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Delineation of the integrase-attachment and origin-of-transfer regions of the symbiosis island ICEM/Sym^{R7A}

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ABSTRACT

Integrative and conjugative elements (ICEs) are chromosomally-integrated mobile genetic elements that excise from their host chromosome and transfer to other bacteria via conjugation. ICEM/Sym^{R7A} is the prototypical member of a large family of "symbiosis ICEs" which confer upon their hosts the ability to form a nitrogen-fixing symbiosis with a variety of legume species. Mesorhizobial symbiosis ICEs carry a common core of mobilisation genes required for integration, excision and conjugative transfer. IntS of ICEM/Sym^{R7A} enables recombination between the ICEM/Sym^{R7A} attachment site *attP* and the 3' end of the *phe*-tRNA gene. Here we identified putative IntS *attP* arm (P) sites within the *attP* region and demonstrated that the outermost P1 and P5 sites demarcated the minimal region for efficient IntS-mediated integration. We also identified the ICEM/Sym^{R7A} origin-of-transfer (*oriT*) site directly upstream of the relaxase-gene *rlxS*. The ICEM/Sym^{R7A} conjugation system mobilised a plasmid carrying the cloned *oriT* to *Escherichia coli* in an *rlxS*-dependent manner. Surprisingly, an in-frame, markerless deletion mutation in the ICEM/Sym^{R7A} recombination directionality factor (excisionase) gene *rdfS*, but not a mutation in *intS*, abolished mobilisation, suggesting the *rdfS* deletion tentatively has downstream effects on conjugation or its regulation. In summary, this work defines two critical *cis*-acting regions required for excision and transfer of ICEM/Sym^{R7A} and related ICEs.

1. INTRODUCTION

Integrative and Conjugative Elements (ICEs) are chromosomally-integrated elements that transfer between bacteria by conjugation (1-3). ICEs are likely the most abundant conjugative element in prokaryotic genomes and carry diverse genetic cargo, which can confer phenotypes such as antimicrobial-resistance, virulence, metabolism and the capacity to enter symbiotic associations with eukaryotes (4-10). After entry of an ICE into a recipient cell, integration into the host chromosome occurs through the actions of an ICE-encoded site-specific recombinase; termed an integrase (11, 12). Integrases belong to either the tyrosine or serine recombinase families; defined by the conserved nucleophilic amino acid that becomes covalently bonded to a DNA strand during the recombination reaction (11).

The recombination events mediated by integrases have largely been defined by studies performed on phages (11-14). Integrases form nucleoprotein complexes consisting of a homodimer structures bound to *cis*-acting DNA regions called attachment (*att*) sites present in the phage and host chromosomes (11). During chromosomal integration, an *attP* site present on the circularised phage genome, is recombined with the host chromosome attachment site *attB*. Upon integration, the integration junctions termed *attL* and *attR* are formed. Each *att* site contains a “catalytic core” site where strand exchange occurs. *att* sites also contain “arm” or “P” sites which flank the core. The P sites facilitate structural organisation of the integrase-DNA complex during recombination (11, 15-17). Recombinases are heterobivalent in that the N-terminal region binds the P sites and the C-terminal region interacts with the *att* core sequence where recombination occurs. During recombination the nucleophilic residue within the C-terminus becomes covalently fused to a DNA strand. An incoming DNA strand then displaces the residue reforming an intact DNA strand resulting in formation of a double-stranded cruciform DNA structure known as a Holliday junction intermediate. A second round of cleavage, strand exchange and ligation resolves this structure to produce the recombination end products (3, 18, 19).

Integrated ICEs are flanked by hybrid *attL* and *attR* sites and the two core sites form a direct repeat bordering the inserted ICE. For both phages and ICEs, excision is catalysed by the integrase, but often an additional factor known as an excisionase or recombination directionality factor (RDF) is required. Excisionases have been shown to bind and bend *att* DNA, shifting the favoured direction of integrase-mediated recombination towards the excision reaction ($attL + attR > attP + attB$) (11, 20). Certain excisionases, particularly the subset *cox* family, have bifunctional roles as transcriptional regulators (12, 21-27). The P2 phage excisionase Cox stimulates the lytic cycle by blocking

transcription of repressor gene *cl*, in addition to its roles as an *att*-binding prophage excision stimulator (25, 26).

