Functional characterization of IScs605, an insertion element carried by tetracycline-resistant *Chlamydia suis*

Jae Dugan,¹ Arthur A. Andersen² and Daniel D. Rockey¹

Correspondence

Daniel D. Rockey
rokeyd@orst.edu

¹Department of Biomedical Sciences, College of Veterinary Medicine and the Molecular and Cellular Biology Program, Oregon State University, Corvallis, OR 97331, USA

²National Animal Disease Center, USDA Agriculture Research Service, Ames, IA 50010, USA

Stable tetracycline resistance in *Chlamydia suis* is mediated by a family of genomic islands [the *tet(C)* islands] that are integrated into the chlamydial chromosome. The *tet(C)* islands contain several plasmid-specific genes, the *tet(C)* resistance gene and, in most cases, a novel insertion element (IScs605) encoding two predicted transposases. The hypothesis that IScs605 mediated the integration of the *tet(C)* resistance islands into the *C. suis* genome was tested using a plasmid-based transposition system in *Escherichia coli*. Both high- and medium-copy-number plasmids were used as carriers of IScs605 in these experiments. IScs605 integrated into a target plasmid (pOX38) when delivered by either donor plasmid, and integration of the entire donor plasmid was common. IScs605-mediated integration occurred at many positions within pOX38, with 36 of 38 events adjacent to a 5'-TTCAA-3' sequence. Deletions in each of the candidate transposase genes within IScs605 demonstrated that only one of the two ORFs was necessary for the observed transposition activity and target specificity. Analysis of progeny from the mating assays also indicated that IScs605 can excise following integration into a target DNA, and, in each tested case, the sequence 5'-AATTC-3' remained at the site of excision. Collectively, these results are consistent with the nucleotide sequence data collected for the *tet(C)* islands, and strongly suggest that a transposase within IScs605 is responsible for integration of these genomic islands into the *C. suis* chromosome.

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INTRODUCTION

Chlamydiae are obligate intracellular bacteria that cause serious disease in humans and many other species (Belland *et al.*, 2004; Pollmann *et al.*, 2005; Wang *et al.*, 2005). One chlamydia of veterinary concern is *Chlamydia suis*, a pathogen that can cause conjunctivitis, abortion and enteritis in swine (Pollmann *et al.*, 2005). Recent work in our laboratory has identified the nature of a set of tetracycline (Tet) resistant (Tet^R) *C. suis* strains isolated from pigs in the midwestern USA (Fig. 1). These isolates are stably resistant to Tet and, in some cases, sulfadiazine (Andersen & Rogers, 1998; Lenart *et al.*, 2001; Suchland *et al.*, 2003). This resistance phenotype is conferred by genomic islands [*tet(C)* islands] that are apparently acquired through horizontal transfer (Dugan *et al.*, 2004).

A major limitation in developing an understanding of the chlamydial host–pathogen interaction process is the lack of a genetic system. Genome sequencing data support the conclusion that these organisms are not naturally receptive

to foreign DNA (Raoult, 2001). While there is a single genomic island within a related obligate intracellular species, the *tet(C)* islands represent the first example of recent horizontal acquisition of DNA in any *Chlamydia* spp. (Greub *et al.*, 2004; Roberts, 2005). It is anticipated that an understanding of the mechanisms associated with acquisition of the *tet(C)* islands by *C. suis* may assist in the development of a stable transformation system in the chlamydiae.

Each of the *tet(C)* islands has several genes found in plasmids from Gram-negative bacteria, including *tet(C)* and *tetR(C)*, which encode an efflux pump for Tet resistance and the cognate repressor protein, respectively (Chopra & Roberts, 2001). Most of these islands also carry a novel insertion element (IS), IScs605 (Dugan *et al.*, 2004). IScs605 is homologous to the IS605 family of insertion sequence elements, which are most commonly found in *Helicobacter pylori* (Kersulyte *et al.*, 1998, 2000, 2002, 2004; Ton-Hoang *et al.*, 2005). This family of IS elements consists of two divergently oriented ORFs that are members of different families of IS elements. One of these (*orfA*) is related to members of the IS200 family of transposases, while the

Abbreviation: IS, insertion element.

