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CONJUGAL TRANSFER OF THE PLASMID pOX38 WITH THE COLICIN E7 GENE

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KONJUGATIVNI PRENOS PLAZMIDA pOX38 Z GENOM ZA KOLICIN E7

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AB Plasmids are widespread and highly diverse extrachromosomal elements of DNA that replicate in an autonomous and self-controlled way. Some plasmids can move between hosts by means of conjugation, a process of genetic transfer whereby DNA is transferred from the donor to the recipient cell by a mechanism requiring cell-to-cell contact. The aim of this thesis was to evaluate the conjugal transfer frequencies of the plasmid pOX38 between different laboratory and wild-type strains of *E. coli* used as both conjugal donors and conjugal recipients. *E. coli* strain SE15i was engineered in analogy to the modified probiotic *E. coli* strain Nissle 1917 with the pOX38a-encoded colicin E7 gene and the chromosomally encoded colicin E7 immunity gene. Conjugal transfer frequencies of the plasmids pOX38:Cm and pOX38a were assessed to evaluate the proposed mechanism of action of the conjugation-mediated colicin E7 delivery system into recipient strains. Lipopolysaccharide (LPS) and capsular polysaccharide profiles were compared between the strains. Analysis of the acquired conjugal transfer frequencies of the plasmids pOX38:Cm and pOX38a (encoding the colicin E7 gene) supported the proposed mechanism of action of the conjugation-mediated colicin E7 delivery system into recipient strains. The conjugal transfer frequencies into wild-type recipient strains were significantly lower compared to the laboratory recipient strains. The laboratory strains exhibited rough LPS profiles and absence of capsular polysaccharides, while the wild-type strains exhibited semi-rough (Nissle 1917) or smooth (other strains) LPS profiles and presence of capsular polysaccharides.

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AI Plazmidi so široko razširjene in raznolike izvenkrosomske molekule DNA z avtonomno replikacijo pod svojo lastno kontrolo. Nekateri plazmidi se lahko med gostitelji premikajo s konjugacijo, procesom izmenjave genetske informacije, pri katerem se DNA prenese iz donorske v recipientsko bakterijsko celico preko fizičnega kontakta med celicama. Cilj magistrske naloge je bil oceniti frekvence konjugacije plazmida pOX38 med različnimi laboratorijskimi, komenzalnimi in patogenimi sevi *E. coli*. Pripravili smo sev SE15i, podoben modificiranemu probiotičnemu sevu *E. coli* Nissle 1917, ki ima na plazmidu pOX38a gen za kolicin E7 in v kromosomu gen za protein imunosti proti kolicinu E7. Da bi ovrednotili delovanje mehanizma dostave gena za kolicin E7 v recipientske seve, smo ocenili frekvence konjugacije plazmidov pOX38:Cm in pOX38a (z zapisom za kolicin E7). Med sevi smo primerjali lipopolisaharide (LPS) in kapsule. Analiza pridobljenih frekvenc konjugacije plazmidov pOX38:Cm in pOX38a je pokazala učinkovito delovanje dostave gena za kolicin E7 v recipientske seve. Frekvence konjugacije v recipientske seve divjega tipa so bile značilno nižje v primerjavi s frekvencami konjugacije v laboratorijske recipientske seve. Pri laboratorijskih sevih smo dokazali LPS tipa »rough« in pri sevih divjega tipa daljše molekule LPS. Pri laboratorijskih sevih nismo zaznali kapsul, pri sevih divjega tipa pa smo kapsule zaznali.

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ABBREVIATIONS AND SYMBOLS

aa	amino acid
ANOVA	analysis of variance
Ap	ampicillin
APS	ammonium persulfate
bp	base pair
CFU	colony-forming unit
Cip	ciprofloxacin
Cm	chloramphenicol
CRISPR-Cas	clustered regularly interspaced short palindromic repeats/CRISPR-associated
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleosyl triphosphate
dPBS	Dulbecco's phosphate-buffered saline
Dtr	deoxyribonucleic acid transfer and replication
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
Eex	entry exclusion
EPS	extracellular polysaccharide
Gm	gentamycin
kb	kilobase pair
kDa	kilodalton
Kdo	2-keto-3-deoxyoctonoic acid
Kn	kanamycin
LB	lysogeny broth
LPS	lipopolysaccharide
MOB	mobility
Mpf	mating pair formation
Mps	mating pair stabilisation
mRNA	messenger ribonucleic acid
nt	nucleotide
NTP	nucleosyl triphosphate
OD_{600}	optical density at 600 nanometres
PCR	polymerase chain reaction
R-LPS	rough lipopolysaccharide
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute or rotations per minute
SDS	sodium dodecyl sulphate

SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sfx	surface exclusion
S-LPS	smooth lipopolysaccharide
Sm	streptomycin
sp.	species
spp.	species (pl.)
SR-LPS	semi-rough lipopolysaccharide
T4CP	type IV coupling protein
T4SS	type IV secretion system
Tc	tetracycline
TEMED	tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
U	enzyme unit
UV	ultraviolet

1 INTRODUCTION

Bacterium *Escherichia coli* (*E. coli*) is a Gram-negative, rod-shaped, motile, facultative anaerobe belonging to the *Enterobacteriaceae* family. It is an almost universal commensal inhabitant of the intestinal tract of humans and warm-blooded animals (Madigan and Martinko, 2006). *E. coli* species consists of many biotypes; most strains are non-pathogenic commensals, serving as a barrier to colonisation by pathogens in the intestinal tract (Maltby et al., 2013) and some *E. coli* strains have also been used as probiotics.

Probiotic bacteria are non-pathogenic and non-toxic bacteria that exert a positive effect on the health of their hosts (FAO/WHO, 2006; Guarner and Schaafsma, 1998). Probiotic bacterial strains have been used in treatment of different intestinal diseases of infectious as well as non-infectious origin, like diarrhoea and chronic inflammatory bowel diseases such as ulcerative colitis and irritable bowel syndrome. Among the most well-characterised *E. coli* probiotic strains is strain Nissle 1917, whose positive effects and biosafety have been continuously proven since its isolation in 1917 (Jonkers et al., 2012; Saad et al., 2013; Sonnenborn and Schulze, 2009).

However, some *E. coli* strains are pathogenic and are among important etiological agents of intestinal as well as extraintestinal infections in humans. *E. coli* has been implicated in diarrhoea and urinary tract infections, as well as in skin and soft tissue infections, neonatal meningitis, and septicaemia (Engleberg et al., 2013; Goering et al., 2008; Madigan and Martinko, 2006).

As multi-drug resistant bacteria pose an emerging risk to public health, alternative antibacterial agents are urgently needed. A particularly interesting class of novel antibacterial agents are bacteriocins, which are ribosomally synthesised molecules produced by a broad range of bacterial species. Bacteriocins synthesised by *E. coli* are called colicins and are widely present in *E. coli* bacterial populations (Cascales et al., 2007). However, there are some drawbacks to the application of colicinogenic probiotic strains for treatment or prevention of infections with pathogenic strains of *E. coli*. First, insensitivity to colicins is a wide-spread characteristic among *E. coli* strains. Second, strains sensitive to colicins can quickly mutate the surface receptors required for colicin entry into the cell and thus become insensitive. Third, colicins are often encoded on large plasmids, which in addition to colicin genes frequently encode a variety of virulence factors (Petkovšek, 2012).

Conjugation is along with transformation and transduction one of the three main mechanisms of horizontal gene transfer. It is a contact-dependent mechanism, mediated by a filament-like structure protruding from the cell surface, called the conjugative pilus (Frost et al., 1994; Snyder and Champness, 2003). Conjugation is an important horizontal gene transfer

mechanism in promoting the spread of virulence factors and antimicrobial resistances among bacterial populations (Smillie et al., 2010).

A genetically modified conjugative plasmid encoding the gene for colicin E7, which possesses DNase activity, has been engineered in the Molecular Genetics and Microbiology Research Group at the Biotechnical faculty of University of Ljubljana (Petkovšek, 2012). Upon conjugal transfer of this plasmid into the recipient strain, the gene encoding the colicin E7 is expressed and its lethal activity occurs, leading to death of the recipient strain. By taking advantage of this conjugal transfer-mediated toxin delivery system, the need for intact colicin receptors on the surface of the recipient strains can be circumvented (Petkovšek, 2012). Donor strains harbouring this conjugative plasmid could be used as probiotic strains in treating and preventing infections with pathogenic strains of *E. coli*.

1.1 AIM AND SCOPE

The aim of this thesis is to evaluate the conjugal transfer frequencies of the plasmid pOX38 with the colicin E7 gene between different laboratory and wild-type strains of *E. coli* used as both conjugal donors and conjugal recipients. A human and animal commensal *E. coli* strain SE15 will be engineered in analogy to the modified *E. coli* strain Nissle 1917, constructed by Petkovšek (Petkovšek, 2012). Conjugal transfer frequencies into different recipient strains as well as the recipient-killing efficacies of donor strains will be evaluated. Possible reasons for the variations in conjugal transfer frequencies between different mating pairs will be explored by lipopolysaccharide (LPS) and capsular polysaccharide profiling of conjugal donor and recipient strains. As bacterial conjugation is present in a wide range of bacterial species and mediates important events such as transfer of virulence determinants and determinants of resistance to antibiotics, understanding the mechanisms of conjugal transfer of genetic material is very important from medical, biotechnological, and ecological points of view.

2 LITERATURE REVIEW

2.1 PLASMIDS

Plasmids are extrachromosomal elements of DNA replicating in an autonomous and self-controlled way (del Solar et al., 1998). Together with bacteriophages, integron cassettes, transposons and integrative conjugative elements, which include conjugative transposons, plasmids make up a group of mobile genetic elements and are very important both as vectors of horizontal gene transfer and as essential tools in genetic engineering (Garcillan-Barcia et al., 2011; Smillie et al., 2010). Plasmids favour genetic exchange between different genetic environments and can incorporate and deliver genes to their host by recombination or transposition. Some of them even possess the ability to pass across genetic barriers between different organisms and hence transfer genetic material to higher eukaryotes (del Solar et al., 1998; Llosa et al., 2002). Plasmids confer to the host a wide range of important genetic traits including resistance to antibiotics, heavy metals and radiation, virulence factors, supplementary metabolic pathways and production of bacteriocins, among others (del Solar et al., 1998; Kado, 1998).

2.2 COLLECTIVE CHARACTERISTICS OF PLASMIDS

Plasmids are present in most Gram-negative and Gram-positive bacterial species and are highly diverse in size, copy number, and genetic structure. They range in size from approximately 300 bp to 2400 kb and are frequently present as covalently closed circular double-stranded DNA molecules, although some plasmids occur as linear DNA molecules (Kado, 1998). The structure of linear plasmids can be of either of the two types: some linear plasmids have terminal hairpin structures on each end of the DNA molecule, while others have a covalently bound protein on each 5′ end of the two strands of DNA (del Solar et al., 1998). Different plasmids are present in cells in different copy numbers with some plasmids being present in a cell in only 1–3 copies and others in over 100 copies. Copy number is controlled by both plasmid-encoded genes and interactions between the plasmid and its host (Madigan and Martinko, 2006). Regardless of their autonomous replication, plasmids extensively use the replication machinery of the host; plasmid-encoded replication genes are concerned primarily with replication control (del Solar et al., 1998; Madigan and Martinko, 2006). Three general mechanisms for the replication of circular plasmids are known: the θ mechanism, the rolling-circle mechanism, and the strand-displacement mechanism. Each circular plasmid replicates by one of these three mechanisms (del Solar et al., 1998). Linear plasmids, on the other hand, have distinctive replication mechanisms; linear plasmids with terminal hairpin structures replicate via concatemeric intermediates, while linear plasmids with terminal 5′ covalently bound proteins replicate by a protein-priming mechanism similar to that of bacteriophage φ29 (Meijer et al., 2001).

According to their mobility, plasmids can be categorised as:

- conjugative or self-transmissible: these plasmids encode the complete set of genes required for conjugation and are capable of transmission to a new host by themselves;
- mobilisable: these plasmids do not encode the complete set of genes required for conjugation and can be transferred to a new host only by using the products of some genes encoded on a conjugative plasmid present in the same donor cell;
- non-mobilisable: these plasmids can move neither by conjugation nor by mobilisation; instead, they are transferred to new hosts by natural transformation or transduction (Smillie et al., 2010).

2.3 CONJUGAL TRANSFER OF PLASMIDS

Some plasmids can move between hosts by means of conjugation. Bacterial conjugation is a process of genetic transfer whereby DNA is unidirectionally transferred from one bacterial cell to another by a mechanism requiring cell-to-cell contact. This process is usually encoded by conjugative plasmids (Madigan and Martinko, 2006; Russell, 2006; Willetts and Skurray, 1980; Willetts and Wilkins, 1984). Any bacterium harbouring a self-transmissible plasmid is a potential donor and any bacterium lacking a self-transmissible plasmid is a potential recipient (Russell, 2006; Snyder and Champness, 2003).

Conjugal transfer of DNA is a highly conserved mechanism (Becker and Meyer, 2012) and occurs within the tightly joint cell envelopes of mating cells, which are referred to as conjugation junctions (Lawley et al., 2002). In gram-negative bacteria, conjugation is a process in which a donor cell elaborates from the cell surface a protruding tube-like structure called the conjugative pilus (Snyder and Champness, 2003), with which it identifies and attaches itself to a recipient cell. Following attachment, the conjugative pilus retracts, bringing the donor and the recipient cell in close contact. After a series of steps, the mating bridge or pore is formed between the donor and the recipient cell. One strand of the transfer DNA in the donor cell is nicked and covalently attached to a relaxase protein. The two plasmid DNA strands then separate and the single-strand DNA-relaxase nucleoprotein complex is transferred to the recipient cell, where the transferred DNA is recircularised, replicated, and established. The two single stranded plasmid DNA molecules in both the donor and the recipient cell therefore serve as templates for the replication of complete double-stranded DNA molecules. After conjugation, the recipient cell has been converted to the donor cell status. A recipient cell that has received DNA as a result of conjugation is called a transconjugant (Arutyunov and Frost, 2013; Snyder and Champness, 2003).

2.4 ORGANISATION OF CONJUGATIVE PLASMID DNA

The genetic material encoded on a conjugative plasmid is typically organised in clusters of genes with related functions. The gene clusters related to plasmid maintenance inside the

host take up a considerable part of plasmid DNA. In addition to these genes, conjugative plasmids consist of clusters of genes responsible for plasmid propagation from one host to the other. Another part of plasmid DNA contains different genes that confer advantage to the host, like antibiotic resistance genes, auxiliary metabolic pathways, and virulence factors. Based on the dissimilar functions of the plasmid-encoded gene products, the plasmid DNA can be viewed as organised in four different modules:

- the replication and stability module;
- the establishment module;
- the propagation module;
- the adaptation module (Garcillan-Barcia et al., 2011).

The replication and stability module of the plasmid determines the absolute copy number of the plasmid and encodes proteins for plasmid replication. The replication and stability module also ensures plasmid stability in different hosts and growth conditions. The establishment module, which is not necessary for plasmid survival in laboratory conditions but is essential in natural environments, encodes a set of genes important when a plasmid enters a new genetic background. Examples of such genes are those coding for single-strand binding proteins, anti-restriction systems, primases and genes supressing the SOS response induced by transferred single-stranded DNA. The propagation module includes two clusters of genes, the first one encoding genes for DNA metabolism during conjugation and the second one encoding the type IV secretion system-like conjugation apparatus for establishing contact with the recipient cell. The adaptation module is the most variable of all and often includes integron gene cassettes encoding traits like antibiotic resistance, bacteriocin production, ecological interaction signals, and virulence factors (Garcillan-Barcia et al., 2011; Smillie et al., 2010).

2.5 F PLASMID

F plasmid is a 100 kb conjugative plasmid found in wild-type *E. coli* strain K-12 and was the first self-transmissible plasmid to be discovered (Frost et al., 1994; Snyder and Champness, 2003). It belongs to the incompatibility subgroup IncFI and together with other plasmids in this incompatibility group makes up an assembly of relatively large, narrow host-range plasmids typically found in the *Enterobacteriaceae* family (Frost et al., 1994; Mulec et al., 2002; Wong et al., 2012). The autonomous replication properties of the F plasmid are primarily conferred by the presence of the replication region RepFIA in the plasmid, which contains the replication origins (the *ori* sites). In addition to RepFIA, F carries a secondary replication region RepFIB, which is independently functional and can sustain plasmid replication in the absence of RepFIA. The third replication region present in F, RepFIC, includes an incomplete remnant of a replication system that is used by some other related plasmids. In F, the RepFIC replication region is interrupted by a γδ sequence (Tn*1000*) (Firth et al., 1996).

A hallmark of the F plasmid is its prototypical F conjugation system. Based on the presence of the F plasmid, two mating types in *E. coli* have been recognised:

- F^+, harbouring the F plasmid and behaving as genetic donors;
- F^-, lacking the F plasmid and behaving as genetic recipients (Adelberg and Pittard, 1965).

The F plasmid also harbours two IS*3* insertion sequences, one IS*2* insertion sequence and a γδ sequence, also known as transposon Tn*1000*, which are responsible for its ability both to integrate into the bacterial chromosome to form Hfr stains, and to excise together with a contiguous chromosomal segment to give rise to F′ plasmids (Deonier and Davidson, 1976; Helmuth and Achtman, 1975; Willetts and Skurray, 1980).

Bacterial conjugation as mediated by the F plasmid has been a topic of study ever since the discovery of the transfer of chromosomal material from one bacterial cell to another by direct cell-to-cell contact in 1946 by Lederberg and Tatum (Adelberg and Pittard, 1965; Lederberg and Tatum, 1946, op cit). The F conjugation system has been the most extensively studied bacterial conjugation system, and consequently detailed genetic and biochemical understanding of the conjugation process and its regulation mechanisms is mainly limited to it. This system is encoded not only by F plasmid itself, but also by a large number of other naturally occurring enterobacterial plasmids (Willetts and Skurray, 1980); examples include the prototypical F plasmid, and the R1, R100 and pED208 plasmids (Wong et al., 2012). F-like replicons and portions of F-like transfer system are found in the majority of large virulence plasmids documented in *E. coli* and *Salmonella* spp. Some F-like replicons possess the capability to transfer themselves to yet other enterobacterial genera; for instance, the F-derived plasmid pOX38 is capable of transfer to *Salmonella* spp., *Klebsiella* spp. and S*higella* spp. (Mulec et al., 2002; Wong et al., 2012). The F plasmid of *E.coli* remains a paradigm for understanding the mechanism of bacterial conjugation (Lawley et al., 2003).

2.5.1 The F transfer (*tra*) region

All the gene sequences required for the transfer of the F plasmid from the donor to the recipient cell are encoded within the plasmid's 33.3 kb transfer (*tra*) region. The *tra* region contains approximately 40 genes: *traA-traI*, *traI**, *traJ-traN*, *traP-traY*, *trbA-trbJ* and *finO* gene sequences in one strand and *finP* and *artA* sequences in the opposite strand, and is organised into three tightly regulated operons. Two monocistronic operons encoding the *traM* and *traJ* are transcribed from promoters P_{traM} and P_{traJ}, respectively. The third polycistronic operon is transcribed from the P_{traY} promoter (Figure 1) and encodes the genes involved in the F pilus synthesis and DNA transfer from the donor to the recipient cell (Frost et al., 1994).

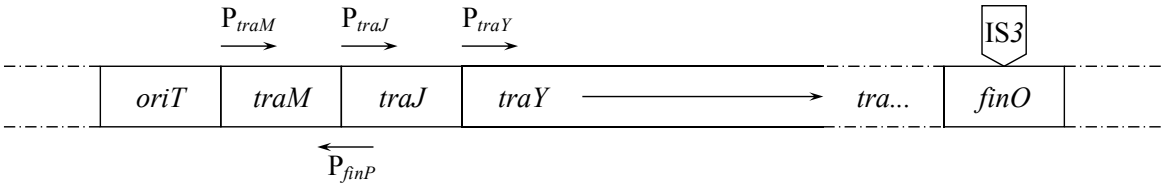

Figure 1: Organisation of the F plasmid transfer (*tra*) region (adapted from Frost et al., 1994)
The *traM* and *traJ* operons are transcribed from promoters P_{traM} and P_{traJ}, respectively. The polycistronic *tra* operon starting with *traY* is transcribed from promoter P_{traY}. The *finO* gene is interrupted by an IS*3* element insertion. The *finP* antisense RNA is transcribed from promoter P_{finP} within and in opposite orientation to the *traJ* gene.
Slika 1: Organizacija regije *tra* pri plazmidu F (povzeto po Frost in sod., 1994)
Operona *traM* in *traJ* se prepisujeta s promotorjev P_{traM} in P_{traJ}. Policistronski operon *tra*, ki se začne z genom *traY*, se prepisuje s promotorja P_{traY}. Gen *finO* je prekinjen z insercijsko sekvenco IS*3*. Protismiselna RNA *finP* se prepisuje s promotorja P_{finP}, ki se nahaja znotraj in v nasprotni orientaciji glede na gen *traJ*.

