Hiroshima University Doctoral Thesis

Identification and Characterization of *Escherichia coli* Chromosomal Genes whose Deficiency in Donor Cells Enhances Bacterial and *Trans*-kingdom Conjugations by IncP1 T4SS Machinery

細菌間接合と生物界間接合の効率が変異によって向上する大腸菌染色体遺伝子の探索と解析

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Table of Contents

Main Thesis

Identification and Characterization of *Escherichia coli* Chromosomal Genes whose Deficiency in Donor Cells Enhances Bacterial and *Trans*-kingdom Conjugations by IncP1 T4SS Machinery

Contents

Chapter 3

Generality Analysis of *frmR, sufA,* and *iscA* Gene Mutations on the Enhancement of Conjugation Efficiency

General Summary

In Chapter 1, I briefly reviewed the background history of the conjugation mechanism, which is known as a major driving force of genetic exchange in eubacteria. This phenomenon depends on the bacterial transmission system of the type IV secretion system (T4SS). As an important mechanism for horizontal gene transfer (HGT), bacterial conjugation occurs ubiquitously and permits the fast dissemination of beneficial genes.

Broad-host-range (BHR) plasmids are known to be transferred through conjugation mechanisms and stably maintained between distinct phylogenetic subgroups of bacteria. In this study, I am focusing on the broad (DNA) transfer range of IncP1-type BHR conjugative plasmid. Therefore, in Chapter 1, I also described the features that confer the broad host range, particularly to the IncP1α (RP4/RK2) plasmid, and the outline of conjugation transfer in Gram-negative bacteria between the IncP1α and narrow host range F plasmids.

In addition to eubacteria, the IncP1-type plasmid is capable to be transferred even to eukaryotes and archaea, known as *trans*-kingdom conjugation. Since the

1970s, studies on the conjugation factors encoded in the IncP1-type plasmids have been extensively analyzed. As reported, the initiation of the conjugation mechanisms requires the expression and regulation of the conjugation factors encoded on the conjugative plasmids. This chapter also described the factors (including the host-encoded proteins) that may affect the conjugation mechanism through the regulation of transfer (*tra*) gene expression, based on the previously reported studies. However, studies on the host chromosomal-encoded genes toward perspective for conjugation are still considered limited. It is also desirable to identify the potential conjugative factor(s) within the host's chromosome (host chromosomal-encoded factor), harboring the IncP1-type plasmids, which may enable us to control the conjugation process in different hosts.

In Chapter 2, I performed a high-throughput genome-wide screening on a comprehensive collection of *Escherichia coli* gene knockout mutants (Keio collection) as donors to *Saccharomyces cerevisiae* recipients to identify the candidates of chromosomal conjugation factors. This screening was performed using a conjugal transfer system mediated by T4SS of the IncP1α plasmids. As a

result, I successfully identified three out of 233 mutants (Δ*frmR*, Δ*sufA*, and Δ*iscA*) which consistently showed higher TKC efficiency compared to the parental strain within triplicate experiments. Similarly, these three mutants also showed an enhancement in *E. coli*-*E. coli* conjugation at least one order of magnitude. The complementation analysis result confirmed the repressing effect on TKC and bacterial conjugation by *frmR, sufA,* and *iscA* wild-type genes. Since *frmR-, sufA-,* and *iscA*-deficiencies positively influence the conjugation mechanism, further validations on these mutants were carried out. Firstly, an assessment of the interaction between *frmR, sufA*, and *iscA* mutants on the conjugation mechanism has been performed. For this purpose, double-KO mutants were constructed by introducing the second gene deletion located within the same or different operon (Δ*frmA*Δ*frmR,* Δ*frmB*Δ*frmR,* Δ*frmR*Δ*sufA,* and Δ*iscA*Δ*frmR*). Mutants within the same operon (formaldehyde sensing (*frm*) operon) showed significantly higher TKC efficiency (at least sevenfold) compared to the parental strain. A non-significant difference was observed between the single-KO (Δ*frmR,* Δ*frmA,* and Δ*frmB*) and the double-KO mutants, indicating that the effect on conjugation efficiency is solely due to *frmR,* neither *frmA* nor *frmB*. This phenomenon is possible in parallel to the

natural regulation of this operon in sensing formaldehyde, where the deficiency of either *frmA* or *frmB* leads to formaldehyde accumulation and inactivates the FrmR protein. Consequently, it may increase conjugation efficiency. Similarly, the TKC efficiency of the double-KO mutants which belong to the different operon (*frm* and iron-sulfur cluster operons), Δ*frmR*Δ*sufA* and Δ*iscA*Δ*frmR* also showed significantly higher (at least 11-fold) compared to the parental strain. However, these mutants showed no significant difference from single-KO Δ*frmR,* Δ*sufA,* and Δ*iscA* mutants. These results suggested that the defects of FrmR, SufA, and IscA independently affect the conjugation but probably act on an identical step of the conjugation machinery of the IncP1α plasmid.

Further assessment was performed to investigate the correlation between the expression of conjugation-related genes and the enhancement of conjugation efficiency by the three mutants. For this purpose, the basal expression level of *traI, traJ, traK,* and *trbL* harbored within the IncP1α-derived pRH220 helper plasmid as well as the wild-type of the three genes in the mutant donors were evaluated and compared with the parental strain. Interestingly, the results confirmed that the

increase in conjugation efficiency by the three mutants was not associated with the mRNA level alteration of conjugation-related genes examined. Following is one plausible explanation for the results mentioned above. SufA and IscA work in repressing other target factors (activators) within the *E. coli* donor either directly or indirectly. At the same time, the inactivation of FrmR, which may also be a repressor of other target factors (activator), will derepress the expression of that factor. The unknown target factors of these three proteins may form a complex to activate the conjugation either directly or indirectly at an identical step of IncP1 conjugation machinery.

In Chapter 3, I performed the generality assessments on the three mutants to examine whether the increased conjugation efficiency was plasmid- or host-specific dependent. Surprisingly, in both prokaryotes and eukaryotes, the three mutants demonstrated an increase in conjugation efficiency of IncP1-type plasmids alone, neither of IncN- nor IncW-type plasmids. This finding suggests that these mutants are likely specific to IncP1-type T4SS. The importance of the three genes was further investigated using a bacterium belonging to a different Pseudomonadota

class. Three knockout mutants of *Agrobacterium tumefaciens* were constructed for this purpose, each lacking a homolog of the three genes: Δ*ATU_RS04380*, Δ*ATU_RS08390,* and Δ*ATU_RS08905*. Similarly, these examined mutants also showed an increase in TKC efficiency. Overall, these results suggest the existence of a specific regulatory system in IncP1 plasmids that enables the control of conjugation efficiency in different hosts.

In conclusion, as the mutants identified in this study showed a clear significant effect on the conjugation mechanism, the development of robust donor strains by these mutants as basis strains mediated by IncP1-type T4SS machinery offers a promising outcome. Although the native function and regulation among the three genes are different, their ability in repressing is common in both bacterial and *trans*-kingdom conjugation. Therefore, this approach could be utilized as a gene introduction tool for bacteria, eukaryotes, and archaea.

Preface

Conjugation is a major driving force of genetic exchange between bacteria, or even to eukaryotes and archaea known as TKC (Dodsworth et al., 2010; Moriguchi et al., 2013b; Garushyants et al., 2015; Lacroix and Citovsky, 2016). This mechanism involves the transfer of genetic materials from donor to recipient cells due to the expression and regulation of the *tra* (transfer) genes encoded within the conjugative plasmids, such as IncP1 (Pansegrau et al., 1994; Haase et al., 1995), IncN (Winans and Walker, 1985), and IncW (Fernández-López et al., 2006) plasmids.

Previously, the IncP1-type broad-host-range (BHR) plasmids have been reported to have the ability to be transferred and replicated in hosts belonging to at least three classes in Pseudomonadota: Alphaproteobacteria (Schmidhauser and Helinski, 1985; Yano et al., 2013), Betaproteobacteria (Kamachi et al., 2006; Suzuki et al., 2010), and Gammaproteobacteria (Schmidhauser and Helinski, 1985; Adamczyk and Jagura-Burdzy, 2003; Suzuki et al., 2010; Norberg et al., 2011), by using a conjugal transfer system mediated by the type IV secretion system (T4SS). In

addition to Pseudomonadota, the IncP1-type plasmid transfer also has been detected in yeast (Hayman and Bolen, 1993) and archaea (Dodsworth et al., 2010), confers its potential as a gene introduction tool for various organisms (Dodsworth et al., 2010; Norberg et al., 2011; Moriguchi et al., 2013a). This phenomenon provides a viewpoint where the conjugation mechanism does not solely rely on genes encoded in the conjugative plasmids. The existence of a specific regulatory system in IncP1 plasmids that enables the control of conjugation efficiency in different hosts was predicted. Therefore, it is desirable to identify the potential chromosomal-encoded conjugation-related genes in the host, harboring the IncP1-type plasmids.

Recently, publications related to the identification of chromosomal-encoded conjugation-related genes in donor cells, which are possibly responsible for promoting horizontal gene transfer of the RP4 IncP1α plasmid, have been reported. However, the process of identifying those conjugation-related genes is limited to the transcriptome data, based on the genome-wide expression analysis of various genes within the treated cells (e.g., antibiotic or heavy metals exposures) (Shun-Mei et al., 2018; Zhang et al., 2019). Without the functional analysis as

further validation, it is still unclear whether those isolated genes are directly correlated to the conjugation mechanism or not.

Therefore, it is essential to correctly isolate the candidate of the conjugation-related gene(s) within the hosts' genome, which possibly correlates to the IncP1-type conjugation mechanism, either directly or indirectly. By isolating those factor(s) using a conjugal transfer system mediated by T4SS of the IncP1α-derived plasmids, useful information regarding the prediction of the existence of a specific regulatory system in IncP1 plasmids that enables to control of the conjugation mechanism in different hosts, can be obtained. I believe the accumulation of such information provides advantages for future studies, particularly for the development of donor strains as gene introduction tools into various organisms, such as bacteria, yeasts, and archaea.

CHAPTER 1

General Background on Conjugation Mechanisms

Abstract

Bacterial conjugation also referred to as bacterial sex, is one of the major mechanisms for horizontal gene transfer. This mechanism involves the exchange of genetic materials from a donor to a recipient bacterium via direct cell-to-cell contact. This phenomenon was first discovered in 1946 in *Escherichia coli,* mediated by the F (fertility) factor. The replicative extra-chromosomal factors including the F factor have been later termed 'plasmid'. Within ecological niches, the conjugation facilitates the adaption of bacteria by mediating the propagation of beneficial properties encoded on a plasmid. It involves the ability of bacteria to utilize a resource material, resistance to heavy metals and antibiotics, etc., resulting in the rapid evolution of bacterial strains. These properties make conjugation a fundamentally important mechanism. Although extensive studies on the influential factors and mechanisms involved in conjugal transfer become the focus of researchers nowadays, the F factor remains the paradigm for understanding the mechanism of type IV secretion system (T4SS) in Gram-negative bacteria. To date, various conjugative plasmids were isolated in Gram-negative bacteria and were classified either as broad or narrow host range plasmids. This chapter described the features that confer the broad host range to the IncP1α

(RP4/RK2) plasmid and the outline of conjugation transfer in Gram-negative bacteria between the IncP1 and narrow host range F plasmids. In addition, the factors that may affect the conjugation mechanism through the regulation of *tra* (transfer) gene expression has also been described.

1.2 Introduction of Bacterial Conjugation

Bacterial conjugation is one of the major horizontal gene transfer mechanisms. Bacteria conjugation is the exchange of genetic information from a donor to a recipient bacterium via cell-to-cell contact. This phenomenon referred to as 'bacterial sex', was first discovered in 1946 (Tatum and Lederberg, 1946) in *Escherichia coli*, mediated by the F (fertility) factor. The F factor is a self-transmissible extra-chromosomal genetic element that can be transferred across the cell membranes of the two parental strains. After this first discovery, various conjugative elements have been found, including conjugative plasmids (Watanabe and Fukusawa, 1961; Datta, 1962; Egawa and Hirota, 1962) and integrative and conjugative elements (ICEs) (Franke and Clewell, 1981; Tribble et al., 1997; Beaber et al., 2002). The formerly termed 'conjugative transposon' has now known as ICE since both elements are known to be present within the bacterial chromosomal genome and carry out the same excision and integration mechanisms (Cury et al., 2017). Although, bacterial conjugation is a universally conserved process of DNA transfer among bacteria. Most often, this type of horizontal gene

transfer occurs via plasmid conjugation. In various habitats, conjugation drives the rapid evolution and adaptation of bacterial strains by mediating the propagation of beneficial properties, such as the ability to utilize a resource material, resistance to heavy metals, antibiotics etc.

Previously, various conjugative plasmids have been isolated from a diverse range of gram-negative bacteria, which reside in various habitats. Following their variability in terms of DNA replication and partitioning systems, the conjugative plasmids have been classified into several different incompatibility groups, such as IncF (Frost et al., 1994), IncRh (Yamamoto et al., 2018), IncP1 (Pansegrau et al., 1994; Haase et al., 1995), IncN (Brown and Willetts, 1981; Winans and Walker, 1985), and IncW (Tait et al., 1982; Llosa et al., 1994).

1.3 Broad Host Range (BHR) Plasmids

Plasmid host range is referred to the spectrum of organisms where a plasmid can be replicated and maintained. The first classification between the broad and narrow host range plasmids was made in 1972 by Datta and Hedges (Datta and Hedges,

1972). Datta and Hedges described BHR plasmids as plasmids that can transfer between *Enterobacteria* and *Pseudomonas* species. Eventually, in 2010, BHR plasmids were predicted able to transfer among bacteria belonging to different classes of taxonomic ranks, according to their genomic signatures (Suzuki et al., 2010). According to the results obtained in this study, the definition of BHR in the latter is more appropriate.

Plasmids belonging to IncP1, IncN, and IncW were classified as broad host range plasmids, while IncF was classified as a narrow host range plasmid (Suzuki et al., 2010). The Ti/Ri plasmids that harbor the pathogenicity determinants encoded by virulence genes (*vir*) belong to the IncRh incompatibility group (Yamamoto et al., 2018). The Ti/Ri plasmids are classified as narrow host range plasmids since their replication is limited among Rhizobia such as *Agrobacterium* and *Rhizobium* species (Sprinzl and Geider, 1988). However, the transfer range of Ti/Ri plasmids to eukaryotes is the widest since these plasmids can transfer T-DNA to various eukaryotes, regardless of the natural environments or limited under laboratory conditions (Furuya et al., 2004; Lacroix et al., 2006; Suzuki et al., 2015; Kang et al.,

2020).

In Gram-negative bacteria, pili and the basement architecture of the pili are structurally and genetically conserved among the bacterial conjugation systems and T-DNA transfer system (Gillespie et al., 2010; Suzuki et al., 2015). As reported by Suzuki et al. (2015), depicts the highly conserved genes for pili and the pilus basement components among Ti plasmid *virB* genes, IncP1α RP4 and IncW R388 BHR plasmids, as well as *Bartonella henselae* chromosomal virulence region **(Figure 1.1(A))**.

1.4 IncP1-type Plasmid as a Representative BHR Plasmid

Among the BHR plasmids, plasmids belonging to the IncP1-type groups were predicted to have a broader host range than those of IncN and IncW. This prediction was made based on the levels of genomic signatures, which advocate how much nucleotide sequences were derived from various host organisms (Suzuki et al., 2010; Norberg et al., 2011). As reported before, the IncP1-type plasmids can be transferred to and replicate in hosts belonging to at least three classes in

Pseudomonadota phylum: Alphaproteobacteria (Schmidhauser and Helinski, 1985; Yano et al., 2013), Betaproteobacteria (Kamachi et al., 2006; Suzuki et al., 2010), and Gammaproteobacteria (Schmidhauser and Helinski, 1985; Adamczyk and Jagura-Burdzy, 2003; Suzuki et al., 2010; Norberg et al., 2011). Moreover, the IncP1-type plasmid can disseminate the mobilizable plasmids from bacteria belonging to Pseudomonadota phylum to other phylum (Musovic et al., 2006), or even to yeast (Heinemann and Sprague, 1989; Nishikawa et al., 1990) and archaea (Dodsworth et al., 2010; Garushyants et al., 2015), known as *trans*-kingdom conjugation. The ability of this IncP1-type plasmid to be adapted to and replicated in different hosts confers its potential as a gene introduction tool.

Regardless of the host ranges and the different incompatibility groups, generally, the backbone of conjugative plasmids carries all genes required for their maintenance and the conjugation processes. The genes involved in the maintenance and conjugation processes are clustered separately in different regions. In the donor cell, the transfer (*tra*) genes encoded on the conjugative plasmid are necessary to be expressed, prior to initiating the conjugation process.

1.5 Outline of Conjugal Transfer of F and RP4/RK2 IncP1α Plasmids

The ability of the donor strain to perform conjugation requires the expression of the *tra* genes on the conjugative plasmid. The *tra* genes are organized and clustered in operons under the control of different promoters, with a clear distinction between those involved in DNA transfer replication (Dtr) and mating pair formation (Mpf). These genes encode all protein factors involved in the relaxosome as well as the elaboration of the conjugative pilus and macromolecule transporter components, T4SS, respectively. For instance, in the RP4/RK2 IncP1α plasmid, the plasmid is organized into two blocks, designated as Tra1 (encoding *tra* genes for Dtr) and Tra2 (encoding *trb* genes for Mpf) regions (Lessl et al., 1992; Pansegrau et al., 1994; Zatyka et al., 1994). **Figure 1.1(B)** illustrates the transport of T-DNA and plasmid DNAs through T4SS machinery.

At the initiation steps of the F-type plasmid conjugation process, some of the Tra proteins involved in the elaboration of the conjugative pilus mediate Mpf. Collectively, Mpf component proteins assemble each other to form the macromolecule transporter (T4SS). While, other Tra proteins (TraI, TraM, and

TraY) form the relaxosome in combination with the integration host factor (IHF) (Gamas et al., 1987; Silverman et al., 1991) will bind to the origin of transfer (*oriT*) sequence and induce the nicking reaction by the TraI relaxase as a preparation for plasmid transfer (Nelson et al., 1995). Interaction between the relaxosome component, TraM, and type IV coupling protein (T4CP), TraD, initiates the transfer of the T-strand by the T4SS, in vivo (Lu et al., 2008a). Similarly, the RP4 IncP1α plasmid also requires three Tra proteins (TraI, TraJ, and TraK) to form the relaxosome. The interaction between the relaxase TraI and TraG T4CP initiates the single-stranded DNA transfer (Szpirer et al., 2000; Schröder et al., 2002) **(Figure 1.2)**.

The transfer of single-stranded plasmid DNA from donor to recipient cells is normally associated with a process known as rolling circle replication (RCR) (Khan, 1997; Khan, 2005; Ruiz-Masó et al., 2015). In the recipient cells, the replication of a complementary strand of the transferred single-stranded DNA is carried out by host-encoded DNA polymerase, resulting in the formation of a double-stranded plasmid. The newly acquired dsDNA of the conjugative plasmid in the recipient

cells will undergo plasmid maintenance through the segregation of daughter cells and replication processes. Unlike F-type and other narrow host range plasmids, broad host range plasmids such as RP4/RK2 IncP1α plasmid are capable to regulate their maintenance by modulating alternative strategies of replication depending on the host (Baxter and Funnell, 2015).

1.6 Replication Control of RP4/RK2 IncP1α Plasmid Depending on Host Factors

The structure of vegetative origin, *oriV,* plays an important feature to ensure the autonomous replication of the plasmids. In the case of the 60 kb RK2 IncP1α BHR plasmid, the structure of *oriV* may influence its replication in various hosts, controlled by the host factors. Iterons are directly repeated sequences located on *oriV*. Iterons served as a binding site for the plasmid-encoded replication protein, TrfA, during the initiation of DNA replication, and are also known to be important in controlling the plasmid copy number (Durland and Helinski, 1990; Konieczny et al., 2015). The RK2 plasmid possesses nine 17 bp-iterons and is separated into three different clusters (one, three and five) (Adamczyk and Jagura-Burdzy, 2003). The

number of iterons required for replication largely depends on the host bacteria. For instance, *E. coli* requires at least five iterons instead of all eight iterons as in *P. putida,* for the *oriV* to be fully functional (Schmidhauser et al., 1983). The binding of the plasmid-encoded TrfA replication protein on the iterons is very often accompanied by the binding of host-encoded DnaA replication protein to DnaA-boxes on the *oriV*. In addition to iterons, the requirement of the host-encoded DnaA binds to the DnaA-boxes are also variable depending on the host bacteria. For instance, in addition to iterons, at least two DnaA-boxes (3 and 4) are required for the replication to be fully functioned in *E. coli* and *P. putida,* while none of the DnaA-boxes but four iterons are required for the replication in *P. aeruginosa* in vivo (Shah et al., 1995; Doran et al., 1999). However, the deletion of all DnaA-boxes except the fourth one can be acceptable for the replication in *Azotobacter vinelandii* (Doran et al., 1999). Both of these elements provide the essential versatility to the RK2 plasmid to enable its replication and maintenance in a promiscuous manner (Schmidhauser et al., 1983; Schmidhauser and Helinski, 1985). Besides the iterons and DnaA-boxes, a 40 bp AT-rich repeat motif within the *oriV* of the RK2 plasmid is also found to be essential for the proper activity of the

origin and provided as a local destabilization of a double-stranded helix region prior to plasmid replication initiation process (Stalker et al., 1981; Konieczny et al., 1997). In the case of *E. coli*, *P. putida*, and *A. vinelandii*, the binding of plasmid-encoded TrfA and host-encoded DnaA on the iterons and DnaA-boxes, respectively, induces the strand opening at the AT-rich region. However, according to the mutational analysis, mutation in either box 3 or 4, or deletion of all DnaA-boxes in *E. coli,* could restore the in vitro replication mechanism by adding a high amount of the purified DnaA protein (Doran et al., 1999). This could be an alternative mechanism of *oriV* initiation where a high level of DnaA protein may recruit the host-encoded DnaB helicase in the presence of TrfA and stimulate the formation of open complex and pre-priming complex at the AT-rich region (Doran et al., 1999). A similar mechanism is expected in *P. aeruginosa* which does not requires DnaA-boxes for the replication (Shah et al., 1995). Therefore, the versatility of RP4/RK2 replication region interacting with different host replication proteins allows alternative strategies for the replication initiation of this plasmid in different bacterial hosts.