Fig. 1. Genomic islands present in Tet^R *C. suis* strains (Dugan *et al.*, 2004). The 5'-AATTCAA and 5'-TTCAA nucleotide sequences found at sites involved in apparent IScs605 transposition and excisions are shown. The individual strains carrying a particular island are indicated on the left. The grey arrows indicate the ORFs within IScs605, and the black arrows indicate the other ORFs within the *tet(C)* islands. The open arrows represent the *inv*-like gene interrupted by the integration of the *tet(C)* island into the chromosome of *C. suis*. The scale in kb is shown in the top island.

second (*orfB*) is similar to a family of proteins related to candidate transposases encoded within IS1341 (Bisercic & Ochman, 1993; Dugan *et al.*, 2004; Murai *et al.*, 1995). There is 100 % sequence identity of IScs605 from the different *C. suis* strains. A pentanucleotide sequence, 5'-TTCAA, is always present adjacent to the *orfA* end of IScs605 (Fig. 1). The *tet(C)* islands integrate into the *C. suis* chromosome within an invasin-like gene, and the site of insertion is also 5'-TTCAA (Dugan *et al.*, 2004).

Nucleotide sequence analysis has suggested that IScs605 may have had a role in the acquisition of the *tet(C)* islands by *C. suis*. The described experiments demonstrate that IScs605 can direct transposition in an *Escherichia coli*-based mating assay, yielding transposition products consistent with those observed in the Tet^R *C. suis* strains.

METHODS

***E. coli* strains, and culture conditions.** All *E. coli* strains were *recA* negative, and were grown in Luria-Bertani (LB) broth, or on LB agar, at 37 °C. *E. coli* RZ212 is a gentamicin (Gen)-resistant strain containing a deletion derivative of the F plasmid [pOX38: *aac(3)-I*], and it serves as a transposition host and a mating donor strain (Guyer *et al.*, 1981). *E. coli* SF800 is resistant to nalidixic acid (Nal), and has a deletion in *polA*, which prevents the strain from supporting ColE1-based origins of replication (Gross & Gross, 1969; Heffron *et al.*, 1977; Stachel *et al.*, 1985). *E. coli* TOP10 (Invitrogen) was used for plasmid rescue, and for constructing the pUC18- and

pBBR1MCS-based vectors. When indicated, particular antibiotics were added to the medium at the following concentrations: Nal, 10 µg ml⁻¹; kanamycin (Kan), 50 µg ml⁻¹; ampicillin (Amp), 100 µg ml⁻¹; chloramphenicol (Chl), 100 µg ml⁻¹; Gen, 10 µg ml⁻¹. All antibiotics were purchased from Sigma.

General PCR conditions. PCR reactions were performed with 0.25 mM dNTP (Fermentas), 0.4 nM forward and reverse primers (Table 1), and *Taq* (NEB) or *Pfx* polymerase (Invitrogen), as indicated. The following reaction parameters were used for all amplifications: templates were denatured for 45 s at 94 °C, the primers were annealed for 45 s at 52 °C, and products were extended for 90 s at 72 °C. All amplifications were performed for 30 cycles. Digoxigenin-labelled PCR products were made using previously described methods (Dugan *et al.*, 2004). The digoxigenin-labelled PCR probe of *aph* was produced with primers JP11 and JP12, using the pCR-Blunt vector (Invitrogen) as the template. The digoxigenin-labelled PCR probe of *bla* was produced with primers JP13 and JP14, using pUC18 as the template. The digoxigenin-labelled PCR probe of *cat* was produced with primers JP15 and JP16, using pBBR1MCS as the template. The digoxigenin-labelled PCR probe of pOX38 was produced with primers JP9 and JP10, using pOX38 as the template.

Plasmid constructs

A collection of pUC18-based and pBBR1MCS plasmids was produced to test the transpositional activity of IScs605. A cartoon of each construct is shown in Fig. 2, and the details for each set of plasmids are described in the following paragraphs.

pUC18 with IScs605. The entire IScs605 sequence was amplified from the Tet^R *C. suis* strain R19 using *Pfx* polymerase PCR