ICEM/Sym^{R7A} is a 502-kb ICE which encodes genes required to form a nitrogen-fixing symbiosis with legumes of the genus *Lotus* (9, 28). Subsequent to the discovery of ICEM/Sym^{R7A}, numerous other mobile symbiosis ICEs have been identified within mesorhizobial strains that confer the ability to form symbioses with a variety of legumes including *Biserrula* and Chickpea (29-31). Recently, more complex tripartite symbiosis ICEs have been discovered, which carry a similar core gene complement to ICEM/Sym^{R7A} but upon integration in a new host they fragment into three physically separate regions within the host chromosome through the action of two additional integrases (31, 32).

ICEM/Sym^{R7A} is a monopartite ICE that integrates into the single *phe*-tRNA gene of the *Mesorhizobium loti* R7A chromosome (9, 20), a feature common to symbiosis ICEs found within mesorhizobia including the tripartite ICEs (31). Integration requires the integrase *IntS*, a tyrosine recombinase related to the P4 bacteriophage integrase family (20). The ICEM/Sym^{R7A} *attP* site contains a 17-bp DNA sequence identical to the last 17-bp of the *phe*-tRNA gene and integration of ICEM/Sym^{R7A} into this *attB* site reconstitutes the *phe*-tRNA gene within the newly-formed *attL* site (9, 20). We have previously demonstrated that a synthetic "mini-ICE" carrying only *intS* and *attP* integrates into the *phe*-tRNA gene in R7ANS; a derivative of R7A from which ICEM/Sym^{R7A} was cured (20). The ICEM/Sym^{R7A} RDF, *RdfS*, is a putative member of the MerR superfamily of winged helix-turn-helix (wHTH) DNA-binding proteins (12, 33, 34). Deletion of *rdfs* abolishes excision of ICEM/Sym^{R7A}, while overexpression of *rdfs* leads to loss of ICEM/Sym^{R7A}, producing the non-symbiotic derivative strain R7ANS (20). Expression of *rdfs* in R7ANS carrying an integrated copy of the mini-ICE stimulated its excision and subsequent loss, suggesting *rdfs* and *intS* together are sufficient for ICEM/Sym^{R7A} excision (20).

Unlike most RDF genes, which are generally positioned directly adjacent to their cognate integrase gene (12), *rdfs* is located within a highly conserved symbiosis-ICE gene cluster, containing conjugation-associated genes including *traF* (encoding a putative prepilin peptidase), a lytic transglycosylase, a VirD2-like conjugative relaxase gene *rlxS* and the putative ICEM/Sym^{R7A} origin-of-transfer (*oriT*) region (20, 28). *oriT* sites are *cis*-acting regions that generally contain inverted repeats and a *nic*/core site targeted by a relaxase protein. Relaxases, often in association with other components, form a protein-DNA complex with the *oriT* called the relaxosome. Within the relaxosome, the relaxase nicks DNA within the *nic* core site and remains covalently attached to the 5'

phosphate of the nicked strand, after which it pilots DNA to the type-IV secretion system for conjugative transfer (35, 36). Relaxosomes often include accessory proteins required for specific recognition of the *oriT* and/or stimulation of *oriT* cleavage (37-39). Mutation of ICEM/Sym^{R7A} *rlxS* abolishes transfer of ICEM/Sym^{R7A}. Moreover, *rlxS* is also required for maintenance of ICEM/Sym^{R7A} during excision, suggesting relaxase-mediated rolling-circle replication contributes to ICEM/Sym^{R7A} maintenance when in the excised form (20).

In this work, we further define two *cis*-acting DNA sites of ICEM/Sym^{R7A}, the IntS attachment site *attP* and the *oriT* site. The minimal *attP* site contained five copies of a directly repeated sequence motif in a pattern resembling the arm sites of other characterised *attP* regions. Removal of a single proximal or distal "P" site severely hampered IntS-mediated integration. Conjugative mobilisation experiments were used to confirm the ICEM/Sym^{R7A} origin-of-transfer (*oriT*) region was located upstream of the *rlxS* gene. Unexpectedly, an intact *rdfs* gene was required for mobilisation, suggesting this mutation has an effect on the expression of the ICEM/Sym^{R7A} conjugation system.

