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RESEARCH ARTICLE

Delineation of a lactococcal conjugation system reveals a restriction-modification evasion system

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Abstract

Plasmid pUC11B is a 49.3-kb plasmid harboured by the fermented meat isolate *Lactococcus lactis* subsp. *lactis* UC11. Among other features, pUC11B encodes a pMRC01-like conjugation system and tetracycline-resistance. In this study, we demonstrate that this plasmid can be conjugated at high frequencies to recipient strains. Mutational analysis of the 22 genes encompassing the presumed pUC11B conjugation cluster revealed the presence of several genes with essential conjugation functions, as well as a gene, *trsR*, encoding a putative transcriptional repressor of this conjugation cluster. Furthermore, plasmid pUC11B encodes an anti-restriction protein, TrsAR, which facilitates higher conjugation frequencies when pUC11B is transferred into recipient strains containing Type II or Type III RM systems. These findings demonstrate how RM mechanisms can be circumvented when they act as a biological barrier for conjugation events.

INTRODUCTION

Conjugative plasmids in lactic acid bacteria have been shown to encode a number of biotechnologically relevant traits, such as lactose utilization, protease activity and bacteriophage resistance (Ainsworth et al., 2014; Mills et al., 2011). Three distinct plasmid-encoded conjugation systems have been described among lactococcal species that allow these conjugative plasmids to self-transfer into a recipient strain at relatively high frequencies, that is, those encoded by plasmids pMRC01, pAF22 and pNP40 (Fallico et al., 2012; Harrington & Hill, 1991; Kelleher et al., 2019; O'Driscoll et al., 2006; Ortiz Charneco et al., 2021). There is substantial gene synteny between the conjugation-associated gene clusters harboured by plasmids pAF22, pMRC01, and related plasmids, which for this reason have been categorized as pMRC01/pAF22-like conjugative plasmids, whereas the conjugation gene cluster associated with pNP40 and pNP40-like plasmids represents a separate and divergent conjugation system (Kelleher et al., 2019; Ortiz Charneco et al., 2021).

The conjugative process is a regulated cascade which commences with a relaxase-mediated cleavage of the phosphodiester bond at the *nic* site within the origin of transfer (*oriT*) recognition sequence, the single stranded 5′ end of which becomes covalently attached to the relaxase (Grohmann et al., 1999). Common components of studied conjugation systems are the relaxase, which is required in the initial and final stages of the DNA transfer process, members of mating channel-related proteins, ATPase-related proteins and peptidoglycan hydrolase proteins (de la Cruz et al., 2010; Goessweiner-Mohr et al., 2014; Smillie et al., 2010). Conjugative transfer of DNA across the membrane of a donor Gram-positive bacterium relies on a Type

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IV secretion system (T4SS), a membrane-associated protein complex, responsible for single-strand DNA (ssDNA) transport when covalently bound to a guiding protein (Cabezón et al., 2015). The plasticity of T4SSs is translated into a high diversity of these systems, which lack a standard nomenclature; however, the names of the *virB* conjugative system from *Agrobacterium tumefaciens* (which is composed of 11 genes, *virB1* to *virB11*) are used without ambiguity since it is the by far the best characterized T4SS, and thus this system is normally used as a model of a conjugation system (Guglielmini et al., 2014). The T4SS is comprised of a transport apparatus, which spans the bacterial cell envelope, and Type IV coupling proteins (T4CP), which recruit the relaxosome (the relaxase-ssDNA complex formed after the relaxase nicks the dsDNA) to the secretion channel (Redzej et al., 2017). The transport apparatus is usually composed of VirB6-, VirB7-, VirB8-, VirB9- and VirB10-like proteins (depending on the system), with VirB4- and VirB11-like proteins being the ATPases of the assembled complex, whereas VirB2- and VirB5-like proteins compose the conjugation pilus in Gram-negative bacterium (thus presenting no homologues in Gram-positive bacterium) (Guglielmini et al., 2014). Upon direct contact with a recipient cell, this being mediated by surface adhesins encoded by the conjugation gene cluster, the relaxosome is transported into the cell in an ATPase-dependent manner and, after internalization, the relaxase recircularizes the DNA strand facilitating complementary strand synthesis (Kohler et al., 2019; Trokter & Waksman, 2018). Despite the significant relevance of conjugation to the dairy industry, this process is still poorly characterized in lactococci.

The presence of genes encoding predicted transcriptional repressors within the conjugation gene cluster indicate that the process of conjugation is tightly regulated to avoid fitness cost to the whole population (Kohler et al., 2019; Koraimann & Wagner, 2014). Therefore, genes that encompass the conjugation cluster are either induced by signalling molecules or constitutively expressed at low concentrations to reduce the metabolic burden (Bañuelos-Vazquez et al., 2017). The TraA relaxase of the *Streptococcus agalactiae* conjugative plasmid pIP501 (Horodniceanu et al., 1976) was shown to bind to the promoter upstream of the conjugation cluster, thereby negatively regulating transcription of the *tra* operon (Kurenbach et al., 2006). TraN acts as an additional repressor of the pIP501-encoded conjugation system, binding to its cognate binding site upstream of the *tra* region promoter and the *oriT*-associated *nic*-site (Kohler et al., 2018). Other conjugation systems and their regulation mechanisms have been studied in considerable detail in Gram-positive bacteria, such as the system encompassed by the *Clostridium perfringens* plasmid pCW3, which encodes a predicted winged helix-turn-helix regulator TcpK, similar to TraN from pIP501 (Kohler et al., 2019; Traore et al., 2018). Plasmid pLS20 from *Bacillus subtilis* possesses a conjugation gene cluster which is kept in a default repressed state by the plasmid-encoded RcoLS20, which is, in turn, deactivated by a conformational change promoted by the binding of the sensor pheromone, anti-repressor RapLS20 (Meijer et al., 2021). Finally, another well-defined pheromone-induced conjugative system is the one present on plasmid pCF10 from *Enterococcus faecalis*, which is regulated by the action of the repressor PrgX, and inducer (cCF10) and inhibitor (iCF10) peptides (Nakayama et al., 1994).

Restriction-modification (RM) systems, typically consisting of a restriction endonuclease (RE) and a cognate methyltransferase (MTase), represent rudimentary yet ubiquitous bacterial immune systems, which pose a biological barrier for horizontal gene transfer processes such as conjugation (Roer et al., 2015). Although it has previously been reported that conjugation is unaffected by these RM systems since most REs were thought to exclusively recognize non-methylated dsDNA (Elhai et al., 1997; Read et al., 1992), more recent studies have shown that the conjugative transfer of plasmids can be significantly reduced between bacterial strains through the action of RM systems (Purdy et al., 2002; Roer et al., 2015).

A previous study classified lactococcal plasmid pUC11B among the pMRC01/pAF22-like conjugative plasmids, representing the second most prevalent type of proven conjugative systems among lactococci to date (Ortiz Charneco et al., 2021). Plasmid pUC11B is present in *L. lactis* strain UC11, which was originally isolated from fermented meat (Kelleher et al., 2017), and whose genome and associated plasmidome have recently been described (Kelleher et al., 2019). Plasmid pUC11B harbours a presumed conjugation cluster comprised of 22 genes with significant homologies to those present in plasmids pMRC01 and pAF22. We recently characterized the pNP40-derived conjugative system, thereby representing the first molecular characterization of a lactococcal plasmid-encoded conjugation system (Ortiz Charneco et al., 2021). Applying our previously optimized conjugation protocol and recombineering approach (Ortiz Charneco et al., 2021), we performed a functional and mutational analysis of the pMRC01-like conjugation machinery encoded by plasmid pUC11B to elucidate the molecular mechanisms behind this conjugational system commonly found in lactococci.