Table 1. Oligonucleotide primers used in this study

Primer	Sequence (5'–3')	Location
JP1	AGCAAAGCTTAAGTGAAGTACTCGCTGCTA	<i>orfB</i>
JP2	AGCAGAATTCCTTGAACGGAGTGCTTGC	<i>orfA</i>
JP3	AGCAGAATTCGGAGTGCTTGCCTCAATCT	<i>orfA</i>
JP4	TCGCAAAACACAAGCCCTG	249 bp from <i>orfB</i>
JP5	AACAACCAAGAACGAACCGCC	71 bp from <i>orfA</i>
JP6	TTGCGTTTCCCTTGTC	<i>aph</i>
JP7	TCCTGTTTGGCTTTCGGC	ISc605
JP8	GCTCACAATCCATCTGCTCACAAG	ISc605
JP9	CACACCCACGCAAAAACAAG	pOX38
JP10	CCATCGGATACATAGGAACCTCC	pOX38
JP11	ACTGGGCTATCTGGACAAGG	<i>aph</i>
JP12	TATCTACACGACGGGGAGTC	<i>aph</i>
JP13	CATCCATAGTTGCCTGACTCC	<i>bla</i>
JP14	GTATTATCCCGTATTGACGCC	<i>bla</i>
JP15	TTAATGAATCGGCCAACG	<i>cat</i>
JP16	CAATGGCATCGTAAAGAACA	<i>cat</i>
JP17	CGGTGATGACGGTGAAAACCTC	pUC18
JP18	CCTGCGTTATCCCCTGATTCTG	pUC18

Fig. 2. Plasmid constructs used in this study. The details of the constructs can be found in Methods. Plasmids pJD1–pJD6 were constructed within pUC18(Amp^R), while pJD7 was built within the medium-copy-number plasmid pBBR1MCS(Chl^R) (Kovach *et al.*, 1994). The plasmid pJD1 contains an intact ISc605 adjacent to a Kan^R gene (*aph*), which is flanked by the ends of ISc605. The plasmid pJD2 contains *aph* with the IS ends, but lacks the intact ISc605. Plasmid pJD3 is pJD1, but with a deletion introduced into the *orfA* ORF. Plasmid pJD4 is identical to pJD1, but carries a deletion that inactivates the *orfB* ORF. Plasmid pJD5 is a modification of pJD4, in which the *aph* coding sequence was placed within the inactivated *orfB*. Plasmid pJD6 is identical to pJD1, except for the lack of the 5'–TTCAA at the 3' end of ISc605. Plasmid pJD7 consists of pBBR1MCS containing the pJD5 insert.

(Invitrogen) with the primers JP1 and JP2 (Table 1). The PCR product was cloned into pUC18, and transformed into Top10 cells, creating pUC18:IScs605. Since there is no antibiotic resistance marker on IScs605, a Kan^R cassette was constructed by placing the resistance gene (*aph*), flanked by approximately 150 terminal nucleotides from each end of the IS element, adjacent to full-length IScs605. The primers JP4 and JP5 were used in a long-PCR protocol to amplify the vector backbone of pUC18:IScs605, which lacked the candidate transposase ORFs. Amplification with these primers produced a linear product containing terminal fragments of each IScs605 end (Table 1) flanking the entire pUC18 molecule, with terminal restriction sites for *NotI* and *EcoRV*. The *aph* sequence was then amplified from the PCR-blunt vector (Invitrogen) using *Pfx* polymerase with primers JP11 and JP12, and this resulted in an *aph* sequence with terminal *NotI* and *EcoRV* restriction enzyme sites. This product was then ligated to the amplification product from the outward PCR reaction. The resulting plasmid (pJD2) was transformed into *E. coli*, and selected by growth on LB agar containing Kan. The IScs605/*aph* fragment from pJD2 was then excised with *EcoRI*, and ligated into the full-length pUC:IScs605 to create the plasmid pJD1. The plasmids pJD1 and pJD2 served as experimental and control plasmids, respectively, for initial studies of the transpositional activity of IScs605.

***orfA* and *orfB* knockouts.** Each of the two ORFs in IScs605 was independently inactivated using a restriction-enzyme-based approach. A 200 bp fragment of *orfA* was deleted by digesting pJD1 with *PsiI* and *PfiI*, which are enzymes that each cut only once within the *orfA* ORF. The restricted plasmid was electrophoresed through 1 % agarose, and the vector-containing fragment was excised from the gel and purified. The DNA was then treated with T4 DNA polymerase (Fermentas) to blunt end the restriction sites. The product was then self-ligated, and transformed into *E. coli* TOP10. PCR was used to demonstrate that the resulting plasmid pJD3 carried an inactivation within *orfA*. A similar strategy was used for *orfB*. In this case, a 1.1 kb deletion within *orfB* was created by restriction digest with the enzymes *NsiI* and *HpaI*, leading to the plasmid pJD4.