1.7 Factors Affecting the Regulation of *tra* Genes Expression

The expression of *tra* genes is regulated by several factors, such as plasmid- and host-encoded proteins, growth phase, and environmental factors **(Figure 1.3).**

1.7.1 Plasmid-encoded Factors

In the F and F-like plasmids, the conjugation mechanism is strictly regulated through activity of plasmid-encoded TraJ (Willetts, 1977) **(Figure 1.4)**. TraJ is expressed from its monocistronic gene, *traJ,* and is thought to be the primary activator for *tra* genes expression in the F-like plasmids (Willetts, 1977). Particularly, during the absence of recipient cells, the activator TraJ in most F-like plasmids is under negative control by plasmid-encoded fertility inhibition FinOP, which is an antisense RNA system. The strict repression of *tra* genes is important to reduce fitness costs in the absence of recipient cells (Frost et al., 1989; Koraimann et al., 1996). However, the missing of Fin circuit in the F and F-like plasmids due to the insertion of the IS*3* element in *finO*, resulting in the constitutive expression of *tra* genes (Yoshioka et al., 1987). Unlike the F plasmid, the negative regulation of the *tra* operon by the FinOP system in the RP4 plasmid is absent, resulting in the

constitutive expression of the *tra* genes at a basal level.

1.7.2 Host-encoded Proteins

In the F and F-like plasmid, the transcriptional activation of *tra* genes by TraJ also requires the host-encoded protein, ArcA (or SfrA) (Silverman et al., 1991). TraJ and ArcA are necessary to activate the F *tra* operon through their binding on the *traY* promoter, P_Y (Silverman et al., 1991; Lu et al., 2019). Transcription of *traY* is normally repressed through the binding of host-encoded histone-like nucleoid structuring protein (H-NS) on P_Y at the stationary growth phase of the *E. coli* donor. Thereby, F plasmid transfer is kept at a low level at the stationary phase (Will et al., 2004; Will and Frost, 2006). However, irrespectively of the exponential nor stationary growth stage of both donor and recipient *E. coli* cells, this factor exhibits no significance in terms of conjugation efficiency by the RP4 IncP1-type plasmid (Moriguchi et al., 2020; Sysoeva et al., 2020).

A. tumefaciens is a unique bacterium in that it can transfer DNA to various eukaryotic organisms, in addition to bacteria (Ohmine et al., 2018; Kiyokawa et al., 2020). Genes for factors that are directly involved to the DNA transfer are coded on

Ti/Ri plasmids **(Figure 1.1(A))**, while chromosomal genes are indispensable to the inter-domain DNA transfer. Chromosomal virulence (*chv*) genes [*chv*A, *chv*B, *chv*D, *chv*E, *chv*G, *chv*H, and *chv*I] and the chromosomal genes affecting the virulence [*acv*B, *pgm* (*exo*C), *glg*P, *mia*A, and *ros*] are coded on the linear or circular chromosomes (Suzuki et al., 2001). Previously, a chromosomally encoded glucose or galactose binding protein, ChvE, was also found to interact with transmembrane sensory protein VirA to enhance *vir* genes activation encoded on the Ti plasmid, upon the induction by acetosyringone (Cangelosi et al., 1990; Shimoda et al., 1993). In addition to *chv*E, a set of two genes must be essential for the *Agrobacterium*'s inter-domain DNA transfer, such as ChvG and ChvI play a role as a two-component system to sense acidity important to the *vir* gene induction.

Therefore, the participation of the chromosomal genes in donor cells is essential to promote the transfer of DNA between bacteria or between bacteria and eukaryotic organisms.

1.7.3 Environmental Factors

The environmental conditions also influence the conjugation mechanism

through the interaction between host- and plasmid-encoded proteins. The Cpx (conjugative plasmid expression) regulon which comprises the host-encoded *cpxA* and *cpxR* in *E. coli* K-12 derivative, was found to be essential for the correct F conjugative plasmid expression, harbored in Hfr cells (McEwen and Silverman, 1980a, 1980b). At the same time, the Cpx regulon is also known to play a role in regulating the protein trafficking in the *E. coli* cell envelope through a signal transduction pathway (Raivio and Silhavy, 1997; Raivio, 2005). This pathway will sense and responds to extracytoplasmic stress by conveying the signal from the cell envelope to the cytoplasm. Thus, the F *tra* operon, which produces a complex transenvelope type IV secretion apparatus is a great candidate for the Cpx system regulation (Sambucetti et al., 1982). Under stressed environments, the Cpx regulon and HslVU heat shock protease-chaperone pair will activate and reduce the TraJ accumulation levels due to the degradation, which largely affects the F plasmid transfer (Lau-Wong et al., 2008). In addition to HslVU, GroEL also is known to be a factor that interacts with TraJ in vivo, in response to heat shock leading to TraJ degradation (Zahrl et al., 2007).

On the other hand, under abiotic stresses such as antibiotics or heavy metal exposures, the host chromosomal-encoded factors of *E. coli* that are particularly involved in SOS response or/and Reactive Oxygen Species (ROS) (e. g., *umuC, recN, yebG,* and *recA*), was reported to be responsible for promoting the RP4 IncP1α plasmid transfer (Shun-Mei et al., 2018; Zhang et al., 2019). However, their plausible explanations are made based on the increase in the expression of essential *tra* genes, at least involved in the relaxosome formation (*traI, traJ,* and *traK*) (Shun-Mei et al., 2018) and pilin formation (*traA, traB, traH, traL,* and *traP*) (Zhang et al., 2019), according to the genome-wide expression screening, without any analyses of conjugation activity. Therefore, it is unclear whether the genes expressed higher or less are correlated directly to the conjugation mechanism or not.

1.7.4 Other Factor (Quorum Sensing)

For some other Gram-negative bacteria, including *Pseudomonas aeruginosa* (Whiteley et al., 1999) and *A. tumefaciens* (Von Bodman et al., 1992; Fuqua and Winans, 1994) conjugation systems, *tra* genes expression is regulated by quorum

sensing (QS) mechanism. QS mechanism is a type of bacterial cell-cell communication system (Miller and Bassler, 2001; Whitehead et al., 2001). This mechanism is used by bacteria to detect and respond to cell population density based on the concentration of signaling molecules, accumulated in the surrounding environments. In general, gram-negative bacteria produce acylated homoserine lactones (AHL) QS molecules as autoinducers to regulate various physiological activities, such as virulence (Diggle et al., 2002; Juhas et al., 2004) and conjugation (Zhang et al., 1993; Lu et al., 2017) mechanisms.

The phytopathogen *Agrobacterium* induces gall formation in a wide range of dicotyledonous plants (Drummond, 1979; Islam et al., 2010). The gall formation results from a genetic transformation process, that relies upon the transfer of T-DNA from bacteria to plant cells (Păcurar et al., 2011; Kado, 2014). The T-DNA, which is a part of a tumour-inducing (Ti) plasmid, such as pTiC58, is transferred to the plant cells due to the activation of virulence (*vir*) genes. To ensure the maintenance of Ti plasmid, the whole Ti plasmid is transferred between agrobacteria in a population via the conjugation process. The conjugation of Ti

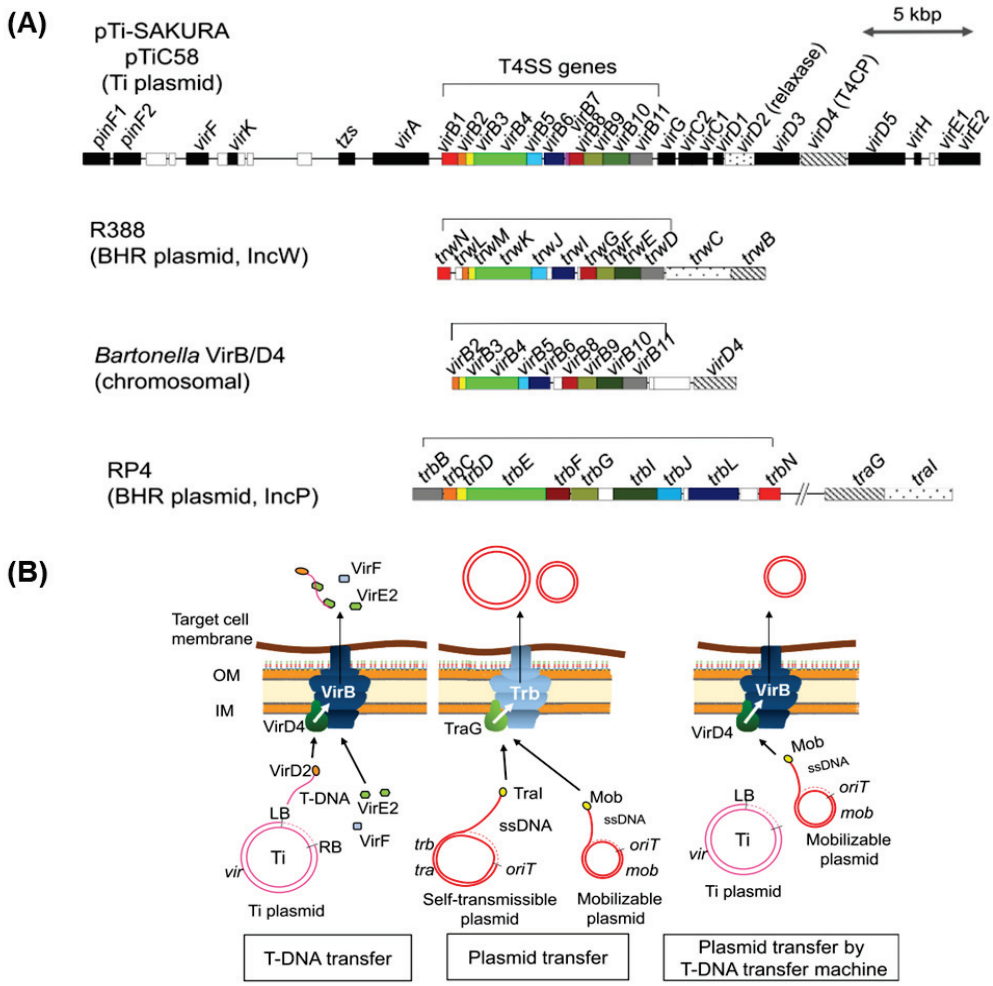
plasmid is regulated by the QS mechanism, due to the activation of *tra* genes (Cook et al., 1997). In *Agrobacterium*, most virulence determinants and *tra* genes are encoded and clustered within the Ti plasmid, at different regions (Dessaux and Faure, 2018). Upon the successful transfer of T-DNA into host plants, the agropines will produce 3-oxo-octanoylhomoserine lactone (OOHL) molecules under the high cellular density of *Agrobacterium* (Piper et al., 1993; Zhang et al., 1993). The binding of LuxR-like protein (TraR) with OOHL triggers the transcription of *tra* (Cook et al., 1997) and *trb* (Li et al., 1998) operons, resulting in the production of T4SS and relaxosome proteins. This regulation provides the coordination between Ti plasmid conjugation and bacterial cell density within the host plants during infection (Dessaux and Faure, 2018).

On the other hand, *P. aeruginosa* uses QS as a virulence determinant (Diggle et al., 2002; Juhas et al., 2004). As reported by Lu et al., (2017), the production of *N*-(3-oxo-dodecanoyl)-$_{L}$-homoserine lactone (OdDHL) AHL molecules by *P. aeruginosa* could suppress the interspecies conjugation with *E. coli* (Lu et al., 2017). Based on their plausible explanation, the binding of AHL molecules to the *E.*

coli LuxR-like SdiA transcriptional factor (AHL-SdiA) to the SdiA-box located on the promoter region of *traI* results to the deactivation of the RP4-encoded *traI* expression, consequently, suppressed the conjugation mechanism. However, according to the sequence given, the authors mistakenly annotated the position of the SdiA-box, which overlapped with the coding sequence of *traJ* (located less than 100 bp downstream of the first nucleotide sequence of *traJ* start codon), but not with the promoter region of *traI.* The authors also did not perform any analysis by looking at the *traI* and *traJ* expression, and the effect on conjugation upon the treatment with QS molecule. Therefore, in this case, the suppression of the RP4-mediated conjugation could be due to the inappropriate translation in TraJ protein synthesis due to the truncation, as a result of the *traJXIH* mRNA extension inhibition upon the binding of AHL-SdiA. Therefore, in the study, it is unclear whether the suppression of interspecies conjugation between *P. aeruginosa* and *E. coli* is due to the direct repression of *traI* transcription from the proposed *traI* promoter or could be due to *traJXIH* mRNA and TraJ protein truncation, resulting in the aberrant mRNA and protein products degradation by SmpB-tmRNA (transfer messenger RNA)-mediated *trans*-translation quality control system in bacteria

(Karzai et al., 1999; Gillet and Felden, 2001; Richards et al., 2008). The degradation of the essential TraJ protein may fail relaxosome formation, resulting in the conjugation mechanism failure since the binding of TraJ has been proposed as a first step of functional relaxosome assembly (Pansegrau et al., 1994).

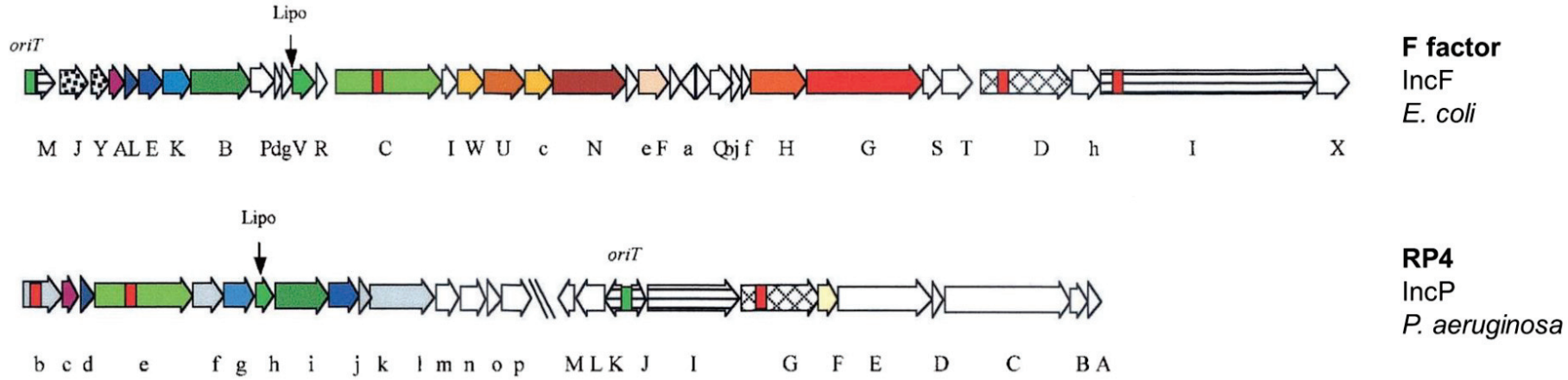
The regulation of the transfer gene expression between different conjugative plasmids harbored in Enterobacteriaceae provides a unique strategy to modulate the transfer efficiency. As described above, the *tra* gene expression is controlled by complex regulatory circuits to modulate the transfer efficiency of a conjugative plasmid. This regulation involves the activities of plasmids in combination with host chromosomal proteins. Moreover, the environmental conditions and physiological states of the bacterial cells also influence this complex regulation.



(Modified from Suzuki et al., 2015)

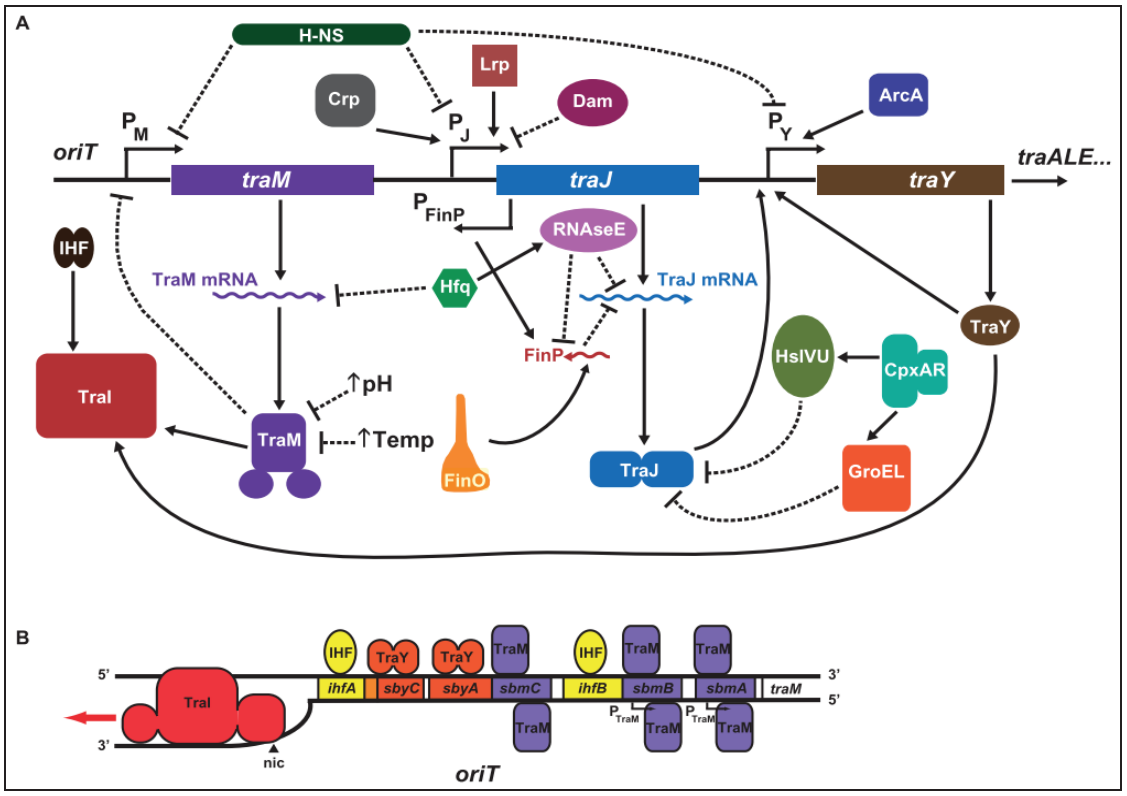
Figure 1.1: Genes coding for the machinery components and transport of T-DNA and plasmid DNAs through T4SS machines. (A) Structure of the virulence gene cluster in Ti plasmid pTi-SAKURA and homologues responsible for VirB/VirD4 T4SS in broad host range (BHR) plasmids. Genes are represented by boxes (spotted box; relaxase gene, hatched box; type IV coupling protein (T4CP) gene, filled box; virulence gene, open box; other genes). The *virB* gene homologues in RP4, R388 and *B. henselae* chromosomal virulence region were estimated based on sharing the same conserved domain with each corresponding agrobacterial VirB protein detected by NCBI conserved domain search program

(http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Each *virB* gene and its homologues are shown by the same color. (B) Transport of DNA and effector proteins by T4SS. A Nickase/relaxase protein, e.g., VirD2, TraI and Mob, releases single-stranded DNA (ssDNA) from plasmids and simultaneously covalently bounds to the 50-end of ssDNA. The nucleoprotein complex is recognized by a coupling protein, such as VirD4 and TraG, then transported to target cells through the transmembrane ultrastructure T4SS consisting of VirB proteins or Trb proteins. Mobilizable plasmids possess *oriT* and a *mob* gene, which product is a nickase special for its *oriT* sequence. Wide-transfer-range conjugation plasmids and the T-DNA transfer machine VirB/VirD4 can deliver the mobilizable plasmids.