RESULTS

pUC11B harbours a pMRC01/pAF22-like conjugation gene cluster

The pMRC01/pAF22-like conjugation systems include those encoded by lactococcal plasmids pUC11B, pUC08B, pIBB477a and pC42 (Ortiz Charneco et al., 2021). Plasmid pUC11B, originally identified in *L. lactis* UC11

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(Kelleher et al., 2017; Kelleher et al., 2019), harbours 53 predicted ORFs and exhibits a G+C content of 34.2%. It harbours three clustered Type II restriction-modification (RM) genes (*M2-M1.LlaUC11I* and *LlaUC11IP*), a tetracycline resistance gene (*tetR*) and a conjugation cluster comprised of 22 genes, downstream of the replication initiator gene, *repB* (Figure 1). The conjugation gene cluster of pUC11B exhibits a similar genetic organization to those of plasmid pMRC01 and, to a lesser extent that of pAF22, while its encoded conjugation-related proteins display various levels of amino acid identity to these two systems (Figure 2). The last three genes of the pUC11B conjugation cluster, *trs24*, *trs25* and *trsAR*, do not have homologous counterparts in plasmid pAF22, although the functionality of pAF22 conjugation system has previously been proven (Fallico et al., 2012). While pUC11B and pMRC01 both possess these genes (with their encoded proteins sharing 31%–50% identity at amino acid level), their absence in pAF22 suggests that they are not essential for conjugation.

Based on comparative sequence analysis using BLAST, the conjugation-associated gene cluster of pUC08B is genetically identical to that of pUC11B, while that of the recently discovered p001F plasmid (Wang et al., 2020) is almost identical to that to pUC11B and pUC08B. Furthermore, pC42 possesses three scattered conjugation-related genes with similarity to those of the pUC11B system, however the limited number of genes that are present likely renders this system non-functional. The pIBB477a conjugation gene cluster resembles that of pMRC01, with an average of 99.77% amino acid identity shared between their encoded proteins (Figure 2).

Beyond their conjugation gene clusters, these plasmids do not share significant similarities (apart from pUC11B and pUC08B, which are identical), thus discarding the possibility of a similar genetic lineage. The significant level of similarity of the pUC11B conjugation gene cluster to the pMRC01/pAF22-like conjugation systems prompted a detailed analysis, to further characterize the functionality of this, as of yet, undefined conjugation system.

pUC11B is self-transmissible via conjugation

While pUC11B is predicted, based on comparison and identity, to harbour an intact conjugation system, the functionality of the system has not been demonstrated.

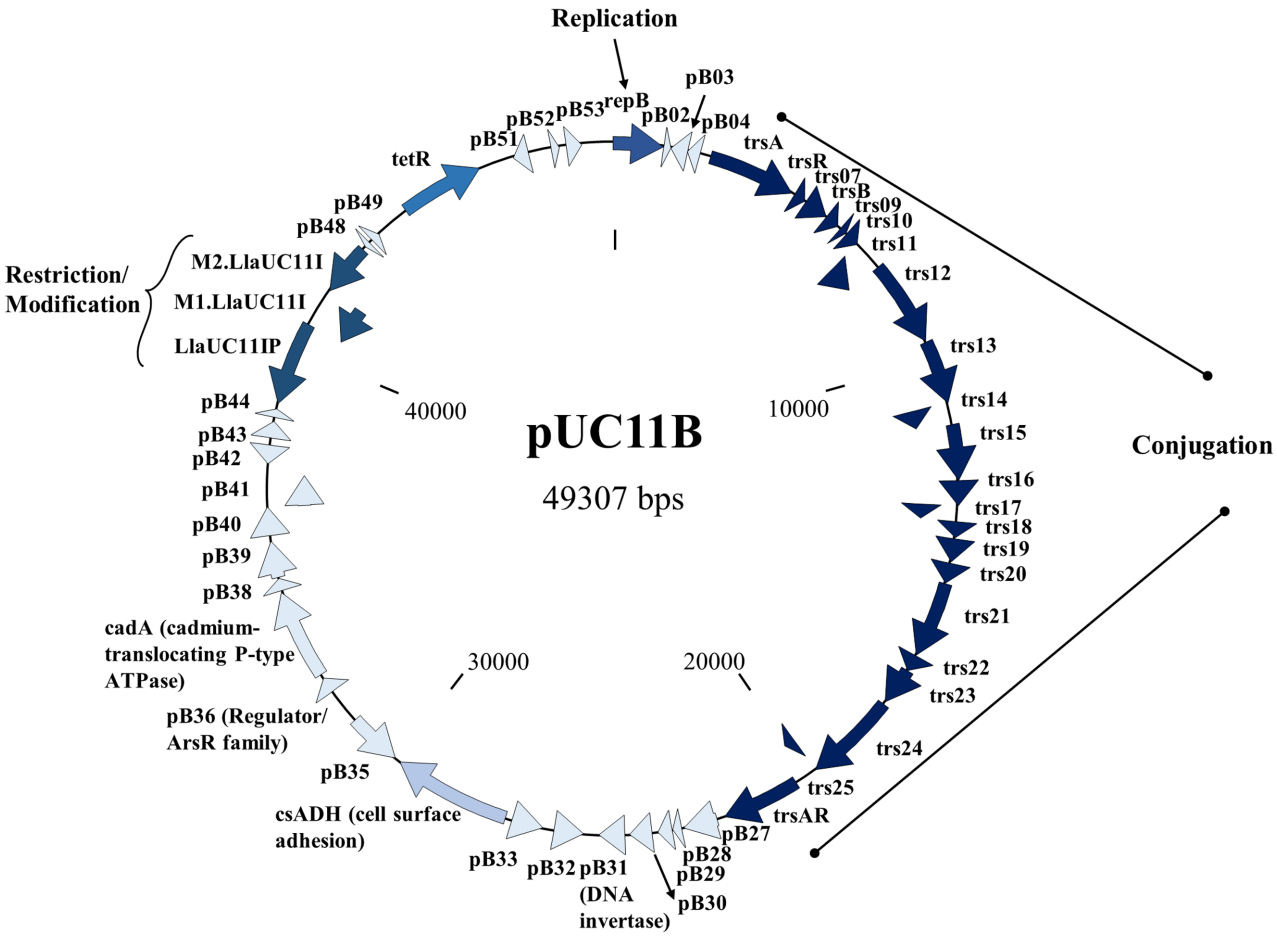


FIGURE 1 Genetic map of lactococcal plasmid pUC11B. Identified ORFs are represented by block arrows and predicted functions are indicated.