Replacement of *orfB* with the Kan^R marker *aph*. The *orfB* sequence within pJD1 was replaced with the Kan^R marker *aph* from the plasmid pCR-Blunt vector (Invitrogen) by restricting pJD1 with *NsiI* and *HpaI*, blunting the single-stranded ends, and ligating *aph* into the vector. The *aph* ORF was amplified with the primers JP11 and JP12 (Table 1). Transformed clones were identified by plating on LB agar containing Kan, and resistant colonies were screened by PCR. This resulted in the plasmid pJD5.

Deletion of the targeting sequence within IScs605. The 5'-TTCAA found at the *orfA* end of IScs605 was deleted within pJD1 by performing *Pfx*-based PCR with the primers JP1 and JP3, using pJD5 as the template. This amplification produced an IS element identical to that found in pJD5, except for a deleted terminal 5'-TTCAA sequence. This PCR product was inserted into pUC18, creating pJD6.

IScs605 insertions into pBBR1MCS. The medium-copy-number vector pBBR1MCS (Kovach *et al.*, 1994) was also used to analyse IScs605 transposition activity. The IScs605 sequence from pJD5 was PCR-amplified using the primers JP1 and JP2. The PCR product was then inserted into pBBR1MCS, producing the plasmid pJD7.

Mating assay. The mating assay was performed by individually introducing each of the plasmids (Fig. 2) into chemically competent *E. coli* RZ212. Following transformation, individual colonies were picked, and cultured overnight in LB broth. These cultures were diluted 50-fold into 10 ml LB broth containing 0.5 % glucose, and grown for 2–4 h to an OD₆₀₀ of 0.4–0.6. The transformed donor

strain was then added to exponentially growing *E. coli* SF800 in equal proportions. This mating mix was incubated at 37 °C for 3 h, without shaking, to allow conjugation between the donor strain and the *polA*-deficient recipient strain. Bacteria were then diluted and spread onto LB agar containing Nal to determine the total number of recipient bacteria, and onto LB agar containing Kan and Nal to determine the number of recipient bacteria that carried pOX38/IScs605 recombinant plasmids. The values reported represent the number of transconjugants (Kan^R Nal^R) per 10⁹ recipient bacteria (Nal^R), and each experiment was repeated at least four times.

Genomic DNA purification. Transconjugants from mating assays were grown overnight in LB at 37 °C, and then concentrated by centrifugation at 3000 *g*. Pellets were resuspended in 50 mM Tris, pH 7.4, 1 % SDS, and 100 units proteinase K (Fermentas). The mixture was incubated at 37 °C for 4–6 h, and extracted three times with 1:1 phenol/chloroform, followed by a single chloroform extraction. The DNA was precipitated with ethanol/ammonium acetate and resuspended in water to a concentration of 1 µg µl⁻¹.

Plasmid rescue. Genomic DNAs from individual transconjugant clones were restricted with the enzymes *MluI* and *BssHII* for 4 h. These restriction enzymes generate the same overhanging ends following digestion and cut many times within pOX38, but do not cut within pJD1, pJD5 or pJD7. Products of these digests were purified using a commercial kit (Qiagen), self-ligated, and then transformed into TOP10 cells. Individual Amp^R Kan^R colonies were transferred to LB, and cultured overnight. Plasmids were purified from these cultures (Qiagen), and the nucleotide sequence of DNA flanking the likely insertion site was determined using primers JP6, JP7 and JP8.

Southern blot analysis. Purified genomic DNA of transconjugants was analysed by Southern blots, as described previously (Dugan *et al.*, 2004). Genomic DNA was digested with *MluI* and *BssHII*, *PsiI* or *PstI*, followed by electrophoresis through a 1 % agarose gel, and then passively blotted to nitrocellulose. The enzymes *MluI* and *BssHII* do not cut within any of the pJD plasmids, and thus *MluI*/*BssHII* restriction fragments analysed by Southern blotting represent DNA not digested within the original plasmid source of IScs605. In contrast, the enzymes *PstI* and *PsiI* cut once within pJD1 through pJD6 (*PstI*) and pJD7 (*PsiI*), and they were useful for identifying multimers of donor plasmids within pOX38. The blots were analysed using probes for *aph*, *bla*, IScs605 *orfB*, *cat* and pOX38.