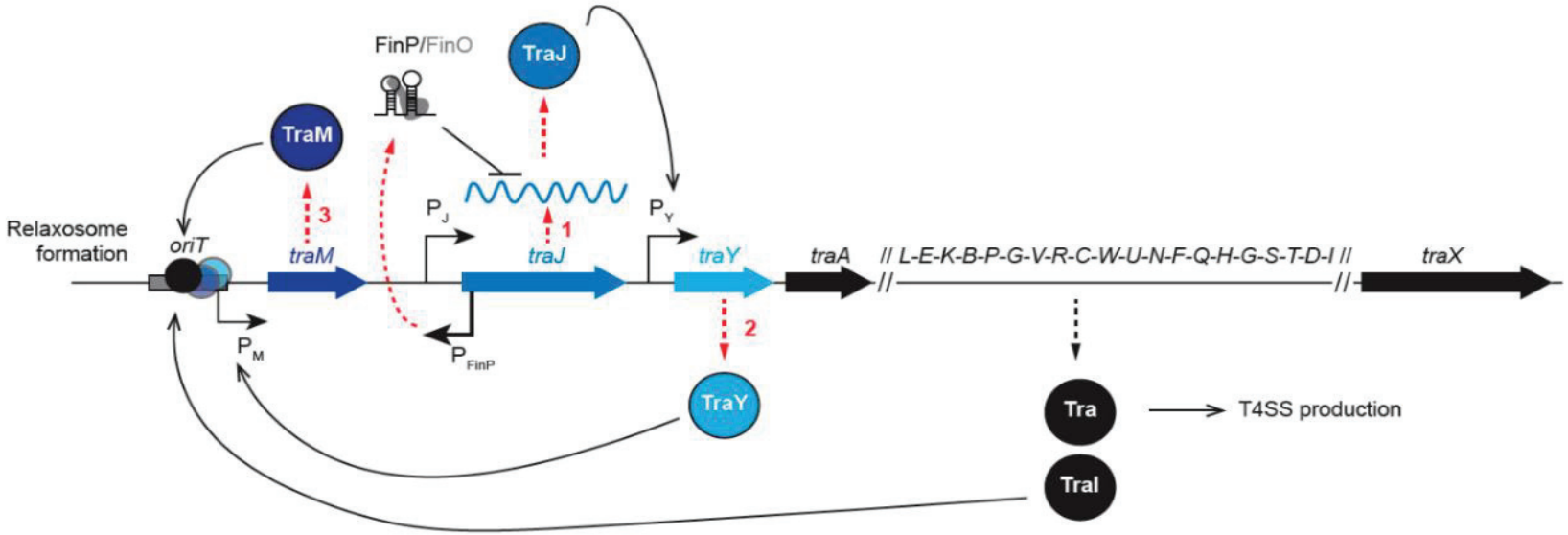
(Modified from Lawley et al., 2003)

Figure 1.2: Comparison between the complete F and RP4 conjugative transfer (*tra*) regions (some regulatory genes may be excluded). Essential *tra* genes represent vary in colours and patterns of the arrow boxes. The same colours and patterns of the arrow boxes represent the homologous gene products between the two conjugative plasmids, while the non-essential *tra* genes are in white. The solid coloured-arrow boxes represent the *tra* genes which encode for Mpf. The patterned-arrow boxes represent the *tra* genes which encode for coupling protein, relaxosome components, and regulatory genes. Light grey arrow boxes represent *tra* gene products with no shared homology. Lipo, lipoprotein motif; Red box, Walker A motif; Green box, the origin of transfer (*oriT*); Capital letters, *tra* genes located at Tra region; Lowercase letters, *tra* genes located at Trb region. The double slash indicates non-contiguous regions. Arrow boxes represent the ORFs and the length is proportional to the length of the ORFs.



(Adopted from Wong et al., 2012)

Figure 1.3: *tra* operon regulation in F and F-like plasmids. (A) Overview of F plasmid *tra* operon regulatory factors. Positive regulatory actions are indicated by solid lines having an arrow, while negative ones are showed by dashed lines. **(B)** *oriT* region with binding sites for DNA-binding proteins. Unwinding of DNA is followed by cleavage at the *nic* site by TraI and covalent attachment of TraI to the 5' end of the nicked DNA. Direction of the unwinding is indicated by a red arrow.



(Adopted from Virolle et al., 2020)

Figure 1.4: Activation cascade of *tra* gene expression. Firstly, P_J promoter drives *traJ* expression **(1)**. TraJ protein binds to the P_Y promoter to notably produce TraY, which activates P_M promoter **(2)**. The P_M promoter activation allows expression of other Tra proteins including components for T4SS, and the relaxase TraI. Once produced **(3)**, TraM autoregulates its own expression through P_M promoter. TraM form the relaxosome that includes *oriT,* TraY, and TraI. The activation of this regulatory cascade is modulated by formation of FinP/FinO complex on *traJ* mRNA. The complex represses the translation of TraJ at the post-transcriptional level. Dotted red arrows illustrate the transcription-translation process.

CHAPTER 2

Isolation and Characterization of the Donor *Escherichia coli* Gene Mutations that Enhance the Conjugal Transfer Mediated by IncP1α Plasmid

Abstract

Conjugal transfer is a major driving force of genetic exchange in eubacteria. By using the system in IncP1-type broad host range (BHR) plasmids, this type of transfer also promotes the transfer of DNA even to eukaryotes and archaea, known as *trans*-kingdom conjugation (TKC). Although the analysis of the conjugation-related genes encoded on the IncP1-type plasmids toward perspective for conjugation mechanism has been extensively progressed, that on the host chromosome remains limited. This chapter reported the potential conjugation-related genes that were isolated from a comprehensive collection of *Escherichia coli* single-gene knockout mutants (Keio collection) via genome-wide screening. This screening was performed using a conjugal transfer system, mediated by the type IV secretion system of the IncP1α plasmid (IncP1-T4SS), between the *E. coli* mutants as donors and *Saccharomyces cerevisiae* as a recipient. Three out of 233 "up"-mutants (over 3,884 mutants) were isolated, namely Δ*frmR*, Δ*sufA*, and Δ*iscA*. These mutants showed an increase by one order of magnitude in both *E. coli*-*E. coli* and *E. coli*- yeast conjugations. The increase in conjugation efficiency did not exhibit an increase in the mRNA expression level of representative conjugation-related genes, namely, *traI*, *traJ*, *traK*, and *trbL*. In addition, the double-knockout mutants for the isolated genes (Δ*frmR*Δ*sufA* and Δ*iscA*Δ*frmR*) did not show any synergistic effects on the conjugation efficiency, suggesting that these factors independently affect an identical step of IncP1α conjugation machinery. The data regarding these isolated mutants could be an appropriate basis for the

development of donor strains as gene-delivery tools to various organisms, such as bacteria, eukaryotes, and archaea.

Introduction

Conjugation is a major mechanism of genetic exchange between bacteria. This mechanism also promotes the transfer of DNA from bacteria to either eukaryote (Bates, 1997; Moriguchi et al., 2013b) or archaea (Dodsworth et al., 2010; Garushyants et al., 2015), known as *trans*-kingdom conjugation (TKC). TKC is a type of horizontal gene transfer (HGT), the opposite of vertical gene transfer (VGT), that promotes the transfer of genetic materials between non-related species (Suzuki et al., 2015). HGT from bacteria to eukaryotes is considered to be much less frequent to occur even though it has been reported and demonstrated previously (Lacroix and Citovsky, 2016). In contrast, HGT occurs ubiquitously and permits the fast dissemination of new beneficial genes within a prokaryotic population. This mechanism has been acknowledged as a driving force for the evolution of bacterial species.

Conjugation is a mechanism which involves the transfer of genetic materials from the donor to the recipient cells via conjugal transfer based on the type IV secretion system (T4SS). This mechanism is activated due to the expression and regulation of the conjugation-related genes, harbored in the conjugative plasmid [e.g., IncP1 (Pansegrau et al., 1994; Haase et al., 1995), IncN (Winans and Walker, 1985), and IncW (Fernández-López et al., 2006) plasmids] in the donor cells. As mobile genetic elements, conjugative plasmids play an important role as a vector for the dissemination of beneficial traits to the hosts, such as antibiotic resistance, heavy metal resistance, and metabolic pathways, which are essential for species

adaptation and survival.

IncP1-type broad host range (BHR) self-transmissible plasmids have been suggested to have a broader host range compared to other BHR self-transmissible plasmids, such as IncN- and IncW-types, as it carries genomic signatures which are predicted to be derived from various host origins (Suzuki et al., 2010; Norberg et al., 2011). This type of plasmid can be transferred to, and replicated in hosts belonging to at least three classes in Pseudomonadota: Alphaproteobacteria (Schmidhauser and Helinski, 1985; Yano et al., 2013), Betaproteobacteria (Kamachi et al., 2006; Suzuki et al., 2010) and Gammaproteobacteria (Schmidhauser and Helinski, 1985; Adamczyk and Jagura-Burdzy, 2003; Suzuki et al., 2010; Norberg et al., 2011). The ability of this IncP1-type plasmid to be adapted to and replicated in different hosts confers its potential as a gene introduction tool.

In a previous study, the IncP1-type conjugation system also was reported to give a detectable DNA transfer to yeasts, in addition to Pseudomonadota (Heinemann and Sprague, 1989; Hayman and Bolen, 1993). This broader transferability of the IncP1-type plasmid employed the usage of this plasmid as a gene introduction tool. Generally, to make it convenient to use as the tool, a native self-transmissible plasmid (e.g., RP4 plasmid) is separated into two parts: a helper plasmid and a shuttle vector. Genes encoded on the helper plasmid are responsible for the biosynthesis of the conjugative pilus and the production of stable mating aggregates (IncP1-T4SS) for the transfer and mobilization. On the other hand, the shuttle vector comprises of origin of transfer (*oriT*) derived from the IncP1 plasmid and genes which encode for the plasmid maintenance and propagation within the donor

and recipient during the conjugation process. Besides the IncP1-type, an IncQ-type mobilizable plasmid is alternatively used as a backbone of the shuttle vector. The transfer of IncQ mobilizable plasmid to the recipient cells is previously known to be facilitated by helper plasmids derived from IncP1α (Moriguchi et al., 2013b) or IncP1β (Willetts and Crowther, 1981; Moriguchi et al., 2016) subfamilies, such as RP4 and R751 plasmids, respectively. To date, this shuttle vector-and-helper system has been used by researchers as a gene introduction tool from proteobacteria to various recipient organisms, such as yeast (Moriguchi et al., 2013a,b; Soltysiak et al., 2019), archaea (Dodsworth et al., 2010), and diatoms (Karas et al., 2015).

Elucidation on the ability of IncP1-type plasmid replication in Pseudomonadota hosts and the dissemination to various recipient organisms has contributed to further investigation, particularly on the regulation mechanism of the IncP1-type transfer system. For instance, the *Agrobacterium* T-DNA transfer system can deliver genes to a broad range of eukaryotic organisms and bacteria (Ohmine et al., 2018; Kiyokawa et al., 2020). Several chromosomal genes are essential for most of the broad-range TKC phenomena. The A*grobacterium* genes for the inter-domain DNA transfer except those on Ti/Ri plasmids are located dispersed over its circular chromosome and linear chromosome (Suzuki et al., 2001).

Therefore, the conjugation mechanism does not solely rely on genes encoded on the conjugative plasmids, but the participation of the chromosomal genes has so far been revealed in bacterium. Thus, it is essential to correctly isolate the candidate of host chromosomal-encoded factor(s) within the donor *E. coli* genome which is possibly correlated to the IncP1-type conjugation mechanism, either directly or

indirectly.

Recently, several publications related to the isolation of chromosomally-encoded factors in donor cells which are responsible for promoting HGT of the RP4 plasmid upon abiotic stress exposures, have been reported (Shun-Mei et al., 2018; Zhang et al., 2019). These include exposure to antibiotics or heavy metals. In these studies, the researchers isolate the factors based on the transcriptome data on the genome-wide expression analysis. The isolated genes include both RP4 plasmid- and chromosomally-encoded genes that are probably responsible for the physiological changes of the donor cell, consequently affecting the HGT. However, in these studies, the screening approach is just based on the expression analysis of the various genes within the stress-exposed donor cells which possibly influence the conjugation mechanism. The functionality analysis was not performed on the isolated candidate gene(s) for further validation. Thus, it is still unclear whether the up- or down-regulated genes are correlated directly to the conjugation mechanism or not.

In this chapter, the chromosomal factor(s) within the *E. coli* genome that possibly influence the conjugative transfer mediated by IncP1α-type plasmid, have been identified from an *E. coli* single-knockout mutant library. The genetic features of that isolated factors also have been characterized. Further characterization of the isolated mutants was performed by examining the possible correlation with the expression of the conjugation-related genes in the IncP1α plasmid.

Materials and Methods

Bacterial strains, yeast, and growth media

Bacterial strains and yeast used in this study are listed in **Table 2.1**. A complete set of *E. coli* non-essential gene deletion clones [(Keio collection) (Baba et al., 2006)] was provided by the National BioResource Project (NBRP) of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. All *E. coli* strains were cultured in Luria Broth (LB) Lennox medium (1% Bacto-tryptone, 0.5% NaCl, and 0.5% yeast extract) at 37°C. In addition, *S. cerevisiae* was cultured in yeast-extract/peptone/dextrose (YPD) medium (2% glucose, 2% polypeptone, 1% yeast extract) at 28°C. Synthetic defined (SD) medium (2% glucose and 0.67% Difco^{TM} yeast nitrogen base without amino acids) containing appropriate individual amino acids (leucine, 0.03 mg/ml; histidine, 0.02 mg/ml; and lysine, 0.03 mg/ml) was used as selection media (SC-Ura) for yeast transconjugants at 28°C. Solid LB Lennox medium was prepared by the addition of 1.5% agar, while solid YPD and SC-Ura media were prepared by the addition of 2% agar. Appropriate antibiotics were added to the media at the following final concentration, corresponding to the selection of bacteria and plasmids: ampicillin (Ap), 50 µg/mL; chloramphenicol (Cm), 30 µg/mL; gentamicin (Gm), 30 µg/mL; kanamycin (Km), 50 µg/mL; rifampicin (Rf), 30 µg/mL; streptomycin (Sm), 50 µg/mL; and tetracycline (Tc), 7.5 µg/mL.

Donor and recipient cell cultures

Details of the genotypes for both donor and recipient cells, as well as the plasmids used in this study, are described in **Tables 2.1 and 2.2**, respectively. For genome-wide screening analysis, donor *E. coli* Keio mutants and the control BW25113 (pBBR122ΔCm^R) harbouring pRH220 and pRS316::$oriT^P$ plasmids **(Figure 2.1)** were inoculated from 96-well frozen stock plate using a 96-pinner tool. The culture was prepared in 96-well flat-bottom plate containing 100 µL medium supplemented with Ap, Cm, and Km and incubated at 37°C for 15 to 18 h.

For standard conjugation assessment, the donor cells of BW25113 wild-type harboring the RP4 plasmid were cultured in media supplemented with Ap and Km. For the complemented strains, single- and double-KO *E. coli* mutants as well as the control harboring pRH220 and pRS316::$oriT^P$ plasmids were cultured in media supplemented with Ap and Cm. These donor cultures were cultured in 5 mL glass tubes and incubated at 37°C for 15 to 18 h. Details for the construction of *E. coli* double-KO mutants and the complemented strains are described below.

The recipient cells of *E. coli* strain SY327 (λ*pir*) or *S. cerevisiae* strain BY4742 were cultured in media supplemented with or without Rf, respectively, in 5 mL glass tubes. The *E. coli* or yeast recipient cells were cultured at 37°C for 16 to 18 h or at 28°C for 18 to 22 h, respectively. Both donor and recipient cultures were pre-cultured with agitation to allow aeration.

Construction of *E. coli* double-KO mutant strains

All primers used in this experiment are listed in **Table 2.3**. The secondary in-frame

gene deletion was performed using the same approach as previously reported by Baba et al., (2006). *Km*^{R} gene cassette was amplified from genomic DNAs of Δ*sufA* and Δ*frmR* mutant strains. Each of the amplified *Km*^{R} gene cassettes includes ≥100 bp of upstream and downstream of the deleted *sufA* and *frmR* genes sequences, by using the primer sets F001-F002 and F003-F004, respectively. The amplified fragments were then introduced into the single-KO mutants (Δ*frmR,* Δ*iscA,* and Δ*frmB*) to generate the double-KO mutants, namely, Δ*frmR*Δ*sufA,* Δ*iscA*Δ*frmR,* and Δ*frmB*Δ*frmR.* The construction of these mutants was done following the instruction manual of Gene Bridges GmbH Red/ET Recombination kit (Heidelberg, Germany). Due to an adjoining position of *frmA* and *frmR* genes within the *frm* operon, the double-KO mutant of Δ*frmA*Δ*frmR* was constructed as follows.

Two fragments were amplified separately, between ≥100 bp upstream region of Δ*frmR* and the stop codon of *Km*^{R} gene cassette and between the start codon of *Km*^{R} gene and ≥100 bp downstream region of Δ*frmA,* by using the primer sets F003-F006 and F005-F007, respectively. Then, the Δ*frmA*Δ*frmR* mutant was generated by using the F003-F007 primer pair through the assembly of these two fragments via PCR. The sequences of Δ*sufA,* Δ*frmR* and Δ*frmA* were obtained from the NBRP *E. coli* strains website.

Construction of *E. coli* complemented strains

All primers used in this experiment are listed in **Table 2.3.** Before constructing the *E. coli* complemented strains, the pJP5603*sacB*Δ*Km*+*Gm* mobilizable suicide vector is essential to be constructed. This vector was constructed by assembling two

fragments, amplified from pJP5603Δ*Km*+*Gm* (containing Gm^R gene (*aacC1*) and R6K gamma *ori*) and pJP5603*sacB* (containing RP4 derived-*oriT* and *mob*, *sacB* and *lac*Z alpha promoter) (Kiyokawa et al., 2020) by using F008-F009 and F010-F011 primer pairs, respectively. This constructed vector was confirmed by performing RE digestion at *Bam*HI and *Hind*III restriction sites, given the product sizes of 1.82 and 3.27 kbp. The constructed mobilizable suicide vector was named pJP5603sacBGmR **(Figure 2.2 (A))**. The pJP5603sacBGmR was amplified inversely by PCR, including the additional sequence of *Eco*RI RE site at both ends of the amplified fragment using F012-F013 primer pairs before assembling with *sufA*, *iscA*, and *frmR* genes.

The *sufA*, *iscA*, and *frmR* genes were amplified from the wild-type BW25113 *E. coli* strain. Each of these fragments was amplified including approximately 1 kb of the upstream and downstream regions of the targeted gene with an additional sequence of *Eco*RI RE site at both ends, by using F014-F015, F016-F017, and F018-F019 primer pairs, respectively. Each of these fragments was then assembled with the pJP5603sacBGmR amplified fragment at the *Eco*RI RE site by using NeBuilder® HiFi DNA Assembly by New England Biolabs (Ipswich, MA, US). The assembled products were confirmed by performing digestion at the *Eco*RI RE sites, given the product size of 2.38 and 5.09 kbp (pJP5603sacBGmR_*sufA*), 3.67 and 5.09 kbp (pJP5603sacBGmR_*iscA*), and 3.73 and 5.09 kbp (pJP5603sacBGmR_*frmR*) (**Figure 2.2 (B), (C), and (D),** respectively).

Next, the successfully constructed vectors were transformed into an *E. coli* strain S17-1 (*λpir*) and the transformants were selected on medium-containing Sm and Gm.

The mobilizable vector was then independently transferred via conjugation from the S17-1 (λ*pir*) to the respective "up"-mutants. In the mutant strains, the homologous recombination will take place between the target gene sequence on the suicide vector and the respective mutated genes, consisting of the homology arms. Primary homologous recombination was induced by culturing the putative transconjugants on LB Lennox medium-containing Gm and Km. The pRH220 and pRS316::$oriT^{P}$ plasmids were then introduced into the primary recombinant via conjugation, prior to inducing the secondary homologous recombination on medium-containing 10% sucrose supplemented with Ap and Cm. The isolation of clones with the complete removal of the kanamycin resistance gene (Km^{R}) cassette was performed on LB Lennox medium-containing Ap and Cm (with and without Km). The clones with Km sensitive (Km^{S}) phenotype represent complemented strains. The representative of the complemented strains of Δ*sufA*+*sufA*, Δ*iscA*+*iscA*, and Δ*frmR*+*frmR* were subjected to PCR confirmation by using F001-F020, F021-F022, and F003-F023 primer pairs, respectively.

***Trans*-kingdom conjugation**

Two-step genome-wide screening was performed to isolate the *E. coli* with high TKC ability candidates ("up"-mutants). The first screening step was performed as reported by Zoolkefli et al., (2021). In the second screening step, the antibiotics and nutrients were excluded during the conjugation reaction process **(Figure 2.3 (A))**. The purpose is to create a stringent condition to select the correct "up"-mutant candidates. The conjugation reaction includes 50 µL overnight culture of each *E.*

coli donor mutant and 50 µL of yeast recipient suspension (containing 2.0 × 10^6 cfu/ 50 µL) in TNB (80mM Tris-HCl [pH 7.5], and 0.05% NaCl). Both donor and recipient were mixed and incubated at 28°C for an hour, followed by the selection of transconjugants. The selection of transconjugants was performed by spotting 15 µL of the conjugation reaction mixture on SC-Ura supplemented with Tc. The culture plate was incubated at 28°C for 48 to 72 h. TKC efficiency for this screening was calculated as the number of transconjugants per median number of transconjugants of the control strains.