In addition to the *tra* region genes, a *cis*-acting *oriT* site, located immediately upstream of the *traM* operon, is required for DNA transfer (Snyder and Champness, 2003). The *oriT* sequence (Figure 1) of the F plasmid is arbitrarily defined as the region between the *Bgl*II restriction site at nt 1 and the beginning of the *traM* gene at nt 463 (Frost et al., 1994). It contains the site where nicking occurs and transfer of single stranded DNA into the recipient cell is initiated (Frost et al., 1994; Ihler and Rupp, 1969). The *oriT* region can be divided into two sections, one of which is important for nicking, and the second for DNA transfer (Frost et al., 1994; Furuya and Komano, 1991).

2.5.2 Regulation of the F transfer (*tra*) region

Transcription of the *tra* region is principally driven by a single promoter P_{traY}, which is regulated by a complex array of plasmid-encoded as well as host-imposed factors. Regulation of P_{traY} largely hinges on the plasmid-encoded transcription factor TraJ, and the host-encoded transcription factor ArcA. TraJ is itself subject to a complex regulatory network involving transcriptional, post-transcriptional, and post-translational regulatory mechanisms (Wong et al., 2012).

Transcription of the *tra* region is mainly regulated by a regulatory array of four *tra*-encoded elements: the *traJ*, *finO* and *finP* gene products and the P_{traY} promoter. The main regulator of conjugation is a 27 kDa protein TraJ, which is required for the initiation of high levels of transcription from the P_{traY} promoter. Its activity is regulated by the fertility inhibition system FinOP, a two-component inhibition system for bacterial conjugation mediated by the F plasmid, encoded by the genes *finO* and *finP*. *finP* encodes a 79 nt *finP* antisense RNA composed of two stem loops, which is transcribed from a promoter P_{finP} (Figure 1) within and in opposite orientation to the *traJ* gene (Jerome et al., 1999). *finP* antisense RNA is complementary to the 5′ untranslated leader of the *traJ* mRNA, and upon binding *traJ*

mRNA, the newly-formed double stranded RNA is degraded by RNase III (Starčič Erjavec, 2003). Regulation of *traJ* mRNA is critically dependent on a 21.2 kDa plasmid-encoded protein FinO, which acts as an RNA chaperone and stabilises *finP* antisense RNA by protecting it from degradation by RNase E. This way, FinO extends the *finP* antisense RNA half-life and accelerates duplex formation between *finP* and *traJ* mRNA. *finP* antisense RNA thus negatively regulates TraJ expression only in the presence of FinO. Once the FinOP complex is bound to the *traJ* mRNA, translation of the TraJ regulator is repressed (Jerome et al., 1999). In most naturally occurring F-like conjugation systems, the level of repression of the *tra* region is highly dependent on the concentrations of FinO and *finP* gene products. Such plasmids transfer with a high efficiency for only a short period after entry into recipient cells; afterwards, expression of the *tra* genes is intermittent and plasmid transfer occurs only sporadically (Snyder and Champness, 2003). In the case of F plasmid, however, the *finO* gene is interrupted by an IS*3* element insertion (Figure 1), leading to constitutive expression of *traJ*, derepression of the *tra* region expression and constitutive pilus synthesis (Frost et al., 1994).

Apart from the FinOP fertility inhibition system, *tra* region is also significantly regulated by additional regulatory elements encoded within the *tra* region itself. In addition, some host-encoded proteins, which are sensitive to cellular conditions like nutrient availability and stress, also play regulatory roles in F plasmid-mediated conjugation (Camacho et al., 2005; Camacho and Casadesus, 2005; Frost et al., 1994; Silverman and Sholl, 1996; Starčič et al., 2003; Starčič Erjavec et al., 2003b; Taki et al., 1998; Will et al., 2004; Wong et al., 2012). Transcription from P_{traY} promoter is therefore very complex, with levels of control imposed both by the host as well as by the *tra* gene products encoded on the conjugative plasmid itself.

2.6 CONJUGATION MACHINERY OF THE F-LIKE PLASMIDS

The central two systems playing essential roles in conjugative transfer of DNA from the donor to the recipient cell are:

- the DNA-processing complex called the relaxosome (also termed in literature as Dtr (DNA transfer and replication) complex or MOB (mobility) complex), which assembles on the plasmid's origin of transfer (*oriT*) and replicates the DNA by a mechanism resembling rolling-circle replication;
- a subtype of type IV secretion system, called the transferosome (also termed in literature as Mpf (mating pair formation) complex), through which the DNA is transferred (Arutyunov and Frost, 2013; Lawley et al., 2003; Smillie et al., 2010).

Conjugation can therefore be visualised as a DNA rolling-circle replication system linked to a type IV secretion system (T4SS) (Llosa et al., 2002). An additional component, a coupling protein, acts as the link between the relaxosome and the transferosome (de la Cruz et al., 2010).

Along with these two essential systems, several important related processes take place during conjugal transfer of DNA:

- mating pair stabilisation (Mps) allows certain transfer systems to function in liquid as efficiently as on solid surfaces;
- surface exclusion (Sfx) prevents donor-donor contacts;
- entry exclusion (Eex) prevents nucleoprotein complex transport between donors (Arutyunov and Frost, 2013).

2.6.1 The relaxosome

The relaxosome is involved in preparing the plasmid DNA for transfer and is composed of several protein components including the plasmid encoded TraI, TraY and TraM, as well as the host factor IHF. Its central part is the plasmid-encoded relaxase TraI – a DNA endonuclease (DNA phosphodiesterase) that specifically cleaves the DNA at the *nic* sequence within *oriT* and initiates the DNA transfer process (Silverman and Sholl, 1996; Taki et al., 1998; Wong et al., 2012). In general, interactions between different components of the relaxosome selectively occur between proteins of the same plasmid (Lawley et al., 2003).

2.6.2 The transferosome – a type IV secretion system

Type IV secretion systems (T4SS) are large cell envelope-spanning protein complexes that form a pore or channel through which macromolecules such as proteins or nucleoprotein complexes can be translocated using nucleosyl triphosphate (NTP) hydrolysis as a transport driving force (Gomis-Ruth et al., 2004; Lawley et al., 2003; Wallden et al., 2010). They are the most versatile and diverse family of secretion systems and have been found in both Gram-negative and Gram-positive bacteria as well as in some members of *Archaea* (Wallden et al., 2010). According to their function, T4SS are divided into three subgroups:

- conjugation systems;
- effector protein translocation systems;
- contact independent type IV secretion systems.

Conjugation systems are the largest and most widely distributed of the T4SS subtypes. These systems are responsible for plasmid conjugation in Gram-negative and Gram-positive bacteria, as well as for transfer of integrative conjugative elements. The *Agrobacterium tumefaciens* Ti plasmid conjugation system, which transfers DNA from a bacterial to a plant cell, also belongs to this first subgroup. The second subgroup consists of effector protein translocation systems, dedicated to translocating effector proteins directly to the cytosol of eukaryotic cells. These systems are important to the infection strategies of many bacteria, including the human pathogens *Helicobacter pylori*, *Legionella pneumophila*, *Bartonella* spp., and *Brucella* spp. The third subgroup consists of contact independent type IV secretion systems, which exchange macromolecules between the cell and its external environment.

This is the only subgroup of T4SS that is not pilus-based. These systems are used by *Neisseria gonorrhoeae* to secrete DNA into the extracellular environment and by *Helicobacter pylori* to mediate DNA uptake from the environment, thus playing an important role during natural transformation (Juhas et al., 2008; Lawley et al., 2003; Wallden et al., 2010; Zechner et al., 2012).

2.6.2.1 The F plasmid type IV secretion system

The F plasmid conjugation system encodes 8 out of 10 highly conserved core genes of T4SS: *traA* (pilin), *traL*, *traE*, *traK*, *traB*, *traV*, *traC*, and *traG*. The products of these genes also require auxiliary gene products of *traF*, *traG*, *traH*, *traN*, *traZ*, *traQ* and *trbC*, which are conserved throughout F-type systems and serve as hallmarks of this family of T4SS. These genes are essential for transfer and appear to be involved in pilus retraction and mating pair stabilisation, which are critical factors for efficient F conjugation in liquid media (Lawley et al., 2003). Additional essential gene products of F conjugation system are the TraD coupling protein, TraI bifunctional relaxase/helicase, TraM and TraY (Lawley et al., 2003).

2.6.2.2 Functional structures of type IV secretion systems

Most T4SS encompass three functional structures:

- the core complex – a transport channel that conducts substrates across the bacterial cell envelope;
- cell surface pili or adhesins that mediate contact between cells;
- a type IV coupling protein (T4CP) that acts as substrate receptor at the cytoplasmic entrance of the secretion channel (Zechner et al., 2012).

2.6.2.2.1 The core complex

The T4SS core complex is a bacterial cell envelope-spanning channel composed of conserved components that form a common central structure for secretion of proteins and nucleoprotein complexes. It is a highly conserved structure among all T4SS, while the peripheral components involved in specific interaction with substrates and target cells differ between different T4SS (Zechner et al., 2012).

2.6.2.2.2 The F-pilus

The structure and function of the F-pilus are not yet well understood. F-pilus is an up to 20 μm long (Wang et al., 2009) cylindrical filament with an outside diameter of 8 nm and a 2 nm central hydrophilic lumen (Silverman, 1997), extending form the surface of donor cells as a tube-like structure (Zechner et al., 2012). F-pilus is assembled from monomers of pilin, which is derived from the 121 aa 13.2 kDa *traA* gene product (Frost et al., 1984; Frost et al.,

1994) by a sequence of processing steps, after which the F-pilin subunits accumulate in the inner membrane. The F-pilus is assembled on the bacterial surface by addition of pilin subunits to the pilus base in a helical array. After contact with the recipient cell, the pilus retracts by depolymerisation, whereby the pilin subunits return to the cell membrane (Lawley et al., 2003).

It is now recognised that the lumen within the pilus is large enough to accommodate the T-DNA-relaxase nucleoprotein complex. It has been shown several times that DNA does travel through an extended pilus, although pilus retraction and tight mating pair formation are most probably preferred for high efficiency mating (Babić et al., 2008; Harrington and Rogerson, 1990; Lawley et al., 2002).

2.6.2.2.3 The type IV coupling protein

The type IV coupling proteins (T4CP) are essential for active secretion. T4CP are multimeric integral inner-membrane proteins that present the link between proteins or nucleoprotein complexes to be transported and the T4SS transport pore. They provide specificity for transport by specifically recognising the relaxase as well as any other proteins to be transferred to the recipient cell. T4CP also communicate the signal from the T4SS conjugative pore, once it has established a connection with the recipient cell, to the relaxosome system by activating the relaxase to nick the plasmid DNA and initiate the transport process. Therefore, the conjugative DNA metabolism does not take place until the conjugative pore between the donor and the recipient cell has not been established. In the F plasmid conjugation system, the T4CP is encoded by the *traD* gene (Gomis-Ruth et al., 2004; Snyder and Champness, 2003; Zechner et al., 2012).

2.6.3 Mating pair stabilisation (Mps)

Mating pair stabilisation system, in F-like conjugation systems conferred by proteins TraN and TraG, allows for equally efficient mating in liquid and on solid media. The products of genes *traN* and *traG* appear to have an important role in building a functional conjugative pore. It has been suggested that TraN interacts with the complex outer membrane protein OmpA (Smith et al., 2007) of the recipient to stabilise the mating pair. TraG is a 102.5 kDa (Frost et al., 1994) protein residing in the cell's inner membrane and protruding into the periplasm, whose exact role in mating pair stabilisation has not yet been entirely clarified (Achtman et al., 1977; Arutyunov and Frost, 2013; Audette et al., 2007; Firth and Skurray, 1992).

2.6.4 Surface (Sfx) and entry (Eex) exclusion

A counterpoint to mating pair stabilisation is provided by two independent processes, namely surface and entry exclusion, involved in the prevention of redundant DNA transfer between donor cells. Surface and entry exclusion refer to the inability of donor cells to mate with other donor cells efficiently. In F plasmid, these traits are conferred by two independently acting proteins, TraT and TraS, encoded in the *traST* locus. TraT and TraS inhibit mating pair formation and DNA transport, respectively (Arutyunov and Frost, 2013; Audette et al., 2007).

2.6.4.1 TraT and surface exclusion (Sfx)

TraT is a 25 kDa outer membrane lipoprotein that decreases mating between donor (F^+) cells by 10- to 50-fold. It has been suggested that TraT acts in the cell's outer membrane to inhibit the formation of stable mating aggregates between donor cells (Achtman et al., 1977; Arutyunov and Frost, 2013).

2.6.4.2 TraS and entry exclusion (Eex)

TraS is an inner membrane protein expressed in much lower quantities than TraT but exhibiting a much greater effect, decreasing mating between donor cells by 500- to 1000-fold. It has been determined that TraG (otherwise part of the Mps discussed in chapter 2.6.3) of the donor cell interacts with TraS of the F^+ recipient cell to prevent DNA synthesis in the donor cell and transfer to the F^+ recipient (Arutyunov and Frost, 2013; Audette et al., 2007; Kingsman and Willetts, 1978).

2.7 GRAM-NEGATIVE CELL SURFACE STRUCTURES: LIPOPOLYSACCHARIDE AND EXTRACELLULAR POLYSACCHARIDES

The Gram-negative cell envelope comprises several structurally and functionally distinct layers. From cytoplasm towards the cell surface, these layers are the cytoplasmic membrane, the periplasmic peptidoglycan, the outer membrane, and frequently additional layers of extracellular polysaccharides like capsules and slime.

Lipopolysaccharide (LPS) is found in the outer membrane of most Gram-negative bacteria. It comprises three basic constituents: the lipid A, the core oligosaccharide region, and the variable O-antigen, which are covalently linked into one large molecule (Caroff and Karibian, 2003) (Figure 2).

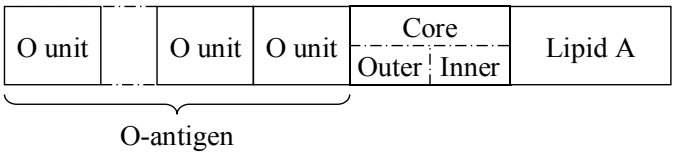

Figure 2: Structural units of the lipopolysaccharide from the outer membrane of Gram-negative bacteria (adapted from Wilkinson et al., 1996)
Slika 2: Strukturne enote lipopolisaharida zunanje membrane Gram-negativnih bakterij (povzeto po Wilkinson in sod., 1996)

Lipid A is the hydrophobic lipid component that anchors the LPS in the outer leaflet of the highly asymmetric outer bacterial membrane and comprises four to seven fatty acid chains bound to a headgroup of two phosphorylated *N*-acetylglucosamines (Alexander and Rietschel, 2001; Reeves et al., 1996). The core oligosaccharide region is covalently attached to lipid A and is formally divided to inner core, generally consisting of 2-keto-3-deoxyoctonoic acid (Kdo) and heptose saccharides, and outer core of hexoses and hexosamines (Le Brun et al., 2013; Wilkinson, 1996). The O-antigen is a highly polymorphic polysaccharide chain of repeating oligosaccharide units (O units). Each O unit comprises one to eight saccharide units, and there can be up to 50 identical O units chained in an O-antigen. The O-antigen is covalently attached to the core region and extends from the bacterial outer membrane out into the environment. While lipid A and the Kdo-containing inner core represent the structurally most conserved region of the LPS, and the outer core oligosaccharide region exhibits only limited variability, O-antigens differ markedly between bacterial strains and define the serotypes of *E. coli* (Caroff and Karibian, 2003; Reeves et al., 1996; Rietschel et al., 1994).

LPS consisting only of the lipid A and the core region is termed rough LPS (R-LPS), while LPS comprising also the O-antigen is termed smooth LPS (S-LPS). R-LPS can be labelled from Ra to Re, relating to the saccharides in the core region where R-LPS terminates, with Ra-LPS terminating at the core saccharides farthest from the lipid A and Re-LPS terminating at the core saccharides closest to the lipid A. Re-LPS consists only of the lipid A and Kdo saccharides and is the minimal LPS required for growth of *E. coli* (Klein et al., 2009; Le Brun et al., 2013).

Typical laboratory strains carry different mutations or deletions preventing the O-antigen addition to the oligosaccharide core and consequently exhibit R-LPS (Anthony et al., 1994). On the other hand, most *E. coli* strains with S-LPS display a basic O-antigen chain length of 10 to 18 O units. However, there is considerable variation, and a number of cases where chain length varies among strains with the same O-antigen have been reported. Different schemes have been proposed for the subdivision of *E. coli* LPSs, according to the predominant O-antigen chain length of each strain's LPS. Hereby, Franco and colleagues have proposed a subdivision of the *E. coli* LPSs into three groups having short (7 to 16),

intermediate (10 to 18), and long (16 to 25) O-antigen chains (Achtman et al., 1986; Brussow and Sidoti, 1992; Franco et al., 1998; Kusecek et al., 1984; Porat et al., 1987).

In wild-type strains displaying S-LPS, the distribution of O-antigen chain lengths is usually clearly bimodal. In these strains, semi-rough LPS (SR-LPS), which contains a single O unit, is very abundant, while molecules containing two, three, and four O units are progressively less abundant up to molecules with about 15 O units, which are normally not present in detectable levels. Above that, the abundance increases again, reaches a plateau of molecules with a strain-specific number of O units, and above this number rapidly declines. Apart from bimodal distributions of O-antigen chain lengths, trimodal and other less common distributions have been observed as well (Aucken and Pitt, 1993; Goldman and Leive, 1980; Goldman and Hunt, 1990; Grossman et al., 1987; Palva and Makela, 1980; Schnaitman and Klena, 1993).

During LPS biosynthesis, O units are polymerised into blocks of varying length and then added to the core as a complete O-antigen. The O-antigen can be transferred to the lipid A-core oligosaccharide moiety at any degree of polymerisation, but with a preference for a certain, strain specific O-antigen chain length. The O-antigen chain length in each bacterial strain is regulated by polysaccharide co-polymerases, which control and determine the degree of O-antigen polymerisation (Caroff and Karibian, 2003; Franco et al., 1998; Goldman and Hunt, 1990; Grossman et al., 1987; Guo et al., 2005; Kalynych et al., 2012; Osawa et al., 2013; Schnaitman and Klena, 1993).

Examples have been reported where the O-antigen is, in addition to being covalently bound to the lipid A-core oligosaccharide moiety, present also in an LPS-unlinked capsular form, termed “O-antigen capsule”, covering the bacterial surface (Goldman et al., 1982; Whitfield and Roberts, 1999). Apart from different forms of LPS, bacterial polysaccharides include also extracellular polysaccharide (EPS). Like O-antigen, EPS is made of repeating units. It can be either released from the cell into the environment as a slime or remain attached to the cell to form a capsule. The distinction between LPS and different forms of EPS is not always clear (Reeves et al., 1996). Capsular polysaccharides represent a major surface K-antigen. Some K-antigens can be expressed on the cell surface of *E. coli* in two different forms: the first form is termed K_{LPS} and comprises low-molecular-weight K-antigenic oligosaccharide of one or a few repeated K units linked to a lipid A core, while the second form comprises high-molecular-weight K-antigen of many repeated K units in an LPS-unlinked capsular form. It is worth noting that not all capsules consist of K-antigen (MacLachlan et al., 1993; Whitfield and Roberts, 1999).

2.8 COLICINS

Colicins are proteins produced by some *E. coli* strains that are lethal for closely related *E. coli* strains. They are a subclass of bacteriocins, toxic proteins produced by a given strain of bacteria and active against related species. *E. coli* is known to produce two types of bacteriocins, classified by their molecular weight into colicins (25 to 80 kDa) and microcins (< 10 kDa) (Budič et al., 2011).

Colicins are produced by *E. coli* strains harbouring colicinogenic plasmids, designated pCol. Such strains are called colicinogenic strains and are widely distributed in nature, with particular abundance in the gut microbiota of animals. Colicinogenic strains usually contain many different plasmids, among them only one specific colicinogenic. Two classes of colicinogenic plasmids pCol have been described:

- type I colicinogenic plasmids are small plasmids of 6 to 10 kb, present in about 20 copies per cell, that can be mobilised in the presence of another conjugative plasmid;
- type II colicinogenic plasmids are large conjugative plasmids of around 40 kb, present in only one copy per cell, that can carry numerous other genes besides the genes for colicin activity.

While type I colicinogenic plasmids encode one colicin, type II colicinogenic plasmids can encode two colicins and transmit them to other strains. Various plasmids can encode a similar colicin (Cascales et al., 2007; Hardy et al., 1973).

The activity spectrum of colicins is confined to closely related organisms such as *Escherichia*, *Salmonella* and *Shigella* strains (Chak et al., 1991). The narrow target range of colicins has been proven to be due to the presence of specific receptors on the surface of sensitive strains, to which colicins bind before exhibiting their lethal action on the cell. The colicin receptors have been shown to be outer membrane proteins that allow the entry of specific nutrients such as vitamins, siderophores and nucleosides into the cell. Mutation of these receptors can lead to the loss of sensitivity to the corresponding colicin (Cascales et al., 2007; Chai and Foulds, 1977; Chai and Foulds, 1979; Di Masi et al., 1973).