Excision analysis. Genomic DNA of transconjugants was used as template for PCR reactions to look for evidence of excision of IScs605 from pOX38 recombinants. Primers for these experiments (JP17 and JP18) were designed to amplify a 750 nt fragment that would be present in genomic DNA if IScs605 had deleted itself from a pJD1 construct. PCR products that were of the appropriate size were excised from gels, and the nucleotide sequence was determined.

Transposition of IScs605 versus integration of entire plasmids. Transconjugants from both pUC18-based (pJD5) and pBBR1MCS-based (pJD7) constructs were analysed for independent transposition of IScs605 into pOX38. Standard mating assays were conducted, and colonies were selected using Kan and Nal. Transconjugants from each mating assay were then individually transferred to plates containing Kan and Nal, and either Amp (for pJD5) or Chl (for pJD7). The ratio of clones that grew on Kan and Nal only, versus those that grew on plates containing Nal and Amp, or Nal and Chl, was determined for each tested donor plasmid. Southern blotting was also conducted using *PsiI*-digested genomic DNAs from pJD7-based mating assays. DNAs from Chl^R and Chl^S transconjugants were used in these blots, and probed with *aph* to monitor the presence and number of IS elements in the transconjugant.

RESULTS

Mating assays

The IS*cs605* mobile genetic element was analysed for functional activity and insertion specificity in an *E. coli*-based mating assay (Kersulyte *et al.*, 1998, 2000). This assay involves transforming a donor strain with IS*cs605*-containing plasmids (Fig. 2), and selecting for transposition into a conjugative plasmid, pOX38. Several different experiments were conducted using independent clones of donors transformed with different pJD1 plasmids, and these assays resulted in a mean of 3265 transconjugants per 10⁹ recipient bacteria (Fig. 3). Control experiments using pJD2 resulted in a mean of <5 background colonies per 10⁹ recipient bacteria. These data demonstrate that the presence of intact IS*cs605* resulted in an approximately 1000-fold increase in resistance to Kan, relative to a control vector, in this transposition system.

The nature of the pJD1 : pOX38 transpositions

Initial experiments showed that all transconjugants using pUC-based donor plasmids were resistant to both Kan and Amp. These data led to the hypothesis that the IS element integrated into pOX38 within its pUC framework. This possibility was examined using Southern hybridizations of genomic DNA from independent transconjugants. Blots of *Mlu*I- and *Bss*HI-digested genomic DNA from >30

transconjugants were probed with the Kan^R gene (*aph*), the Amp^R gene (*bla*), *orfA* from IS*cs605*, and a fragment from pOX38. All transconjugants were positive for each probe, and each of *aph*, *bla* and IS*cs605* was present on an identical band in each individual transconjugant. These data support the conclusion that the entire pJD1 plasmid was integrated into pOX38 following pJD1-mediated transposition.

ORF knockouts

The two ORFs within IS*cs605* are members of different families of IS elements. To assess the possible role of each ORF in the integration of pJD1 into pOX38, plasmids containing interrupted *orfA* or *orfB* (pJD3 and pJD4 respectively) were constructed and used in mating assays. The results showed that inactivation of *orfA* led to a reduction of transconjugants to control levels, while the inactivation of *orfB* had a minimal effect on the formation of Amp^R Nal^R transconjugants (Fig. 3). Mating experiments using pJD5, in which the *orfB* ORF was replaced by *aph*, produced identical results to pJD4. This construct was also capable of facilitating integration of DNA into pOX38 (Fig. 3). These experiments supported the conclusion that *orfA* was essential for transposition, while inactivation of *orfB* had no detectable effect on transposition frequency.