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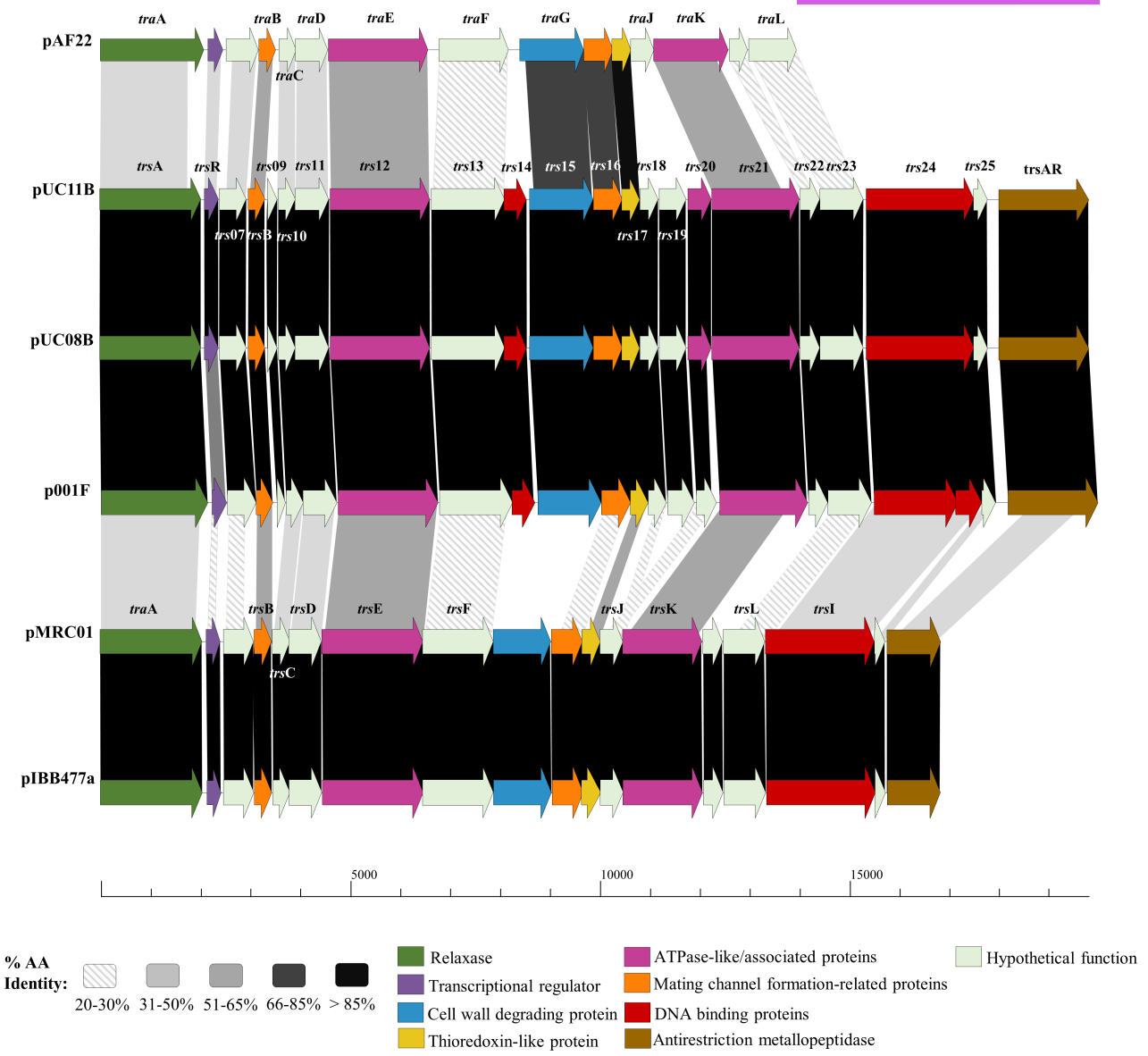


FIGURE 2 Comparison of organization of the conjugative transfer regions of plasmids pMRC01, pAF22, pUC11B, pUC08B, pIBB477a and p001F. The amino acid identity of the encoded proteins is indicated by the shaded boxes. Coloured arrows indicate predicted conjugative functions, based on a functional analysis of the pUC11B conjugation gene cluster.

To evaluate if pUC11B can undergo self-transfer, conjugation trials using a spread solid mating protocol were performed with *L. lactis* UC11 (as the donor strain) and *L. cremoris* MG1614 (as the recipient strain) (a complete list of the strains used in this study is presented in Table 1). Conjugation frequencies of 1.28% were achieved through this combination of donor and recipient (Figure 3), expressed as the percentage of transconjugants per recipient (details of the experimental procedures followed in the present study are described in the Appendix S1). To facilitate mutagenesis of the pUC11B conjugation system, the plasmid was transferred via conjugation (at low frequency < 0.1%) to the prototypical lactococcal strain *L. cremoris* NZ9000 harbouring the recombineering-associated plasmid pJP005 (van Pijkeren & Britton, 2012) (the full list of plasmids and constructs used in this study is summarized in Table 2). *L. cremoris* NZ9000 harbouring pUC11B and pJP005 was subsequently assessed as a donor of pUC11B to the *L. cremoris* MG1614 and yielded significantly ($p < 0.05$) higher pUC11B conjugation frequency of 3.98%, compared to that obtained for *L. lactis* UC11 as a donor. In all the cases, PCR-based verification was performed using oligonucleotides targeting the *tetR* gene to support the tetracycline-resistance phenotype associated with pUC11B (Table S1).

Predictive functional analysis of the pUC11B conjugation operon

The predicted gene cluster associated with conjugation in pUC11B spans 19,829 bps (Kelleher et al., 2019),

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TABLE 1 Lactococcal strains used in this study: *L. lactis* UC11 (Kelleher et al., 2017), was the principal donor strain employed in the mating experiments; *L. cremoris* MG1614 (Gasson, 1983) was used as recipient; *L. cremoris* NZ9000 pJP005 was used for the recombineering approach (van Pijkeren & Britton, 2012).

Strain	Plasmids present in the strain	Relevant properties
L. lactis UC11	pUC11A, pUC11B, pUC11C, pUC11D, pUC11E, pUC11F	Principal donor strain, harbouring the conjugative plasmid pUC11B, which confers tetracycline resistance.
L. cremoris MG1614	-	Principal recipient strain, plasmid-free and resistant to streptomycin.
L. cremoris NZ9000	pJP005	Strain harbouring plasmid pJP005, resistant to chloramphenicol and encoding *RecT*.
L. cremoris NCDO700	pNCDO700A, pNCDO700B, pNCDO700C, pNCDO700D, pNCDO700E, pNCDO700F,	Rifampicin-resistant mutant of *L. cremoris* NCDO700, with predicted type I RM system.
L. lactis I11	pI11A, pI11B, pI11C, pI11D, pI11E, pI11F	Rifampicin-resistant mutant of *L. lactis* I11, with predicted type I RM system.
L. cremoris UC047	pUC047A, pUC047B, pUC047C, pUC047D, pUC047E, pUC047F, pUC047G	Rifampicin-resistant mutant of *L. cremoris* UC047, with predicted type I and II RM system.
L. cremoris F27	pF27A, pF27B, pF27C, pF27D, pF27E, pF27F, pF27G	Rifampicin-resistant mutant of *L. cremoris* F27, with predicted type I and II RM system.
L. lactis UC073	pUC073A, pUC073B, pUC073C, pUC073D, pUC073E, pUC073F, pUC073G	Rifampicin-resistant mutant of *L. lactis* UC073, with predicted type I and III RM system.
L. lactis UL021	pUL021A, pUL021B, pUL021C, pUL021D	Rifampicin-resistant mutant of *L. lactis* UL021, with predicted type I and III RM system.

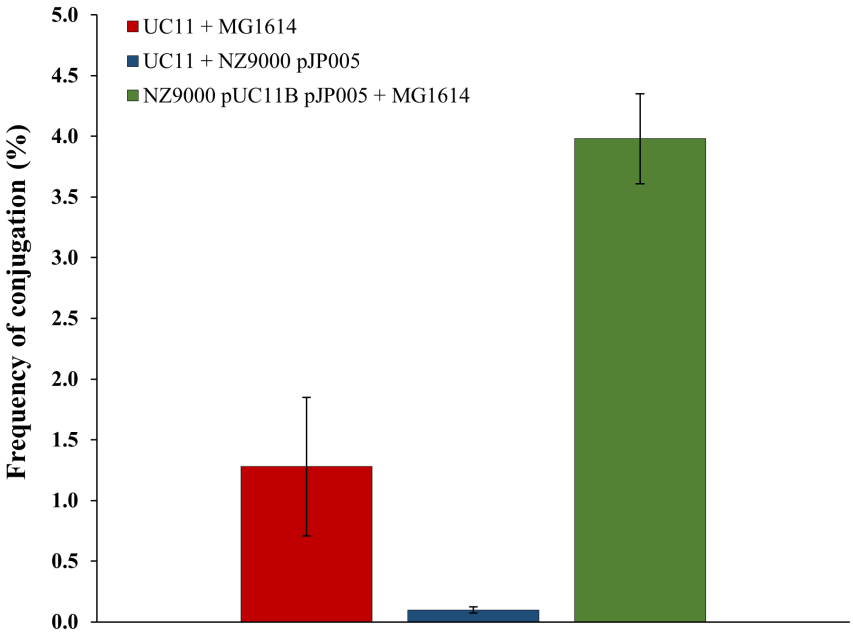


FIGURE 3 Conjugation frequencies achieved using the spread solid mating protocol and different donor–recipient combinations: *L. lactis* UC11 and *L. cremoris* MG1614, *L. lactis* UC11 and *L. cremoris* NZ9000 pJP005 (intermediate step, to achieve a strain both capable of conjugation and suitable for the recombineering approach), and *L. cremoris* NZ9000 pUC11B, pJP005 and *L. cremoris* MG1614. Presented data are the mean of three replicates ± standard deviation.