Colicins act on target cell in three steps. After binding to a specific receptor in the outer membrane of the target cell, the colicin is translocated through the cell envelope to its target location. Based on the mechanism of translocation, colicins are classified as either group A colicins, which utilise the Tol pathway, or group B colicins, which utilise the TonB pathway. In addition, group A colicins are mainly encoded on group I plasmids and are released from the producing cell to the medium, while group B colicins are mainly encoded on group II plasmids and are not secreted into the environment. According to their target location and mechanism of action, colicins can be classified as either ionophoric colicins, whose target is the cell's inner membrane where they form a pore to allow ion leakage, or nuclease colicins,

whose target is the cytoplasm where they act by degrading the target cell's nucleic acid. A unique colicin M acts by inhibiting the peptidoglycan synthesis (Cascales et al., 2007; El Ghachi et al., 2006).

Colicins do not act on their own producing bacteria since each bacterium produces one or more specific colicin inhibitors called the immunity proteins. Immunity proteins of ionophoric colicins are located in the inner membrane of the producing cells (Weaver et al., 1981), where they block the colicin as it reaches the target after entry into the cell. In contrast, the immunity proteins of nuclease colicins form a complex with the cognate colicin in the producing cell, neutralising its catalytic activity. The colicin-immunity protein complex is subsequently released from the producing cell and dissociates only during colicin action on the sensitive cell (Cascales et al., 2007; Duche et al., 2006).

All colicin molecules are organised into three domains, each corresponding to one of the three steps of colicin action. The N-terminal domain is involved in translocation through the cell envelope, the central domain is involved in binding to the colicin receptor, and the C-terminal domain is involved in colicin activity. Ionophoric colicins exist as monomers, whereas nuclease colicins are heterodimers of colicin and its immunity protein (Cascales et al., 2007).

2.8.1 Organisation and regulation of colicin genes

The colicin operons are, depending on the group of the colicin and its mechanism of action, comprised of one to three genes. In all colicin operons, the first gene is the gene encoding colicin, called *cxa*, for colicin X activity. In the operons encoding a nuclease colicin, the gene encoding the immunity protein, designated either *cxi*, for colicin X immunity, or *immX*, is located downstream from the colicin activity gene. It is regulated by two promoters: the LexA promoter of the whole colicin operon and its own constitutive promoter, located within the structural gene of the nuclease colicin that allows a constant production of the immunity protein in order to ensure that there is never free colicin present, which would kill the producing cell. In operons encoding an ionophoric colicin, there is no immunity protein; it is instead encoded in the opposite DNA strand of the intergenic space between the colicin activity and colicin lysis genes and is transcribed from its own constitutive promoter. The last gene of colicin operons is the gene encoding the colicin lysis protein, designated *cxl*, for colicin X lysis protein, whose product allows colicin release into the medium and is responsible for cell death after induction. It is mainly present colicins of group A (Akutsu et al., 1989; Cascales et al., 2007; Chak et al., 1991; Curtis et al., 1989; Jakes and Zinder, 1984).

Transcription of the colicin operons is under normal conditions highly repressed by the LexA protein, a repressor of SOS response genes. Agents able to trigger the SOS response and therefore to induce colicin production are numerous and versatile, encompassing

UV radiation, chemical substances, physical agents and stress conditions. Although LexA is a common repressor of colicin transcription, other activators or repressors play a role in modulating the colicin operon expression (Cascales et al., 2007). Colicin synthesis has thus been shown to be stimulated in various cases by stringent response, catabolite repression, the stationary phase of growth, anaerobiosis and other factors (Cascales et al., 2007; Ebina and Nakazawa, 1983; Eraso and Weinstock, 1992; Eraso et al., 1996; Lotz, 1978; Pugsley, 1984; Salles et al., 1987). Regulation of the colicin operons is complex and varies form one operon to the other. However, colicins are primarily under control of SOS response (Cascales et al., 2007).

2.8.2 Colicin E7

Colicin E7 is a 61 kDa nuclease colicin with non-specific DNase-type activity, belonging to group A colicins. It is encoded on a 6.2 kb plasmid ColE7, together with the 9.9 kDa E7 immunity protein (ImmE7) and the 4.9 kDa E7 lysis protein in a single operon (Chak et al., 1991). To enter the target cells, it parasitizes the high-affinity cobalamin (vitamin B_{12}) import system of *E. coli*. An essential component of this system is a 66 kDa protein BtuB (Heller and Kadner, 1985), a minor component of the *E. coli* outer membrane present in about 200 copies per cell, to which colicin E7 binds prior to entry into the target cell (James et al., 1996; Lazdunski et al., 1998).

2.8.2.1 Organisation and regulation of the colicin E7 operon

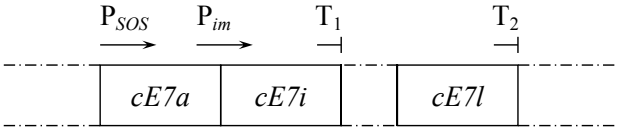

Figure 3: Organisation of the colicin E7 operon (adapted from Cascales et al., 2007)
P_{SOS} = SOS promoter; P_{im} = constitutive promoter of the E7 immunity protein ImmE7; T_1 = terminator 1; T_2 = terminator 2.
Slika 3: Organizacija operona kolicina E7 (povzeto po Cascales in sod., 2007)
P_{SOS} = promotor SOS; P_{im} = konstitutivni promotor proteina imunosti na kolicin E7 ImmE7; T_1 = terminator 1; T_2 = terminator 2.

Transcription from the SOS promoter of the colicin E7 operon (represented in Figure 3), as well as of other group A nuclease colicin operons, results in the formation of two mRNA transcripts due to presence of two terminators of transcription. The major mRNA corresponds to the colicin gene *cE7a* and its immunity gene *cE7i*. Colicin and immunity genes are transcribed and translated co-ordinately, and their products associate into a dimeric complex immediately after translation to inhibit the colicin's toxic enzymatic activity and protect the producing cell. An additional constitutive promoter is present upstream of the immunity gene within the *cE7a* gene, allowing a higher production of the immunity gene as

compared to the colicin itself. The minor mRNA is the largest one and corresponds to a transcript of the entire operon comprised of the colicin, the immunity protein, and the lysis protein genes. Thus, the lysis gene is transcribed at lower levels than the colicin gene (Cascales et al., 2007). Autoregulation of the translational expression of the colicin E7 operon has been suggested through cleavage of its mRNA transcript by its immunity protein ImmE7, indicating specific RNase activity of ImmE7 (Hsieh et al., 1997).

2.9 PROBIOTICS

Probiotics are defined as living microorganisms that, when administered in adequate amounts, exert health benefits on the host beyond inherent basic nutrition (FAO/WHO, 2006; Guarner and Schaafsma, 1998). They are used as dietary supplements in a wide spectrum of applications, especially for treatment of chronic inflammatory bowel diseases such as ulcerative colitis, irritable bowel syndrome (Jonkers et al., 2012), and infectious diseases including viral, bacterial, or antibiotic associated diarrhoea, among others (Saad et al., 2013). The mechanisms of action of probiotics consist principally of:

- competition with pathogens for sites of adhesion;
- innate immune system stimulation;
- competition for nutrients;
- production of antimicrobial compounds and therefore pathogen antagonism;
- protection of intestinal barrier integrity;
- regulation of anti-inflammatory cytokine and inhibition of pro-inflammatory cytokine production (Saad et al., 2013).

Most microorganisms recognised to date as probiotics are Gram-positive, with *Lactobacillus* and *Bifidobacterium* being the main species used (Marco et al., 2006). However, some Gram-negative bacteria are also used as probiotics, the best example being *E. coli* Nissle 1917 (EcN) (Gordon, 2009; Jacobi et al., 2012).

2.9.1 *E. coli* Nissle 1917 (EcN)

E. coli Nissle 1917 was isolated by Alfred Nissle in 1917 from the faeces of a soldier during the First World War, who in contrast to his comrades, did not develop infectious diarrhoea during a *Shigella* outbreak (Behnsen et al., 2013). Since then, the probiotic effect and biosafety of *E. coli* Nissle 1917 have been extensively shown in numerous trials and underlined by its long medical history as a microbial remedy. *E. coli* Nissle 1917 has been used as the active component of the pharmaceutical preparation Mutaflor®, a microbial drug licensed for use in human medicine in Germany and some other European countries (Sonnenborn and Schulze, 2009). Oral administration of *E. coli* Nissle 1917 has been shown to be highly protective against infection with *Salmonella* spp., *Shigella* spp., and others (Sonnenborn and Schulze, 2009). Moreover, recombinant strains of *E. coli* Nissle 1917 have

been engineered to express and secrete human defensins as an alternative approach to treat patients with Crohn's disease (Seo et al., 2012), and to express and secrete human epidermal growth factor to promote wound healing on human intestinal epithelial cells (Choi et al., 2012).

E. coli Nissle 1917 belongs to serotype O6:K5:H1, exhibits semi-rough LPS phenotype, and is serum sensitive (Grozdanov et al., 2002). Its complete 5023 kb genome sequence is known (Cress et al., 2013). In addition to being capable of modulating the host immune response, *E. coli* Nissle 1917 exhibits several fitness factors that contribute to its colonisation efficiency and survival within the host, among them production of the microcins MccH47 and MccM (Behnsen et al., 2013; Grozdanov et al., 2002; Grozdanov et al., 2004; Patzer et al., 2003; Trebichavsky et al., 2010; Wehkamp et al., 2004).

2.10 BACTERIAL CONJUGATION-BASED TECHNOLOGIES

Bacterial conjugation-based technologies are an alternative form of application of antimicrobial agents to the target bacterial cells. They exploit conjugative plasmids to deliver plasmid-encoded antimicrobial agents into target bacterial cells. The main advantage of such technologies is their ability to circumvent the barriers caused by the impermeability of bacterial cell envelope to most antimicrobial compounds. Their potential is based on a universal property of conjugative systems: plasmid-encoded information, even information encoding self-destruction, is expressed upon conjugal transfer of plasmid DNA to a recipient cell. Generally, two different types of plasmid-encoded destructive elements have been employed to act against the recipient cells:

- mutations in plasmid copy-control mechanisms;
- antimicrobial agents.

Mutations in plasmid copy-control mechanisms disrupt the function of the repressors controlling plasmid copy numbers and cause over-replication of plasmids, producing a copy-up phenotype. In extreme cases, such mutations lead to complete loss of plasmid copy-control mechanisms and result in runaway plasmid replication. Plasmid over-replication takes up all the host cell resources and finally leads to cell death. As a second approach, genes encoding different antimicrobial agents, such as bacteriocins, may be present in a plasmid. The plasmid donor cell may be protected from antimicrobial agents either by chromosomally encoded antitoxins, which neutralise the toxic effect of the plasmid-encoded antimicrobial agent. Alternatively, the donor may be rendered insensitive to a killer plasmid by using a tightly regulated promoter-operator system in which the expression of a lethal gene is prevented by a repressor made only in the donor cell (Filutowicz et al., 2008).

2.10.1 *E. coli* Nissle 1917 pOX38a – strain ŽP

The genetically modified probiotic strain *E. coli* Nissle 1917 pOX38a with the plasmid pOX38-encoded colicin E7 synthesis gene *cE7a* and the chromosomally encoded colicin E7 immunity gene *cE7i* is an example of a bacterial conjugation-based technology. It employs the colicin E7 as an antimicrobial agent and the chromosomally encoded colicin E7 immunity gene as the antotoxin that protects the donor cell from the colicin. The strain *E. coli* Nissle 1917 pOX38a – strain ŽP has been engineered in the Molecular Genetics and Microbiology Research Group at the Biotechnical faculty of the University of Ljubljana in 2012.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Bacterial strains

Table 1: *E. coli* strains used in this study
Preglednica 1: Sevi *E. coli* uporabljeni v tej raziskavi

Strain	Selected genotype and phenotype features	Source or reference
MC4100	*ara*D139 Δ(*argF-lac*)U169 *rps*L150 *rel*A1 *flb*B5301 *pts*F25 *deo*C1	Casadaban, 1976
RU4405	MM294::Tn*1731* Tc^r *thi end*A *hsd*R	Ubben and Schmitt, 1986
DH5α pOX38:Cm	Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF)*U169 *endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1* pOX38:Cm	M. Starčič Erjavec
DH5α pUC19i	Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF)*U169 *endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1* pUC19 *immE7*	Petkovšek, 2012
HB101 pRK100	*hsdR hsdM recA13 supE44 leuB6 lacZ proA2* pRK100 Ap^r, Tc^r	M. Starčič Erjavec
J5 pOX38:Kn	pOX38:Kn	M. Starčič Erjavec
MS252 pGZ1	pGZ	M. Starčič Erjavec
MS253 pGZ2	pGZ *traJ*	M. Starčič Erjavec
S17-1 λpir pAG408i	*recA thi pro hsdR-M*$^+$ *RP4-2(Km::Tn7,Tc::Mu-1) λpir* Tp^r pAG408 *immE7*	Petkovšek, 2012
N4i	Nissle 1917 (EcN) *immE7* Gm^r	Petkovšek, 2012
N4i pOX38a (ŽP)	Nissle 1917 (EcN) *immE7* Gm^r pOX38:Cm *colE7*	Petkovšek, 2012
SE15	Sm^r	E. Oswald
TA131	$F^–$; Cm^s Cip^r	M. Starčič Erjavec
DL94	O21:H–; $F^–$; Cm^s Tc^r	M. Starčič Erjavec

3.1.1.1 Clinical *E. coli* isolates – TA collection

Clinical *E. coli* isolates in TA collection were isolated from patients with skin and soft tissue infections (SSTI) at the Institute of Microbiology and Immunology of the Medical Faculty of the University of Ljubljana. This collection includes 102 biochemically identified *E. coli* strains.

3.1.1.2 Clinical *E. coli* isolates – DL collection

Clinical *E. coli* isolates in DL collection were isolated from patients with urinary tract infections (UTI) at the Institute of Microbiology and Immunology of the Medical Faculty of the University of Ljubljana. This collection includes 110 biochemically identified *E. coli* strains.

3.1.2 Plasmids

3.1.2.1 pOX38

pOX38 is a conjugative 55 kb derivative of F plasmid, obtained through restriction of the F plasmid with *Hind*III. It includes the full *tra* operon and the RepFIA replication region. In this study, the following pOX38 derivatives were used:

- pOX38:Cm conferring chloramphenicol resistance;
- pOX38a carrying the *colE7* gene and conferring chloramphenicol resistance;
- pOX38:Kn conferring kanamycin resistance.

3.1.2.2 pRK100

pRK100 is a 145 kb conjugative plasmid. It includes a full F-like plasmid *tra* operon and the RepFIB and RepFIIA replication regions. It confers ampicillin and tetracycline resistance.

3.1.2.3 pUC19i

pUC19 is a 2686 bp non-conjugative ampicillin resistance conferring plasmid, commonly used as a cloning vector. In this study, the *immE7* gene-carrying derivative designated pUC19i was used.

3.1.2.4 pGZ

pGZ is a 4500 bp non-conjugative plasmid conferring chloramphenicol resistance. In this study, the following pGZ derivatives were used:

- pGZ1 without the cloned insert;
- pGZ2 carrying the traJ gene.

3.1.2.5 pAG408i

A 5700 bp suicidal plasmid lacking the *pir* gene, which disables survival of the plasmid outside the host strain expressing the Pir protein. pAG408i plasmid carries the IS*50* transposition region, inside which the *immE7* gene and the gentamycin resistance marker are cloned. The Tn*5* transposase and the ampicillin resistance marker are located outside the IS*50* transposition region.

3.1.3 Culture media

3.1.3.1 Liquid LB medium

Liquid LB medium was prepared with 25 g of LB powder dissolved in 1000 ml of dH_2O. Medium was sterilised by autoclaving at 121 °C for 15 min. If desired, medium was supplemented with stock solutions of antibiotics to final concentrations (Table 2) once cooled to 55 °C.

Table 2: Final concentrations of antibiotics in media
Preglednica 2: Končne koncentracije antibiotikov v gojiščih

Antibiotic	Final concentration (µg/ml)
Ampicillin	120
Chloramphenicol	50
Ciprofloxacin	1.4
Gentamycin	15
Kanamycin	50
Streptomycin	150
Tetracycline	10

3.1.3.2 Solid LB medium

Solid LB medium was prepared with 25 g of LB powder and 15 g of agar dissolved in 1000 ml of dH_2O. Medium was sterilised by autoclaving at 121 °C for 15 min. If desired, medium was supplemented with stock solutions of antibiotics to final concentrations (Table 2). Medium was aseptically poured into disposable plastic Petri dishes once cooled to 55 °C.

3.1.3.3 Solid minimal medium A

To prepare 10×A stock solution, salts (Table 3) were dissolved in 500 ml of dH_2O. Volume was then filled up to 1000 ml with dH_2O. Stock solution was autoclaved at 121 °C for 15 min and stored at room temperature until use.

Table 3: 10×A stock solution
Preglednica 3: Založna raztopina 10×A

Salt	Quantity (g)
$(NH_4)_2SO_4$	10
K_2HPO_4	105
KH_2PO_4	45
Na-citrate × 5 H_2O	5
$MgSO_4$ × 7 H_2O	1

100 ml of 10×A stock solution (Table 3) were diluted in 200 ml of dH_2O and autoclaved at 121 °C for 15 min. 12 g of agar were separately dissolved in 700 ml of dH_2O, autoclaved at

121 °C for 15 min, cooled to 55 °C and mixed with the prepared salt solution. Medium was supplemented with 5 ml 40 % sterile glucose and, if desired, with 4 ml of 10 mg/ml sterile amino acids or stock solutions of antibiotics to final concentrations (Table 2), once cooled to 55 °C. Medium was aseptically poured into disposable plastic Petri dishes.

3.1.4 Chemicals

- $(NH_4)_2SO_4$, Merck, Germany
- 1×Dulbecco's phosphate-buffered saline (1×dPBS) without Ca^{2+} and Mg^{2+}, PAA, Austria
- 10 mM dNTPs, Thermo Scientific, USA
- 10×*Taq* DNA Polymerase Buffer, Thermo Scientific, USA
- 10×Tris-glycine SDS Running Buffer, Thermo Scientific, USA
- 100 ml L Broth, Biotrading, the Netherlands
- 25 mM EDTA (pH 8.0), Invitrogen, USA
- 29.4 % NH_4OH, Sigma-Aldrich, USA
- 37 % formaldehyde, Merck, Germany
- 50 mM $MgCl_2$, Thermo Scientific, USA
- 6×Loading dye, Thermo Scientific, USA
- Acetic acid, Merck, Germany
- Acryl-/bisacryl-amide 30 % (19:1), Bio-Rad, USA
- Acryl-bisacryl-amide 40 % (19:1), Bio-Rad, USA
- Acryl-bisacryl-amide 40 % (29:1), Bio-Rad, USA
- Agar no.1, Oxoid, UK
- Agarose, Sigma-Aldrich, USA
- $AgNO_3$, Merck, Germany
- Alcian blue, Merck, Germany
- Ammonium persulfate (APS), Merck, Germany
- Ampicillin, Sigma-Aldrich, USA
- Boric acid, Merck, Germany
- Bromophenol blue, Merck, Germany
- $CaCl_2$, Sigma-Aldrich, USA
- Chloramphenicol, Sigma-Aldrich, USA
- Ciprofloxacin, Sigma-Aldrich, USA
- Citric acid, Merck, Germany
- dH_2O
- dNTP mix, Thermo Scientific, USA
- Ethylenediaminetetraacetic acid (EDTA), Sigma-Aldrich, USA
- Ethanol, Merck, Germany

- Ethidium bromide, Sigma-Aldrich, USA
- Gentamycin, Lek, Slovenia
- Glucose, Merck, Germany
- Glycerol, Sigma-Aldrich, USA
- HCl, Merck, Germany
- K_2HPO_4, Merck, Germany
- Kanamycin, Sigma-Aldrich, USA
- KH_2PO_4, Merck, Germany
- LB Medium Lennox, MP Biomedicals, USA
- Methanol, Merck, Germany
- $MgCl_2$, Merck, Germany
- $MgSO_4 \times 7\ H_2O$, Merck, Germany
- $MgSO_4$, Merck, Germany
- MiliQ
- Na-citrate $\times 5\ H_2O$, Merck, Germany
- NaCl, Merck, Germany
- NaOH, Merck, Germany
- Periodic acid, Merck, Germany
- Streptomycin, Sigma-Aldrich, USA
- Tetracycline, Sigma-Aldrich, USA
- Tetramethylethylenediamine (TEMED), Bio-Rad, USA
- Tricine, Sigma-Aldrich, USA
- Tris(hydroxymethyl)aminomethane (Tris), Sigma-Aldrich, USA
- UltraPure™ 10 % SDS Solution, Invitrogen, USA
- β-mercaptoethanol, Merck, Germany

3.1.5 Primer oligonucleotides

- chuA.1b and chuA.2, Macrogen, South Korea
- yjaA.1b and yjaA.2b, Macrogen, South Korea
- TspE4C2.1b and TspE4C2.2b, Macrogen, South Korea
- AceK.f and ArpA1.r, Macrogen, South Korea
- ArpAgpE.f and ArpAgpE.r, Macrogen, South Korea
- trpAgpC.1 and trpA gpC.2, Macrogen, South Korea
- trpBA.f and trpBA.r, Macrogen, South Korea
- ColE7a+p f and ColE7a+p r, Jena Bioscience, Germany
- ColE7i+p f and ColE7i+p r, Jena Bioscience, Germany
- TraD f and TraD r, Jena Bioscience, Germany
- RepFIA-f and RepFIA-r, Jena Bioscience, Germany

- FIC FW and FIC RV, Jena Bioscience, Germany
- Sal14-a and Sal14-b, Jena Bioscience, Germany
- RepFIB-E1 and RepFIB-E2, Jena Bioscience, Germany
- PtraJ-1 and PtraJ-2, Jena Bioscience, Germany
- traT-1 and traT-2, Jena Bioscience, Germany

3.1.6 Enzymes and ladders

- GeneRuler™ 1 kb Plus DNA Ladder, Thermo Scientific, USA
- GeneRuler™ 100 bp Plus DNA Ladder, Thermo Scientific, USA
- Proteinase K, Sigma-Aldrich, USA
- Spectra™ Multicolor Broad Range Protein Ladder, Thermo Scientific, USA
- *Taq* DNA Polymerase, Thermo Scientific, USA

3.1.7 Buffers and reagents

3.1.7.1 5×TBE buffer

5×TBE buffer was prepared with 54 g of Tris, 27.5 g of boric acid and 20 ml of 0.5 M EDTA (pH 8.0) in 1000 ml of dH_2O.