Insertion-site specificity

Nucleotide sequence analysis was performed to determine the site of insertion of the IS*cs605* plasmids into pOX38. These data demonstrated that integration of pJD1 occurred throughout pOX38, and that insertions occurred in both orientations (Fig. 4). Certain regions appeared to lack recombined plasmid, but it is likely that integrations at these sites (i.e. critical genes for conjugation during the mating assay) may have resulted in a pOX38 : pJD1 plasmid that did not conjugate efficiently into recipient *E. coli* (Frost *et al.*, 1994). These data also demonstrated that 36 out of 38 independent insertion events were adjacent to a 5'-TTCAA sequence present within pOX38 (Fig. 5), and that the two alternate target sequences (5'-GTCAA and 5'-TTTTCAG) were similar to 5'-TTCAA. This insertion-site specificity was also observed in experiments using the *orfB*-deletion plasmid pJD5. There was no evidence of insertion specificity at the *orfB* end of IS*cs605* (Fig. 5). The sequencing also demonstrated that there were no direct or inverted repeats at the sites of plasmid integration. These data were consistent regardless of which particular plasmid containing the IS element was used for analysis, as the plasmids pJD1 (pUC-based, intact *orfA* and *orfB*), pJD5 (pUC-based, *orfB* deletion) and pJD7 (pBBR1MCS-based *orfB* deletion) consistently targeted the 5'-TTCAA site (Fig. 5). Thus, the presence of *orfA* within the IS element is responsible for both the transpositional activity and insertion specificity of IS*cs605*.

Fig. 3. Efficiency of transconjugant formation using different IS*cs605* constructs as donors in the mating assays. The vertical axis represents the number of transconjugants per 10⁹ recipient bacteria resulting from mating assays using the pUC18-based pJD plasmids as a source of different IS element constructs. The error bars show the standard deviation based on at least three replicate experiments for each plasmid.

Fig. 4. Sites of integration of the pJD plasmids within pOX38, based on nucleotide sequence analysis of recovered plasmid DNA. The arrowheads represent all insertions within pOX38, derived from analysis of pUC18-based donor plasmids. Some sites had multiple independent insertion events, represented by open arrowheads. Arrowheads above and below the plasmid map represent insertions oriented to the left and right, respectively. Filled arrowheads represent selected genes or collections of genes within pOX38. Asterisks indicate sites of insertion in mating assays that used pJD7 as a donor. Open circles indicate products in which the donor DNA is not inserted at a 5'-TTCAA.

Fig. 5. Sequence analysis of the pOX38 insertion site, and excision events. (a) Sequences adjacent to the sites of insertion within pOX38 showing association with the pentanucleotide 5'-TTCAA-3' (underlined). Asterisks indicate sites of insertion in mating assays that used pJD7 as a donor plasmid. Open circles indicate products in which the donor DNA is not inserted at a 5'-TTCAA-3'. (b) Sequences at the margins of IScs605 before and after apparent excision in transconjugants. Note the sequence 5'-AATTCAA-3' remaining at the site of excision. This sequence was identical in eight independent transconjugants containing the plasmid pJD5.

Concatamer-based insertion, and secondary excision events, in the mating assay

The nucleotide sequencing was often complicated by the presence of apparent overlapping electropherogram profiles. Several clones that yielded these overlapping sequence reads were further analysed by using Southern blotting and PCR. Southern blots of *Pst*I-digested DNA from

Fig. 6. Southern blots of *Pst*I-digested genomic DNA from transconjugants. Genomic DNA from transconjugants was restriction digested with *Pst*I. Results showed the presence of multiple copies of the *bla*, IScs605 and *aph* elements in the transconjugants. Nucleotide sequencing of genomic DNAs, using a primer within *aph*, resulted in overlapping sequence reads for genomic DNAs shown in lanes 1, 2, 3, 4 and 6. In contrast, there were no overlapping sequence reads in the analysis of genomic DNAs shown in lanes 5 and 7. Southern blots were probed for *bla* (a), *aph* (b) and *orfB* of IScs605 (c). Molecular mass standards are listed to the right of the blots.

transconjugants were probed with *bla*, *aph* and IS*cs605* *orfB*. The data demonstrated that there were multiple copies of each of these genes within most genomic DNAs generated in mating assays using pUC-based donor plasmids (Fig. 6). These experiments suggested several different scenarios: (1) more than one pOX38 recombinant may be present in a single cell, (2) secondary transposition may have occurred inside either the donor or the recipient cell, or (3) concatamers of pJD1 may be inserted into single pOX38 within the donor. As mentioned above, Southern blots were also performed using DNA digested with the enzymes *Mlu*I and *Bss*III, which do not have recognition sites within pJD1. These blots demonstrated that the *bla*-specific probe reacted with single positive restriction fragments in all tested clones (not shown). These data indicate that, in *E. coli*, the IS*cs605* element can drive the integration of entire plasmids and plasmid concatamers into a target DNA.