For the standard TKC reaction, the suspension of donor *E. coli* strains in LB Lennox medium and recipient yeast in TNB containing 1.8 × 10^7 and 4.0 × 10^6 cfu/ 300 µL, respectively, were mixed. The low-living cell ratio of donor overnight cultures for all *E. coli* double- and two single-KO mutants (Δ*frmA* and Δ*frmB*) were concentrated to four times their original concentration. The purpose is to adjust the living cell number to an input cell number of the wild-type control. The conjugation reaction was performed for up to 6 h for the assessment of IncP1α conjugation by the *E. coli* "up"-mutants. The conjugation reactions for the other TKC experiments including the complementation analysis were performed for 1 h. The TKC efficiency was determined based on the recovery of uracil phototrophic transconjugants and the supplemented Tc in the selection medium to inhibit the growth of donor *E. coli*. For these TKC experiments, the TKC efficiency was calculated as the number of transconjugants per recipient cell.

Bacterial conjugation

The identical protocol was used as described in standard TKC, where 300 µL of the overnight culture suspensions in LB Lennox medium of both the “up"-mutant donor and SY327 (λ*pir*) recipient, were used during the conjugation reaction. In the conjugation reaction, the donor and recipient containing 1.8×10^7 and 7.1×10^7 cfu/ 300 µL, respectively, were mixed and the co-cultivation was performed for up to 6 h. As for the autoinducer assessment, 40 µM of *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) (Merck, Germany) was added exogenously into the reaction mixture and was substituted with 0.6 µL of DMSO as for control. The conjugation reaction was performed up to 6 h at 37°C. The conjugation efficiency was determined based on the selective transconjugants on the medium supplemented with Rf and in addition of Ap and Km for the autoinducer assessment. The conjugation efficiency was calculated as the number of transconjugants per number of recipient cells.

RNA isolation and quantitative RT-PCR

For the preparation of RNA purposes, identical culture conditions as conjugation experiments were used for the preparation of donor *E. coli* cell cultures. Total *E. coli* RNA was extracted using a NucleoSpin® RNA kit, purchased from Macherey-Nagel GmbH & Co. KG (Dueren, Germany). As for DNA removal and cDNA conversion, 2 µg of the harvested total RNA was used as a template according to the manufacturer’s instructions, using a PrimeScript™ RT reagent kit with gDNA Eraser purchased from TaKaRa Bio Inc. (Shiga, Japan). One hundred ng of cDNA with appropriate primer sets were included in the pre-mixed FastStart Essential DNA Green Master and

subjected to RT-qPCR on a LightCycler® 96 Instrument. The PCR conditions include pre-incubation at 95°C for 10 mins, followed by 45 cycles of 3-step amplification at 95°C for 10 s, 60°C for 10 s, and extension at 72°C for 10 s. For melting curve construction, 95°C for 10s, 65°C for 1 min, and 97°C for 1 s. Both the instrument and reagent were purchased from Roche Diagnostics Corporation (Indianapolis, United States). The expression levels of the target genes were normalized to the expression of the internal reference genes, *cysG* and *rrsA* (Zhou et al., 2011). All primers used in this experiment are listed in **Table 2.4**.

Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM) of at least three independent biological experiments. The differences between groups were analyzed using Student's *t*-test when two groups were compared by two-tailed, and one-way ANOVA (Tukey HSD analysis) for multiple-group comparison. Analyses were performed using SPSS IBM Software for Windows Version 17.0 (SPSS Inc. Chicago, II, United States). Tests were considered statistically significant when $p < 0.05$, $p < 0.01$, or $p < 0.001$.

Results

Isolation and identification of high-TKC *E. coli* mutants' donor by genome-wide screening

To identify mutants with high TKC ability, two-step genome-wide screening of donor *E. coli* single gene deletion mutants on plasmid transfer to yeast recipients, was performed. Mutants that showed a $\log_2$ substituted relative TKC value equal to or greater than three (eightfold compared to parental strain) during the first screening step, were isolated for the second screening step. In total, 233 out of 3,884 mutants were isolated (Zoolkefli et al., 2021). In the second screening step, the top three out of 233 mutants were selected (Δ*sufA*, Δ*iscA*, and Δ*frmR*) and named "up"-mutants. These mutants stably showed high TKC efficiency compared to the parental strain within triplicate experiments (sum $\log_2$ value ≥ 2.48) **(Figure 2.3 (B))**. The *sufA* and *iscA* genes encode the proteins within the iron-sulfur cluster assembly machinery (Lu et al., 2008b), while the *frmR* gene encodes FrmR transcriptional repressor protein on formaldehyde-sensing (*frm*) operon (Higgins and Giedroc, 2014; Denby et al., 2016).

The enhancement of both *E. coli*-yeast and *E. coli*-*E. coli* conjugations by the three "up"-mutants

To confirm the enhancement effect of TKC efficiency by Δ*sufA*, Δ*iscA*, and Δ*frmR* mutant donors, the TKC efficiency between these mutants and the parental strain was compared, at different co-cultivation times **(Figure 2.4).** At 1 h co-cultivation, at least 17-fold increases in TKC efficiency were observed in these mutants

compared to the parental strains **(Figure 2.4 (A)).** At 6 h co-cultivation, at least ninefold increases in TKC efficiency were observed in these mutants compared to the parental strain **(Figure 2.4 (A)).**

To assess the effect of these mutations on the bacterial conjugation efficiency, the corresponding conjugation reaction was performed with *E. coli* strain SY327 recipient cells. At 1 h co-cultivation, at least ninefold increases in conjugation efficiency were observed in these mutants compared to the parental strain **(Figure 2.4 (B)).** At 6 h co-cultivation, at least fourfold increases in conjugation efficiency were observed in these three "up"-mutants compared to the parental strain **(Figure 2.4 (B)).** Based on these results, it may be concluded that these three "up"-mutants (Δ*sufA*, Δ*iscA*, and Δ*frmR*) increased in both *E. coli*-yeast and *E. coli*-*E. coli* conjugations. Between the "up"-mutants and parental strain, the efficiency of IncP1α plasmid in both conjugation types consistently showed significant differences at both 1 and 6 h co-cultivation **(Figure 2.4).** Thus, the conjugation reaction was integrated at 1 h co-cultivation after these experiments.

Complementation analysis was performed to confirm the repressing effect of *sufA*, *iscA*, and *frmR E. coli* chromosomal genes on the TKC of the IncP1α plasmid. This analysis was performed by integrating the wild-type genes into the Δ*sufA*, Δ*iscA*, and Δ*frmR* donors via homologous recombination, followed by TKC to yeast recipient **(Figure 2.5).** As expected, the result of the complementation analysis shows that the repressing effect on TKC was restored within the complemented donor strains.

Deficiency of *frmR, sufA,* and *iscA* genes can affect independently, but not synergistically to activate the IncP1α plasmid transfer

To assess the correlation between the interaction of the isolated KO genes and the conjugation efficiency, the double-KO mutant strains were constructed, by introducing the second gene mutation within the "up"-mutants, located within the same or different operon.

frmR, within the *frm* operon of *E. coli* K-12 derivatives, encodes a transcriptional repressor protein, FrmR (as a negative regulator). The FrmR specifically deactivates the transcription of this operon in the absence of formaldehyde (Higgins and Giedroc, 2014; Denby et al., 2016; Osman et al., 2016). In the presence of formaldehyde, the expression of this operon is activated due to the binding mechanism between the FrmR repressor protein and the formaldehyde. This mechanism consequently induced the formaldehyde detoxification machinery by FrmA and FrmB, encoded by the downstream genes, *frmA* and *frmB,* respectively (Denby et al., 2016; Osman et al., 2016) **(Figure 2.6).** In this experiment, the FrmR protein has been hypothesized to be related to the increase in conjugation efficiency, either due to its absence or inactivation as the result of a deletion mutation or the binding of formaldehyde, respectively. As reported by Zoolkefli et al., (2021) **(Figure 2.7 (A))**, an effect on the conjugation efficiency of the parental strains with the wild-type gene of *frmR* was observed upon the addition of formaldehyde during the conjugation reaction. According to the TKC results, in comparison to the non-treated parental strain, no significant difference was observed between the treated and non-treated Δ*frmR* mutant with formaldehyde. These results support the

hypothesis.

The effect of the downstream genes of *frmR* on TKC efficiency was also examined. According to the result, the TKC efficiency of both Δ*frmA* and Δ*frmB* mutants was significantly increased, compared to the parental strain and at a comparable efficiency as Δ*frmR* **(Figure 2.7 (B))**. This can be attributed to the accumulation of endogenous ligands, including formaldehyde, as a result of the detoxification mechanism failure, resulting in the inactivation of the FrmR protein. To further examine the interaction between the *frmA* or *frmB* and *frmR* genes as well as the relation to the TKC efficiency, the double-KO mutants, namely, Δ*frmA*Δ*frmR* and Δ*frmB*Δ*frmR,* were constructed. As a result, no significant increase in the TKC efficiency was observed between the double-KO and single-KO mutants but significantly increased (at least sevenfold) compared to the parental strain **(Figure 2.7 (B)).** This result indicates neither the *frmA* nor *frmB* alone directly affects the conjugation efficiency of the IncP1α plasmid. This phenomenon could be the effect which solely comes from *frmR*.

The construction of the double-KO mutant, Δ*iscA*Δ*sufA,* was unsuccessful probably because of its synthetic lethality (Vinella et al., 2009). Due to that, the KO of either of these genes with Δ*frmR* was constructed to discover the genetic interaction. The TKC efficiency of the double-KO mutants Δ*iscA*Δ*frmR* and Δ*frmR*Δ*sufA* showed significantly higher compared to the parental strain by at least 11-fold. However, no significant difference compared to the single-KO Δ*frmR,* Δ*sufA*, and Δ*iscA* was observed **(Figure 2.7 (C))**. These results indicate that the *frmR, sufA,* and *iscA* genes affect the conjugation independently but probably act on

an identical step of the conjugation machinery of the IncP1α plasmid.

To validate the correlation between the conjugation efficiency by these "up"-mutants and *tra/trb* gene expression, the basal expression levels of the selected *tra* and *trb* genes (*traI, traJ, traK*, and *trbL*) were assessed. The basal expression levels of these genes, which harbored within the IncP1α-derived pRH220 helper plasmid in the donor cells, were compared between the mutants and parental strain. These genes were selected as representatives of the three major operons within the RP4 IncP1α plasmid under the regulation of three major promoters: $P_{traJ,}$ P_{traK}, and P_{trbB} (Pansegrau et al., 1994). As shown in **Figure 2.8 (A)**, the expression of *traI, traJ, traK*, and *trbL* in the donor mutant strains of Δ*frmR,* Δ*sufA*, and Δ*iscA* showed no significant increase in comparison with the parental strain. These results confirmed the increase in both *E. coli*-yeast and *E. coli*-*E. coli* conjugations by the three mutants were not directly influenced by the selected conjugation-related genes.

In addition, the correlation between the basal gene expression of *sufA, iscA,* and *frmR* with conjugation efficiency was also assessed, by evaluating the expression of these genes within the "up"-mutant donor strains **(Figure 2.8 (B))**. Real-time PCR analysis revealed that the expression level of the *iscA* gene in both Δ*frmR* and Δ*sufA* mutants, as well as the *frmR* gene in both Δ*sufA* and Δ*iscA* mutants, were not significantly different and was comparable with that of the parental strain. These results indicate that *iscA* and *frmR* genes in the mutants expressed at the same level as in *E. coli* parental strain. In addition, the expression of the *sufA* gene in both Δ*iscA* and Δ*frmR* was significantly higher (approximately six- and ninefold,

respectively) compared to the parental strain. However, no complementary effect on the repression of conjugation efficiency in these two mutants was observed **(Figure 2.7 (C))**. Therefore, no clear correlation between the transcriptional interaction among these three genes with conjugation efficiency was observed.

Based on the results obtained from the single- and double-KO mutant analyses, it may be suggested that the defects of FrmR, SufA, and IscA affect independently to terminate the repression of IncP1α plasmid conjugation, but probably act on the identical step(s) of conjugation machinery **(Figures 2.6 and 2.10).**

Discussion

The enhancing effect on conjugation efficiency, mediated by IncP1α-T4SS by the three mutants (Δ*sufA*, Δ*iscA*, and Δ*frmR*) **(Figure 2.3)** to both prokaryote and eukaryote **(Figure 2.4)** was observed and confirmed since the complemented strains of these mutants showed a normal phenotype on TKC **(Figure 2.5).**

A previous study reported that the autoinducer molecules, *N*- acyl homoserine lactones (AHLs), produced by *P. aeruginosa* can suppress interspecies conjugation (Lu et al., 2017). The AHL molecule, such as *N*-(3-oxododecanoyl)-$_{L}$-homoserine lactone (OdDHL), produced by *P. aeruginosa* PAO1, can suppress the mobilizable plasmid conjugation to *E. coli*, mediated by the RP4 IncP1α plasmid. Based on the plausible explanation by Lu et al. (2017), the repressing mechanism caused by the deactivation of RP4-encoded *traI* expression, as a result of transcriptional repression upon the binding of AHLs to the SdiA-box, followed by the binding of AHL-SdiA to the upstream region of the *traI* (Lu et al., 2017). However, according to the sequence given, the authors mistakenly annotated the position of the SdiA-box, which overlapped with the coding sequence of the *traJ* (located less than 100 bp downstream of the first nucleotide sequence of *traJ* start codon), but not with the promoter region of *traI*. The authors also did not perform any analysis by looking at the *traI* and *traJ* expression and the effect on conjugation, upon the treatment with QS molecule. Therefore, it is unclear whether the suppression of interspecies conjugation mediated by the QS signal is directly correlated to the *traI* expression deactivation or the TraJ protein truncation as a result of *traJXIH* mRNA

extension inhibition upon the binding of AHL-SdiA. In this case, the *traJXIH* mRNA and TraJ protein truncation may results in the aberrant mRNA and protein products degradation by the SmpB-tmRNA (transfer messenger RNA)-mediated *trans*-translation quality control system in bacteria (Karzai et al., 1999, 2000; Gillet and Felden, 2001; Richards et al., 2008). The degradation of the essential TraJ protein may fail the relaxosome formation, resulting in the conjugation mechanism failure since the binding of TraJ has been proposed as a first step of functional relaxosome assembly (Pansegrau et al., 1994). Although there is a published article reported that the truncation of F plasmid-encoded *traI* doesn't affect the relaxosome formation, the truncated TraI protein is somehow unable to support the DNA transfer (Matson and Ragonese, 2005).

Previously, there are published reports on the QS regulator of LuxR homolog, SdiA, in *E. coli* (Wang et al., 1991; Yamamoto et al., 2001). To date, no report was available on the autoinducer synthase, LuxI-like homolog in *E. coli* confirmed its inability to produce AHLs. In this study, no significant effect was observed on the conjugation efficiency, even though the OdDHL was exogenously supplied in the conjugation reaction **(Figure 2.9)**. This phenomenon is mostly due to the novel mode of the LuxR-like SdiA in *E. coli* which controls the expression of the *ftsQAZ* operon, particularly for cell division (Yamamoto et al., 2001). Based on this status, at least under the experimental conditions used in this study, the increase in conjugation efficiency by the "up"-mutants is not related to the autoinducer-mediated mechanism. In addition, the enhancing phenotype by these mutants on the conjugation efficiency also did not directly influence by the

selected conjugation-related genes encoded within the IncP1α-derived plasmid according to the result shown in **Figure 2.8 (A)**.

FrmR is a formaldehyde-sensing transcriptional repressor of the *frm* operon (Denby et al., 2016; Osman et al., 2016). As reported by Zoolkefli et al. (2021), no additional effect on conjugation efficiency was observed in the Δ*frmR* mutant due to the addition of formaldehyde **(Figure 2.7 (A))**. This result suggests that the absence or inactivation of FrmR from the *frm* operon due to gene deletion or the binding of this protein to the excessive formaldehyde, respectively, results in an increased conjugation efficiency. In the case of TKC efficiency by the gene mutation within the *frm* operon **(Figure 2.7 (B))**, single-KO of both Δ*frmA* and Δ*frmB* conferred no significant difference compared to Δ*frmR*. This was probably due to the accumulation of endogenous ligands, including formaldehyde, caused by the failure of the detoxification mechanism of FrmA and FrmB within the cells, which possibly inactivated the FrmR, leading to the increase in conjugation efficiency **(Figures 2.6 and 2.7 (A))**. Also, FrmR is predicted to repress the expression of other target factor(s) (activator) within the *E. coli* donor which leads to an increase in conjugation efficiency. Previously, a study reported that the deletion of *frmA* causes an increase in basal *frmR* promoter activity as well as improved sensitivity to formaldehyde, which supports our expectation (Woolston et al., 2018). In the case of double-KO mutants, Δ*frmA*Δ*frmR* and Δ*frmB*Δ*frmR*, no additional increase in TKC efficiency was observed between these mutants and single-KO mutants (Δ*frmR,* Δ*frmA* and Δ*frmB*). Based on this status, it may propose that neither *frmA* nor *frmB* alone directly affects the conjugation efficiency of the IncP1α plasmid.

This phenomenon could be the effect which solely comes from *frmR*.

In *E. coli*, the iron-sulfur cluster carrier protein genes *iscA, sufA*, and *erpA* are paralogs and have overlapping functions (Loiseau et al., 2007; Roche et al., 2013). The KO mutant of *erpA* is not included in the Keio library because of its essentiality (Loiseau et al., 2007). In addition, the double-KO mutant of Δ*iscA*Δ*sufA* genes results in synthetic lethality under an aerobic condition (Vinella et al., 2009). This is due to the complementary roles constituted by SufA and IscA in the biogenesis of the iron-sulfur cluster in *E. coli*, particularly under aerobic conditions (Lu et al., 2008b). Thus, this double-KO mutant was excluded from this conjugation assessment, and the KO mutant with the inclusion of either of these genes with Δ*frmR* was constructed to confirm the gene interaction. The conjugation efficiency of double-KO mutants Δ*iscA*Δ*frmR* and Δ*frmR*Δ*sufA* did not exhibit any synergistic increase in conjugation efficiency and were comparable with that of the single-KO mutants (Δ*sufA*, Δ*iscA*, and Δ*frmR*) **(Figure 2.7 (C))**. Also, the loss of expression of any three genes in a mutant did not lead to attenuated expression of the other two genes **(Figure 2.8 (B)).**

Based on this status, it is predicted that SufA, IscA, and FrmR target different unknown factor(s) (e.g., activator or repressor) within the *E. coli* donor cells, and independently affect the conjugation mechanism. The defect of SufA, IscA, and FrmR probably targets the activator(s) which may directly or indirectly activate the conjugation mechanism. In addition, SufA and IscA are also predicted to work indirectly, either in activating or repressing the conjugation factor by repressing or activating the unknown target factor(s), respectively. This prediction was made

since no decreasing effect in conjugation efficiency was observed in single-KO Δ*sufA* and Δ*iscA,* as well as in double-KO Δ*iscA*Δ*frmR* and Δ*frmR*Δ*sufA* mutants, regardless of the presence of Δ*frmR* mutation. This is probably due to the absence of a complementation effect between both SufA and IscA. Thus, it is predicted that both SufA and IscA are probably necessary for activating or repressing the conjugation mechanism indirectly with an unknown target factor of FrmR.

On the basis of the results obtained from the single- and double-KO conjugation experiments and the relation with basal gene expression, the models for the repression mechanism of the IncP1-type conjugation system are proposed, based on the known function of SufA, IscA, and FrmR **(Figure 2.10)**. According to these models, SufA and IscA are deduced to work in repressing or activating other target factors (activators or repressors, respectively) within the *E. coli* donor, either indirectly **(Figure 2.10 (A))** or directly **(Figure 2.10 (B))**, respectively. At the same time, the FrmR which may also be a repressor of other target factor(s) (activator), will deactivate and derepress the expression of that factor. The unknown target factor(s) of SufA, IscA, and FrmR may form a complex to indirectly activate or repress the conjugation mechanism. Based on these model mechanisms, the SufA, IscA, and FrmR proteins are possible to repress the conjugation at the identical step(s) of IncP1 conjugation machinery, although the exact mechanism beyond this phenomenon remains unknown.

