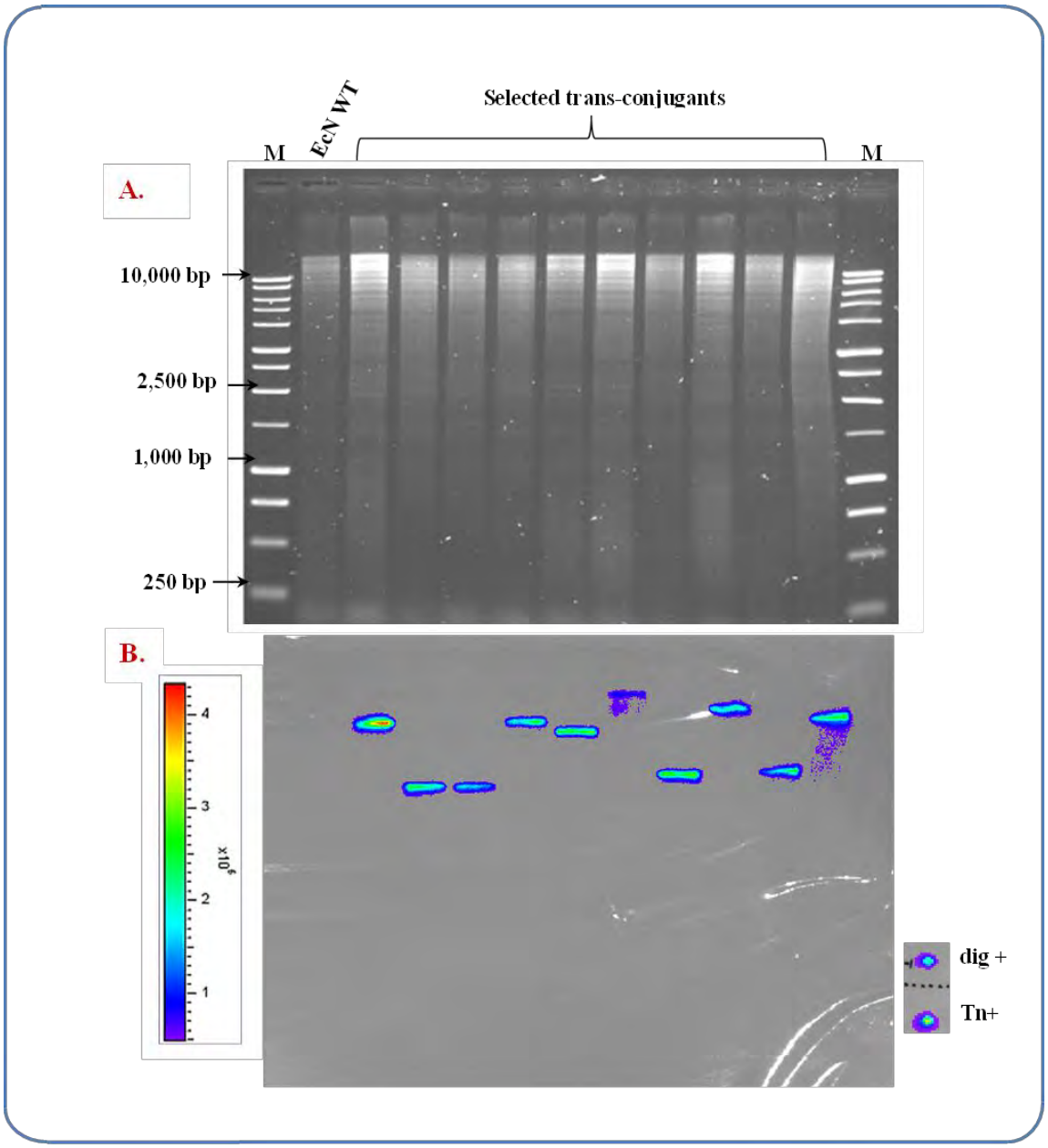


Figure 3.12: Assessment of single, random insertion of mini-Tn*5* in EcN trans-conjugants genomic DNA by Southern blotting

A: Total chromosomal DNA was extracted from trans-conjugants and EcN wild-type then was digested (2µg DNA from each sample) with *Pst*I and fragments separated on a 1% (w/v) agarose gel.
B: separated fragments were transferred onto nitrocellulose membrane then subjected by Southern hybridisation with a digoxigenin (DIG) - labelled *npt*II probe, and visualized using IVIS50.

M = 1kb Promega DNA ladder
EcN WT= genomic DNA extracted from EcN wild-type (negative control)
dig+ = digoxygenin labelled probe (assay positive control)
Tn+ = pRL27 plasmid DNA (sample positive control)

3.4.2.4 Identification of transposon-flanking regions in *E. coli* Nissle mutants

The mutants confirmed to possess single and random insertion of mini-Tn*5* by Southern hybridisation (designated JN1-10), were also used to ensure that identities of mini-Tn5 flanking regions could be revealed. Genomic DNA extracted from the mutants were used as tem late in a "cloning-free" AP-PCR –based and the results are shown in Figure 3.13. Sequences were obtained from the PCR products using Tn-specific primer (pLR27Primer3), analysed and identities of the gene disrupted in selected mutants are presented in Table 3.3. The PCR products produced abundant bands of products ranging in size from ~150-1100 bp on an agarose gel, each producing unique DNA banding patterns (indicative of Tn insertion at distinct chromosomal locations). After sequencing, nine out of the ten samples produced Tn-junction nucleotide sequences of lengths from 141-614 bp. Analysis of the sequences (using the BioEdit software) revealed good quality chromatograms, showing well-resolved peaks and with no ambiguities (indicative of the AP-PCR methods ability to identify genes flanking the transposon). Moreover, the sequences predominantly showed homology to other *E. coli* strains, with different gene identities (Table 3.3).

Taken together, these results from the Blastn search of the entire non-redundant database, and a wide range of functions represented reinforce: i) random insertion of mini-Tn*5*, ii) single insertion, and iii) pRL27 delivery vector was lost completely and did not co-integrate with transposon, which could have been revealed by gene identities.

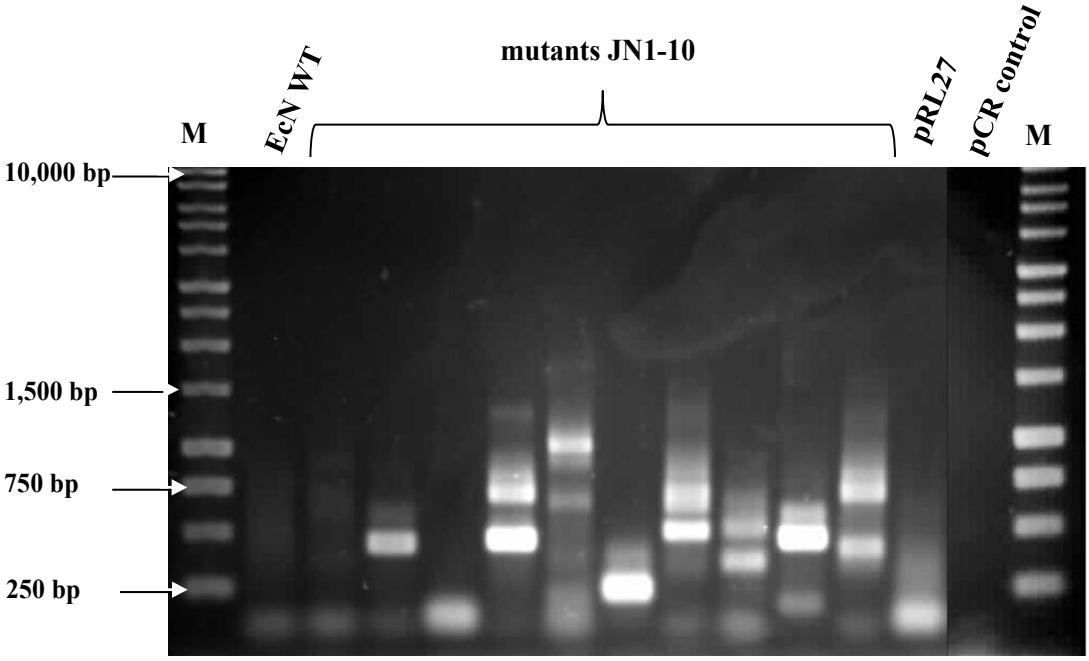


Figure 3.13: PCR amplification of regions flanking mini-Tn*5* insertion sites in *E. coli* Nissle mutants

The PCR products are of a second run of two PCRs of the AP-PCR method (detailed in Section 3.2.2.5). Primers pRL27Primer3 and Primer4 were used and DNA template obtained from products of first PCR. DNA fragments were separated on 1 % (w/v) agarose gel.

EcN WT= genomic DNA from EcN wild-type (negative control)
JN1-10 = genomic DNA extracted from mutants
pRL27 = purified plasmid pRL27 (positive control)
PCR control =sterile deionised water
M=1kb Promega DNA ladder

Table 3.3: Identification of genes flanking mini-Tn*5* insertion sites in *E. coli* Nissle mutants

Mutant	Length* (bp)	Homology (Blastn) ≠	Identity (%)	E-value§
JN1	141	DNA-binding transcriptional activator CadC: *E. coli* D i14	114/123 (93 %)	1e-40
JN2	347	Hypothetical protein: *E.coli* D i14	345/346 (99 %)	2e-178
JN3	16	No significant similarity found	-	-
JN4	407	Acetyl-CoA synthetase: *E.coli* D i14	398/402 (99 %)	0.0
JN5	517	Hypothetical protein: *E.coli* D i14	513/517 (99 %)	0.0
JN6	157	Membrane protein: *E. coli* JJ1886	157/158 (99 %)	6e-74
JN7	411	Putative symporter YidK: *E.coli* D i14	387/395 (99 %)	0.0
JN8	437	Conserved hypothetical protein: *E.coli* D i14	417/433 (96 %)	0.0
JN9	373	Hypothetical protein: *E.coli* D i14	365/368 (99 %)	0.0
JN10	614	Membrane protein FdrA : *E.coli* D i14	606/615 (98 %)	0.0

*: length of sequence searched (bp), ≠: "best hit" was selected based on highest bit score;

§ : the Expect (E) value is a parameter that describes the number of hits "expected" to see by chance when searching a database of a particular size. It decreases exponentially as the score (S) of the match increases.

3.5 Discussion

3.5.1 Optimisation of plasmid transformation in *E. coli* Nissle 1917

The introduction of plasmids into cells is an essential requirement for molecular cloning experiments in general and genetic manipulation of bacteria. Efficient transfer of plasmid into bacterial cells is highly critical in those vectors used for the construction of large-scale genomic libraries. In order to generate a good quality large random transposon mutant library, highly reliable and efficient strategies for the transfer of plasmid vectors into target cells are required. Chemical transformation is a good choice for this purpose in *E. coli* strains, especially when the conventional approach of conjugal transfer is not feasible.

The chemical transformation is a well-standardised method for a wide variety of *E. coli* host strains used in recombinant DNA experiments (Hanahan 1983, Van Die *et al.* 1983, Inoue *et al*. 1990, Sambrook and Russel 2001). However, the established standard transformation conditions for these cloning strains might not be suited for optimum transformation of individual wild-type strains. In this study, plasmid transformation efficiency in EcN wild-type was optimized by evaluating the effect of culture growth temperature, culture density and duration of heat-shock treatment on EcN transformability using pUC19 plasmid DNA. These three parameters were selected because of their widely reported critical significance to the transformation of *E .coli* (Hanahan 1983, Chung *et al.* 1989, Inoue *et al*. 1990, Casali, 2003, Tu *et al*. 2005).

Overall, the results obtained in this study indicate that CCCs produced from low-density cultures grown at RT, and treated with a prolonged heat-shock (180 seconds) were able to yield an optimum number of pUC19 transformants in EcN. A combination of RT growth and low-density cultures of OD_{600} of 0.5 at harvesting resulted in approximately 5-fold increase in transformation efficiency as compared to those grown at 37°C to the same

density (Figure 3.7). These findings are consistent with those of other studies involving some *E. coli* cloning strains which have shown that lowering culture growth temperature from the conventional 37°C to 18-20°C enhances DNA uptake (Van Die *et al*. 1983, Inoue *et al*. 1990); greater susceptibility to transformation were demonstrated in low-density (OD_{600}< 1) cultures harvested during an early phase of growth (Chung *et al.* 1989, Inoue *et al*. 1990, Tu *et al*. 2005).

Amongst the three parameters examined in this current study, the duration of the heat-shock treatment showed a substantial deviation from the standard transformation protocol in EcN cells. Various durations of heat-shock treatment ranging from 30 to 120 seconds at 42°C have been described (Chung *et al.* 1989, Sambrook and Russell, 2006, Roychoudhury *et al*. 2009) with an optimum transformation efficiency in most standard laboratory strains generally accepted to be achieved with a 30-50 seconds heat-shock (Chung *et al*. 1989, Sambrook *et al.* 1989, Inoue *et al*. 1990, Singh *et al*. 2010). This was also confirmed in for *E. coli* strains EPI300 and S17.1 in this current study, as both cloning strains demonstrated the highest transformation efficiency when a 50 seconds heat-shock was applied. Contrary, EcN required a longer heat-shock treatment of 180 seconds to achieve optimal transformation efficiencies. The observed "individual transformability r ofile" of EcN could be otentiall y associated either with this strains inherent ability to withstand harsh treatment during transformation or the expression of strain-specific features which might affect the uptake of foreign DNA.

Although heat-shock at 42°C is known to enhance the uptake of free DNA in *E .coli* CCCs (Mandel and Higa 1970, Inoue *et al*. 1990, Singh *et al*. 2010), it can also affect the viability of CCCs, which are already fragile following harsh treatments that include exposure to Ca^{2+} cations. Chung *et al*. (1989) have reported a decrease in *E. coli* JM109 transformation efficiency when cells were treated with a heat-shock of 45°C. They concluded that the

heat-shock step was deleterious to cells, and could even be unnecessary. This might explain the observed decrease of transformation efficiency in *E. coli* strains EPI300 and S17.1 in heat-shock duration dependent pattern, in this study. By contrast, the increase in EcN transformability when the heat-shock was extended to 180 seconds could suggest that the strain was robust enough to survive the process better than EPI300 and S17.1. This assumption could have been verified if the experiment used for this current study would have been designed to investigate the transformation frequency (transformants per total viable cells) rather than the efficiency (transformants per unit weight of DNA).

Strain-specific features such as components of the cell surface structures can potentially play a role in DNA binding and translocation across the membrane of CCCs (Sarkar et *al.* 2002). EcN expresses a special truncated variant of lipopolysaccharide (LPS) at the cell surface that was also found to be responsible for a semi-rough colony morphology of this strain (Grozdanov *et al*. 2002). This phenotype might offer a protective structure, maintaining the integrity of the cell membrane during the transformation process, without blocking the DNA uptake. The structure and length of LPS have been demonstrated to play an important role in chemical transformation of *E.coli*, where a deep-rough colony morphology tend to be more protective but less permeable to foreign DNA as compared to those with a smooth colony morphotype (Chang *et al.* 2010). Furthermore, EcN produces a K5 capsule type belonging to group 2 capsules, which can only be expressed in cultures grown at temperatures above 20°C (Whitfield and Roberts 1999, Rowe *et al*. 2000). The role of the capsule in *E. coli* transformation is not clear yet, but it can be suggested that decapsulated cells, like those grown at RT could be more competent, leading to higher transformation efficiency.

The less domesticated nature of EcN as a wild-type strain as compared to the widely used laboratory strains could also help to explain its transformability profile. These strains have

been explicitly selected for amenability to molecular genetic manipulations (Sambrook and Russell 2001, Casali 2003), whereas the primary interest in EcN is the reported probiotic effect (Boudeau *et al*. 2003, Sonnenborn and Schulze 2009). The most obvious difference between wild-type and standard cloning strains of *E .coli* is that the latter have often undergone extensive manipulations in order to improve their cloning and transformation efficiencies, as well as their ability to maintain the integrity of the transformed plasmid (Casali 2003).

Such improvements may include removal of the endogenous restriction enzymes, methylation systems, foreign DNA recognition systems, and some regulatory gene systems that hinder replication of foreign plasmids (Russell and Zinder 1987, Raleigh 1992, McClelland *et al.* 1994, Casali 2003). These features are likely to be intact in *E. coli* Nissle wild-type, which was found to have a particularly high abundance of genes coding for type I and II restriction-modification systems (Sun *et al*. 2005, Vejborg *et al.* 2010b). A prolonged heat-shock has been demonstrated to temporarily inactivate some of these systems in a variety of bacterial strains, allowing efficient uptake of foreign DNA (Raja and Dharmalingam 1991, Edwards *et al.* 1999). Extending heat-shock treatment of EcN cells to 180 seconds could have resulted in the disruption of these restriction-modification systems, therefore improving the efficiency of transformation as observed in this current study.

3.5.2 Mutagenesis of *E. coli* Nissle

The availability of Tn*5*-based random mutagenesis systems (Simon *et al.* 1989, de Lorenzo *et al.* 1990, Herrero *et al.* 1990, Larsen *et al.* 2002) has revolutionised bacterial functional genomics by facilitating the discovery of novel genes in bacteria associated with human health, mainly those involved in virulence (Hensel and Holden 1996, Jones *et al*. 2004, Autret and Charbit 2005, Hadjifrangiskou *et al*. 2012), and most recently in probiotic effects (Lasaro et *al.* 2009, Ito *et al.* 2010, Ruiz *et al.* 2013). In this study, two mini-transposon delivery systems, pUTmini-Tn*5*Km2 and pRL27::mini-Tn*5*, were evaluated for use in probiotic *E. coli* Nissle using two methods of plasmid transfer into cells, transformation and conjugation. While pRL27::mini-Tn*5* was able to produce kanamycin-resistant colonies of *E. coli* Nissle in both conjugation and chemical transformation, the pUT system failed to produce any such colonies by transformation. The observed difference in the ability of both systems to deliver the transposon into *E. coli* Nissle cells by transformation is likely to reflect their distinct functional features.

The pUTmini-Tn*5*Km2 system is a member of the pUT-mini transposon series constructed by de Lorenzo *et al.* (1990) and Herrero *et al.* (1990). It consists of a mini-Tn*5* kanamycin transposable unit (Km^R gene flanked by two 19-bp inverted repeats) that is contained in pUT vector. The vector carries genes that encode functions for the Tn delivery into the target strain, including transposition functions (*tnp* gene placed outside of the transposable unit), mobilization functions (*oriT* for conjugal transfer), and suicide delivery properties based on the conditional origin of replication, the *ori*R6K that allows stable maintenance of the plasmid vector in λ ir lysogenic *E. coli* host strains, but not recipient target strains (Herrero *et al.* 1990). This system has been widely used to generate chromosomal insertion mutations in a variety of wild-type *E. coli* strains (Martindale *et al.* 2000, Tatsuno *et al.* 2000, Dziva *et al.* 2004, Li *et al.* 2005, van Diemen *et al.* 2005, Roca *et al*. 2008). In all

these studies, pUTmini-Tn*5*Km2 has been delivered into cells by conjugation. There is no report on the pUTmini-Tn*5*Km2 transfer by transformation.

The inability of this system to produce kanamycin-resistant transformants in EcN (despite using an optimised transformation protocol for this strain, described in Section 3.3.1) could suggest that chemical transformation was unsuitable for the transfer of the pUT delivery vector into cells. However, because this plasmid does not replicate in the EcN recipient, the suitability of the transformation method could not be determined. Since no evidence of successful transfer of pUTmini-Tn*5*Km2 into EcN cells was obtainable, the failure of transposition can not be ruled out.

Low transposition efficiency of pUT mini-Tn*5* constructs has been reported in some bacteria, for which random mutagenesis remains difficult and unpredictable (Larsen *et al.* 2002). It was suggested that this problem could be a result of a combination of poor enzymatic activity of the Tn*5* transposase and low binding affinity between the enzyme and inverted repeats (IRs) recognition sequences, which are used by these systems (Zhou et *al.* 1998, Goryshin and Reznikoff 1998). Larsen *et al.* (2002) addressed these issues with the development of pRL27::mini-Tn*5*. The pRL27 construct features the majority of genes found in pUTmini-Tn5Km2 (*oriT*, *ori*R6K, Km^R) but carries a modified, hyperactive mutant version of the *tnp* gene encoding a transposase that is 1,000-fold more active than the wild-type transposase (Zhou and Reznikoff 1997, Goryshin and Reznikoff 1998). In addition, the IRs ending the mini-Tn*5* element used in pRL27, have been optimised to improve the binding efficiency of the Tnp, resulting in additional efficiency of the transposition (Zhou *et al*. 1998).

The main distinctive features of the pUT and pRL27 delivery vectors and potential effects of these on mutagenesis are summarised in Table 3.4. Besides the hyperactive

Table 3.4: Summary of distinctive features of pLR27::mini-Tn*5* and pUTmini-Tn*5*Km2 and impact on transposon mutagenesis of *E.coli*

	pLR27::mini-Tn5	pUTmini-Tn5Km2	Impact on mutagenesis	reference
Transposable element	(Km^R + *ori*R6K) flanked by optimized* inverted repeats (IRs), size of Tn ~1.5 kb	Km^R flanked by IRs, size of Tn ~1.7 k	- pUT delivery vector likely to co-integrate with transposon - Optimised IRs increases Tnp binding efficiency and minimize the probability of pRL27 co-integration - The *ori*R6K inside Tn facilitates cloning and identification of the regions flanking Tn insert	de Lorenzo *et al.* 1990; Larsen *et al*, 2002 ; Zhou et *al.* 1998 ; Dennis and Zylstra 1998
Transposase	hyperactive ‡ *tnp* gene 1,000-fold more active than the wildtype Tnp *tetA* promoter (*tetA*p) for expression of *tnp*	*tnp* gene, same activity as Tn*5* wild-type	- Higher transposition efficiency and frequency in pRL27 system - *tetA*p::*tnp* allows transposition in in a wide variety of bacteria	Goryshin and Reznikoff 1998
Host strain	*E. coli* S17.1, λpir lysogen producing π protein for replication of vector plasmids (with *ori*R6K) in host but not recipient	*E. coli* BW29427, auxotrophic to DAP, carries :: *pir* gene in chromosome also providing π protein to host	- *E. coli* S17.1 can release λ*pir* phage to infect the recipient strain by integrating its chromosome then allow replication of the plasmid - The :: *pir* gene remains stable in host strain - DAP auxotrophy allows counter-selection for donor	de Lorenzo and Timmis 1994 ; Ferrières *et al.* 2010

*: optimised by point mutations into 19bp IRs sequences of Tn*5* wild-type, screening of mutants and isolation of those showing enhanced recognition and binding to Tnp (Zhou et *al*. 1998) ‡**:** hyperactive Tnp is a result of three mutation in *tnp* wild-type: i) enhancing binding activity of Tnp, ii) blocking the synthesis of Inh, and iii) enhances Tnp activity possibly by increasing dimerization of the bound Tnp (Goryshin and Reznikoff 1998).

transposition, the improved affinity of IRs for Tnp can enhance the excision of transposon from the pRL27 replicon resulting in authentic transposition of the mini-Tn*5* into the target chromosome, as opposed to co-integration of the whole plasmid vector. Formation of co-integates of the pUT delivery vector into the bacterial chromosome has been reported in mutagenesis-based studies (Herrero *et al.* 1990, de Lorenzo *et al*. 1993, Jungnitz *et al.* 1998), and is therefore a drawback to the use of pUTmini-Tn5Km2.

In addition to the cleavage of the donor replicon, the hyperactive transposase increases the efficiency of the Tn chromosomal integration in the pRL27 system. The improved efficiency of transposon integration can increase the chances of getting Tn insertion mutations, even when the delivery vector is transferred into cells using other methods than the conventional conjugal transfer. This accounts for the successful use of pRL27 in mutagenesis-based studies of *E. coli* strains using electroporation (Bradshaw *et al.* 2003, Kouzminova and Kuzminov 2004, Shi et *al*. 2005, Lukas and Kuzminov 2006, Ting *et al*. 2008, Rotman *et al*. 2009), in stark contrast to the use of electroporation with pUT systems (de Lorenzo *et al*. 1998, de Lorenzo *et al*. 1994).

Southern blot analysis of chromosomal DNA from ten trans-conjugants selected at random and not based on phenotype, confirmed a single transposition event per genome as well as randomness of transposon insertion. Both characteristics are required for good quality mutant libraries, and could reflect the suitability of the pRL27 system for the generation of a large-scale random transposon mutant library of EcN. Furthermore, the 'cloning free' A-PCR method was proven successful in revealing the identity of transposon-flanking genes showing homology to sequences from *E. coli* strains within a range of 93-99 % identity.

Whereas these results confirm single and random insertion of the mini-Tn*5*, the small simple size of mutant used in this study may not indicate the occurrence of hotspots in the

EcN genome. Ting *et al.* (2007) and Rotman *et al*. (2009) reported hotspots for the pRL27 mini-Tn*5* insertion in the *E.* coli chromosome, following high-throughput screens of more than 200,000 mutants in each case. The hotspots consisted of preferential insertion of the transposon in a segment of DNA rich in genes associated with homologous genetic recombination and DNA repair (Ting *et al.* 2007), and lipopolysaccharide (LPS) biosynthesis genes (Rotman *et al*. 2009). However, this problem was not considered to be a major limiting factor for the use of pRL27, since the mutants of interest were successfully isolated and characterised in both studies mentioned.

Another important consideration from previous studies that have employed pRL27 for random mutagenesis of *E coli*, is the characteristics of mutations and consequences of the Tn insert on gene expression in various mutants. Chromosomal integration of the mini-Tn*5* has been demonstrated to cause complete gene inactivation, leaky mutation, polar effects on the downstream genes, antisense effects on the upstream genes, inactivation of the regulatory C-terminal domains of proteins, and gene overexpression, in several mutants showing phenotypic alterations **(**Bradshaw *et al.* 2003, Kouzminova and Kuzminov 2004, Kuzminov *et al*. 2004, Shi *et al*. 2005, Lukas and Kuzminov 2006, Ting *et al*. 2008). The ability of Tn insertional mutagenesis to generate such a diversity of mutations can be an advantage, especially in discovering the unexpected association of multiple genes in a given phenotype or pathway. However, establishing definitive functions from putative functions of the gene of interest requires supplementary molecular-based techniques. These can include gene deletion and complementation assays, as well as gene expression studies in order to close the gap in the data generated by random transposon mutagenesis.

Having discussed the benefits and suitability of mini-Tn5 systems, it is also important to address a potential disadvantage of this approach for their utilization in elucidating host-microbe interactions in EcN. Transposons can be lethal to cells when inserted into

essential-genes for bacterial growth under standard laboratory conditions. This makes essential genes hard to study genetically as insertion mutations in them result in non-viability. Essential genes can also be involved in other functions including those related to host-microbe interactions in probiotics. For example, Gardes *et al.* (2003), using a genetic footprinting technique for a genome-wide screening, reported 17 % of 3,746 functional genes of *E. coli* MG1655 to be essential for robust aerobic growth of this strain in rich media. By employing single gene deletion strategies, Baba *et al.* (2006) identified 7 % of 4,288 targeted genes of the *E. coli* MG1655 to be essential for aerobic growth at 37ºC in complex media. Genes included in bacterial cell surface biosynthesis are likely to be dispensable for bacterial growth (Thanassi *et al*. 2002, Sperandeo *et al*. 2007, Babu *et al*. 2011) but can also potentially play a role in host-microbe interactions (Lebeer *et al.* 2010).

Overall, these findings prescribe an optimised protocol for efficient plasmid transformation of probiotic *E. coli* Nissle based on standard chemical transformation, and confirms the functionality and suitability of the pRL27 mini-Tn*5* system for large-scale random transposon mutagenesis of this strain, as well as the approach to identify the genes disrupted in mutants. This outcome provides a powerful tool for genetic manipulation of *E. coli* Nissle that can facilitate studies investigating host-microbe interactions in this strain.

3.6 Conclusions

- An optimised protocol for low-cost, efficient transformation of *E. coli* Nissle 1917 (EcN) based on standard chemical transformation has been established. This protocol was confirmed to improve the transfer of pRL27::mini-Tn*5* into EcN cells.

- The pRL27 suicide delivery vector and the mini-Tn*5* transposon can be used to generate random, single-insert transposon mutants in *E. coli* Nissle 1917 and 'cloning free' arbitrary PC -based protocol can also be used to identify transposon insertion sites.

Chapter 4: Phenotypic characterisation of *E. coli* Nissle 1917 wild-type and identification of genes relevant to gut survival and host-microbe interactions

4.1 *E. coli* Nissle 1917 probiotic phenotypes

Numerous studies have investigated phenotypic characteristics of the *E. coli* Nissle 1917 (EcN) (Blum-Oehler and Harker 1995, Blum-Oehler *et al.* 2003, Grozdanov *et al*. 2004, Sun *et al*. 2005, Hancock *et al*. 2010a, Hancock *et al*. 2010b, Vejborg *et al*. 2010). Surprisingly, this probiotic strain has demonstrated high levels of homology (at genome level) to some members of the uropathogenic *E.* coli (UPEC) but without expressing virulence factors common among this group (Grozdanov *et al*. 2004, Sun *et al*. 2005). Instead, comparative studies involving EcN and closely related *E. coli* strain, have shown this probiotic to exhibit enhanced abilities in the following characteristics: i) attachment to abiotic and biotic surfaces at 37ºC (Hancock *et al*. 2010a, Hancock *et al*. 2010b, Vejborg *et al.* 2010), ii) colonisation of germ-free (GF) mice gut (Schulze *et al.* 1992, Denou *et al*. 2009, Leatham *et al*. 2009), and iii) higher expression of anti-inflammatory activity on human epithelial cells (Kamada *et al*. 2008).

Moreover, EcN has been shown to survive and transiently colonise animal models as well as human host during passage through the gastro-intestinal tract (GIT) (Lodinova-Zadnikova and Sonnenborn 1997, Prilassnig *et al*. 2007, Schultz *et al*. 2009). Combined together, these characteristics are most likely to contribute to the EcN's performance in the gut and delivery of beneficial effects on host. Unfortunately, the genetic determinants of survival, colonisation and host-interactions are still poorly understood in this probiotic strain. A clearer knowledge of these aspects is fundamental to the application of probiotics as health-promoting agents, and further development of novel, more effective and targeted probiotic therapies.

4.1.1 Genetic determinants of the phenotypes specific to *E. coli* Nissle 1917

The probiotic nature of EcN is determined by a unique genetic make-up that distinguishes this strain from the rest of the *E. coli* group. Table 4.1 highlights basic genetic and phenotypic characteristics of EcN, including those contributing to its probiotic effects. Genomic analysis of EcN has revealed the presence of several determinants (genes) encoding virulence (i.e. adhesions, proteases, toxic protein common to pathogens) but without being functionally expressed at phenotypic level (Grozdanov *et al*. 2004, Vejborg *et al*. 2010). It was suggested that such genes, possess premature stop codons, deletions, or insertions, hence rendering them non-functional (Grozdanov *et al*. 2004, Vejborg *et al*. 2010). This indicates that EcN had evolved from a virulent parent into an avirulent progeny through minor genetic variations and subsequent loss of gene expression, rather than a major shift in genomic content.

Nevertheless, the genomic content cannot be disregarded when studying the beneficial effects of EcN. Using genome sequencing and *in silico* reconstruction appproches, Sun *et al.* (2005) have revealed a total of 108 strain-specific coding sequences (CDs) in EcN as compared to five closely related *E. coli* strains. The enzymatic functions assigned to those CDs included the biosynthesis of components and proteins associated with the bacterial outer membrane, as well as regulation of secretory systems that could be underlying beneficial traits in EcN (Sun *et al.* 2005).These findings indicate that further phenotypic characterisations of EcN could help to elucidate genes regulating host-probiotic interactions.

Table 4.1: Basic microbiological and molecular genetic characteristics of *E. coli* Nissle 1917

Characteristics	Gene products/ biosynthesis results	Genes	Phenotype present
Adhesion	Type I fimbriae (F1A)	*fim*	+
	F1C fimbria	*foc*	+
	Curli fimbria	*sfa*	+
Iron acquisition (siderophores)	Enterobactin	*ent*	+
	Salmochelin	*iro*	+
	Aerobactin	*aer*	+
	Yersiniabactin	*ybt*	+
	Hemin uptake system	*chu*	+
	Citrate uptake system	*cit*	ND
Other characteristics associated with probiotic effects and survival in gut	Capsule(K5-type) ‡	*kfi/kps*	+
	Flagella (H1-type)	*fla*	+
	Arginine dighydrolase¥	*NA*	+
	Cellulose biosynthesis	*bcs*	+
	Microcin H47	*mch*	+
	Microcin M	*mcm*	+
	LPS core	*wa**	+
	LPS O6 repeating unit	*wb**	+
	O6 antigen polymerase, non functional	*wzy*§	+

+, present; ND, not determined; NA, no specific DNA probe available; ‡: present in 1 % of *E.* coli isolates;: ¥ present in 7 % of *E.* coli isolates; §:wzy gene truncated because of integrated stop codon.

This table was adapted from Sonnenborn and Schulze (2009**)**.

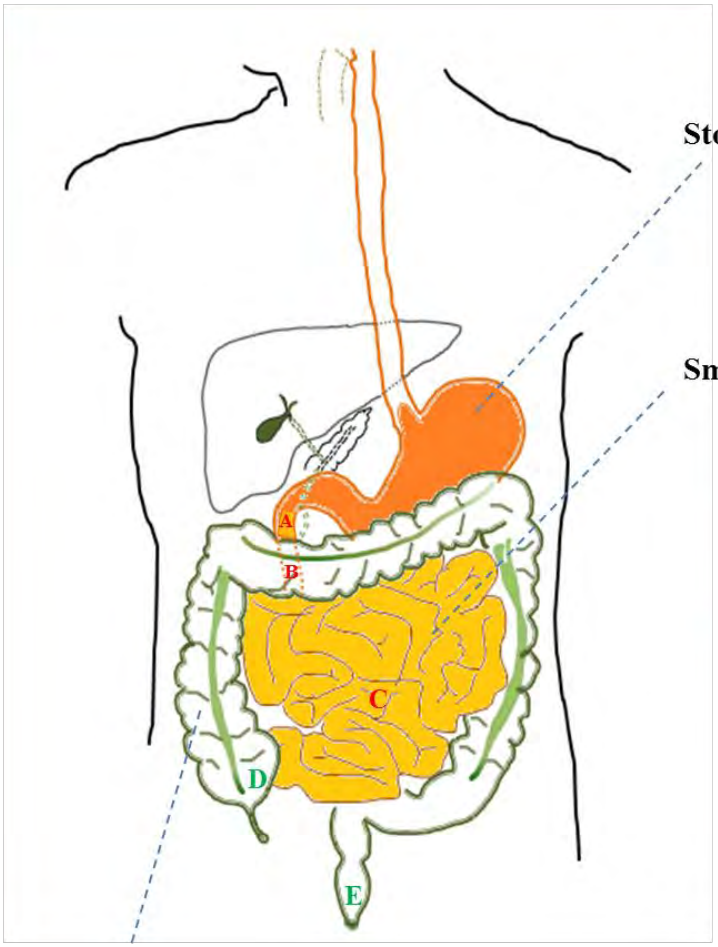
4.1.2 The human gastro-intestinal milieu and survival probiotic bacteria

The physiology and composition of the human gastro-intestinal tract (GIT) is important to consider when studying host-microbe interactions in probiotics, and EcN. In particular, these can help to determine the survival and performance of the probiotic *in vivo*. In the healthy adult, the GIT consists of an epithelium-lined tube of approximately 5.5 metres long and is highly complex throughtout, both in digestive processes and in chemical composition of the milieu (Pocock *et al*. 2013). Its accessory organs (i.e. the pancreas, liver, and the biliary system) as well as gastric epithelial cells (parietal cells), contribute to the composition of the gastro-intestinal milieu through secretion of enzymes, gastric acids, and bile.

4.1.2.1 Characteristics of the GIT with relevance to probiotic survival

The characteristics of the human gut that may create a hostile environment for probiotic survival and establishment are highlighted in Figure 4.1. Of particular interest is the low pH of the stomach (1.5- 3.0) from secretion of hydrochloric acid (HCl) by parietal cells. HCl permeabilises Gram-negative bacteria (including EcN) by disrupting the outer membrane of cells (Kanjee and Houry 2013). Bile also is a serious obstacle to probiotic survival. It is secreted by the liver then released into the duodenum of the small intestine up to as much as a litre each day (Begley *et al*. 2005). The major constituents of bile include bile acids that primarily exert their effects on the bacterial cell membrane, by emulsifying and solubilizing its lipids (Begley *et al*. 2005).

EcN has shown the ability to survive passage through the stomach and establish the intestinal tract of human infants for up to 24 months after administration (Sonnenborn and Schulze 2009). However, the genetic determinants of this robustness in the gut milieu (acids and bile remains) remains poorly investigated overall. Bacterial mechanisms of tolerance /resistance to bile acids and major acids of the human gut have been studied in



Stomach:
gastric juices, HCl
pH 1-3
0-10^2 bacteria/ mL
Lactobacillus, Candida, Streptococcus, Helicobacter pylori, Peptostreptococcus

Small intestine:
bile, pancreatic juices

A: Duodenum (25 cm)
pH 5.7-6.4
~10^3 bacteria/ mL
Lactobacillus, Streptococcus

B: Jejunum (1.0 m)
pH 5.9-6.8
~10^4 bacteria/ mL
Lactobacillus, Streptococcus, Veillonella

C: Ileum (2.0 m)
pH 7.3-7.7
10^6 - 10^8 bacteria/ mL
Enterobacteria, Enterococcus, Bacteroides, Clostridium, Lactobacillus, Veillonella

Large intestine:
fermentation of indigestible carbohydrates

D: Cecum/ colon (150 cm)
pH 5.7-6.8
~10^{11} bacteria/ g

E: Rectum
pH 6.6-6.8

Bacteroides, Eubacterium, Bifidobacterium, Ruminococcus, Peptostreptococcus, Propionibacterium, Clostridium, Lactobacillus, Escherichia, Streptococcus, Methanobrevibacter

Figure 4.1: Characteristics of the human gastro-intestinal tract milieu

Anatomic, physiologic and microbiologic aspects affecting the survival and gut establishment of the ingested probiotics.

This diagram was adapted from Ridlon et *al.* (2006) and Sartor (2008).

various enteropathogenic bacteria including *E. coli* (Lin *et al*. 1996, Gunn 2002, Begley *et al.* 2005, Kanjee and Houry 2013). Nevertheless, such investigations are yet to be conducted for EcN.

While the survival of enteropathogens in the human gut is undesirable, that of a probiotic is an important characteristic relevant for long-term persistence in the gut and delivery of beneficial effects to the host. For example, the expression of RfaH protein (a well-known virulence regulator of many enterobacteria) by EcN was found to enhance both this strain resistance to bile and the efficient colonisation of the mouse gut (Nagy *et al.* 2002, Nagy *et al.* 2005). Thus, strategies that facilitate elucidation of further genetic determinants of EcN survival in the gut are required.

4.1.2.2 Effects of dietary factors on probiotics

Dietary components constitute another important factor that might help to determine the genetic basis of probiotic survival during their transit through the different sections of the GIT. These might include food supplements such as salts that might exert osmotic effect on the probiotic cells, resulting in plasmolysis (Koch 1984). Complex non-digestible dietary carbohydrates (CHOs) (resistant to gastric acidity and enzymatic hydrolysis) as well as host-derived glycans (i.e mucin) in the human intestine can have a huge effect on probiotic survival and proliferation in the gut. Those probiotics that are able to produce degradative enzymes and other metabolic or fermentative capabilities for these components can compete better in the gut with the rest of the gut microbial communities, during periods of reduced energy supply (Flint *et al*. 2012).

Members of bifidobacteria and lactobacilli are most known to ferment non-digestible CHOs such as fructooligosaccharides (FOS) (Gibson and Roberfroid 1995). In contract, EcN (like other members of *E. coli*) is thought to lack metabolic capacities for non-

digestible CHOs (Sonnenborn and Schulze 2009). Nevertheless, metabolic capabilities of EcN for gastro-intestinal mucin are still not yet clearly investigated and a better understanding on this property could help to clarify the mechanisms of host-microbe interactions.

4.1.3 Bacterial components with relevance to host-probiotic interactions

Besides the ability to survive the human gut environment, probiotics must become established transiently in the intestine in order to effectively interact with the human host. Probiotics can easily colonise the mucus layer overlaying epithelial cells of the small and large intestines (Kaper and Sperandio 2005). Gut microbes interact with the epithelium using various components, including bacterial surface structures, extracellular secreted molecules (often proteins), fermentation products (such as short-chain fatty acids), and bacterial DNA (Walker 2008, Lebeer *et al*. 2010, Madsen 2011, Gagic *et al*. 2013).