3.1.7.2 2×Laemmli buffer

2×Laemmli buffer was prepared with 4 % SDS, 20 % glycerol, 0.004 % Bromophenol blue and 0.125 M Tris pH 6.8 in dH_2O. If needed, 15 % β-mercaptoethanol was added to the buffer.

3.1.7.3 LPS extraction

3.1.7.3.1 Lysis buffer

Lysis buffer was prepared with 60 mM Tris-HCl and 1 mM EDTA, pH 6.8.

3.1.7.3.2 Lysis buffer with SDS

Lysis buffer with SDS was prepared with 2 % SDS, 60 mM Tris-HCl and 1 mM EDTA, pH 6.8.

3.1.7.3.3 2×Sample buffer

2×Sample buffer was prepared with 6 % SDS, 6 % β-mercaptoethanol, 46 % glycerol, 60 mM Tris-HCl pH 8.0, and 0.1 % bromophenol blue.

3.1.7.4 Silver stain for LPS

3.1.7.4.1 Fixation buffer

Fixation buffer for LPS silver stain was prepared with 40 % ethanol and 5 % acetic acid in MiliQ.

3.1.7.4.2 Oxidation buffer

Oxidation buffer for LPS silver stain was prepared with 40 % ethanol, 5 % acetic acid and 0.7 % periodic acid in MiliQ.

3.1.7.4.3 Staining buffer

2 ml of 29.4 % NH_4OH were mixed with 28 ml of 0.1 N NaOH. 5 ml of 20 % $AgNO_3$ in MiliQ were then added dropwise to prevent precipitation. The solution was made up with MiliQ to 150 ml.

3.1.7.4.4 Developing buffer

100 µl of 10 % citric acid in MiliQ and 100 µl of 37 % formaldehyde were added to 100 ml of MiliQ.

3.1.7.4.5 Stop buffer

Stop buffer was prepared with 7 % acetic acid in MiliQ.

3.1.7.5 Tris-glycine SDS-PAGE

3.1.7.5.1 Tris-glycine SDS-PAGE gels

Table 4: Preparation of gels for Tris-glycine SDS-PAGE with 30 %, 19:1 acryl-/bisacryl-amide
Preglednica 4: Priprava gelov za Tris-glicin SDS-PAGE s 30 %, 19:1 akril-/bisakril-amidom

5 % stacking gel	**10 % separating gel**	**15 % separating gel**
0.68 ml dH_2O	1.9 ml dH_2O	1.1 ml dH_2O
0.17 ml acryl-/bisacryl-amide (30 %, 19:1)	1.7 ml acryl-/bisacryl-amide (30 %, 19:1)	2.5 ml acryl-/bisacryl-amide (30 %, 19:1)
0.13 ml 1.0 M Tris pH 6.8	1.3 ml 1.5 M Tris pH 8.8	1.3 ml 1.5 M Tris pH 8.8
0.01 ml 10 % SDS	0.05 ml 10 % SDS	0.05 ml 10 % SDS
0.01 ml 10 % APS	0.05 ml 10 % APS	0.05 ml 10 % APS
0.001 ml TEMED	0.002 ml TEMED	0.002 ml TEMED

Table 5: Preparation of gels for Tris-glycine SDS-PAGE with 40 %, 29:1 acryl-/bisacryl-amide
Preglednica 5: Priprava gelov za Tris-glicin SDS-PAGE s 40 %, 29:1 akril-/bisakril-amidom

5 % stacking gel	**13 % separating gel**
0.73 ml dH_2O	2.0 ml dH_2O
0.125 ml acryl-/bisacryl-amide (40 %, 29:1)	1.65 ml acryl-/bisacryl-amide (40 %, 29:1)
0.125 ml 1.0 M Tris pH 6.8	1.25 ml 1.5 M Tris pH 8.8
0.01 ml 10 % SDS	0.05 ml 10 % SDS
0.01 ml 10 % APS	0.05 ml 10 % APS
0.001 ml TEMED	0.002 ml TEMED

3.1.7.5.2 10×Tris-glycine SDS-PAGE running buffer

10×Tris-glycine SDS-PAGE running buffer was prepared with 250 mM Tris, 1.92 M glycine and 1 % SDS.

3.1.7.6 Alcian blue capsule stain

3.1.7.6.1 Alcian blue staining buffer

Alcian blue staining buffer was prepared with 2 % acetic acid, 40 % methanol and 0.5 % alcian blue in dH_2O. Alcian blue was first dissolved in absolute methanol and 96 % acetic acid. The solution was centrifuged and the supernatant was filled up to final volume and concentrations of acetic acid and methanol.

3.1.7.6.2 Alcian blue destaining buffer

Alcian blue destaining buffer was prepared with 2 % acetic acid and 40 % methanol in dH_2O.

3.1.8 Kits

- GeneJET Plasmid Miniprep Kit, Thermo Scientific, USA
- QIAGEN Plasmid Midi Kit, QIAGEN, the Netherlands

3.1.9 Laboratory equipment

- 15 ml and 50 ml tubes, Corning, USA
- Automatic pipettes, Biohit, Finland
- Automatic pipettes, Eppendorf, Germany
- Automatic pipettes, Gilson, USA
- Benchtop centrifuge 1-15P, Sigma, Germany
- Benchtop centrifuge 5417C, Eppendorf, Germany
- Boxer Autoclave, Lab3, UK
- Centrifuge Rotanta 460R, Hettich AG, Switzerland
- Clean bench, CleanAir, USA
- Cooled centrifuge 5402, Eppendorf, Germany
- DNA and protein electrophoreses, Bio-Rad, USA
- Eclipse TS1000 inversion microscope, Nikon, Japan
- Electroporator 2510, Eppendorf, Germany
- GeneAmp PCR System 2400, Perkin Elmer, Canada
- Incubating shaker, Infos HT, Switzerland
- Microcentrifuge tubes, Eppedorf, Germany
- Multifuge 3SR+, Thermo Scientific, USA
- MyCycler™ thermal cycler, Bio-Rad, USA
- NanoDrop ND-1000 spectrophotometer, Thermo Scientific, USA
- Novaspec Plus spectrophotometer, Amersham Biosciences, UK
- Rocking platform, VWR International, USA
- Safety cabinet, CeanAir, USA
- Thermoblock Constantemp, Technilab, USA
- Thermolyne Dri-Bath Type 1650, Thermo Scientific, USA
- Universal Hood III, Bio-Rad, USA
- Water bath ISOTEMP 215, Fisher Scientific, USA
- Water bath, GFL, Germany

3.2 METHODS

3.2.1 Polymerase chain reaction (PCR)

3.2.1.1 Template DNA preparation

A single colony of bacterial strain was suspended in 40 µl of dH_2O, incubated at 95 °C for 10 min and centrifuged at 14000 rpm for 10 min. 20 µl of supernatant were stored at −80 °C and subsequently used as a DNA template for PCR.

3.2.1.2 Oligonucleotide primers and PCR conditions

The polymerase chain reactions were performed in 20 µl reaction mixtures with 10 pmol of forward and reverse primers, 1 µl of template DNA sample, 4 nmol of each dNTP, 0.6 U of *Taq* DNA polymerase, 50 nmol of $MgCl_2$ and 1×*Taq* DNA polymerase buffer in dH_2O.

The phylogenetic quadruplex polymerase chain reactions were performed in 20 µl reaction mixtures with 20 pmol of forward and reverse primers chuA.1b, chuA.2, TspE4C2.1b, TspE4C2.2b, yjaA.1b and yjaA.2b, 40 pmol of forward and reverse primers AceK.f and ArpA1.r, 3 µl of template DNA sample, 40 pmol of each dNTP, 2 U of *Taq* DNA polymerase and 1×*Taq* DNA polymerase buffer in dH_2O.

The phylogenetic duplex polymerase chain reactions were performed in 20 µl reaction mixtures with 20 pmol of either forward and reverse primers ArpAgpE.f and ArpAgpE.r or trpAgpC.1 and trpAgpC.2, 12 pmol of forward and reverse primers trpBA.f and trpBA.r, 3 µl of template DNA sample, 40 pmol of each dNTP, 2 U of *Taq* DNA polymerase and 1×*Taq* DNA polymerase buffer in dH_2O.

Table 6: Oligonucleotide primers and PCR conditions
Preglednica 6: Oligonukleotidni začetniki in pogoji PCR

Oligonucleotide primer	**Nucleotide sequence**	**PCR conditions**	**Source or reference**
for *cE7a+p* PCR:			
ColE7a+p f	5′-GCGAAGCTTAATATCC CTCCCTGACTTGACAG-3′	95 °C, 5:00 min ×1 94 °C, 1:30 min	Petkovšek, 2012
ColE7a+p r	5′-GCGAAGCTTGCCTCTGTGTA ATCACTAATACTATTTTTC-3′	65 °C, 0:45 min ×30 72 °C, 2:10 min 72 °C, 1:00 min ×1	
for *cE7i+p* PCR:			
ColE7i+p f	5′-GCCCCGGGAAAAGTTT CGATGATTTTCGTAAG-3′	95 °C, 4:00 min ×1 94 °C, 1:00 min	Petkovšek, 2012
ColE7i+p r	5′-GCGGTACCTATTTCAGC CCTGTTTAAATCCTGGC-3′	69 °C, 1:00 min ×30 72 °C, 1:00 min 72 °C, 7:00 min ×1	
for *traD* PCR:			
TraD f	5′-GGAATTCCAGATTGC GTCCATGCGTATCC-3′	94 °C, 4:00 min ×1 94 °C, 1:00 min	Starčič Erjavec,
TraD r	5′-GGAATTCATCACCAC ACATATCACCGCGC-3′	63 °C, 1:00 min ×30 72 °C, 1:00 min 72 °C, 10:00 min ×1	2003
for *traJ* PCR:			
PtraJ-1	5′-TCCAAAAAATGATGATGAAT-3′	94 °C, 4:30 min ×1	Starčič et al.,
PtraJ-2	5′-ATAGGAACCTCCTCACAAAG-3′	94 °C, 0:30 min 60 °C, 0:30 min ×30 72 °C, 0:30 min 72 °C, 7:00 min ×1	2003
for *traT* PCR:			
traT-1	5′-GGTGTGGTGCGATGAGCACAG-3′	94 °C, 4:30 min ×1	Johnson and
traT-2	5′-CACGGTTCAGCCATCCCTGAG-3′	94 °C, 0:30 min 63 °C, 0:30 min ×30 72 °C, 0:30 min 72 °C, 7:00 min ×1	Stell, 2000
for RepFIA PCR:			
RepFIA-f	5′-CTCACTGAGGCGGCATATAGTC-3′	94 °C, 4:30 min ×1	Starčič
RepFIA-r	5′-ATGGAAGTGAT ATCGCGGAAGG-3′	94 °C, 0:30 min 63 °C, 0:30 min ×30 72 °C, 1:00 min 72 °C, 7:00 min ×1	Erjavec et al., 2003a
for RepFIB PCR:			
RepFIB-E1	5′-GGAATTCTCGCTGC AAACCTTGTCACT-3′	94 °C, 4:30 min ×1 94 °C, 0:30 min	Starčič Erjavec et
RepFIB-E2	5′-GGAATTCGGAGATCC TGCGTACACTGCCT-3′	63 °C, 0:30 min ×30 72 °C, 1:30 min 72 °C, 7:00 min ×1	al., 2003a

(continued)

(continuation)

Table 6: Oligonucleotide primers and PCR conditions

Oligonucleotide primer	Nucleotide sequence	PCR conditions	Source or reference
for RepFIC PCR:			
FIC FW	5′-GTGAACTGGCA GATGAGGAAGG-3′	94 °C, 4:30 min ×1 94 °C, 0:30 min	Carattoli et al., 2005
FIC RV	5′-TTCTCCTCGTCG CCAAACTAGAT-3′	60 °C, 0:30 min ×30 72 °C, 0:30 min 72 °C, 7:00 min ×1	
for RepFIIA PCR:			
Sal14-a	5′-CCCTGAAGTGACCTCCTCTG-3′	94 °C, 4:30 min ×1	Starčič
Sal14-b	5′-GAAAGGCGGCACTCTGTTGT-3′	94 °C, 0:30 min 63 °C, 0:30 min ×30 72 °C, 1:00 min 72 °C, 7:00 min ×1	Erjavec et al., 2003a
for phylogenetic quadruplex PCR:			
chuA.1b	5′-ATGGTACCGGACGAACCAAC-3′	94 °C, 4:30 min ×1	Clermont et al., 2013
chuA.2	5′-TGCCGCCAGTACCAAAGACA-3′	94 °C, 0:30 min	
TspE4C2.1b	5′-CACTATTCGTAAGGTCATCC-3′	59 °C, 0:30 min ×30	
TspE4C2.2b	5′-AGTTTATCGCTGCGGGTCGC-3′	72 °C, 0:30 min	
yjaA.1b	5′-CAAACGTGAAGTGTCAGGAG-3′	72 °C, 5:00 min ×1	
YjaA.2b	5′-AATGCGTTCCTCAACCTGTG-3′		
AceK.f	5′-AACGCTATTCGCCAGCTTGC-3′		
ArpA1.r	5′-TCTCCCCATACCGTACGCTA-3′		
for phylogenetic group C PCR:			
trpAgpC.1	5′-AGTTTTATGCCCAGTGCGAG-3′	94 °C, 4:30 min ×1	Clermont et al., 2013
trpAgpC.2	5′-TCTGCGCCGGTCACGCCC-3′	94 °C, 0:30 min	
trpBA.f	5′-CGGCGATAAAGACATCTTCAC-3′	59 °C, 0:30 min ×30	
trpBA.r	5′-GCAACGCGGCCTGGCGGAAG-3′	72 °C, 0:30 min 72 °C, 5:00 min ×1	
for phylogenetic group E PCR:			
ArpAgpE.f	5′-GATTCCATCTTG TCAAAATATGCC-3′	94 °C, 4:30 min ×1 94 °C, 0:30 min	Clermont et al., 2013
ArpAgpE.r	5′-GAAAAGAAAAAG AATTCCCAAGAG-3′	57 °C, 0:30 min ×30 72 °C, 0:30 min	
trpBA.f	5′-CGGCGATAAAGACATCTTCAC-3′	72 °C, 5:00 min ×1	
trpBA.r	5′-GCAACGCGGCCTGGCGGAAG-3′		

3.2.2 Agarose gel electrophoresis

Agarose gel electrophoresis was performed in TBE buffer. Agarose gel was prepared with 0.7 μg/ml ethidium bromide and 0.7 to 2.0 % agarose, depending on the size of the DNA fragments analysed. DNA samples were mixed with the loading dye. Depending on the size of the DNA fragments analysed, voltage between 80 and 120 V was chosen for the electrophoresis run.

3.2.3 Preparation of strain RU4405 pUC19i

3.2.3.1 Isolation of plasmid pUC19i

Plasmid pUC19i was isolated from strain DH5α pUC19i using "QIAGEN Plasmid Midi Kit" following manufacturer's instructions.

3.2.3.2 Preparation of chemically competent cells

A single colony of bacterial strain RU4405 was inoculated in 3 ml of LB with the appropriate antibiotic and incubated at 37 °C and 160–180 rpm overnight. 500 µl of the overnight culture were inoculated into 50 ml of preheated LB without the antibiotic and incubated at 37 °C and 160–180 rpm to OD_{600} of 0.3–0.4. The whole volume was transferred to a centrifuge tube and incubated on ice for 5–10 min. Cells were harvested by centrifugation at 7000 rpm and 4 °C for 7 min. Supernatant was discarded and cells were resuspended in 10 ml of ice cold 60 mM $CaCl_2$ with 15 % glycerol. Centrifugation and resuspension steps were repeated twice. After second wash, cells were incubated on ice for 30 min. Subsequently, cells were harvested by centrifugation at 7000 rpm and 4 °C for 7 min and resuspended in 1 ml of ice cold 60 mM $CaCl_2$ with 15 % glycerol. Cells were then stored at −80 °C in 150 µl aliquots.

3.2.3.3 Transformation of chemically competent cells

100 µl of chemically competent cells were mixed with 1 µl of isolated plasmid pUC19i DNA and incubated on ice for 30 min. After 30 min, cells were incubated in a 42 °C water bath for 90 s and immediately transferred on ice for 1–2 min. 400 µl of liquid LB were added and the mixture was incubated at 37 °C and 160–180 rpm for 1 h. 100 µl of the mixture were plated on an LB plate with ampicillin. The rest of the cells were harvested by centrifugation at 13000 rpm for 1 min, resuspended in 100 µl of liquid LB and plated on a second LB plate with ampicillin. LB plates were incubated at 37 °C overnight.

The presence of the plasmid pUC19i in the strain RU4405 was confirmed by plasmid isolation and the presence of the *immE7* gene was confirmed by PCR with ColE7i+p f and ColE7i+p r oligonucleotide primers. Isolated and amplified DNA fragments were analysed with agarose gel electrophoresis.

3.2.4 Preparation of strain SE15i

The *immE7* gene was integrated into the chromosome of the strain SE15 by conjugation and transposition. Plasmid pAG408i was transferred from strain S17-1 λpir to strain SE15 by filter conjugation, as described below. Both the donor strain S17-1 λpir pAG408i and the recipient strain SE15 were separately inoculated in 5 ml of liquid LB with the appropriate

antibiotic and incubated at 37 °C and 160–180 rpm overnight. 50 µl of each overnight culture were transferred to 5 ml 10 mM $MgSO_4$, mixed by vortexing and filtered through a 0.45 µm filter. Filter was then transferred to an LB plate with cells facing upwards and incubated at 37 °C overnight. Subsequently, filter was transferred to 5 ml of 10 mM $MgSO_4$ and cells were resuspended by vortexing. 300 µl of cell suspension were plated on solid minimal medium with gentamycin and incubated at 37 °C overnight.

Phylogeny of the strains S17-1 and SE15 was determined by phylogenetic PCR. Strain SE15 was found to belong to phylogenetic group B2, while strain S17-1 belongs to group A. Phylogenetic PCR was also performed on transconjugant colonies. Transconjugant colonies belonging to the phylogenetic group B2 were further plated on minimal medium with ampicillin to confirm that the λ phage was not induced during conjugation. Ampicillin sensitive colony was designated SE15i and was used for all further work.

3.2.5 Preparation of donor strains MC4100, N4i, and SE15i harbouring pGZ1 or pGZ2

3.2.5.1 Isolation of plasmids pGZ1 and pGZ2

Plasmids pGZ1 and pGZ2 were isolated from strains MS252 and MS253 using "QIAGEN Plasmid Midi Kit" following manufacturer's instructions.

3.2.5.2 Preparation of electrocompetent cells

A single colony of each bacterial strain MC4100, N4i and SE15i was separately inoculated in 3 ml of liquid LB with the appropriate antibiotic and incubated at 37 °C and 160–180 rpm overnight. 500 µl of the overnight culture were inoculated into 50 ml of preheated liquid LB without the antibiotic and incubated at 37 °C and 160–180 rpm to OD_{600} of 0.5. The whole volume was transferred into a centrifuge tube and incubated on ice for 15 min. Cells were harvested by centrifugation at 7000 rpm and 4 °C for 7 min. Supernatant was discarded and cells were resuspended in 10 ml of ice cold dH_2O. Centrifugation and suspension steps were repeated twice. After second wash, cells were harvested by centrifugation at 7000 rpm and 4 °C for 7 min and resuspended in 1 ml of ice cold dH_2O with 15 % glycerol. Cells were then stored at −80 °C in 150 µl aliquots.

3.2.5.3 Electroporation

50 µl of electrocompetent cells of strains MC4100, N4i and SE15i were mixed with 1 µl of isolated plasmid pGZ1 or pGZ2 DNA in a 100 µl electroporation cuvette. Electroporation was performed at 1700 V for 5 ms. Following electroporation, cells were immediately transferred to preheated liquid LB and incubated at 37 °C and 160–180 rpm for 60 min.

100 µl of each electroporation mixture were plated on an LB plate with chloramphenicol. The rest of the cells were harvested by centrifugation at 13000 rpm for 1 min, resuspended in 100 µl of liquid LB and plated on a second LB plate with chloramphenicol each. LB plates were incubated at 37 °C overnight.