Table 2.1: Strains used in this study

Strains	Relevant characteristics or genotype	Source or reference
E. coli		
Keio collection	An in-frame single-gene knockout mutant collection derived from BW25113, Km^R	NBRP Japan
BW25113Δ*frmR*Δ*sufA*	*frmR* and *sufA* double-gene knockout mutant, constructed from Δ*frmR* derived from Keio collection, Km^R	This study
BW25113Δ*iscA*Δ*frmR*	*iscA* and *frmR* double-gene knockout mutant, constructed from Δ*iscA* derived from Keio collection, Km^R	This study
BW25113Δ*frmA*Δ*frmR*	*frmA* and *frmR* double-gene knockout mutant, constructed from Δ*frmA* derived from Keio collection, Km^R	This study
BW25113Δ*frmB*Δ*frmR*	*frmB* and *frmR* double-gene knockout mutant, constructed from Δ*frmB* derived from Keio collection, Km^R	This study
BW25113Δ*frmR*+*frmR*	Complemented strain of *frmR* mutant, harbouring *frmR* wild-type gene	This study
BW25113Δ*sufA*+*sufA*	Complemented strain of *sufA* mutant, harbouring *sufA* wild-type gene	This study
BW25113Δ*iscA*+*iscA*	Complemented strain of *iscA* mutant, harbouring *iscA* wild-type gene	This study
BW25113	*F^- Δ(araD-araB)567 ΔlacZ4787(::rrnB-3) λ-rph-1 Δ(rhaD rhaB)568 hsdR514*	NBRP Japan
SY327 (λ*pir)*	Δ*(lac pro) argE(*Am*) recA56 λpir* Rif^R Nal^R	NBRP Japan
S17-1 (λ*pir)*	*F^- RP4-2(Km^R::Tn7,Tc^R::Mu-1) pro-82λpir recA1 endA1 thiE1 hsdR17 creC510*	NBRP Japan
S. cerevisiae		
BY4742	*MAT*α *SSD1-V his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Invitrogen

Table 2.2: Plasmids used in this study

Plasmids	Relevant characteristics or genotype	Source or reference
pBBR122Δ*Cm*^{R}	Derivative of a commercially provided plasmid vector pBBR122; *Rep*$^{pBBR'}$(non-transmissible) *Km*^{R} Δ*Cm*^{R}	Moriguchi et al., 2020
pRH220	Helper plasmid; *tra*$^{P1\alpha}$ *trb*$^{P1\alpha}$ *oriT*$^{P1\alpha}$ *ori*-pSC101 *Cm*^{R}	*AB526840; Nishikawa and Yoshida, 1998
pRS316::*oriT*^{P}	Mobilizable plasmid; *URA3 CEN6/ARSH4 ori*-pMB1 *Ap*^{R} *oriT*^{RP4}	Moriguchi et al., 2013
RP4	IncP1α-type conjugative broad host range plasmid; *Km*^{R}, *Tc*^{R}, *Ap*^{R}	Pansegrau et al., 1994
pJP5603sacBGmR	Mobilizable plasmid; *sacB oriT*$^{P1\alpha}$ *Gm*^{R} Used for the construction of *E. coli* complemented strains	This study *LC599391
pJP5603sacBGmR_*sufA*	Partial *menI*, *ydiH*, *RydB*, *sufA*, and *sufB* integrated within pJP5603sacB*Gm*^{R}	This study
pJP5603sacBGmR_*iscA*	*iscS, iscU, iscA, hscB,* and *hscA* integrated within pJP5603sacB*Gm*^{R}	This study
pJP5603sacBGmR_*frmR*	Partial *yaiX*, *yaiO, frmR, frmA*, and *frmB* integrated within pJP5603sacB*Gm*^{R}	This study

*DDBJ/EMBL/GenBank accession number

Table 2.3: Primers used for the construction of *E. coli* double-knockout mutants and complemented strains

Primer name	Primer sequence (5′-3′)	Purpose
F001	CGGTAAAGCCCCTGCGTTTG	Double KO construction (Δ*sufA*) and Δ*sufA*+*sufA* confirmation
F002	GTGGCTAACTGGGTGAAGAATC	Double KO construction (Δ*sufA*)
F003	GGGGTATGCATTGACATATAG	Double KO construction (Δ*frmR*) and Δ*frmR*+*frmR* confirmation
F004	CAACGATTTCCAGCGGTTTAC	Double KO construction (Δ*frmR*)
F005	ATGATTGAACAAGATGGATTGCAC	Double KO construction (Km^R)
F006	TTAGAAGAACTCGTCAAGAAGG	Double KO construction (Km^R)
F007	GTTCCATCAGTCATTATCTCAG	Double KO construction (Δ*frmA*)
F008	GATGCGTCCGGCGTAGAG	pJP5603sacBGmR construction
F009	GGTCATTTCGAACCCCGGCGTTGTGACAATTTACC	pJP5603sacBGmR construction
F010	GGGTTCGAAATGACCGACCA	pJP5603sacBGmR construction
F011	TACGCCGGACGCATCGT	pJP5603sacBGmR construction

Table 2.3: (continued)

F012	AATTCACTGGCCGTCGTTTTAC	pJP5603sacBGmR_*Eco*RI construction
F013	CGAGCTCGGTACCCGGG	pJP5603sacBGmR_*Eco*RI construction
F014	CGGGTACCGAGCTCGAATTCCTGGAAATCAATGCTAACCA	pJP5603sacBGmR_*sufA* construction
F015	AACGACGGCCAGTGAATTCCTGGGATATTTCCACGTAATC	pJP5603sacBGmR_*sufA* construction
F016	CGGGTACCGAGCTCGAATTCTGCATTGAGTGATGTACG	pJP5603sacBGmR_*iscA* construction
F017	AACGACGGCCAGTGAATTCGTTCACGCACCAGCGGCAC	pJP5603sacBGmR_*iscA* construction
F018	CGGGTACCGAGCTCGAATTCGGCGCTGGAGTTCTTTAATC	pJP5603sacBGmR_*frmR* construction
F019	AACGACGGCCAGTGAATTCACATATTCATCTGGGTTAC	pJP5603sacBGmR_*frmR* construction
F020	TAACGCCCATCGACAAACAC	Δ*sufA*+*sufA* confirmation
F021	ACTGTTTTCCCGTCTGCGTA	Δ*iscA*+*iscA* confirmation
F022	TGCGATCCGCCGCGATAAC	Δ*iscA*+*iscA* confirmation
F023	AGATAAGTCGTGCGCAGATG	Δ*frmR*+*frmR* confirmation

Table 2.4: Primers for quantitative real-time PCR

Gene	Primer sequence (5′-3′)		Amplicon size	Reference
	Forward	Reverse		
traI	GCTGAAATGCTATTGCCGCG	TATCGAAGCCGTTTAGCCGC	178 bp	This study
traJ	AGGGCTACAAAATCACGGGC	TGCTTCTCTTCGATCTTCGCC	178 bp	This study
traK	TCTCCTACGAGACGTTCCGC	TTGGGTTGAAGGTGAAGCCG	195 bp	This study
trbL	TCGACAACGTATTGCAGCGC	CGGTGAAGATGGTGAACCGC	189 bp	This study
sufA	GGCGATACACATCCGTGAGC	AGCGGGACAAACAGCTTCG	171 bp	This study
iscA	AGAACCTCCGGGTGTTCAGG	AACCCTTCGTTCAGGCCTTC	173 bp	This study
frmR	GGGGCAGATTGATGCTCTGG	AACGGATTGGCTGACTTCGC	181 bp	This study
cysG	CGCAGTCGCTGGCAAACAACGA	TTCAGTGTCAGCAGCCCGGCAT	136 bp	This study
rrsA	CTCTTGCCATCGGATGTGCCCA	CCAGTGTGGCTGGTCATCCTCTCA	105 bp	Zhou et al., 2011

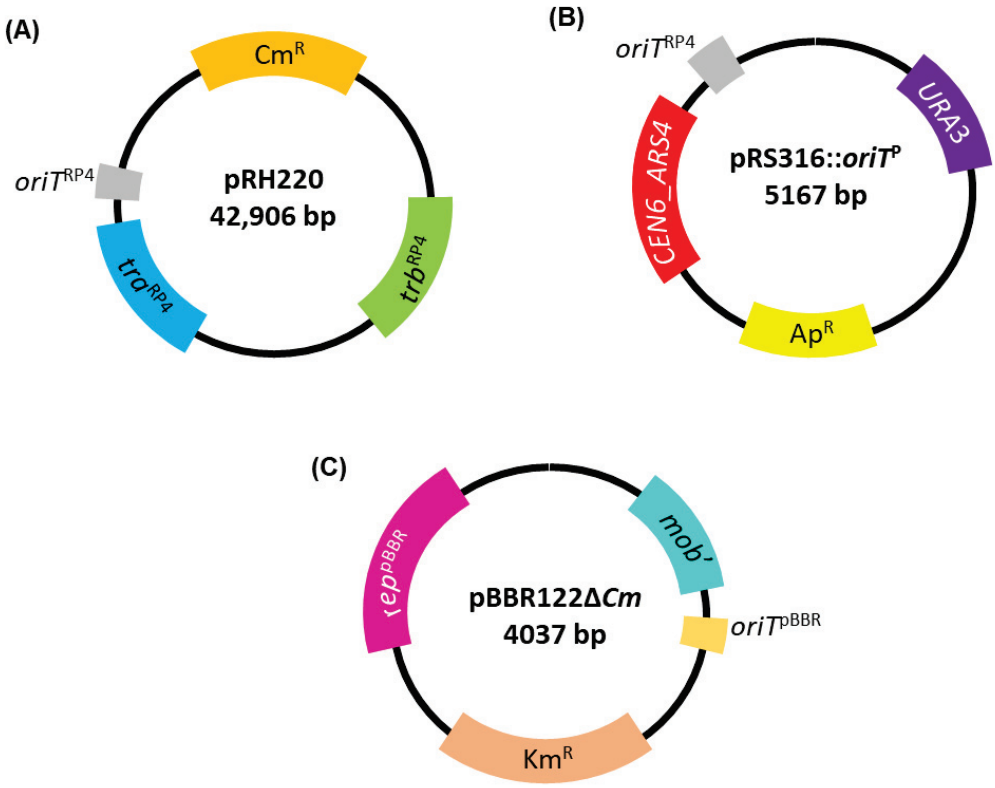


Figure 2.1: Plasmids used during the genome-wide screenings. (A) pRH220 helper plasmid, **(B)** pRS316::*oriT*^{P} TKC shuttle vector, and **(C)** pBBR122Δ*Cm*. pRH220 and pRS316::*oriT*^{P} plasmids were introduced into the *E. coli* Keio single-KO mutants prior to the genome-wide screening analysis. In addition to the pRH220 and pRS316::*oriT*^{P} plasmids, pBBR122Δ*Cm* plasmid was introduced into the control strain of BW25113. All mutants and control strains used in the screening were selected using Ap, Cm, and Km antibiotics.

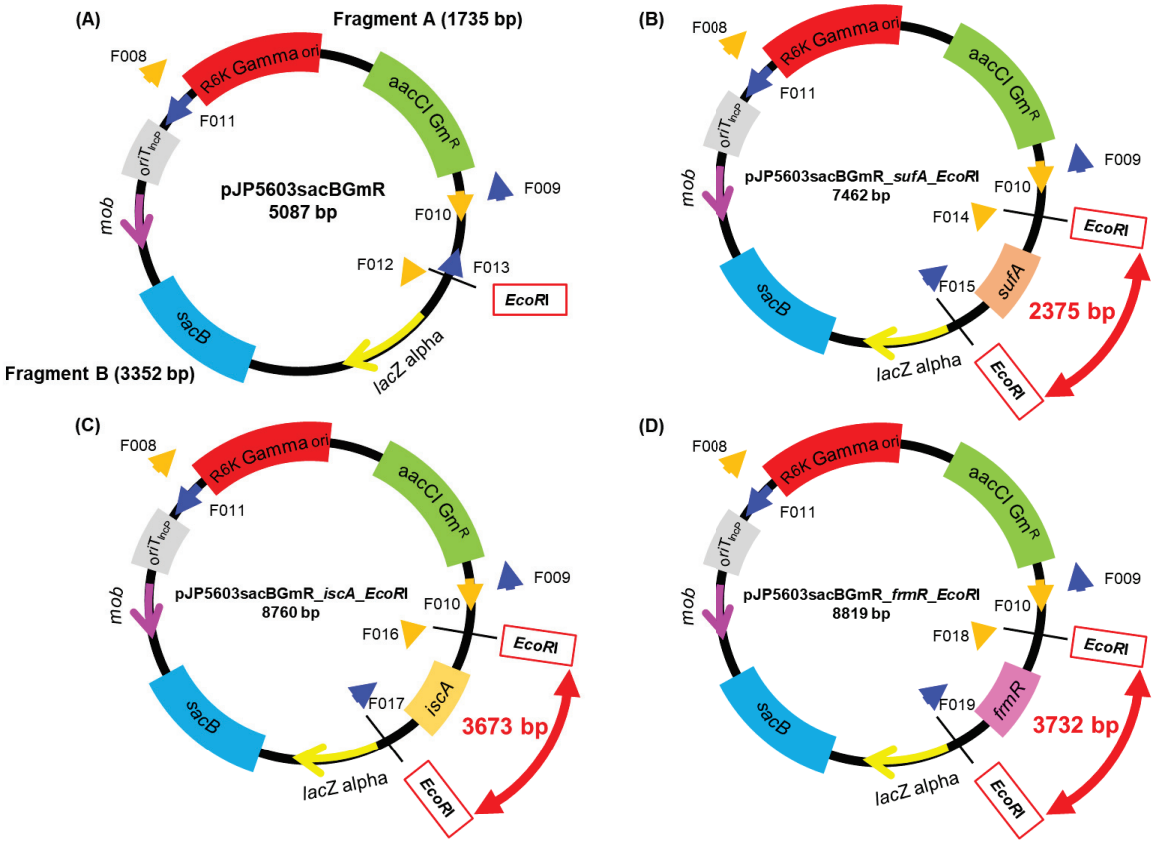


Figure 2.2: A mobilizable suicide vector and plasmids used for the construction of *E. coli* complemented strains. (A) The backbone of the suicide vector, pJP5603sacBGmR, was constructed by assembling two amplified fragments (fragments A and B), which contain selectable markers; *aacC*1 (Gm^R) and *sacB* genes, respectively. **(B)** pJP5603sacBGmR_*sufA*, **(C)** pJP5603sacBGmR_*iscA*, and **(D)** pJP5603sacBGmR_*frmR* plasmids were used for complementation of Δ*sufA*, Δ*isc*A, and Δ*frmR* mutations, respectively.

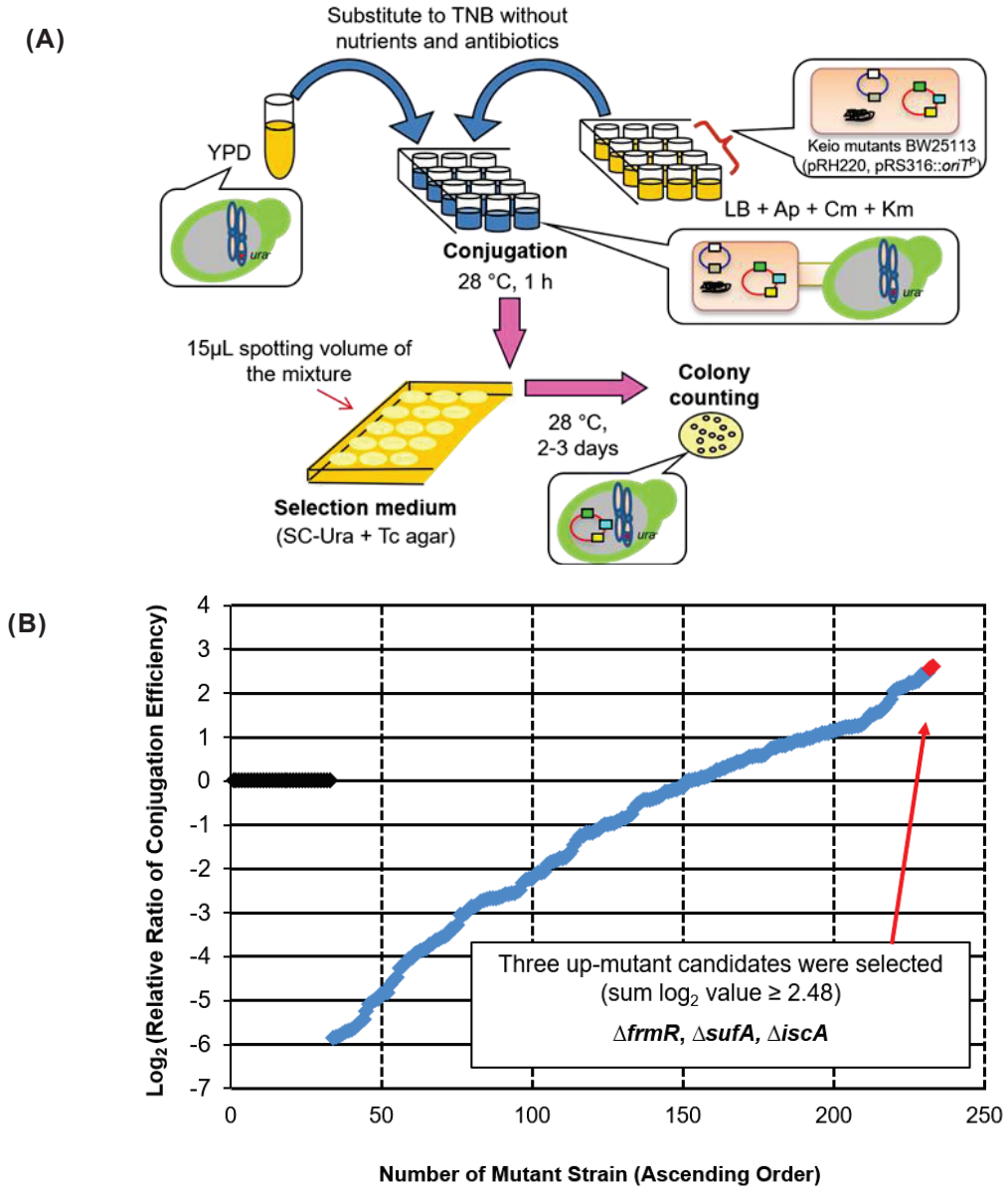


Figure 2.3: Second step of genome-wide screening of *E. coli* Keio single-KO mutants (233 mutants). (A) Overall flowchart of the second screening. **(B)** Distribution pattern of the relative TKC efficiency within triplicate data (n = 3) by the mutant donors in 1 h conjugation reaction, normalized using the median value of control in every experimental replicate. Log_2 [number of transconjugants/ median number of transconjugants of the control strains] values for each mutant are plotted in ascending order. In this screening, 33 mutants showed values lower than the detection limit (indicated as black diamonds). Three mutants with increased conjugation efficiency within the triplicate experiments (sum log_2 value ≥2.48) were isolated and subjected to characterization analysis. BY4742 was used as the recipient.

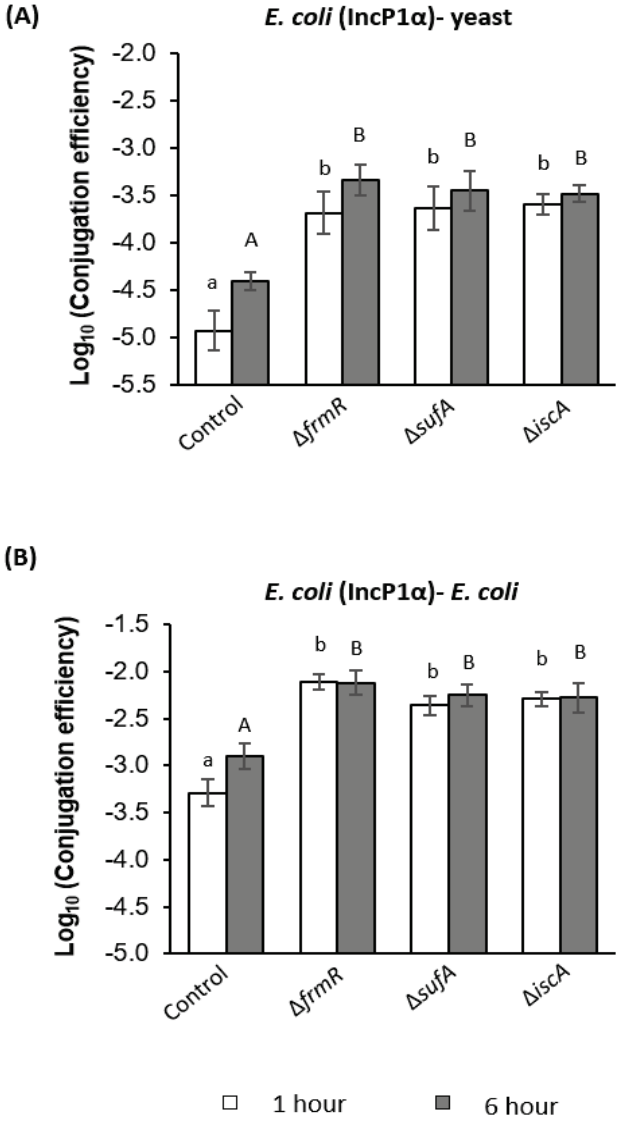


Figure 2.4: Effect of *frmR*, *sufA*, and *iscA* mutations in *E. coli* on IncP1α conjugations. (A) TKC efficiency of IncP1α transfer from *E. coli* to yeast within four experimental replicates (n = 4). **(B)** Conjugation efficiency of IncP1α transfer from *E. coli* to *E. coli* within seven experimental replicates (n = 7). Both conjugation reactions were performed for 1 h (white bar) and 6 h (black bar). Data are presented as mean ± standard error of the mean (SEM). Different letters indicate significant differences between mutants and wild-type control at $p < 0.05$ using Tukey HSD multiple comparison analysis. BY4742 and SY327 (λ*pir*) were used as the recipients in **(A)** and **(B)**, respectively. BW25113 parental strain was used as the control.