Bacterial surface structures have been suggested to play a significant role in probiotic – host intestinal epithelial cell (IEC) interactions (Lebeer *et al*. 2010). This is partly because various macromolecules of the bacterial cell surface can serve as ligands that interact with host pattern recognition receptors (PRRs) of the IECs. PRR is a primitive part of the innate immune system and thought to have evolved before other types of immune system (i.e adaptative) are in place Medzhitov (2007). Moreover, bacterial cell surface structures can play an important role in probiotic gut colonisation by acting as adhesions.

Many *E. coli* (commensal and pathogens) expresses surface adhesins, non-adhesive cell surface com onents and secretory r oteins that mediate interactions with host's e it helial cells. These include cellulose, fimbriae adhesins, short adhesins such as protein Ag43, flagella, capsules, intimin, lipopolysaccharides (LPS), and many other components that to

date remain uncharacterised (Jarvis *et al*. 1995, Kenny *et al*. 1997, Oshima *et al*. 2008). Interestingly, probiotic EcN produces most of these components, and these are thought to be involved in this strain's successful colonisation of the gut and interaction with the host (Grozdanov *et al.* 2004, Sun *et al*. 2005, Lasaro *et al*. 2009, Hafez *et al*. 2005). There is currently a lack of strategies and tools that can facilitate studies of unknown genetic basis of probiotic cell surface structures using *in vitro* cells or animal models. Hoverer various cell surface molecules and structures of *E. coli* have been implicated in biofilm formation on abiotic surface (Van Houdt and Michiels 2005). Therefore, investigating biofilm formation may be one strategy by which genetic determinants of EcN relevant to host microbe interactions may also be studied.

4.2 Aims

The aims of the work presented in this chapter are to explore the genetic basis of phenotypes relevant to host-microbe interaction in *E. coli* Nissle 1917 using a random transposon mutagenesis approach.

4.3 Material and methods

Bacterial growth media and supplements were purchased from Fisher Scientific, UK unless otherwise specified. Media were supplemented with 50 μg/mL kanamycin (Fisher Scientific) for the routine culture of mini-Tn*5* transposon mutants. Microplates and trays obtained from Genetix (Wokingham, UK).

4.3.1 Random transposon mutagenesis of *E. coli* Nissle 1917

E. coli Nissle 1917 was subjected to random transposon mutagenesis using the pRL27:: mini-Tn*5* system (Larsen *et al*. 2002), as represented in Figure 3.5.B. Suicide delivery vector (pRL27) hosted in *E. coli* BW29427 (a DAP auxotrophic strain) was transferred to EcN by conjugation, according to the method described in Section 2.2.13. Trans-conjugants harbouring transposon inserts were selected on LB agar containing kanamycin (50 μg/mL) but without diaminopimelic to eliminate the donor strain. Individual mutants were picked and subcultured in LB broth containing kanamycin (30 μg/mL) in 96-well microtiter polystyrene plates. Plates were incubated overnight at 37°C, then cultures were supplemented with 10 % (v/v) sterile glycerol, and the resulting mutant bank stored at –80ºC.

4.3.2 Isolation of mutants sensitive to acidic pH

High-throughput screenings of EcN mutants with sensitivity to acidic pH were performed by growing cultures in sterile acidic pH (LB-HCl) media. Prior to the screenings, a pH-based minimum inhibitory concentration (MIC) of HCl against EcN wild-type was established.

4.3.2.1 Establishment of MIC of acidic pH against *E. coli* Nissle

LB-HCl medium at pH 2.0–7.5 (in increments of 0.5 pH units) was prepared, sterilised by autoclaving and used to test growth of the EcN wild-type. An aliquot (5 mL, for each pH level being tested) was transferred to a sterile 15 mL plastic tube then inoculated with overnight cultures of EcN wild-type at a ratio of 1:1,000 (inoculum density of ~ 2 x 10^6 Cfu/mL). The inoculated medium was then dispersed in 96-well plates, which were then sealed with breathable plastic film to prevent evaporation, and incubated for up to 24 hours at 37°C. Bacterial growth was monitored over time using a plate reader at an optical density of 600 nm (OD_{600}). The MIC was determined as the pH level inhibiting the bacterial growth (0.2 > OD_{600}) after 20 hours incubation of cultures at 37ºC statically in 96-well plates.

4.3.2.2 High-throughput screening of mutants for sensitivity to acidic pH media

To identify mutants with sensitivity to acidic pH media, high-throughput screenings of mutants were established in 96-well plates in two-step screening processes.

First screening: In the first step (preliminary screening), plates were filled with fresh LB-HCl medium (supplemented with kanamycin, 50 μg/ mL) at pH level slightly above the MIC value (against the wild-type). The medium was then inoculated with mutants (from the mutant bank, Section 4.3.1) using a sterile 96-pin replicator (Genetix). The inoculated plates were sealed in plastic films then incubated at 37°C for 20 hours, statically. Bacterial culture growth was recorded (OD_{600}) and used to calculate the average growth per plate (all well containing mutants considered). To identity mutants with potential sensitivity to acidic pH media, individual mutants (OD_{600} readings) were compared to the pre-calculated average plate (96-well) readings. Those showing absorbance readings of 0.2 OD_{600} below the plate average were selected for second-round screenings.

Second screening: To confirm acidic-sensitive phenotype of those mutants isolated in first-round screening, the sensitivity to acid pH of the mutants was compared directly with that of the wild-type EcN in 96-well plate assay. Each plate contained positive control wells inoculated with the wild-type, as well as uninoculated medium (negative control) at each level of the pH being investigated. To determine the effect of mutation on overall growth of the mutant strain, mutants were also assessed for ability to grow in plain LB broth and compared to the wild-type. Mutants displaying statistically significant differences in sensitivity to acidic pH ($P< 0.05$), but no significant differences in growth in plain LB ($P>0.05$), were determined as acidic-sensitive mutants and identities of disrupted genes were revealed.

4.3.3 Isolation of osmosensitive mutants in NaCl medium

To investigate osmosensitivity phenotype of EcN, high-throughput screenings (in 96-wellplate format) for mutants were performed. The MIC of NaCl against EcN wild-type was established in LB broth medium containing 0-20 % w/v NaCl using the protocol used for acidic pH (Section 4.3.2.1). Also, mutants were screened and isolated for sensitivity to NaCl using the two-step screening process described in Section 4.3.2. Those mutants determined osmosensitive were selected and identities of disrupted genes were revealed.

4.3.4 Isolation of bile-sensitive mutants

Bile-sensitive mutants were isolated in high-throughput screenings for bacterial growth on LB agar supplemented with unfractionated bile bovine oxgall (Sigma-Aldrich). Before the screenings, the minimum inhibitory concentration (MIC) of bile against EcN wild-type was first determined.

4.3.4.1 Establishment of MIC of bile oxgall against *E. coli* Nissle

LB agar was supplemented with 1 to 20 % w/v bile oxgall, sterilised by autoclaving then poured into Perti dishes. The LB-bile agar was allowed to set at room temperature for 30 minutes then spotted with 2 µL overnight cultures of EcN WT. After inoculation, plates were sealed in plastic bags and incubated at 37ºC for 24 hours. The MIC was defined as the % of bile (w/v in LB) inhibiting the growth of visible colonies on the agar plate after 24 hours incubation 37ºC.

4.3.4.2 High-throughput screening of mutants for sensitivity to bile oxgall

To identify mutants with sensitivity to bile, high-throughput screenings were established in sterile bioassay polystyrene dishes (242 x 240 x 20 mm) plates in two-step screening processes

First screening: In first-round screening, mutants with potential sensitivity to bile were first isolated on LB-bile agar medium (with % bile slightly below the MIC value), su l emented with 50 µg/ mL kanamycin. The agar was inoculated with mutants, using a sterile 96-pin replicator (Genetix). The inoculated plates were then incubated for 24 hours at 37ºC, and mutants showing poor growth or no growth on this medium were selected for second-round screenings.

Second screening: To confirm bile-sensitive phenotype of those mutants isolated in first-round screen, the mutants' sensitivity to bile was compared with that of EcN wild-type. For this second screening, the concentration of bile used in growth medium was lower than that used in first-round screen. This allowed the establishment of the level of mutant's sensitivity to bile. After mutants were confirmed to be bile-sensitive, they were compared to the wild-type EcN for growth in plain LB broth, at 37ºC for 24 hours. Bile-sensitive mutants showing no significant difference in growth rate in plain LB as compared to the wild-type ($P > 0.05$), were selected for sequencing and identification of disrupted genes.

4.3.5 High throughput screening for *E. coli* Nissle mutants with altered biofilm forming abilities

To identify mutants of EcN with altered biofilm forming abilities, the crystal violet (CV) staining-based assay using microtitre (O'Toole and Kolter 1998a) was employed. The assay was performed in two-round screening steps.

First screening: For this preliminary screening, 96-well plates were first filled with sterile LB (100 µL/well) then inoculated with mutants using a 96-pin replicator. Plates were sealed in plastic films then incubated at 37°C for 24 hours, statically. After the incubation, the medium was removed from all wells, which were subsequently washed twice with SDW (150 µL/ well) to remove non-adherent bacterial cells. After washing, plates were dried by incubation at room temperature for 5 minutes. The wells were then filled with 120 µL of a 1% w/v CV solution (Pro-Lab Diagnostics, Neston, UK), and the plates were then incubated at room temperature for 15 minutes. After the incubation, the staining solution was decanted and wells were washed three times with SDW (150 µL) to remove the unbound stain. The bound stain was then eluted by addition of dimethyl sulfoxide (150 µL) and measured by spectrophotometry at 595 nm (OD_{595}). The recorded readings were used to calculate the average OD_{595} per plate (all wells containing mutants were considered). To detect mutants with potential alterations in biofilm formation, the pre-calculated average OD_{595} per plate was compared to individual readings of mutants, and those showing +/- 0.1 OD_{595} of the plate average were selected for second-round screening.

Second screening: this screening was performed to confirm the observed biofilm formation phenotypes in mutants isolated in first-round screening. The selected mutants were compared with the wild-type EcN for biofilm formation in 96-well plate, using the CV staining assay used in first-round screening. Each plate contained wells inoculated with mutants (n=4, for each one) the wild-type (n = 4) and control uninoculated LB both (n = 3).

Moreover each plate assay was performed in triplicate. Prior to the biofilm staining, mutants were assessed for their ability to grow in LB as compared to the wild-type. Growth was measured at 600 nm (OD_{600}) using a plate reader. Mutants showing statistically significant differences in biofilm formation (CV at OD_{595}, $P< 0.05$), but without significant differences in bacterial growth (cultures at $OD_{600,}$ $P > 0.05$), were confirmed biofilm altered mutants of EcN and defined as biofilm enhanced (BFE) or biofilm deficient (BFD) mutants.

4.3.6 Identification of disrupted genes in mutants of interest

Genes disru ted in mutants of interest were identified using a "cloning free" arbitrary PCR-based approach to amplify transposon flanking regions as described by Manoil (2000) using primers and protocol detailed in Section 3.2.2.5. Total genomic DNA was extracted from mutants as described in Section 2.2.7. The resulting amplicons were sequenced by GATC Biotech Ltd. (London, UK) and putative identity of disrupted genes was assigned using BLASTX based on correlation of mini-Tn*5* junction sequences in EcN mutants, to the EcN wild-type genome (Cress *et al*. 2013).

4.4 Results

A large-scale random bank of EcN mini-Tn*5* insertion mutants was generated and used to isolate *E. coli* Nissle mutants exhibiting acid-sensitive, salt-senstitive, bile-sensitive phenotypes, as well as alterations in biofilm formation.

4.4.1 Isolation of acid-sensitive mutants

To investigate the ability of EcN to grow in acidic media, the MIC of acidic pH (in LB-HCl broth) against EcN wild-type was first determined at pH 4.0 as shown in Figure 4.2. A total of 3,542 mutants were then screened for growth in LB-HCl broth at pH 4.2, and the results are presented in Figure 4.3. Seven mutants (designated JNpH1-7) were confirmed to be acid-sensitive as they displayed statistically significant difference in growth at pH 4.2 as compared to the wild-type ($P < 0.05$), but no significant differences in growth in plain LB, pH 7.1 ($P > 0.05$). They were therefore taken as acid-sensitive mutants and identities of disrupted genes were revealed (Table 4.3).

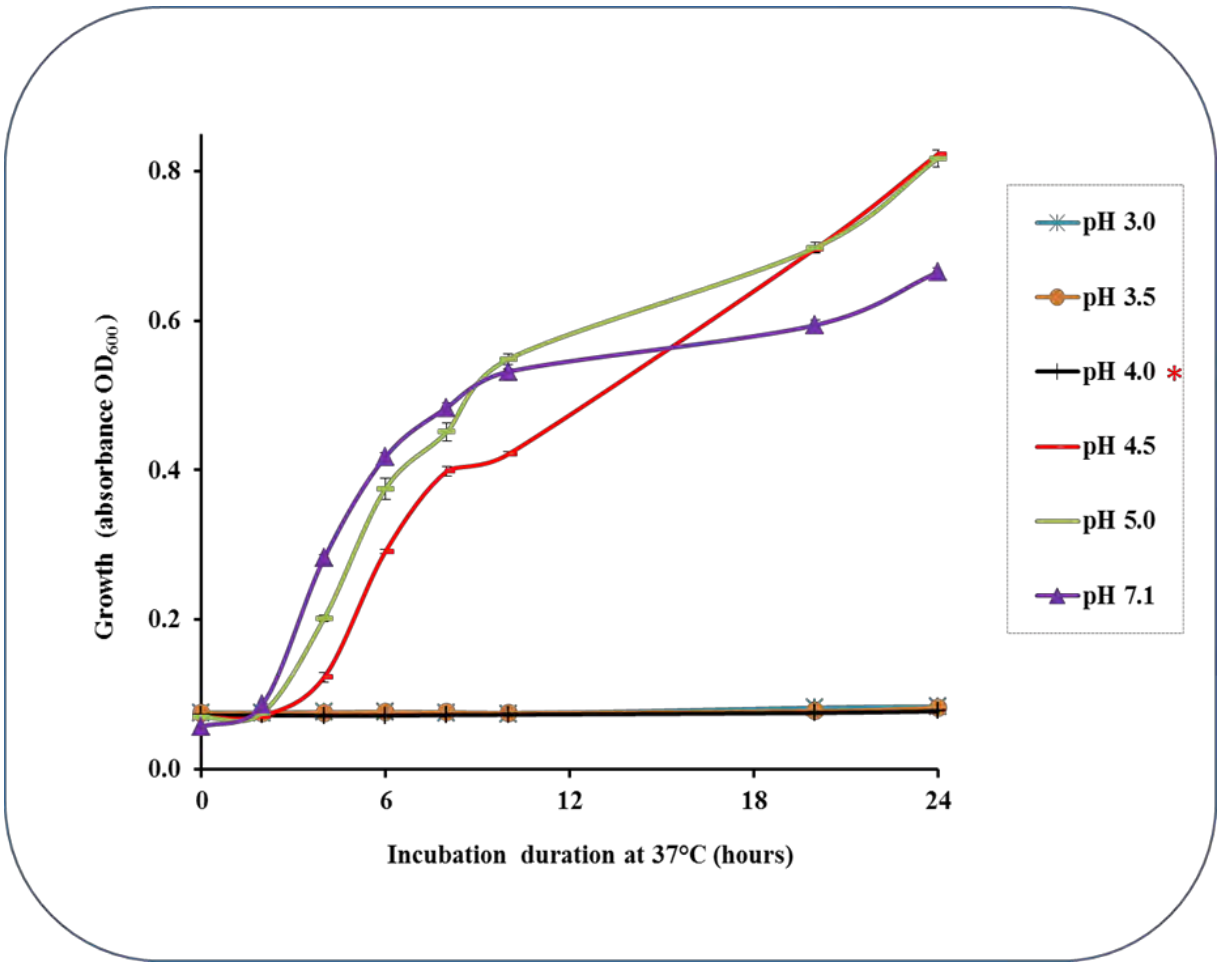


Figure 4.2: Determination of acidic pH MIC of HCl against *E. coli* Nissle 1917 wild-type

Cultures were grown in LB-HCl broth at pH levels ranging from 2.0 to 5.0 and in plain LB (pH 7.1) pH levels in 96-well plate, each. Growth was monitored by absorbance at 600 nm (OD_{600}) at 0, 2, 4, 6, 10, 20 and 24 hours. Absorbance was calculated as mean of 4 well readings per plate. Control wells (uninoculated medium) were included for each pH level. Results shown are the mean ± SEM of three independent experiments (3 plates).

*The pH-based MIC was determined at pH 4.0.

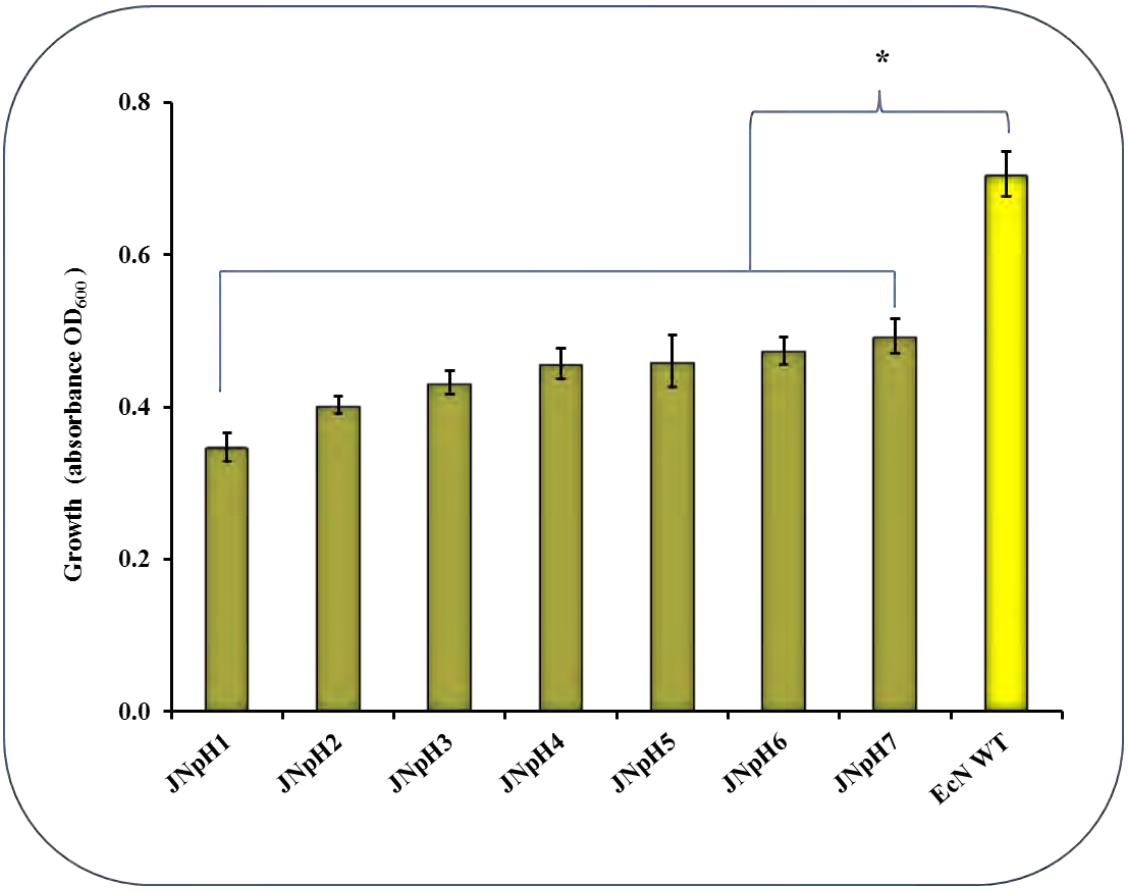


Figure 4.3: Growth of *E .coli* Nissle acidic-sensitive mutants at pH 4.2

Mutants and control *E. coli* Nissle wild-type were grown at 37°C for 20 hours in LB-HCl broth (pH 4.2) in 96-well plate format.

Growth medium for mutants was supplemented with kanamycin (50 µg/ mL) except for EcN WT.

All experiments were undertaken in triplicate (n=4 for each strain, per plate assay). Results shown are the mean ± SEM of three independent experiments (3 plates; n=12).

*: significant difference in growth rate at pH 4.2 ($P < 0.05$) but without a significant difference in growth at pH 7.1 (plain LB).

4.4.2 Isolation of osmosensitive mutants

To study the osmosensitivity of EcN, the MIC of NaCl against the wild-type was first established at 8 % w/v NaCl in LB broth as shown in Figure 4.4. A total of 3, 324 mutants were then screened for growth in LB-NaCL broth containing 7 % NaCl and one mutant (designated JNNaCl1) was demonstrated sentitive to salt at this concentration. Figure 4.5 displays the salt-sensitivity profile of mutant JNNaCl1 in LB without salt and with salt at concentration ranging from 1 – 8 % w/v, in comparison to the wild-type EcN. Interestingly, JNNaCl1 displayed enhanced growth in salt-free medium, which was statistically significantly different to that of EcN wild-type ($P < 0.05$), but the opposite was true when a small % of salt was added to the growth medium. Mutant JNNaCl1 was then taken as an osmosensitive mutant, and gene disrupted was revealed in (Table 4.3).

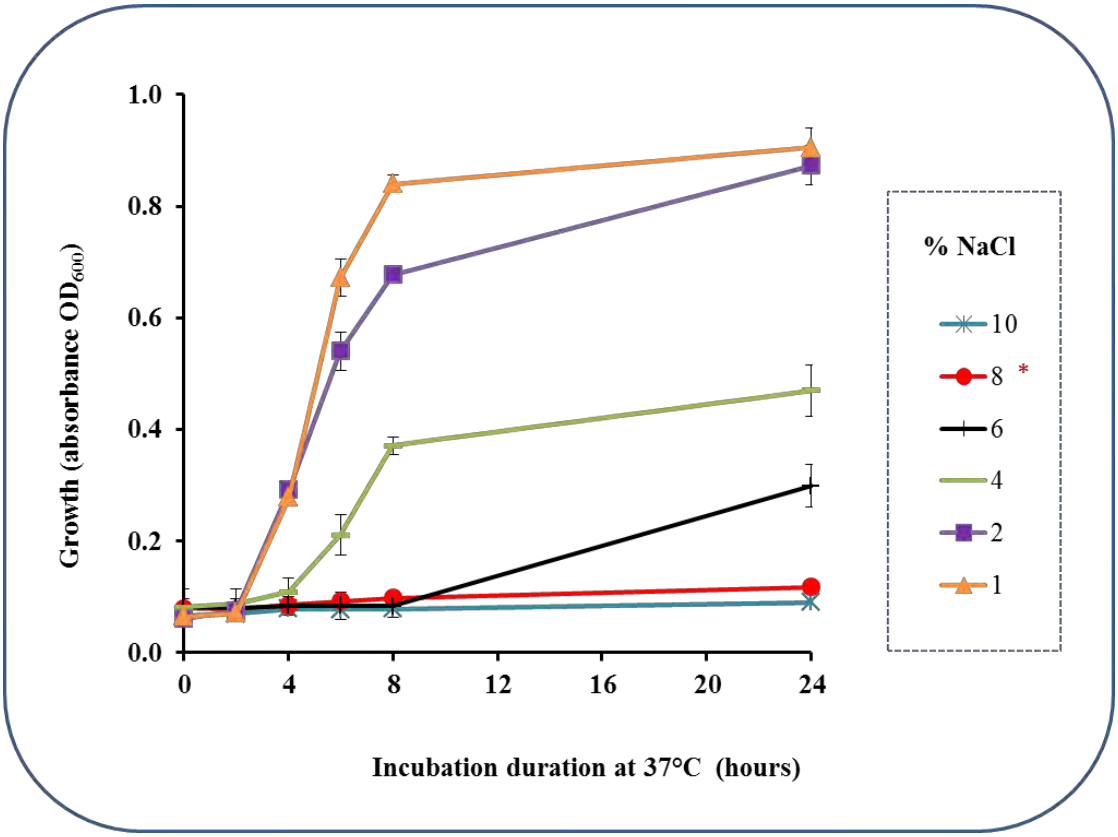


Figure 4.4: Determination of NaCl MIC against *E. coli* Nissle 1917 wild-type

Cultures were grown in LB broth supplemented with NaCl levels ranging from 1 to 10 % w/v and bacterial growth was monitored by absorbance at 600 nm (OD_{600}) at 0, 2, 4, 6, 8, and 24 hours. Absorbance was calculated as mean of 4 well readings per plate. Control wells (uninoculated medium) were included for each salt level. Results shown are the mean ± SEM of three independent experiments (3 plates; n=12).

*The NaCl MIC was determined at 8 % after 24 hours incubation.

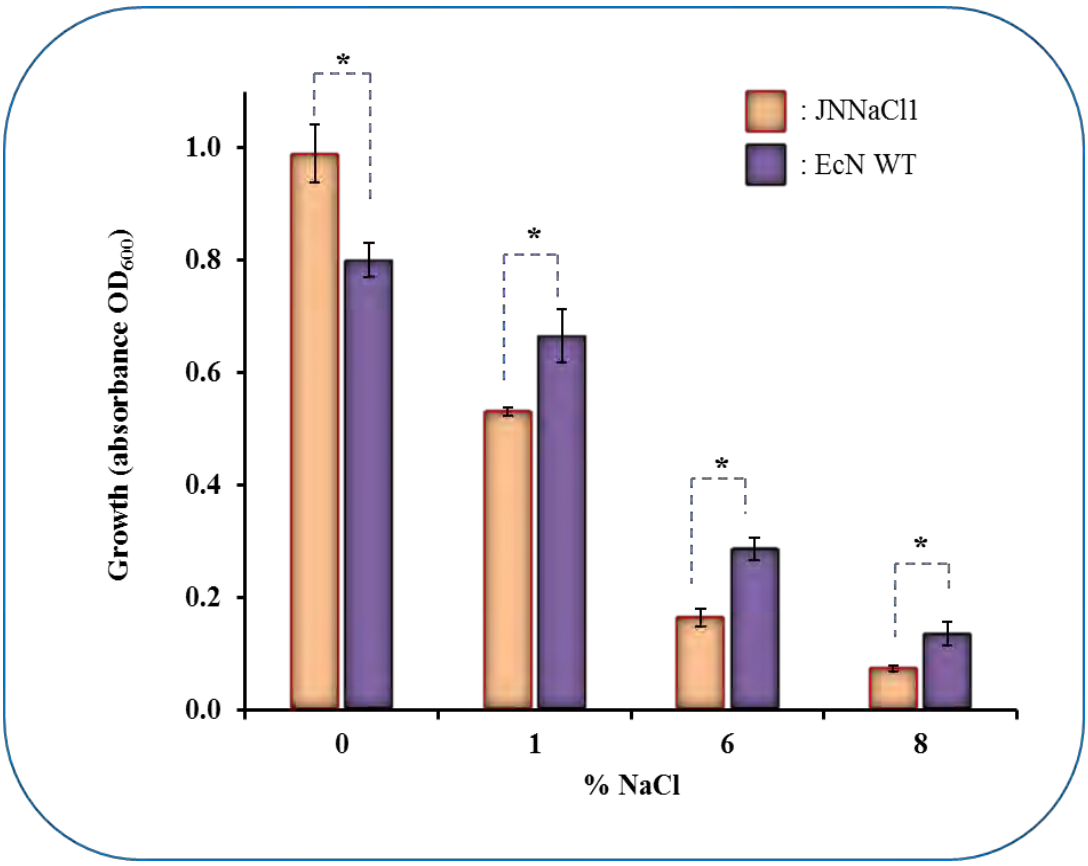


Figure 4.5: Growth of *E .coli* Nissle osmosensitive mutant

Mutant and control *E. coli* Nissle wild-type were grown at 37°C for 20 hours in LB-NaCl medium in 96-well plates.

Growth medium for mutants was supplemented with kanamycin (50 µg/ mL) except for EcN WT.

All experiments were undertaken in triplicate (n=4 for each strain, per plate assay). Results shown are the mean ± SEM of three independent experiments (3 plates; n=12).

*: significant difference in bacterial growth at OD_{600} ($P< 0.05$).

4.4.3 Isolation of bile-sensitive mutants

To investigate the genetic basis of EcN ability to grow in the presence of bile, the MIC of bile against EcN wild-type was first determined at 12 % w/v bile oxgall in LB agar. A total of 3,404 mutants were then screened for growth on LB-bile agar containing 0 - 11 % w/v oxgall, and seven mutants were found to be bile-sensitive but without a significant difference in bacterial growth in plain LB broth as compared to the wild-type EcN ($P < 0.05$) (Table 4.2). Visible bacterial growth on agar plates was detectable at 9 % w/v oxgall for six out of the seven mutants whereas the wild-type EcN was able to grow well in medium containing up to 11 % w/v oxgall. One particular mutant, JNBL12 exhibited a bile "super-sensitive" phenotype following its inability to grow only on bile agar of up to 4 % w/v oxgall. Identities of genes disrupted by the pRL27 mini-Tn*5* were revealed and presented in Table 4.3.

Table 4.2: Selection of bile-sensitive mutants of *E. coli* Nissle 1917

Mutant	Bile tolerance phenotype*	Growth rate compared to the wild-type in plain LB broth
JNBL3	Bsen	Normal
JNBL4	Bsen	Normal
JNBL5	Bsen	Normal
JNBL6	Bsen	Normal
JNBL7	Bsen	Normal
JNBL12	BSsen**	Normal
JNBL13	Bsen	Normal

* Bsen: bile-sensitive mutants: those not growing on 10 % w/v oxgall agar medium and above

** BSsen : bile super-sensitive mutants with no visible growth on 5 % w/v oxgall agar medium but growing on oxgall 4 % w/v on LB agar.

Growth rate was determined by spectrophotometer at OD_{600}. Mutant strains and *E. coli* Nissle wild-type were grown in 5 mL LB broth with and without kanamycin (50 µg/mL), respectively then standardised to an OD_{600} of 1.0, and inoculated in fresh LB in 96-well plates. Plates were incubated at 37°C for 24 hours and growth was measured at OD_{600} and compared to the wild-type.

Normal growth: no significant difference in growth rate as compared to *E coli* Nissle wild-type ($P > 0.05$).

4.4.4 Isolation of mutants with altered abilities to form biofilms in 96-well plate

A total of 4,116 EcN mini-Tn*5* were screened in a high-throughput crystal violet (CV) based assay for biofilm formation in microtitre-plates and 21 mutants were found to be significantly different in their ability to form biofilms as compared to EcN wild-type (WT) ($P < 0.05$) grown under the same conditions, but without a significant difference in bacterial growth rate. The majority of these (n=15) exhibited a biofilm formation enhanced (BFE) whereas six exhibited biofilm formation deficient (BFD) phenotype, as compared to the wild-type (Figure 4.6).Identities of genes disrupted in these mutants were predicted and putative functions assigned based on correlation of sequences flanking mini-Tn*5* insertions, to the EcN chromosome (Table 4.3).

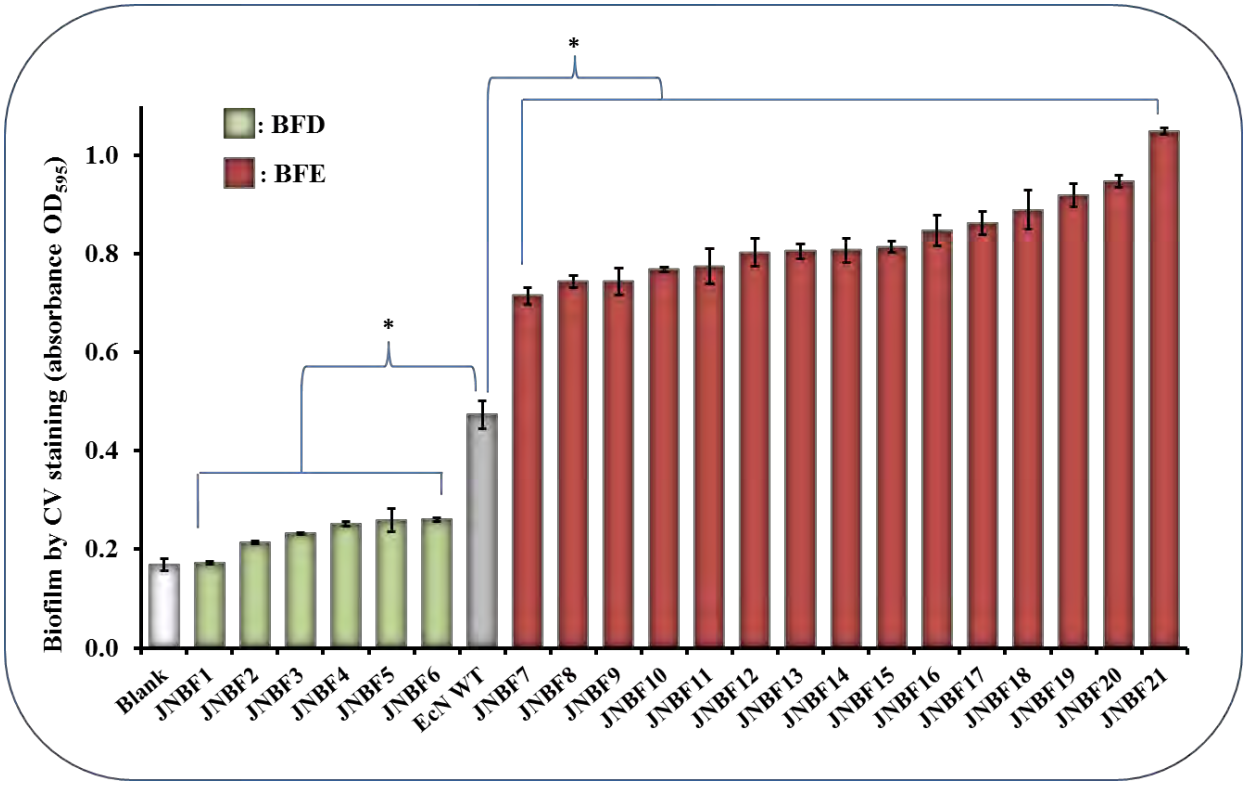


Figure 4.6: Quantitation of biofilm formed by mutants in 96-well plate

Strains were grown in LB broth statically for 24 hours at 37°C, assessed for bacteria growth rate by spectrophotometry OD600 then stained with crystal violet (CV), eluted with DMSO and quantified at O_{D595}.

Data are expressed as the mean of three independent replicates, each with n=4 wells per plate. Results shown are the mean ± SEM of three independent experiments (3 plates).

BFD: biofilm formation deficient phenotype
BFE: biofilm formation enhanced phenotype

*: indicates statistically significant differences ($P < 0.05$) in biofilm formation but without significant difference in bacterial growth rate at ($P > 0.05$) as compared to *E. coli* Nissle wild-type.

Table 4.3: Putative functions of genes disrupted in *E. coli* Nissle mini-Tn*5* mutants of acid-sentitive, salt sensitive bile-sensitive, and biofilm formation phonotypes

Mutant	Phenotype [a]	Putative function/product of disrupted genes *	Function category	Cellular function
JNpH1	Acid-sensitive	Molybdopterin biosynthesis protein MoeA (127/127)	-	-
JNpH2	Acid-sensitive	Formate dehydrogenase chain D (124/133)	Anaerobic respiration	-
JNpH3	Acid-sensitive	Cytosol aminopeptidase (39/44)	Metabolism	-
JNpH4	Acid-sensitive	Bipartite regulatory protein (31/31)	Transcription	DNA binding
JNpH5	Acid-sensitive	2-octaprenyl-6-methoxyphenol hydroxylase, partial (83/84)	-	-
JNpH6	Acid-sensitive	Guanosine polyphosphate pyrophosphohydrolases/synthetases (57/59)	-	-
JNpH7	Acid-sensitive	Isopentenyl-diphosphate delta-isomerase (59/60)	-	-
JNNaCl1	Osmosensitive (NaCl)	tRNA pseudouridine synthase A (64/65)	-	-
JNBL3	Bile-sentitive	Protein YjgF, putative endoribonuclease L-PSP (82/92)	-	-
JNBL4	Bile-sentitive	UDP-glucose:(heptosyl) LPS alpha1,3-glucosyltransferase WaaG (191/203)	Cell surface components	LPS biosynthesis
JNBL5	Bile-sentitive	FIG00637882: hypothetical protein (47/50)	-	-
JNBL6	Bile-sentitive	Hypothetical lipoprotein YajG precursor (45/45)	-	-
JNBL7	Bile-sentitive	N-acetylmuramoyl-L-alanine amidase (38/52)		
JNBL12	Bile- super sentitive	2,3-bisphosphoglycerate-independent phosphoglycerate mutase (107/111)	-	-

Mutant	Phenotype [a]	Putative function/product of disrupted genes *	Function category	Cellular function
JNBL13	Bile-sentitive	MltA-interacting protein MipA (133/133)	Membrane-associated protein	Peptidoglycan biosynhtesis
JNBF1	BFD	Ornithine carbamoyltransferase (55/55)	-	-
JNBF2	BFD	Type 1 fimbriae anchoring protein FimD (58/58)	Cell surface components	Fimbriae biogenesis
JNBF3	BFD	Flagellar hook-associated protein FliD (104/118)	Cell surface components	Flagellar biosynthesis
JNBF4	BFD	Flagellar motor switch protein FliM (224/227)	Cell surface components	Flagellar biosynthesis,
JNBF5	BFD	Flagellar biosynthesis protein FliC (166/174)	Cell surface components	Flagellar biosynthesis,
JNBF6	BFD	Periplasmic thiol: disulfide interchange protein DsbA (135/135)	Membrane-associated protein	A Disulfide Bond–Forming Enzyme
JNBF7	BFE	CidA-associated membrane protein CidB (26/27)	Membrane-associated protein	Cell envelope biogenesis, outer membrane
JNBF8	BFE	DUF1706 domain-containing protein (142/143)		
JNBF9	BFE	Hypothetical protein (119/119)	-	-
JNBF10	BFE	FIG00638001:hypothetical protein (200/225)	-	-
JNBF11	BFE	General secretion pathway protein C (59/74)	Secretion	Protein transport
JNBF12	BFE	FIG004016: Uncharacterised protein YggN (105/105)	-	-
JNBF13	BFE	Putative inner membrane protein YqgA (71/73)	Membrane-associated protein	possible Na+ channel or pump
JNBF14	BFE	Chaperone HdeB (20/23)	Periplasmic –associate dprotein	Acid resistance

Mutant	Phenotype [a]	Putative function/product of disrupted genes *	Function category	Cellular function
JNBF15	BFE	RNA polymerase sigma factor RpoD (107/117)	Transcription	Promotion of RNA attachement
JNBF16	BFE	KfiB protein, involved in biosynthesis of K5 capsule (64/64)	Cell surface components	K5 capsule biosynthesis
JNBF17	BFE	Polysialic acid transport ATP-binding KpsT (54/58)	Cell surface components	K5 capsule export
JNBF18	BFE	Type VI protein secretion system component Hcp (174/174)	Secretion	-
JNBF19	BFE	Signal transduction histidine-protein kinase BarA (29/29)	Signalling	Regulation of carbon metabolism
JNBF20	BFE	Probable transcriptional activator for leuABCD operon (47/49)	Transcription	Transcriptional regulator/DNA binding
JNBF21	BFE	TsgA protein homolog (208/208)	Inner-membrane associated	Putative transpoter

[a:] BFE**:** Biofilm Ehanced phenotype, BFD**:** Biofilm Deficient phenotype

*Putative identity of disrupted genes was assigned using BLASTX based on correlation of mini-Tn*5* junction sequences in EcN mutants, to the *E. coli* Nissle wild-type genome (Cress et al. 2013).

4.5 Discussion

4.5.1 Designing of high-throughput screenings for isolation of *E. coli* Nissle mutants

Despite the fact that *E. coli* Nissle 1917 has been the most commonly used and studied Gram-negative probiotic bacteria for decades (Sonnenborn and Schulze 2009, Behnsen *et al.* 2013), the mechanisms underlying host-microbe interactions in this strain are still not clearly understood. Mutagenesis systems have become easily available for studies of unknown genes from known phenotypes (de Lorenzo *et al*. 1990, Hansen and Holden 1996, Alberts *et al*. 2002). However, the design and application of selective screens for isolation of mutants with desired phenotypes is often key to the success of mechanistic studies of host-microbe interactions (Hansen and Holden 1996, Alberts *et al*. 2002). Screenings of mutant libraries must be simple, rapid and high-throughput (if possible) in order to achieve clear and meaningful results (Hansen and Holden 1996, Alberts *et al*. 2002). Moreover, the phenotypes of interest must be well defined and if possible simple in order to facilitate the designing of the screens (Alberts *et al*. 2002).