2. MATERIAL AND METHODS

2.1 Strains and growth conditions

Strains used in this study are listed in **Supplemental Table 1**. *M. loti* strains were cultured at 28°C or 30°C in Tryptone Yeast (TY) media (0.3% w/v yeast extract, 0.5% w/v tryptone, 0.6% v/v CaCl₂) or in glucose rhizobium defined medium (G/RDM) (40) supplemented with vitamins as described previously (20). *Escherichia coli* DC10B (41) cultures were grown in Lysogeny broth (LB) at 37°C. Media was supplemented with antibiotics where required at the following concentrations: 2.0 µg/mL (*M. loti*) and 5.0 µg/mL (*E. coli*) tetracycline, 50 µg/mL gentamicin and 50 µg/mL ampicillin.

2.2 Molecular biology and cloning

Plasmid construction is detailed in **Supplemental Table 1** and oligonucleotides and QPCR probes used are described in **Supplemental Table 2**. QPCR was carried using the ViiA 7 Real-time PCR system (Applied Biosystems) in 20-µl reactions using the SensiFAST Probe Lo-ROX Kit (Bioline), using the standard-speed running conditions (i.e. non-fast). DNA was extracted from stationary-phase non-IPTG induced TY cultures (64 h) using PrepMan Ultra (ThermoFisher) reagent as previously described (20). Primers and probes used were purchased from Integrated DNA Technologies (IDT - Singapore) and probes were modified with a 5' FAM fluorophore and 3' ZEN quencher. The *melR* gene probe was lengthened to increase binding and compensate for the lack of a minor-groove-binder ligand on the IDT probes. The primer/probe combinations exhibited QPCR efficiencies of 2.04, 2.09 and 2.03 for *attP*, *attB* and *melR*, respectively. Reactions were normalised using the standard template as previously described (20).

2.3 Integration assays for attP-carrying plasmids

Two-hundred nanogram aliquots of plasmid DNA were electroporated into electrocompetent R7ANS(pJJ611) cells as previously described (42). Cells were recovered in 1 mL TY broth with shaking at 28°C for 3 hours. Serial dilutions were then plated on G/RDM containing vitamins (thiamin, biotin and nicotinate) (20) and gentamicin. Colonies were enumerated after 5 days of growth at 28°C. pFUS conjugation experiments of *E. coli* ST18 and *M. loti* R7ANS(pJJ611) were performed as described in section 2.4. Confirmation of pFUS vector integration sites were done by PCR and sequencing (primers used listed in **Supplemental Table 2**).

2.4 Conjugative mobilisation experiments

M. loti donor strains carrying pFAJ2013 (strains used are listed in **Supplemental Table 1**) (20) and the *E. coli* recipient DC10B were grown to stationary phase (OD_{600} 0.8-1.0) in 5 mL TY (R7A) or LB-broth (DC10B). Five-hundred microlitres of *M. loti* TY culture were pelleted and resuspended in 500 μ L *E. coli* DC10B LB culture and centrifuged for an additional 2 minutes at 17,000 g. The pellet was resuspended in 100 μ L TY broth. Thirty-microlitres of the cell mixture spotted in triplicate onto TY agar media and incubated at 30°C overnight. Strains containing pSRKKm or pSRKrdS were spot plated on TY agar media. Mobilisation experiments with R7A Δ rdS(pFAJ2013)(pSRKrdS) were carried out both with and without 1 mM IPTG, however no mobilisation was observed in either experiment. Spots were recovered and resuspended in 1 mL sterile water and serially diluted 10-fold to 1×10^{-9} . One-hundred microlitre aliquots were plated onto LB agar containing tetracycline and incubated overnight at 37°C, and colonies were enumerated. Controls consisting of the donor strains only were prepared similarly, with 500 μ L culture resuspended in 100 μ L TY broth and 30 μ L spots grown overnight at 30°C on TY agar media.