3.2.6 Plate mating protocol and preparation of conjugal donor strains

The donor and the recipient strain cultures were streaked one over the other on an LB plate and incubated overnight at 37 °C. Following incubation, the mixture of donor, recipient and transconjugant strains was streaked on an appropriate LB plate supplemented with antibiotics for the selection of transconjugants and incubated overnight at 37 °C. Selectivity of LB plates was tested by streaking the donor and the recipient strains separately on two selective LB pates and incubating at 37 °C overnight.

Strains MC4100 pOX38:Cm, MC4100 pOX38a pUC19i, MC4100 pGZ1 pRK100, MC4100 pGZ2 pRK100, N4i pOX38:Cm, SE15i pOX38:Cm, SE15i pOX38a, N4i pGZ1 pOX38:Kn, N4i pGZ2 pOX38:Kn, SE15i pGZ1 pOX38:Kn, SE15i pGZ2 pOX38:Kn and Nissle 1917 pOX38:Cm were prepared by conjugal transfer of plasmids pOX38:Cm, pOX38a and pOX38:Kn from donor strains DH5α pOX38:Cm, HB101 pRK100, N4i pOX38a and J5 pOX38:Kn to recipient strains MC4100, MC4100 pUC19i, MC4100 pGZ1, MC4100 pGZ2, N4i, SE15i, N4i pGZ1, N4i pGZ2, SE15i pGZ1, SE15i pGZ2 and Nissle 1917.

3.2.7 Assessment of conjugal transfer frequencies in liquid medium – protocol I

Single colonies of donor and recipient strains were inoculated in 3 ml of liquid LB with the appropriate antibiotics and incubated at 37 °C and 160–180 rpm overnight. 900 µl of liquid LB were transferred to a microcentrifuge tube and incubated at 37 °C in a water bath for 15 min. After 15 min, 40 µl of the donor overnight culture were added to liquid LB and incubated at 37 °C in a water bath for 30 min. As the negative control, 40 µl of liquid LB were added instead of the donor overnight culture. After 30 min, 100 µl of the recipient overnight culture were added to the incubated donor culture and incubated at 37 °C for another 30 min. Subsequently, the microcentrifuge tube was incubated on ice for 2 min and mixed on vortex for 1 min. Tenfold serial dilutions were prepared with 0.9 % NaCl and plated on selective LB plates for CFU counts of the transconjugants and the recipient strain. LB plates were incubated at 37 °C overnight and CFUs of the transconjugants and the recipient strain were assessed. Selectivity of LB plates was tested by streaking the donor and the recipient strains separately on selective LB pates and incubating over night at 37 °C. The conjugation frequency was calculated using the following formula (1):

$$\text{conjugal transfer frequency} = \frac{\text{CFU transconjugants}}{\text{CFU recipient strain}} \qquad \ldots(1)$$

Experiment was repeated three times and arithmetic means of the conjugation frequencies were calculated.

3.2.8 Assessment of conjugal transfer frequencies on solid medium – protocol II

Single colonies of donor and recipient strains were inoculated in 3 ml of liquid LB with the appropriate antibiotics and incubated at 37 °C and 160–180 rpm overnight. Overnight cultures were diluted in a 1:100 ratio in 5 ml of liquid LB without antibiotics and incubated at 37 °C and 160–180 rpm for 2 h. 1 ml of recipient strain culture was centrifuged at 5000 rpm for 10 min, the supernatant was discarded and the harvested cells were resuspended in 400 µl of the donor strain culture. The whole volume was spread over an LB plate and incubated at 37 °C for 4 h. Subsequently, conjugation mixture was collected from the LB plate by resuspension in 1 ml of 1×dPBS. Tenfold serial dilutions were prepared with 1×dPBS and plated on selective LB plates for CFU counts of the transconjugants and the recipient strain. LB plates were incubated at 37 °C overnight and CFUs of the transconjugants and the recipient strain were assessed. Selectivity of LB plates was tested by streaking the donor and the recipient strains separately on selective LB pates and incubating overnight at 37 °C. The conjugation frequency was calculated using formula (1). Experiment was repeated three times and arithmetic means of the conjugation frequencies were calculated.

3.2.9 Lipopolysaccharide (LPS) profiling

SDS-PAGE profiling of the LPS, followed by silver staining, is a convenient method of differentiating between LPSs of different bacterial strains. In this method, LPS molecules are first separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). In this technique, the lipid A moiety of LPS binds to SDS and moves through the gel together with the bound O-antigen moiety. Since O-antigens in each strain occur in different lengths, the result is a ladder-like profile in which the lowest rungs represent rough LPS, while the higher rungs represent smooth LPS with increasing numbers of repeating O units. The size of the repeating O unit is reflected in the spacing between the rungs. Following electrophoresis, the carbohydrate constituents of the LPS can be stained with silver. The majority of saccharides include at least one pair of adjacent carbon atoms with bound hydroxyl groups. In this technique, such adjacent hydroxyl groups are selectively oxidised with periodic acid to yield aldehyde groups. Subsequently, ammoniacal silver cations $[Ag(NH_3)_2]^+$ are added. In this reaction, the aldehyde groups are oxidised to carboxyl groups, while the silver cations are reduced to elemental silver Ag^0, which forms dark brown to black deposits in the polyacrylamide gel.

3.2.9.1 LPS extraction and electrophoresis

A single colony of each strain was inoculated into 5 ml of liquid LB with the appropriate antibiotics and incubated at 37 °C and 160–180 rpm overnight. 3 ml of overnight culture were pelleted by centrifugation at 14000 rpm for 5 min and washed 3 times with 0.9 % NaCl. After the third wash, cells were pelleted and resuspended in 125 µl lysis buffer with SDS and boiled for 5 min. Cells were vortexed before and after boiling. Lysed cells were resuspended in 875 µl lysis buffer solution without SDS, 100 µg proteinase K were added to lysed cells and incubated at 65 °C for 2 h. LPS extracts were stored at 4 °C until analysis.

An equal volume (50 µl) of 2×sample buffer was added to the LPS extracts and boiled for 5 min. Samples were vortexed before and after boiling. After boiling, 10 µl of each sample were analysed by Tris-glycine SDS-PAGE using 1×Tris-glycine SDS-PAGE running buffer, 5 % (29:1) stacking and 13 % (29:1) running gels. Tris-glycine SDS-PAGE was run at 90 V.

3.2.9.2 Silver stain for LPS

The SDS-PAGE gels were incubated in fixation buffer for 30 min and in oxidation buffer for 5 min. After oxidation, gels were washed with MiliQ three times for 10 min. Following washing, gels were incubated in staining buffer for 10 min with vigorous agitation and washed with MiliQ three times for 10 min. Gels were then incubated in developing buffer until sufficiently developed. When sufficiently developed, staining was stopped by addition of stop buffer and agitation. The whole protocol was performed in extremely clean glassware or disposable plastic.

3.2.10 Capsule detection

Polysaccharides of Gram-negative cell surface structures are usually acidic, as they harbour carboxyl groups. In water solutions, they are therefore present as anions and can be effectively stained with cationic dyes. Alcian blue is a cationic dye most often used for selectively staining acidic mucosubstances. In this method, the polyacrylamide gel is stained with alcian blue stain after SDS-PAGE separation of the prepared samples.

3.2.10.1 Sample preparation

3.2.10.1.1 Sample preparation from liquid medium

A single colony of each strain was inoculated into 5 ml of liquid LB with the appropriate antibiotics and incubated at 37 °C and 160–180 rpm overnight. OD_{600} of the overnight bacterial culture was measured and the appropriate amount was centrifuged at 15000 rpm for 5 min to harvest 1×10^9 bacterial cells. Supernatant was discarded and the pellet was

resuspended in 100 µl of 2×Laemmli buffer with 2 mg/ml proteinase K. The suspension was incubated at 65 °C for 1 h, followed by 96 °C for 10 min. Samples were then allowed to cool to room temperature.

3.2.10.1.2 Sample preparation from solid medium

A single colony of each strain was streaked over an LB plate and incubated at 37 °C overnight. A portion of the solid medium bacterial culture was streaked from the plate and suspended in 1×dPBS. OD_{600} of the suspension was measured and the appropriate amount was centrifuged at 15000 rpm for 5 min to harvest 1×10^9 bacterial cells. Supernatant was discarded and the pellet was resuspended in 100 µl of 2×Laemmli buffer with 2 mg/ml proteinase K. The suspension was incubated at 65 °C for 1 h, followed by 96 °C for 10 min. Samples were then allowed to cool to room temperature.

3.2.10.2 Tris-glycine SDS-PAGE

5 % (19:1) stacking gels, 10 % (19:1) separating gels and 1×Tris-glycine SDS running buffer were used for Tris-glycine SDS-PAGE. 10 µl of samples were loaded onto gels and the electrophoresis was run at 75 V until the samples were through the stacking gel and then at 125 V until the end of the run.

3.2.10.3 Alcian blue capsule stain

The gels were washed with dH_2O, stained with alcian blue staining buffer overnight and destained with alcian blue destaining buffer until bands appeared.

3.2.11 Statistical analysis

The data were logarithmically transformed and analysed using one-way analysis of variance (ANOVA). Statistical analysis was performed using R.

4 RESULTS

4.1 POLYMERASE CHAIN REACTIONS

In order to confirm the suitability of the selected wild-type strains to act as recipients in conjugation experiments, we used a PCR-based method to detect the presence of replication regions RepFIA, RepFIB, RepFIC and RepFIIA, as well as the *traJ* and *traT* fragments of the *tra* region in the candidate recipient strains. Detection of these PCR products indicates the presence of F-like conjugative plasmids in these strains and thus makes them inappropriate conjugal recipients. In addition, the strains were phylotyped to confirm their identity.

Table 7: The results of the PCR reactions performed
Preglednica 7: Rezultati izvedenih reakcij PCR

strain	phylogenetic PCR	RepFIA	RepFIB	RepFIC	RepFIIA	*traJ*	*traT*
DL94	A	–[a]	–	–	–	–	–
TA131	B2	–	–	–	–	–	–

[a] –, no product was detected; produkta nismo zaznali.

The phylogenetic groups of the two wild-type recipient strains were determined. The results of the PCRs for RepFIA, RepFIB, RepFIC and RepFIIA confirm that the strains do not include plasmids of the incompatibility group F. The results of the PCRs for *traJ* and *traT* confirm that the strains do not include F-like conjugal plasmids.

4.2 ASSESSMENT OF CONJUGAL TRANSFER FREQUENCIES

Conjugal transfer frequencies of plasmids pOX38:Cm and pOX38a were assessed. Laboratory *E. coli* strain MC4100 and two commensal *E. coli* strains N4i and SE15i were used as conjugal donors, and laboratory *E. coli* strain RU4405 and two pathogenic *E. coli* strains DL94 and TA131 were used as conjugal recipients. Strain DL94 was isolated from a urinary tract infection, and pathogenic recipient strain TA131, isolated from a skin and soft tissue infection. The mating pairs as well as the conjugal frequencies acquired following experimental protocol I (chapter 3.2.7) are presented in Table 8.

Table 8: Conjugal transfer frequencies of plasmids pOX38:Cm and pOX38a, acquired following experimental protocol I
Preglednica 8: Frekvence konjugacije plazmidov pOX38:Cm in pOX38a po protokolu I

donor plasmid × recipient strain	**donor strain**		
	MC4100 (pUC19i)	**N4i**	**SE15i**
pOX38:Cm × RU4405	$(3.7 \pm 0.4) \times 10^{-2}$	$(2.2 \pm 0.8) \times 10^{-2}$	$(2.6 \pm 1.2) \times 10^{-2}$
pOX38:Cm × RU4405 pUC19i	$(1.5 \pm 0.5) \times 10^{-3}$	$(7.2 \pm 4.5) \times 10^{-3}$	$(2.6 \pm 2.3) \times 10^{-3}$
pOX38a × RU4405	$(2.0 \pm 1.1) \times 10^{-4}$	0 ± 0	$(3.1 \pm 2.0) \times 10^{-6}$
pOX38a × RU4405 pUC19i	$(5.2 \pm 3.8) \times 10^{-2}$	$(7.9 \pm 7.9) \times 10^{-4}$	$(4.9 \pm 3.3) \times 10^{-5}$
pOX38:Cm × DL94	0 ± 0	0 ± 0	0 ± 0
pOX38:Cm × DL94 pUC19i	0 ± 0	0 ± 0	0 ± 0
pOX38a × DL94	0 ± 0	0 ± 0	0 ± 0
pOX38a × DL94 pUC19i	0 ± 0	0 ± 0	0 ± 0
pOX38:Cm × TA131	0 ± 0	0 ± 0	0 ± 0
pOX38:Cm × TA131 pUC19i	0 ± 0	0 ± 0	0 ± 0
pOX38a × TA131	0 ± 0	0 ± 0	0 ± 0
pOX38a × TA131 pUC19i	0 ± 0	0 ± 0	0 ± 0

Conjugal transfer frequencies of plasmids pOX38:Cm and pOX38a, acquired following experimental protocol I (chapter 3.2.7), were assessed from conjugal donor strains MC4100 (pUC19i), N4i and SE15i to conjugal recipient strains RU4405 (pUC19i), DL94 (pUC19i) and TA131 (pUC19i). Experiments were performed in triplicates. Standard error was used as an indicator of variation.
Frekvence konjugacije plazmidov pOX38:Cm in pOX38a po protokolu I (poglavje 3.2.7) smo ocenili iz donorskih sevov MC4100 (pUC19i), N4i in SE15i v recipientske seve RU4405 (pUC19i), DL94 (pUC19i) in TA131 (pUC19i). Poskuse smo izvedli v triplikatih. Kot mero variabilnosti rezultatov smo uporabili standardno napako.

Statistical analysis of the results of the experimental protocol I shown in Table 8 has shown that the conjugal transfer frequencies of pOX38:Cm from each of the three donor strains into RU4405 were not significantly different ($P >> 0.1$). Further, it was shown that with each of the three donor strains, the conjugal transfer frequencies of pOX38:Cm into RU4405 were not significantly different from the frequencies of pOX38:Cm into RU4405 pUC19i ($P >> 0.1$). Comparing plasmids pOX38:Cm and pOX38a, the conjugal transfer frequencies of pOX38a into RU4405 were significantly lower than the frequencies of pOX38:Cm into RU4405 with each of the three donor strains ($P < 0.01$). With donor strains MC4100 and N4i, the conjugal transfer frequencies of pOX38a into RU4405 were significantly lower than the frequencies of pOX38a into RU4405 pUC19i ($P < 0.1$ and $P < 0.05$, respectively), while with donor strain SE15i, we did not observe statistically significant differences in the same matings ($P >> 0.1$). The donor strain MC4100 was the only one where the conjugal transfer frequency of pOX38a into RU4405 pUC19i was not significantly different from the frequency of pOX38:Cm into RU4405. Since no transconjugants were detected in any of the matings with recipients DL94 and TA131 following experimental protocol I, these results were not included in statistical analysis.

As no transconjugants could be detected in any of the mating experiments performed with wild-type recipient strains DL94 and TA131 following experimental protocol I, attempts

were made to modify the experimental protocol with the aim of determining the experimental conditions, at which the plasmid pOX38 successfully transfers into the recipient strains. Either liquid or solid LB medium and minimal medium A were used and matings were performed during different time periods. The final experimental protocol II is presented in chapter 3.2.8. Due to high variability in observed frequencies, matings employing conjugal recipients harbouring pUC19i were abandoned in all subsequent experiments, except for matings MC4100 × RU4405, which were performed to be able to compare the results acquired following both experimental protocols. The mating pairs as well as the conjugal transfer frequencies acquired following experimental protocol II are presented in Table 9.

Table 9: Conjugal transfer frequencies of plasmids pOX38:Cm and pOX38a, acquired following experimental protocol II
Preglednica 9: Frekvence konjugacije plazmidov pOX38:Cm in pOX83a po protokolu II

donor plasmid × recipient strain	**donor strain**		
	MC4100 (pUC19i)	**N4i**	**SE15i**
pOX38:Cm × RU4405	$(1.1 \pm 0.7) \times 10^{-2}$	$(5.0 \pm 2.0) \times 10^{-3}$	$(2.8 \pm 1.6) \times 10^{-3}$
pOX38:Cm × RU4405 pUC19i	$(5.5 \pm 0.5) \times 10^{-3}$	—[a]	—
pOX38a × RU4405	$(1.8 \pm 0.6) \times 10^{-5}$	0 ± 0	$(3.4 \pm 2.0) \times 10^{-6}$
pOX38a × RU4405 pUC19i	$(1.6 \pm 0.8) \times 10^{-3}$	—	—
pOX38:Cm × DL94	$(3.8 \pm 2.6) \times 10^{-5}$	$(5.8 \pm 3.9) \times 10^{-7}$	$(4.8 \pm 3.1) \times 10^{-7}$
pOX38a × DL94	$(1.2 \pm 0.2) \times 10^{-8}$	0 ± 0	0 ± 0
pOX38:Cm × TA131	$(3.3 \pm 2.0) \times 10^{-6}$	$(5.6 \pm 3.0) \times 10^{-6}$	$(1.3 \pm 0.2) \times 10^{-5}$
pOX38a × TA131	$(2.3 \pm 0.3) \times 10^{-6}$	0 ± 0	0 ± 0

Conjugal transfer frequencies of plasmids pOX38:Cm and pOX38a, acquired following experimental protocol II (chapter 3.2.8), were assessed from conjugal donor strains MC4100 (pUC19i), N4i and SE15i to conjugal recipient strains RU4405 (pUC19i), DL94 and TA131. Experiments were performed in triplicates. Standard error is used as an indicator of variation.
Frekvence konjugacije plazmidov pOX38:Cm in pOX38a po porotkolu II (poglavje 3.2.8) smo ocenili iz donorskih sevov MC4100 (pUC19i), N4i in SE15i v recipientske seve RU4405 (pUC19i), DL94 in TA131. Poskuse smo izvedli v triplikatih. Kot mero variabilnosti rezultatov smo uporabili standardno napako.
[a] —, experiment was not performed; poskusa nismo izvedli

Statistical analysis of the results of the experimental protocol II shown in Table 9 has shown that with donor strain MC4100, the conjugal transfer frequencies of pOX38:Cm into RU4405, pOX38:Cm into RU4405 pUC19i, and pOX38a into RU4405 pUC19i, were not significantly different ($P >> 0.1$), while the conjugal transfer frequency of pOX38a into RU4405 was significantly lower ($P < 0.01$). The conjugal transfer frequencies of pOX38:Cm from each of the three donor strains into RU4405 were not significantly different ($P >> 0.1$). Further, the conjugal transfer frequencies of pOX38:Cm from all three donor strains into RU4405 were significantly higher than the frequencies into recipients DL94 and TA131 ($P < 0.01$ in all cases). The conjugal transfer frequencies of pOX38:Cm from MC4100 into DL94 were significantly higher than the frequencies of pOX38:Cm from the other two donor strains (N4i and SE15i) ($P < 0.05$); the latter two were not significantly different ($P >> 0.1$). The conjugal transfer frequencies of pOX38:Cm from all three donor strains into recipient TA131 were not significantly different ($P >> 0.1$). Comparing the two recipient strains, the

conjugal transfer frequencies of pOX38:Cm from MC4100 into DL94 and TA131 were not significantly different ($P >> 0.1$); the same was observed with donor strain N4i ($P >> 0.1$). In contrast, the conjugal transfer frequencies of pOX38:Cm from SE15i into DL94 were significantly lower than the frequencies of pOX38:Cm into TA131 (($P < 0.1$). Comparing the plasmids pOX38:Cm and pOX38a, we observed that the conjugal transfer frequencies of pOX38a from all three donor strains into DL94 were significantly lower than the frequencies of pOX38:Cm into DL94 ($P < 0.01$). Likewise, the conjugal transfer frequencies of pOX38a from donor strains N4i and SE15i into TA131 were significantly lower than the frequencies of pOX38:Cm from the same donors into TA131 ($P < 0.01$). On the contrary, the conjugal transfer frequencies of pOX38:Cm and pOX38a from MC4100 into TA131 were not significantly different ($P >> 0.01$).

In cases where, even by experimental protocol II, no transconjugants could be detected, plate matings were performed as described in chapter 3.2.6. The results are presented in Table 10.

Table 10: Survival of transconjugants acquired by plasmid transfer from conjugal donor strains N4i pOX38a and SE15i pOX38a.
Preglednica 10: Preživetje transkonjugant, pridobljenih s prenosom plazmida iz donorskih sevov N4i pOX38a in SE15i pOX38a

recipient strain	**donor strain**	
	N4i pOX38a	**SE15i pOX38a**
RU4405	–[a]	+[b]
DL94	–	–
TA131	–	±[c]

Experiments were performed in triplicates.
Poskuse smo izvedli v triplikatih.
[a] –, no transconjugants were isolated; transkonjugant nismo izolirali
[b] ±, a small number of transconjugants were isolated; izolirali smo nizko število transkonjugant
[c] + transconjugants were isolated already following protocol II and this experiment was therefore not performed; transkonjugante smo izolirali že po protokolu II, zato tega poskusa nismo izvedli

4.2.1 Comparison of observed conjugal transfer frequencies acquired following experimental protocol I and experimental protocol II

Statistical analysis of all comparable conjugal transfer frequencies acquired following experimental protocols I (Table 8) and II (Table 9) has shown that none of the frequencies acquired following protocol I were statistically different from those acquired following protocol II ($P >> 0.1$ in all cases).