The simplicity of both the screening and target phenotype is a vital factor in studies of host-microbe interactions, which are already convoluted enough considering both the complexity of host gastro-intestinal milieu and of bacteria cell compounds involved in the process. The screens used in this study were designed to look at those simple phenotypes that might be directly or indirectly relevant to host-microbe interactions and satisfied the requirement for high throughput screenings. For the isolation of EcN mutants showing sensitivity to major compounds of the gut antimicrobial medium (bile, acidic pH, and dietary salts), crude bile (oxgall), hydrochloric acid, and sodium chloride were selected. The bovine bile (oxgall) used in this study is similar to human bile and has been commonly employed to assess the *in vitro* bile resistance/tolerance of bacterial strains (Begley *et al*. 2003, Begley *et al*. 2005, Jones *et al*. 2008). HCl was also considered as the best-known

component of the gastric juice and secreted by the parietal cells in the stomach (Samuelson and Hinkle 2003, Yao and Forte 2003).

4.5.2 Recovery in phenotypes relevant to survival in the gut and host-microbe interactions

Out of a total of 3,542 mutants screened for bile, salt, and acid sensitivity phenotypes, 7 mutants showed sensitivity to bile, 1 osmosensitive mutant (NaCl), and 7 mutants were sensitive to acidic pH of the growth media. Also, these mutants demonstrated the ability to grow in plain LB medium at same levels as the wild-type suggesting that the genes disrupted in these mutants were important in the tested phenotypes and not general growth of EcN. When putative functions of gene disrupted in these mutants were assigned and analysed, some were shown to play roles in different stress responses confirming that bile, salt and HCl are important for EcN survival in the gut. At least two of the bile-sensitive mutants were predicted to be defective in LPS and peptidoglycan biosynthesis. This confirms previous findings on the LPS to be a main factor in Gram-negative tolerance to bile acids (Begley *et al*. 2005).

In the case of isolation of EcN mutants with altered abilities to form biofilm on abiotic surfaces, the crystal violet (CV) staining assay was used (O'Toole and Kolter 1998a). This was based on the hypothesis that through identification of mutants with alterations in biofilm formation, it would be possible to recover a high proportion with defects in cell surface structures, and that these would also be relevant to the way EcN interacts with host cells. Given the lack of high throughput *in vitro* screens utilising human cells, it was rational that the biofilm assay would provide an indirect way of enriching for mutants altered in host-microbe interactions.

This hypothesis was formulated based on three main facts from published works that: i) various cell surface molecules and structures of EcN were suggested to be key factors in probiotic- host crosstalk, through their interactions with host cells pattern recognition receptors (PRRs) (Lebeer *et al*. 2010); ii) cell surface components of *E. coli* are largely implicated in biofilm formation on abiotic surfaces (Schembri *et al*. 2003, Van Houdt and Michiels, 2005) ; and iii) EcN has been demonstrated to be a better biofilm former on abiotic surface as compared to many members of *E. coli* (Hancock *et al*. 2010a, Vejborg *et al.* 2010). Moreover, the high-throughput screens using CV staining assay for biofilm formation in plastic plates (O'Toole and Kolter 1998a) have already been proven to be efficient for isolation of transposon mutants with relevance to host-microbe interactions in Gram-negative pathogens including *E. coli* (O'Toole and Kolter 1998a, Pratt and Kolter 1998, Hadjifrangiskou *et al*. 2012, Holling *et al*. 2014). As well as host-probiotic interactions in *E. coli* Nissle itself (Lasaro *et al.* 2009).

Most of the genes disrupted in BFD mutants have been previously characterised and associated with biofilm formation in *E. coli* and attachment to the host mucosa. These include *fimD* involved in ex ort and assembly of ty e 1 fimbriae (lumer *et al*. 2005, Lasaro *et al*. 2009), *fli*C*, fliD* and *fliM* associated with flagella which plays a crucial role in cell motility and initiation of attachment to surfaces in *E. coli* (Pratt and Kotler 1998, Chilcott and Hughes 2000; Troge *et al*. 2012), and *dsb*A encoding for periplasmic protein disulfide isomerase (Lasaro *et al*. 2009). Contrarily, the genes in BFE phenotypes have not been directly associated with biofilm formation or interaction with host cells in previous studies. Classification of the mutants based on putative functions of the disrupted genes revealed 6 with defects in cell surface structures, and at least 3 in bacterial membrane associated proteins. Therefore these mutants were good candidates for studies exploring the mechanisms of host-probiotic interactions.

4.6 Conclusion

- A bank of *E. coli* Nissle mini-Tn*5* mutants has been generated

- High-throughput screening protocols have been developed and implemented for mutants exhibiting acid-sensitive, bile-sensitive and osmosensitive phenotypes. These protocols are preliminary steps towards elucidating the mechanisms used by probiotic *E. coli* Nissle to survive in the gut.

- High-throughput screening for mutants with altered biofilm forming abilities was established and were able to recover mutants predicted to encode proteins involved in generation of cell surface structures. These mutants could be be relevant to the studies exploring the mechanisms of host-probiotic interactions.

Chapter 5: Host-microbe interactions in *E. coli* Nissle 1917 surface structure mutants

5.1 Experimental models for studying the effects of probiotics on host

Functional, efficacy and safety aspects of probiotics must be substantiated using experimental laboratory models prior to undertaking human trials. Several types of *in vitro* human-derived intestinal cell models as well as *in vivo* animal systems have been developed and used in studies of host-microbe interactions (Pavan *et al*. 2003, Lee and Salminen 2009). *In vitro* intestinal cell models can be classified into two main categories, immortalised cell lines and primary cells, whereas laboratory animal systems consist mainly of conventional models and gnotobiotic models (any associated microbial community, if present, is fully defined) (Quaroni and Beaulieu 1997, Lee and Salminen 2009, Chougule *et al*. 2012).

Both intestinal cell and animal models have allowed the investigation of various properties of probiotics including safety (Pavan *et al*. 2003, Sanders *et al*. 2010); protective activities against enteropathogens (Bibiloni et *al*. 1999, Boudeau *et al*. 2003, Likotrafiti *et al*. 2013); development of intestinal immune system (Shroff *et al*. 1995, Cebra 1999); immunomodulatory and anti-inflamatory properties (Haller *et al*. 2000, Cross *et al*. 2004, Hafez *et al*. 2009, Taverniti and Guglielmetti 2011); antitumor effects (Hirayama and Rafter 1999, de Roos and Katan 2000, McFarland 2000); and effects of both probiotics and prebiotics on gut microbial community (Mountzouris *et al*. 2006, Scholz-Ahrens *et al*. 2007).

The application of human intestinal cells and animal models in host-probiotic studies is compared in Table 5.1. While animal models can adequately help to investigate the overall

Table 5.1: Application of intestinal cells and animal models to study probiotics properties relevant to host interactions

Group	Advantages	Limitations	Reference
Human derived intestinal cells	**Cell lines**: - High level of control of experimental parameters and variables - Rapid, cost effective, easy culture maintenance and scalability - Suitable for high- throughput studies - Can provide indication of mechanisms that may be evaluated further in more complex *in vivo* models	- May not fully recapitulate *in vivo* intestinal phenotypes - Requires supplementary methods (*in vivo* animal models) to substantiate the obtained results	Falk *et al*. (1998), Balimane and Chong (2005), Vizoso Pinto *et al*. (2007), Likotrafiti *et al.* (2013)
	Primary cells: - Retains majority of the *in vivo* functionality - Usually composed of mixtures of cell types, reflecting host physiology	- As for cell lines but also: - Limited life span - Difficult to give good reproducibility between samples - Not suitable for high-throughput assays	Whitehead *et al*. (1993), Quaroni and Beaulieu (1997), Panja (2000)
***In vivo* animal models**	**Gnotobiotic animal:** - Allow investigation of defined microbial community - Useful for studying development of immune system and inflammatory bowel diseases - Used to confirm mechanistic studies obtained in cell lines	- Requirement for special facilities and special trained personnel - Expensive, time consuming - May not reflect human host physiology	Shroff *et al*. (1995), Falk *et al*. 1998, Cebra (1999), Clancy (2003)
	Conventional animal: - Adequate for studying overall function, efficacy and safety of probiotics - Consideration of gut microbiota, nutrition-related and disease factors permitted	- As for gnotobiotic animals but also: - Probiotic-indigenous microbiota interactions likely to confound results	Sanders *et al*. (2010), Lee and Salminen (2009)

function and efficacy of r obiotics in host's health, they often fail to elucidate the molecular mechanisms underlying the probiotic effects at the cellular level. This is mainly because of high complexity of the factors associated with the intestinal milieu of animal models. For instance, the intestinal milieu includes a complex and dense microbial community, luminal secretions (such as digestive and immune mediators) as well as food components. These elements, when encountered by probiotics *in vivo*, can exhibit independent or synergistic effects on the host intestinal physiology. This can in turn hinder the ability of the animal models to clearly reveal the mechanisms of host-probiotic interactions without any prior knowledge. When there is some prior knowledge about the cell line model or bacterial strains being investigated, the *in vitro* models of human intestinal cells can be relatively simple to use and provide the possibility of: i) controlling the experimental parameters and variables, ii) easy culture maintenance and scalability, iii) conducting rapid tests and high- throughput assay formats. These parameters make intestinal cell models a good choice in mechanistic studies of probiotics.

5.1.1 Intestinal epithelial cells

Human intestines play a major role in the digestion and absorption of foods. Moreover, the intestinal mucosal surfaces represent the first line of defence against luminal pathogen infection and is also a site of action for beneficial microbes. A basic structure of human intestinal epithelium is represented in Figure 5.1. Intestine walls present a laminar structure. Its inner side is lined by a thin, strongly corrugated layer of epithelial cells, consisting predominantly of the enterocytes. Moreover, this layer comprises other types of cells that perform various functions as outlined in the legend of Figure 5.1. These include Paneth cells, endocrine cells, goblet cells, microfold cells and stem cells (Cheng *et al*. 1974, Potten 1998, Brown 2011).

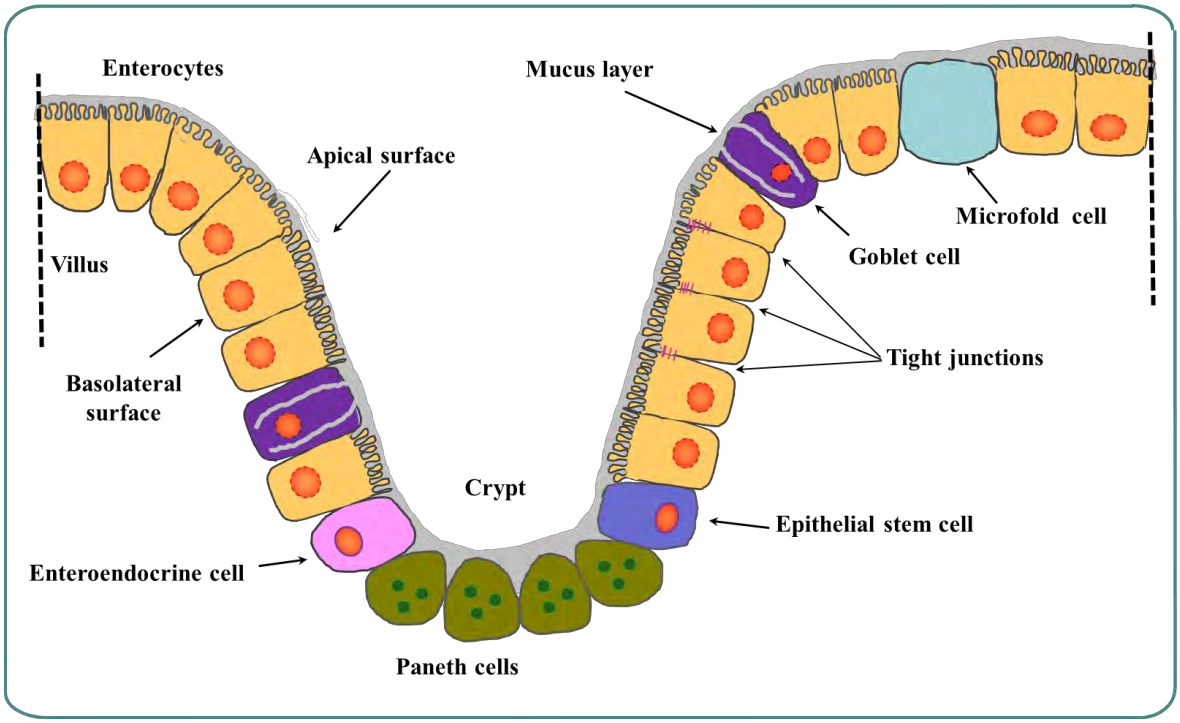


Figure 5.1: Schematic diagram of human small intestine epithelial cells

- Enterocytes (with brush-border structure) are the main sites for nutrient absorption and host interaction with luminal content
- Goblet cells secrete a protective mucus barrier
- Enteroendocrine cells release gastro-intestinal hormones
- Paneth cells secrete antimicrobial peptides
- Microfold or membranous cells (M cell) play a role in intestinal immune system by mounting both immunological and mucosal tolerogenic responses to foreign antigens
- Stem cells fuel a self-renewal process of the intestinal epithelium

Adapted from Abreu (2010) and Umar (2010).

Enterocytes differentiate to form a polarised structure with distinct basolateral and apical domains, which are separated by the tight junctions. The apical surface of enterocyte consists of a 'brush-like' border com risi ng thousands of microvilli per cell, and is covered with mucus secretion forming an interface between the body and the luminal environment (Clatworthy and Subramanian 2001). This microvillar structure provides the human small intestine with a massive surface area estimated to be ~200 m^2 in healthy adults (Wilson 2011).

Functionally, the microvilli serve as a conduit for nutrient absorption and enzyme secretion. The basolateral surface contains extracellular matrix receptors, channels and transporters regulating ion/solute transfer from the intestinal lumen to the interstitium (Yeaman *et al*. 1999). Moreover both the apical and basolateral surfaces of the enterocytes express a variety of host pattern recognition receptor (PRR) systems, which play a crucial role in immune-cell activation in response to specific microbial-associated molecular patterns (MAMPs) (Cario 2005, Kyd and Cripps 2008). The PRRs are thought to be key in host-probiotic interactions through the recognition of specific surface molecules of probiotic bacteria (Lebeer 2010). The predominant distribution of the enterocytes across the human intestines together with their structural and functional characteristics, make these cells good candidates for investigating host-microbe interactions in probiotic bacteria.

5.1.2 Cell cultures of intestinal epithelium *in vitro*

Techniques of culturing epithelial tissues were developed in the mid–1970's, and technological advances are still emerging in this field. Major approaches that have been employed to develop *in vitro* models of the intestinal epithelium include: i) culturing of normal enterocytes (primary cells), and ii) inducing differentiation of malignant tumour cells into enterocyte-like cells (also referred as maturation-induction) (Quaroni *et al.* 1979, Pinto *et al.* 1983). The primary intestinal cells are freshly harvested from a living organism

then maintained *in vitro*. Although the primary cells more closely represent the physiological characteristics of the host donor cells *in vivo*, their application can mainly be hampered by their short life span soon after they become adapted to *in vitro* culture conditions.

The maturation-induction approach involves treating normal or neoplastic cells with maturation-inducing factors such as galactose, dimethylsulfoxide and sodium butyrate, leading to morphological and functional differentiation of the cells (Dexter and Hager, 1980, Pinto *et al*. 1983, Dzierzewicz *et al*. 2002). The resulting cell line is often immortal and can proliferate indefinitely under specified culturing conditions. This approach has been used to develop various enterocyte-like cell models from human colorectal cancer cells, such as Caco-2, HT-29, LS174T, SW116 and SW-480 cell lines (Fogh *et al*. 1977, Pinto *et al*. 1983, Chung *et al*. 1985, Lenaerts *et al*. 2007).

Caco-2 is among the best characterised intestinal cell lines and has been widely used in mechanistic studies involving host-microbe interactions and drug absorption and metabolism (Lee and Salminen 2009, Cencic and Langerholc 2010). Upon reaching confluence, Caco-2 cells have been demonstrated to express morphological and functional characteristics of the enterocytes (Pinto *et al*. 1983, Lee *et al*. 2009, Uchida *et al*. 2009). Caco-2 cells are able to grow as a monolayer that adhere strongly to plastic surfaces and differentiate under standard conditions of tissue culture in laboratory. The HT-29 cell line is another important enterocyte-like cell line that was also isolated from colon adenocarcinoma tissue like Caco-2 (Pinto *et al*. 1983). However, the HT-29 cell line is essentially undifferentiated and is capable of secreting the mucus as compared to the non-mucus producing Caco-2 cells (Huet *et al*. 1995). Both Caco-2 and HT-29 have been proven to be good models of studying bacterial effects on host, enteropathogens as well as probiotics (Lee and Salminen 2009).

5.1.3 Bacterial adherence onto intestinal epithelial cells

Adherence to intestinal epithelial cells (IECs) is an important property for intestinal colonisation, and survival of bacteria in the gut environment. This applies particularly to the small intestine, where the high peristaltic flow rate is most likely to dislodge the colonising bacteria (Ouwehand and Salminen 2003). For invasive enteropathogens, successful adherence to IECs is often a necessity for virulence (Beachey *et al.* 1981, Kline *et al.* 2009). Conversely, adherence of probiotic bacteria to the intestinal mucosa is a desirable characteristic and usually a prerequisite for the selection of candidate probiotic strains (Tuomola *et al*. 2001, Lee and Salminen 2009).

Adhesion properties of several probiotic bacteria have been investigated using *in vitro* models of IECs, including Caco-2 and HT29 (Tuomola *et al*. 2001, Ouwehand and Salminen 2003). In many cases, bacterial adherence to IECs has been studied simply as a screening criterion for probiotics, and an indicator on how these might be capable of colonising the gut *in vivo* (Tuomola *et al*. 2001, Fioramonti *et al*. 2003, Lasaro *et al*. 2009). Some other studies have looked at adherence as a strategy by which probiotics inhibit the attachment of enteropathogens to IECs, by competitive exclusion (Adlerberth *et al.* 2000, Fons *et al*. 2000). Modulation of the immune system has also been explored in conjunction with adherence to IECs in probiotics (Morita *et al.* 2002, Hafez *et al*. 2009, Bahrami *et al*. 2011). Despite the availability and use of a single cell line and pure culture of a probiotic strain, adherence involving two living organisms is a complex process (at the molecular level) and still not clearly understood. The mechanisms underlying EcN interaction with host cells remain less well characterised than for many other probiotic species.

5.2 Aims

The aims of the work presented in this chapter are to investigate mechanisms of host-probiotic interactions in *E. coli* Nissle 1917 mutants with deficiencies in surface structures using an *in vitro* co-culture model with the human intestinal Caco-2 cell line.

5.3 Material and methods

5.3.1 Optimisation of i*n vitro* co-culture model of bacteria and human intestinal epithelium cell-line

The host-microbe interactions in probiotic *E. coli* Nissle 1917 (EcN) were investigated using monolayers of the human colon carcinoma cell line Caco-2 (Fogh *et al*. 1977, Pinto *et al.* 1983). This co-culture model was optimised by determining the effect of the bacterial culture density and duration of exposure period on Caco-2 cell viability, using the trypan blue exclusion test (Freshney 2000) as described in Section 2.4.4. Caco-2 cells were grown in complete DMEM growth medium in six-well plates at 37°C, 5 % CO_2 to reach 75- 80 % confluence (~ 1 x 10^6 cells /well). Bacteria cultures to be used as inocula were grown in LB broth and harvested in mid-log-phase (OD_{600} of 0.5; ~2 x 10^7 Cfu/mL), washed in PBS then resuspended in DMEM at 5 x 10^7 Cfu/mL.

The monolayers were rinsed once with sterile P S and 2 mL bacteria cell suspensions were added to each well, corres ondi ng to multi licities of infection (MOIs) ranging from 0.1:1 to 100:1 (bacteria:Caco-2). The late s were incubated for 2, 4, and 6 hours to allow the cells to grow and interact. Two wells of untreated monolayers (Caco-2 cells with medium only) were included in each ex e riment as controls e r late . At the end of the ex e riments, the co-culture medium was removed by gentle as ir ation and monolayers were then rinsed once with P S to remove the bacterial sus ensions and any detached Caco-2 cells. The rinsed monolayers were try sinised and the cells were assessed for viability by try a n blue staining. The viability of cells in treated sam l es was ex r essed as % of viable untreated cells (maximum viability) using the following formula:

% viability = (viable cells in treated sample/ viable cells in untreated control) x 100

The optimal conditions of the co-culture model were determined as the bacterial cell densities (multiplicity of infection levels) and co-incubation durations producing ≥ 95 % viability of Caco-2 cells.

5.3.2 Bacterial adherence to and internalisation in Caco-2 cells

Bacterial adherence to and internalisation in Caco-2 cells were investigated using a modified protocol of the gentamicin protection assay described by Albiger *et al.* (2003). Monolayers of Caco-2 cells and EcN inocula were prepared as described in Section 5.3.1. Bacterial suspensions were added to monolayers in 6-well plates at an MOI of 1:1 and plates were incubated for 4 hours to allow bacterial adherence.

After the incubation, non-adherent cells were removed by gentle aspiration of the co-culture medium and washing the monolayers 3 times with PBS. Each time, 3 mL of PBS were added to each well and the cells were washed gently by swirling the plate. The washed monolayers were then treated with a lysis solution consisting of 1 % w/v saponin (Sigma-Aldrich) in trypsin-EDTA (0.05 % w/v trypsin /0.5 mM EDTA). One mL of the lysis solution was added to each well and the plate was incubated at 37°C and 5 % CO_2 for 5 minutes to allow permeabilisation of the Caco-2 cells and recovery of total cell-associated bacteria. The sensitivity of EcN strains to the lysis solution was examined by incubating bacterial cultures 5 to 20 minutes at 37°C and 5 % CO_2 followed by viable counts in comparison with controls (treated with SDW).

After the lysis, cells were mixed gently by pipetting (using 200-µL pipette tip), serially diluted in PBS, plated onto LB agar, and incubated at 37°C overnight. The obtained viable count represented the total number of adherent and internalised bacteria. Internalised bacteria were quantified using a similar protocol, except that the 4-hours treated monolayers were washed twice with PBS then incubated for an extra 2 hours in complete

growth medium su l emented with gentamicin (Sigma) at 250 μg/mL to kill extracellular bacteria, prior to the addition of the lysis solution.

The number of adherent bacteria was determined using the following formula:

Adherent bacteria = total bacteria count – internalised bacteria count

The results were expressed as the number of adhering or internalised bacteria per Caco-2 cell, and those from mutants were compared as % of the wild-type levels.

5.3.3 Hoechst propidium iodide staining of Caco-cells in co-culture with bacteria strains

Health status (death and viability) of Caco-2 cells in co-cultures with EcN strains was initially determined using the Hoechst 33342/ Propidium iodide (HPI) double fluorescence staining technique (Section 2.4.5). Monolayers of Caco-2 cells and EcN inocula were prepared as described in Section 5.3.1. Bacterial suspensions were added to monolayers in 6-well plate at a range of MOIs (10:1 to 1:1) and plates were incubated for 4 hours. Untreated cells (with complete growth medium only) were included in the experiment as controls.

After the incubation, the medium was removed from the wells by aspiration and monolayers were washed once with PBS then stained with the HPI staining solution. The cells were visualised by fluorescence microscopy. Viable healthy cells, apoptotic cells and necrotic cells were identified based on the appearance of chromatin as light blue, bright blue and bright pink, respectively.

5.3.4 Caspase activity analysis in Caco-2 cells in co-culture with bacterial strains

Activity of caspase 3/7 was measured in Caco-2 cells treated with bacterial strains using the Promega Caspase-Glo® 3/7 assay as described in section 2.4.6. Caco-2 cells were

cultured in 96-well plates at 37°C and 5 % CO_2 for 60 hours to give a semi-confluent monolayer (~ 60 % confluence ~ 10,000 cells/ well). Complete growth medium was replaced with serum-free medium for the last 12 hours of the incubation. The cells were then washed with PBS then co-cultured with either bacterial suspensions or bacterial supernatants.

Bacterial suspensions were prepared in serum-free medium from mid-log-phase cultures (grown in LB to an OD_{600} of 0.5) then added to Caco-2 cells at an MOI of 10:1 in a final volume of 100 µL/ well. The plates were incubated for 12 hours at 37°C and 5 % CO_2. After 2 hours of co-incubation, the medium (containing bacterial suspensions) was removed from each well then replaced with serum-free medium supplemented with gentamicin (200 µg/mL) to stop bacterial growth. Control wells were included on each plate. These consisted of : i) untreated cells (cells and serum-free medium without bacteria), ii) bacteria control (bacteria in serum-free medium, without Caco-2 cells), iii) medium control (serum-free medium only without cells) and iv) cells treated with serum-free medium containing 0.1 mM camptothecin (sigma-Aldrich) as a positive control for apoptosis.

Bacterial supernatants were obtained from bacterial cells grown in 5 mL serum-free DMEM at 37°C overnight, with shaking. The supernatants were collected by centrifugation (1,500 *g* for 10 minutes), pH- adjusted to 7.2 using 5 M HCl and filter-sterilised (0.4µm pore size filter). The supernatants were diluted in fresh serum-free medium at 1:1 ratio then added to Caco-2 cells in place of cells suspensions. The plates were then incubated for 12 hours at 37°C and 5 % CO_2.

After the 12 hours incubation, the Caspase-Glo® 3/7 assay was performed and luminescence proportional to caspase 3/7 activity was measured as relative light units (RLUs) using a Synergy Multi-Mode Plate Reader (BioTek, Potton, UK) operated with BioTek Gen5.20 software.

5.3.5 Genetic characterisation of *E. coli* Nissle *kfiB* transposon mutant by cloning

The putative JNBF16 *kfib* mutant (showing enhanced adherence to Caco-2 cells and elevated induction of apoptosis) was further characterised to analyse the transposon insertion by subcloning and sequencing. PCR amplification of the *kfiB* locus was performed using a template of genomic DNA from JNBF16 mutant and EcN wild-type (as a control), and primers EcNkfi _F (5'-TAGTCGGACATCCTGGCTCA-3') and EcNkfiB _ (5'- GCCCTTGATTTTAGCTCTCC-3'). The primers were designed using the sequence of the *E. coli* Nissle 1917 Genomic Island II (Grozdanov *et al*. 2004; Genbank accession no: AJ586888). The obtained amplicon was cloned into pGEMT vector (Promega) and sequenced as described in section 2.2.13. The nucleotide sequence was correlated with the *E. coli* Nissle 1917 Genomic Island II as well as the mini-Tn*5* sequence (Larsen *et al*. 2002) using the ARTEMIS Comparison Tool (http://www.sanger.ac.uk).

5.3.6 Construction of *kfiB* and *kfiC* deletion mutants

To further investigate the function of the *kfiB* gene in host-*E. coli* Nissle interactions, mutants with deletions in either *kfiB* or the associated *kfiC* gene were generated by homologous recombination, and subsequently compared in the studies of interactions with Caco-2 cells. Both genes are involved in the biosynthesis of the K5 capsular polysaccharide in EcN (Figure 5.2). This polysaccharide is a polymer consisting of repeating units of -4)-β-D-glucuronic acid (GlcA)-(1,4)-α-D-*N*-acetyl-glucosamine (GlcNAc)(1- (Vann *et al*. 1981). The *kfiC* gene has often been a target in generating acapsular ($K5^-$) mutants *of E. coli* (Griffiths *et al.* 1998, Burns and Hull 1999, Hafez *et al*. 2009, Hafez *et al.* 2010); as it encodes a well-characterised glycosyltransferase KfiC, which is involved in the polymerisation of the polysaccharide (Griffiths *et al.* 1998). In contrast, *kfiB* has no clear function in this process but its mutation has been confirmed to abolish the capsule (Whitfield and Roberts 1999, Sugiura *et al*. 2010).

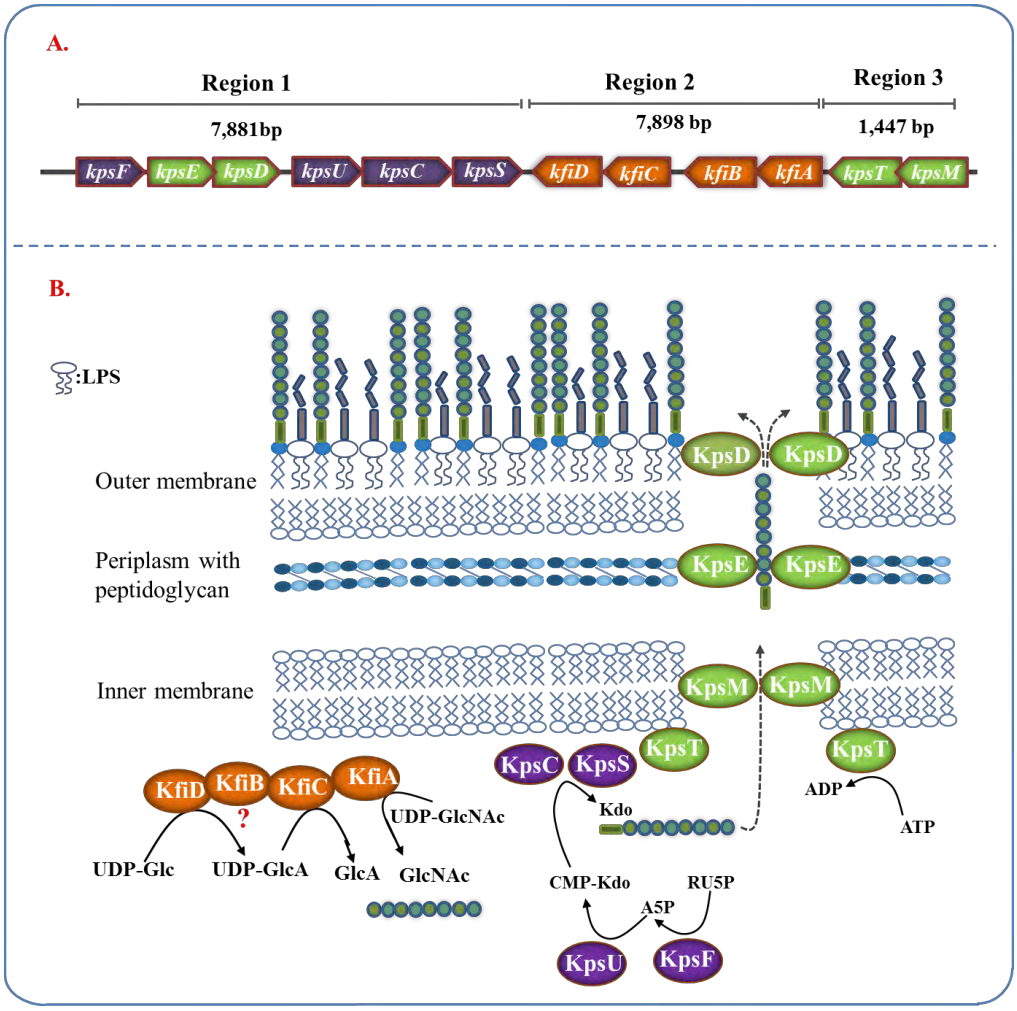


Figure 5.2: Biosynthesis of *E. coli* K5 capsular polysaccharide

A. Genetic organisation of the K5 gene cluster in *E. coli* Nissle 1917 (adapted from Grozdanov *et al.* 2004**)**. Central Region 2 encodes genes ***kfiA-D*** for biosynthesis of the capsular polysaccharide; Region 1 and 3 include genes ***kpsF, U, C, S*** involved in polysaccharide modifications and ***kps E, D, T, M*** involved in exportation of the polysaccharide chains across the plasma membrane. **?**: specific function of *kfiB* is unknown in the biosynthesis but mutation in this gene abolishes the capsule.

B. Model for K5 capsular polysaccharide biosynthesis and export (Adapted from Whitfield and Roberts 1999, Corbett and Roberts 2008)

- Dehydrogenase KfiD converts UDP-Glc into UDP-GlcA. From substrates UDP-GlcA and UDP-GlcNAc, glycotransferases KfiA and KfiC elongate the polymer chain by alternative addition of GlcA and GlcNAc, respectively, to the non-reducing end of the nascent polysaccharide chain.

- KpsF converts D-ribulose 5-phosphate (Ru5P*)* to D-arabinose 5-phosphate *(*A5P*),* which is then isomerised by KpsU into cytosine monophosphate keto- 3-deoxy-manno-2-octulosonic acid (CMP-Kdo). CMP-Kdo serves as substrate for KpsC and KpsS to produce Kdo that is subsequently transferred to the reducing terminus of the polysaccharide.

- The finished polymer is transported across the cytoplasmic membrane by KpsM and KpsT that form an ABC-2 transporter complex, and then exported to the bacterial cell surface by KpsE and KpsD.

Gene deletion was performed using the Xer-cise™ chromosomal modification system (Cobra Biologics, Keele, UK). The system permits replacement of target genes in bacterial host chromosome with a markerless deletion cassette. The main components of the systems are plasmid vectors pTOPO-DifCAT and pLGBE, providing the recombination machinery required for gene replacement as illustrated in Figure 5.3. Three key steps were involved for the functionality of the system: i) providing target bacterial cells with recombination functions; ii) construction of the insertion cassette, specific to the chromosomal target locus; iii) chromosomal integration of the cassette and deletion of the target locus (Bloor and Cranenburgh 2006).

5.3.6.1 Provision of recombination functions

Prior to performing gene deletion, recipient cells of EcN wild-typewere first provided with the λ Red recombination functions from genes *bet, exo* and *gam*, and encoded on the tetracycline-selectable plasmid pLGBE (Figure 5.3.a). These functions allow homologous recombination as well as protection of foreign linear DNA from degradation by host exonucleases.

Plasmid pLGBE DNA was transferred into the cells via electroporation as described in Section 2.2.12. The resulting transformants (designated EcN-pLGBE) were isolated on LB agar supplemented with 12 μg/mL tetracycline. To confirm the presence of pLGBLE in EcN, the tetracycline-resistant colonies were picked and used to grow cultures for isolation of the plasmid DNA. This was subsequently digested using restriction enzyme then visualised on agarose get to confirm replication of pLGBE in host cells. Successful transformants (replicating pLGBE) were stored as aliquots in 20 % glycerol at -80ºC freezers until needed for further steps of the gene deletion process.

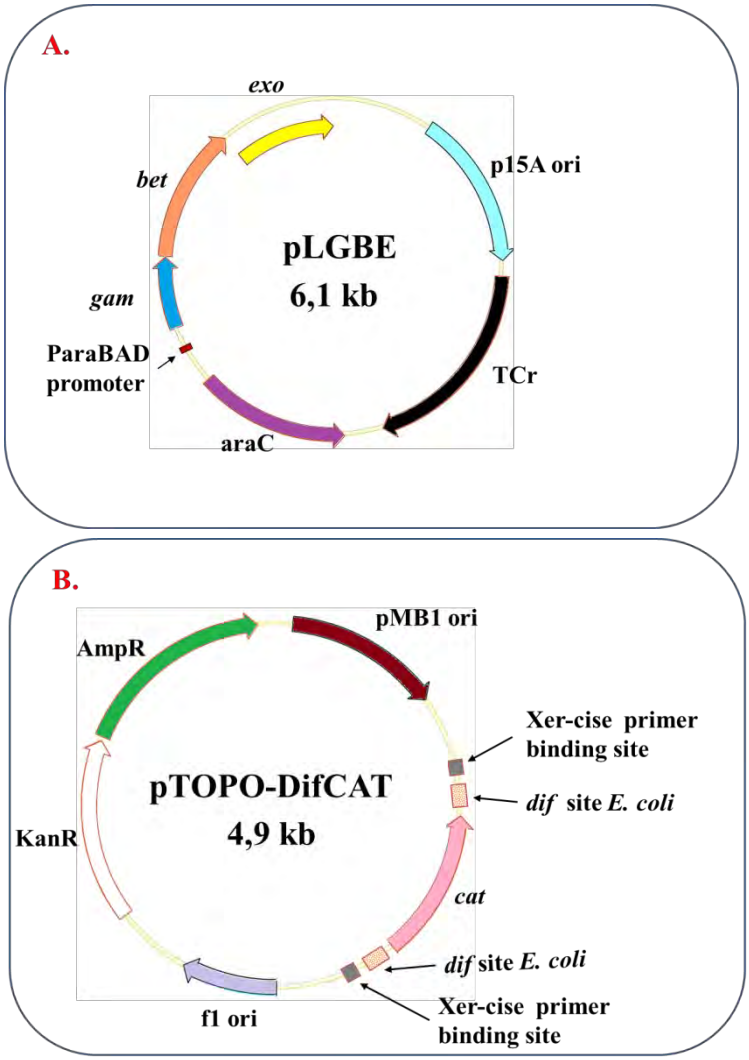


Figure 5.3: Genetic tools used in Xer-cise™ chromosomal modification system

A. Plasmid pLGBE containing the *bet*, *exo* and *gam* genes from bacterio hage λ, and ex ressed from the arabinose-induced ParaBAD promoter. *Gam* encodes functions that inhibit linear DNA degradation by native exonucleases in host bacterial strain; *Bet* and *exo* enable chromosomal integration. The plasmid also carries the tetracycline resistance gene TCr and the p15A origin of replication, which help to maintain a low copy number of plasmids per cell.

B. Plasmid pTOPO-DifCAT containing the *dif*-flanked chloramphenicol resistance gene (*cat*) The fragment terminated by the Xer-cise ends provides gene replacement cassette, a main functional component in chromosomal gene replacement. The cassette can be extended further by adding short sequences overhangs, to either ends of the cassettes and homologous of the chromosomal region to be modified
The *dif* elements are natural chromosomal sites found in *E. coli* and upon which native Xer recombinases act in resolving the chromosome and plasmid dimers generated by RecA before segregation at cell division. This structure allows the excision of the *cat* gene and subsequent recombination following chromosomal integration of the cassette.
The plasmid carries additional resistance genes *bla* (ampicillin) and *kan* (kanamycin), Pmb1 origin offering high copy number of the plasmid per cell and F1 origin of replication which permits generating single stranded DNA in bacteria infected with bacteriophage

5.3.6.2 Construction of gene deletion cassettes

To delete *kfiB* or *kfiC* from the EcN chromosome, the *dif* $_{E.\ coli}$ *-cat-dif* $_{E.\ coli}$ cassettes were amplified by PCR from pTOPO-DifCAT DNA template, using various sets of primers as listed in Table 5.2. For each target gene, a set of 70-nucleotide (nt) primers, *kfiB*.int_F and *kfiB*.int_R, or *kfiC*.int_F and *kfiC.* int_R, were used in the PCR for deletion of *kfiB* or *kfiC*, res ec tively. For each rimer, 50 nt of the 5' ends consisted of a sequence homologous to the chromosomal regions terminating the target gene and 20 nt of the 3' ends were homologous to pTOPO-DifCAT. The *dif* $_{E.\ coli}$ *-cat-dif* $_{E.\ coli}$ control cassette was also constructed using primers *Xer-cise*_F and *Xer-cise*_R, consisting only of the 20 nt 3' end (the TOPO-DifCAT homologous) described above. Sizes of the DNA fragments obtained from the PCRs of deletion cassettes were compared to those of the control cassette on agarose gel. A successful PCR should produce a larger DNA fragment, with an extra 100 nt as compared to the control PCR product size. PCR fragments showing the right product size were purified using the Qiagen gel extraction kit (as described in Section 2.2.5) then used directly or kept in freezer at -20ºC until needed.

5.3.6.3 Chromosomal integration of the gene deletion cassette

Figure 5.4 and 5.5 illustrate the process used to generate *kfiB* and *kfiC* deletion mutants from EcN. Recipient cells for gene deletion consisted of the *E. coli* Nissle–pGLBE (EcN-pLGBE) transformants (prepared in section 5.3.6.1) whereas the integration cassette for gene deletion consisted of linear DNA fragment from the PCR product of the *dif* $_{E.\ coli}$ *-cat-dif* $_{E.\ coli}$ cassette (prepared in section 5.3.6.2).