2.5 Bioinformatic analyses of attP and oriT regions.

The ICEM/Sym^{R7A} sequence is available from GenBank accession AL672111.1 (28). A 352-bp putative attP region (assembled from 501647-501801[attR] and 1-157[attL]) was used in MEME (43)(version 5.0.2) alignments with non-mesohizobial sequences identified by PSI-BLAST (44) as described in section 3.1. GenBank accessions used; *Magnetospirillum* sp. (FXXN01000020.1), *Marinicaulis flavus* (PJCH01000010.1), *Nitrospirillum amazonense* (AFBX01000767.1), *Acidisphaera* sp. (CP029176.1), Rhizobiales sp. (PIGG01000031.1), *Prosthecomicrobium hirschii* (LJYW01000001.1), *Sphingomonas* sp. (CH959307.1) and *Rhodopseudomonas palustris* (CP000301.1). The putative 352-bp attP region was also submitted to MEME with BLAST-identified *Mesorhizobium* spp. phe-tRNA-adjacent intergenic regions (GenBank accessions: CCNB01000043.1, AYWT01000014.1, KI421454.1, MDLH01000001.1, CAAF010000028.1, AYVN01000010.1, CCNA01000045.1, MDFL01000146.1, NPKJ01000019.1, PZJX01000011.1, FNEE01000009.1, NNRI01000015.1, AHAM01000208.1 and MZXV01000055.1) to confirm the presence of the identified P-site motif.

ICEM/Sym^{R7A} putative oriT sequence was collected from the upstream region of *rlxS* (*msi106*) (133491-133680) and used in PSI-BLAST investigations described in section 3.2. GenBank accessions used; *Sphingobium lactosutens* (ATDP01000088.1), *Oceanibaculum nanhaiense* (MPOB01000010.1), *Phaeosporillum fulvum* (FNWO01000021.1), *Rhodospirillum photometricum* (HE663493.1), *Thalassospira xiamenensis* (LPXL01000008.1), *Niveispirillum cyanobacteriorum* (CP025612.1) and *Erythrobacter xanthus* (QXFM01000025.1). HMMER hmmscan described in section 3.2 used hmm

profiles downloaded from https://github.com/gem-pasteur/Macsyfinder_models/tree/master/models/Conjugation/profiles.

3. RESULTS

3.1 Identification of the minimal ICEM/Sym^{R7A} *attP* site required for integration

intS is located directly downstream of the ICEM/Sym^{R7A} *attL* site, which also forms part of the *attP* site on the circularised element. To identify potential IntS P sites within the ICEM/Sym^{R7A} *att* sites, we initially used PSI-BLASTP (44) searches to identify divergent IntS homologues outside the *Mesorhizobium* genus. Nucleotide sequences corresponding to each IntS-homologue gene were inspected to determine if the integrase was preceded by a tRNA gene. Intergenic regions between each tRNA and *intS* homologue were collected and analysed along with the ICEM/Sym^{R7A} *attL* region using the MEME motif identifier software (43, 45). MEME was set to search for multiple repetitions (per sequence) of a 5-20-bp motif. A motif with consensus “TGKTGGTATC” was present as 2-3 direct repeats in each of the putative *attL* sequences (**Fig. 1A**). Inspection of the ICEM/Sym^{R7A} *attP* revealed two additional copies of this motif upstream of the core region (on *attP* / *attR*). The five sites were named P1-P5 (**Fig. 1B**). All five instances of the suspected P-site motif were conserved on the IntS *attP* regions of *Mesorhizobium* spp. strains: WSM1284, WSM1497, NZP2037 and WSM1271 (**Fig. 1B**).

Fig. 1. The ICEM/Sym^{R7A} attP site. (A) Putative P-site sequence motif derived from an alignment of attL regions corresponding to divergent IntS homologues (see Section 2.5). (B) ICEM/Sym^{R7A} attP sequence aligned (using MAFFT (46)) with the attP regions of *Mesorizobium* spp. WSM1284 (accession: CP015064.1), WSM1497 (accession: CP021070.1), NZP2037 (accession: CP016079.1) and WSM1271 (accession: CP002447.1) Asterisks (*) denotes alignment difference from consensus. Putative P-sites P1-P5 are boxed. ICEM/Sym^{R7A} 17-bp core-containing region is underlined. The 5' start site of primers used to PCR-amplify the attP regions used in pFUS2 integration assays are shown as arrows.