covering over 40% of the overall plasmid and incorporating 22 genes. This pMRC01/pAF22-like conjugation cluster is delimited at its 5′-end by three genes (pB02/03/04) encoding products of unknown function and a replication initiator-encoding gene (*repB*), and at its 3′-end by a putative transporter-encoding gene (pB27 - Figure 1). Similar to pNP40, pMRC01 and pAF22, the replication initiator-encoding gene of pUC11B is located close to the relaxase-encoding gene of its conjugation cluster (Figure 1); however, it is noteworthy that the position of the relaxase-encoding gene may differ in a system-specific manner. Protein families that are represented in all functional conjugation systems are the peptidoglycan hydrolases, T4SS transport channel core component family, ATPases and relaxases (Goessweiner-Mohr et al., 2014; Zhang et al., 2012). Nucleotide and amino acid sequence comparisons and analysis of the pUC11B conjugation-associated genes and their related products was performed to identify possible functions for each component of the system

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TABLE 2 Plasmids and plasmid constructs used in this study.

Plasmid	Characteristics	Reference
pJP005	Plasmid resistant to chloramphenicol and encoding *RecT*, required for recombineering.	van Pijkeren & Britton, 2012
pUC11B	Conjugative plasmid, resistant to tetracycline.	Kelleher et al., 2019
$trsR_{pUC11B}$::Ter	pUC11B derivative presenting a single mutation in the *trsR* gene, causing an increased conjugation phenotype.	This study
trs13/17/18/22/23/24/25/AR_{pUC11B}::Ter	pUC11B derivatives presenting a single mutation in one of eight genes encompassing its presumed conjugation gene cluster, causing no impact in conjugation frequency.	This study
$trs07/16/21_{pUC11B}$::Ter	pUC11B derivatives presenting a single mutation in one of three genes encompassing its presumed conjugation gene cluster, causing a mild impact in conjugation frequency.	This study
$trs15_{pUC11B}$::Ter	pUC11B derivative presenting a single mutation in the *trs15* gene, causing a major impact in conjugation frequency.	This study
$trsA/B/09/10/11/12/14/19/20_{pUC11B}$::Ter	pUC11B derivatives presenting a single mutation in one of nine genes encompassing its presumed conjugation gene cluster, causing a fully absent conjugation.	This study
pPEPi	Erythromycin version of the low-copy-number *E. coli-L. lactis* shuttle vector, pPTPi, containing a nisin-inducible promoter P*nisA*.	This study
pNZ44E	Erythromycin-resistant, *E. coli-L. lactis* shuttle vector pNZ44, P44 constitutive promoter.	Draper et al., 2009
pPEPi::trsA-21	pPEPi derivatives containing an intact copy of one of the 22 genes that encompass the pUC11B conjugation cluster.	This study
pPEPi::F27M	pPEPi derivatives containing a copy of the RM methylase encoding gene of *L. cremoris* F27.	This study
pPEPi::UC073M	pPEPi derivatives containing a copy of the RM methylase encoding gene of *L. lactis* UC073.	This study
pNZ44E::trsR	pNZ44E derivative containing gene *trsR* from pUC11B.	This study

(Table 3; this table lists HHPred results, the PDB identifier, as well as the probability of structural homology). The nomenclature applied to the pUC11B conjugation cluster was either function-based in the case of *trsR* (regulator) and *trsAR* (anti-restriction protein), following the traditional nomenclature of pMRC01-like conjugation systems (*trsA* and *trsB*) (Dougherty et al., 1998) or referred to the arrangement among the pUC11B annotated ORFs.

Identification of the minimal components and a putative transcriptional regulator of the pUC11B conjugation system

Individual mutational analysis of the 22 genes that comprise the presumed pUC11B conjugation gene cluster was performed to validate and complement the in silico functional analysis, using a recombineering approach (see Appendix S1). Each mutant derivative was employed as a donor in conjugation assays with the recipient strain *L. cremoris* MG1614 in order to assess the impact of individual mutations on conjugation efficiency relative to the positive control donor (i.e. *L. lactis* NZ9000 pUC11B, pJP005) (Figure 4). The different pUC11B derivatives are summarized in Table 2.

Following this examination, genes were classified based on the impact of their individual mutation on conjugation frequency (Table 2). The effects included a >2-fold increase in conjugation frequency when mutating gene *trsR*; no apparent impact in conjugation frequency (i.e. mutations in *trs13*, *trs17*, *trs18*, *trs22*, *trs23*, *trs24*, *trs25* and *trsAR*); mild impact with <7-fold decrease in conjugation frequency (i.e. mutations in *trs07*, *trs16* and *trs21*); major impact with 4500-fold decrease in conjugation frequency (i.e. mutation in gene *trs15*) and, finally, those that were determined to be essential for conjugation to occur (i.e. mutations in genes *trsA*, *trsB*, *trs09*, *trs10*, *trs11*, *trs12*, *trs14*, *trs19* and *trs20*) (Figure 4).

To validate that the observed effects were due to the incorporated mutations or to establish if they were due to polar effects on downstream genes, gene complementation studies were performed. Mutants that displayed a reduced or inhibited conjugation phenotype were complemented by reintroducing the intact version of the mutated gene cloned into pPEPi and complete restoration of conjugation frequencies was achieved for all assessed mutants (Table 4). Plasmid pPEPi was constructed using the backbone of plasmid pPTPi without the tetracycline-resistance gene (O'Driscoll et al., 2004) and the erythromycin-resistance gene from pNZ44E (plasmid construction is detailed in Appendix S1). Nisin-mediated induction of the cloned

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TABLE 3 Functional analysis of the protein-encoding genes encompassing the presumed pUC11B conjugation cluster. The analysis is based on different software: amino acid and protein sequence comparison performed with BLAST and Pfam v34.0, prediction of transmembrane helices in proteins using TMHMM and structure prediction based on HHpred analysis.

Gene	BLAST	Pfam	HHpred	TMHMM (transmembrane domain)
trsA	MobA/MobL family protein/ nickase	MobA/MobL family protein/ nickase	DNA relaxase (PDB: 4HTE_A 100%)	No
trsR	Hypothetical	Transcriptional regulator	DNA binding/Repressor/TraN similarity (PDB: 4P0Z_A 99.2%)	No
trs07	Hypothetical	Insignificant	Unknown	No
trsB	TraB conjugation protein (mating channel formation)	T4SS CagC/TrbC/VirB2 family	Membrane protein complex (PDB: 7O3V_F 92.2%)	Yes (3 domains)
trs09	Hypothetical	Insignificant	Unknown	No
trs10	Conjugal transfer protein	Insignificant	Unknown	Yes (2 domains)
trs11	Conjugal transfer protein	Insignificant	Unknown	No
trs12	ATP-binding protein	AAA-like domain/TrbE, VirB family	VirB4-like ATPase (PDB: 7043_B 100%)	No
trs13	Hypothetical/TrsF similarity	Insignificant	Membrane protein/Essb similarity (PDB: 4ANO_A 100%)	Yes (1 domain)
trs14	ssDNA-binding protein	Single strand binding family	Single-stranded DNA binding protein (PDB: 7F5Y_B 99.8%)	No
trs15	CHAP domain-containing protein	CHAP domain/ Glucosaminidase	Amidase/Peptidoglycan protease (PDB: 5T1Q_D 70.79%)	Yes (1 domain)
trs16	Hypothetical	Insignificant	Membrane protein (PDB: 6H9N_B 99.47%)	Yes (1 domain)
trs17	Thioredoxin family protein	Thioredoxin	Thioredoxin-like protein (PDB: 1GH2_A 97.9%)	Yes (1 domain)
trs18	Hypothetical	Insignificant	Unknown	Yes (2 domains)
trs19	Hypothetical	Insignificant	Unknown	Yes (3 domains)
trs20	Hypothetical	Insignificant	ATPase-like (PDB: 2KSE_A 67.1%)	Yes (2 domains)
trs21	T4SS DNA transfer family protein	T4SS, TraG-like coupling protein	T4SS coupling protein, VirB4-like ATPase (PDB: 1E9R_G 99.96%)	No
trs22	Hypothetical	Insignificant	Unknown	Yes (1 domain)
trs23	Hypothetical	Insignificant	Unknown	Yes (6 domains)
trs24	DNA topoisomerase III	DNA topoisomerase	DNA topoisomerase I/III (PDB: 1I7D_A 100%)	No
trs25	Hypothetical	Insignificant	Unknown	No
trsAR	ImmA/IrrE family metallo-endopeptidase	Metallopeptidase, IrrE N-terminal-like domain	ArdC-like protein; Antirestriction, metal binding protein (PDB: 6I89_A 99.82%)	No

genes was achieved by adding 5 ng/ml of nisin to the growth medium.