Cultures of EcN-pLGBE were grown in LB broth supplemented with 0.2 % (w/v) arabinose (Sigma) and 12 µg/mL tetracycline at 37ºC overnight with shaking, then used to produce electrocompetent cells. Arabinose was used to induce the expression of

Table 5.2: Primers used in deletion of *kfiB* or *kfiC* from the *E. coli* Nissle 1917 chromosome

Primer	Sequence (5'→3') [a]	AT (°C)	PCR product (nt)	Comments	Source
EcN*kfiB*_F EcN*kfiB*_R	TAGTCGGACATCCTGGCTCA GCCCTTGATTTTAGCTCTCC	56.3	1,667	*kfiB* locus of EcN K5 biosynthesis cluster	This study
EcN*kfiC*_F EcN*kfiC*_R	GGGACAAATATTGGTGCTCTTG GTCTTGCCGCGATATCACTA	52.2	1,417	*kfiC* locus of EcN K5 biosynthesis cluster	This study
kfiB.int_F *kfiB*.int _R	CAACAACAATTAACTTAAATGGAAGGGTAATGAATCC AAATATCGAATTA**AGTGTGCTGGAATTCGCCCT** TCAGATAGTTGAACATTTTTTGAAAGAAATTGGCATG AACTCACCAAATT**CTGCAGAATTCGCCCTTCCT**	70.8	1,190	Construction of the *dif* $_{E.\ coli}$*-cat-dif* $_{E.\ coli}$ cassette for deletion of a 1, 552 -nt segment of *kfiB*	This study
kfiC.int_F *kfiC*.int _R	CTTCGAGATTGTGATATATATCCATCAGGTAGAGCTT CTGTTCCTTTATT**AGTGTGCTGGAATTCGCCCT** CAATTCATAAGGAGAAAGTTGATCTTCAACATAAAAA CTCGCCTTTAAAA**CTGCAGAATTCGCCCTTCCT**	70.8	1,190	Construction of the *dif* $_{E.\ coli}$*-cat-dif* $_{E.\ coli}$ cassette for deletion of a 1,257-nt segment of *kfiC*	This study
*Xer-cise*_F *Xer-cise*_R	**AGTGTGCTGGAATTCGCCCT** **CTGCAGAATTCGCCCTTCCT**	58.4	1,090	*dif* $_{E.\ coli}$*-cat-dif* $_{E.\ coli}$ cassette control	Bloor and Cranenburgh (2006)
RT*kfiB*_F RT*kfiB*_R	GGCTGCATTATGGGAGGTAG TGTTCCAACTGCTCTTGCAC	56.3	456	internal segment of *kfiB*	This study
RT*kfiC*_F RT*kfiC*_R	ATTGTCGCCCAAACAAAAAG ACAATCATCGCACACGAGAA	52.2	463	internal segment of *kfiC*	This study

[a]For primers *kfiB*.int_F/R and *kfiC*.int_F/R (70-nt long each), the homology regions terminating target genes being deleted are underlined whereas the pTOPO-DifCAT homologous regions are bold faced.

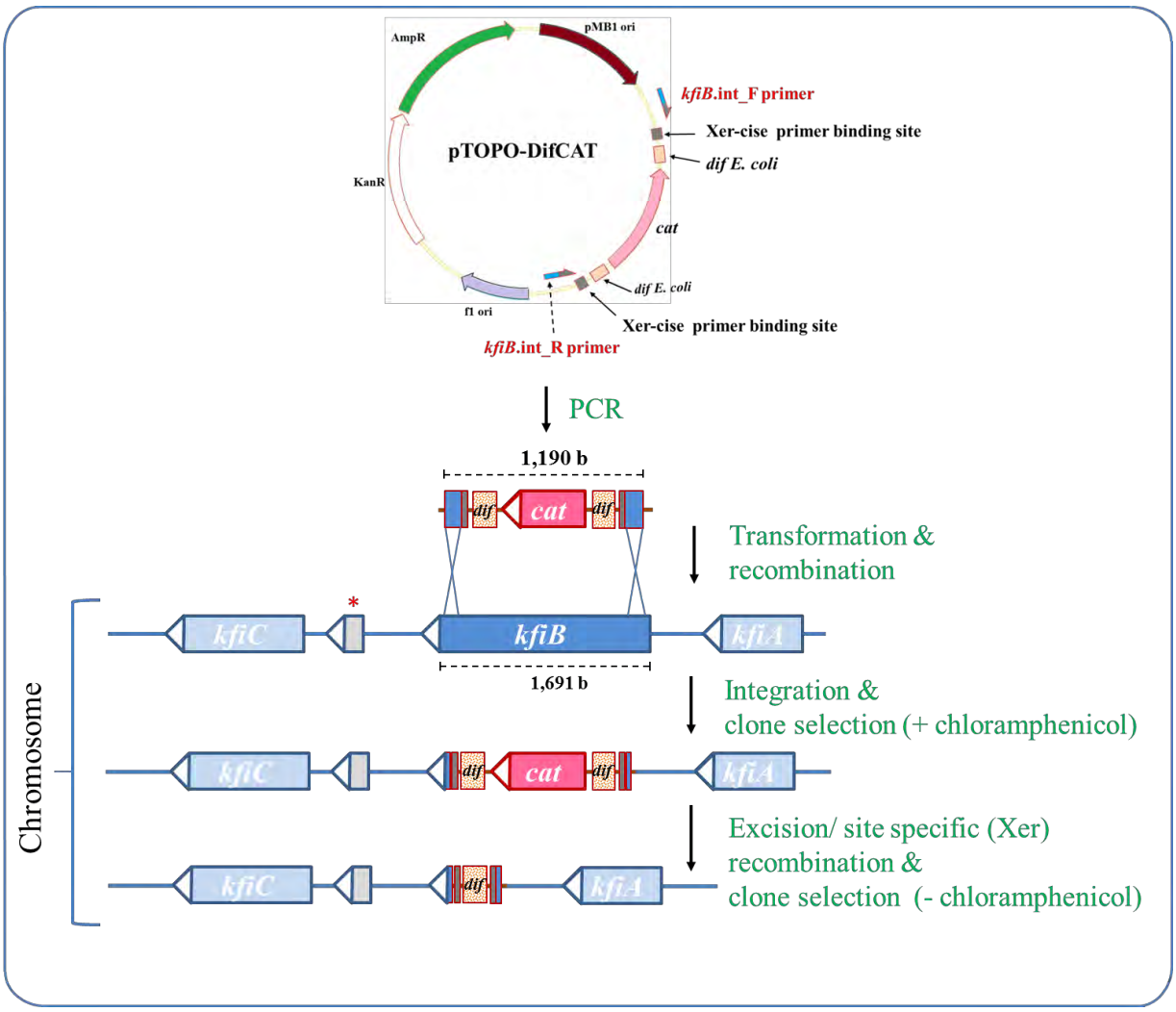


Figure 5.4: Deletion of *kfiB* using a PCR product *dif* $_{E.\ coli}$ *-cat-dif* $_{E.\ coli}$ cassette

The cassette has homology to the regions terminating chromosomal *kfiB* in order to generate EcNΔ*kfiB*.

* intergenic ORF with playing unknown functions but not for K5 capsule biosynthesis (Corbett and Roberts 2008).

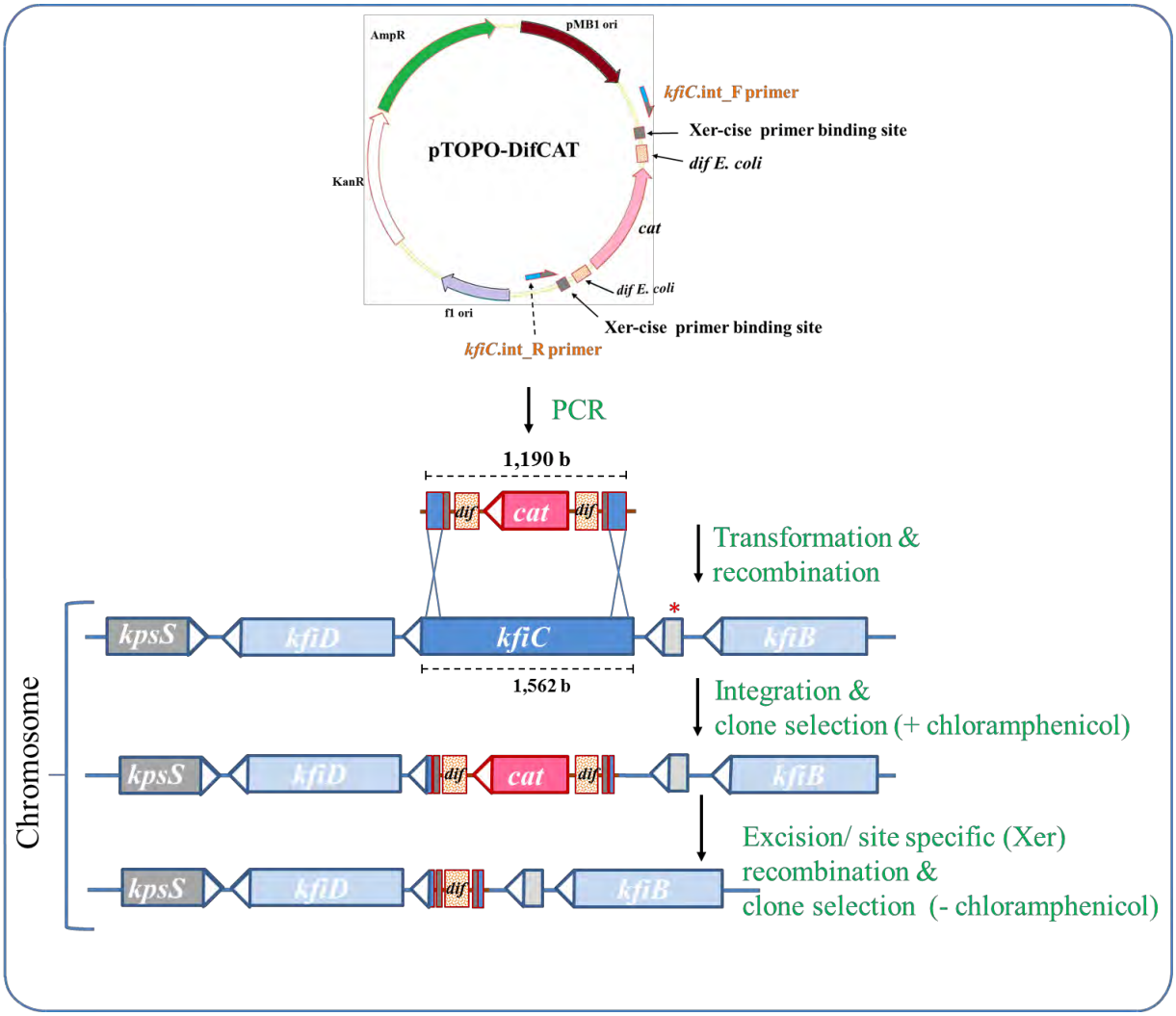


Figure 5.5: Deletion of *kfiC* using a PCR product *dif* $_{E.\ coli}$ *-cat-dif* $_{E.\ coli}$ cassette

The cassette has homology to the regions terminating chromosomal *kfiC* in order to generate EcNΔ*kfiC*.

* intergenic ORF with playing unknown functions but not for K5 capsule biosynthesis (Corbett and Roberts 2008).

the Red λ recombination functions from the pLGBE plasmid. Electrocompetent cells (100-µL aliquot) were transformed with 2.5 µg DNA of the gene cassette PCR products (either specific for *kfiB* or *kfiC* deletion).

After electroporation, cells were allowed to recover in SOC broth medium supplemented with 0.2 % (w/v) arabinose and 8 µg/mL tetracycline for 3 hours at 37ºC with shaking. Cultures were then plated onto LB agar supplemented with 20 µg/mL chloramphenicol and 0.2 % (w/v) arabinose followed by overnight incubation at 37ºC to allow chromosomal integration of the *dif* $_{E.\ coli}$*-cat-dif* $_{E.\ coli}$ cassette to take place. The resulting chloramphenicol-resistant colonies were considered as putative integrants of the *kfiB* deletion cassette or *kfiC* deletion cassette.

Putative integrants were first screened by PCR using primers flanking the chromosomal insertion site to confirm that the target gene was being replaced and that the cassette has integrated in the right site. Total DNA extract was obtained from 10 fresh colonies of the putative integrants (from each category) and control EcN-pLGBE (non-transformed with the gene cassette DNA) by colony boil technique (Section 2.2.8). The DNA extract was used as template in PCR screening for the *kfiB* or *kfiC* locus using primer sets EcN*kfiB*_F/R and EcN*kfiC*_F/R, respectively. The PCR products obtained from putative integrant samples were compared to those of controls, and successful chromosomal integration of the cassette was determined based on size of the DNA fragments.

Cultures showing successful integration of the cassette were further subcultured (up to 10 times) in LB broth without chloramphenicol to allow excision of the *dif-cat* fragment of the integrant cassettes, and recombination by native Xer enzymes of *E. coli*. Each time, liquid culture was spread on LB agar plates with and without chloramphenicol to determine loss of the resistance gene and formation of the *dif-cat* resolvants (potential deletion mutants). Successful resolvants were also checked for loss of the pLGBE

plasmid by analysing their sensitivity to tetracycline (selective for pLGBE) on LB agar media. The loss of pLBGE was expected through the subculturing of the cells in tetracycline-free medium.

Successful mutants (EcNΔ*kfiB* or EcNΔ*kfiC*) were further confirmed by: i) PCR screening the target locus (using primer sets EcN*kfiB*_F/R or EcN*kfiC*_F/R), ii) PCR screering internal fragments of the target locus to confirm gene deletion (using primer sets RT*kfiB*_F/R or RT*kfiC*_F/R), and iii) PCR screening the Xer-cise cassette to confirm excision of the *dif*-cat fragment. All the primers are listed in Table 5.2.

5.3.7 Examination of the polar effects in *kfiB* and *kfiC* mutants

The effect of gene deletion or disruptions in *kfiB* and *kfiC* mutants, on the expression of downstream genes (polar effects) was assessed using reverse transcriptase (RT-) PCR and compared with the wild-type. Total RNA was extracted from mid-log-phase bacterial cells via the RNeasy Protect Cell Mini Kit (Qiagen) and treated using the Ambion® TURBO DNA-*free*™ system (Ambion-Life technologies) to remove any potential DNA contamination according to manufacturers' instructions. The treated NA concentration was determined using *NanoDrop 2000* (Thermo Scientific) then used as template in RT-PCR reaction which was carried out with the One Step RT-PCR kit (Qiagen) to produce cDNA according to the manufacturer's instructions. Control reactions were added for each experiment and included: i) negative control (all RT components minus RNA template), ii) control for DNA contamination (all RT components minus reverse transcriptase enzyme). Primers series RT*kfi*A-D_ (F/R*)* were used in standard PCR and are listed in Table 5.3. In addition to the RT reaction controls (mentioned above), the PCR positive control (chromosomal DNA used as template) and standard PCR negative control (all reagents without template) were also included in the PCR.

Table 5.3: Primers used in RT-PCR for analysis of the K5 capsule biosthesis genes in strains in *E. coli* Nissle 1917

Primer	Sequence (5'→3')	AT (°C)	PCR product (nt) [a]	Source
RT*kfiA*_F	TGTTGGGATTCATGGCTGTA	52.2	355	This study
RT*kfiA*_R	TGCGATTGCTTGTGTTTCTT			
RT*kfiB*_F	GGCTGCATTATGGGAGGTAG	56.3	456	This study
RT*kfiB*_R	TGTTCCAACTGCTCTTGCAC			
RT*kfiC*_F	ATTGTCGCCCAAACAAAAAG	52.2	463	This study
RT*kfiC*_R	ACAATCATCGCACACGAGA A			
RT*kfiD*_F	TTGGTCTGAATACGCGTCAG	56.3	393	This study
RT*kfiD*_R	CCCGTTCCAAAGGTGAGTTA			

[a] PCR amplification of internal fragments of the target genes

5.3.8 Cell surface expression of K5 polysaccharide in EcNΔ*kfiB* or EcNΔ*kfiC* mutants

To assess the effect of *kfiB* or *kfiC* deletion on the ability of EcN to express the capsule, the bacteriophage (ΦK5) sensitivity assay was employed (Gupta *et al.* 1983). The ΦK5 encodes a tail-associated lyase protein, which specifically attaches and subsequently degrades the K5 capsule, thus infecting strains producing this surface structure (Gupta *et al.* 1983, Hanfling *et al*. 1999).

The bacteriophage was diluted and maintained in phage dilution buffer (PDB) (100 mM NaCl, 8 mM $MgSO_4$, 0.01 % w/v gelatine, 50 mM Tris; pH 7.5). Soft agar (1 % w/v NaCl, 0.5 % w/v yeast extract, 1 % w/v tryptone, 0.75 % w/v agar) was used as top layer agar in bacteriophage plaque assay. Cultures of EcNΔ*kfiB*, EcNΔ*kfiC,* positive control EcN wild-type and negative control *E.coli* MG1655 were grown in LB with shaking at 37°C to an OD_{600} of 0.3, e lleted by centrifugation (10,000 x *g* for 10 minutes) then resus ende d in ice-cold 10 mM $MgSO_4$. Aliquots of cell sus ension (100 µL) were mixed with 100 µL of the a r o ria te bacterio h age dilution (ranging from 10^1 to 10^9 Pfu/mL from stock sus ension of 2.1x 10^8 Pfu/mL) in sterile 1.5 mL E e ndorf tubes were then incubated at T for 30 minutes, statically. The cell-bacteriophage suspension was added to a volume (3 mL) of the soft agar held at 50°C in 15 mL sterile glass tube, and the content of the tubes were mixed gently by swirling. The inoculated soft agar was poured on top of LB agar and incubated for 16 hours at 37°C to allow formation of plaques

5.3.9 Adherence of EcNΔ*kfiB* or EcNΔ*kfiC* mutants to Caco-2 cells and abiotic surfaces

The effect of *kfiB* or *kfiC* deletion on EcN strains' ability to adhere to Caco-2 cells and to form biofilms in microtitre were assayed using the gentamicin protection assay described (Section 5.3.2) and crystal violet staining assay for biofilm formation (Section 4.3.5),

respectively. Adherence index and biofilm formation index of mutants were calculated as the percentage of adherence and biofilm formation in the EcN wild-type.

5.3.10 Analysis of apoptosis and cytotoxicity in Caco-2 cells co-cultured with EcNΔ*kfiB* or EcNΔ*kfiC* mutants

The effect of gene deletion in EcNΔ*kfiB* or EcNΔ*kfiC* on induction of apoptosis and cytotoxicity in Caco-2 cells was assessed by measuring the activity of caspase 3/7 (using a protocol detailed in Section 5.3.4) and lactate dehydrogenase (LDH), respectively. The LDH assay and was performed to determine the amount the LDH leaked into the co-culture medium, an indicator of cytoplasmic membrane damage. The CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega, Southampton, UK) was used according to the manufacturers' instructions.

Both assays were performed in parallel using 96-well plate format co-culture model as detailed in Section 5.3.4. After treatment of Caco-2 cells with bacterial strains, supernatants were collected from plate wells using a multichannel pipette then transferred to fresh 96-well plates at 50 µL/well. The supernatant was diluted further in serum-free culture then mixed with the CytoTox 96® substrate at 1:1 ratio. Plates were incubated in dark at room temperature for 30 minutes and absorbance at 490 nm (OD_{490}) was recorded. The percentage of cytotoxicity was calculated as LDH released in treated cells (OD_{490})/maximum LDH release (OD_{490}) x 100. Maximum release was determined as the amount released by total lysis of untreated Caco-2 cells with the CytoTox 96® Lysis Solution (10 x).

5.3.11 Analysis of cellular and nuclear morphology in Caco-2 cells co-cultured with EcNΔ*kfiB* or EcNΔ*kfiC* mutants

Membrane integrity and nuclear morphology of Caco-2 cells was analysed by phalloidin staining of *F-actin* cytoskeleton and confocal fluorescence microscopy (Barak *et al*. 1980). Cells were grown on sterile glass cover slips (Oncor *Inc.,* Dallas, USA) in 6-well plates then co-cultured with inocula of EcNΔ*kfiB* or EcNΔ*kfiC* mutants and controls EcN wild-type and *E. coli* MG1655 for 12 hours as described in Section 5.3.1. After the treatments, the Caco-2 cells on coverslips were washed with PBS then fixed with 4 % formaldehyde (Sigma) in PBS for 20 minutes at RT. The fixed cells were washed three times with PBS and made permeabile by treating with 0.5 % Triton X-100 (Sigma) in PBS for 10 minutes at RT. The cells were washed three times with PBS, 5 minutes each on a rocker, then treated with a 0.1 µg/mL solution of fluorescein isothiocyanate-phalloidin (Sigma- Aldrich) in PBS for 1 hour at RT in the dark. The cells were washed twice with PBS and were mounted with the Fluoroshield™ DAPI medium (Sigma) and examined under a Leica TCS SP5 Confocal Laser Scanning microscope (Leica Microsystems, Wetzlar, Germany).

5.3.12 Statistical analyses

All experiments were performed a minimum of three times and data were assessed by a one way analysis of variance (ANOVA) or t-test. Differences were considered statistically significant if $P<0.05$.

5.4 Results

5.4.1 Optimisation of co-culture model of E. *coli* Nissle and Caco-2 cells

To evaluate the effect of tissue culture medium on bacterial growth, EcN was assayed for growth in Caco-2 cell growth medium. The results are presented in Figure 5.6 and show that both complete and serum-free DMEM media supported the growth of EcN cultures. These media were therefore confirmed to be useful in co-culture models.

Optimisation of a co-culture model of EcN-Caco-2 was performed to determine ranges of bacterial cell densities and co- incubation period affecting the viability of the Caco-2 cells. The results are presented in Figure 5.7. Overall, Caco-2 cell viability was found to be unaffected in co-cultures with EcN at multiplicity of infections (MOIs) below or equal to 10:1 for up to 4 hours incubation. Extending co-incubation to 6 hours was found to reduce Caco-2 cell viability by 60 % at the lowest MOI value (0.1:1) used in this study. MOIs above 20:1 for 2 hours co-incubation were also found to be detrimental to Caco-2 cells. Therefore the optimum conditions of EcN in Caco-2 cell model were determined at MOIs of 10:1 and below and co-incubation duration of up to 4 hours.

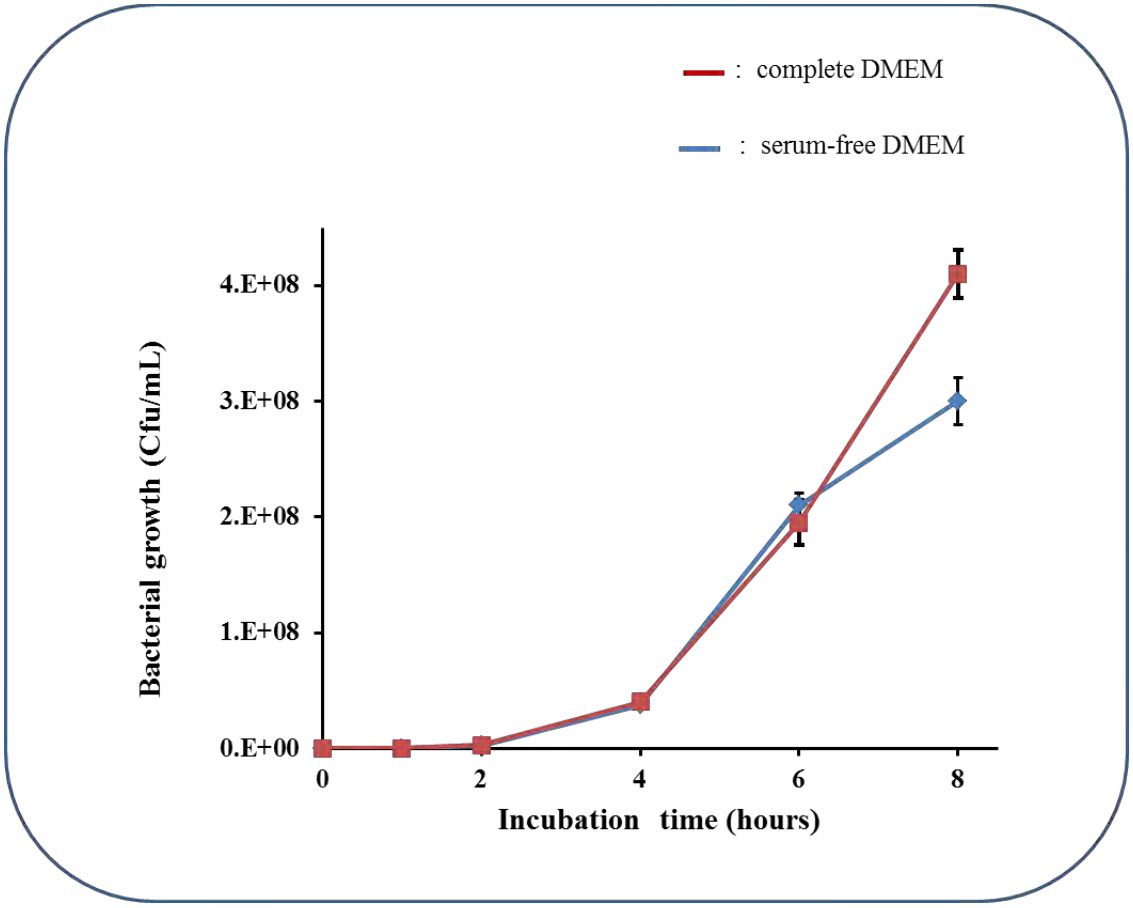


Figure 5.6: Growth of *E. coli* Nissle in DMEM media

Cultures were grown statically at 37°C, 5 % CO_2 and bacteria growth was monitored by plating cultures on agar plates. Results shown are the mean ± SEM of three independent experiments.

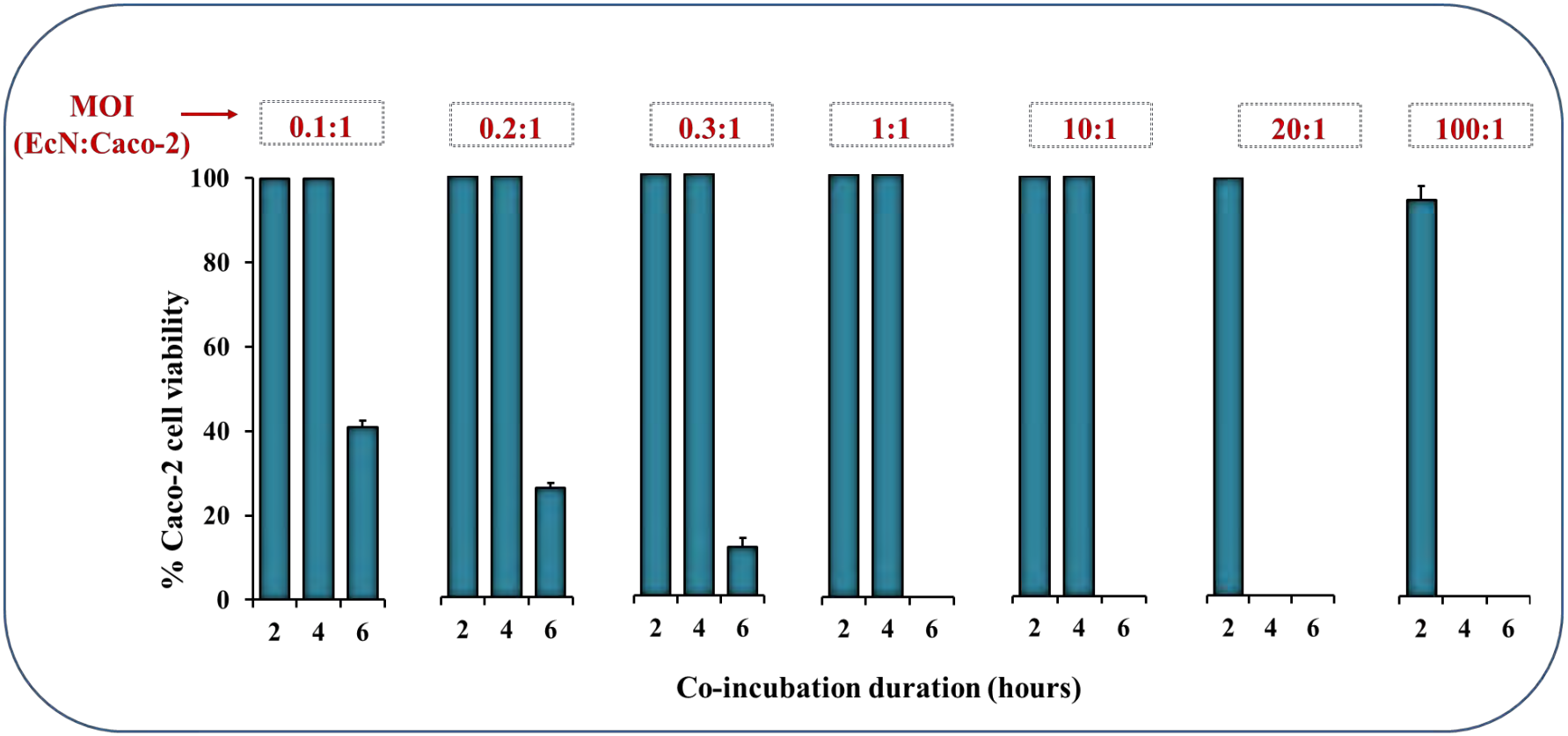


Figure 5.7: Assessment of Caco-2 cell viability in co-culture with *E. coli* Nissle 1917

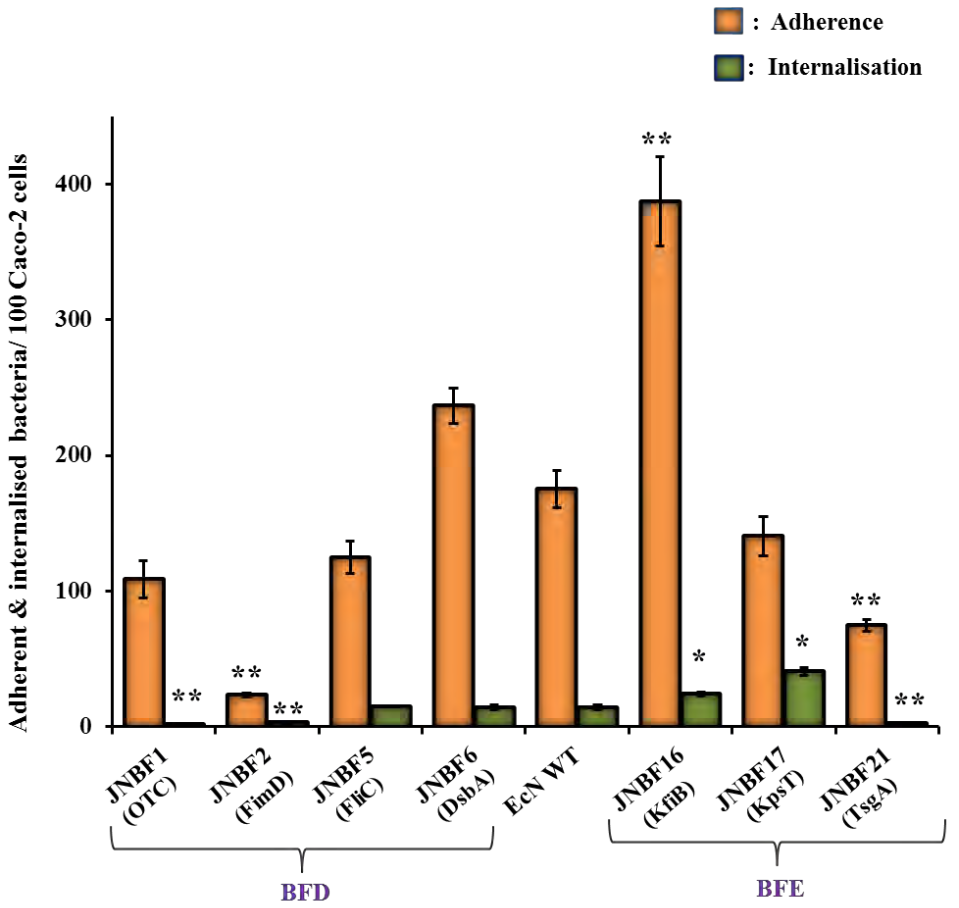
Monolayers of Caco-2 cells (75-80 % confluent, ~ 1 x 10^6 cells /well in 6-well plate) were co-cultured with mid-log-phase cultures of EcN wild-type at a range of multiplicity of infection (MOI), and incubation durations, at 37 °C and 5 % CO_2. Cell viability was determined by trypan blue exclusion assay and % viability of Caco-2 cells was calculated in comparison with control wells (with medium only). Data is expressed as the mean of three independent replicates. Error bars show standard error of the mean.

5.4.2 Adherence to and internalisation of *E. coli* Nissle cell surface structure mutants to Caco-2 cells

Five EcN mini-Tn*5* mutants disrupted in genes predicted to encode for cell surface structures (referred to as 'surface structure' mutants) were used in this study. These mutants had been recovered in biofilm formation screens of the EcN transposon mutant library as described in Section 4.3.5. The mutants as well as the wild-type were found to be non-sensitive to the lysis solution (1 % w/v saponin in trypsin-EDTA) that was used to dissolve Caco-2 cells in gentamicin protection assay (Section 5.3.2).

To determine adhesion properties of the selected mutants to Caco-2 cells, the number of adherent and internalised bacteria was assayed, and compared to values obtained for EcN wild-type. The results are displayed in Figure 5.8. Overall, levels of bacterial adherence to Caco-2 cells showed no direct correlation with biofilm phenotype in 96-well plates among the selected mutants. Only mutants JNBF2 (with defect in *fimD* involved in fimbriae polymerisation) and JNBF16 (defect in *kfiB* involved in biosynthesis of capsule K5) demonstrated a direct correlation in both parameters. JNBF2, a biofilm formation deficient (BFD) mutant was demonstrated to be adhesion-deficient to Caco-2 cells while JNBF16, a biofilm formation enhanced (BFE) mutant was shown to exhibit enhanced adherence to and internalisation in Caco-2 cells.

Of particular interest were two mutants defective in genes involved in the biosynthesis and translocation of K5 capsule but which exhibited contrasting phenotypes in adherence to Caco-2 cells. These were JNBF16 and JNBF17 mutants disrupted in *kfiB* and *kpsT* genes, respectively; both genes are featured in Figure 5.2 illustrating capsule K5 biosynthesis. The *kfiB* mutant exhibited the highest levels of adhesion to Caco-2 cells with a significant difference in adhesion as compared to the wild-type ($P < 0.01$). Conversely, the *kpsT* mutant (JNBF17) demonstrated no significant differences in adhesion to Caco-2 cells as compared to the wild type ($P > 0.05$).



Mutant	Putative function/product of disrupted gene
JNBF1 #	Ornithine carbamoyltransferase OTC
JNBF2	Type 1 fimbriae anchoring protein FimD
JNBF5	Flagellar biosynthesis protein FliC
JNBF6	Periplasmic thiol:disulfide interchange protein DsbA
JNBF16	KfiB protein, involved in K5 biosynthesis
JNBF17	Polysialic acid transport ATP-binding KpsT
JNBF21 #	TsgA protein homolog

Figure 5.8: Adherence characteristics of EcN 'surface structures' mutants to Caco-2 cells

Monolayers of Caco-2 cells grown in 6-well plate (75-80 % confluent, ~ 1 x 10^6 cells /well) were co-cultured with mid-log-phase bacteria at an MOI of 1:1 for 4 hours at 37°C, 5 % CO_2. The number of adherent and internalised bacteria was determined using the gentamicin protection assay.

BFD: biofilm formation deficient, **BFE**: biofilm formation enhanced mutants.

#: genes disrupted in mutants JNBF1 and JNBF21 were not directly related to bacterial cell surface structures, but exhibited lowest and highest levels of adherence to abiotic surfaces (biofilm formation) , respectively. They were added to this experiment as controls.
*: significant difference as compared to *E. coli* Nissle wild-type (P <0.05), ** (P <0.01). Data are expressed as the mean of three replicates. Error bars show SE of the mean.

5.4.3 Caco-2 cell health analysis in co-cultures with *E. coli* Nissle 'surface structure' mutants using the Hoechst propidium iodide staining

To analyse Caco-2 cell health status in co-culture with cell 'surface structure' mutants of *E. coli* Nissle, the HPI staining was performed (Section 2.3.5). Applying the HPI stain to Caco-2 cells treated with bacterial cultures at an MOI value of 10:1 showed a detrimental effect on the epithelial cells and a lower value of MOI 5:1 and a 4-hours co-incubation were used for the assay. Results are presented in Figure 5.9. Overall there were no major differences in Caco-2 cell viability between co-cultures with mutants and those with controls (EcN wild-type and MG1655). However a subjective assessment indicated more apoptotic cells (bright-blue stain) in co-cultures with mutants JNBF1, JNBF16, JNBF17 and JNBF21. Overall these results indicate that EcN mutants may increase levels of apoptosis (a programmed cell death) rather than necrosis in some cases. Therefore, apoptosis of Caco-2 cells in co-culture with EcN strains was investigated further using quantitative techniques.

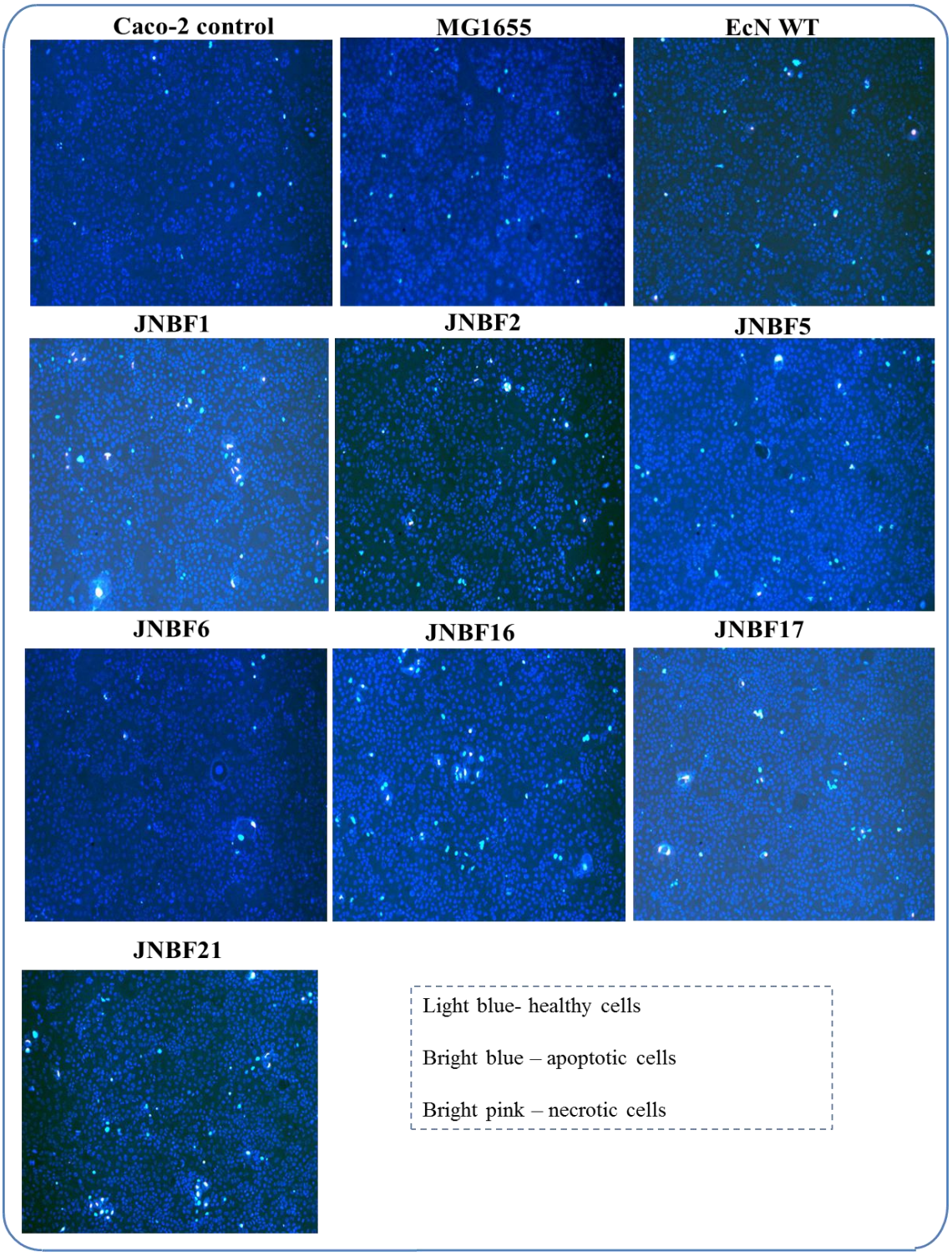


Figure 5.9: HPI staining of Caco-2 cells treated with EcN 'surface structure' mutants

- Monolayers of Caco-2 cells grown in 6-well plate (75-80 % confluent, ~ 1 x 10^6 cells /well) were co-cultured with mid-log-phase bacteria at an MOI of 5:1 for 4 hours at 37°C, 5 % CO_2.