intS and *attP* can function together to recombine *attP* and *attB* when cloned on separate plasmids. An introduced suicide vector carrying a 628-bp *attP* region (pJJ610) is efficiently integrated into the *phe*-tRNA gene, but only in the presence of a functional *intS* expressed by a coresident plasmid pJJ611 (20). We extended this approach here to define the minimal *attP* region. A smaller 283-bp region of *attP* containing only the P1-P5 region was cloned into the gentamicin-resistance suicide plasmid pFUS2, producing pFUSP1-P5. pFUS plasmids lacking either P1, P5 or both P1 and P5, were additionally constructed. Each construct was then transferred by conjugation from *E. coli* ST18 (47) to R7ANS(pJJ611). Apparent transfer produced rates for the P1-P5 construct were on average 3.8×10^{-3} exconjugants per donor, but experiments using the pFUS2 clones lacking the outermost P1 or P5 sites were reduced 190- and 140-fold, respectively (**Table 1**). No colonies were isolated following transfer of the clone lacking both P1 and P5, suggesting removal of both sites abolished IntS-mediated integration. The *attR* sites formed by examples of the three integrated pFUS2 constructs were amplified by PCR and sequenced, which confirmed the constructs integrated downstream of the *phe*-tRNA gene (9, 20). We additionally electroporated the same pFUS2 constructs into R7ANS(pJJ611), which produced a similar pattern of results. Therefore, these experiments both defined the minimal *attP* site for IntS and suggested that the outermost P1 and P5 sites are required for efficient IntS-mediated recombination.

Table 1. Conjugation and electroporation assays for IntS-mediated *attP* integration.

<i>attP</i> region [†]	Apparent conjugation rate [§]	Apparent electroporation efficiency cfu/ml/ng DNA
P1-P5	$3.8 \times 10^{-3} \pm 8.8 \times 10^{-4}$	935 $\pm$ 420
P1-P4	$1.9 \times 10^{-5} \pm 3.0 \times 10^{-6}$	3.1 $\pm$ 1.3
P2-P5	$2.5 \times 10^{-5} \pm 8.7 \times 10^{-6}$	4.0 $\pm$ 2.2
P2-P4	ND [§]	0
none	ND [§]	0

[†]*attP* regions cloned into non-replicative plasmid pFUS2 and conjugated or electroporated into R7ANS(pJJ611).

[‡]Conjugation rates are expressed as exconjugants per donor and all data are the average of three replicates of the entire experiment.

[§]Limit of detection for conjugation approximately 3×10^{-8} exconjugants per donor.

3.2 Identification of ICEM/Sym^{R7A} origin-of-transfer region

The relaxase RlxS is essential for transfer of ICEM/Sym^{R7A} and extrachromosomal maintenance of ICEM/Sym^{R7A} when excised (20). A hmmscan search (48) of relaxase protein family hmm profiles revealed RlxS was related to members of the MOB_{p1} family (37, 38). *oriT* sites of MOB_p relaxases generally contain an inverted-repeat sequence upstream of a conserved *nic* core region. *oriT* sites are often located upstream of their cognate relaxase gene (49, 50). To identify potential conserved sequence motifs upstream of the *rlxS* gene, the RlxS protein was used as a query in PSI-BLASTP searches (44) of non-mesozothobial genomes. The NCBI-BLAST taxonomy viewer guided selection of a diverse collection of representatives from the PSI-BLASTP matches. Approximately 500-bp of DNA that preceded each relaxase was collected and analysed using MEME (43). MEME was set to search for 10-60-bp motifs present once on each sequence. Two large motifs were identified, which flanked an inverted-repeat which consisted of a variably-sized central region flanked by short regions of stronger conservation (**Fig. 2A**). One of the regions adjacent to the inverted-repeat bore resemblance to the consensus core region for MOB_p relaxases and contained a conserved 'GC' *nic* sequence (**Fig. 2B**). Interestingly, an insertion sequence related to the IS1111 family is present within this site in the *M. japonicum* MAFF303099 symbiosis-ICE sequence (**Fig. 2A**) (also noted in (28)), suggesting the MAFF303099 ICE may be non-mobile. It has been noted that members of the IS1111 family preferentially target regions adjacent to DNA stem-loop structures for their insertion (35, 51-53).