Interestingly, mutation of *trsR* caused a more than 2-fold increase in conjugation frequency compared to the wild type (WT) control (unmutated pUC11B), indicating that this gene plays a regulatory repressing role. To further evaluate the role of this gene in pUC11B conjugation, the *trsR* gene was cloned under the control of the constitutive P44 promoter in the high copy expression plasmid pNZ44E (construct pNZ44E::*trsR*) to allow overexpression in *L. cremoris* NZ9000 pUC11B, pJP005 to determine the impact on conjugation frequency in this host background. As a negative control, *L. cremoris* NZ9000 pUC11B, pJP005 harbouring the empty vector pNZ44E was used. The generated NZ9000 pUC11B pNZ44E::*trsR* strain was then employed as a donor of pUC11B with the recipient *L. cremoris* MG1614 following the spread solid mating protocol. Overexpression of

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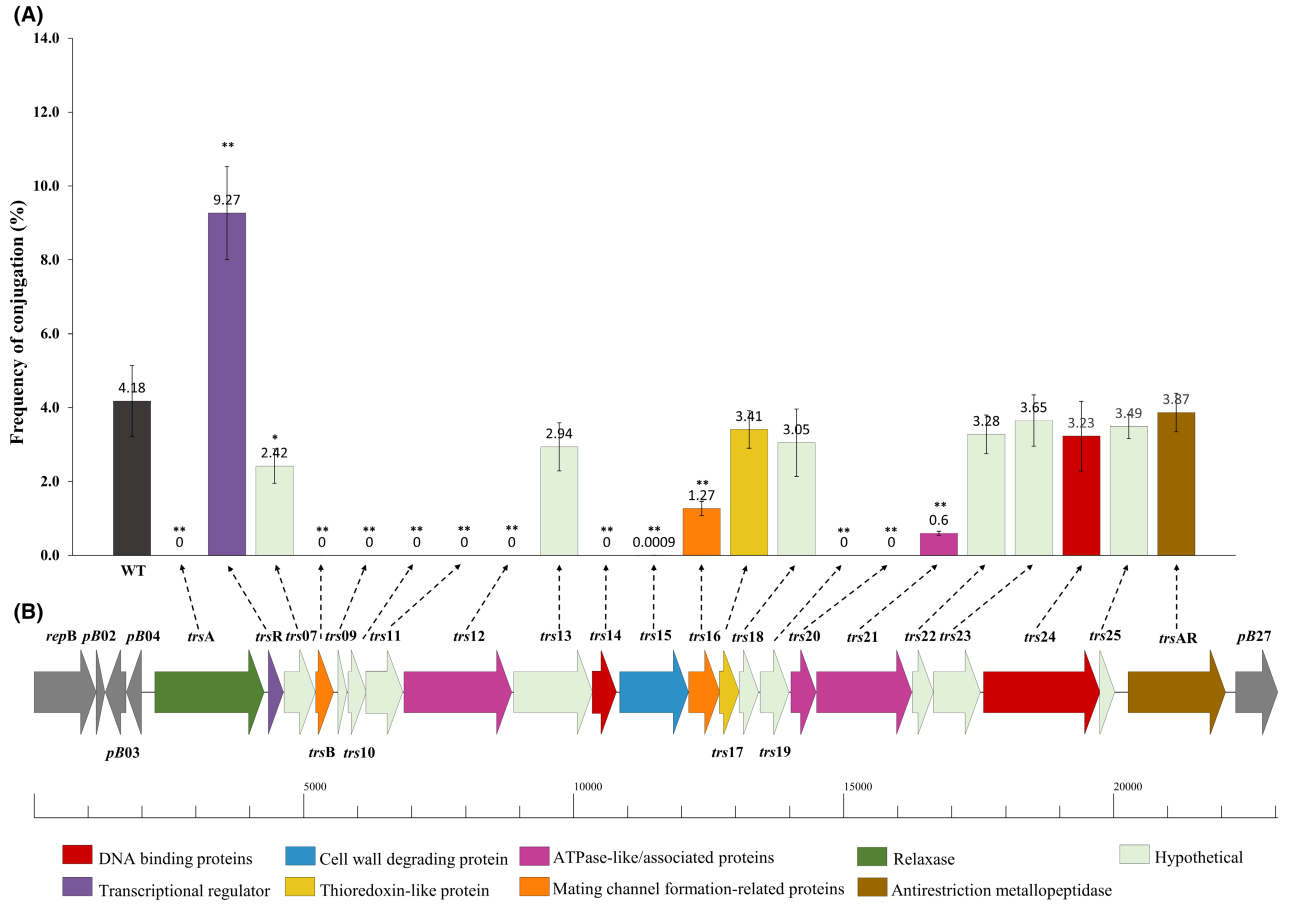


FIGURE 4 Deletion analysis of the pUC11B transfer region. Conjugations were performed following the spread solid mating protocol (Ortiz Charneco et al., 2021), using as donor the relevant mutant strain (NZ9000 pUC11B, pJP005 carrying one of the 22 individual mutations) and *L. cremoris* MG1614 as the recipient. Conjugation between a WT *L. cremoris* NZ9000 pUC11B pJP005 and *L. cremoris* MG1614 was used as reference for conjugation frequency. A p-value < 0.05 was considered significant and is represented in the graph with a single asterisk "*", whereas a p-value ≤ 0.001 was considered very significant and is represented in the graph by two asterisks "**". Data are the mean of three replicates ± standard deviation. The genetic organization of the pUC11B-encoded conjugation cluster and assigned functions are represented.

trsR caused a significantly ($p < 0.05$) reduced conjugation phenotype (0.28%) compared to the control (4.22%). This phenomenon of reduced conjugation frequency when overexpressed in a WT strain is consistent with the observed increase in conjugation frequency when *trsR* is mutated, indicating that this gene is involved in downregulating (repressing) the expression or function of the pUC11B conjugation machinery.