- Bacterial cultures consisted of surface structure mutants of EcN, wild-type EcN and *E. coli* MG1655 (human gut commensal strain, as a bacterial control). Untreated Caco-2 cells as experimental control.

- HPI stain was performed and cells were visualised under fluorescent microscope, using Zeiss Axiovert 25 inverted at 5x magnification.

- These images are representative of four independent experiments.

5.4.4 Analysis of apoptosis in Caco-2 cells treated with *E. coli* Nissle surface structure mutants and supernatants by caspase activity

To quantitatively analyse the induction of apoptosis in Caco-2 cells treated with *E. coli* Nissle 'surface structure' mutants and their supernatants, the activity of caspase-3/7 was measured. The results are displayed in Figure 5.10 and 5.11 and show that co-culturing bacteria but not supernatants of mutant JNBF16 (*kfiB* mutant) increased caspase 3/7 activity in Caco-2 cells. The obtained caspase activity in this co-culture was significantly different from the one obtained in untreated cells or those treated with EcN wild-type ($P<0.05$).

Although mutants JNFB1, JNFB17, and JNFB21 also appeared to exhibit increased level of apoptosis in HPI staining (Section 5.4.3), this was not supported by quantitative assays here. There was no significant difference in caspase activity between the cells treated with these mutants, and controls (untreated caco-2 cells, cells treated with EcN wild-type and those treated with *E. coli* MG1655).

Co-incubation with bacterial supernatants did not show any significant difference between caspase 3/7 activities of untreated Caco-2 cells control and samples (Figure 5.11). This could indicate that the observed apoptosis induction exhibited by mutant JNBF16 was cell-to-cell contact dependent rather than due to secreted products. This mutant was therefore selected to investigate the role of *kfiB* gene and of K5 capsule in host-microbe interactions.

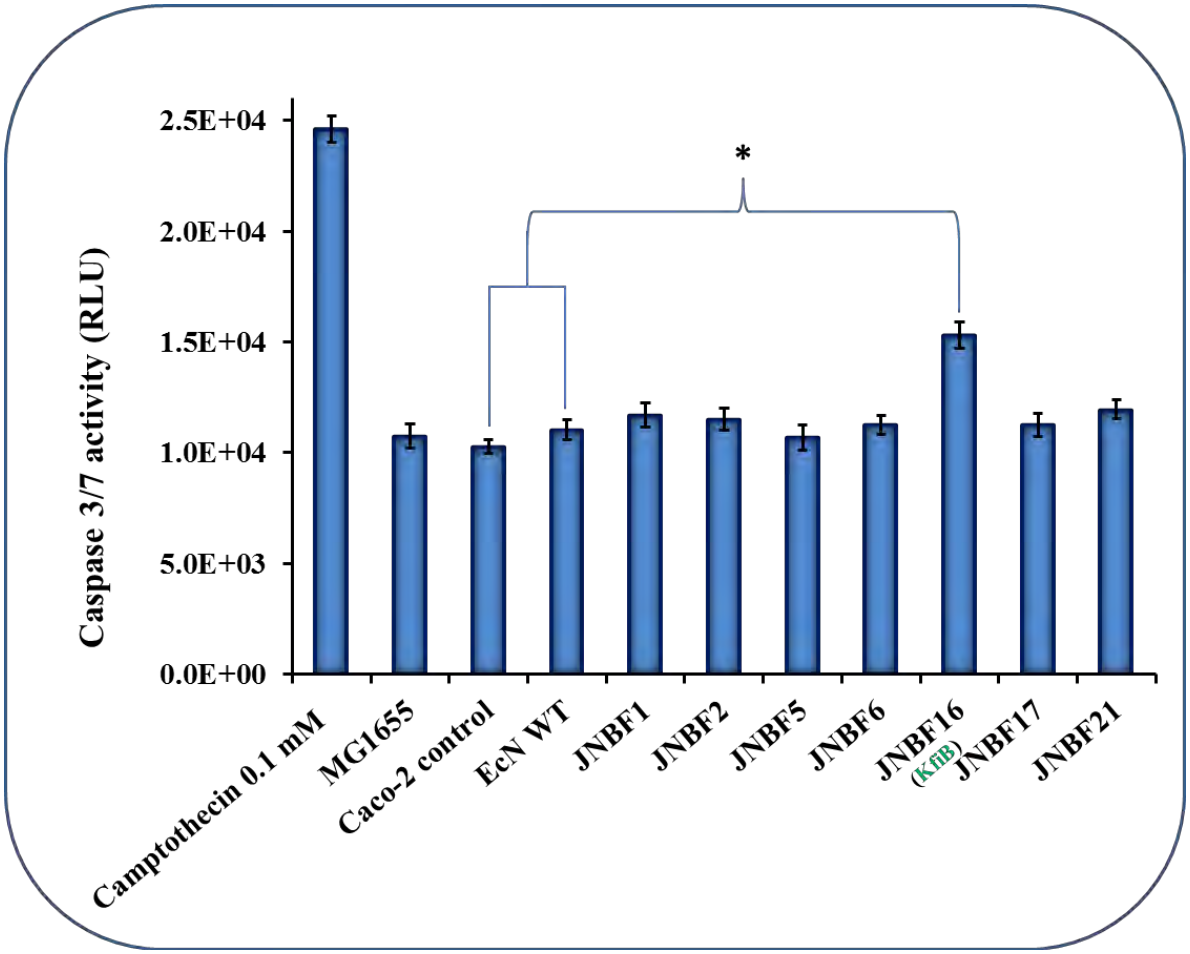


Figure 5.10: Analysis of apoptosis in Caco-2 cells treated with *E. coli* Nissle 'surface structure' mutants

- Caco-2 cells were grown in 96-well plates until 60 % confluence (~ 10,000 cells/ well) then treated with mid-log-bacterial strain suspensions at an MOI of 10:1. Bacterial suspensions were prepared in serum –free DMEM.

- The plates were incubated for 12 hours at 37°C and 5 % CO_2. After 2 hours of co-incubation, the medium (containing bacterial suspensions) was removed from each well then replaced with serum-free DMEM supplemented with gentamicin at 200 µg/mL.

- Caspase activity 3/7 assay was performed and luminescence proportional to the caspase activity was recorded as relative light units (RLUs) using microplate-luminometer.

*: significant difference as compared to *E. coli* Nissle wild-type ($P < 0.05$). Data are expressed as the mean of four independent replicates. Error bars show SE of the mean.

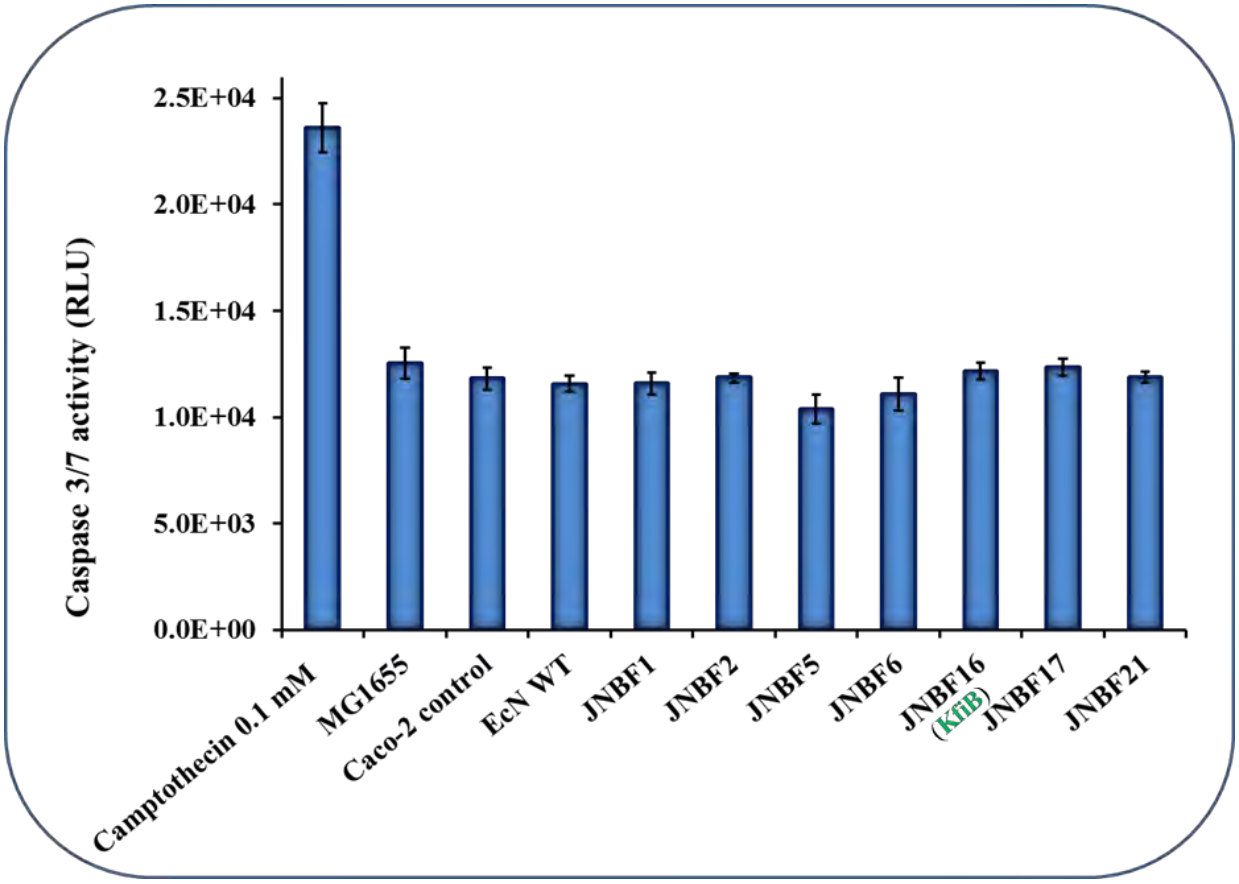


Figure 5.11: Analysis of apoptosis in Caco-2 cells treated with supernatant obtained from *E. coli* Nissle 'surface structure' mutants

- Caco-2 cells were grown in 96-well plates until 60 % confluence (~ 10,000 cells/ well) then treated with bacterial supernatants. Bacterial cultures were grown in serum –free DMEM overnight. Supernatants were then collected by centrifugation, pH adjusted to 7.2, filter sterilised and diluted in fresh serum-free medium before they were added to Caco-2 cells.

- The plates were incubated for 12 hours at 37°C and 5 % CO_2.

- Caspase activity 3/7 assay was performed and luminescence proportional to the caspase activity was recorded as relative light units (RLUs) using microplate-luminometer.

- Data are expressed as the mean of four independent replicates. Error bars show SE of the mean.

5.4.5 Confirmation of transposon insertion site in JNBF16 (*kfiB* mutant) using gene cloning approach

To confirm that *kfiB* was the gene disrupted by mini-Tn*5* in JNBF16 mutant (showing enhanced adherence to Caco-2 cells and elevated induction of apoptosis), PCR amplification of the entire *kfiB* locus was performed and the resulting product was cloned into pGMET then sequenced. Results are displayed in Figure 5.12. When genomic DNA extract from JNBF16 mutant was used as template in the PCR, a product of ~ 3,400 bp was obtained whereas template from EcN wild-type resulted in ~ 1,600 bp PCR products. Size difference between both DNA fragments is ~ 1,800 bp which is the approximate length of the mini-Tn*5* mobile element (Larsen *et al.* 2002) used in transposon mutagenesis of *E. coli* Nissle in this study. Sequence analysis of the 3,400-bp fragment confirmed the genetic identity of the mini-Tn*5* element and revealed its insertion site in JNBF16 mutant in *kfiB* gene at 220 nt downstream the gene start codon as shown in Figure 5.12.

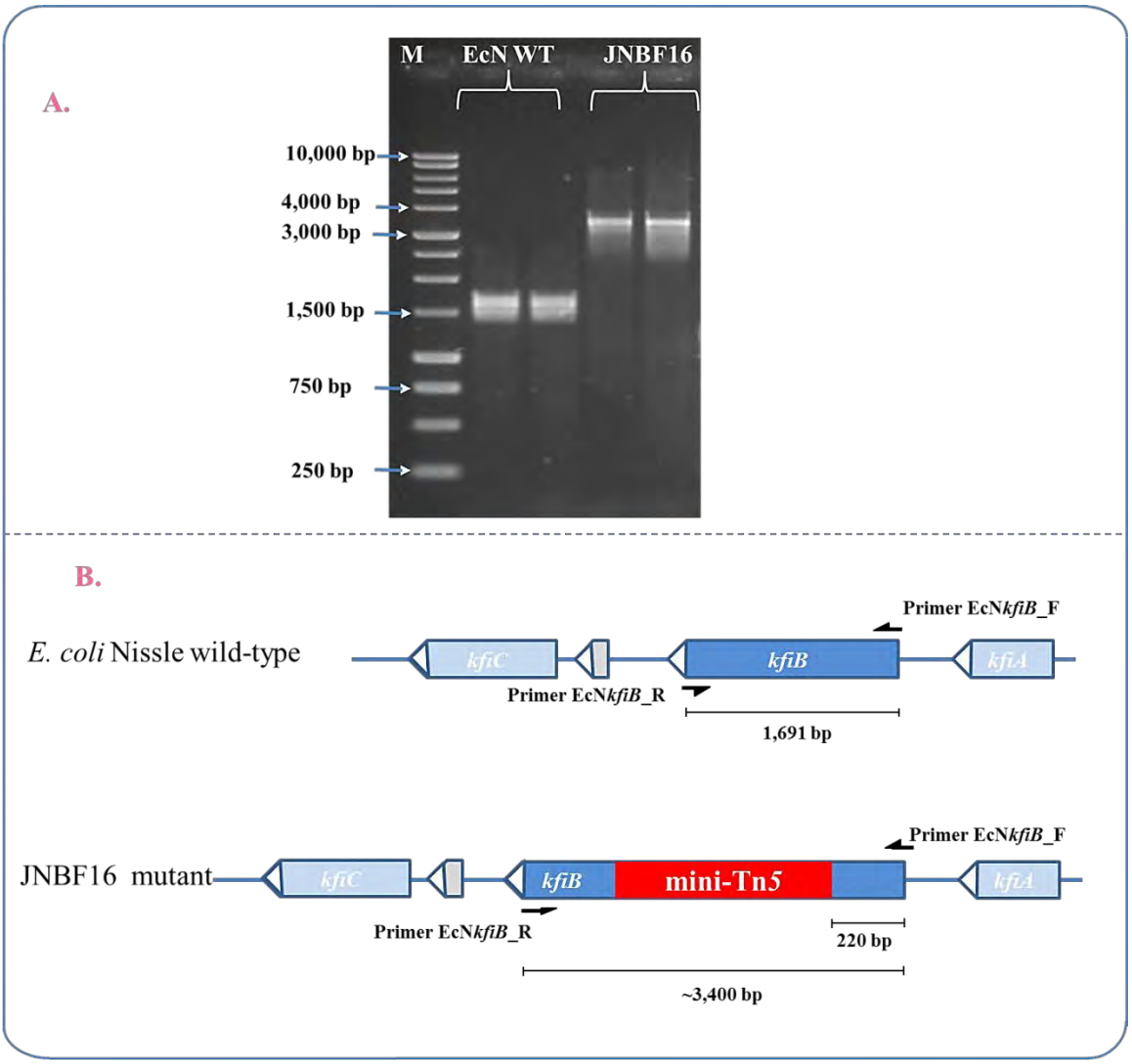


Figure 5.12: PCR screening *kfiB* locus in JNBF16 mutant

A. PCR amplification *kfiB* in JNBF16 mutant and *E. coli* Nissle wild-type chromosome.

Genomic DNA was extracted from bacterial strain and used as template in PCR screening *kfiB* locus using primers EcN*kfiB*_F and EcN*kfiB*_R. The obtained PCR product was separated on 1 % agarose.

M= 1 kb DNA ladder marker

B. Mapping of transposon insertion site in JNBF16 mutant

The ~ 3,400- bp DNA fragment obtained in the PCR above was purified then cloned into pGEMT easy vector then sequenced from SP6 and T7 Promoter Primers. Sequences were analysed and the site of mini-Tn*5* insert was determined and mapped in the *E. coli* Nissle genome.

5.4.6 Construction of deletion mutants EcNΔ*kfiB* and EcNΔ*kfiC*

Markerless deletion mutants EcNΔ*kfiB* or EcNΔ*kfiC* were constructed in order to confirm the role of *kfib* gene and K5 capsule in host-*E.coli* Nissle interactions. EcN wild-type cells were first provided with the λ ed recombination functions encoded on lasmi d LG E. Results are shown in Figure 5.13 and confirm that plasmid pLGBE was able to replicate in EcN and generate transformants (EcN-pLGBE).

Furthermore chromosomal integration cassettes *dif* $_{E.\ coli}$ *-cat-dif* $_{E.\ coli}$ for deletion of *kfiB* or *kfiC* were assembled by PCR and results are shown in Figure 5.14. Product size of the cassettes was shown to be ~1,200 bp, which is a 100-bp larger as compared to control cassette (without overhangs regions specific to target gene).

DNA fragments of these cassettes were introduced to EcN-pLGBE cells by electroporation. The resulting integrants (expressing chloramphenicol resistance from *cat* gene) and resolvants (integrants that had lost *dif-cat*) were analysed by PCR amplification of the entire *kfiB* or *kfiC* locus. The results are presented in Figures 5.15 and 5.16 and confirm that i) the cassettes integrated the chromosomal target , ii) replaced target genes, and iii) lost the *dif-cat* integration fragment followed by recombination and generation of mutants. Excision of the *dif-cat* fragment and generation of markerless mutants EcNΔ*kfiB* and EcNΔ*kfiC* was also confirmed by PCR amplification of the entire *dif* $_{E.\ coli}$ *-cat-dif* $_{E.\ coli}$ fragment in mutants as shown in Figure 5.17.

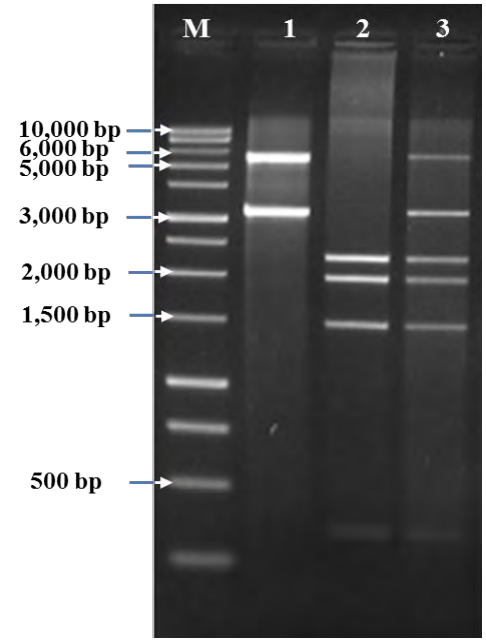


Figure 5.13: Transformation of *E. coli* Nissle with pLGBE plasmid

Plasmid pLGBE was transferred to *E. coli* Nissle Wild-type by chemical transformation. Colonies of tranformants were used to grow cultures which were used for plasmid preps.

Plasmids were digested by double restriction consisting of *Hind*III and *Bam*HI mix which linearises EcN cryptic plasmids pMUT1 and pMUT2, respectively (Lane 1).

M= 1kB ladder marker

1= Plasmid DNA isolated from *E. coli* Nissle wild-type

2= plasmid pLGBE

3-6= EcN pLGBE

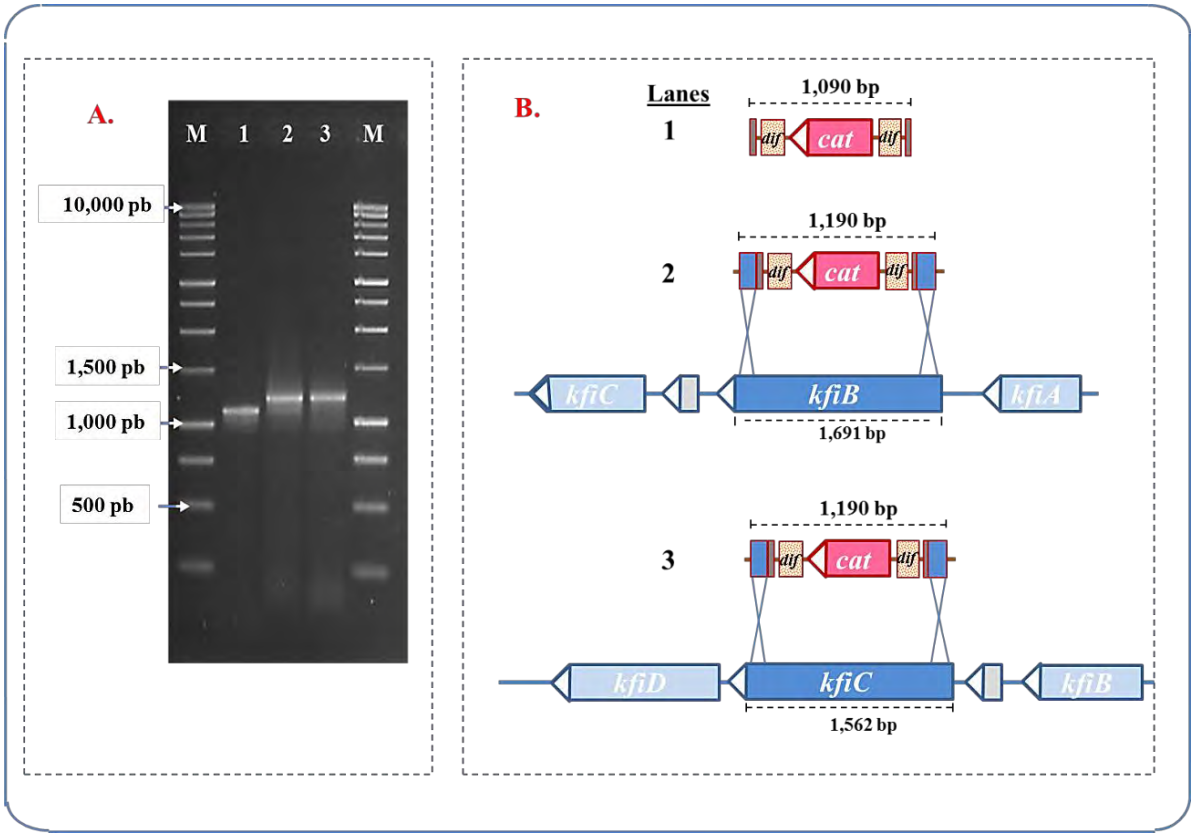


Figure 5.14: Construction of *dif* $_{E.\ coli}$ *-cat-dif* $_{E.\ coli}$ cassettes for deletion of *kfiB* and *kfiC*

A. Assembled of deletion cassettes by PCR. plasdmid pTOPO-DifCAT DNA as template and the 70-bp primers (with 50 pb homologous to target gene and 20 bp to pTOPO-Dif region) (Table 5.2)

B. Diagram showing the *dif* $_{E.\ coli}$ *-cat-dif* $_{E.\ coli}$ cassettes and their chromosomal targets

Lanes:

M= 1kB ladder marker

1= *dif* $_{E.\ coli}$ *-cat-dif* $_{E.\ coli}$ control, using primers Xer-cise_F&R specific

2= *dif* $_{E.\ coli}$ *-cat-dif* $_{E.\ coli}$ for *kfiB* deletion using 70-bp long primers *kfiB*.int_F&R

3= *dif* $_{E.\ coli}$ *-cat-dif* $_{E.\ coli}$ for *kfiC* deletion using 70-bp long primers *kfiC*.int_F&R

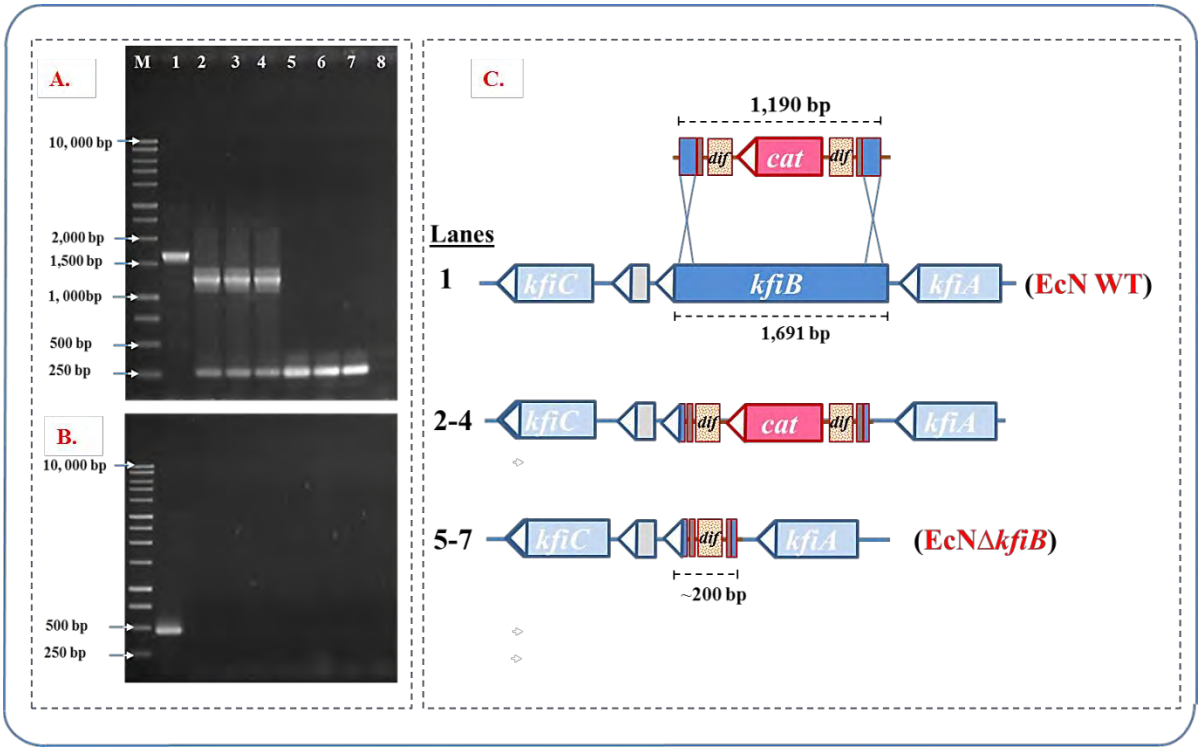


Figure 5.15: Chromosomal integration of the *dif* $_{E.\ coli}$ *-cat-dif* $_{E.\ coli}$ and replacement of the *kfiB* gene

A. PCR amplification of *kfiB* locus in chromosomal DNA to cassette integration

B. PCR amplification of internal fragment of *kfiB* locus to confirm gene deletion

C. Diagram showing the steps involved in generation of EcNΔ*kfiB* mutant

Lanes:

M = 1kB ladder marker

1 = Templates for PCR were obtained *E. coli* Nissle wild-type

2-4 = Templates for PCR were obtained from chloramphenicol resistant transformants/integrant (obtained by transformation of EcN-pLGBE cells with DNA from *dif* $_{E.\ coli}$ *-cat-dif* $_{E.\ coli}$ *KfiB* cassette)

5-7 = Templates for PCR were obtained from resolvants (integrants that have been subcultured on chloramphenicol-free medium up to 9 times to allow excision of the antibiotic resistance gene and recombination)

8 = PCR negative control (sterile deionized water)

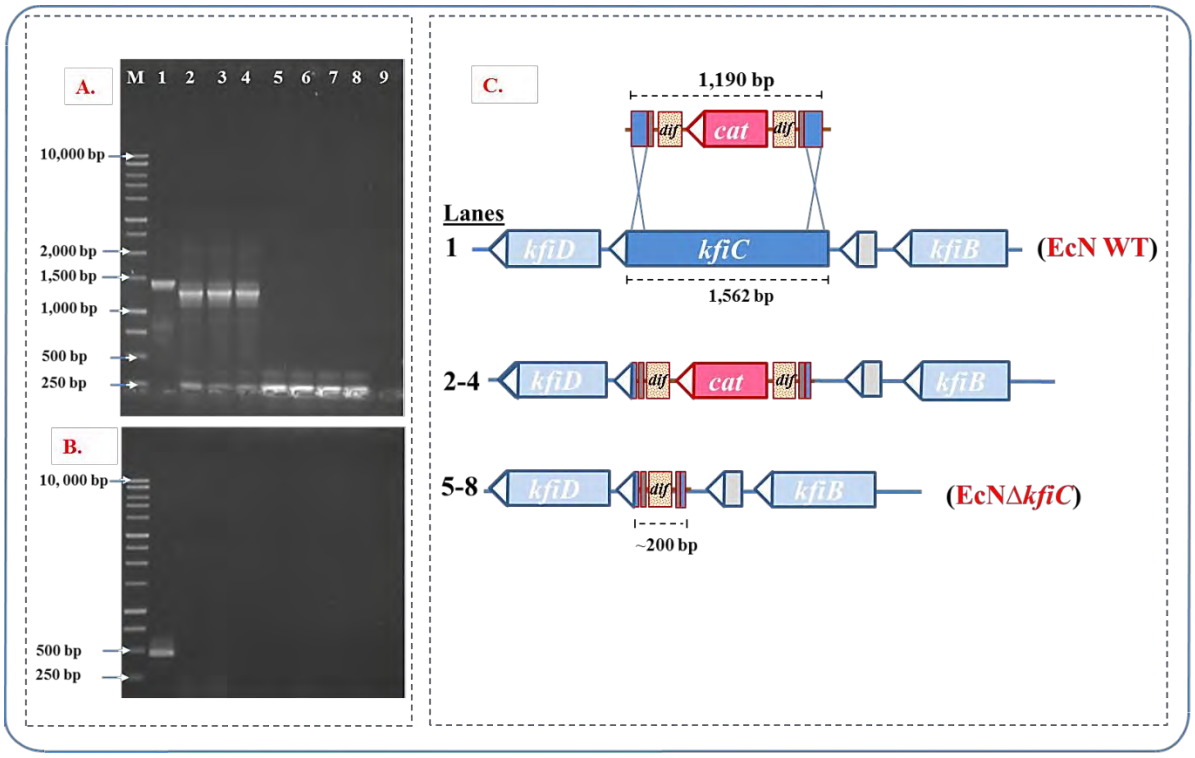


Figure 5.16: Chromosomal integration of the *dif* $_{E.\ coli}$*-cat-dif* $_{E.\ coli}$ and replacement of the *kfiC* gene

A. PCR amplification of *kfiC* locus in chromosomal DNA to cassette integration

B. PCR amplification of internal fragment of *kfiC* locus to confirm gene deletion

C. Diagram showing the steps involved in generation of EcNΔ*kfiC* mutant

Lanes:

M = 1kB ladder marker

1 = Templates for PCR were obtained *E. coli* Nissle wild-type (EcN-pLGBE cells).

2-4 = Templates for PCR were obtained from chloramphenicol resistant transformants/integrant (obtained by transformation of EcN-pLGBE cells with DNA from *dif* $_{E.\ coli}$*-cat-dif* $_{E.\ coli}$ *KfiC* cassette)

5-8 = Templates for PCR were obtained from resolvants (integrants that have been subcultured on chloramphenicol-free medium up to 9 times to allow excision of the antibiotic resistance gene and recombination)

9 = PCR negative control (sterile deionized water)

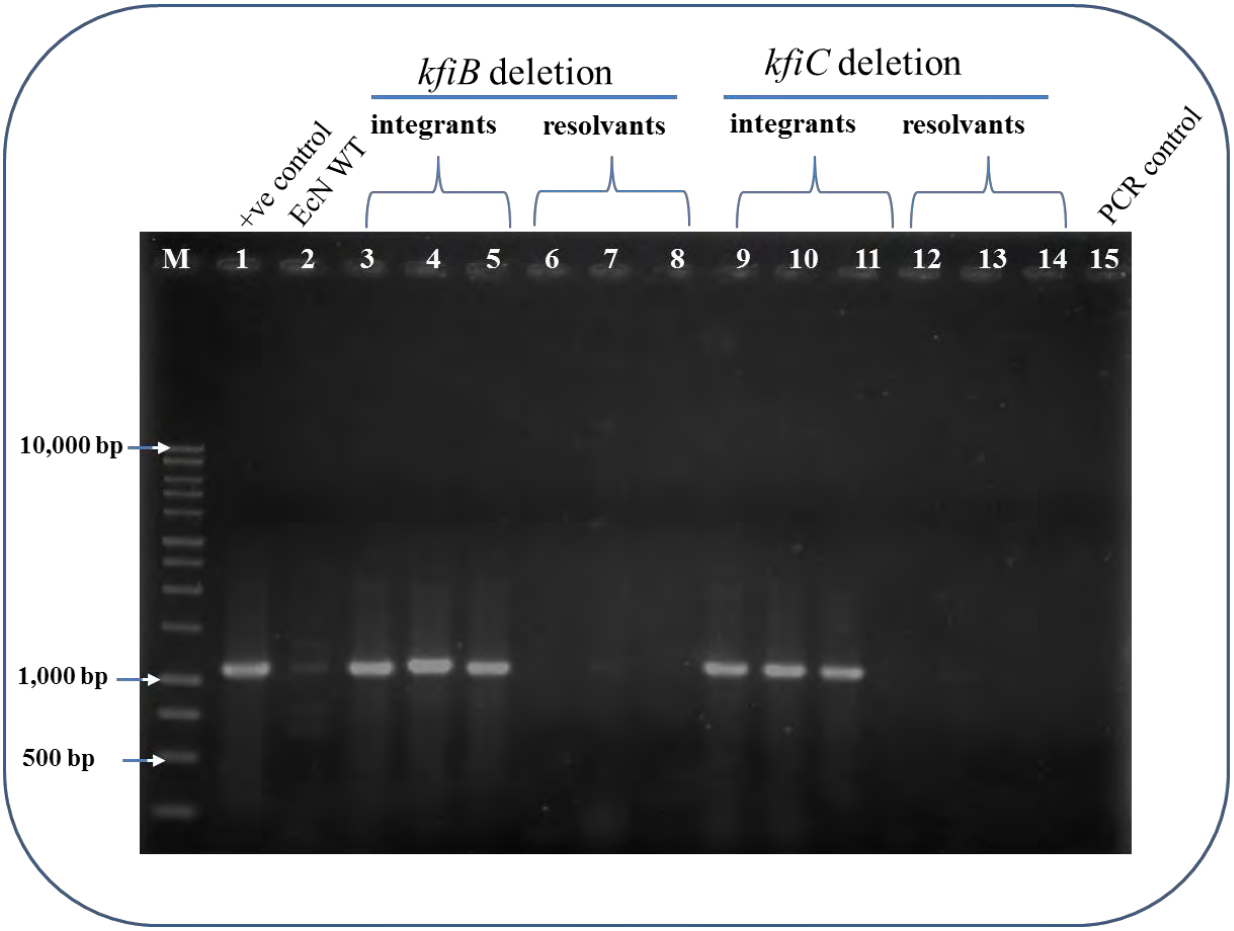


Figure 5.17: PCR screening the *dif* $_{E.\ coli}$ *-cat-dif* $_{E.\ coli}$ fragment to confirm generation of markerless mutations of *kfiB* and *kfiC*

Primers Xer-cise_F&R were used and templates consisted of chromosomal DNA obtained from integrants (obtained by transformation of EcN-pLGBE cells with DNA from *dif* $_{E.\ coli}$ *-cat-dif* $_{E.\ coli}$ cassette) and resolvants (integrants that have been subcultured on chloramphenicol-free medium up to 9 times to allow excision of the antibiotic resistance gene and recombination) .

+ve control= plasmid pTOPO-DifCAT DNA used as template
EcN WT= chromosomal DNA from *E. coli* Nissle wild-type
PCR control PCR negative control (Sterile deionized water)

5.4.7 Verification of polar effects in K5 caplules mutants

To confirm the effect of gene deletion in mutants EcNΔ*kfiB* or EcNΔ*kfiC* on the expression of downstream genes, RT-PCR analysis was performed and the results are presented in Figure 5.18. In both mutants, deletion of the target gene was confirmed to cause no polar effect on expression of downstream genes.

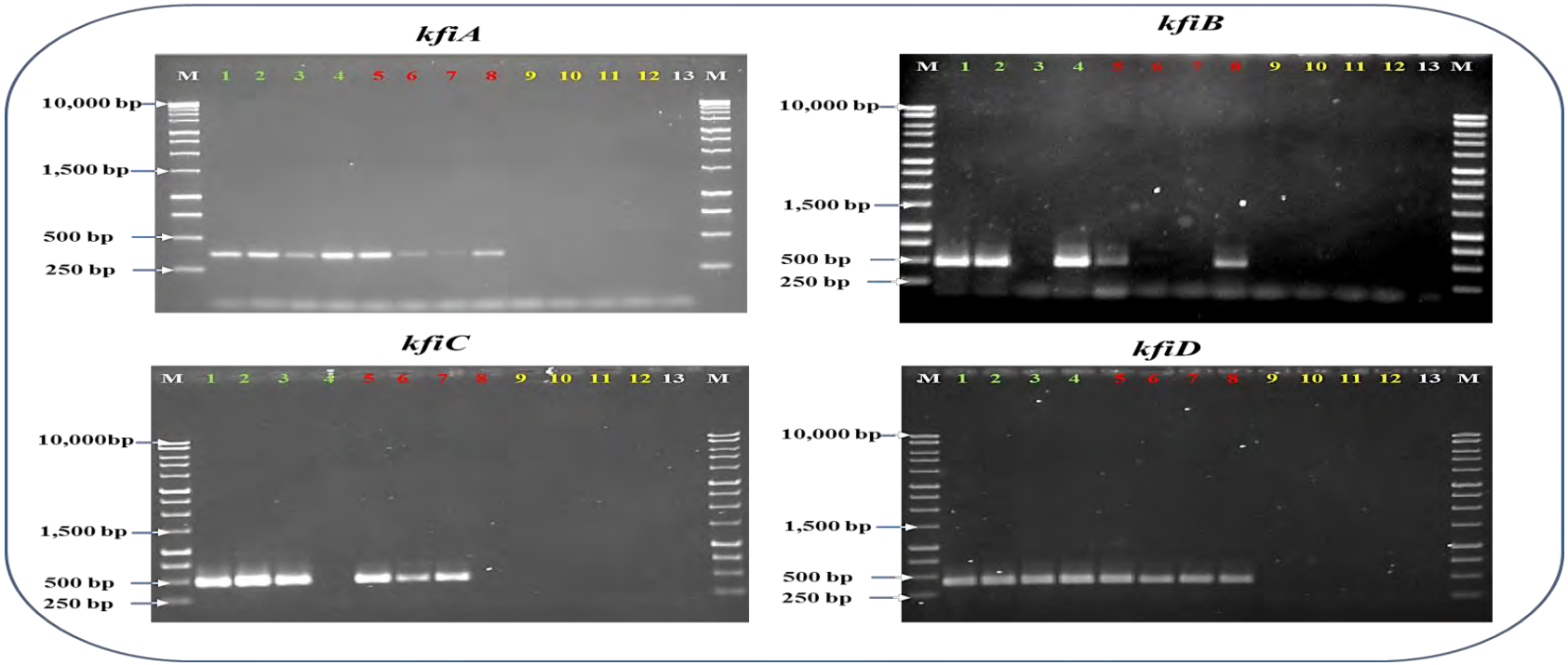


Figure 5.18: RT-PCR analysis for *kfiA-D* gene expression in *E. coli* Nissle capsule mutants

Genomic DNA (positive control)	**cDNA (from RT reactions)**	**Contamination control***
Lane 1: EcN wild-type	**Lane 5**: EcN wild-type	**Lane 9**: EcN wild-type
Lane 2: EcN *KfiB* mini-Tn5	**Lane 6:** EcN *KfiB* mini-Tn5	**Lane 10:** EcN *KfiB* mini-Tn5
Lane 3: EcNΔ*kfiB*	**Lane 7:** EcNΔ*kfiB*	**Lane 11:** EcNΔ*kfiB*
Lane 4: EcNΔ*kfiC*	**Lane 8:** EcNΔ*kfiC*	**Lane 12:** EcNΔ*kfiC*

Lane 13: Standard PCR negative control (Sterile deionized water) **; *Contamination control:** (RT reactions without enzyme)

5.4.8 Verification of the expression of K5 caplule in EcNΔ*kfiB* or EcNΔ*kfiC* mutants

To confirm the role of *kfiB* and *kfiC* in K5 capsule biosynthesis, mutants EcNΔ*kfiB* and EcNΔ*kfiC* were analysed for sensitivity to the K5 bacterio ha ge (ΦK5) and results are presented in Figure 5.19. While EcN wild-type was demonstrated to be highly susceptible to the ΦK5 infection in dose-dependent manner, mutants EcNΔ*kfiB* and EcNΔ*kfiC* as well as control *E. coli* MG1655 showed resistance against the ΦK5 infection. These results confirm the importance of *kfiB* and *kfiC* gene in K5 capsule biosynthesis in EcN.