3.3 *rlxS* and *rdfS* are essential for mobilisation of a plasmid carrying the ICEM/Sym^{R7A} *oriT*

A 95-bp region encompassing the predicted *oriT* region was cloned into the broad host-range plasmid pFAJ1700, producing pFAJ2013. R7A(pFAJ2013) was used as a donor to mobilise pFAJ2013 by conjugation to *Escherichia coli* DC10B. pFAJ2013, but not the vector-only control pFAJ1700, was mobilised to DC10B at an average rate of 1.2×10^{-4} exconjugants per donor (**Table 2**). Next, pFAJ2013 was introduced into non-symbiotic derivative of R7A, R7ANS, and the *rlxS*-mutant R7ArlxS⁻ (20) and the resulting strains were used as donors in conjugation experiments. No transfer was observed from either of these strains. To genetically complement the *rlxS* mutation, a region carrying both the *oriT* and the *rlxS* gene was cloned into pFAJ1700, producing plasmid pFAJCMV. In contrast to pFAJ2013, this plasmid was mobilised to *E. coli* from the R7ArlxS⁻ background at an average frequency of 2.1×10^{-4} exconjugants per donor (**Table 2**). These data confirmed that mobilisation was dependent on the ICEM/Sym^{R7A} conjugation machinery and *rlxS*.

rdfs is located directly upstream of the *oriT* and *rlxS*, suggesting it has evolved closely with the DNA transfer region of ICEM/Sym^{R7A}. Therefore, we tested if a previously characterised strain carrying an in-frame deletion in *rdfs* (20) was able to mobilise pFAJ2013. Surprisingly, pFAJ2013 mobilisation was completely abolished in this background (**Table 2**). The lack of mobilisation was not related to a deficit in ICEM/Sym^{R7A} excision per se, as pFAJ2013 transfer was observed from the *intS* mutant strain R7AΔ*intS* (in which ICEM/Sym^{R7A} does not excise (20)), albeit at a reduced frequency. In order to complement the *rdfs* deletion, a vector compatible with pFAJ2013 was constructed, using pSRKKm (54). The *rdfs* coding sequence was placed downstream of the *lac* promoter in pSRKKm and the resulting plasmid pSRKrdfS was introduced into R7AΔ*rdfs*(pFAJ2013). To confirm pSRKrdfS was able to complement the ICEM/Sym^{R7A} excision defect in R7AΔ*rdfs*, QPCR was carried out to measure the abundance of the excision products *attP* and *attB*. As expected, the R7AΔ*rdfs*(pFAJ2013)(pSRKKm) strain exhibited around 100-fold reduced abundance of *attP* and *attB* excision products compared to R7A carrying the same plasmids (**Supplemental Fig. 1**). Strain R7AΔ*rdfs*(pFAJ2013)(pSRKrdfS) however, exhibited close to wild-type levels of excision. Attempts to complement the deficit in pFAJ2013 transfer using the same strains were unsuccessful.

Table 2. Conjugation assay data and colony counts for pFAJ1700 vectors.

Strain	Vector	Region cloned	Transfer frequency ^a	Standard deviation
R7A	pFAJ1700		ND*	
	pFAJ2013	<i>oriT</i>	1.24 x 10 ⁻⁴	2.62 x 10 ⁻⁵
R7ANS	pFAJ1700		ND*	
	pFAJ2013	<i>oriT</i>	ND*	
R7ArlxS	pFAJ1700		ND*	
	pFAJ2013	<i>oriT</i>	ND*	
	pFAJCMV	<i>oriT-rlxS</i>	2.07 x 10 ⁻⁴	6.29 x 10 ⁻⁵
R7AΔ <i>intS</i>	pFAJ1700		ND*	
	pFAJ2013	<i>oriT</i>	7.12 x 10 ⁻⁶	3.88 x 10 ⁻⁶
	pFAJCMV	<i>oriT-rlxS</i>	6.64 x 10 ⁻⁶	2.97 x 10 ⁻⁶
R7AΔ <i>rdfs</i>	pFAJ1700		ND*	
	pFAJ2013	<i>oriT</i>	ND*	
	pFAJCMV	<i>oriT-rlxS</i>	ND*	

^aPer-donor transfer frequencies are the average of three independent experiments.