4.2.2 Overexpression of *traJ* and its effect on conjugal transfer frequency

Attempt was made to raise the observed conjugal transfer frequencies from donor strains N4i and SE15i. We introduced an additional plasmid pGZ2, encoding the main transcriptional activator of the *tra* region *traJ*, into both donor strains. Experiments were

performed following experimental protocol I (chapter 3.2.7). The results are presented in Table 11.

Table 11: The effect of overexpression of *traJ* on conjugal transfer frequency into recipient strain RU4405
Preglednica 11: Učinek povečanega izražanja gena *traJ* na frekvenco konjugacije v recipientski sev RU4405

mating pair	**conjugal frequency**
N4i pGZ1 pOX38:Kn × RU4405	$(1.5 \pm 0.7) \times 10^{-2}$
N4i pGZ2 pOX38:Kn × RU4405	$(1.1 \pm 0.5) \times 10^{-2}$
SE15i pGZ1 pOX38:Kn × RU4405	$(9.8 \pm 7.7) \times 10^{-5}$
SE15i pGZ2 pOX38:Kn × RU4405	$(2.0 \pm 0.8) \times 10^{-4}$
MC4100 pGZ1 pRK100 × RU4405	$(1.6 \pm 0.6) \times 10^{-6}$
MC4100 pGZ2 pRK100 × RU4405	$(6.8 \pm 0.9) \times 10^{-3}$

Strains N4i and SE15i were used as conjugal donors. Strain MC4100 and plasmid pRK100 were used as a positive control. Experiments were performed in duplicates. Standard error was used as an indicator of variation. pGZ1, control plasmid; pGZ2, plasmid encoding *traJ* gene.
Seva N4i in SE15i smo uporabili kot konjugativna donorja. Sev MC4100 in plazmid pRK100 smo uporabili kot pozitivno kontrolo. Poskuse smo izvedli v duplikatih. Kot mero variabilnosti rezultatov smo uporabili standardno napako. pGZ1, kontrolni plazmid; pGZ2, plazmid z vstavljenim genom *traJ*.

Neither donor strain N4i nor SE15i exhibited significant change of conjugal transfer frequency when overexpressing the *traJ* gene in the donor, compared to conjugal transfer frequency without the additional copy of *traJ* present ($P >> 0.1$ in both cases). The positive control showed significant increase in conjugal transfer frequency when overexpressing the *tra J* gene in the donor, compared to conjugal transfer frequency without the additional copy of *traJ* present ($P < 0.01$).

4.2.3 Conjugation between genetically identical donor and recipient strains

This experiment was performed following experimental protocol II (chapter 3.2.8). The results are presented in Table 12.

Table 12: Conjugal transfer frequency between genetically identical conjugal donor and conjugal recipient strains
Preglednica 12: Frekvenca konjugacije med genetsko identičnima konjugativnim donorjem in konjugativnim recipientom

mating pair	**conjugal frequency**
Nissle 1917 pOX38:Cm × N4i	$(9.0 \pm 0.3) \times 10^{-8}$

Experiments were performed in triplicates. Standard error was used as an indicator of variation.
Poskuse smo izvedli v triplikatih. Kot mero variabilnosti rezultatov smo uporabili standardno napako.

The result was compared to the ones presented in Table 9. Statistical analysis has shown that the determined average frequency observed in this experiment was not significantly different than any of the frequencies presented in Table 9.

4.3 LIPOPOLYSACCHARIDE (LPS) PROFILING OF THE CONJUGAL DONOR AND RECIPIENT STRAINS

LPS profiling was performed as described in chapter 3.2.9 and the results are presented in Figure 4.

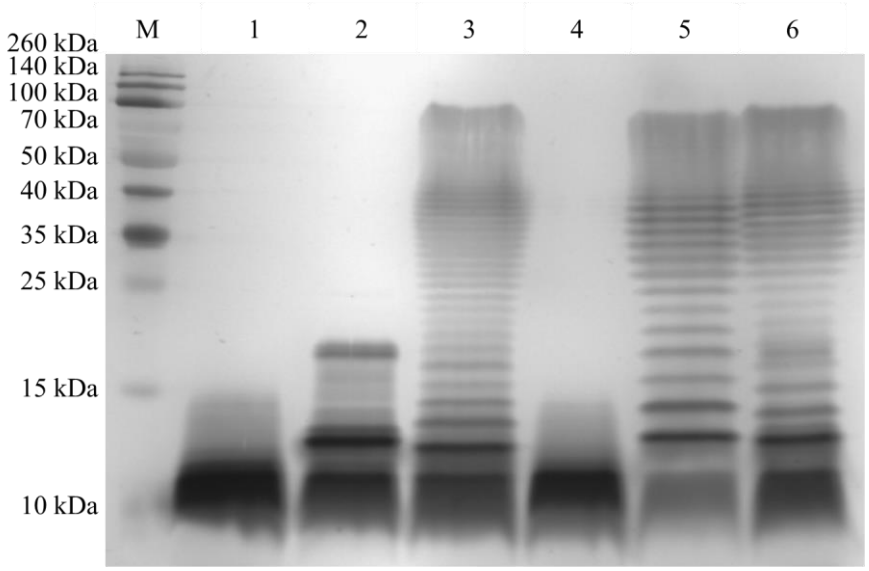

Figure 4: Silver-stained SDS-PAGE profiles of the LPS of the conjugal donor and recipient strains
M, Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific); 1, MC4100; 2, N4i; 3, SE15i; 4, RU4405; 5, DL94; 6, TA131.
Slika 4: S srebrom obarvani SDS-PAGE profili LPS konjugativnih donorjev in recipientov
M, Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific);1, MC4100; 2, N4i; 3, SE15i; 4, RU4405; 5, DL94; 6, TA131.

The two laboratory strains, MC4100 and RU4405 exhibit rough LPS, while strain N4i exhibits a semi-rough LPS. The other three strains (SE15i, DL94 and TA131) possess smooth LPS-types with a medium number of O units comprising the O-antigen. Strain SE15i exhibits a modal number of O units of more than 10. Strains DL94 and TA131 exhibit very similar LPS profiles with a modal number of O units around 8 to 15. Based on the spacing between the rungs in the profiles of each of the smooth LPS strains, strain SE15i exhibits the O unit with the lowest molecular weight and strain DL94 the O unit with the largest molecular weight among the analysed smooth LPS-type strains. Larger polyacrylamide gels are required to be able to more precisely assess the number of O units in the O-antigen in each strain. In all three strains with smooth LPS, SE15i, DL94 and TA131, a high molecular weight polysaccharide without an LPS-specific rung profile is present between 50 kDa and 100 kDa markers.

4.4 CAPSULE PROFILING OF THE CONJUGAL DONOR AND RECIPIENT STRAINS

Capsular polysaccharide was profiled as described in chapter 3.2.10.

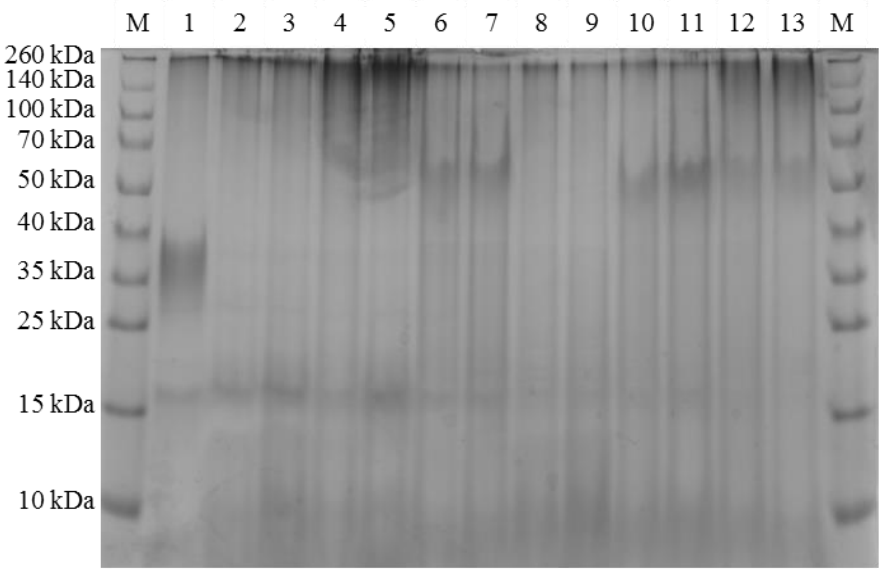

Figure 5: Alcian blue-stained SDS-PAGE profiles of the capsules of the conjugal donor and recipient strains
M, Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific); 1, staining control (*Campylobacter jejuni)*; 2, MC4100 incubated in liquid LB; 3, MC4100 incubated on solid LB; 4, N4i incubated in liquid LB; 5, N4i incubated on solid LB; 6, SE15i incubated on liquid LB; 7, SE15i incubated on solid LB; 8, RU4405 incubated in liquid LB; 9, RU4405 incubated on solid LB; 10, DL94 incubated in liquid LB; 11, DL94 incubated on solid LB; 12, TA131 incubated in liquid LB; 13, TA131 incubated on solid LB.
Slika 5: Z alcianskim modrilom obarvani SDS-PAGE profili kapsul konjugativnih donorjev in recipientov
M, Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific); 1, kontrola barvanja (*Campylobacter jejuni*); 2, MC4100 inkubiran v tekočem LB; 3, MC4100 inkubiran na trdnem LB; 4, N4i inkubiran v tekočem LB; 5, N4i inkubiran na trdnem LB; 6, SE15i inkubiran v tekočem LB; 7, SE15i inkubiran na trdnem LB; 8, RU4405 inkubiran v tekočem LB; 9, RU4405 inkubiran na trdnem LB; 10, DL94 inkubiran v tekočem LB; 11, DL94 inkubiran na trdnem LB; 12, TA131 inkubiran v tekočem LB; 13, TA131 inkubiran na trdnem LB.

Capsule profiling has shown that laboratory strains MC4100 and RU4405 lack high molecular weight capsular polysaccharides; wild-type strains N4i, SE15i, DL94 and TA131, however, possess high molecular weight capsular polysaccharides. In strains SE15i, DL94 and TA131, capsular polysaccharide was detected between 50 kDa and 100 kDa markers. Capsular polysaccharides of the highest molecular weight were observed in strains N4i (between 50 kDa marker and end of gel) and TA131 (between 100 kDa and 260 kDa markers).

5 DISCUSSION

As a first step in the development of an alternative approach to the treatment and prevention of infections with *E. coli* in humans and animals, and to prevention of colonisation of animal reservoirs by zoonotic *E. coli* strains, a recombinant probiotic *E. coli* strain Nissle 1917 has been created and the aim of this work was to test and evaluate the antimicrobial efficacy of the previously engineered recombinant probiotic strain *E. coli* Nissle 1917 (N4i). In addition to strain N4i, a second conjugal donor strain SE15i was prepared and the conjugal transfer frequencies of the colicin E7-encoding plasmid pOX38 were evaluated into different laboratory and wild-type *E. coli* strains. Wild-type strains that do not carry F-like conjugative plasmids were selected as conjugal recipient strains. The absence of F-like conjugative plasmids in wild-type strains was evaluated using a PCR-based method, whereby fragments of the replication regions and the *tra* region were amplified. We also evaluated the killing efficacy of the donor strains and assessed possible reasons for the differences in conjugal transfer frequencies between different donor and recipient strains by profiling LPS and capsular polysaccharides.

The PCRs performed have shown that the two chosen wild-type recipient strains, DL94 and TA131, harbour neither F-like conjugative plasmids (PCR products *traJ* and *traT* were not detected) nor plasmids of the F incompatibility group (PCR products RepFIA, RepFIB, RepFIC and RepFIIA were not detected). As such, the two chosen strains DL94 and TA131 were appropriate recipient strains for pOX38 in all following experiments. The absence of F-like conjugative plasmids enables pOX38 to enter the recipient cells via conjugal transfer as there are no F-like surface and entry exclusion systems present, while the absence of an additional plasmid of the F incompatibility group enables uninterrupted replication of pOX38 inside the recipient cell, and consequently enables transconjugant detection by cultivation on selective media following mating experiments.

Conjugal transfer frequencies of plasmids pOX38:Cm and pOX38a, encoding the colicin E7 gene, were first assessed with strains MC4100, N4i, and SE15i as conjugal donors, and laboratory strain RU4405 as conjugal recipient in liquid medium. It was expected that the observed conjugal transfer frequencies in plasmid-recipient mating combinations: donor pOX38:Cm × recipient; donor pOX38:Cm × recipient pUC19i; and donor pOX38a pUC19i × recipient pUC19i would be approximately equal, as either the control plasmid pOX38 without the ColE7 activity gene or an immune recipient strain (pUC19i – is a pUC19 vector carrying the ColE7 immunity gene) were used. Further, it was expected that the observed conjugal transfer frequency in the mating combination: donor pOX38a × recipient would be significantly lower as in this mating combination the pOX38 with the ColE7 activity gene and a sensitive recipient were used. In this last mating combination, the actual conjugal transfer frequency was expected to be the same as in other three matings; however, viable transconjugants were not expected to be isolated, due to the lethal activity of the plasmid-

encoded colicin E7 gene delivered into the non-immune recipient. The expressed product of the colicin E7 gene would kill any transconjugants, which would lead to a lower conjugal transfer frequency.

The acquired conjugal transfer frequencies of the plasmid pOX38:Cm from the three donor strains MC4100, N4i, and SE15i into the laboratory recipient strain RU4405, performed in liquid medium (protocol I), were not significantly different and were comparatively high. Comparison of the conjugal transfer frequencies of pOX38:Cm into RU4405 and pOX38a into RU4405 from all three donors (MC4100, N4i and SE15i), as well as the frequencies of pOX38a into RU4405 pUC19i from the conjugal donors MC4100 and N4i supported the proposed mechanism of action of the conjugation-mediated colicin E7 delivery system into the recipient strain. In this regard, two important observations could be made. First, a statistically significant decrease in observed conjugal transfer frequencies of pOX38a, as compared to that of pOX38:Cm (both from all three donor strains), suggested that the lethal activity of the plasmid-encoded colicin E7 killed the majority of transconjugants (viable transconjugants could not be isolated). Second, after rendering the recipient strain immune to colicin E7 by introducing a non-conjugative plasmid pUC19i, encoding the ImmE7 colicin E7 immunity protein, into the recipient strain, an increase in conjugal transfer frequency of pOX38a was observed with donor strains MC4100 and N4i. Altogether, the results supported the proposed lethal activity of the conjugal transfer-delivered colicin E7 toxicity into non-immune recipient cells.

The results also showed that introducing pUC19i into the recipient in order to convey ColE7 immunity to the recipient strain immune did not completely restore the conjugal transfer frequency. This indicated that other factors were also affecting the conjugal transfer frequency. One such factor might be the presence of this additional plasmid pUC19i in the recipient cell, as the plasmids are known to be a metabolic burden. Although these results were not expected, they do not disprove the proposed mechanism of action of the conjugal transfer-delivered colicin E7 toxicity into non-immune recipient cells.

Performing conjugation in liquid medium, significant differences in the occurrence of conjugal transfer were observed between laboratory and pathogenic recipient strains. We were able to evaluate the conjugal transfer frequencies from all three donor strains into the laboratory recipient strain RU4405. In contrast, we were not successful in evaluating the conjugal transfer frequencies into the pathogenic recipients following the same experimental protocol. Despite several attempts, we were unable to isolate any transconjugants. The possible reasons for this are discussed below.

Very little is known about the steps required for mating pair formation during conjugation. Early studies revealed that the outer membrane protein OmpA and the core LPS were important components of the recipient cell, provided that mating occurred in liquid culture.

Experiments conducted with mutants in either *ompA* or the locus responsible for the core LPS synthesis showed that neither of the two mutations were completely effective at blocking the F plasmid transfer. Instead, they caused a 100- to 1000-fold decrease in mating efficiency (Achtman et al., 1978b; Havekes and Hoekstra, 1976). Achtman et al. (1978) therefore concluded that neither OmpA nor the core LPS were recognised by the F pilus and suggested that these structures may instead play roles in mating pair stabilisation. Achtman et al. (1978) also concluded that the lack of functional OmpA or the core LPS in the recipient cell could be overcome if the matings were performed on solid media, where the mating pair stabilisation system might play a less important role (Achtman et al., 1978a; Achtman et al., 1978b; Anthony et al., 1994). In 1994, Anthony et al. evaluated the effects of the mutations in *ompA* and the loci responsible for LPS synthesis on the conjugation of pOX38 and other plasmids. They suggested that the initial step in mating pair formation in liquid media might involve interaction of the pilus with the recipient LPS, followed by pilus retraction and interaction of components of the transfer system with OmpA in the recipient cell. During mating on solid surfaces, these initial steps, which are required to bring the donor and recipient cells together in liquid media, would be redundant,, since the cells would be sufficiently close to establish contact.. They also suggested that the contact establishment might involve TraN in the donor cell outer membrane, perhaps interacting with the LPS, as well as TraG, the other *tra* product involved in this process (Anthony et al., 1994). In 2007, Smith et al. suggested that protein TraN, which forms the mating-pair stabilisation system (discussed in chapter 2.6.3), interacted with the OmpA of the recipient to stabilise the mating pair (Smith et al., 2007). Perez-Mendoza and de la Cruz performed a related study in 2009, in which the addressed the question of the recipient cell contribution to the process of conjugation. In a genome-wide screen, they employed an automated conjugation assay to systematically analyse individual contribution of each essential *E. coli* gene to conjugation. They did not find any non-essential *E. coli* genes playing essential roles in conjugation. However, mutations in the LPS synthesis pathway showed drastic inhibition of F-plasmid transfer, but only in liquid matings, supporting the results of the preceding studies. They also concluded that recipient cells could not avoid being used as conjugal recipients and suggested that conjugation acted with little regard to the constitution of the recipient cell (Perez-Mendoza and de la Cruz, 2009).

It is now thought that in the F plasmid conjugation system, the OmpA and certain groups in the LPS are required for successful conjugation as they both interact with TraN (Arutyunov and Frost, 2013). Plasmid-mediated conjugation can be severely limited by the presence of the O-antigen of the LPS as well as capsular polysaccharide on recipient cells (but not donor cells), which are both thought to physically block mating (Anthony et al., 1994).

Based on our observations and careful literature review, we hypothesised that the long O-antigens of the smooth LPS as well as the capsular polysaccharide, which are present in wild-type pathogenic recipient strains used in our experiments, were the limiting factors in

conjugation. They could mask the OmpA or other important components in the outer membrane of the recipient cell and physically prevent it from interacting with the TraN in the outer membrane of the donor strain. This would prevent the mating pair stabilisation system from stabilising the mating pairs in liquid medium and therefore prevent conjugal transfer of the plasmid. We also hypothesised that the capsular polysaccharide, which is thought to physically block mating (Anthony et al., 1994), played an analogous role to the O-antigens of the smooth LPS.

Laboratory strains generally exhibit a rough LPS profile consisting of only low-molecular weight LPS. In contrast, wild type strains can also exhibit smooth LPS profiles. In wild-type strains that produce smooth LPS the distribution of O-antigen chain lengths is usually clearly bimodal, with the majority of O-antigens comprising 10 to 18 O units.

To evaluate the differences in LPS structures of the donor and the recipient strains, SDS-PAGE and oxidative silver staining of the LPS were used. We profiled the LPS of all the donor and recipient strains. While both laboratory strains, MC4100 and RU4405, exhibit rough LPS profiles, all the wild-type strains exhibit bimodal ones: strain N4i exhibits semi-rough LPS (corresponding to the observations reported in literature), while strains SE15i, DL94 and TA131 exhibit clearly bimodal smooth LPS profiles. The observed smooth LPS profile of the strain DL94 is in accordance with the strain's smooth serotype (O21:H−).

The LPS profiles of the recipient strains support our hypothesis on obstruction of contact between the donor and the recipient strain. A significant difference in LPS profiles was observed between the donor strains N4i (semi-rough LPS) and SE15i (smooth LPS), showing the strain specificity of the LPS profiles. Interestingly, the O-antigens of the LPS of the two wild-type donor strains, N4i and SE15i, did not seem to interfere with mating in liquid media. This might be due to the presence of the conjugative pilus, which as discussed earlier, plays a central role in liquid matings. The pilus might be important for protruding through long-chain LPS O-antigens and capsular polysaccharide of the donor cell surface, thus facilitating contact establishment with the recipient cell and bringing the components of mating pair stabilisation system of the donor and the recipient cells in close proximity. Further analysis should be carried out to support these observations and conclusions.

Capsular polysaccharide profiling revealed additional differences between strains. While we did not detect any extracellular polysaccharides in laboratory strains MC4100 and RU4405, all other strains were shown to possess capsular polysaccharides of different molecular weights. Due to difficulties experienced throughout staining and consequentially inferior quality of the profiles, further analysis has to be carried out before any conclusions can be drawn regarding the effects of capsular polysaccharide on the conjugal plasmid transfer.