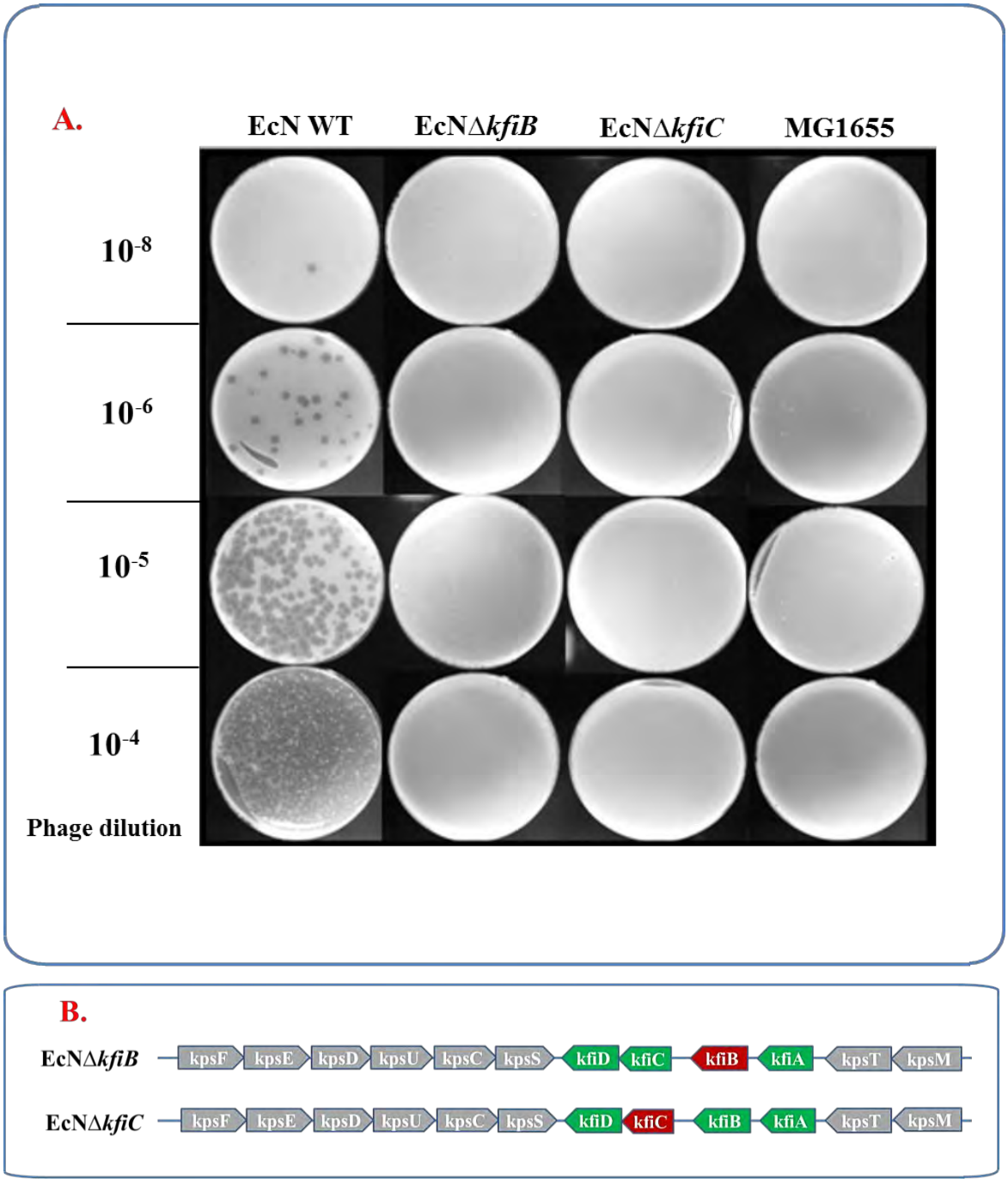


Figure 5.19: Effect of *kfiB* and *kfiC* mutations on ΦK5 sensitivity

A. Sensitivity to ΦK5: bacteria cultures were grown at 37°C to mid-log-phase, infected with phage dilutions from stock of 2.1 x10^8 Pfu/ mL, and then incubated for 16 h at 37°C to allow formation of plaques.

B. RT-PCR analysis of genes in K5 capsule biosynthesis gene cluster.

: gene deleted : expression confirmed

5.4.9 Verification adherence, induction of apoptosis and cytotoxicity in EcNΔ*kfiB* or EcNΔ*kfiC* mutants

To confirm the role of *kfiB* and *kfiC* genes in adherence to Caco-2 cells, and induction of apoptosis and cytotoxicity mutants EcNΔ*kfiB* and EcNΔ*kfiC* were used in appropriate co-culture assays, investigating these aspects and results are presented in Figures 5.20 and 5.21. Mutant EcNΔ*kfiB* was demonstrated to show significantly enhanced adherence to Caco-2 cells, and elevated apoptosis as compared to EcNΔ*kfiC* or control *E. coli* Nissle wild-type ($P<0.05$). This was shown by a significant activation of caspase 3/7 and cell damage as indicated by the amount of lactate dehydrogenase (LDH) released into the medium in EcNΔ*kfiB* co-cultures but not in EcNΔ*kfiC,* EcN WT or *E. coli* MG1655 ($P<0.05$). Moreover, Caco-2 cells treated with the *kfiB* mutant were found to show high levels of condensed chromatin, nucleus defragmentation and cell damage, all parameters indicative of apoptotic cell death (Figure 5.22). Co-culture with mutant's su er natants did not affect Caco-2 cell health suggesting that bacterial contact was essential for the observed effects.

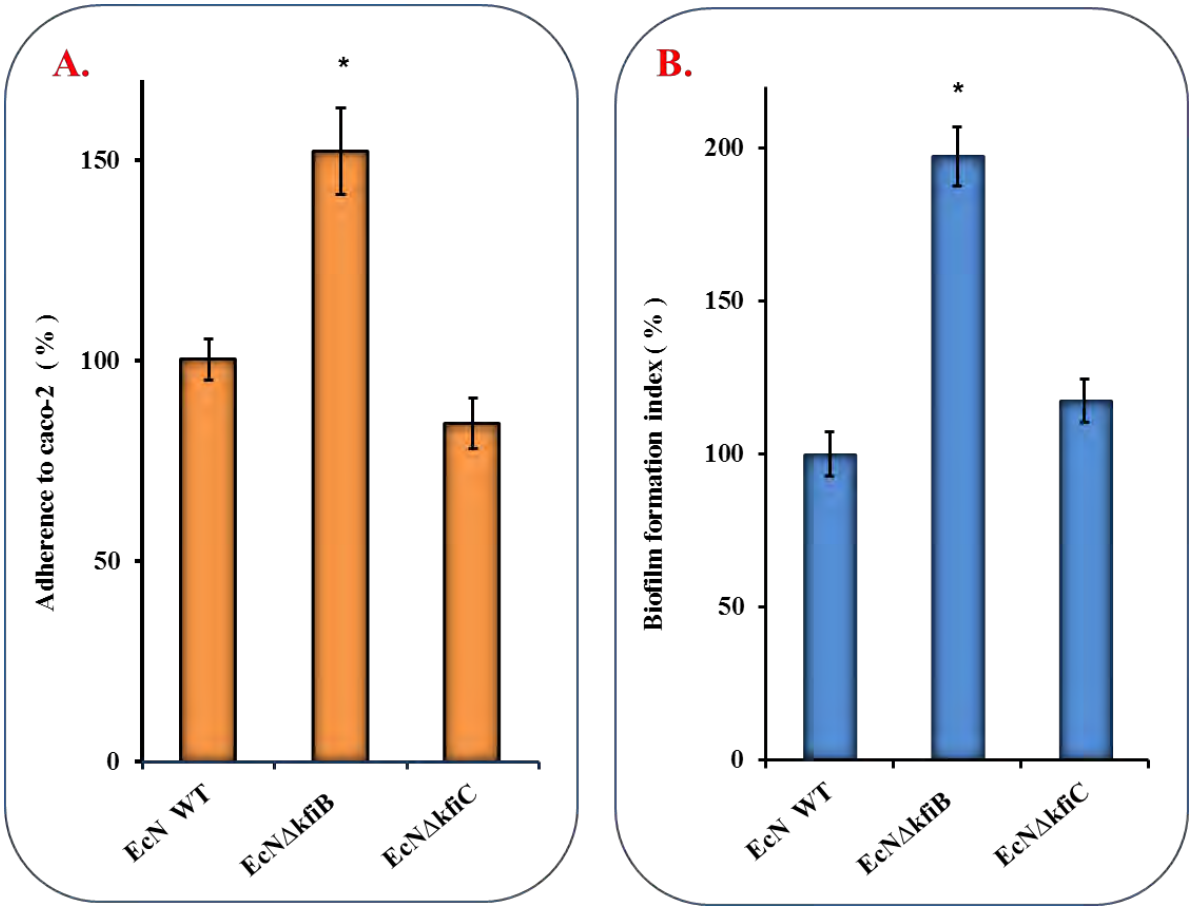


Figure 5.20: Effect of *kfiB* and *kfiC* mutations on adherence to Caco-2 and biofilm formation

A. Adherence of bacterial strains to Caco-2 cells was expressed as % of EcN WT adherent to Caco-2 using the gentamicin protection assay.

B. Biofilm formation index as % of biofilm formed by EcN WT in microtitre-plate and quantified by the CV staining assay.

***:** significant difference as compared to EcN WT at P <0.05. Data are expressed as the mean of four replicates. Error bars show SE of the mean.

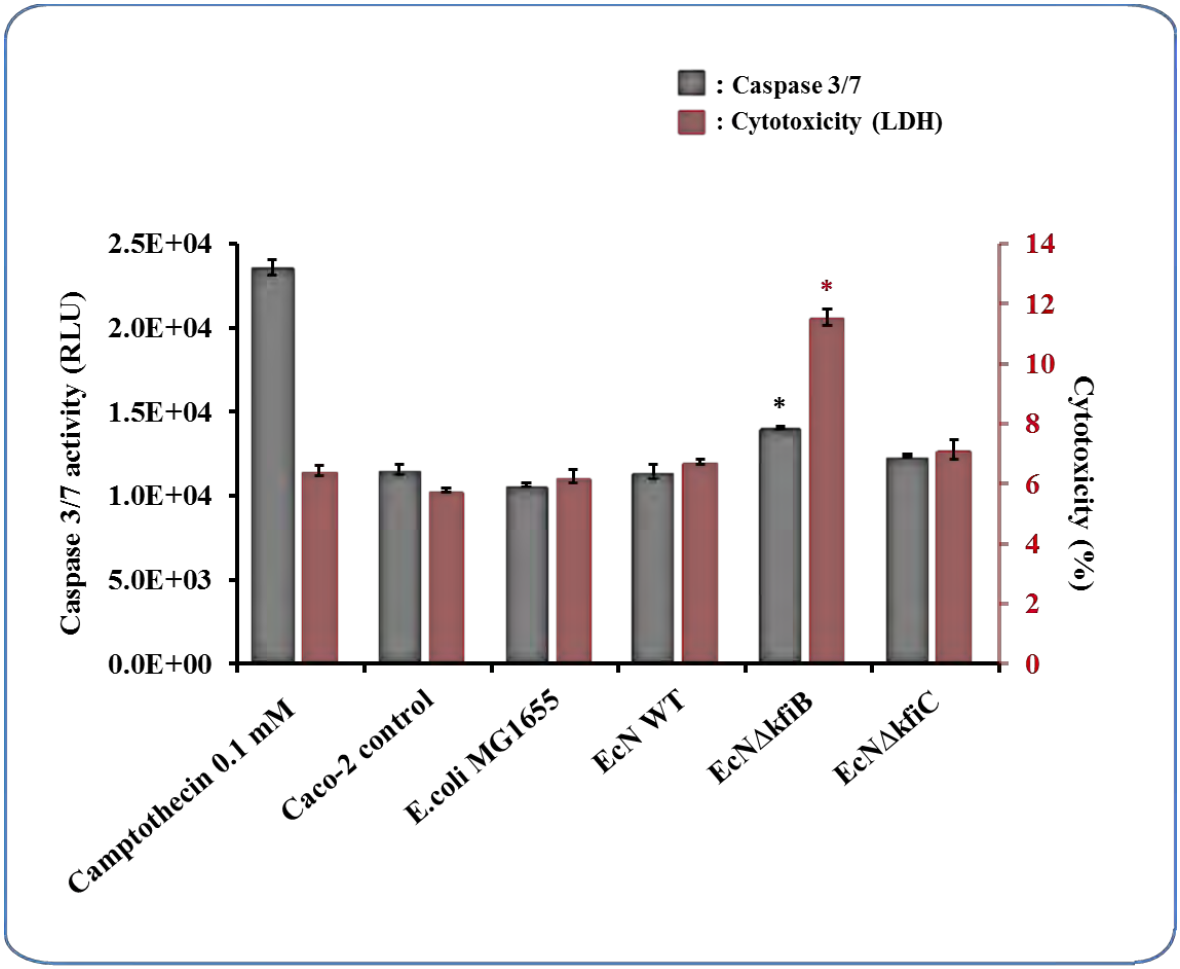


Figure 5.21: Effect of *kfiB* and *kfiC* mutations on Caco-2 cell apoptosis and cytotoxicity

Monolayers of Caco-2 cells were treated with mid-log-phase bacteria at an MOI of 10:1 and positive control 0.1 mM camptothecin for 12 hours at 37°C, 5 % CO_2. Activity of caspase 3/7 was measured in whole-cell lysate and expressed as relative luminescence units (RLU); the LDH released into culture medium was quantified and expressed as % of total LDH obtained from whole-cell lysate of untreated Caco-2 cells *: significant difference as compared to EcN WT with EcN WT ($P<0.05$). Data are expressed as the mean of three replicates. Error bars show SE of the mean.

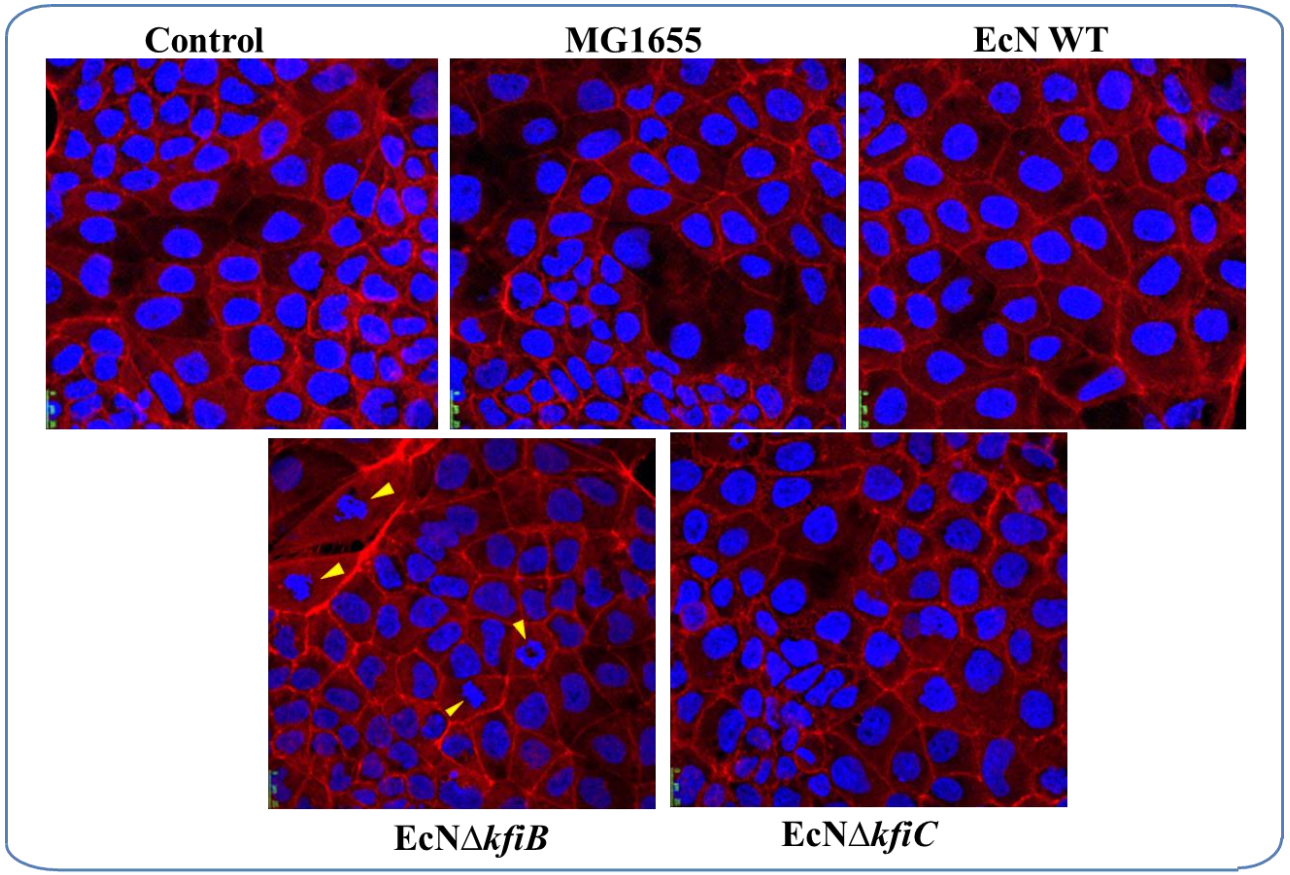


Figure 5.22. Effect of *kfiB* and *kfiC* mutations on Caco-2 cell membrane integrity and nuclear morphology

Monolayers of Caco-2 cells were grown on sterile glass cover slips in 6-well plates then treated with with mid-log-phase bacteria at an MOI of 10:1 for 12 hours at 37°C, 5 % CO_2. The treated cells were rinsed with PBS, fixed with 4 % formaldehyde, permeabilised with 0.5 % Triton X-100, stained with phalloidin and DAPI. The cells were examined by confocal microscopy using the Leica TCS SP5 Confocal Laser Scanning microscope with an oil immersion 63×/1.4 63×/1.4 objective lenses. Yellow arrows show condensed chromatin of cells undergoing apoptosis. These images are representative of three independent experiments.

5.5 Discussion

5.5.1 A co-culture model for bacterial stimulation

The use of *in vitro* cultures of human epithelial cells can provide an effective approximation to the *in vivo* situation as regard to host microbe interactions. Most often, co-cultures of human intestinal cell lines with probiotics are carried out to study gut colonisation properties (Jacobsen *et al.* 1999, Nagy *et al*. 2005, Lasaro *et al.* 2009), antagonism against pathogens (Jacobsen *et al.* 1999, Resta-Lenert and Barrett 2003, Nagy *et al*. 2005), abilities to promote gut maturation and integrity (Wehkamp *et al*. 2004, Zyrek et *al*. 2007, Fink and Frokiaer 2008), as well as stimulation of the immune system (Wehkamp *et al*. 2004, Vinderola *et al*. 2005, Kamada *et al*. 2008, Hafez *et al*. 2009).

In such studies, a number of parameters are to be considered when designing the required experiments matching the research questions. Key parameters include the choice of the intestinal cell line (IECs) type, culturing vessels, and medium supporting the growth of both mammalian and bacterial cells, without interfering with the assays involved (Lee and Salminen 2009). Moreover, experimental parameters such as cell densities and co-incubation durations are critical, especially for maintenance of viability and integrity of the IECs in probiotic studies.

Many of the *in vitro* assays involving IECs are carried out in static co-culture configuration and do not necessarily incorporate a continuous flow of culture media as found *in vivo.* Such a state of stagnation can cause bacterial overgrowth which can subsequently result in destruction of the intestinal cells. Patterns of bacterial growth in the co-culture media are also crucial especially for *E. coli* Nissle 1917, a facultative anaerobe which can grow very fast at body temperature and in the presence of oxygen. Considering the importance of these parameters, it was surprising to find out that the aspects of IECs cell health (i.e.

viability) have not been discussed or even mentioned in the majority of published works that had employed co-culture model of IECs in probiotic studies.

The results of the optimisation of Caco-2-*E. coli* Nissle co-culture model (Section 5.4.1) show optimum bacterial culture densities and durations of exposure that permit stimulation of the IEC without affecting their viability. Moreover, the optimised parameters provided a broad range of co-incubation durations of up to 12 hours. This period can allow the host cells-probiotic interactions to take place and induce a large number of relevant cellular responses. Several studies have reported a range of 1 to 48 hours of co-incubation/stimulation of Caco-2 monolayers by strains of EcN or their supernatants at 37ºC (Wehkamp *et al*. 2004, Ukena *et al*. 2005, Kamada *et al*. 2008, Hafez *et al*. 2009, Hafez *et al*. 2010). Interestingly, a 6-hours co-incubation of Caco-2 cells with EcN at a multiplicity of infection (MOI) of 1:1 was found allow regulation of 126 genes (relevant to probiotic effects) in Caco-2 genome, using microarrays analysis (Ukena *et al*. 2005).

Sensitivity of EcN strains to the Caco-2 cell growth medium (DMEM, also used in co-culture) was also investigated in this current study. Although complete DMEM contains several nutrients required to support the growth of many bacteria including *E. coli*, it also contains 10 % of foetal bovine serum (FBS), a potential inhibitor of EcN. EcN is known to be serum-sensitive due to its special truncated LPS, a property that makes this strain avirulent to human or animal hosts (Blum *et al*. 1995, Grozdanov *et al*. 2002).

Interestingly, the growth of EcN was unaffected in complete DMEM when compared to serum-free DMEM medium (Figure 5.6). It can be suggested that the amount of FBS present in DMEM was not sufficient to challenge the probiotic, in this cell growth medium which also contained high glucose content (4.5 mg/L) and non-essential amino acid supplements, all favouring the bacterial growth. Moreover, the observed ability to grow in

serum-free medium is an important attribute for its use in co-culture experiments requiring the omission of serum (for technical or physiological reasons).

5.5.2 Adherence to Caco-2 cells

The ability of probiotics to adhere to IECs is thought to be important to gut colonisation, interaction with the host and subsequent delivery of beneficial effects (Dunne *et al.* 2001, Lasaro *et al*. 2009). Whether adherence of EcN to IECs might or might not be associated with the probiotic's effects, a clear understanding of the bacterial components involved in adherence might help to illuminate the mechanisms of host-microbe interactions.

Components of the cell surface structure of many enteropathogenic *E. coli*, as well as probiotic EcN, have been demonstrated to contribute to bacterial attachment to intestinal mucosa as well as IECs (Klemm and Schembri 2000, Lasaro 2009). Site-directed mutants of *E. coli* Nissle with defects in cell surface structures have been previously used to investigate the role of these features in adherence to IECs (Hafez *et al*. 2009, Lasaro 2009, Troge *et al.* 2012). It was revealed that some surface structures can directly facilitate adherence to IECs, whereas others could indirectly mediate host-probiotic interactions by inducing the expression of cytokines. However, by using site-directed mutagenesis, the focus has been on investigating the genes that are already known to encode for adhesive functions in non-probiotic *E. coli*. It can be hypothesised that a random transposon mutagenesis approach could help to illumine novel genes with relevance in both cell surface structures and interactions with host cells; because this approach required no prior knowledge of genes that may be involved and makes no assumptions in this regard.

In this study, adherence of EcN transposon insertion mutants to Caco-2 cells was explored. The selected mutants had originally been isolated based on their biofilm formation phenotypes in microtitre-plates, which permitted identification of mutants more likely to be

attenuated in synthesis of surface structures. Genes disrupted in selected mutants included those involved in fimbriae (*fimD*), flagella (*fliC*), lipopolysaccharides (*dsbA*), as well as capsule biosynthesis (*kfiB* and *kpsT*) Section 5.4.2. Moreover these mutants have demonstrated their suitability to be used in adherence experiments in comparison with EcN wild-type. Their suitability was confirmed based on their ability to grow in the co-culture medium (DMEM) and non-susceptibility to saponin-trypsin lysis buffer (used in gentamicin protection assay). Both saponin (a detergent-like glycoside) and trypsin (proteolytic enzyme) are potential bactericidal agents that can act by disrupting the bacterial membrane (Killeen *et al*.1998).

The results obtained (Section 5.4.2) lend strong support to previous studies on EcN adherence to IECs, but also provide new and important insights. For example, the observed deficiency in adhesion of the *fimD* mutant (*fimD* is involved in anchoring type 1 fimbriae on the cell surface) to Caco-2 cells supports previous findings by Lasaro *et al*. 2009 who studied the role of the EcN fimbriae in gut colonisation. Their work demonstrated that mutation in *fimA* gene (that participates in production of type 1 fimbriae) was important for the probiotic to adhere intimately to both abiotic and IECs surfaces *in vitro* as well as for persistent colonisation of mouse intestine.

Another interesting observation is on adhesion of the *fliC* (flagella) transposon insertion mutant that was similar to the EcN wild-type, despite the well-documented role of flagella in initiating bacterial adhesion to host intestine in *E. coli* and other bacterial groups (Moens and Vanderleyden 1996, Girón *et al*. 2002, Haiko and Westerlund-Wikström 2013). These results support findings from a recent study on the role of EcN flagella in adherence to host in *fliC*-deletion mutant by Troge *et al*. (2012). Their study also employed a Caco-2 cell co-culture model, and reported that flagella played no role in adherence to Caco-2 cells. However when an *ex vivo* model with cryosections of human gut biopsies was used, a

dramatic decrease in adhesion was observed in the *fliC*-deletion mutant but not the wild-type. This indicated that the presence of mucus was crucial for the EcN flagellum to mediate adhesion with intestinal cells. Moreover, Troge *et al*. (2012) demonstrated gluconate (component of mucin 2) as a specific receptor for the binding of *E. coli* Nissle flagella to human intestines.

One unanticipated finding was the enhanced adherence phenotype in the *kfiB* mutant which showed a 2.2-fold increase in adherence to Caco-2 cells as compared to the wild-type. The *kfiB* gene is part of the K5 capsule biosynthesis gene cluster in EcN but with no confirmed function (Corbett and Roberts 2008) as detailed in K5 biosynthesis diagram (Figure 5.2). These current results were unexpected as this gene has not been previously associated with adherence to IECs in *E. coli*.

Furthermore, the predicted decapsulation of the *kfiB* mutant would be more likely to lead in deficiency in adherence to Caco-2 cells rather than the observed enhanced phenotype in this strain. This is because capsular polysaccharides that coat the outside of the *E.coli* cell are conventionally thought to mediate bacterial attachments to host cells rather than preventing/hindering this event. Moreover, when coincubated, the *kfiB* mutant was found to induce apoptosis in Caco-2 cells. This detrimental effect has not been previously reported in *in vitro* or *in vivo* studies involving EcN or its derivative mutant strains. Considering these controversies, the *kfiB* mutant was investigated further to confirm the putative function of this gene in the observed phenotypes and provide possible insight into the mechanism involved in enhanced adherence.

5.5.3 The effect of *kfiB* mutation on host-*E. coli* Nissle 1917 interactions

Functional verification of computationally predicted genes can be important in elucidating host-microbe interactions. One of many benefits of transposon mutagenesis based approach is that it offers a possibility to easily identify the disrupted gene. The *kfiB* mutant has been preliminary characterised by analysis of sequences flanking regions flanking the transposon insert in comparison to public genome databases. In order to confirm that *kfiB* gene was responsible for the enhanced adherence to Caco-2 cells and elevated induction of apoptosis, and that the expression of the K5 capsule was involved in these properties: i) disruption of *kfiB* by mini-Tn*5* was verified by gene cloning, ii) deletion mutants EcNΔ*kfiB* and EcNΔ*kfiC* were constructed, iii) attenuation of K5 capsule production was verified in both mutants, and iv) polar effects from gene disruption on expression of downstream genes was ruled out in both mutants.

The observation that both deletion mutants EcNΔ*kfiB* and EcNΔ*kfiC* demonstrated resistance to the capsular K5- bacteriophage shows loss or attenuation of the K5 capsule in these mutants. The loss of capsule in *kfiB* mutant confirms a role for *kfiB* in the synthesis of the K5 capsule (Corbett and Roberts 2008). Moreover, deletion of the *kfiB* gene was confirmed to cause no polar effect on expression of downstream genes. This indicates that *kfiB* could be responsible for any phenotypic characteristic that was observed in the EcNΔ*kfiB* as compared to the wild-type. Also, RT-PCR analysis confirmed that deletion of the *kfiC*gene in EcNΔ*kfiC* mutant did not affect the expression of downstream *kfiD*. This was important to verify before both mutants could be compared in co-culture studies with Caco-2 cells.

5.5.4 Mutation of *kfiB* enhances adherence of *E. coli* Nissle to Caco-2 cells

To further investigate the function of the *kfiB* in host-microbe interaction, deletion mutants EcNΔ*kfiB* and EcNΔ*kfiC* were compared in their ability to adhere to Caco-2 monolayers and to induce apoptosis. While EcNΔ*kfiB* was confirmed to show significantly enhanced adherence to Caco-2 cells and elevated induction of apoptosis and cytotoxicity, EcNΔ*kfiC* didn't show any significant difference in these properties as compared to *E. coli* Nissle wild-type.

The results of the EcNΔ*kfiC* support two findings that have been recently published. The first was by Hafez *et al.* (2009) who investigated the effect of K5 capsule (using mutant *kfiC* knockout) on adherence to Caco-2 cells and cytokine induction. While these studies demonstrated a change in cytokine profiles between co-cultures with the *kfiC* mutant and those with EcN wild-type, they found a comparable pattern of adhesion to Caco-2 cells between both strains. A second study was conducted by Nagy *et al*. (2005) who also looked at the adhesion of the EcN capsular (*kps*) mutants to intestinal cell lines HCT-8 and INT407. They also found that the loss of the K5 capsule in these mutants didn't affect the adhesion capabilities of EcN to these cells.

Apoptosis, also called programmed cell death, is the crucial machinery by which multicellular organisms control cell numbers to ensure that damaged or potentially harmful cells are removed (Arends and Wyllie 1991). The early stages of apoptosis are marked by the activation of a series of cysteine-aspartic acid specific proteases (caspases). These enzymes are synthesized by cells as dormant precursors and can be activated by different types of stimuli such as cytotoxic agents, TNF-α, or other cytokines, leading to the cell death and its elimination from tissue (Gnesutt and Minden 2003). Human apoptotic caspases are classified into two major groups based on their functions as well as structures (Pop and Salvesen 2009). These are: i) monomeric initiator caspases (caspase-8, -9 and -

10), which after receiving proapoptotic signals will activate effector caspases, ii) dimerized effector caspases (caspase-3, -6 and -7), which execute the apoptosis. Following their activation, the effector caspases (especially caspase-3 and -7) target various cytoskeletal/ structural proteins, leading into a cascade of biochemical and morphological changes (i.e.cell shrinking, convolution, internucleosome cleavage) and subsequent cell death (McStay *et al*. 2008).

The results obtained in this current study indicate that the apoptosis was induced in Caco-2 cells when co-incubated with strains of EcN *kfiB* mutants but not supernatants. This suggests that the host–microbe interactions required a cell-cell contact rather than a secretory dependent mechanism. The observed apoptosis in Caco-2 cells was characterised by an increased membrane permeability and subsequent damage (shown by LDH release), as well as damage to cell DNA. Interestingly, these characteristics have been reported previously in IECs co-incubated with various strains of enteropathogenic *E. coli* (EPEC) (Crane *et al*. 1999, Foster *et al*. 2000, Figueiredo *et al*. 2012). In particular, studies conducted by Foster *et al*. (2000) and Crane *et al.* (1999) on enterohemorrhagic *E. coli* (EHEC) and other diarrheagenic *E. coli* (DEC) have demonstrated their induction of apoptosis in IECs (including Caco-2 cells). Both studies confirmed that bacterial attachment or strong adherence to IECs (instead of production of toxin) was key to causing the apoptosis in IECs as well as diarrhoeal and haemorrhagic colitis in animal models.

While the observed apoptosis-inducing properties of *kfib* mutants are yet to be investigated for their effects on host health using animal models, it is not clear if this mutant would be beneficial or detrimental to host in this regard. A study conducted by Altonsy *et al.* (2010) has suggested that a mild-apoptotic effect of probiotic bacteria on IECs might be beneficial to the host, especially in protection against colon cancer. Altonsy *et al.* (2010) investigated the apoptosis induction in Caco-2 cells by *Atopobium minutum, Lactobacillis* and

Bifidobacterium, pathogenic *E. coli* (EPEC and verotoxin-producing), and commensal *E. coli* MG1655 in a 6-hour co-culture model and a range of multiplicity of infections (MOIs). Interestingly, they found that all the tested probiotics exhibited a mild –apoptotic effect, enteropathogens a major apoptotic effect, but no effect for commensal MG1655. Moreover, the researchers demonstrated that the mild apoptotic effects observed in these probiotics were consistent with their beneficial roles in protection against colon cancer. Although there are no reports on the EcN apoptotic induction or its mutants *in vitro* or *in vivo* this probiotic has been demonstrated to upregulate the expression of at least two proapoptotic genes in Caco-2 co-cultures (Ukena *et al.* 2005) shown in Figure 5.23. It would be interesting to investigate the apoptotic induction of EcN capsule mutants on host health.

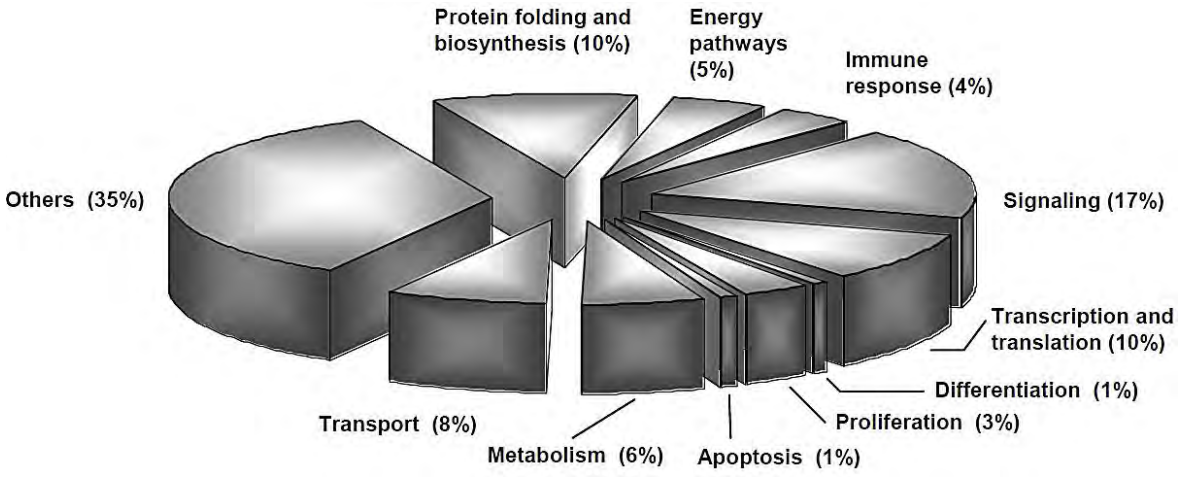


Figure 5.23: Classification of genes regulated in confluent Caco-2 cells by co-culture with *E. coli* Nissle for 6 hours

(Source Ukena *et al.* 2005).

Analysis of microarray identified 126 genes being regulated. These genes were assigned to different classes based on their involvement in biological processes (percent by number of genes per class).

Considering the observed phenotypic characteristics of EcNΔ*kfiB* mutant (enhanced adherence and induction of apoptosis and cytotoxicity in Caco-2 cells) which are missing in EcNΔ*kfiC* it can be suggested that: i) mutation in distinct *kfi* genes might result in distinct phenotypes of capsule mutants, ii) decapsulation of EcN in EcNΔ*kfiB* might be facilitating intimate attachment to host-cells unblocking the surface expression of protein ligands i.e Ag43, iii) *KfiB* might be encoding for another function in addition to involvement in K5 expression.

5.6 Conclusions

- Random transposon mutagenesis is a powerful tool in elucidating the roles of unexpected genes in host-microbe interactions

- Optimisation of co-culture model of host-microbe interactions is crucial in probiotic studies

- *kfiB* based capsule mutants display distinct differences in effects on host cells compared with other capsule mutants, and elevated apoptosis in the Caco-2 co-culture model.

Chapter 6: Effect of the probiotic *E. coli* Nissle 1917 on neurotransmitter release from intestinal epithelial cells

6.1 Probiotic interactions with host intestinal epithelium

The intestinal epithelium is the main medium of host-probiotic interactions. Currently, there are three major modes of action by which probiotics contribute to human health (Lebeer *et al.* 2010). These are: i) exclusion or inhibition of pathogens, ii) enhancing the function and integrity of the intestinal epithelial barrier (i.e by modulating defensin production or preventing apoptosis) and iii) modulation of host immune responses. These modes of action have been studied and confirmed in *E. coli* Nissle 1917 using *in vitro* models as well as animal models (Sonnenborn and Schulze 2009). Nevertheless, in most cases such models have been designed and used to investigate a limited number of intestinal cell types. These are mainly the enterocytes (Ukena *et al*.2005, Hafez *et al.* 2009, Lasaro *et al*. 2009) and rarely immune cells (Mandel *et al.* 1995, Sturm *et al*. 2005, Duncker *et al.*2006, Guzy *et al*. 2008). Currently, there are no reports on how probiotics might affect other important cell types of the intestinal epithelium such the endocrine cells, which are known to play a key role in neurotransmitter synthesis and release. This is also likely to be relevant to any impact of probiotics on functions of the intestine, such as motility.

6.1.1 Endocrine cells of the intestinal epithelium

The human intestinal epithelium comprises several types of specialised cells, playing vital functions in the maintenance of health. Major cell types include absorptive enterocytes, Paneth cells, goblet cells, and enteroendocrine cells, and all have been shown to develop from the same stem cells (Gordon 1993, Höcker and Wiedenmann 1998). The enteroendocrine cells produce hormones and neurotransmitters in response to various

stimuli in the lumen. Despite comprising less than 1 % of all intestinal epithelial cells, the enteroendocrine cells represent the largest mass of endocrine cells in the body (Vella and Drucker 2011). The frequency of enteroendocrine cells is highest in proximal regions of the small intestine and in rectum, but falls in the distal sections of both small and large intestines (Stramsuddin *et al.* 1982, Sjolund *et al*. 1983). The diffused localisation of the enteroendocrine cells in the gastro-intestinal tract (GIT) as well as their diversity (at least 15 cell subtypes) constitute a challenge for studies exploring their roles in human health and disease (Moran *et al.* 2008, Gunawardene *et al*. 2011).

6.1.2 Enterochromaffin cells

Enterochromaffin (EC) cells constitute the most abundant and well characterised enteroendocrine cell subtype and are distributed all along the GIT. They were first described by Heidenhain in 1870, based on their ability to retain a brownish colour when stained by chromium salts. These cells were then named 'enterochromaffin' by Ciaccio in 1907 who confirmed their location in the intestinal epithelium (Plockinger 2008). Morphologically, EC cells are about 8 μm in size and triangular or pyramidal in shape, and access the luminal milieu throughout the apical cytoplasm (Modlin *et al*. 2006). Also, they possess specialised microvilli, and some specialised transporters and enzymes known to be common in the apical sections of the enterocytes (Buchan 1999). Functionally, they play a crucial role in gut secretory, motility, and sensory functions (Sjolund *et al*. 1983, Gershon 1999). These functions are accomplished though the biosynthesis, storage and release of the biogenic amine serotonin (5-hydroxytryptamine) as well as a variety of peptides (Plockinger 2008).

6.1.3 Serotonin

The EC cells produce the largest pool of the neurotransmitter serotonin (5-HT) in the body. This accounts for more than 90 % of serotonin, with the remainder mainly synthesised in the central nervous system (CNS) (Kim and Camilleri 2000, Gershon and Tack 2007). Figure 6.1 shows key steps involved in biosynthesis and transmission of 5-HT from EC cells. The biosynthesis step is initiated from the precursor tryptophan, an essential amino acid obtained from the diet. Two steps are involved in this process. The first is the rate-limiting conversion of tryptophan to an intermediate 5-hydroxytryptophan (5-HTP), catalysed by tryptophan hydroxylase-1 (Tph-1) (Walther *et al*. 2003). The second step is the conversion of 5-HTP to serotonin, 5-hydroxytryptamine (5-HT) which is catalysed by aromatic L-amino acid decarboxylase (L-AADC).