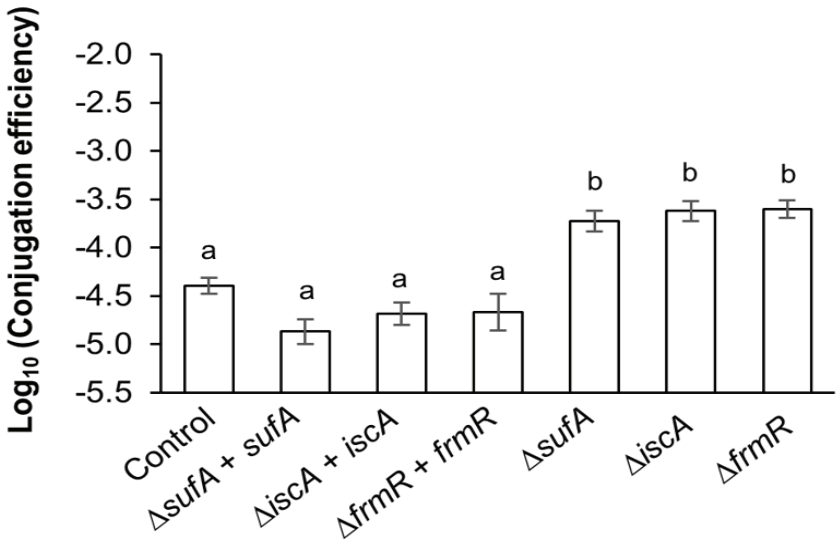
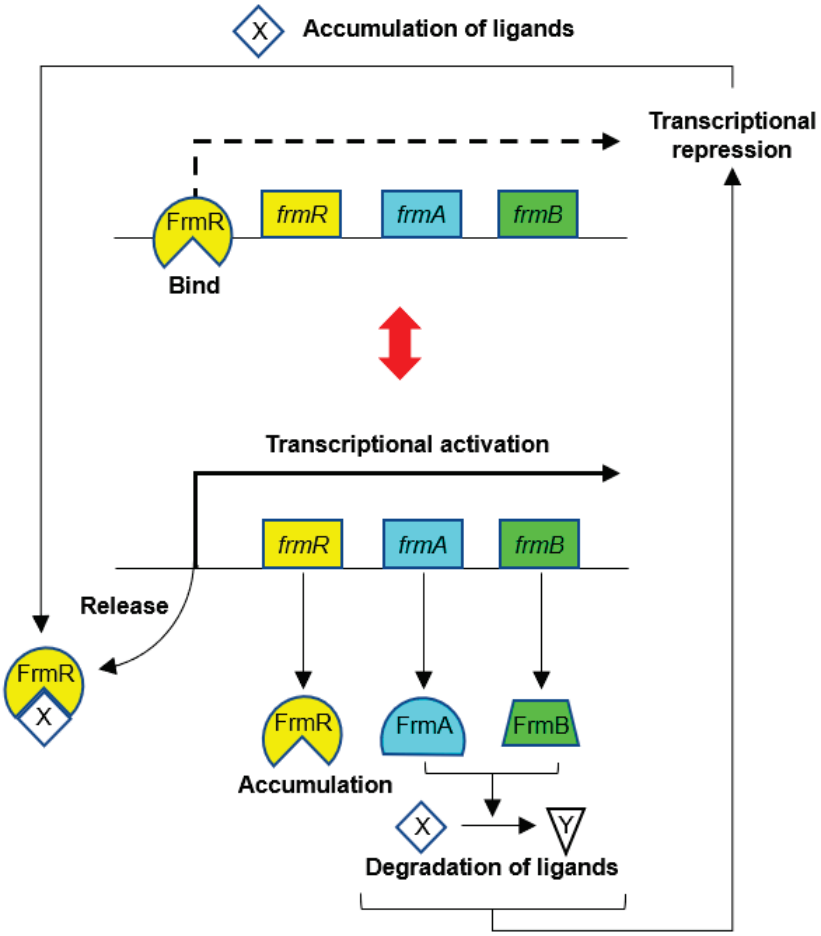


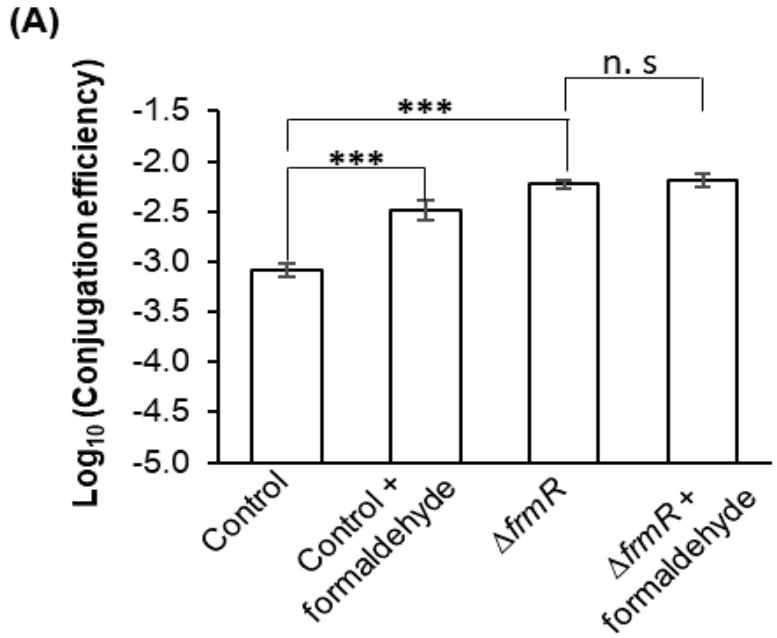
Figure 2.5: Complementation analysis of *sufA, iscA,* and *frmR* mutants. *E. coli* single-KO mutants (Δ*sufA*, Δ*iscA*, and Δ*frmR*) were transformed with pJP5603sacBGmR_*sufA,* pJP5603sacBGmR_*iscA,* or pJP5603sacBGmR_*frmR* (each of which includes their adjacent sequences) by S17-1 (λ*pir*) via conjugation method. The primary homologous recombination was then induced within the genome of the Δ*sufA*, Δ*iscA*, and Δ*frmR*, respectively, by culturing on LB Lennox medium-containing Gm and Km. Prior to the secondary homologous recombination step, pRH220 and pRS316::$oriT^{P}$ plasmids were introduced into the primary recombinant strains, followed by the induction of secondary homologous recombination by culturing the strains on the LB Lennox medium-containing Ap, Cm and 10% sucrose. The successfully complemented strains with the complete removal of the kanamycin resistance gene (Km^{R}) cassette were isolated on LB Lennox medium containing Ap and Cm. Following that, an assessment of the TKC efficiency (within 1 h co-cultivation) by these complemented strains, in comparison to wild-type and single-KO mutants of Δ*sufA*, Δ*iscA,* and Δ*frmR*, was performed. Data are presented as mean ± standard error of the mean (SEM) for nine experimental replicates (n = 9) (control, Δ*sufA* + *sufA*, and Δ*frmR* + *frmR*); eight experimental replicates (n = 8) (Δ*frmR,* Δ*iscA,* and Δ*sufA*); six experimental replicates (n = 6) (Δ*iscA* + *iscA*). Different letters indicate significant differences between mutants and wild-type control at $p < 0.05$ using Tukey HSD multiple comparison analysis. BY4742 was used as the recipient. BW25113 parental strain was used as the control.



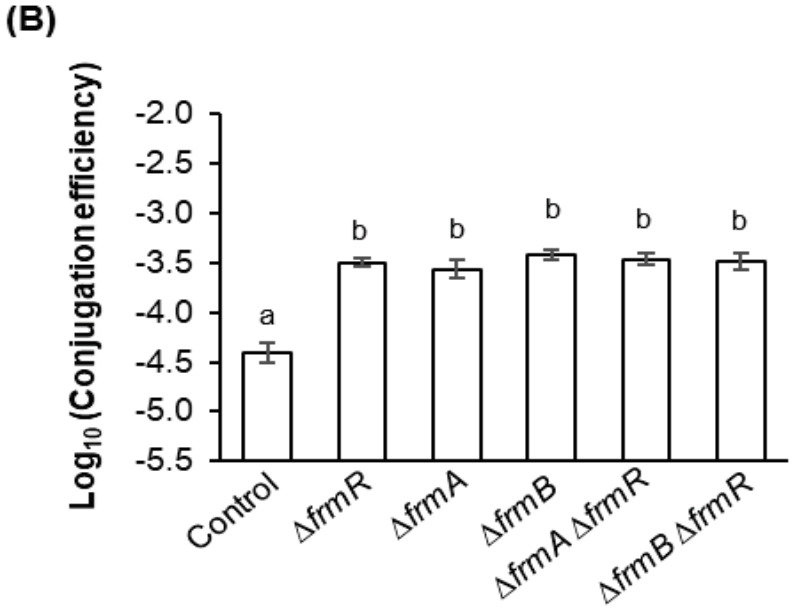
Feedback regulation of *frm* operon

(Adopted from Zoolkefli et al., 2021)

Figure 2.6: Feedback regulation of *frm* operon. FrmR is a transcriptional repressor of the *frm* operon. The accumulation of ligands (e.g., formaldehyde) causes the inactivation of FrmR repressor activity due to the binding of ligands, consequently activating the transcriptional activity of this operon. This transcriptional activation leads to the expression of the downstream genes, *frmA* and *frmB,* which encode FrmA and FrmB, respectively, for formaldehyde detoxification.



(Adopted from Zoolkefli et al., 2021)



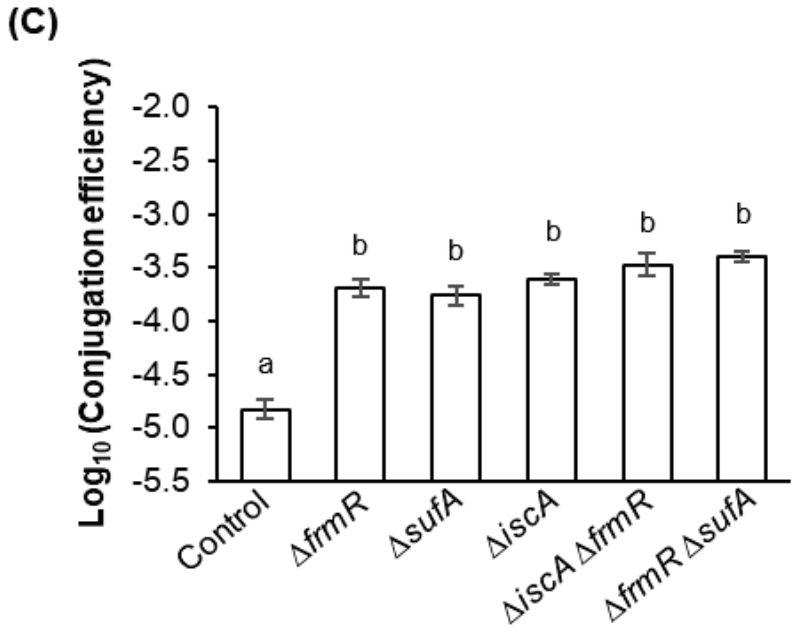


Figure 2.7: Confirmation analysis of the high conjugation efficiency in *frmR*, *sufA*, and *iscA* mutants. (A) Effect of formaldehyde (250μM) on the conjugation efficiency of IncP1α plasmid transfer by Δ*frmR* mutants and wild-type control to *E. coli* recipient within five experimental replicates (n = 5)**. (B)** TKC efficiency of IncP1α transfer by genes-deficient *E. coli* donor, belonging to the same operon (*frm* operon) within five experimental replicates (n = 5). **(C)** TKC efficiency of IncP1α transfer by genes-deficient *E. coli* donor, belonging to the different operons. This experiment was performed within 12 experimental replicates (n = 12) for single-KO mutants and wild-type control, while five experimental replicates were performed for double-KO mutants (n = 5). All conjugation reactions were performed for 1 h. Data are presented as mean ± standard error of the mean (SEM). Different letters indicate significant differences between mutants and wild-type control at $p < 0.05$ using Tukey HSD multiple comparison analysis. BW25113 parental strain was used as the control. BY4742 was used as the recipient.

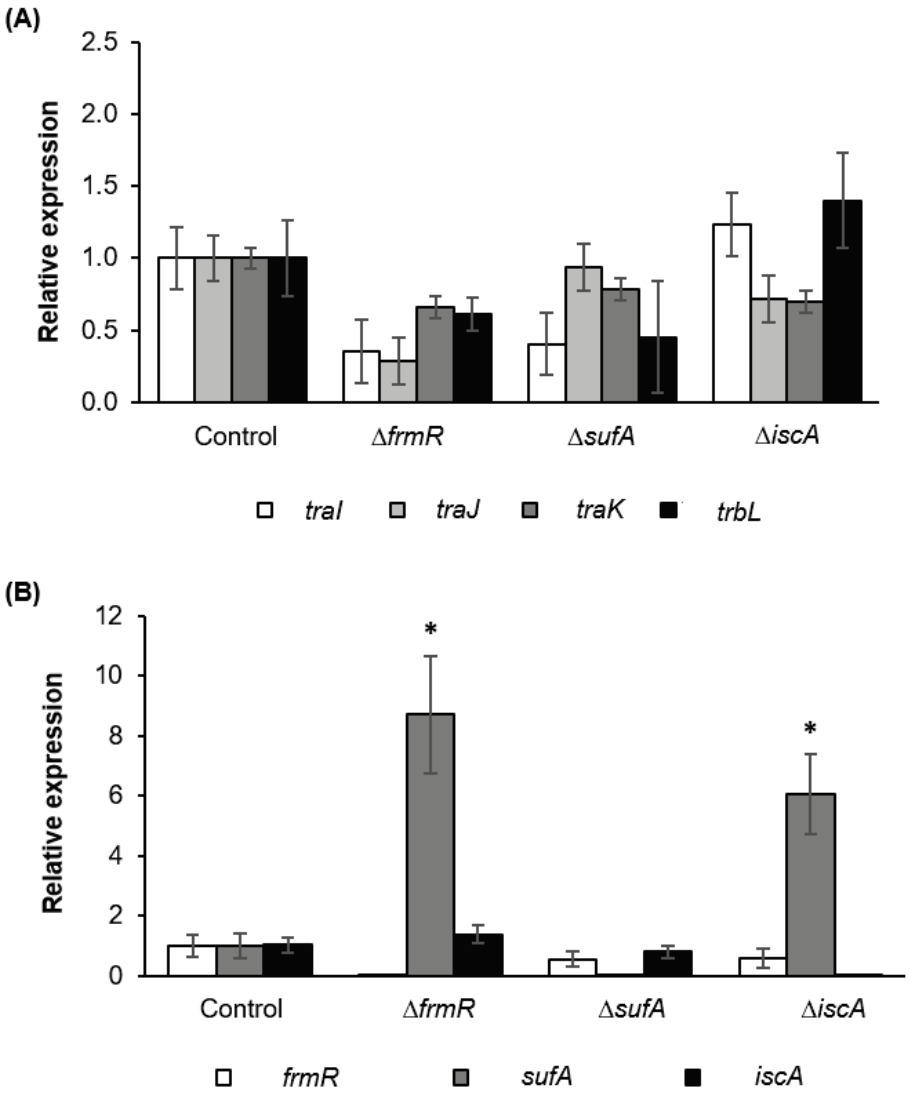


Figure 2.8: Expression analysis of conjugation-related and "up"-mutant genes by RT-qPCR. (A) Expression of *traI*, *traJ*, *traK*, and *trbL* genes within the helper plasmid, IncP1α-pRH220, harboured in the "up"-mutants and wild-type control donor strains, within triplicate experiments (n = 3). **(B)** Expression of *frmR*, *sufA*, and *iscA* genes within the "up"-mutant and wild-type control donor strains, within six experimental replicates (n = 6). Data are presented as mean ± standard error of the mean (SEM). Asterisk (*) indicates statistically significant differences at $p < 0.05$ (two-tailed *t*-test) compared to the wild-type control. BW25113 parental strain was used as the control.

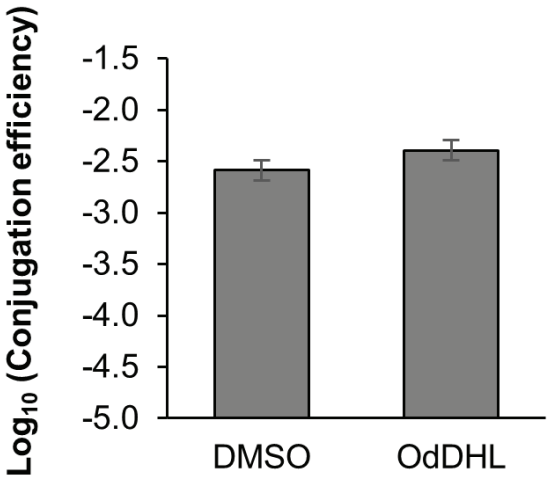
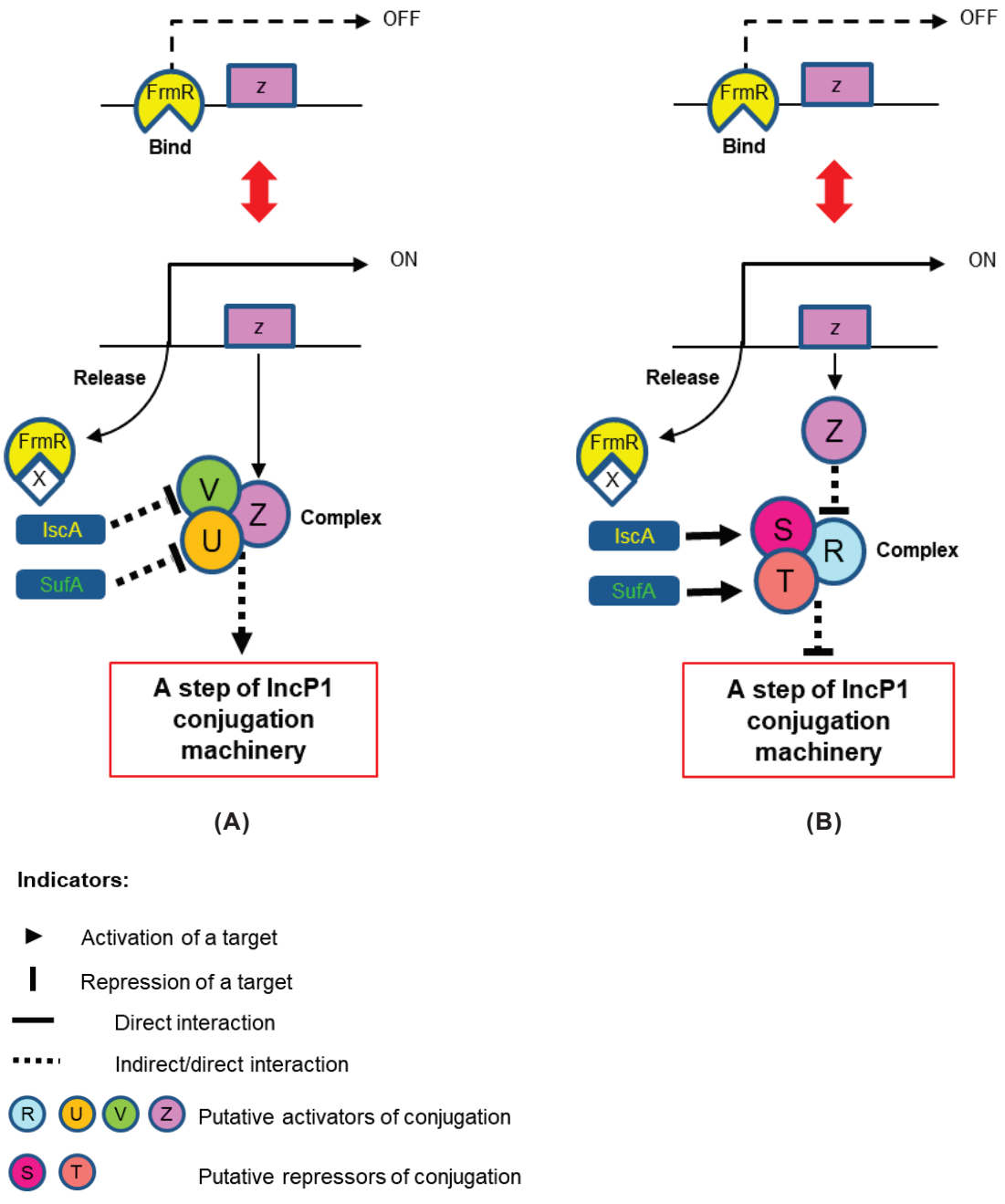


Figure 2.9: Assessment of the effect of conjugation efficiency of RP4 IncP1α plasmid from wild-type *E. coli* BW25113 strain in the absence or presence of *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) in a 6 h conjugation reaction. Data are presented as mean ± standard error mean (SEM) for four experimental replicates (n = 4). Asterisk (*) indicates statistically significant differences at $p < 0.05$ (two-tailed *t*-test) compared to the control (without exogenously supplied OdDHL) in the conjugation reaction mixture of the corresponding donor *E. coli* strain. SY327 was used as the recipient.