While spread solid mating was the optimized method applied in the described complementation and overexpression assays, the mutant strain $trsR_{pUC11B}$::Ter was also evaluated for conjugative transfer capabilities using the liquid mating protocol, a suboptimal condition for pUC11B conjugation. This was performed to ascertain if the stimulatory effect that the mutation of *trsR* caused in the conjugation frequency of pUC11B under optimal conditions would be more noticeable in a suboptimal scenario. As demonstrated in Table 5, the liquid mating approach using the strain *L. cremoris* NZ9000 pUC11B, pJP005 as donor (with *L. cremoris* MG1614 as recipient) yielded conjugation at levels below the detection threshold ($<1 \times 10^{-5}$%). However, when strain *L. cremoris* NZ9000 $trsR_{pUC11B}$::Ter was used as the donor of plasmid pUC11B with the recipient strain *L. cremoris* MG1614, conjugation was achieved at a frequency of 6.71×10^{-5}%. While this is a low conjugation frequency, it represents a crossing of the detection threshold which could not be achieved by the control, in which no conjugation was observed. This further substantiates the previous reduced conjugation frequency phenotype presented by the mutant strain $trsR_{pUC11B}$::Ter and provides additional evidence to support that the product of gene *trsR* acts as a repressor of the pUC11B conjugation gene cluster and that, when this gene is mutated, conjugation frequencies significantly ($p < 0.05$) increase.

pUC11B and pNP40 encode divergent but functionally similar conjugation systems

As previously stated, plasmids pUC11B and pNP40 represent genetically divergent conjugation systems with little

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TABLE 4 Complementation and overexpression results. *L. cremoris* MG1614 was used as the recipient strain in all cases, and the spread solid mating protocol was applied. Results represent conjugation frequencies using either the mutant (trs_{pUC11B}::Ter), the complemented (trs_{pUC11B}::Ter pPEPi::*trs*) or the overexpressed (NZ9000 pUC11B pNZ44E::*trsR*) strains as donors. Data are the mean of three replicates ± standard deviation (SD).

Donor strains	Conjugation frequency (% ± SD)	Donor strains	Conjugation frequency (% ± SD)
$trsA_{pUC11B}$::Ter	0.00 ± 0.00	$trs14_{pUC11B}$::Ter	0.00 ± 0.00
$trsA_{pUC11B}$::Ter pPEPi::*trsA*	3.88 ± 0.83	$trs14_{pUC11B}$::Ter pPEPi::*trs14*	3.48 ± 0.66
$trs07_{pUC11B}$::Ter	2.28 ± 0.61	$trs15_{pUC11B}$::Ter	$7.05 \times 10^{-4} \pm 5.87 \times 10^{-5}$
$trs07_{pUC11B}$::Ter pPEPi::*trs07*	3.49 ± 0.77	$trs15_{pUC11B}$::Ter pPEPi::*trs15*	3.83 ± 1.03
$trsB_{pUC11B}$::Ter	0.00 ± 0.00	$trs16_{pUC11B}$::Ter	1.34 ± 0.21
$trsB_{pUC11B}$::Ter pPEPi::*trsB*	4.26 ± 1.08	$trs16_{pUC11B}$::Ter pPEPi::*trs16*	4.30 ± 0.92
$trs09_{pUC11B}$::Ter	0.00 ± 0.00	$trs19_{pUC11B}$::Ter	0.00 ± 0.00
$trs09_{pUC11B}$::Ter pPEPi::*trs09*	3.39 ± 0.76	$trs19_{pUC11B}$::Ter pPEPi::*trs19*	3.84 ± 1.05
$trs10_{pUC11B}$::Ter	0.00 ± 0.00	$trs20_{pUC11B}$::Ter	0.00 ± 0.00
$trs10_{pUC11B}$::Ter pPEPi::*trs10*	4.09 ± 0.91	$trs20_{pUC11B}$::Ter pPEPi::*trs20*	3.63 ± 0.68
$trs11_{pUC11B}$::Ter	0.00 ± 0.00	$trs21_{pUC11B}$::Ter	0.57 ± 0.09
$trs11_{pUC11B}$::Ter pPEPi::*trs11*	4.17 ± 0.88	$trs21_{pUC11B}$::Ter pPEPi::*trs21*	4.08 ± 1.19
$trs12_{pUC11B}$::Ter	0.00 ± 0.00	$trsR_{pUC11B}$::Ter	9.39 ± 2.09
$trs12_{pUC11B}$::Ter pPEPi::*trs12*	3.69 ± 0.72	NZ9000 pUC11B pNZ44E::*trsR*	0.28 ± 0.08

TABLE 5 Conjugation frequencies achieved when using the WT strain *L. cremoris* NZ9000 pUC11B, pJP005 or the mutant *L. cremoris* NZ9000 $trsR_{pUC11B}$::Ter as donors for conjugation with the recipient *L. cremoris* MG1614. Two different conjugation approaches were undertaken, the optimized spread solid mating and the suboptimal liquid mating. Data are the mean of three replicates ± standard deviation.

Donor strain	Spread solid mating (% ± SD)	Liquid mating (% ± SD)
NZ9000 pUC11B, pJP005	4.26 ± 1.19	$<1 \times 10^{-5} \pm 0.00$
NZ9000 $trsR_{pUC11B}$::Ter	9.39 ± 2.09	$6.71 \times 10^{-5} \pm 9.58 \times 10^{-6}$

to no sequence similarity. Although the T4SSs of these two plasmids may have lost any detectable sequence similarity, they may have retained similar functionalities. A previous study (Ortiz Charneco et al., 2021) identified the minimal set of components of the pNP40-encoded conjugation machinery required for conjugation, and a similar assay was performed in this study. These mutational analyses, paired with predictive analyses based on sequence and structural similarities performed in both systems, allowed to uncover certain activities and protein functions shared between both the pNP40- and pUC11B-encoded conjugation systems despite low or undetectable sequence homology (Figure 5). A detailed description of these findings and associated functional implications is presented below in the discussion section.

pUC11B encodes an anti-restriction system that affects conjugative processes

Inactivation of *trsAR* did not cause any discernible effect in conjugation frequency compared to the WT strain. Due to its structural similarities to the ArdC-like family of anti-restriction proteins, further conjugation assays were performed using *L. cremoris* NZ9000 $trsAR_{pUC11B}$::Ter and the WT control as donors of pUC11B with strains harbouring different RM systems as recipients (i.e. Type I, II or III RM systems). For this purpose, spontaneous rifampicin-resistant derivatives of the *L. cremoris* NCDO700 and *L. lactis* I11 (with predicted Type I RM systems); *L. cremoris* UC047 and F27 (harbouring predicted Type I and II RM systems); *L. lactis* UC073 and UL021 (with predicted Type I and III RM systems) were isolated and used as recipients in conjugation trials using the spread solid mating protocol. An overview of their predicted RM systems and cognate recognition sequences (where known or predicted) is indicated in Figure S1.

No difference in conjugation frequencies was observed between the control (WT *L. cremoris* NZ9000 pUC11B, pJP005) and the mutant strain (*L. cremoris* NZ9000 $trsAR_{pUC11B}$::Ter) when strains *L. cremoris* NCDO700 and *L. lactis* I11 (both harbouring

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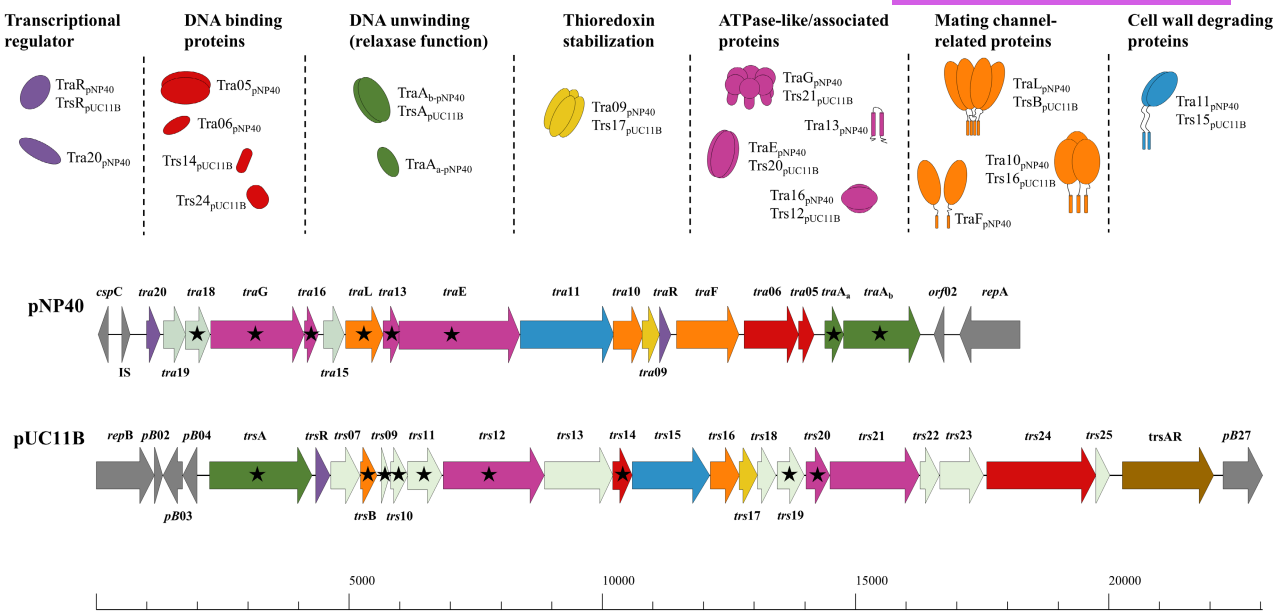