The newly synthesised 5-HT is stored in vesicles and is released following mechanical and chemical stimulation of the mucosa (Erickson *et al*. 1996). Therefore the EC cells act as transducers to luminal stimulant. Once released, into the extracellular matrix, 5-HT interacts with receptors located on intrinsic primary afferent neurons (IPANs) within the submucosal and myenteric plexus that help to direct motility and ion transport (Filip and Bader 2009). The majority of the released 5-HT (from EC cells) is cleared into the neighbouring enterocytes by the serotonin transporter (SERT) protein (Fuller and Wong 1990). After clearance, 5-HT is rapidly metabolised to various metabolites of which 5-hydroxyindole acetic acid (5-HIAA) is the most abundant. The production of 5-HIAA is catalysed by monoamine oxidase (MOA).

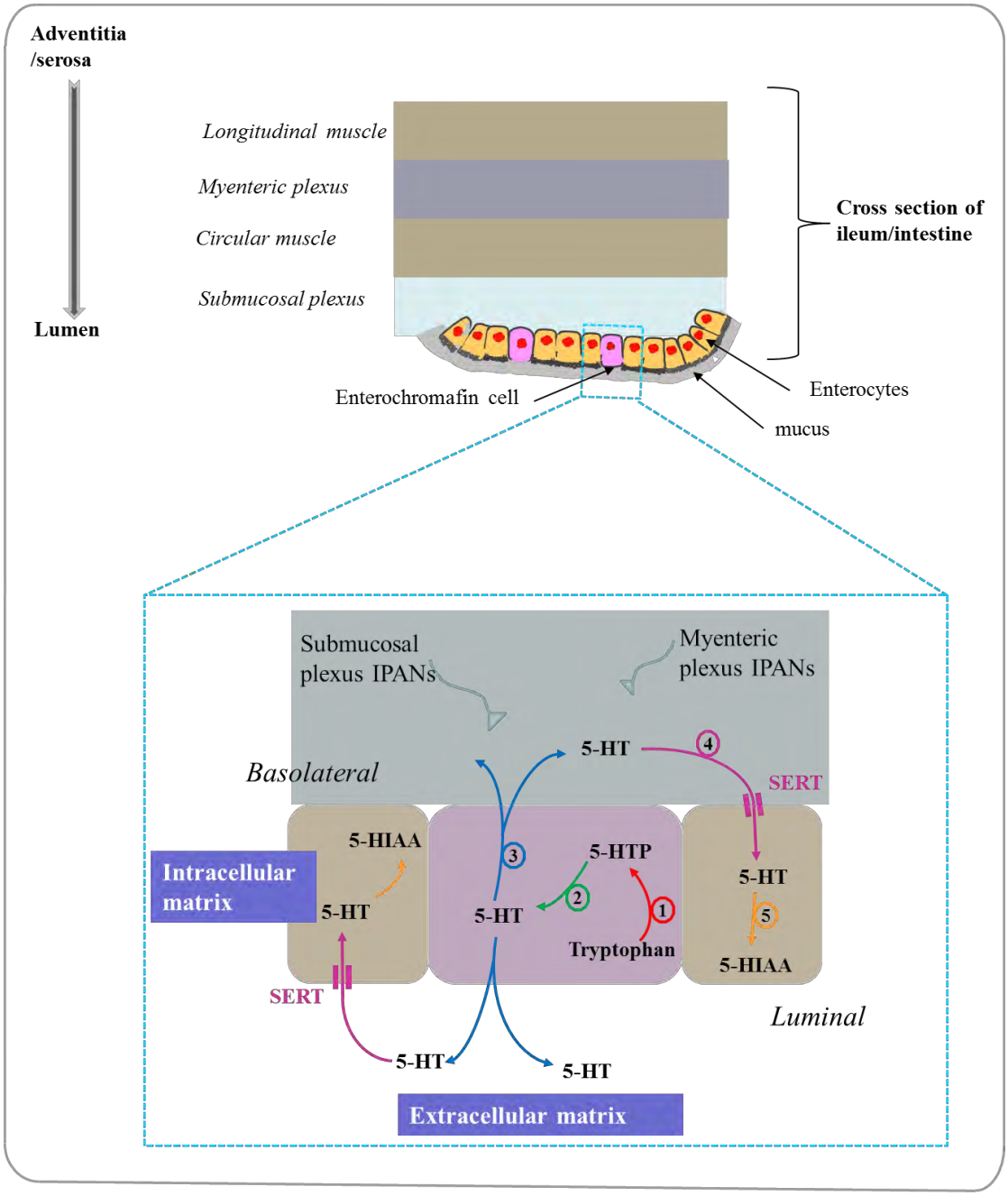


Figure 6.1: Serotonin synthesis and transmission from enterochromaffin cells

Top: Cross-section of the view of the intestine

Bottom: 5-HT signalling mechanism. Steps 1–5 show the process involved in 5-HT biosynthesis (from precursor tryptophan) and release from enterochromafin (EC) cells, clearance and metabolism to 5-HIAA.

Step 1: Tryptophan hydroxylase-1 (Tph-1) converts tryptophan into 5-HTP; **Step 2**: L-amino acid decarboxylase (L-AADC) produces 5-HT; **Step 3**: 5-HT is released into the extracellular matrix following stimulation of EC cells. The released 5-HT can bind to 5-HT receptors on intrinsic primary afferent neurons (IPANs); **Step 4**: clearance of 5-HT into enterocytes via the serotonin reuptake transporter (SERT); **Step 5**: metabolism of 5-HT into 5-HIAA by the enzyme monoamine oxidase A (MAO).

This diagram was adapted from Parmar *et al*. (2012)

6.1.4 Alteration of serotonin synthesis and metabolism in diseases

Alteration in serotonin bioavailability has been found to be associated with a variety of gastro-intestinal (GI) diseases, including colon carcinoma, inflammatory bowel diseases (IBD), enteric infections and functional disorders such as irritable bowel syndrome (IBS) (Hansen and Witte 2008, Manocha and Khan 2012). Although the aetiology of many of the GI disorders remains obscure, parameters related to 5-HT availability in these conditions can help: i) to establish possible causative agents (role of stimulates such as gut microbiota, cell secretions, and dietary components), and ii) establish therapeutic targets for the treatment/alleviation of the symptoms in these conditions.

Table 6.1 highlights some example studies showing alterations in 5-HT bioavailability and in the EC cells populations in GI diseases. As summarised in Table 6.1, both Kidd *et al*. (2009) and Coates *et al.* (2004) have reported an increase and decrease in 5-HT bioavailability in Crohn's disease (CD) and ulcerative colitis (UC), res e ctively. In both studies, the activity of tryptophan hydroxylase (Tph-1) was found to be directly related to the observed alteration in 5-HT availability. Consequently a possible pathogenetic role for Tph-1 was established and could be pharmacologically controlled by either: i) oral intake of the Tph inhibitor parachlorophenylamine, or reserpine which diminishes the already synthesised 5-HT from cells, or by ii) injection of tryptophan.

In some other GI disorders, malfunctions of SERT have been found to cause 5-HT-induced symptoms, such as pain, and diarrhoea. This has been confirmed in patients with IBD or IBS with defects in gene encoding for SERT (Coates *et al.* 2004); and in patients suffering from dyspepsia in association with low SERT expression (Foxx-Orenstein *et al*. 2007).

Table 6.1: Alteration of enterochromaffin cells and serotonin in gastro-intestinal diseases

Gastro-intestinal disorders	Enterochromaffin cells	Serotonin	Tph-1 mRNA	Reference
CD		Increased	Increased	Kidd *et al*. 2009
CD			Increased	Minderhoud *et al*. 2007
UC		Decreased	Decreased	Coates *et al.* 2004
CD and UC		Decreased		Magro *et al*. 2002
CD and UC	Increased			El-Salhy *et al*. 1997
UC	Decreased			Ahonen *et al*.1976
IBS	Increased			Ahonen *et al*.1976
IBS	Unchanged	Decreased		Coates *et al*. 2004
IBS-C		Increased		Miwa *et al.* 2001

CD, Crohn's disease; UC, ulcerative colitis; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; IBS-C irritable bowel syndrome with constipation; Tph-1, tryptophan hydroxylase-1.

This table was adapted from Manocha and Khan (2012)

While probiotic EcN has been used as a treatment of a variety of GI-associated disorders (Sonnenborn and Schulze 2009, Verna and Lucak 2010, Behnsen *et al*, 2013), its effect on 5-HT release by the enterochromaffin (EC) cells of the gut is yet to be explored. Elucidation of host- EC cells probiotic interactions in EcN could provide new insights into the functioning of probiotic bacteria

6.2 Aims

The aims of the work presented in this chapter were to investigate the effect of the probiotic *E. coli* Nissle 1917 on neurotransmitter synthesis and metabolism by intestinal mucosal cells.

6.3 Materials and methods

6.3.1 Analysis of neurotransmitter synthesis in co-cultures of the intestinal mucosal layer cells and bacteria

The analysis of neurotransmitter synthesis in mouse intestinal epithelial cells co-cultured with probiotic EcN was performed using High Performance Liquid Chromatography (HPLC) with Electrochemical Detection (ED) as described by Parmar *et al*. (2011).

Standard chemicals tryptophan, 5-hydroxytryptamine (serotonin, 5-HT), 5-hydroxy-3-indoleacetic acid (5-HIAA), 5-hydroxytryptophan (5-HTP). Sodium acetate and citric acid were used in sample preparation experiments. The assay buffer consisted of Krebs buffer solution pH 7.4 (made up of the following components in mM: 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, and 11 glucose). All these chemicals were obtained from Sigma. The Bradford Protein Assay kit (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) was used for measurement of proteins. Solutions were prepared and kept in Class A volumetric flasks.

6.3.1.1 Animal experiments

The C557BL/6 mouse, which is a multipurpose model (Crabe *et al.* 1999, Hansen *et al*. 2008), was used to provide intestinal tissue samples in this study. Animal experiments (handling and care) were carried out in accordance with the national as well as institutional guidelines for animal usage in research. Three-month-old male C557BL/6 mice were weighed then euthanized via cervical dislocation. The abdomen was subsequently opened, intestines removed. Sections of distal ileum and distal colon were collected using dissecting scissors and placed in ice-cold oxygenated (95 % O_2 5 % CO_2) Krebs buffer solution. These intestinal sections were used for the preparation of cells.

6.3.1.2 Intestinal cell sample preparation

Mucosa samples were isolated from freshly harvested intestines using the intracellular sampling approach (Parmar *et al*. 2011). This allows monitoring of intracellular neurochemicals. Sections of distal and proximal colon were divided into 1 cm long segments. Each segment was transferred to an inverted glass Petri dish (pre-chilled on ice) then opened longitudinally along the mesenteric border, and laid out with the mucosal surface facing up. The flattened tissue consisted of approximately 1 cm^2 segment was subsequently rinsed twice with Krebs buffer solution to remove any visible faecal matter. The rinsed mucosa was gently scrapped off using a scalpel and transferred into a sterile chilled 1.5 mL Eppendorf tube. The harvested mucosal scraping (of ~ 150 µL per 1 cm^2 tissue segment) was homogenised gently using a plastic microfuge pestle, and the resulting cell suspensions were then used in co-culture assays.

6.3.1.3 *Ex-vivo* co-cultures with bacterial cells

The homogenised mucosal suspensions were co-incubated with bacterial cells of *E. coli* Nissle 1917 or *E. coli* MG1655 in Krebs buffer solution. Prior to performing the co-culture assay, bacterial strains were investigated for their ability to grow in Krebs buffer solution at 37°C and 5 % CO_2 , statically over a period of 8 hours. Aliquots of the growing culture were plated out on LB agar and colony counting was performed.

Bacterial inocula were obtained from overnight cultures in LB broth, which were adjusted to an OD_{600} of 5.0, ~5 x 10^9 Cfu/mL, harvested by centrifugation and the pellets resuspended in sterile Krebs buffer solution. The bacterial suspensions were either used neat or 10-fold dilutions. An aliquot of 100 µL bacterial cell suspension was then mixed with the 150 µL intestinal mucosal suspension (prepared in Section 6.3.1.2) then transferred into a 6-well plate and incubated at 37°C, 5 % CO_2 for 1 hour. Controls were

included in the experiment and consisted of: i) only Krebs buffer solution, ii) mucosa without bacteria and iii) bacterial cells without mucosa.

After the co-incubation, samples and controls were immediately transferred (individually) to prechilled 1.5 mL sterile Eppendorf tubes and homogenised in 250 µL of ice-cold 0.1 M $HClO_4$ by vortexing. The samples were then centrifuged at 13,000 *g* at 4°C for 10 minutes. The obtained supernatants (mucosa extracts) were collected and filtered (through Minisart RC membrane 0.2-µm-pore-size filter) then stored on before analysis with HPLC. The pellets were stored at -80°C and used later for protein analysis.

6.3.1.4 HPLC analysis of neurochemicals

The HPLC system consisted of a Jasco HPLC pump (Model: PU-980) and Rheodyne manual injector equi e d with a 20 µL sam l e loo . Se a ration column consisted of a Kinetic® ODS 2.6 µm 100 mm x 2.1 mm internal and in-line filter was a KrudKatcher™ Ultra model (Phenomenex, Macclesfield, UK). Isocratic mode was used to separate and quantify the molecules of interest at a rate of 100 µL e r minute. A 20- µL sample volume was injected into the system. Detector voltage and current record were controlled using CHI1001A potentiostat (CH Instruments, Austin, TX, USA). A 3 mm glassy carbon electrode (flow cell, BAS) was employed as the working electrode, a Ag|AgCl as reference electrode, and a stainless steel block as the auxiliary electrode. Amperometric measurements were taken and data were collected and processed using the CHI1001A software.

The mobile phase stock buffer consisted of 1 M sodium acetate, 0.1 M citric acid and 27 µM disodium ethylene-diamine-tetra-acetate (EDTA) in deionised water, and adjusted to

pH 3.0. The mobile phase solution was prepared by mixing the stock buffer with methanol in the ratio of 4:1 (v/v) then degassed by sonication.

Stock solutions of individual standards were prepared at 1 mM in 10 mL volume of 0.1 M $HClO_4$ .The mixture of standard analytes consisted of 1 μM 5-HTP, 1 μM 5-HT, 60 μM tryptophan and 2 μM 5-HIAA in 0.1 M $HClO_4$. This was used to plot a calibration curve. Samples (perchloric acid extracts obtained from mucosa bacterial co- cultures, Section 6.3.1.3) were subsequently injected and run as well. The peak areas corresponding to the neurochemicals of interest were converted to concentrations based on the calibration responses obtained from standard analytes.

6.3.1.5 Analysis of protein content

The concentration of the neurochemicals was normalised between samples to the protein content of these samples. The mucosal pellets (Section 6.3.1.3) were defrosted on ice and subsequently used to obtain protein extracts. These were first washed twice with methanol. Each time, the pellet was homogenised in 400 μL ice-cold methanol by pipetting then centrifuged for 15 minutes at 12,000 *g* and 4°C. Tubes containing the washed pellets (with cap open) were placed upright in rack for 5 minutes at room temperature, allowing the pellets to air-dry and remaining methanol to evaporate. Protein extracts were then obtained by resuspending the pellet in 50 μL thiourea/urea lysis buffer (2 M thiourea, 7 M urea, 4 % (w/v) CHAPS, 10 mM dithiothreitol).

The protein was then quantified using the Quick Start Bradford protein assay kit (Bio-Rad) according to the manufacturer's instructions. ovine γ-globulin was used to establish the standard calibration curve. The concentration of protein in standard and sample solutions was measured spectrophotometrically at 595 nm on a microplate reader.

6.3.1.6 Data analysis

Peak values of neurochemicals of interest were normalised to the protein content of the sample. The normalised data were expressed as molar concentration of the neurochemical being tested per milligram protein of the mucosa sample (mM mg^{-1} protein). Data were plotted on diagrams and shown as mean ± standard error of the mean (SEM) and *n* referring to the number of single tissue samples per animal used in the experiment.

Whenever required, levels of clearance and metabolism of serotonin were calculated as follows:

$$\textbf{Serotonin clearance/metabolism per sample group} = \frac{\text{5-hydroxy-3-indoleacetic acid (5-HIAA) content}}{\text{5-hydroxytryptamine (5-HT) content}}$$

Overall one way ANOVA test was used for comparison between group samples, assuming populations were Gaussian ($H_{0:}$ means are equal, 95 % confidence interval level). Whenever H_0 was rejected ($P<0.05$), a post-test (post-hoc) taking into account all comparisons was employed. Data were analysed using GraphPad Prism V5.03.

6.3.2 Analysis of short-chain fatty acids (SCFAs) produced in co-culture medium

Analysis of SCFAs was performed using an adapted reversed phase HPLC (RP-HPLC) adopted from Schiffels *et al*. (2011), whereby SCFAs are derivatised to 4-nitrophenyl esters prior to chromatographic analysis. The HPLC consisted of a Waters 2695 Alliance quaternary gradient system, linked to a Waters dual wavelength UV/Vis absorbance detector. Data acquisition was made via a PE Nelson 900 series interface connected to a pc running Totalchrom (PerkinElmer, Shelton, CT) dual channel software. As a modification/simplification of the Schiffels *et al* (2011) HPLC separation method, mobile phase A consisted of 20 mM triethlyamine and 20 mM ammonium acetate, adjusted to pH 4.7 with formic acid and mobile phase B was acetonitrile only. Ethanoic (acetic), propanoic (propionic) c and butanoic (butyric) acids served as analytical (quantitation) standards and *hexanoic (caproic) acid* as internal (IS, normalisation/recovery) standard. Hexanoic acid was selected to replace the nonanoic IS used by Shiffels *et al.* (2011) as it did no co-elute, appeared earlier (shortening run-time) and peaked from a flat baseline rather than a rising one, thereby improving quantitation precision standards and samples were separated using a Fortis C_{18} (ODS) column (150 mm x 3.0 mm ID – 3 μM) connected directly to a Phenomenex 0.5 μM Krudcatcher® pre-filter. The standards, sample preparation reagents and solvents were purchased Fisher Scientific, UK.

6.3.2.1 Sample preparation

Co-cultures experiments of intestinal mucosa cells with *E. coli* Nissle and MG1655 were conducted as described in sections 6.3.1.2 and 6.3.1.3 excepting that the collected intestinal mucosal scrapings were pooled together, mixed properly then aliquoted (150 μL) and used in co-cultures. Controls were included in co-culture experiments. These consisted of: i) only Krebs buffer solution, ii) mucosa without bacteria and iii) bacterial cells in Krebs buffer solution without mucosa. The co-cultured samples were then centrifuged at 13,000 *g*

at 4°C for 2 minutes. The obtained supernatants were collected and filtered through Minisart RC membrane 0.2-µm-pore-size filter then used for the analysis of SCFAs.

6.3.2.2 Extraction and derivatization of SCFAs

Samples aliquots (300 µL) were transferred to 1.5 mL Eppendorf tubes then 0.2 g of NaCl and 100 µL of concentrated HCl were added to each tube. The tubes contents were mixed by vortexing. To extract total SCFAs from sam l es, 800µL of diethyl ether (containing 35 mM hexanoic acid as internal standard) was added to the sample mixture, then mixed by vortexing and centrifuged for 1 minute at 12,000 g to remove residual water from the ether ha se. An aliquot (200 µL) of the obtained ether ha se was transferred to 1.5 mL screw capped glass vials and treated with 200 µL of oxalyl chloride solution [250 mM oxalyl chloride in N,N-dimethyl formamide/acetonitrile 1:100 (v/v)]. Tubes were incubated at room tem e rature for 5 minutes then, 800 µL of derivatization reagent (50 mM 4-nitrophenol in 500 mM pyridine/acetonitrile) was added and mixed by swirling. These samples containing 4-nitrophenyl esters, derivatives of SFCAs were directly used for analysis of the compounds of interest.

6.3.2.3 HPLC calibration

For HPLC calibration, the method of standard additions (MOSA) was employed (Snyder *et al*. 1997). Instead of preparation of calibration standard in a blank matrix (Krebs buffer solution) sample solution consisting of bacterial supernatants (grown in Krebs buffer solution then filter-sterilised) were used. A mixture of standard analytes consisting of acetic, propionic, and butyric acid, in the same molar ratio was added to the sample solution to concentrations ranging from 0 to 50 mM. Aliquots (300 µL) from these solutions were used for extractions and preparation of the 4-nitrophenly esters according to the procedure described in Section 6.3.2.2.

6.3.2.4 HPLC analytical procedure for short-chain fatty acids

Autosampler injections (20 μL of each SCFA standard or sam l e solution) were sequenced and separations were achieved using the following gradient (A/B): 80/20 (no isocratic hold) to 20/80 (in 18 minutes, no isocratic hold) to 0/100 (in 1 minute, hold for 3 minutes) before returning to 80/20 (in 3 minutes, hold for 5 minutes); 30 minutes between injections. Mobile phase flow was programmed at 0.6 mL/minute throughout for optimal separation and safe (pressure, 3000 psi cut-off) operation. Samples and standards were kept at 8°C, to reduce evaporation, by the onboard chiller unit, whilst the column was maintained at 45°C by the integral column oven. Settings for the detector, in dual channel mode, were 295 nm, 60 mV offset and sensitivities of 0.1 and 0.5 absorbance units full scale (AUFS), channel B (0.1 AUFS) permitting the increased detection of low levels of the carboxylic acids (ethanoic, propanoic, butanoic) of interest. The Totalchrom acquisition was ceased at 20 minutes for each run, around two minutes after elution of the hexanoic acid IS, recommencing with the next injection signal from the Waters 2695. Retention times for the ethanoic, propanoic, butanoic and hexanoic (IS) acids were approximately 10, 12, 14.7 and 17.6 minutes, respectively.

6.3.2.5 Data analysis

Peak areas of analytes in standard additions were plotted on the curve to represent the analyte in the presence of the sample matrix. The measured peak areas were first corrected to the peaks obtained from internal standard (hexanoic acid). The linearity of the curve was then obtained by linear regression analysis equation of $y=mx+b$; y being the peak area, m the slope, and b the intercept of the curve on the y-axis. Peak areas corresponding to the target analyte were recorded in samples, normalised to the internal standard (hexanoic acid) then molar concentration of these determined using the MOSA calibration curve. Data were analysed using MS Excel 2010. Significant differences between sample means were assessed using two sample t-tests.

6.4 Results

6.4.1 Establishment of *ex-vivo* co-culture model of intestinal mucosal layer cells and bacteria

To establish an *ex-vivo* co-culture model of intestinal mucosa cells (freshly harvested from mice) and probiotic EcN, bacterial cultures were first assayed for their ability to grow in the co-culture medium and the results are shown in Figure 6.2. Both *E. coli* strains Nissle 1917 and MG1655 were found to grow in the Krebs buffer solution. Moreover, bacterial inocula ranging from 2 $x10^8$ to 2 $x10^9$ Cfu/mL and 1-hour co-incubation duration were demonstrated to allow stimulation of cells (scraped from ~1 cm^2 intestinal layer segment) and synthesis of neurotransmitters from precursor tryptophan (Figure 6.3). Chromatographic analysis of bacterial control samples (bacteria without mucosa) confirmed the absence of contaminants, or peaks corresponding to the neurochemicals of interest in these controls. These parameters were subsequently used to investigate the effect of EcN on serotonin synthesis in intestinal mucosa isolated from mice.

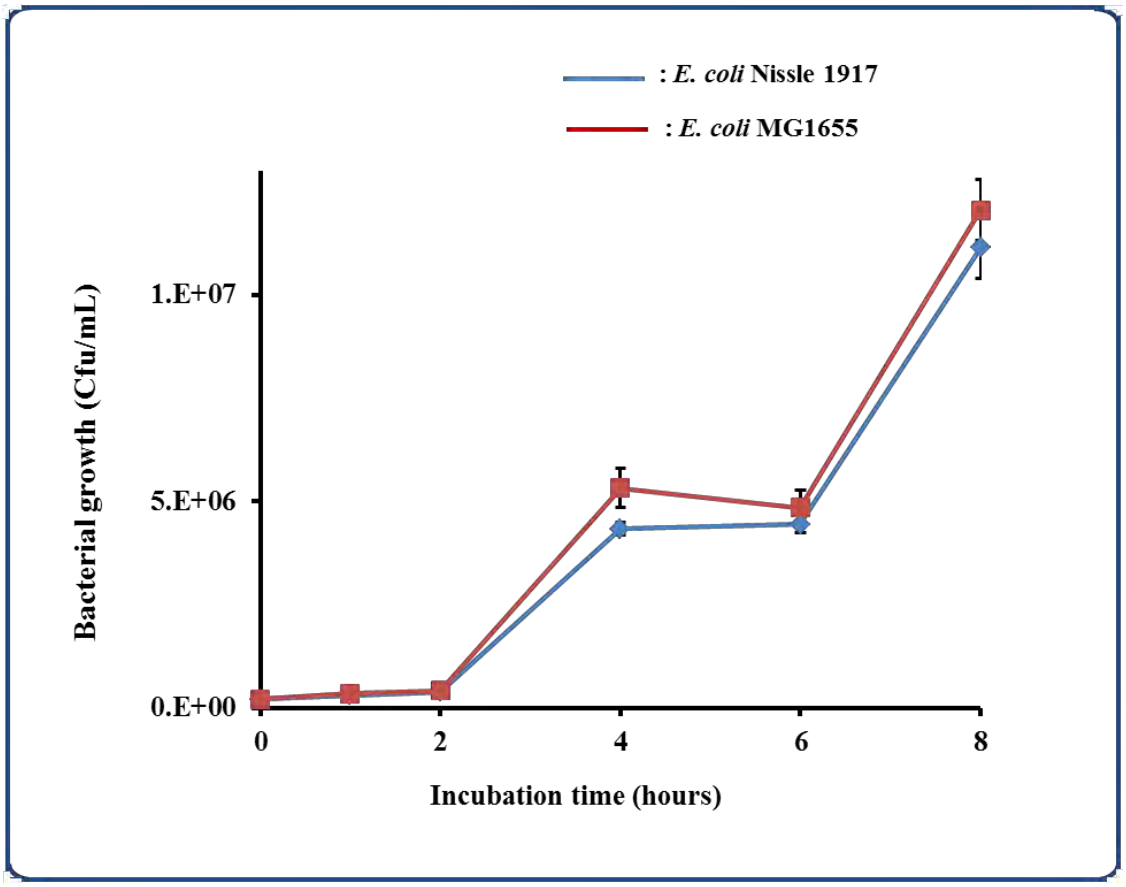


Figure 6.2: Growth of *E. coli* strains in Krebs buffer solution

Cultures were grown overnight in LB broth, centrifuged then resuspended in 5 mL sterile Kreps buffer at 2 x 10^5 Cfu/mL. The fresh culture was grown statically at 37°C, 5 % CO_2 and bacteria growth was monitored by plating cultures on agar plates. Results shown are the mean ± SEM of three independent experiments.

6.4.2 Effect of *E. coli* Nissle on neurotransmitter release from enterochromaffin cells

To gain an overview of possible effects of probiotic EcN on neurotransmitter release from mouse enterochromaffin cells, an *ex-vivo* co-culture model was employed (a 1-hour co-incubation) then neurochemicals were measured by chromatography.

6.4.2.1 Intracellular levels of tryptophan and 5-HTP

Figures 6.3.A and B show the levels of precursor tryptophan, and 5-HTP in cell samples following treatment with EcN or non-pathogenic gut commensal *E. coli* strain MG1655. Levels of 5-HTP were shown to be significantly higher in mucosa treated with as compared to non-treated control and MG1655 ($P<0.05$, ANOVA). Assuming that intracellular levels of tryptophan were initially similar in mucosa samples prior to the treatment with bacteria, high levels of 5-HTP in EcN samples can indicate that the probiotic was stimulating the synthesis of serotonin through the steps highlighted in Figure 6.3.C.

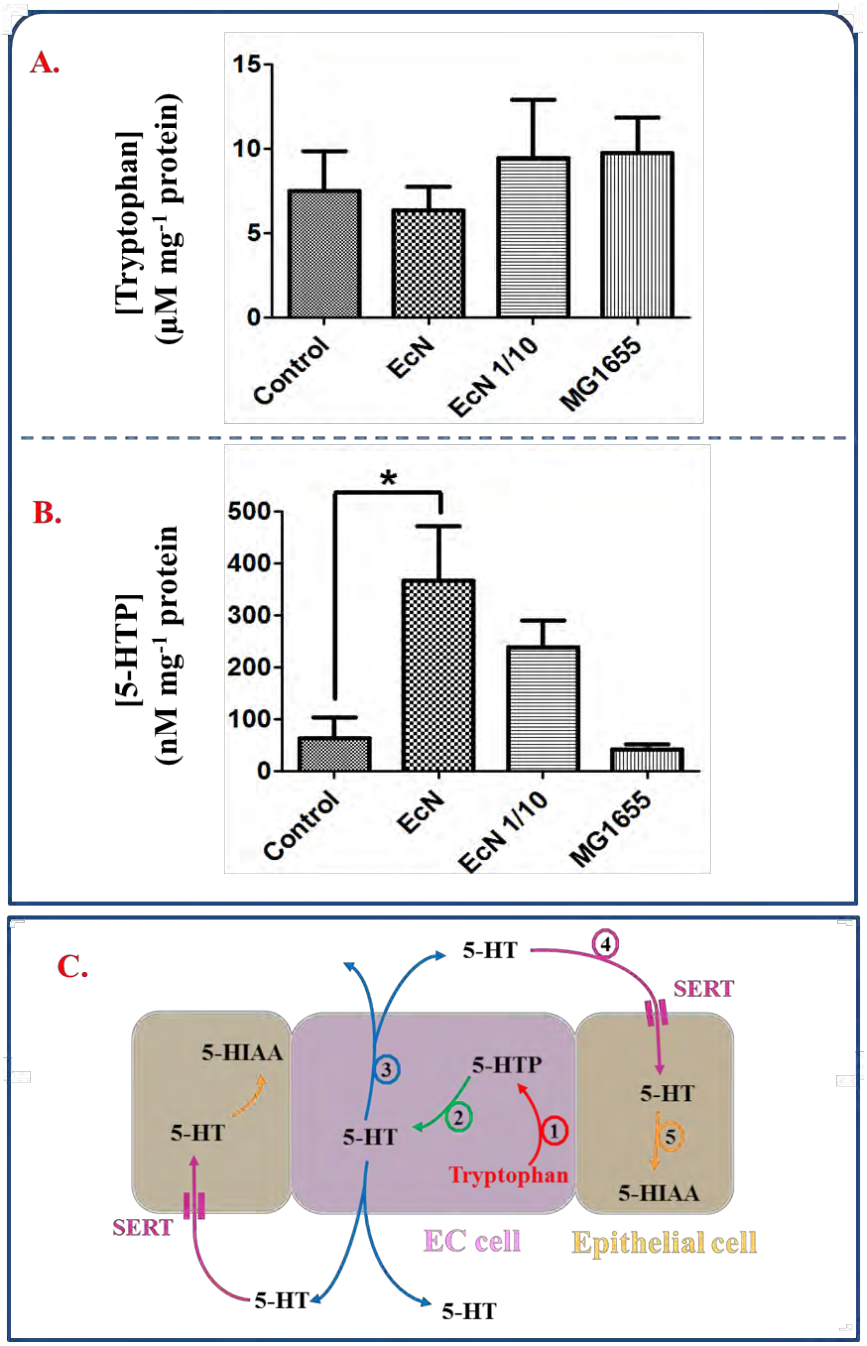


Figure 6.3: Intracellular tryptophan and 5-HTP levels of mucosa treated with *E. coli* strains

A. Tryptophan levels; **B** . 5-HTP levels: Mucosa cells (isolated from ~ 1 cm^2 intestinal layer segment) were co-cultured with bacterial cells at $2x10^9$ Cfu/mL ($2x10^8$ Cfu/mL for EcN 1/10) in 6-well plate at 37°, 5 % CO_2 for 1 hour. Control samples (untreated with bacteria). Cell supernatants were then analysed for tryptophan content using HPLC and electrochemical detection.
Data were recorded based on levels obtained from intracellular sampling and normalised to protein content per surface area of the intestinal tissue segment used to extract the mucosa. Data are shown as mean ±SEM, n=6 (six independent experiments).

C. Mechanism of 5-HT signalling from the mucosa of the gastro-intestinal tract, tryptophan being a precursor.

6.4.2.2 Serotonin content

Figure 6.4.A show serotonin content in co-cultures. These results show that stimulation of the mucosal cells with EcN increased serotonin (5-HT) content in the co-cultures significantly ($P<0.01$, $n=6$) as compared to the non-treated control. This was a 5-fold increase in 5-HT content in the EcN co-culture compared with the inoculated control. This was not observed in co-culture with control MG1655 ($P> 0.05$, $n=6$). Moreover the stimulatory effect of EcN was dose-dependent as serotonin production was reduced by half when a 10-fold smaller inocula was used (EcN 1/10), resulting in levels comparable to those observed in MG1655 experiments. Overall, serotonin content was found to be elevated in bacterial co-cultures as compared to controls, suggesting that both EcN and MG1655 were stimulating the enterochromaffin (EC) cells for biosynthesis of serotonin from tryptophan, and the intermediate 5-hydroxytryptophan (5-HTP), as indicated by steps 1 and 2 on Figure 6.3 C. These results also support the data on increased levels of 5-HTP in co-cultures with EcN (Figure 6.3.B) suggesting that Ecn could be exhibiting higher stimulatory effects onto the EC cells, considering that tryptophan was not a limiting factor.

6.4.2.3 Intracellular levels of metabolite 5-hydroxy-3-indoleacetic acid (5-HIAA)

The content of 5-hydroxy-3-indoleacetic acid (5-HIAA) (a metabolite of serotonin) was also determined in co-culture samples and results are shown in Figure 6.4.B. There was a statistically significant difference in 5-HIAA content between group sample means from co-cultures with EcN and MG1655 or non-treated control ($P<0.05$, ANOVA). As the conversion of 5-HT into 5-HIAA takes place mainly in the enterocytes (neighbouring to 5-HT producing EC cells) as shown in Figure 6.3.C steps 4 and 5, low levels of this metabolite in co-cultures with EcN could explain the high 5-HT content observed in Section 6.4.2.2, and probably indicate that the 5-HT was not metabolised into 5-HIAA as expected.

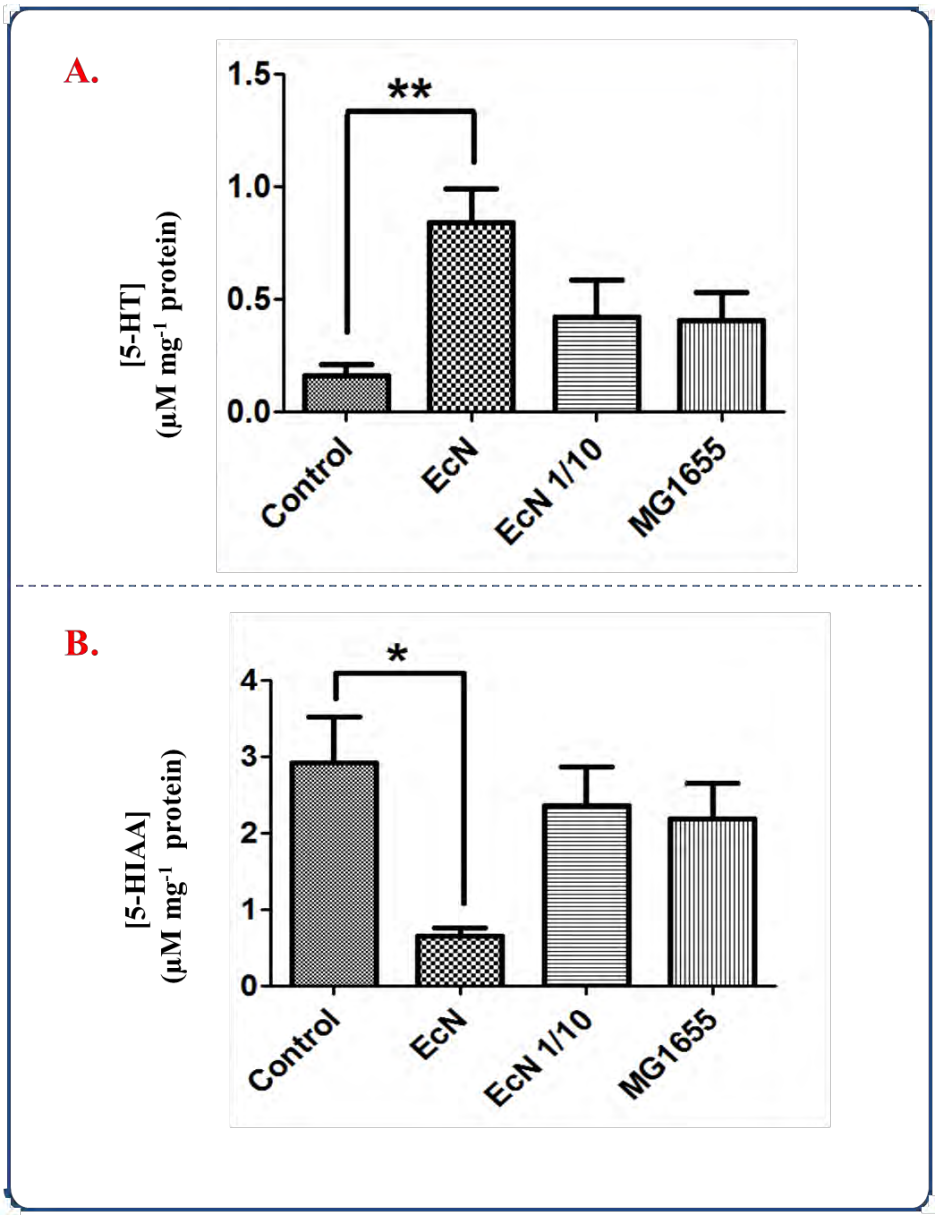


Figure 6.4: Production and metabolism of serotonin in mucosa cells treated with *E. coli* strains

Mucosa cells (isolated from ~ 1 cm^2 intestinal layer segment) were co-cultured with bacterial cells at $2x10^9$Cfu/mL ($2x10^8$ Cfu/mL for EcN 1/10) in 6-well plate at 37°, 5 % CO_2 for 1 hour. Control samples (untreated with bacteria). Cell supernatants were then analysed for: A. Serotonin content and B**.** metabolite 5-hydroxy-3-indoleacetic acid (5-HIAA)

Data were recorded based on levels obtained from intracellular sampling and normalised to protein content per surface area of the intestinal tissue segment used to extract mucosa cells.

One-way ANOVA was used to compare means between all the 4 groups (n=6 for each group) Bonferroni's multiple comparison tests was subsequently used to establish the groups showing statistical differences.

Data are shown as mean ±SEM, ** P<0.01, n=6 (six independent experiments).

6.4.2.4 Measurement of 5-HIAA: 5-HT ratio

Figure 6.5 shows results of responses that could reflect on the clearance of 5-HT from EC cells into neighbouring enterocytes and subsequent metabolism. These results show that stimulation of cells with EcN led to a significantly smaller 5-HIAA: 5-HT ratio as compared to uninoculated control ($P<0.01$, $n=6$). The ratios were 1.2 ± 0.2 and 15.4 ± 1.6, in EcN samples and control, respectively. These could suggest either: i) poor clearance of the produced 5-HT or ii) inactivity of the monoamine oxidase (MOA) enzyme that catalyse the oxidative deamination of 5-HT into 5-HIAA.