Based on the above stated observations, the experimental protocol for the assessment of the conjugal transfer frequency was modified. Conjugation was performed at higher cell densities, allowing mating aggregation to occur more efficiently. Instead of liquid LB, we performed conjugation on LB plates in an attempt to minimalize the need for efficient mating pair stabilisation. In addition, the time available for conjugal transfer to occur was increased. Following this protocol, we were able to isolate transconjugants of pathogenic recipient strains.

On solid medium, we were able to evaluate the frequencies of conjugation into wild-type recipient strains. There were some differences among the donor as well as among the recipient strains. Overall, the observed conjugal transfer frequencies were rather low (10^{-7}–10^{-5}). Comparison of both experimental protocols for the assessment of the conjugal transfer frequencies has shown that if the plasmid transfer occurs and transconjugants can be isolated following both protocols, the acquired results following both protocols are not significantly different.

Results of the conjugal transfer frequency assessments also indicate that the lethal activity of the conjugation-mediated colicin E7 delivery system is efficient against laboratory as well as against pathogenic strains. Using laboratory strain RU4405 as the conjugal recipient, donor strain N4i was the only donor with which no pOX38a transconjugants could be isolated in any of the matings. Using pathogenic recipient strains DL94 and TA131, donor strain N4i was the most efficient as well, as no pOX38a transconjugants were isolated in any of the matings, in contrast to donor strain SE15i, where a small number of TA131 pOX38a transconjugants were isolated from plate mating experiments. Donor strain MC4100 was inefficient against recipient TA131, as no significant differences were observed between frequencies of pOX38:Cm and pOX38a.

As we observed relatively low conjugal transfer frequencies into pathogenic recipient strains, we attempted to raise the frequency by overexpressing *traJ*, the main transcriptional regulator of the *tra* region, by introducing an additional copy of the plasmid pGZ-encoded *traJ* into the donor strains. It was expected that overexpression of *traJ* would result in higher levels of expression of the *tra* region, synthesis of additional pili and thus raised conjugal transfer frequency. We did not observe any significant change in conjugal transfer frequency after introducing pGZ into the donor strains, while the positive controls showed significant increase in conjugal transfer frequency using donor strains overexpressing *traJ*, compared to control donors without additional *traJ* copies present.

The conjugative ability of the F plasmid is extremely growth phase dependent: plasmid transfer efficiency drops rapidly as donor cells progress through the growth cycle towards stationary phase. The factors responsible for this decline in mating ability have not yet been fully explained, but they appear to be sensitive to the physiological state of the host cell (Will

et al., 2004). F-pilus synthesis increases through exponential growth and reaches the maximal level in early stationary phase. In stationary phase, the F-pilus synthesis and mating efficiency decrease to undetectable levels and the transfer ability of the donor is lost, so that the donor cells can act as recipients in $F^+ \times F^+$ matings and thus behave as F^- phenocopies (Frost and Manchak, 1998; May et al., 2010). As cells enter stationary phase, the host nucleoid-associated protein H-NS acts to repress the expression of *traM* and *traJ*. H-NS is a 15.4 kDa cytoplasmic protein that preferentially binds to segments of intrinsically curved DNA. However, at high concentrations *in vitro*, it is capable of binding non-specifically to extended segments of DNA. Because of this, H-NS is thought to act as a transcriptional silencer that binds preferentially to a region of curved DNA and then proceeds along the DNA, thereby repressing nearby promoters. The H-NS mediated repression of the *tra* region expression appears to be crucial in down-regulating F plasmid transfer as the host cell enters stationary phase (Owen-Hughes et al., 1992; Rimsky et al., 2001; Will et al., 2004; Williams and Rimsky, 1997). The occurrence of F^- phenocopies could hence result in redundant conjugal plasmid transfer between donor cells and this way decrease the number of true recipient cells receiving the plasmid, affecting the conjugal transfer frequency.

Restriction-modification systems of the recipient cell present another barrier to successful plasmid conjugation. These systems consist of a restriction endonuclease, which recognises a specific nucleotide sequences prior to DNA cleavage, and a corresponding modification enzyme – a DNA methyltransferase, which modifies the DNA by methylation of specific adenine or cytosine residues within the specificity site of the restriction endonuclease, thus protecting the DNA from degradation. These enzymes operate on double stranded DNA molecules. Hemimethylated DNA, as generated by replication of fully methylated DNA, is protected from cleavage and represents a substrate for the modification enzyme. Restriction-modification systems have evolved as a cellular defence mechanism against entry of foreign DNA, which is recognised as such by lack of specific methylation. Any plasmid DNA entering the recipient cell from a donor using a different type of restriction-modification system will not be appropriately modified by methylation and will thus be degraded (Wilkins, 2002). Although the DNA that is transferred during conjugation is single stranded and is thus believed to be resistant to cleavage, it is subsequently converted to a double stranded molecule that can be then either protected or restricted by the host's own restriction-modification system (Filutowicz, 2008). In addition, several restriction endonucleases of different types have been shown to cleave single stranded DNA molecules (Bischofberger et al., 1987; Nishigaki et al., 1985; Reckmann and Krauss, 1987). Laboratory strains, in contrast to most wild-type strains, usually have mutated restriction-modification systems, which allow them to be efficiently used in cloning experiments. It is thus possible that the conjugal donor and conjugal recipient strains that we used have different restriction-modification systems, which degrade the plasmid DNA upon entry into the recipient cell.

To test whether restriction-modification systems inhibit successful conjugation, we carried out a mating experiment employing strain *E. coli* Nissle 1917 as both the conjugal donor and conjugal recipient. In such an experimental setting, the restriction-modification systems in both the donor and the recipient strains are the same and therefore cannot affect conjugal DNA transfer. The results have shown that frequencies of pOX38 in this experiment were not different or were even significantly lower than any of the observed frequencies of pOX38:Cm into RU4405, DL94 and TA131. This result indicates that other factors besides restriction-modification systems may obstruct conjugation in wild-type recipients.

Restriction-modification systems are not the only mechanism by which bacteria protect themselves from potentially harmful foreign DNA invasion. Additional systems that could present a challenge for conjugative plasmid DNA migration from donor to recipient cells are prokaryotic CRISPR-Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated) immune systems. These systems are widespread adaptive and heritable immune systems that specifically degrade non-self DNA. A universal property of CRISPR-Cas systems is that they make use of a genomic CRISPR locus for integration of short sequences derived from invader genetic elements. These invader sequences (spacers) are separated from each other by host-derived repeating sequences of approximately the same size. The acquisition of new spacer sequences during the CRISPR-adaptation stage provides resistance against genetic elements containing cognate sequences. CRISPR-Cas systems function by employing RNA transcripts of the invader sequences to recognise and degrade invader DNA, and in this way provide protection against potentially harmful mobile genetic elements (Westra et al., 2013). CRISPR systems appear to be active against all forms of invading DNA and have been reported to mediate resistance against conjugative plasmids (Marraffini and Sontheimer, 2008). However, a study by Westra et al. indicates that single stranded DNA may not be a target for at least some types of CRISPR-Cas systems (Westra et al., 2013).

A wide range of biotic and abiotic factors also affect conjugal transfer of plasmids. Such factors are, in addition to the above stated, the initial cell density of the donor and recipient strains, the ratio of donor to recipient strain cells, the amount of nutrients available, pH and temperature of the environment, and mating time (Fernandez-Astorga et al., 1992). An additional problem is also the possibility of erroneously interpreting non-detectable levels of conjugal transfer as inhibition or absence of conjugal gene transfer. Furthermore, comparison of the results obtained to those published in literature is difficult, as different authors employ different methodologies to evaluate conjugal transfer frequency and calculate it in different ways.

5.1 CONCLUSION

The results of our work suggested that the proposed mechanism of action of the conjugation mediated colicin E7 based toxicity delivery system was functional. Employing laboratory strain MC4100 as conjugal donor and laboratory strain RU4405 as conjugal recipient, we have shown that the pOX38a-encoded colicin E7 efficiently killed recipient cells, and that the conjugation between these two strains occured equally well in liquid and on solid media. We observed significant differences between different laboratory and wild-type strains in the ability to act as conjugal recipients. Conjugal transfer into wild-type pathogenic recipients did not take place under the same experimental conditions as into the laboratory recipient strain used. Results suggested that the genetically modified donor strain Nissle 1917 (strain N4i) was somewhat more efficient in killing pathogenic strains than donor strain SE15i. Attempts to raise the conjugal transfer frequency were not successful. Evaluation of the LPS profiles and capsular polysaccharides of the donor and recipient strains implied significant differences in the composition of the outer membrane of the strains. Considerably higher molecular weight LPS, as well as the presence of capsular polysaccharides, of the wild-type recipient strains could interfere with the contact establishment between the conjugal donor and recipient strains. Further research is needed in order to evaluate and verify different aspects of *in vivo* activity of the genetically modified colicinogenic probiotic strain *Escherichia coli* Nissle 1917 pOX38a.

Conjugation is a ubiquitous mechanism of horizontal gene transfer, present in a wide range of species belonging to all three domains of life. Understanding the underlying mechanisms of conjugal transfer of genetic material is very important from medical, biotechnological, and ecological points of view. Plasmids delivered to new hosts by conjugation could be effective antimicrobial agents in prevention and treatment of infections with multi-drug resistant pathogens.

6 SUMMARY

Probiotic bacteria are non-pathogenic and non-toxic bacteria that exert a positive effect on the health of their hosts (FAO/WHO, 2006; Guarner and Schaafsma, 1998). Probiotic bacterial strains have been used in treatment of different intestinal diseases of infectious as well as non-infectious origin. Among the most well-characterised *Escherichia coli* probiotic strains is strain Nissle 1917, whose positive effects and biosafety have been continuously proven since its isolation in 1917 (Jonkers et al., 2012; Saad et al., 2013; Sonnenborn and Schulze, 2009). As multi-drug resistant bacteria pose an emerging risk to public health, alternative antibacterial agents are urgently needed. A genetically modified conjugative plasmid encoding the gene for colicin E7, which possesses DNase activity, has been engineered in the Molecular Genetics and Microbiology Research Group at the Biotechnical faculty of University of Ljubljana (Petkovšek, 2012). Upon conjugal transfer of this plasmid into the recipient strain, the gene encoding the colicin E7 is expressed and its lethal activity occurs, leading to death of the recipient strain. By taking advantage of this conjugal transfer-mediated toxin delivery system, the need for intact colicin receptors on the surface of the recipient strains can be circumvented (Petkovšek, 2012). Donor strains harbouring this conjugative plasmid could be used as probiotic strains in treating and preventing infections with pathogenic strains of *Escherichia coli*.

The aim of this thesis was to evaluate the conjugal transfer frequencies of the plasmid pOX38 with the colicin E7 gene between different laboratory, commensal, and pathogenic strains of *Escherichia coli* used as both conjugal donors and conjugal recipients. Conjugal transfer frequencies of plasmids pOX38:Cm and pOX38a were assessed with laboratory *E. coli* strain MC4100 and two commensal *E. coli* strains N4i and SE15i as conjugal donors, and laboratory *E. coli* strain RU4405 and two pathogenic *E. coli* strains DL94 and TA131 as conjugal recipients. Strain DL94 was isolated from a urinary tract infection, and the strain TA131 was isolated from a patient with a skin and soft tissue infection. Possible reasons for the variations in conjugal transfer frequencies between different mating pairs were explored by LPS and capsular polysaccharide profiling of conjugal donor and recipient strains. Conjugal transfer frequencies were assessed by mating in liquid and solid media. LPS profiling was performed using tris-glycine SDS-PAGE and silver staining. Capsular polysaccharide profiling was performed using tris-glycine SDS-PAGE and alcian blue staining.

The acquired conjugal transfer frequencies of the plasmids pOX38:Cm and pOX38a (encoding the colicin E7 gene) from the three donor strains MC4100, N4i, and SE15i into the laboratory recipient strain RU4405 were comparatively high. Comparison of the conjugal transfer frequencies of pOX38:Cm into RU4405, pOX38a into RU4405, and pOX38a into RU4405 pUC19i from all three conjugal donors supported the proposed mechanism of action of the conjugation-mediated colicin E7 delivery system into the recipient strain. Significant

differences in the occurrence of conjugal transfer were observed between laboratory and pathogenic recipient strains. We were able to evaluate the conjugal transfer frequencies from all three donor strains into the laboratory recipient strain RU4405, where the observed conjugal transfer frequency was approximately 10^{-2}. In contrast, we were not successful in evaluating the conjugal transfer frequencies into the pathogenic recipients following the same experimental protocol. We hypothesised that the long O-antigens of the smooth LPS, present in wild-type pathogenic recipient strains used in our experiment, could mask important components in the outer membrane of the recipient cell. This would physically prevent them from interacting with the components in the outer membrane of the donor strain important for contact establishment prior to conjugation in liquid medium. On solid medium, we were able to evaluate the frequencies of conjugation into wild-type recipient strains. There were some differences among the donor as well as among the recipient strains. Overall, the observed conjugal transfer frequencies were rather low (10^{-7}–10^{-5}). The commensal donor strain N4i showed to be the most efficient in killing both laboratory and pathogenic recipients, as no pOX38a transconjugants were isolated in any of the matings, in contrast to donor strain SE15i, where at least a small number of pOX38a transconjugants were isolated in all mating experiments. The results of LPS profiling showed significant differences between the laboratory and the wild-type strains. While both laboratory strains, MC4100 and RU4405, exhibited rough LPS profiles, all the wild-type strains exhibited bimodal smooth LPS-profiles. Capsular polysaccharide profiling also revealed some differences between strains. While the laboratory strains MC4100 and RU4405 lacked high molecular weight extracellular polysaccharides, we could detect such polysaccharides in all wild-type strains, namely N4i, SE15i, DL94 and TA131.

The results of our work suggest that the proposed mechanism of action of the conjugation mediated colicin E7 based toxicity delivery system is functional. We observed significant differences between different laboratory and wild-type strains in the ability to act as conjugal recipients. Further research is needed in order to evaluate and verify different aspects of *in vivo* activity of the genetically modified colicinogenic probiotic strain *Escherichia coli* Nissle 1917 pOX38a. Plasmids delivered to new hosts by conjugation could be effective antimicrobial agents in prevention and treatment of infections with multi-drug resistant pathogens.

7 POVZETEK

Escherichia coli (*E. coli*) je po Gramu negativna fakultativno anaerobna gibljiva bakterija iz družine *Enterobgacteriaceae*. Pri človeku in živalih je zelo pogosto prisotna kot komenzal v gastrointestinalnem traktu. Vrsto *E. coli* sestavlja veliko različnih biotipov; večina sevov je nepatogenih komenzalov, ki gostitelju služijo predvsem kot zaščita pred kolonizacijo gastrointestinalnega trakta s strani patogenih bakterijskih sevov. Nekateri sevi *E. coli* so patogeni in jih prištevamo med najpomembnejše povzročitelje črevesnih kot tudi zunajčrevesnih infekcij pri človeku. Določeni sevi bakterije *E. coli* so v uporabi tudi kot probiotiki (Madigan in Martinko, 2006; Maltby in sod., 2013).

Plazmidi so ekstrakromosomski elementi DNA z avtonomnim podvajanjem (del Solar in sod., 1998). Skupaj z bakteriofagi, integroni, transpozoni, integrativnimi konjugativni plazmidi, kamor prištevamo konjugativne transpozone, in drugimi sorodnimi elementi sestavljajo skupino mobilnih genetskih elementov in so pomembni kot vektorji horizontalnega genskega prenosa ter tudi kot nepogrešljivo orodje v genskem inženiringu (Garcillan-Barcia in sod., 2011; Smillie in sod., 2010). Omogočajo prenos genskega materiala med različnimi gostitelji, preko mehanizmov transpozicije in rekombinacije pa lahko tudi spreminjajo gostiteljev genom. Nekateri plazmidi omogočajo prenos DNA tudi v evkariontske organizme (del Solar in sod., 1998; Llosa in sod., 2002). Za gostitelja so izredno pomembni, saj lahko imajo gene rezistenc proti antibiotikom, težkim kovinam, sevanju, virulentne faktorje, dodatne metabolne poti, gene za sintezo bakteriocinov in tudi gene za mnogo drugih lastnosti, ki gostitelju zagotavljajo prednost (del Solar in sod., 1998; Kado, 1998). Prisotni so v veliki večini po Gramu negativnih in po Gramu pozitivnih bakterij in so po svoji velikosti in genetski strukturi zelo raznoliki. Veliki so od 300 do 2400 bp in so najpogosteje prisotni kot krožne molekule DNA, nekateri plazmidi pa se pojavljajo tudi kot linearne molekule DNA (Kado, 1998). Plazmidi so v celicah prisotni v različnem številu kopij: nekateri so prisotni le v 1 ali nekaj kopijah, drugi so v celici lahko prisotni tudi v več kot 100 kopijah. Število kopij plazmida v celici določajo tako geni kodirani na plazmidu, kot tudi lastnosti gostiteljske celice (Madigan in Martinko, 2006). Glede na njihovo mobilnost lahko plazmide razdelimo na konjugativne plazmide, ki kodirajo celoten nabor genov, potreben za konjugacijo in se lahko prenesejo v nove gostiteljske celice; mobilizirajoče plazmide, ki ne kodirajo celotnega nabora genov, potrebnih za konjugacijo in se tako lahko v nove gostitelje celice prenesejo le ob prisotnosti drugega konjugativnega plazmida, ki jim zagotovi potrebne gene za konjugacijo; in na ne-konjugativne plazmide, ki se v nove gostitelje ne morejo prenesti ne s konjugacijo ne z mobilizacijo, ampak le z naravno transformacijo ali transdukcijo (Smillie in sod., 2010).

Bakterijska konjugacija je proces, pri katerem se DNA prenese iz donorske v recipientsko celico po vzpostavitvi fizičnega kontakta med njima. Vsaka bakterija, ki ima konjugativni plazmid, je potencialni donor, in vsaka, ki ga nima, je potencialni recipient (Madigan in

Martinko, 2006; Russell, 2006; Snyder in Champness, 2003; Willets in Skurray, 1980; Willetts in Wilkins, 1984) . Pri po Gramu negativnih bakterijah se konjugacija začne s sintezo konjugativnega pila na površini donorske celice, s katerim donor prepozna in se pritrdi na recipientsko celico. Po pritrditvi se konjugativni pil skrajša in celici privede bližje skupaj. V naslednjih korakih se med celicama vzpostavi konjugativna pora, preko katere se ena veriga plazmidne DNA skupaj s proteinom relaksazo prenese v recipientsko celico. Po prenosu v obeh celicah pride do sintez komplementarne verige plazmidne DNA. Recipientsko celico, ki je s konjugacijo sprejela konjugativni plazmid, imenujemo transkonjuganta (Arutyunov in Frost, 2013; Snyder in Champness, 2003). Bakterijska konjugacija je široko prisoten proces in je skupaj s transformacijo in transdukcijo eden izmed treh glavnih mehanizmov horizontalnega genskega prenosa. Z medicinskega vidika je zelo pomembna zaradi širjenja virulentnih faktorjev in antibiotskih rezistenc med bakterijskimi populacijami, zanimiva pa je tudi z biotehnološkega in ekološkega vidika (Smillie in sod., 2010).

Plazmid F je 100 kb velik konjugativni plazmid, prisoten v sevu *E. coli* K-12 divjega tipa in je prvi odkriti konjugativni plazmid (Frost in sod., 1994; Snyder in Champness, 2003). Spada v inkompatibilnostno podskupino IncFI in skupaj z drugimi plazmidi inkompatibilnostne skupine IncF tvori skupino velikih plazmidov z ozkim gostiteljskim območjem, ki so prisotni v družini *Enterobacteriaceae* (Frost in sod., 1994; Mulec in sod., 2002; Wong in sod., 2012). Geni, potrebni za avtonomno replikacijo plazmida F, se nahajajo v replikacijski regiji RepFIA, ki je glavna replikacijska regija plazmida F. Plazmid F poseduje tudi replikacijsko regijo RepFIB, ki je od RepFIA neodvisna in lahko v celici vzdržuje plazmid tudi v odsotnosti regije RepFIA. Poleg teh dveh replikacijskih regij ima plazmid tudi nepopoln ostanek replikacijske regije RepFIC, ki je prekinjena s sekvenco γδ (transpozon Tn*1000*) in je nefunkcionalna. Poleg tega plazmid ima še dve insercijski sekvenci IS*3* ter eno insercijsko sekvenco IS*2* (Firth in sod., 1996).