*Not detected. Detection limit approximately 3.4 x 10⁻⁹

Fig. 2. The ICEM/Sym^{R7A} origin-of-transfer (*oriT*).

(A) Organisation of *rdxS-rlxS* gene cluster, with relative location of *oriT* indicated (20, 28). Conserved sequences based on non-*Mesorhizobium* sp. relaxase-upstream regions (underlined) and inverted repeat location (arrows). Suspected *nic* core site bolded for ICEM/Sym^{R7A} based on similarity to core sites shown in B. MEME-generated (43) 15-bp conserved motifs shown under respective underlined location. Location of IS1111-family transposon insertion within the related MAFF303099 ICE is indicated. (B) Inverted-repeat regions (arrows), partial *nic* core sites (bold) and *nic* sites (arrowhead) of *oriT* within MOB_P-carrying elements; conjugative transposon Tn1549 (49), plasmid RP4 (IncPα) (55), pIP501 (56) and plasmid R64 (57).

4. DISCUSSION

Regulation of ICEM/Sym^{R7A} excision and transfer has been extensively studied (9, 20, 58-60), however, the *cis*-acting sites integral to these processes, *attP* and the *oriT*, have remained relatively uncharacterised. In this work, we defined the minimal ICEM/Sym^{R7A} *attP* region and showed that a 283-bp region containing five putative arm, or "P" sites, P1-P5, were sufficient for efficient integration by IntS. We also confirmed the location of the ICEM/Sym^{R7A} *oriT* and demonstrated that a plasmid carrying the *oriT* region was mobilised from R7A to *E. coli* and this was dependent on the presence of ICEM/Sym^{R7A} and *rlxS*.

Integrase P-site sequences and their positions relative to the core region vary but they are typically short, loosely conserved repeats positioned 50-140-bp from the *attP* core (11, 61) (**Fig. 3**). We identified a conserved 10-bp motif sequence following bioinformatic comparisons of putative *attL* sites from distantly related IntS-gene homologues. The consensus motif sequence "TGKTGGTATC" was present five times in each of the *attP* regions of ICEM/Sym^{R7A}, ICEMcSym¹²⁷¹, ICEM/Sym²⁰³⁷ (32) and on putative ICEs in *Mesorhizobium* spp. strains WSM1284 and WSM1497. The arrangement of the likely P sites relative to the core site was similar to that observed for other well-characterised *attP* sites including Tn916 and λ bacteriophage, despite the fact that these elements encode distantly related integrases (**Fig. 3**) (20, 62, 63). Removal of either of the outermost putative P sites significantly reduced the efficiency of integration, presumably due to reduced binding, or incorrect positioning of IntS molecules within the nucleoprotein complex during recombination (13, 14, 63). Integration was abolished when both outermost putative P sites were removed.

Fig. 3: Comparison of the ICEM/Sym^{R7A} *att* sites with those of Tn916 and bacteriophage λ.

Schematic of ICEM/Sym^{R7A}, Tn916 (34) and bacteriophage λ *attP* sites (63). Black lines representing each element are to scale, indicated below the DNA (in base-pairs relative to the core). The black rectangles show the *attP* site core regions (not to scale). Relative orientation of ICEM/Sym^{R7A} putative P sites, and Tn916 and bacteriophage λ P sites shown as the black arrows (not to scale). Start location of ICEM/Sym^{R7A} *IntS* CDS shown with grey arrow.