FIGURE 5 Functional comparison of the conjugation gene clusters of plasmids pNP40 and pUC11B. Solid black stars represent the essential genes required for conjugation in pNP40 (Ortiz Charneco et al., 2021) and pUC11B. A schematic and colour-coded representation of the shared protein functionalities between the clusters is shown. In brown is the *trsAR* gene, encoding an accessory function of the pUC11B conjugation cluster, whereas hypothetical genes are indicated as light green arrows. Grey arrows represent genes presumed not to be associated with conjugation gene clusters.

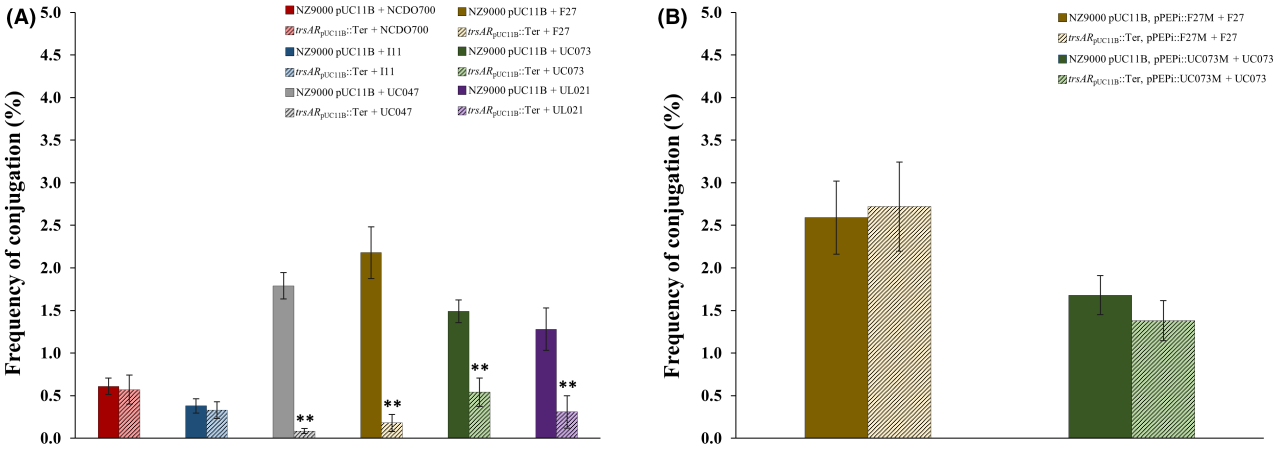


FIGURE 6 Conjugation frequencies observed using the spread solid mating protocol and different donor/recipient combinations. (A) *L. cremoris* NZ9000 pUC11B or *L. cremoris* NZ9000 *trsAR*$_{pUC11B}$::Ter as donors with the rifampicin-resistant derivatives *L. cremoris* NCDO700, *L. lactis* I11 (with predicted Type I RM systems), *L. cremoris* UC047, F27 (with predicted Type I and II RM system), *L. lactis* UC073 and UL021 (with predicted Type I and III RM systems) as recipients. (B) *L. cremoris* NZ9000 pUC11B, pPEPi::F27M-UC073M or *L. cremoris* NZ9000 *trsAR*$_{pUC11B}$::Ter, pPEPi::F27M-UC073M as donors against the rifampicin-resistant derivatives *L. cremoris* F27 and *L. lactis* UC073 as recipients. All values were compared against their respective wild-type control, and a p-value ≤ 0.001 was considered very significant and is represented by two asterisks "**". Presented data are the mean of three replicates ± standard deviation.

predicted Type I RM systems) were used as recipients (Figure 6A). However, using recipient strains *L. cremoris* UC047 and F27, and the strain carrying the *trsAR* mutant version of pUC11B as donor, significant ($P < 0.05$) differences in conjugation frequencies were observed compared to the control. *L. cremoris* UC047 and F27 contain predicted Type I and II RM systems. When *L. cremoris* NZ9000 *trsAR*$_{pUC11B}$::Ter was applied as a donor with *L. cremoris* UC047, the conjugation frequency decreased 20-fold compared to the control, whereas a 12-fold reduction in conjugation frequency was observed using the recipient strain *L. cremoris* F27. In a similar manner, when using *L. lactis* UC073 and UL021 as recipient strains for conjugation with the *trsAR* mutant strain, moderate, yet significant ($p < 0.05$) differences compared to the unmutated control were observed. *L. lactis* UC073 (2.75-fold decrease) and UL021 (4-fold decrease) harbour predicted Type I and III RM systems. From the above results it can be inferred that the product of

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trsAR does not facilitate circumvention of Type I RM systems, but it appears to circumvent the negative effects of Type II and III RM systems on conjugation.

To further ascertain if strain-specific differences in conjugation frequencies were due to the presence of a specific RM system in the recipient strain, the corresponding MTase-encoding genes of some of these systems were cloned into the low-copy, inducible plasmid pPEPi, and transformed into both donor strains. The MTase-encoding genes of strains *L. cremoris* F27 and *L. lactis* UC073 were selected as representatives of Type II and III RM systems respectively. Resulting donor strains, harbouring one of the pPEPi constructs (pPEPi::F27M or pPEPi::UC073M), were subsequently assayed for conjugation with the recipient strain from which the relevant MTase gene was derived. As a control, the Type II MTase from *L. cremoris* F27 (*M2.LlaF270RDP*) was selected since this methylase is predicted to be part of the ScrFI RM system (Figure S1) (Davis et al., 1993; Twomey et al., 1997). The empty and unmethylated pPEPi, as well as the methylated construct pPEPi::F27M (both previously induced with the appropriate amount of nisin) were treated separately with the RE ScrFI. The presence of the *L. cremoris* F27 Type II MTase-encoding gene in plasmid pPEPi protects it against the restriction activity of ScrFI (Figure S2), which manifests that this gene is active in the aforementioned plasmid and is shielding any DNA present in the same strain against the restriction activity of the *L. cremoris* F27-encoded Type II RM system.

Through this approach, it was ascertained that expression of the Type II or III MTase-encoding genes in the donor strain enhanced the conjugation of the premethylated plasmid pUC11B with gene *trsAR* mutated into the relevant recipients compared to the same donor that does not express the associated MTase-encoding gene (Figure 6B).

DISCUSSION

Plasmid pUC11B possesses a pMRC01/pAF22-like conjugation system, which represents the second most abundant type of plasmid-encoded conjugation systems among lactococcal strains. Despite the relevance of lactococcal species in the dairy industry and the importance of conjugation in the potential enhancement of starter cultures, relatively few studies have interrogated the functionality of these systems.

Plasmid pMRC01 has previously been reported to be capable of self-transfer to a recipient cell at different frequencies, dependent on the recipient strain used, with up to 0.2% conjugation frequency per donor cell when employing strain *L. cremoris* MG1363 as both donor and recipient of the plasmid (Coakley et al., 1997). In the present study, we achieved conjugation frequencies of 1.28% and 4% (of the recipient cell population) when using either *L. lactis* UC11 or *L. cremoris* NZ9000 pUC11B pJP005 as donor strains, respectively, with recipient *L. cremoris* MG1614. The increased conjugation frequency achieved when employing similar donor and recipient strains, observed both here and with pMRC01 (Coakley et al., 1997), was similar to that found for pNP40-mediated conjugation (Ortiz Charneco et al., 2021). This suggests that more related donor–recipient systems may increase successful conjugative transfer, as is the case with strains *L. cremoris* NZ9000 and *L. cremoris* MG1614, both plasmid-free derivatives of the parental strain *L. cremoris* MG1363 (Gasson, 1983).