(Adopted from Zoolkefli et al., 2021)

Figure 2.10: Predicted model mechanisms of the FrmR, SufA, and IscA proteins interactions within *E. coli* donor in repressing the conjugation of IncP1α plasmid. FrmR is also predicted to be a transcriptional repressor on the operon of another target factor (activator) within the *E. coli* donor which represents as factor Z. IscA and SufA are predicted to work in repressing the activators (factors V and U, respectively), either by directly or indirectly **(A)** or directly activate the repressors (factors S and T, respectively) **(B).** At the same time, both activators may form a

complex with the FrmR target factor (Z) to activate conjugation **(A)** or the repressor may form a complex with other factors (factor) repressed by factor Z, either directly or indirectly, resulting to the repression of conjugation **(B)**. Based on these model mechanisms, the FrmR, SufA, and IscA proteins are predicted to repress the conjugation at the identical step(s) of IncP1 conjugation machinery.

CHAPTER 3

Generality Analysis of *frmR*, *sufA*, and *iscA* Gene Mutations on the Enhancement of Conjugation Efficiency

Abstract

Conjugation is a mechanism that permits the DNA transfer from donor to recipient cells via a bacterial type IV secretion system (T4SS). According to the analyses performed in Chapter 2, three isolated chromosomally-encoded genes of *E. coli* K-12 derivative, namely, *sufA, iscA,* and *frmR,* are found to repress the IncP1α conjugation efficiency by 10-fold. This result provides a point of view that the conjugation mechanism does not solely rely on genes encoded on the conjugative plasmids but is also controlled by genes in a host's genome. Concerning the effect of those isolated genes in the conjugation mechanism of other broad-host-range (BHR) plasmids, a conjugation experiment was performed between *E. coli* donor mutants (Δ*sufA*, Δ*iscA*, and Δ*frmR*), harboring either R46 (IncN) or pSa (IncW), and *E. coli* recipient. Surprisingly, the three mutants demonstrated a comparable conjugation efficiency as the wild-type control, in both IncN- and IncW-type plasmid transfer, but not in IncP1β. All three mutants showed an increase in the conjugation efficiency of an IncQ plasmid mediated by IncP1β, in both the bacterial and TKC conjugations. This result suggested that the repression mechanism by the three isolated host chromosomally-encoded genes is likely specific to the IncP1-type T4SS. The importance of the three genes was further investigated in *Agrobacterium tumefaciens*, belonging to a different class in Pseudomonadota phylum. Three chromosomal gene knockout mutants were constructed for this purpose, each lacking a homolog of the three isolated genes. Also, these examined mutants showed an increase in TKC efficiency mediated by the IncP1-type transfer

system. Overall, the data suggest the existence of a specific regulatory system in IncP1 plasmids, that enables the control of conjugation efficiency in different hosts.

Introduction

Conjugation is a major driving force of genetic exchange in eubacteria. Besides between non-related species bacteria, this mechanism also promotes the transfer of DNA from a bacterium to an organism belonging to other kingdoms, such as eukaryotes (Bates, 1997; Moriguchi et al., 2013a) and archaea (Dodsworth et al., 2010), known as *trans*-kingdom conjugation (TKC). The TKC is a type of horizontal gene transfer (HGT), in addition to transformation (Gelvin, 2003; Keen et al., 2017) and transduction (Zinder and Lederberg, 1952). Within a prokaryotic population, HGT occurs ubiquitously and permits fast dissemination of new genes, which is essential for species adaptation and survival. Therefore, this mechanism has been acknowledged as a driving force for the evolution of bacterial species (Richard et al., 2017; Ward et al., 2018). In the case of HGT from bacteria to eukaryotes, it was predicted to occur to a certain extent, and play a role in adaptive evolution (Keeling and Palmer, 2008; Schönknecht et al., 2014).

The conjugation mechanism involves the transfer of genetic material from a donor to a recipient via a bacterial type IV secretion system (T4SS) (Alvarez-Martinez and Christie, 2009; Wallden et al., 2010). The activation of this mechanism is due to the expression and regulation of the transfer (*tra*) genes encoded in a self-transmissible plasmid. The self-transmissible plasmids which can be transferred and stably maintained in a wide range of bacteria are known as broad host range (BHR) plasmids. In addition to the BHR self-transmissible plasmids, the BHR mobilizable plasmids such as IncQ and pBBR1 were also found able to

replicate in various hosts (Szpirer et al., 2001; Suzuki et al., 2010). These so-called BHR plasmids provide special interest by researchers for interspecies gene exchange. The molecular biology of the BHR IncP1 (Figurski and Helinski, 1979; Smith and Thomas, 1984; Pansegrau et al., 1994; Zatyka et al., 1994; Jagura-Burdzy and Thomas, 1995), IncN (Coupland et al., 1987; Krishnan et al., 1990; Kim et al., 1994), IncW (Llosa et al., 1991, 1994; Okumura and Kado, 1992), as well as the mobilizable IncQ (Scherzinger et al., 1984; Derbyshire and Willetts, 1987; Scholz et al., 1989; Frey et al., 1992; Sakai and Komano, 1996) and pBBR1 (Szpirer et al., 2000, 2001) plasmids, have been discovered intensively since the beginning of the 1980s. Later, miniderivative plasmids of pCU785, pSW29T, and pLR303R, derived from pCU1 IncN (Krishnan and Iyer, 1988), pSW29 IncW (Demarre et al., 2005), and pBP136 IncP1β (Yano et al., 2013), respectively, were developed. Notably, the replication origin of pCU785 and pSW29T was derived from the RK2 IncP1α plasmid, as a strategy to overcome the host range limitation of the transfer system. The host range within the IncP1 plasmid group is known to be diverse (Yano et al., 2013), supporting the prediction that the promiscuous IncP1 plasmid has a broader host range than IncN- and IncW-type plasmid groups (Suzuki et al., 2010; Norberg et al., 2011). Since the 1980s, a BHR mobilizable IncQ plasmid, RSF1010, was found to mobilize to *E. coli* and *Pseudomonas aeruginosa*, facilitated by IncP1, but not that IncN and IncW plasmids (Willetts and Crowther, 1981). Nowadays, many applications of the conjugation study have been done by mobilizing the IncQ plasmid to even eukaryote recipient cells, facilitated by the IncP1 T4SS transfer system (Mizuta et al., 2012; Moriguchi et al., 2013b;

Moriguchi et al., 2016; Zoolkefli et al., 2021).

There are several features which have been discovered by researchers that confer the broad host range to the plasmids, including the number and structure of replication origins (*ori*). In the case of naturally occurring BHR pJD4 (IncW) (Pagotto and Dillon, 2001) and pCU1 (IncN) (Krishnan and Iyer, 1988), both plasmids present with three origins. For instance, the pCU1 plasmid required at least two origins (*oriB* and *oriS*) with host-encoded replication protein, RepA, to replicate and maintain in hosts, whether the DNA Polymerase I-dependent mechanism existed or absent ($PolA^+$ or $PolA^-$, respectively) (Banerjee et al., 1992; Kim et al., 1994). Therefore, having multiple *ori* will ensure the survival of the plasmid, even if the replicon sequences become altered or inactivated due to the mutations.

Notably, the RP4/RK2 (IncP1α) plasmid presents with a single *cis*-acting origin of replication (*oriV*) and a *trans*-acting replication protein (TrfA) (Schmidhauser and Helinski, 1985; Shah et al., 1995). This plasmid also possesses nine 17 bp iterons with directly repeated sequences located on *oriV* and is organized into three clusters (Adamczyk and Jagura-Burdzy, 2003). Iterons serve as the TrfA binding region during the initiation of DNA replication (Toukdarian et al., 1996). The binding of the plasmid-encoded TrfA replication protein on the iterons is very often accompanied by the binding of host-encoded DnaA replication protein to the DnaA-boxes located on the *oriV* (Doran et al., 1999). Intriguingly, the requirement of the TrfA and host DnaA bind to the iterons and DnaA-boxes, respectively are known to be varied depending on the host bacteria (Schmidhauser et al., 1983;

Schmidhauser and Helinski, 1985; Shah et al., 1995; Doran et al., 1999). Therefore, both of these elements provide the necessary versatility to the RK2 plasmid to allow it to replicate and maintain in a promiscuous manner (Schmidhauser et al., 1983; Schmidhauser and Helinski, 1985).

Taken together, the host factor(s) also plays an essential role in the plasmid replication and maintenance in various hosts, in addition to the plasmid-encoded factor. As reported before, host chromosomally-encoded factors are also essential, even for the activation of the conjugation mechanism, through the regulation of the *tra* genes (Silverman et al., 1991; Starčič-Erjavec et al., 2003; Lu et al., 2019). This is evidence that the conjugation mechanism does not solely rely on plasmid-encoded factors. In Chapter 2, three isolated host chromosomally-encoded genes of *E. coli*, namely, *sufA, iscA,* and *frmR,* are found to repress the IncP1α conjugation machinery. Concerning the effect of those isolated genes in the conjugation mechanism of other BHR conjugative plasmids, a conjugation experiment was performed between *E. coli* mutant donors (Δ*sufA*, Δ*iscA*, and Δ*frmR*), harboring either R46 (IncN) or pSa (IncW), and *E. coli* recipient. Since *E. coli* is one of the hosts of the naturally occurring R46 and pSa plasmids, the effect of these plasmid transfers by the three isolated mutants is investigated. Previously, the BHR RP4 IncP1α was found able to replicate in a broad range of bacteria belonging to the phylum of Pseudomonadota. Therefore, the importance of the three isolated genes in repressing the IncP1 conjugation mechanism was further investigated in *A. tumefaciens*, belonging to a different taxonomic class: Alphaproteobacteria.

Materials and Methods

Bacterial strains, yeast, and growth media

Bacterial strains and yeast used in this study are listed in **Table 3.1**. All *E. coli* strains and *A. tumefaciens* were cultured in LB Lennox medium at 37°C and 28°C, respectively. In addition, *Saccharomyces cerevisiae* yeast was cultured in a yeast-extract/peptone/dextrose (YPD) medium at 28°C. Synthetic-defined (SD) medium containing appropriate individual amino acids lacking uracil (SC-Ura) was used as the selection media for yeast transconjugants at 28°C. All components included in each medium were described in Chapter 2. Appropriate antibiotics were added to the media at the following final concentration, which corresponded to the selection of bacteria and plasmids: ampicillin (Ap), 50 µg/mL; chloramphenicol (Cm), 30 µg/mL; kanamycin (Km), 50 µg/mL; meropenem (Me), 10 µg/mL; rifampicin (Rf), 30 µg/mL; streptomycin (Sm), 50 µg/mL; and tetracycline (Tc), 7.5 µg/mL.

Donor and recipient cell cultures

Details of the genotypes for both donor and recipient cells as well as the plasmids used are described in **Tables 3.1 and 3.2**, respectively.

For standard conjugation assessment, the donor *E. coli* BW25113: Δ*frmR,* Δ*sufA*, Δ*iscA,* and wild-type control, harbouring either R46 (IncN) or pSa (IncW) plasmid, was cultured in media supplemented with Gm or Ap, respectively. These donor

cultures were cultured in 5 mL glass tubes and incubated for 15 to 18 h at 37°C.

In addition, the donor *A. tumefaciens* C58C1: Δ*ATU_RS04380*, Δ*ATU_RS08905*, Δ*ATU_RS08390*, and wild-type control, harboring RP4 and pYN402 plasmids were cultured in media supplemented with Gm, Km, and Rf at 28°C. These donor cultures were cultured in 5 mL glass tubes and incubated for 16 to 18 h at 28°C. Each of these mutant strains lacks a gene that homolog to *frmR* as well as *sufA,* and *iscA* genes in *E. coli*, respectively.

The recipient cells of *E. coli*, strain SY327 (λ*pir*) or *S. cerevisiae,* strain BY4742, were cultured in media supplemented with or without Rf, respectively, in 5 mL glass tubes. The *E. coli* or yeast recipient cells were cultured for 16 to 18 h at 37°C or 18 to 22 h at 28°C, respectively. Both donor and recipient cultures were pre-cultured with agitation to allow aeration.

Construction of *A. tumefaciens* single gene-deletion mutants, the homolog of Δ*sufA*, Δ*iscA*, and Δ*frmR E. coli* mutants

All primers used in this experiment are listed in **Table 3.3**. To construct the single gene-knockout mutants of *A. tumefaciens*, the homologous recombination system of the pK18mobsacB mobilizable suicide vector **(Figure 3.1 (A))** was used. A backbone vector of pK18mobsacB was initially amplified inversely by PCR, flanking between *Eco*RI and *Bam*HI restriction sites, by using F101-F102 primer pairs. The homolog gene of *frmR*, *sufA*, and *iscA*, namely, *ATU_RS04380* (*atu0890*) [NCBI accession number: NP_353911.2 (WP_010971229.1)], *ATU_RS08905* (*atu1819*) [NCBI accession number: NP_354803.1 (WP_010971889.1)], and

ATU_RS08390 (*atu1713*) [NCBI accession number: NP_354701.1 (WP_010971818.1)], respectively, were also amplified. The amplification (approximately 1000-1500 bp fragment) was performed by PCR, using F103-F104, F105-F106, and F107-F108 primer sets, flanking between the target gene fragment of *ATU_RS04380, ATU_RS08905,* and *ATU_RS08390*, respectively, with the additional sequence of *Eco*RI and *Bam*HI RE sites at both ends of each fragment. These genes showed high homology with those isolated *E. coli* genes, based on a BlastP search in the NCBI database. The backbone vector and target gene fragment were then assembled by using NeBuilder® HiFi DNA Assembly by New England Biolabs (Ipswich, MA, US) at the *Bam*HI and *Eco*RI restriction sites by using, given to new plasmid constructs, namely, pK18mobsacB-*ATU_RS04380,* pK18mobsacB-*ATU_RS08905,* and pK18mobsacB-*ATU_RS08390,* respectively.

To construct in-frame target gene deletion in *A. tumefaciens*, at least two plasmid constructs are necessary for each gene. In addition to the first plasmid constructs described above, second plasmids were constructed through the removal of the target gene sequence (*ATU_RS04380, ATU_RS08905,* and *ATU_RS08390*) from the first plasmid construct by inverse PCR, using F109-F110, F111-F112, and F113-F114 primer sets, respectively. Each fragment was then re-circularized by using NEBuilder® HiFi DNA Assembly Cloning Kit, given the plasmid construct of pK18mobsacB-Δ*ATU_RS04380* **(Figure 3.1 (B))**, pK18mobsacB-Δ*ATU_RS08905* **(Figure 3.1 (C))**, and pK18mobsacB-Δ*ATU_RS08390* **(Figure 3.1 (D))**. This methodology was performed similarly to a previously reported study by Schäfer et al. (1994).

Trans-kingdom conjugation

As for the TKC reaction, the suspension culture of donor *A. tumefaciens* strain C58C1 in LB Lennox medium and recipient yeast in TNB, containing 5.0×10^7 cfu/ 300µL and 4.0×10^6 cfu/ 300µL, respectively, were mixed. The reaction scale was increased up to sevenfold to detect the transconjugant. TKC efficiency was determined based on the recovery of uracil prototrophic transconjugants on a selection medium supplemented with Me to inhibit the growth of *A. tumefaciens*. TKC efficiency was calculated as the number of transconjugants per recipient cell and compared with the control.

Bacterial conjugation

An identical method was used as *trans*-kingdom conjugation, as described above. 1.8×10^7 cfu/ 300 µL of donor and 7.1×10^7 cfu/ 300 µL of recipient suspensions in LB Lennox medium were mixed, and the co-cultivation was performed for an hour at 37°C. The transconjugants were selected on LB Lennox solid medium, supplemented with Rf and the addition of either Gm or Ap for selecting the transconjugants, harbouring either pSa (IncW) or R46 (IncN), respectively. The conjugation efficiency was calculated as the number of transconjugants per recipient cell and compared with the control.

Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM) of at least

three independent biological experiments. The multiple group comparison was analyzed using one-way ANOVA (Tukey HSD analysis). Analyses were performed using SPSS IBM Software for Windows Version 17.0 (SPSS Inc. Chicago, II, United States). Tests were considered statistically significant when $p < 0.05$, $p < 0.01$, or $p < 0.001$.

Results

The enhancement of conjugation efficiency by up-mutants specifically affects IncP1-type plasmid transfer

To examine the effect on the conjugation efficiency of other broad-host-range plasmids, such as IncW and IncN due to the deletion of *sufA, iscA,* and *frmR* genes in donor *E. coli*, an experiment has been performed. In this experiment, the conjugation reaction was performed between the *E. coli* mutant donors (Δ*sufA*, Δ*iscA*, and Δ*frmR*), harbouring either R46 (IncN) or pSa (IncW) plasmids, and the *E. coli* recipient. The conjugation efficiency was then compared with the parental strain.

According to the results, no significant difference was observed in the conjugation efficiency of the pSa and R46 plasmids, between the mutant and parental strains **(Figures 3.2 (A) and (B)).** It could be predicted that the repressing effect by the *frmR*, *sufA,* and *iscA* genes in *E. coli* is specific to the IncP1 conjugation mechanism. As expected, a significant increase in the conjugation efficiency by the three mutants was observed when the conjugation mechanism is driven by the IncP1β transfer system, in both *E. coli*-*E. coli* and *E. coli*-yeast conjugations (seven- to ninefold and three- to fivefold, respectively), compared to the parental strain (Zoolkefli et al., 2021) **(Figures 3.2 (C) and (D))**. According to Zoolkefli et al. (2021), the conjugation efficiency was measured by measuring the transfer of IncQ-derived *E. coli*-yeast shuttle vector (pAY205), facilitated by the IncP1β-derived helper plasmid (pDPT51).

Mutation of the homologous *E. coli* genes in *A. tumefaciens, ATU_RS04380*, *ATU_RS08390*, and *ATU_RS08905* enhance the *trans*-kingdom conjugation

To further examine the enhancing effect of the *E. coli* Δ*frmR*, Δ*sufA*, and Δ*iscA* gene mutation on the IncP1-type T4SS-mediated plasmid transfer in other bacterial species, TKC analysis was performed by combining an IncQ-derived mobilizable plasmid (pYN402), mediated by an IncP1α-type plasmid (RP4) transfer system and mutants of *A. tumefaciens*.

The homologous *E. coli* "up"-mutant genes in *A. tumefaciens* strain C58 were selected based on BlastP score analysis, phylogenetic trees, and previously reported studies (Higgins, and Giedroc, 2014; Chen *et al.*, 2016; Heindl *et al.,* 2016). According to the BlastP analysis, the homologous gene of *frmR* (*ATU_RS04380*) had the highest similarity (46.03% amino acid identity and 2e–15 e-value). In addition, the shared homologous genes of *sufA* and *iscA*: *ATU_RS08390* and *ATU_RS08905* (*sufA*:37.14% and 43.69% amino acid identity; 6e–23 and 9e–26 e-value, respectively) as well as (*iscA*: 37.14% and 39.62% amino acid identity; 3e–23 and 4e–24 e-value, respectively) carry out the same function in *E. coli* and are representative members of the iron-sulfur cluster assembly (Lu et al., 2008b).

All examined mutants showed significantly higher TKC efficiency compared to the parental strain **(Figure 3.3)**. The Δ*ATU_RS08390* mutant showed a fourfold increase in TKC efficiency compared to the parental strain. In addition, threefold increases in conjugation efficiency were observed in Δ*ATU_RS04380* and Δ*ATU_RS08905* mutants **(Figure 3.3)**. These results suggest that these homologous mutant genes (*ATU_RS04380*, *ATU_RS08390*, and *ATU_RS08905*) have similar

characteristics in terms of enhancing the TKC efficiency facilitated by IncP1-type T4SS machinery to the recipient cell.

Discussion

As described in Chapter 2, the enhancing effect on conjugation efficiency, mediated by IncP1α-T4SS by the three *E. coli* mutants (Δ*sufA*, Δ*iscA*, and Δ*frmR*) to both prokaryote and eukaryote was observed. This phenomenon indicates the repressing effect of the IncP1α conjugation mechanism by the three genes.

Concerning the repressing effect of these three genes on the conjugation mechanism of other BHR plasmids, a conjugation experiment was performed between the *E. coli*, where the mutants as a donor, harboring either R46 (IncN) or pSa (IncW). Surprisingly, no enhancement of conjugation efficiency of both IncN- and IncW-type plasmids by the mutants was observed, suggesting that the repressing mechanism by the three genes is likely specific to the IncP1-type T4SS **(Figure 3.2 (A) and (B))**. As previously reported by Zoolkefli et al. (2021), a significant increase of both bacterial and TKC conjugations by the mutants mediated by pDPT51 plasmid derived from R751 IncP1β plasmid was observed, supporting our prediction **(Figure 3.2 (C) and (D))**.

Comparative sequence analysis between the RP4 IncP1α and R751 IncP1β plasmids revealed a similarity of 74% at the nucleotide sequence level, indicating that these plasmids have a common ancestor (Zieglin et al., 1991). The genes in members of the IncP1β subgroup, including R751 plasmids, which involve in plasmid maintenance and conjugal transfers were found to have homology to RP4, revealing the similar genetic organization between both plasmids (Smith and Thomas, 1985). The fact that IncP1β plasmids share a common arrangement of

transfer loci with the IncP1α plasmid RK2/RP4 (Pansegrau et al., 1994) supports the assumption that they rely on similar conjugational transfer mechanisms. Therefore, the repressing mechanism by the three chromosomal genes towards the conjugal transfer of IncP1α and IncP1β plasmids suggests the existence of a specific regulatory system in IncP1 plasmids, which could be at the identical target region or protein.