RlxS is a member of the MOB_P family, a large and diverse family of relaxases found in a diverse collection of plasmids and ICEs. *oriT* sites of MOB_P relaxases typically display considerable sequence diversity, however they often contain a moderately conserved *nic* core sequence adjacent to a less conserved inverted-repeat region (49, 64). The ICEM/Sym^{R7A} *oriT* region contained an inverted-repeat region and interestingly, strongly conserved motifs that flanked it were identified. One of these motifs resembled a MOB_P *nic* core site (**Fig. 2B**), however, it is possible that the core region could be positioned on the other side of the inverted repeat. Further work is required to fully dissect the functional components of the ICEM/Sym^{R7A} *oriT*.

rdfS is peculiar for an RDF gene in that it is present upstream of genes involved in conjugation; *traF*, *msi107* (a lytic transglycosylase) and *rlxS*, rather than adjacent to its cognate integrase *intS* (28). Here we demonstrated that deletion of *rdfS* abolished the ability of the ICEM/Sym^{R7A} conjugation system to mobilise a plasmid carrying the *oriT*. Strangely, ectopic expression of *rdfS* restored the excision defect observed in the *rdfS* mutant, as previously demonstrated (20), but did not restore the mobilisation defect (**Supplemental Fig. 1**). *rdfS* is the first gene in a polycistronic operon encoding *traF*, *msi107* and probably *rlxS* (33, 58). The *traF* start site also overlaps with the *rdfS* stop codon suggesting they may be translationally coupled. We cannot exclude the possibility that loss of mobilisation was due to disruption of downstream expression or translation of *traF* and/or *msi107*. However, the R7AΔ*rdfS* deletion is markerless, in-frame and the deletion terminates 59-bp upstream of the *traF* open-reading frame (20).

It is possible that RdfS is required for transcriptional activation of other conjugation genes on ICEM/Sym^{R7A} such as the type-IV secretion system (*trb*) gene cluster or *rlxS*. We have previously demonstrated that RdfS activates expression of both *rdfG* and *rdfM* on ICEMcSym¹²⁷¹ (33), so a role for RdfS as a transcriptional activator of conjugation genes is conceivable. The TraN protein of the *Enterococcus* sp. plasmid pIP501, like RdfS, is a WHTH protein and it binds upstream of the pIP501 *oriT* and represses transcription of the *tra*-operon, repressing transfer (65, 66). Alternatively, RdfS could have a more direct role in *oriT* processing or relaxosome assembly. The *Clostridium perfringens* plasmid pCW3 encodes a WHTH transcription factor necessary for conjugation, TcpK, which binds the *oriT* utilising a novel binding mechanism for WHTH proteins (67). However, these possibilities aside, it remains unclear why mobilisation of pFAJ2013 was not restored by pSRKrdfS, unless the appropriate physiological levels of RdfS protein were not achieved using this vector. In future experiments investigating the downstream effects of the *rdfS* deletion, including possible polarity of the mutation on expression and translation of *traF* and *msi107*, will be required to resolve this.

ICEM/Sym^{R7A} transfer requires coordination of IntS-mediated excision with expression of the type-IV secretion system and processing of the relaxosome. It is clear from the positioning of *rdfS* as the first gene in a cluster containing essential transfer genes *traF* and *rlxS* that coregulation of these processes has become linked over the course of evolution and our results suggest that they may be mechanistically intertwined. The work described herein defines the *attP* and *oriT* sites ICEM/Sym^{R7A} and demonstrates a potentially complex link between the regulation of excision and transfer through expression of the *rdfS-traF-msi107* region.

DATA AVAILABILITY

All data have been provided in-text and accession numbers listed where appropriate.

SUPPLEMENTARY DATA

Supplemental Table 1: Bacterial strains and plasmids utilised in this study.

Supplemental Table 2: Oligonucleotides used in this study.

Supplemental Fig. 1: Excision percentages of R7A and *rdfS* mutant strains containing pFAJ2013 and pSRK or pSRKrdfS vectors.

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CONFLICT OF INTEREST

The authors have declared that no competing interests exist.

ACCEPTED MANUSCRIPT

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HIGHLIGHTS

Verdonk, CJ, *et al.* **Delineation of the integrase-attachment and origin-of-transfer regions of the symbiosis island ICEM/Sym^{R7A}**

- We define the minimal ICEM/Sym^{R7A} integrase attachment site *attP* region sufficient for efficient IntS-mediated integration
- We define the origin-of-transfer (*oriT*) region for ICEM/Sym^{R7A}
- The ICEM/Sym^{R7A} conjugation system can mobilise an *oriT*-carrying plasmid to *Escherichia coli*