Based on a functional and mutational comparative analysis of the prevalent and divergent conjugative systems encoded by lactococcal plasmids pUC11B and pNP40, we determined the minimal requirements of such a DNA transfer apparatus (Figure 5). Both pUC11B and pNP40 conjugation systems require a relaxase, two ATPase-like/associated proteins and one mating channel component to function. Furthermore, both systems encode a highly conserved thioredoxin-like protein whose elimination did not impact on conjugation, one putative transcriptional repressor and a peptidoglycan hydrolase which, although not essential for conjugation, was deemed to play a major role in the conjugative transfer of both pNP40 (Ortiz Charneco et al., 2021) and pUC11B. It would be feasible now, by looking at raw sequence data, to assess what functionalities are present in lactococcal plasmids and thus determine whether a presumed lactococcal conjugation system is likely to be active or not.

The predictive functional and mutational analysis of the pUC11B conjugation cluster (paired with the complementation and overexpression results) allowed for a better understanding of the pUC11B conjugation machinery. Similar to the situation for plasmid pNP40 (Ortiz Charneco et al., 2021), the relaxase of the pUC11B conjugation system, TrsA, which is presumed to cut one strand of the *nic*-site located within the *oriT*, and unwind the supercoiled DNA (Dostál et al., 2011), was shown to be essential for conjugation. The relaxase-encoding gene, *trsA*, is located close to the presumed origin of plasmid pUC11B replication. This proximity of the relaxase to the plasmid replication origin can also be observed for plasmids pMRC01, pAF22 and pNP40. This co-location may serve to couple conjugative DNA transfer and plasmid replication, which both contribute to plasmid dissemination, being either vertical or horizontal, respectively (Guzmán-Herrador & Llosa, 2019). In this context, it is worth mentioning that replication proteins and relaxases have been proposed to be, at least to some degree, functionally interchangeable (Wawrzyniak et al., 2017).

Apart from the relaxase, four major groups of conjugation-related proteins were found in plasmid

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pUC11B, displaying a similar impact on conjugation frequency when their encoding gene was mutated as their counterparts in plasmid pNP40 (Ortiz Charneco et al., 2021): the ATPase-like proteins Trs12 and Trs20 were observed to be absolutely required for conjugation, while mutation of the other ATPase-like-encoding gene *trs21* did not cause complete abrogation of conjugation but a significant reduction. This suggests that its role may be auxiliary or partially complemented by other protein(s). The function of mating channel-related proteins TrsB and Trs16 was in the case of the former deemed essential for conjugation, while in the case of the latter was shown to play a minor role in conjugation. The peptidoglycan hydrolase Trs15 was shown to play a major role in the conjugation process, although it is not absolutely required. Finally, a proposed repressor of this conjugation cluster, TrsR, is similar to members of the MerR family of transcriptional regulators, particularly to the TraN repressor protein of the pIP501 conjugation operon (Kohler et al., 2019). A similar MerR family repressor-encoding gene is present in both pMRC01 and pAF22 plasmids (Figure 2) and although the amino acid identity similarity between these proteins is not high, this suggests a similar mechanism of action repressing the conjugation of these systems. Furthermore, overexpression of this protein caused a reduced conjugation frequency phenotype in the WT *L. cremoris* NZ9000 pUC11B, pJP005 strain, a phenomenon also displayed by the regulator of plasmid pNP40, TraR (Ortiz Charneco et al., 2021). Overall, this analysis and the discovered similarities between two prevalent and distinct conjugation systems represented by the pNP40 and pUC11B plasmids brings us to the most up to date understanding of lactococcal conjugation systems.

The essential genes for pUC11B conjugation, *trsA*, *trsB*, *trs10*, *trs11* and *trs12*, have counterparts in plasmids pMRC01 and pAF22 while other seemingly essential genes (*trs09*, *trs14* and *trs19*) lack counterparts in either of these plasmids (Figure 2). Since both pMRC01 and pAF22 have previously been reported to be capable of self-transfer by conjugation (Coakley et al., 1997; Fallico et al., 2012), it would seem that the role played by these four genes is supported by other, non-homologous genes within their conjugation clusters. The pUC11B-encoded conjugation-related proteins present a low level of amino acid identity to those encoded by pAF22 and pMRC01, while also displaying high similarity to those of the recently annotated p001F. Therefore, lactococcal plasmid-encoded conjugation clusters could be divided in pNP40-like, being these prevalent among public databases (Ortiz Charneco et al., 2021), and pMRC01-like, with pMRC01, pAF22 and pUC11B presenting significant similarities between them but being still distinct from each other.

Our results implicate the *trsAR* gene product in overcoming Type II and III RM systems present in the recipient cell during conjugation, thus increasing the successful conjugative transfer of plasmids, as shown for pUC11B, and possibly broadening its host range and its ability to transfer properties to the recipient cell. It is known that RM systems play a major role as barriers to horizontal gene transfer events, recognizing and cleaving DNA not modified by the corresponding MTase of the system (Roer et al., 2015). The TrsAR protein presents high structural similarities to the ArdC anti-restriction protein from plasmids R388 and pSa. ArdC is believed to act as an ssDNA-binding protein with a metalloprotease domain, and this binding interferes with the actual RM system binding, thereby hindering the degradation of its target DNA (Belogurov et al., 2000; González-Montes et al., 2020; Wilkins, 2002). Based on the similarities with this ArdC protein, a similar mechanism of action of TrsAR is proposed here, although further functional and structural studies should be performed to determine the precise function and mode of action of TrsAR. Similarly, plasmids pMRC01 and pIBB477a harbour homologues of the gene encoding the anti-restriction system, albeit truncated versions and with reduced levels of sequence identity. This TrsAR protein would be expected to function in the recipient cell, and thus it would be feasible to presume that it is differently expressed from the relaxase and the T4SS-encoding genes. Additionally, the *oriT* regions of pUC11B and pNP40 are currently not well defined. A transcriptional analysis of both conjugation gene clusters is currently ongoing, which is expected to provide further insights into the precise location of these *oriT* regions and the expression of the conjugation-related genes.

This functional and mutational mapping of the conjugation operon of plasmid pUC11B has allowed the genetic depiction of this and other pMRC01-like conjugation clusters. However, further studies should be considered in order to determine the precise function of each gene present in this conjugation cluster, as well as the location of any transcriptional starting sites and the mechanism by which transcriptional repressors present within these clusters regulate conjugation.

EXPERIMENTAL PROCEDURES

Details of the experimental procedures followed in the present study are described in the Supplemental text S1.

AUTHOR CONTRIBUTIONS

Guillermo Ortiz Charneco: Conceptualization (equal); formal analysis (lead); investigation (lead); validation (equal); writing – original draft (lead); writing – review and editing (equal). **Philip Kelleher:** Data curation (equal). **Andrius Buivydas:** Investigation (supporting). **Sofia Dashko:** Writing – review and editing (equal). **Paul P. de Waal:** Writing – review and editing

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(equal). **Noël N. M. E. van Peij:** Writing – review and editing (equal). **Richard John Roberts:** Data curation (equal); writing – review and editing (equal). **jennifer mahony:** Conceptualization (equal); funding acquisition (equal); supervision (equal); validation (equal); writing – review and editing (equal). **Douwe van Sinderen:** Conceptualization (equal); funding acquisition (equal); supervision (equal); validation (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST

S.D., P.P.d.W. and N.N.M.E.v.P. were employed by the company DSM. R.J.R. was employed by the company New England Biolabs. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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