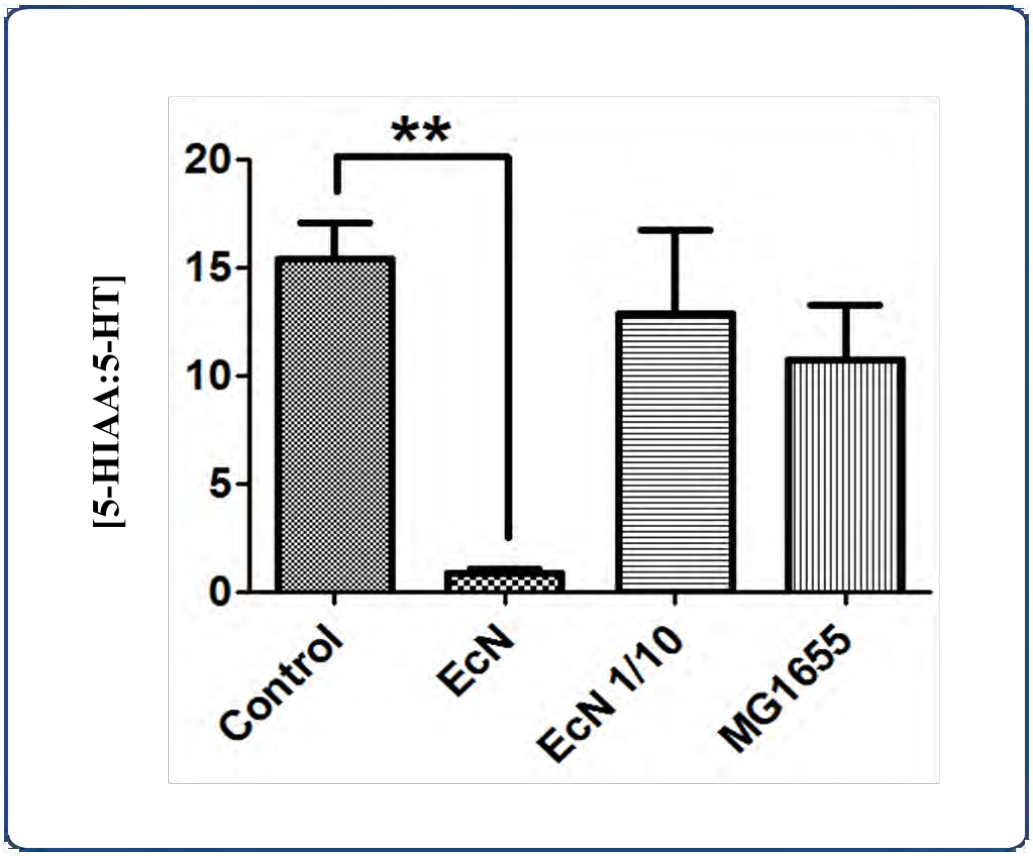


Figure 6.5: Alterations in the clearance and metabolism of serotonin between co-cultures of *E. coli*

Ratios of 5-HIAA:5-HT were calculated using data presented in Figure 6.4

Non-parametric Kruskall-Wallis test was performed to compare serotonin clearance ratios between all the 4 groups, (n=6 for each group). Dunn's multiple comparison test was subsequently used to establish the groups showing statistical differences.

Data are shown as mean ±SEM, ** $P<0.01$, n=6 (six independent experiments).

6.4.3 Analysis of short short-chain fatty acids (SCFA) in mucosa co-cultures with bacteria

To determine the profile of SCFA production by *E. coli* Nissle 1917 and control MG1655 in mice mucosa co-culture media, HPLC analysis of SCFA was performed. Acetic acid, propionic acid and butyric acid, were analysed based on their potential to stimulate serotonin release in enterrochromaffin cells (Fukumoto *et al*. 2003). Only acetic acid was found to be detectable with levels of propionic and butyric acids below the detection limit (0.5 mM) in all samples (bacterial and uninoculated control) (Figure 6.6). There was a statistically significant difference in acetic acid production between MG1655 and EcN mucosa co-cultures at the same inoculum density ($P < 0.05$, n=3), and in EcN 1/10 ($P < 0.01$, n=3). Using same inoculum density in co-cultures, *E. coli* MG1655 produced more acetic acid with 11.6 ± 1.2 mM whereas *E.coli* Nissle 1917 produced 7.7 ± 1.1 mM acetic acid. Acetic acid yield was 3.1 ± 0.3 mM when a 1:10 of the *E. coli* Nissle 1917 inoculum density was used (EcN 1/10). These results suggest that the observed increase in serotonin content in EcN co-cultures (Figure 6.4.A) was not mediated by acetic acid production.

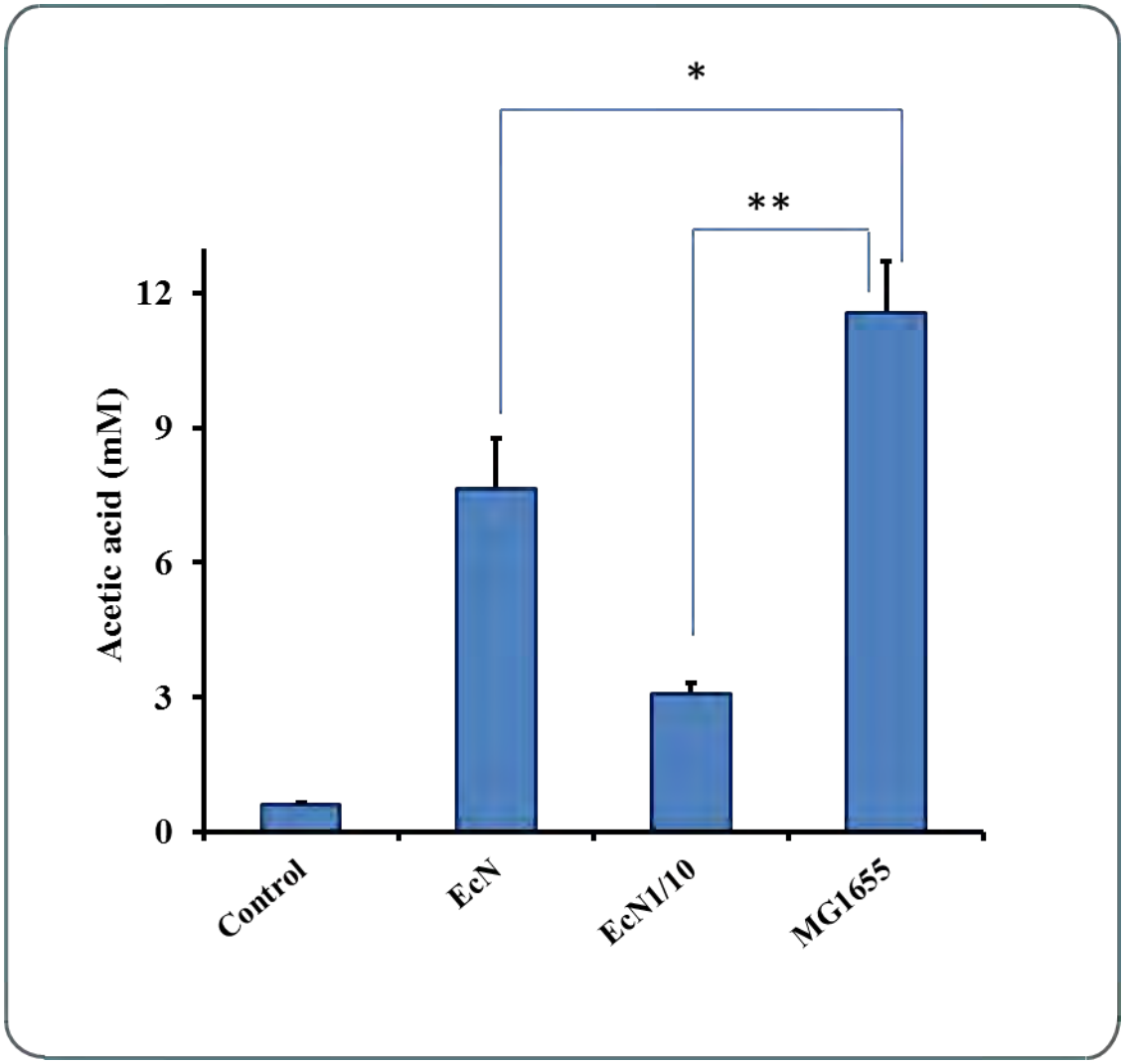


Figure 6.6: Production of acetic acid in intestinal mucosa co-cultured with bacteria

The mucosa was harvested from mouse ileum then resuspended in Krebs Buffer solution. This was co-cultured with bacterial cells at $2x10^9$ Cfu/mL ($2x10^8$ Cfu/mL for EcN 1/10) in 6-well plate at 37°, 5 % CO_2 for 1 hour. Control samples consisted of mucosa only (incubated without bacterial treatment). Co-culture supernatants were collected and short-chain fatty acids content was analysed using HPLC. The HPLC was calibrated using the method of standard addition (MOSA) ($y = 0.1494x + 0.0196$ with $R^2 = 0.9982$).

Data are shown as mean ±SEM, n=3 (three independent experiments). * $P<0.05$, ** $P<0.01$ (t-test).

6.5 Discussion

Gut mucosal enterochromaffin (EC) cells are the major sensory transducers of the gut neuroendocrine system, through the regulation of gastro-intestinal absorption, secretion and motility (Bülbring and Lin 1958, Sjolund *et al*. 1983, Grider *et al*. 1996, Cooke *et al*. 1997, Furness *et al*. 1999, Gershon 1999, Pan and Gershon 2000). These functions are mainly induced by serotonin (5-HT) released by EC cells, in response to various chemical and mechanical luminal stimuli (Gershon 1999, Lundgren 2002, Sharkey and Mawe 2002). Mechanical stimulation consists mainly of a food bolus-mediated pressure against the intestinal wall, whereas chemical stimuli may include bile acids, food nutrients and bacterial by-products, and toxins (Gershon 1999, Zhu *et al*. 2001, Lundgren 2002, Smith *et al*. 2006).

While the effects of bacterial enterotoxin (i.e. cholera toxin and lipopolysaccharides) on serotonin production by EC cells have been investigated (Racke *et al*. 1996, Fukumoto *et al.* 2003, Kidd et al 2009), those of live gut-associated bacterial cells, and of probiotics, remain unclear in this process. However, probiotics are known to possess various strategies (secretory as well as cell-to-cell dependent) of interacting with host intestinal mucosa (Lebeer 2010), and it is reasonable to hypothesise this may encompass strategies involving modulation of neurotransmitter release, re uptake or metabolism.

In the current study, the effect of probiotic *E. coli* Nissle 1917 on neurotransmitter production was examined using an *ex-vivo* co-culture model of mouse intestinal mucosal cells. The mucosal cells were harvested from fresh intestinal tissue using intracellular sampling approaches that were recently developed by Parmar *et al*. (2011). This allowed the investigation of the effect of co-incubated bacteria on: i) the synthesis of individual neurochemicals by EC cells and ii) metabolism of the synthesised serotonin. The obtained

results demonstrate the ability of *E. coli* Nissle 1917 to enhance 5-HT production by the EC cells from precursor tryptophan. Moreover the produced serotonin was not metabolised into 5-hydroxy-3-indoleacetic acid (5-HIAA) as expected under normal physiological conditions.

The non-conversion of 5-HT was not observed in mucosa co-cultured with *E. coli* MG1655, where the produced 5-HT was subsequently inactivated by monoamine oxidase (MOA) into 5-HIAA. MG1655, is a human gut commensal strain (Blattner *et al*. 1997), and has been used as a control in several studies involving *E. coli* Nissle 1917 (Grozdanov *et al*. 2004, Ukena 2005, Bleich *et al*. 2008, Maltby *et al*. 2013). Therefore it can be suggested that the enhanced production and non-conversion of serotonin that were observed in mucosal cells co-cultured with *E. coli* Nissle 1917 is specific to this probiotic strain. It was also important to determine the role of bacterial cultures in the production of short-chain fatty acids (SCFAs) in the co-culture media. This was useful in establishing a possible mechanism by which *E. coli* Nissle could be mediating serotonin synthesis in EC cells. It was revealed that MG1655 produced more acetic acid in the co-culture media than *E. coli* Nissle, ruling out the possibility that the serotonin stimulatory effect of *E. coli* Nissle probiotic was solely mediated through acetic acid production.

6.5.1 High serotonin levels in *E. coli* Nissle co-culture, an indication of serotonin biosynthesis, release, clearance and metabolism

Figure 6.1 shows the basic mechanisms involved in serotonin synthesis and release from EC cells, under normal physiologic conditions in humans. It is clear that the production and levels of serotonin in the intestinal mucosa depend mainly upon the availability of precursor tryptophan. In this current study, when mucosal cells were stimulated with *E. coli* Nissle, levels of 5-HTP and serotonin increased whereas those of metabolite 5-HIAA were reduced. This indicates a substantial alteration in 5-HT metabolism (Figure

6.6). While it seemed clear that intracellular tryptophan was being used up for the biosynthesis of 5-HT, it not clear if the produced serotonin was: i) released from the EC cells into the extracellular matrix; ii) cleared into the neighbouring enterocytes; or iii) metabolised into 5-HIAA once inside the enterocytes. All these three scenarios could have an implication in revealing possible mechanisms by which *E. coli* interact with the mucosa cells and the EC cells in particular.

The newly synthesised serotonin is first stored into vesicles of the EC cells by the vesicular monoamine transporter 1 (VMAT1) before it can be released into the extracellular matrix *via* an external Ca^{++}-dependent process (Bertrand and Bertrand 2009). This release is mainly thought to be induced by mechanical stimulation or acidification of the gut lumen (Kellum *et al.* 1999, Yu *et al*. 2001). In order for the newly synthesised 5-HT not to be released from EC cells, EcN could either be blocking calcium channels of EC cells or producing a compound that inhibits the release.

Bacterial by-products in the gut mucosa have been previously implicated in the inhibition of 5-HT. For exam l e, γ-Aminobutyric acid (GABA) produced by enteric bacteria (i.e *Lactobacillus, Bifidobacterium, Streptococcus*), from the precursor glutamic acid, has been found to inhibit the release of 5-HT secreted by EC cells (Schwörer *et al*. 1989, Lyte 2011). Interestingly, GABA has been found in to be produced by EC cells as an endocrine mediator and a neurotransmitter (Hyland and Cryan 2010). However this neurochemical can inhibit 5-HT release when it is produced from outside the EC cells (Bertrand and ertrand 2009). It's not clear if *E. coli* Nissle could be producing any inhibitory compound such as GABA or blocking 5-HT release through inhibition of upstream enzymatic processes (i.e. the rate limiting tryptophan hydroxylase-1). However, neither scenario is

congruent with the observed reduction of tryptophan and high levels of serotonin generated upon EcN exposure of mucosal cells.

Assuming that 5-HT was released from EC cells (treated with bacteria and controls) into the extracellular matrix, the majority would be expected to be cleared into the neighbouring enterocytes by the serotonin reuptake transporter (SERT) (Fuller and Wong 1990, Wade *et al*. 1996, Gill *et al*. 2008). This uptake of 5-HT is essential for its degradation into 5 hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase (MAO), a mitochondrial enzyme of the epithelial intestinal cells (Egashira and Waddell 1984, Rodriguez *et al*. 2001). The observed elevated 5-HT content and low levels of 5-HIAA in mucosal cells treated with *E. coli* Nissle could reflect the inhibition of 5-HT clearance (SERT inhibition), inhibition of MAO activity, or both. Any of these three scenarios could explain the significant decrease in 5-HIAA: 5-HT ratio ($P<0.01$, $n=6$) that was observed in *E. coli* Nissle co-cultures (Figure 6.5).

In the case of putative SERT inhibition, the non-clearance of 5-HT could lead into an increased bioavailability of 5-HT and, into the gut tissues, and into the lumen with impact on gut function (i.e motility and secretion) (Hansen 2003). Such an increased availability of 5-HT is not always considered beneficial. Increased 5-HT can either increase or desensitize 5-HT receptors, leading to abnormal motility and also alter physiological levels in the vasculature. Desensitised receptors have been found to be associated with peristaltic reflex impairment and dysmotility of inflammatory bowel disease (Linden *et al.* 2003). Moreover, high levels of 5-HT have been associated with symptoms such as diarrhoea (in cholera) (Lundgren 1998, Turvill *et al*. 2000), and chemotherapy-induced nausea (Roila and Fatigoni 2006). However, the increased availability of 5-HT that is recorded in gut diseases/conditions (mentioned above) also accompanies many other pathologic factors

and it is difficult to clearly determine if 5-HT is the main protagonist of these conditions. It is not known if the observed high-levels of 5-HT induced by EcN could be beneficial or detrimental to the host. Nevertheless, there is no report of EcN causing those conditions in clinical trials. In contrast, the potential ability of EcN to interact with SERT could be beneficial in normal physiologic conditions or even in alleviating some disease symptoms.

SERT is the target for key therapeutic agents such as paroxetine and fluoxetine, which are selective serotonin reuptake inhibitors (SSRIs) (Bertrand and Bertrand 2009). Also, SERTs are expressed at both the apical and basolateral surfaces of the enterocytes (Gill *et* al. 2008), thus easily accessible to the probiotics colonising the intestinal mucosa. The crucial role of SERT in gastro-intestinal disorders was explored by Chen, J.J. *et al*. (2001) using transgenic mice lacking SE T (SE T −/− mice). They have re o rted the SE T −/− mice to exhibit increased colon motility and frequent diarrhoea interspersed with phases of constipation, whereas these symptoms were absent in normal mice (SERT +/+ mice). The authors suggested that the watery diarrhoea was caused by enhanced bioavailability of 5-HT while periodical constipation was attributable to the 5-HT receptor desensitisation.

E. coli Nissle has been shown to be therapeutically effective in a variety of gut disorders including diarrhoea (Henker *et al*. 2007, Henker *et al*. 2008) and constipation (Möllenbrink and Bruckschen 1994, Sonnenborn and Schulze 2009). However, its potential to induce 5-HT synthesis or alter SERT functions in these therapeutic effects has not been investigated. Administration of *E. coli* Nissle for 8 weeks to chronically constipated patients in a randomised double-blind study (n=35, for each group) has been proven effective for the treatment of this condition (Möllenbrink and Bruckschen 1994). The results from this

study showed a significant increase in number of bowel movements in experimental group (6 movements/week) as compared to placebo group (1.6 movements/ week).

Bar *et al*. (2009) have related the anti-constipation effect of *E. coli* Nissle *in vivo* to this strain's ability to induce human colonic motility *in vitro*. From their experiments, Bar *et al*. (2009) suggested that these effects were due to the production of short-chain fatty acids (SCFAs), especially acetic acid. While motor effects of SCFAs in the gastro-intestinal tract have been established (Scheppach 1994, Cherbut *et al.* 1997, Cherbut 2003), it's not clear whether probiotic EcN would outcompete the indigenous community of the gut in acetic acid production. If this was the case, then the anti-constipation effects of *E*ecN *in vivo* could be mainly due to the production of SCFAs (Möllenbrink and Bruckschen 1994). Otherwise the SCFAs formed at fermentation of carbohydrates in the colon by indigenous members of the gut microbiota would be probably enough to cure the constipation.

The results obtained in this current study comparing the ability of *E. coli* Nissle and control MG1655 to produce SCFAs in the co-culture media, do not contradict the possibility of acetic acid production as a mechanism of probiotic action. However they suggest that EcN has the ability to increase the bioavailability of 5-HT, which can also contribute to the colonic motility amd anti-constipation effects exhibited by this strain in the studies dissussed above. It can be suggested that the probiotic effects of EcN in the treatment of constipation and probably of diarrhoea could involve 5-HT induction and SERT blockage. Still, this can not rule out the possibility of a failure in 5-HT metabolism to 5-HIAA, probably through the inactivation of the MAO enzyme in enterocytes. However, this possibility is much less likely as MAO is expressed and active intracellularly, therefore more difficult to perturb as compared to SERT. Nevertheless, even if EcN was inhibiting

the MAO, it is possible that the non-metabolised 5-HT could lead into a high bioavailability of 5-HT in the extracellular matrix, as the enterocytes are not specialised in storing 5-HT. This could thus cause similar physiological reactions in the host as those observed in SERT blockage. Having suggested these possible scenarios and probable implications, it is clear that the current results are only preliminary and leave more questions unanswered and the mechanisms involved in EcN are yet to be clarified.

6.5.2 Confirmation of serotonin release clearance and metabolism in *E. coli* Nissle

The gut neurotransmission system involves four major processes: biosynthesis, release, clearance and metabolism (Parmar *et al*. 2011). The biosynthesis and metabolism of 5-HT are both intracellular events mainly taking place in enterochromafin (EC) cells and enterocytes, respectively. Both events can be easily monitored using intracellular sampling and analysis of neurochemicals from mucosa tissue samples. The release of 5-HT as well as its clearance into the adjacent enterocytes might be monitored more precisely by using both intracellular and extracellular sampling approaches.

The experimental design that was utilised in this current study had involved the intracellular sampling approach (Parmar *et al*. 2011) prior to co-cultures. This model seemed to be suited for measuring all the four process combined together because: i) intracellular levels of neurochemicals are assumed to be equal between mucosa samples prior to co-incubation with bacteria; ii) the model allows assessment of the effect of bacterial treatment on 5-HT synthesis and release in the co-cultures, by comparing levels of 5-HT, precursors (tryprophan and 5-HTP) and metabolite (5-HIAA) between the treated mucosa samples and the untreated but incubated controls; iii) after the co-culture, the mucosa is homogenised allowing quantification of total neurochemicals including 5-HT that might be released into the extracellular matrix or cleared into the enterocytes.

However, a more precise measure and rate of 5-HT release and clearance can be achieved by combining both the intracellular and extracellular approaches (Parmar *et al*. 2011) prior to co-cultures with *E. coli* Nissle 1917 and MG1655 as proposed in Figure 6.7. The extracellular sampling approach could also help to determine the impairment of the SERT or MAO by adding fluoxetine (SERT inhibitor) to the co-culture medium (Chen *et al*. 1998, Linden *et al*.2003) as shown in Figure 6.8.

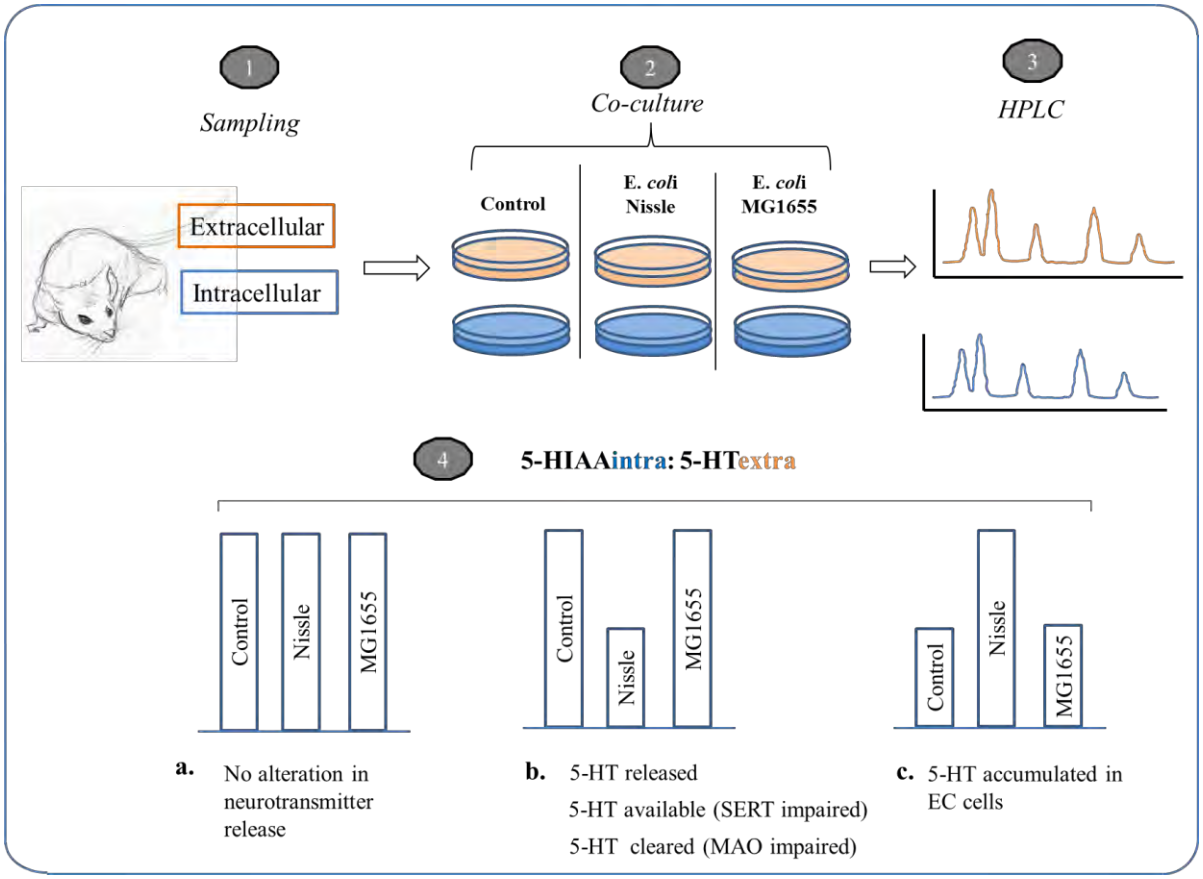


Figure 6.7: Confirmation of 5-HT release in EC cells stimulated by *E. coli* Nissle

This schematic diagram illustrates an extension of the co-culture model and experimental set-up utilised to generate the results described in this chapter. It also includes extracellular sampling approach which could help to measure the rates of 5-HT synthesis and release.

Step 1: Intracellular (intra) and extracellular (extra) sampling to allow monitoring intracellular and extracellular levels of 5-HT

Step 2: Co-cultures with bacteria and controls

Step 3: analysis of neurochemicals in both intra and extracellular fractions

Step 4: Calculation of 5-HIAA*intra*:5-HT*extra* ratio considering that all the available 5-HT *extra* is the result of 5-HT release during co-culture duration

Possible outcomes and implication:

- **4.a**: 5-HIAA*intra*:5-HT*extra* ratios are proportionally similar between treatment groups and controls. *Implicatio*n: No alteration in neurotransmitter release.

- **4. b**: HIAA*intra*:5-HT*extra* ratios confirmed to be significantly smaller in *E. coli* Nissle samples. *Implication*: 5-HT release is confirmed and increased availability of 5-HT also confirmed either in extracellular matrix (predicting SERT impairment) or in adjacent enterocytes (MAO impairment).

- **4.c**: HIAA*intra*:5-HT*extra* ratios bigger in *E. coli* Nissle samples. *Implication*: 5-HT synthesised by accumulated /entrapped inside EC cells

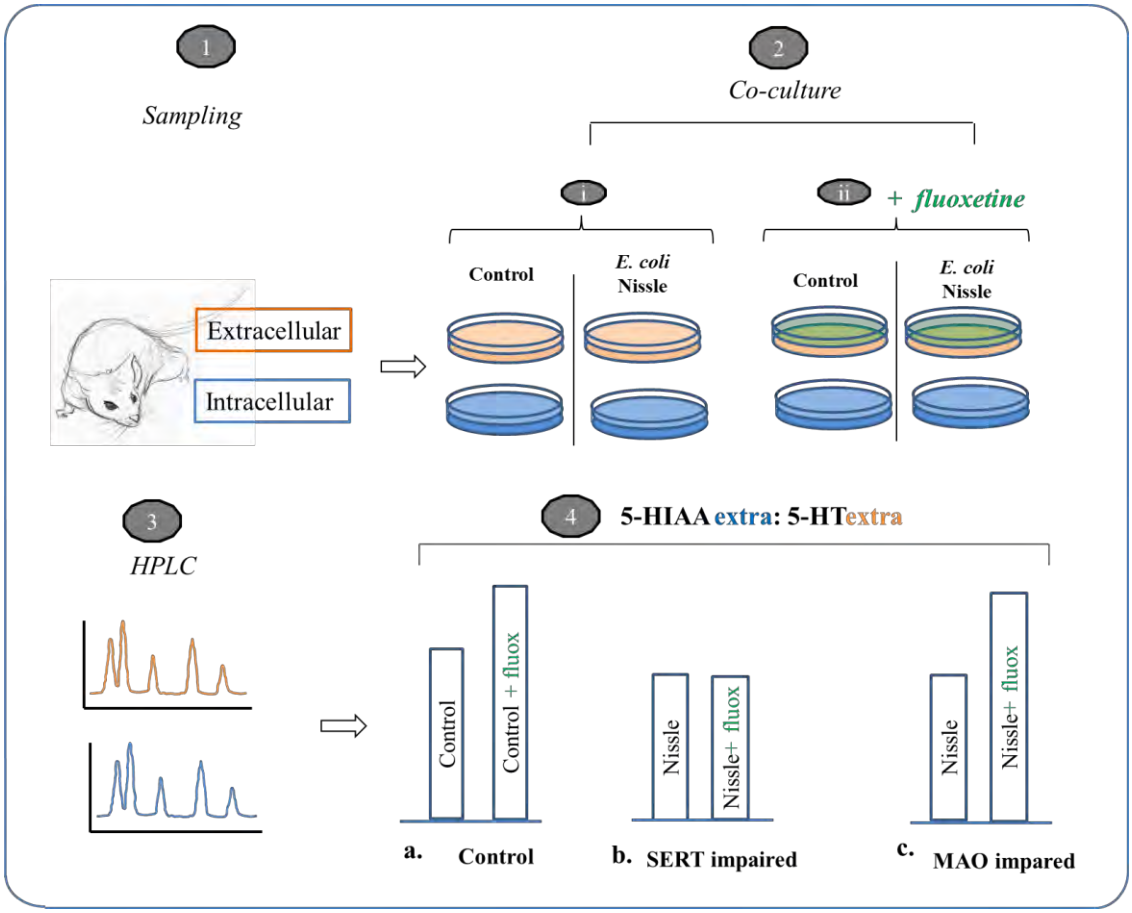


Figure 6.8: Confirmation of SERT /MAO impairment in mucosa treated with *E. coli* Nissle

This schematic diagram illustrates a proposed modification to the co-culture experiments described in this chapter, in order to test the potential for E. coli Nissle to increase 5-HT bioavailability through SERT inhibition.

Step 1: Intracellular (intra) and extracellular (extra) sampling to allow monitoring intracellular and extracellular levels of 5-HT

Step 2: Co-culture of *E. coli* Nissle Vs untreated controls, with or without fluoxetine (SERT inhibitor)

Step 3: analysis of neurochemicals in both intra and extracellular fractions

Step 4: Calculation of 5-HIAA*intra*:5-HT*extra* ratio considering that all the available 5-HT *extra* is the result of 5-HT available in extracellular matrix (in fluoxetine sample) or released + cleared in other samples

Possible outcomes and implication:

- **4.a**: HIAA*intra*:5-HT*extra* ratios high in control treated with fluoxetine. *Implication* :SERT inhibitory effect of fluoxetine confirmed

- **4. b**: no change in HIAA*intra*:5-HT*extra* ratios between *E. coli* Nissle samples with or without fluoxetine. *Implication*: clearance of 5-HT is blocked and *E. coli* Nissle confirmed to inhibit SERT activity

- **4.c**: High HIAA*intra*:5-HT*extra* ratio in *E. coli* Nissle samples + fluoxetine. *Implication:* 5-HT clearance is happening but MAO likely to be impaired as a results of *E. coli* Nissle treatment.

6.5.3 Summary

Taken together, the results obtained in this study show for the first time tangible evidence on the ability of *E. coli* Nissle 1917 to enhance 5-HT synthesis in EC cells of the gut. The increased levels of serotonin in the *ex-vivo* co-culture model of intestinal mucosa and *E. coli* Nissle could suggest that this probiotic does enhance the bioavailability of 5-HT. It is important to note that bioavailability of 5-HT can be therapeutically relevant in many gastro-intestinal diseases or could help to keep a good balanced level of this neurotransmitter in healthy individuals. Beside this fundamental new insight into host-microbe interactions, this study provides tools, parameters and rationale, which could be used: i) to direct further studies of modulation of neurotransmitter-based signalling in probiotics, and ii) aid the rational bioengineering of more effective probiotic based therapies and interventions.

6.6 Conclusion

- A co-culture model for exploring the effect of *E. coli* Nissle 1917 and other bacteria with relevance to human health has been established.

- The ability of *E. coli* Nissle to enhance 5-HT synthesis and metabolism has been explored and could be used to explain probiotic effects (i.e anti-constipation effect) which have already been observed in clinical trials.

- Further studies are required in order to determine the mechanisms of *E. coli* Nissle 1917 in modulation of serotonin release, as well as those exploring new therapeutic opportunities of probiotics.

Chapter 7: General discussion

The gastro-intestinal tract (GIT) mucosal surface offers an interface between the host and bacterial members of the gut microbiota, which perform many functions relevant to human health. A growing interest in the beneficial functions of the gut microbiota led to the isolation and application of probiotic bacteria to enhance human health or treat diseases. Probiotic *E. coli* Nissle 1917 has been proven to be efficient for the treatment or alleviation of symptoms in GIT-related disorders, in which the gut microbiota or its optimal functioning has been disturbed (Lee and Salminen 2009, Sonnenborn and Schulze 2009, Verna and Lucak 2010, Behnsen *et al*. 2013). However, little is known regarding the mechanisms underlying the effects of this strain, or how it interacts with the human host. The overall hypothesis of the current study was that exploring the genetic basis of EcN survival in the gut, and interactions with intestinal cells would provide mechanistic insight into the effects of probiotics on human hosts. This would not only contribute to novel, improved design of probiotic interventions, but also support of EcN clinical efficacy.

7.1 Genetic manipulation of *E. coli* Nissle 1917 and isolation of mutants showing altered abilities to interact with the human intestinal cells

Application of genomics-based approaches to the study of probiotic effects is a powerful way in which individual genes, and the processes that they regulate, can be explored with regard to their role during host-probiotic interactions. In the case of EcN , adherence to Caco-2 cells and subsequent effects on cell health and apoptosis, random transposon mutagenesis provides many benefits over other molecular approaches. Issues associated with studying gene expression using secondary host laboratory strains, and the current lack of knowledge about genes involved in host-probiotic interactions support the use of this mutagenesis approach.

The results obtained in this study indicate that random transposon mutagenesis is a powerful tool to investigate *E. coli* Nissle survival in the gut milieu as well as adherence and effect on Caco-2 cell health. This can allow the identification of novel genes and processes that might be important to gut colonisation and maintenance of the intestinal epithelial cell integrity by the probiotic EcN. Modifications to the methods described here may allow different therapeutic and prophylactic aspects EcN and other beneficial bacteria to be studied.

In this study, the mutagenesis approach was used to identify EcN mutants showing altered tolerance to bile, elevated osmolality (NaCl) and reduced pH. These three elements constitute a massive challenge to the establishment of the ingested bacteria in the gut. Interestingly, disrupted genes in these mutants included those playing putative roles in stress responses and maintenance of the cell envelope. Addition of phenotypic characterisations of these mutants would allow the investigation of the mechanisms underlying EcN survival and adaptation to the gut stresses. For example, the parameters associated with survival (kill-curve) could be investigated in: i) gastric juices and pepsin by adapting bacterial survival assays developed by Zhu *et al.* (2006); ii) in crude bile and individual bile acids, using kill-curve experiments proposed by Jones *et al*. (2008), or iii) pre-exposure to a GIT stress and subsequent investigation of another (cross-adaptation) (O'Driscoll *et al*. 1996, Begley *et al.* 2002).

Another key strategy of the mutagenesis approach was the use HTP screening for mutants with altered biofilm forming abilities in 96-well plate using the crystal violet (CV) staining assay (O'Toole and Kolter 1998a). This intermediate screening was able to recover mutants with disruptions in putative cell surface structures, which demonstrated relevance to host-probiotic interactions and adherence to the human intestinal Caco-2 cells. However the biofilm formation model could be improved by including some elements of the intestinal mucosa. For example, coating the 96-well plate with mucin from the GIT prior to performing the biofilm assays could help to recover more mutants with alterations in host-probiotic interactions. Mucin-coated plates were found to facilitate the characterisation of bacterial strains and mutants in host-pathogen interactions (Silva *et al*. 2006, Landry *et al*. 2006, Staquicini *et al*. 2010). Moreover, components of mucin 2 of the human intestinal mucosa have been recently demonstrated to be specific receptors for binding of the EcN (Troge *et al*. 2012). This suggests that the biofilm screen could be adapted (by adding mucin to the assay) in order to facilitate studies of genetic basis of EcN attachment to surfaces. Moreover, further insight into the adhesion of EcN strains to intestinal epithelial cells (IECs) could be obtained by using mucus secreting cell line such as HT-29 (Pinto *et al*. 1983) in addition to the Caco-2 cell line which was used in this current study.

7.2 Importance of K5 capsule and kfiB gene in host-*E. coli* Nissle 1917 interactions

The studies on adherence of 'surface structure' mutants of EcN to Caco-2 and effect on cell-health revealed, for the first time, the role of *kfiB* gene in host intestinal cell-EcN interactions. The *kfiB* is part of the K5 capsule biosynthesis gene cluster but with no confirmed function in this process. This is important for Caco-2 cell health in co-culture with *E. coli* Nissle, as mutation in *kfiB* but not in the downstream *kfiC*, resulted in apoptosis induction and cytotoxicity in Caco-2 cells. Production of capsule K5 was also

demonstrated to be attenuated (not detectable using the ΦK5 bacteriophage sensitivity assay) in both EcNΔ*kfiB* and EcNΔ*kfiC* mutants. This indicates that different genes associated with capsule biosynthesis in EcN may mediate distinct aspects of host-microbe interaction.

The EcNΔ*kfiB* ability to induce apoptosis and cytotoxicity in Caco-2 cells was coupled with enhanced adherence to Caco-2 cells, a he noty e that was not observed in EcNΔ*kfiC*. From these observations it was surmised that mutation in *kfiB* could lead to: i) a more pronounced decapsulation than EcNΔ*kfiB*, unblocking the surface expression of protein ligands such as protein Ag43, which could facilitate the observed enhanced adherence to host-cells, or ii) *kfiB* could be encoding for another function in addition to involvement in K5 expression. The first hypothesis can be verified by comparing mutants EcNΔ*kfiB* and EcNΔ*kfiC* using a range of methods including: i) electron microscopy (Bronner *et al*. 1993); autoaggregation and hydrophobicity assays which have been previously used to confirm loss of capsule (Davey and Duncan 2006); quantitative analysis of the self-recognising Ag43, an adhesion likely to be expressed at the bacterial cell surface in decapsulated mutants (Shembri *et al.* 2004, Ulett *et al*. 2007). The second hypothesis regarding *kfiB* involvement in functions other than K5 biosythesis could be verified using comparative analysis of the outer membrane protein (OMP) profiles of EcNΔ*kfiB* and EcNΔ*kfiC* mutants (Carlsohn *et al.* 2006, Aguilera *et al.* 2014). The obtained OMP profiles could then help to direct more advanced studies if necessary.

7.3 Stimulation effect of *E. coli* Nissle on neurotransmitter release from intestinal epithelial cells

The results of neurotransmitter release from enterochromaffin cells in this study show the ability of the probiotic EcN to enhance 5-HT synthesis from EC cells and inhibits its metabolism into 5-HIAA. These results suggest that *E. coli* increases the bioavailability of serotonin within the gut mucosa. This can be therapeutically relevant in several gastro-intestinal disorders in which 5-HT signalling is altered such as chronic constipation (Zhao *et al*. 2003). Chronic constipation is difficult to treat as patients have often been found to fail to respond to the conventional therapies of constipation (Voderholzer *al*.1997, Costedio *et al.* 2006). However, *E. coli* Nissle 1917 has been demonstrated to be therapeutically effective for treatment of chronic constipation, thus a potential alternative therapy (Möllenbrink and Bruckschen 1994, Sonnenborn and Schulze 2009). Nevertheless, it is important that mechanisms underlying the EcN anticonstipation effect are fully elucidated prior to considering this as a conventional therapy. The results presented in this study could be useful in direct mechanistic studies of neurotransmitter-based signalling in *E. coli* Nissle 1917.

7.4 Future work

The following should be investigated in future studies:

- Development of additional phenotypic and genotypic characterisations (i.e. kill curves, cross-adaptation assays, gene deletion and RT-PCR) of *E. coli* Nissle bile-sensitive, salt sensitive and acid-sensitive mutants isolated in this study. This should allow allow investigation the strategies used by *E. coli* Nissle to survive gastro-intestinal stresses.

- Investigate the use of additional proteomic analysis approaches and microarrays, in conjunction with the *in vitro* co-culture models of *E.coli* Nissle with Caco-2 model to identify more genes and gene products involved in host-probiotic interactions.

- The importance of capsule K5 in in host-probiotic interactions should be investigated further through the analysis of cytokines induction/ expression (mainly NF-kappaB and COX-2) using *in vitro* co-culture models of intestinal cell lines and EcNΔ*kfiB* and EcNΔ*kfiC* mutants.

- *In silico* analysis of K5 gene cluster and *kfiB* in a large cohort of human gut microbiota. This could help to get more insight into the origin, functions and pathways involving host-*E. coli* Nissle 1917 interactions in the K5 capsule.

- Investigation of the effect of *E. coli* Nissle 1917 supernatants and cell surface structure mutants (capsule, flagella, fimbriae and LPS) on neurotransmitter release from intestinal mucosa cells. This could help to establish the mechanisms of enterochromafin cell-microbe interactions.

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