Previously, it was reported on a study that the effect of the growth stage towards the conjugation efficiency of a representative plasmid belonging to IncP1α, IncN, and IncW incompatibility groups, namely, RP4, pCU1, and R388 plasmids, respectively (Sysoeva et al., 2020). In the article, the authors revealed that both IncP1 and IncW but not IncN conjugal transfers are growth-independent regulations since no effect on conjugation efficiency was observed between the exponential and stationary donor cultures. This characteristic would be beneficial for the three isolated mutants to serve as the appropriate model for the development of powerful donor strains, mediated by the RP4-T4SS transfer system. Since the RP4 and R751 plasmids are presumed to share a common ancestor according to their similarity in nucleotide sequence (Zieglin et al., 1991), generally, this characteristic may also be applicable in the R751-T4SS transfer system. On the other hand, the representative plasmids belonging to the IncN and IncW incompatibility group used by the authors are different from this study, thus, their findings cannot be applied to this study.

Lastly, IncP1-type T4SS carries a high potential for the application of a gene introduction system into various organisms (Dodsworth et al., 2010; Norberg et al., 2011; Moriguchi et al., 2013a). The data regarding the mutants isolated in this study

should be an appropriate basis for the breeding of donor strains from various Pseudomonadota, each of which carries high cytological affinity with target organisms in addition to high conjugation ability. Further characterization in terms of possible gene interaction within the chromosomal mutants based on physiological analysis with the possible regulators within the IncP1 plasmids will lead to a better understanding of the isolated genes' diversity with the TKC mechanism. Additionally, it will be interesting to determine the specificity of T4SS-mediated IncQ conjugal transfer in the isolated mutant strains by using Ti plasmid (VirB/D4-T4SS system) in comparison to IncP1-type system as it has been reported to serve as a DNA delivery system by *Agrobacterium* (Bohne et al., 1998; Kiyokawa et al., 2020).

Table 3.1: Strains used in this study

Strains	Relevant characteristics or genotype	Source or reference
E. coli		
BW25113Δ*sufA*	An in-frame *sufA* single-gene knockout mutant, derived from BW25113, Km^R (Keio collection)	NBRP Japan
BW25113Δ*iscA*	An in-frame *iscA* single-gene knockout mutant, derived from BW25113, Km^R (Keio collection)	NBRP Japan
BW25113Δ*frmR*	An in-frame *frmR* single-gene knockout mutant, derived from BW25113, Km^R (Keio collection)	NBRP Japan
BW25113	*F^- Δ(araD-araB)567 ΔlacZ4787(::rrnB-3) λ-rph-1 Δ(rhaD-rhaB)568 hsdR514*	NBRP Japan
SY327 (λ*pir)*	Δ*(lac pro) argE(*Am*) recA56 λpir* Rif^R Nal^R	NBRP Japan
S17-1 (λ*pir)*	*F^- RP4-2(Km^R::Tn7,Tc^R::Mu-1) pro-82λpir recA1 endA1 thiE1 hsdR17 creC510*	NBRP Japan
A. tumefaciens		
C58C1	pTiC58-cured and Rif^R derivative of C58	Yamamoto et al., 2007
C58C1Δ*ATU_RS04380*	*ATU_RS04380* (*atu0890*) single-gene knockout derived from C58C1, Rif^R	This study
C58C1Δ*ATU_RS08905*	*ATU_RS08905* (*atu1819*) single-gene knockout derived from C58C1, Rif^R	This study
C58C1Δ*ATU_RS08390*	*ATU_RS08390* (*atu1713*) single-gene knockout derived from C58C1, Rif^R	This study
S. cerevisiae		
BY4742	*MATα SSD1-V his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Invitrogen

Table 3.2: Plasmids used in this study

Plasmids	Relevant characteristics or genotype	Source or references
RP4	IncP1α-type conjugative broad-host-range plasmid; Km^R, Tc^R, Ap^R	Pansegrau et al., 1994
pSa	IncW-type conjugative broad-host-range plasmid; Cm^R Su^R Sp^R Sm^R Km^R Gm^R Tb^R	Tait et al., 1982
R46	IncN-type conjugative broad-host-range plasmid; Tc^R Sm^R Su^R Ap^R	Brown and Willetts, 1981
pDPT51	Helper plasmid; *tra*$^{P1\beta}$ *trb*$^{P1\beta}$ ori-CoIE1 Tp^R Ap^R	Taylor et al., 1983
pYN402	Mobilizable plasmid; *oriV*^{Q} *oriT*^{Q} *mob*^{Q} URA3 2μ-ori Gm^R	*AB531984
pAY205	Mobilizable plasmid; *oriV*^{Q} *oriT*^{Q} *mob*^{Q} *URA3 TRP1 ARS1* Km^R Tc^R	*AB526841
pK18mobsacB	Mobilizable plasmid; *sacB oriT* Km^R used for the construction of *A. tumefaciens* knock-out mutant strains	Schäfer et al., 1994
pK18mobsacB-*ATU_RS04380*	Partial *ATU_RS04365*, *ATU_RS04370*, *ATU_RS04375*, *ATU_RS04380*, and *ATU_RS04385,* integrated within pK18mobsacB; Km^R	This study
pK18mobsacB-*ATU_RS08905*	Partial *ATU_RS08895*, *ATU_RS08900*, *ATU_RS08905*, *ATU_RS08910* and partial *nifS,* integrated within pK18mobsacB; Km^R	This study
pK18mobsacB-*ATU_RS08390*	*dgt, ATU_RS08390,* and partial *ATU_RS08395,* integrated within pK18mobsacB; Km^R	This study
pK18mobsacBΔ*ATU_RS04380*	*ATU_RS04380* single-gene knockout within pK18mobsacB-*ATU_RS04380*; Km^R	This study
pK18mobsacBΔ*ATU_RS08905*	*ATU_RS08905* single-gene knockout within pK18mobsacB-*ATU08905*; Km^R	This study
pK18mobsacBΔ*ATU_RS08390*	*ATU_RS08390* single-gene knockout within pK18mobsacB-*ATU_RS08390*; Km^R	This study

*DDBJ/EMBL/GenBank accession number

Table 3.3: Primers used for the construction of *A. tumefaciens* mutants, the homolog of *E. coli* up-mutants

Primer name	Primer sequence (5′-3′)	Purpose
F101	GGATCCTCTAGAGTCGACC	pK18mobsacB-*Bam*HI
F102	GAATTCGTAATCATGTCATAGC	pK18mobsacB-*Eco*RI
F103	CATGATTACGAATTCCGCCGTGGTAGGAGGCAA	pK18mobsacB-*ATU_RS04380* construction
F104	GACTCTAGAGGATCCTTAGGCACTTGCCGGGCTGA	pK18mobsacB-*ATU_RS04380* construction
F105	CATGATTACGAATTCGGCACAGGACGGCGTTTC	pK18mobsacB-*ATU_RS08905* construction
F106	GACTCTAGAGGATCCTCATCCGCGAACGGCAGG	pK18mobsacB-*ATU_RS08905* construction
F107	CATGATTACGAATTCCTATCGCAAATCGGGAGTGTG	pK18mobsacB-*ATU_RS08390* construction
F108	GACTCTAGAGGATCCCAGCCGATCGGACTGAAATC	pK18mobsacB-*ATU_RS08390* construction
F109	ATTTTTAACTGAAGACCTACATCCGGTA	pK18mobsacBΔ*ATU_RS04380* construction
F110	GTCTTCAGTTAAAAATGCTCCATCAGAA	pK18mobsacBΔ*ATU_RS04380* construction
F111	ATAGCTGAGTCATGATCGCAAAGCCCAT	pK18mobsacBΔ*ATU_RS08905* construction

Table 3.3: (continued)

F112	ATCATGACTCAGCTATCGAGGTGCGACG	pK18mobsacBΔ*ATU_RS08905* construction
F113	CTATGATCATGCACGAGCTTTTCGATTTGA	pK18mobsacBΔ*ATU_RS08390* construction
F114	GCTCGTGCATGATCATAGACCAGCGCGC	pK18mobsacBΔ*ATU_RS08390* construction

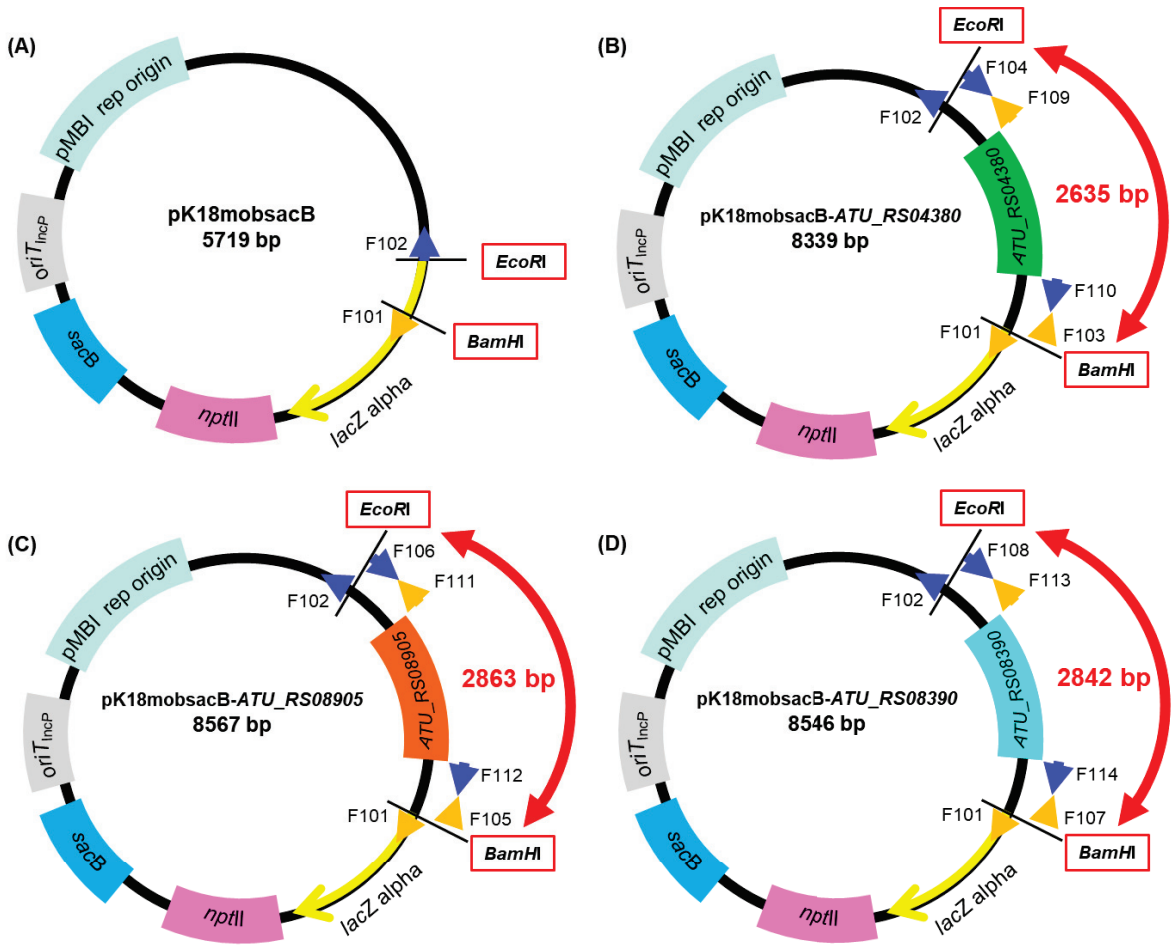


Figure 3.1: Mobilizable suicide vectors used for the construction of the homologous "up"-mutant gene deletion mutation in *A. tumefaciens* strain C58C1. (A) The backbone of the mobilizable suicide vector pK18mobsacB, which comprises a selectable marker gene *npt*II (Km^R) and a counter-selectable marker gene *sacB* (levan sucrase). **(B)** pK18mobsacB-*ATU_RS04380*, **(C)** pK18mobsacB-*ATU_RS08905*, and **(D)** pK18mobsacB-*ATU_RS08390* plasmids were used for the construction of *A. tumefaciens* homolog mutants, through homologous recombination.

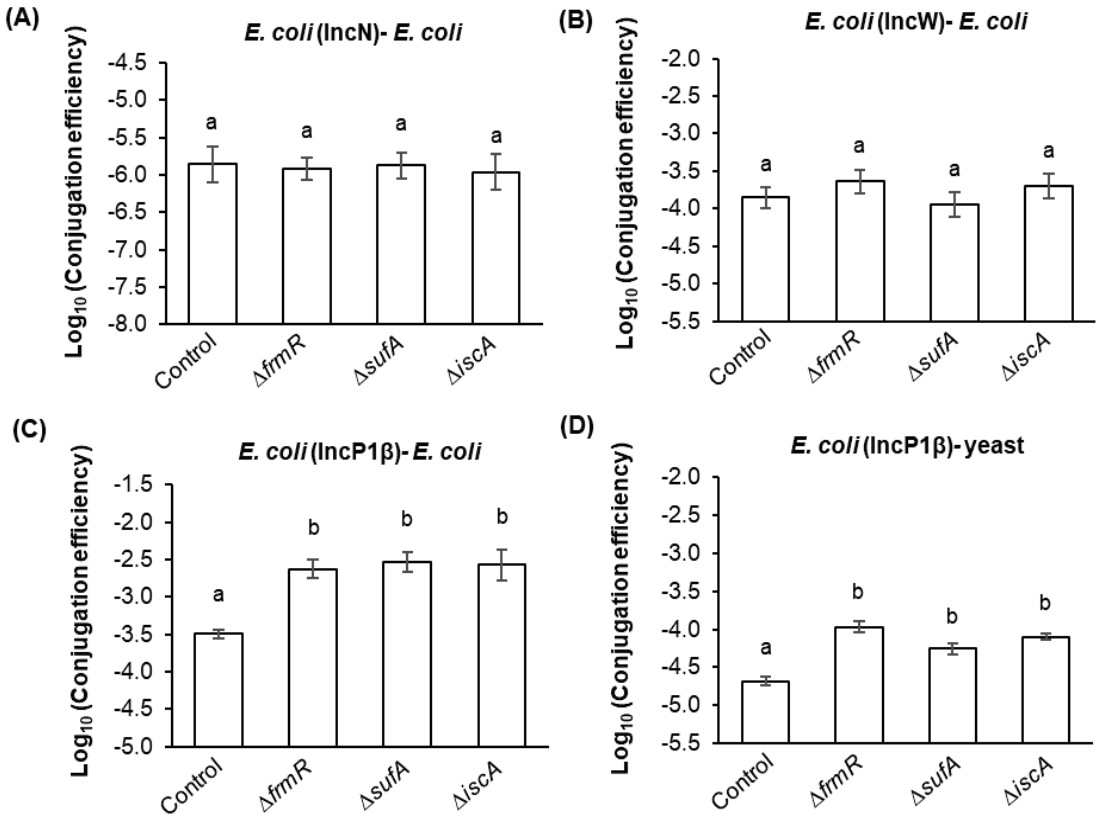


Figure 3.2: Generality assessment of *frmR*, *sufA*, and *iscA* mutations on the conjugation of broad-host-range plasmids. (A) Conjugation efficiency of IncN (R46) plasmid transfer to *E. coli* recipient cells within five experimental replicates (n = 5). **(B)** Conjugation efficiency of IncW (pSa) plasmid transfer to *E. coli* recipient cells within seven experimental replicates (n = 7). **(C)** Conjugation efficiency of IncP1β (pDPT51)-mediated shuttle vector (pAY205) transfer to *E. coli* and **(D)** yeast within triplicate experiments (n = 3). **(C)** and **(D)** data were adopted from Zoolkefli et al. (2021). All conjugation reactions were performed for 1-h. Data are presented as mean ± standard error of the mean (SEM). Different letters indicate significant differences between mutants and wild-type control at $p < 0.05$ using Tukey HSD multiple comparison analysis. BW25113 parental strain was used as the control. BY4742 and SY327 were used as the recipients.

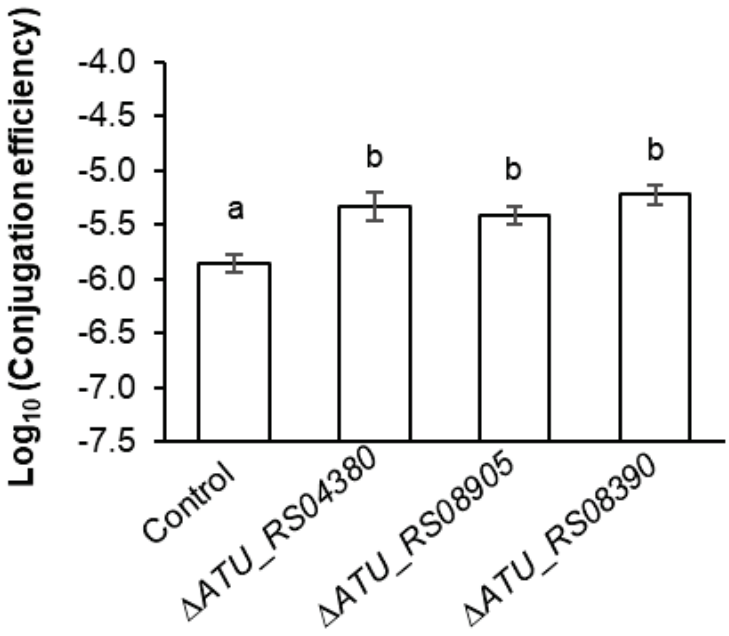


Figure 3.3: Effect of the "up"-mutant homologous gene-knockout in *A. tumefaciens* on IncP1-type conjugation. The conjugation reaction was performed for 1-h. Data are presented as mean ± standard error of the mean (SEM) within six experimental replicates (n = 6). Different letters indicate significant differences between mutants and wild-type control at $p < 0.05$ using Tukey HSD multiple comparison analysis. C58C1 parental strain was used as the control. BY4742 was used as the recipient.

General Conclusions and Perspectives

In Chapter 1, I briefly reviewed the background history of conjugation as a major driving force of genetic exchange in eubacteria. The conjugation mechanism facilitates the adaptation and survival of bacteria by mediating the propagation of beneficial properties, encoded on the conjugative and mobilizable plasmids. Since it was discovered in 1946, studies were performed extensively on the influential factors and mechanisms involved in the conjugal transfer. Outcomes of the studies on the F factor have been a paradigm for understanding the mechanism of T4SS in Gram-negative bacteria.

After the discovery, the researchers found out that the conjugation mechanism does not solely rely on the plasmid-encoded gene(s), but also on the host chromosomally-encoded gene(s). However, the involvement of chromosomal genes in donor cells remains elusive in the conjugation phenomena. Therefore, in Chapter 2, the potential conjugation-related genes were identified via a genome-wide screening from a comprehensive collection of single-KO mutants of *E. coli* K-12 derivative (Keio collection). This screening was performed using a conjugal transfer system, mediated by the type IV secretion system of the IncP1α. In this screening, three out of 233 “up”-mutants were isolated, namely, Δ*frmR*, Δ*sufA*, and Δ*iscA*, which showed an increase of efficiency by about 10-fold, in both *E. coli*-*E. coli* and *E. coli*-yeast conjugations. The increase in conjugation efficiency by the three mutants also did not correlate with an increase in the mRNA expression levels of *tra* genes on the plasmid. The double-KO mutants, namely, Δ*frmR*Δ*sufA* and

Δ*iscA*Δ*frmR* did not show any synergistic effects on the conjugation efficiency, suggesting that these factors affect independently but at an identical step in the conjugation machinery, through the repressing mechanism.

In Chapter 3, the transfer of other BHR plasmids by the three mutants was conducted in order to investigate how widely the three genes affect the conjugal transfer systems among the plasmids. The three mutants demonstrated an increase in conjugation efficiency of IncQ mobilizable plasmid, mediated by IncP1β plasmid, but not that of IncN and IncW plasmids, suggesting the possibility that the repression mechanism of the three genes is IncP1-type T4SS specific. The importance of the three genes was further investigated using a bacterium belonging to a different class in Pseudomonadota. Three knockout mutants of *Agrobacterium tumefaciens* were constructed for this purpose, each lacking a homolog of the three genes. Similarly, these examined mutants also showed an increase in TKC efficiency. Overall, these results suggest the existence of a specific regulatory system in IncP1 plasmids that enables the control of conjugation efficiency in different hosts.

In conclusion, the mutants identified in this study showed a significant effect on the conjugation mechanism mediated by the IncP1-type T4SS transfer system. Although the native function and regulation are different among the three genes, their ability in repressing conjugation is common. The identification of the presumptive chromosomal gene(s) which could genetically interact with *frmR, sufA,* and *iscA* genes through physiological analysis would be noteworthy. Further characterization of the interaction between the three genes and the presumptive

regulatory genes on the IncP1 plasmids will later lead to a better understanding of how chromosomal genes control the conjugation and TKC mechanisms. From a practical viewpoint, this approach could be utilized for the development of robust donor strains mediated by the IncP1-type T4SS machinery as a gene introduction tool for bacteria, eukaryotes, and archaea.

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