Glavna značilnost plazmida F je njegova 33,3 kb velika regija *tra*, v kateri so kodirani vsi geni potrebni za konjugatvni prenos plazmida iz donorske v recipientsko celico. Regijo *tra* sestavljajo trije operoni, dva monocistronska in en policistronski, ki se prepisujejo iz treh promotorjev. Poleg genov, kodiranih v regiji *tra*, je za konjugacijo potreben še element *oriT*, ki se nahaja navzgor od regije *tra* in vsebuje mesto začetka prenosa DNA iz donorja v recipienta (Frost in sod., 1994). Transkripcija regije *tra* je primarno regulirana s štirimi elementi: *traJ, finO, finP* in promotorjem P_{traY}. Glavni regulator konjugacije je 27 kDa protein TraJ, ki je potreben za začetek prepisovanja genov regije *tra* s promotorja P_{traY}. Njegova aktivnost je regulirana z dvokomponentnim inhibicijskim sistemom FinOP. Gen *finP* kodira 79 nt protismiselno RNA, komplementarno 5′ neprevedenemu koncu *traJ* mRNA. Protismiselna RNA *finP* se tako poveže z mRNA za TraJ, nastane dvoverižna DNA, ki jo razgradi RNaza III. Na ta način je preprečena sinteza TraJ. FinO je 21,2 kDa velik protein, ki deluje kot šaperon RNA in stabilizira RNA *finP* tako, da jo zaščiti pred razgradnjo

z RNazo E. Na ta način FinO podaljša življenjsko dobo *finP* in pospešuje formacijo kompleksov *finP-traJ* mRNA. *finP* torej negativno regulira izražanje TraJ le v prisotnosti FinO (Jerome in sod., 1999; Starčič Erjavec, 2003). V večini naravno prisotnih plazmidov podobnih plazmidu F, je nivo represije regije *tra* močno odvisen od koncentracij FinO in *finP*. Taki plazmidi se z visoko frekvenco prenašajo le kratek čas po vstopu v recipientsko celico; kasneje je ekspresija genov regije *tra* nestalna in konjugacija poteka le sporadično. V plazmidu F pa je gen *finO* prekinjen z insercijsko sekvenco IS*3*, kar vodi v konstitutivno izražanje *traJ*, derepresijo izražanja genov regije *tra* in konstitutivno sintezo konjugativnih pilov (Frost in sod., 1994).

Konjugacijski aparat plazmidov podobnih F je sestavljen iz dveh med seboj povezanih sistemov, ki opravljata ključni vlogi pri prenosu konjugativne DNA iz donorske v recipientsko celico. Prvi sistem, ki sestavlja konjugacijski aparat, je DNA-procesivni kompleks oz. relaksosom, ki se po začetku konjugacije sestavi na mestu *oriT* na konjugativnem plazmidu in pred prenosom v recipienta podvoji plazmidno DNA po mehanizmu kotalečega se kroga. Glavna komponenta relaksosoma je relaksaza, ki reže DNA v mestu *oriT* in iniciira prenos DNA. Drugi za konjugacijo pomemben sistem pa je podtip sistema izločanja tipa IV, ki se imenuje transferosom. Transferosom je sestavljen iz kompleksa, ki v bakterijski celični steni tvori trasportni kanal za prenos DNA iz donorske v recipientsko celico, in iz pilov na površini celice, ki posredujejo kontakt med donorsko in recipiensko celico. Oba sistema med seboj povezuje povezovalni protein T4CP (type IV coupling protein) (Arutyunov in Frost, 2013; de la Cruz in sod., 2010; Lawley in sod., 2003; Llosa in sod., 2002; Smillie in sod., 2010).

Poleg dveh že omenjenih sistemov v konjugaciji igrajo pomembno vlogo tudi s konjugacijo povezani sistemi. Sistem Mps (mating pair stabilisatoin) skrbi za stabilizacijo povezave med donorsko in reipientsko celico in pri nekaterih konjugacijskih sistemih zagotavlja enako učinkovit prenos DNA v tekočinah in na trdnih površinah. Glavna sestavna dela sistema Mps sta proteina TraG in TraN, ki interagirata s površino recipientske celice in pomagata stabilizirati med njima vzpostavljeni kontakt. Sistem Sfx (surface exclusion) preprečuje vzpostavitev konjugativnega kontakta med dvema F^+ celicama, njegov glavni sestavni del je protein TraT. Sistem Eex (entry exclusion) pa preprečuje prenos DNA konjugativnega plazmida med dvema F^+ celicama. Njegov glavni sestavni del je protein TraS. Če pride do vzpostavitve kontakta med dvema F^+ celicama, se TraS recipientske F^+ celice poveže s TraG (ki je sicer sestavni del kompleksa Mps) donorske F^+ celice in prepreči sintezo DNA v donorski celici in njen prenos v recipientsko F^+ celico (Achtman in sod., 1997; Arutyunov in Frost, 2013; Audette in sod., 2007; Kingsmann in Willetts, 1978).

Celično steno po Gramu negativnih bakterij sestavlja več strukturno in funkcionalno različni slojev. Citoplazmo obdaja citoplazemska membrana, ki je najbolj notranja plast celične stene. Sledi ji peptidoglikan, ki se nahaja v periplazemskem prostoru, zunanji sloj pa

predstavlja lipopolisaharid (LPS). LPS pogosto prekrivajo dodatni sloji ekstracelularnih polisaharidov, kot so kapsule in drugi izločeni polisaharidi.

LPS je sestavljen iz treh strukturnih enot: v zunanji del zunanje membrane je usidran s hidrofobnim lipidom A, ki je sestavljen iz verig maščobnih kislin, vezanih na skupino dveh fosforiliranih *N*-acetilglukozaminov. Na lipid A je kovalentno vezan oligosaharid sredice LPS, nanj pa je pri nekaterih sevih vezan še zunanji O-antigen. O-antigen je zelo raznolika veriga polisaharidov, sestavljena iz ponavljajočih se O enot. Vsaka O enota je sestavljena iz enega od osmih molekul saharidov, v O-antigen pa je običajno lahko povezanih do 50 O enot. Lipid A je strukturno najbolj ohranjena regija LPS, v sredici je variabilnost omejena na nekaj različic, O-antigen pa je visoko variabilen in je osnova za določevanje serotipov *E. coli*. LPS sestavljen le iz lipida A in sredice imenujemo »rough« LPS (R-LPS), LPS, ki ga sestavlja tudi O-antigen, pa imenujemo »smooth« LPS (S-LPS) (Alexander in Rietschel, 2001; Caroff in Karibian, 2003; Klein in sod., 2009; Le Brun in sod., 2013; Reeves in sod., 1996; RIetschel in sod., 1994).

Pri sevih s S-LPS je število v O-antigen povezanih O enot značilno za vsak posamezen sev. Pri večini sevov se to število giblje med 10 in 18, lahko pa je tudi večje ali manjše. V sevih s S-LPS pa O-antigen ni vezan na vse molekule LPS, ki so prisotne v zunanji membrani. Običajno je v takih sevih v membrani prisotno tudi visoko število molekul LPS, pri katerih je v O-antigen vezana le ena O enota. Število molekul, ki imajo v O-antigen vezani dve, tri ali več O enot, se postopoma zmanjšuje, tako da v molekul LPS z dolžino O-antigena okrog 15 enot navadno ne zaznamo. Količina LPS molekul z O-antigenom, ki ga sestavlja še več O enot nato skokovito naraste in doseže svoj maksimum pri številu v O-antigen vezanih O enot, značilnih za posamezni sev, nad tem pa strmo upade. Večina sevov ima tako značilno bimodalno razporeditev dolžin O-antigenov v svojem LPS, poznamo pa tudi trimodalne in druge manj pogoste razporeditve (Aucken in Pitt, 1993; Franco in sod., 1998; Goldman in Lieve, 1980; Goldman in Hunt, 1990; Grossman in sod., 1987; Palva in Makela, 1980; Schnaitman in Klena, 1993).

Sestavo LPS različnih bakterijskih sevov lahko enostavno opazujemo z metodo SDS-PAGE, ki ji sledi oksidativno barvanje LPS s srebrom. Pri tej metodi LPS najprej ekstrahiramo iz kulture seva, nato pa molekule LPS s SDS-PAGE ločimo po velikosti. Manj O enot kot je vezanih v O-antigen LPS, hitreje celotna molekula LPS potuje v poliakrilamidnem gelu. Po elektroforezi sledi oksidativno barvanje LPS s srebrom. Pri tej metodi najprej s perjodovo kislino hidroksilne skupine sladkorjev v LPS oksidiramo v aldehidne skupine. Aldehidne skupine pa lahko nato selektivno detektiramo z dodatkom srebrovih ionov, ki aldehidne skupine oksidirajo do karboksilnih, pri tem pa se tvori elementarno srebro, ki je v poliakrilamidnem gelu vidno v obliki temno rjavih do črnih depozitov. Po barvanju je na gelu viden profil LPS bakterijskega seva. Iz števila lis na gelu lahko ocenimo število O enot, vezanih v O-antigen, iz razmika med lisami pa lahko primerjalno ocenimo velikost O enot,

vezanih v O-antigen. Metoda nam omogoča identifikacijo sevov z R-LPS in s S-LPS in relativno primerjavo različnih S-LPS.

Poleg LPS lahko celično steno tvorijo tudi ekstracelularni polisaharidi, ki so lahko na površino celice vezani v obliki kapsule ali izločeni v okolje v obliki sluzi. Kapsularni polisaharidi predstavljajo K-antigen *E. coli* (Goldman in sod., 1982; MacLahlan in sod., 1993; Reeves in sod., 1996; Whitfield in Roberts, 1999). Podobno kot LPS lahko tudi kapsularne in druge izvencelične polisaharide opazujemo z metodo ločevanja molekul s SDS-PAGE, ki ji sledi barvanje z alcianskim modrilom. Večina izvenceličnih polisaharidov vsebuje karboksilne skupine, ki so v vodnih raztopinah deprotonirane in imajo negativni naboj. Alciansko modrilo pa je kationsko barvilo, ki se selektivno veže na negativno nabite karboksilne skupine polisaharidov in jih v poliakrilamidnem gelu tako obarva modro. Po barvanju so na gelu vidne široke modre lise, ki predstavljajo polisaharide celične stene in kapsule.

LPS in polisaharidi celične stene bi lahko igrali pomembno vlogo v konjugaciji plazmidov podobnih F. Nekateri avtorji predpostavljajo, da so strukture v sredici LPS in morebitni proteini v zunanji membrani, kot je na primer OmpA, pomembne strukture recipientske celice za stabilizacijo kontakta med celicama (Mps) med konjugacijo (Achtman in sod., 1978a; Achtman in sod., 1978b; Anthony in sod., 1994; Arutyunov in Frost, 2013; Havekes in Hoekstra, 1976; Smith in sod., 2007; Perez-Mendoza in de la Cruz,a 2009).

Patogeni bakterijski sevi z večkratnimi odpornostmi na različna protimikrobna sredstva predstavljajo vedno večjo grožnjo zdravju ljudi, zato se je pojavila potreba po alternativnih antibakterijskih agensih. Zelo zanimiva skupina snovi s protibakterijskim delovanjem so bakteriocini, ribosomsko sintetizirane molekule, ki jih proizvaja široka paleta bakterijskih vrst. Pri *E. coli* lahko ločimo dva tipa bakteriocinov, in sicer bakteriocine z visoko molekulsko maso (25 do 85 kDa), ki jih imenujemo kolicini, in tiste z nizko molekulsko maso (< 10 kDa), ki jih imenujemo mikrocini (Budič in sod., 2011). Sinteza bakteriocinov je med sevi *E. coli* široko razširjena lastnost. Najpogosteje so geni za sintezo kolicinov zapisani na kolicinogenih plazmidih pCol. Delovanje kolicinov je omejeno na ozko sorodne organizme rodov *Escherichia*, *Salmonella* in *Shigella*, saj je za vstop kolicina v celico potrebna prisotnost speifičnega površinskega receptorja, ki omogoča vezavo in translokacijo kolicina skozi celično membrano v notranjost celice. Glede na mehanizem delovanja lahko kolicine delimo na ionoforne kolicine, ki ustvarijo poro v notranji celični membrani in s tem povzročijo uhajanje ionov, ter na nukleazne kolicine, katerih tarča je nukleinska kislina v citoplazmi, ki jo ti kolicini razgradijo. Celice, ki producirajo kolicine, so pred delovanjem lastnih kolicinov zaščitene z geni, ki kodirajo proteine imunosti za posamezne kolicine (Cascales in sod., 2007; Chai in Foulds, 1977; Chai in Foulds, 1979; Chak in sod., 1991; Di Masi in sod., 1973).

Kljub temu, da so kolicini kot protimikrobne snovi primerni za uporabo proti patogenim sevom za kolicine občutljivih rodov, pa obstaja nekaj zadržkov pri uporabi kolicinov kot alternativnih protimikrobnih snovi. Prav tako kot kolicini, je tudi neobčutljivost nanje med sevi *E. coli* zelo široko razširjena lastnost. Tudi na kolicine občutljivi sevi pa lahko hitro pridobijo mutacije, s katerimi spremenijo strukturo površinskih receptorjev, potrebnih za vstop kolicinov v notranjost celice. Poleg tega so zapisi za sintezo kolicinov pogosto prisotni na velikih konjugativnih plazmidih, ki poleg genov za sintezo kolicinov imajo tudi gene za različne virulentne dejavnike (Petkovšek, 2012). Eden izmed možnih načinov, da se izognemo problemom s pojavljanjem neobčutljivosti za kolicine preko spremembe površinskih receptorjev, je uporaba na bakterijski konjugaciji temelječih tehnologij za doseganje toksičnega učinka na recipientsko celico (Filutowicz in sod., 2008). Možna sta dva pristopa, za katera je oba značilno, da plazmid na recipientsko celico deluje letalno in jo vodi v propad. Na konjugativnem plazmidu so tako lahko prisotne mutacije v genih, ki skrbijo za regulacijo replikacije plazmida, kar po vstopu v recipientsko celico vodi v prekomerno podvajanje pazmida. To v končni fazi izčrpa vse celične metabolne vire in celico vodi v propad. Druga možnost pa je zapis genov s toksičnimi produkti na konjugativnih plazmidih, ki se po vstopu v recipienta začnejo izražati in celico vodijo v propad. Pri obeh strategijah je donorsko celico, ki ima konjugativni plazmid, treba zaščititi pred škodljivim delovanjem plazmida (Filutowicz in sod., 2008).

Na Katedri za molekularno genetiko in biologijo mikroorganizmov Oddelka za biologijo Biotehniške fakultete Univerze v Ljubljani so pripravili gensko spremenjen probiotični sev *E. coli* Nissle 1917. Sev ima konjugativni plazmid pOX38 z zapisom za sintezo kolicina E7, ki ima DNazno aktivnost, v kromosom pa je vstavljen gen, ki kodira protein imunosti proti kolicinu E7 in sevu zagotavlja zaščito pred toksičnim genom, kodiranim na plazmidu (Petkovšek, 2012). Po konjugativnem prenosu plazmida v recipientsko celico, se v njej sintetizira kolicin E7, ki s svojo DNazno aktivnostjo razgradi recipientovo DNA, kar vodi v propad celice. Na ta način lahko uporabljamo kolicine kot protimikrobne snovi tudi proti sevom, ki na svoji površini nimajo receptorjev za vstop kolicina v celico. Donorski sevi, ki imajo tak konjugativni plazmid, bi se lahko uporabljali kot probiotični sevi za preprečevanje ali zdravljenje okužb s patogenimi sevi *E. coli*.

Cilj magistrske naloge je oceniti frekvence konjugacije plazmida pOX38 z genom za kolicin E7 med različnimi laboratorijskimi, komenzalnimi in patogenimi sevi *E. coli*. Pripravili smo sevu N4i 1917 pOX38a soroden sev SE15i pOX38a in ocenili frekvence konjugacije in učinkovitost protimikrobnega delovanja sistema dostave toksičnega gena v recipientsko celico. Z ugotavljanjem strukture LPS in prisotnosti kapsularnih polisaharidov smo poskušali razjasniti nekatere vzroke za variabilnost v frekvencah konjugacije med različnimi recipientskimi sevi.

Frekvence konjugacij med izbranimi donorskimi in recipientskimi sevi smo ocenili v tekočem in na trdnem gojišču. Frekvenco konjugacije smo izračunali kot razmerje med titroma transkonjugant in recipientskih celic ob koncu poskusa. Statistično analizo rezultatov smo izvedli z analizo variance (ANOVA). V tekočem gojišču smo titra transkonjugant in recipientskih celic ugotavljali po 30 min skupne inkubacije, na trdnem gojišču pa po 4 h skupne inkubacije donorskega in recipientskega seva. Kot donorje smo uporabili laboratorijski sev MC4100 ter komenzalna seva N4i in SE15i, kot recipiente pa smo uporabili laboratorijski sev RU4405 in klinična izolata DL94 iz okužbe sečne poti in TA131 iz okužbe mehkih tkiv. Pred začetkom poskusov smo v obeh patogenih izolatih, DL94 in TA131, z metodo PCR potrdili odsotnost replikacijskih regij RepFIA, RepFIB, RepFIC in RepFIIA, ter genov *traJ* in *traT*, ki sta oba del za konjugacijo esencialne regije *tra*. Na ta način smo v obeh sevih zagotovili odsotnost konjugativnih plazmidov podobnih F, kar omogoča plazmidu pOX38 konjugacijski prenos v celico in replikacijo v bakterijski celici. Frekvence konjugacij med laboratorijskima donorjem in recipientom so v obeh primerih statistično značilno potrdile toksično delovanje konjugativnega kolicinogenega plazmida pOX38a. Rezultati so pokazali še, da med različnimi donorskimi sevi ni bilo statistično značilnih razlik v frekvencah konjugacije v laboratorijski recipientski sev. Izkazalo se je tudi, da v primerih, kjer konjugacija v tekočem gojišču lahko poteče, tj. pri uporabi laboratorijskega seva kot recipienta, ni bilo statistično značilnih razlik v frekvencah konjugacije v tekočem gojišču in na trdnem gojišču. Frekvence konjugacij iz vseh treh donorskih sevov v oba patogena seva v tekočem gojišču nismo uspeli določiti, saj v nobenem primeru nismo uspeli izolirati transkonjugant. Nasprotno pa smo na trdnem gojišču zaznali konjugacijo, ki pa je potekala v patogene seve z značilno nižjo frekvenco kot v laboratorijski sev. Izdelali smo LPS in kapsularne elektroforetske profile vseh donorskih in recipientskih sevov. Pri obeh laboratorijskih sevih, MC4100 in RU4405, smo zaznali R-LPS, pri donorskem sevu N4i smo zaznali SR-LPS, pri donorskem sevu SE15i in pri obeh patogenih sevi pa smo opazili prisotnost S-LPS. Kapsularne polisaharide smo zaznali pri vseh ne-laboratorijskih sevih. Navedena opažanja so bila v skladu s pričakovanji. Predpostavili smo, da so te strukture odgovorne za preprečitev stabilizacije kontakta (Mps) med donorsko in recipientsko celico v tekočem gojišču, kjer je zaradi manjše stabilnosti okolja potreben trdnejši stik med celicama. Dolgi O-antigeni in drugi polisaharidi celične stene recipienta bi lahko prekrivali za potek konjugacije pomembne strukture in tako fizično onemogočali stabilizacijo ali tudi vzpostavitev konjugacijskega aparata med F^+ in F^- celicama.

Kot možni vzrok za opažene nizke frekvence konjugacij v patogene seve smo raziskali tudi vplive restrikcijsko-modifikacijskih sistemov. V ta namen smo kot konjugacijska donorja in recipienta uporabili genetsko identični sev *E. coli* Nissle 1917. Izkazalo se je, da se opažena frekvenca konjugacije ni statistično značilno razlikovala od frekvenc konjugacije v patogene seve.

Ker smo ob uporabi patogenih recipientskih sevov opazili nizke frekvence konjugacije, smo frekvence poskušali zvišati preko povečane ekspresije glavnega regulatorja konjugacije TraJ v donorski celici. Dodatne kopije gena smo v sev uvedli na nekonjugativnem plazmidu pGZ in s takim donorjem opazovali frekvenco konjugacije. Statistično značilnih razlik v frekvencah konjugacije nismo opazili.

Na konjugacijo poleg navedenega lahko vpliva še več biotskih in abiotskih faktorjev, kot so začetni celični gostoti donorja in recipienta, razmerje med donorskimi in recipientskimi celicami, količina dostopnih hranil, pH in temperatura okolja ter čas, ki je na voljo za potek konjugacije (Fernandez-Astorga in sod., 1992). Dodaten problem predstavlja dejstvo, da lahko frekvence konjugacije, ki so tako nizke, da so pod detekcijsko mejo poskusa, napačno interpretiramo kot odsotnost konjugacije. Težave se pojavljajo tudi ob primerjavi dobljenih rezultatov z že objavljenimi raziskavami v znanstveni literaturi, saj različni avtorji za oceno frekvence konjugacije uporabljajo različne metodologije in izračune, kar močno omeji primerljivost rezultatov.

Rezultati dela potrjujejo delovanje toksičnega plazmida pOX38a in nakazujejo razlike v sposobnosti sprejema plazmidne DNA med laboratorijskimi recipientskimi sevi in recipientskimi sevi divjega tipa. Za razjasnitev vzrokov za opaženo variabilnost med posameznimi bakterijskimi sevi so potrebne dodatne raziskave. Razumevanje mehanizmov bakterijske konjugacije je zelo pomembno, saj konjugacija kot mehanizem horizontalnega genskega prenosa pospešuje izmenjevanje genskih informacij med bakterijskimi populacijami in omogoča bakterijskim sevom učinkovitejše prilagajanje na okolje, pridobivanje virulentnih dejavnikov, rezistenc proti antibiotikom in širokega spektra drugih lastnosti. Nenazadnje so lahko konjugativni plazmidi tudi učinkoviti protimikrobni agensi, ki jih lahko uporabljamo za preprečevanje in zdravljenje okužb s patogenimi sevi z večkratnimi odpornostmi proti antibiotikom.

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