In some cases, minor bands present within the Southern blots suggested the possibility of secondary transposition following the original integration event (Figs 6a and 7b). This was supported by detailed analysis of several products recovered for nucleotide sequence analysis (not shown). These observations led us to hypothesize that IS*cs605* may have excised itself in subpopulations from many of the primary recombinants. This was tested by PCR-based analysis of selected genomic DNAs. Oligonucleotide primers flanking IS*cs605* in recipient pOX38 were used to successfully amplify sequences consistent with excision events in 40 out of 40 tested transconjugants. Sequence analysis of a subset of these PCR products showed that products lacking the entire IS element were present in the population, with a signature sequence 5'-AATTCAA at the original location of the IS element (Fig. 7b). Evidence of such excision events was found in both pJD1- and pJD5-based transconjugants, suggesting that the excision event was also *orfA* mediated.

Therefore, IS*cs605* can excise from cointegrates produced in this *E. coli* system.

Importance of the pentanucleotide sequence

The sequencing analysis demonstrated that the sequence 5'-TTCAA was commonly a target for integration in this system. This pentanucleotide is present at the *orfA* end of each IS*cs605* found in the Tet^R chlamydiae (Fig. 1). The importance of this sequence in IS*cs605*-mediated transposition was examined by conducting mating assays with a plasmid construct that had the 5'-TTCAA sequence deleted from the IS element (pJD6, Fig. 2). This deleted plasmid was not able to facilitate integration into pOX38 (Fig. 3). Therefore, the 5'-TTCAA sequence at the *orfA* end of IS*cs605* is essential for transposition in *E. coli*.

IS*cs605* transposition activity in pBBR1MCS

To examine the possible role of the vector in defining integration events in the mating experiments, parallel assays were conducted with plasmid pJD7, in which the medium-copy-number plasmid pBBR1MCS was the vector for delivery of *orfA*⁺ *orfB* IS*cs605* in the mating assay. Analysis of the transconjugants using pJD7 as the source of IS*cs605* showed both similarities and differences to experiments with the pUC-based plasmids. First, there were similar rates of transposition observed using either plasmid donor (not shown). Sequencing of the transconjugants showed that pJD7 also inserted adjacent to 5'-TTCAA (Fig. 4) and that there was evidence of concatamers in the inserted products (Fig. 7b, lanes 1–4). However, in contrast to the observations with pUC-based mating assays, transconjugants from the pBBR1MCS matings routinely resulted in only the IS element – and not the vector – being incorporated into pOX38 (Fig. 7a). This was measured using donor plasmids that contained the *aph* marker within

Fig. 7. Analysis of transposition using IS*cs605* donor plasmids based on different plasmid vectors. (a) The percentage of Kan^R transconjugants that also contained the resistance marker found in the vector used in plasmids pJD5 (pUC18) and pJD7 (pBBR1MCS). Colonies growing on Kan were then screened for Amp resistance (pJD5-based assays) and Chl resistance (pJD7-based assays). The data represent the percentage of Kan^R colonies that were also resistant to the second antibiotic, and the error bars represent the standard deviation of three independent assays. One hundred colonies were examined for each replicate. (b) Southern blot for *aph* of *Pst*I-digested genomic DNA from Chl^R and Chl^S colonies resulting from pJD7-based mating assays. Lanes 1, 2, 3 and 4 contain DNA from Chl^R colonies. Lanes 5, 6, 7 and 8 contain DNA from Chl^S colonies.

the IS element (pJD5 and pJD7). Selection could then be measured first with Amp, and then transconjugants examined for a resistance marker in the vector. pUC-based mating assays universally resulted in recombinants containing both markers, while pBBR1MCS-based mating assays resulted in a high frequency of products that contained only the marker in the IS element, and not the marker in the vector. Therefore, in some plasmid backgrounds, IS*Scs605* *orfA* can facilitate transposition of the IS element in the absence of the donor plasmid.

DISCUSSION

The results of the described experiments support the hypothesis that IS*Scs605* is responsible for the integration of the *tet*(C) islands into the *C. suis* genome. The IS element was capable of inserting entire plasmids into the target plasmid pOX38 (Figs 4, 7). Both medium- and high-copy-number plasmids were successfully used in these assays (Fig. 7). Nucleotide sequencing showed that integration of the donor plasmids occurred at many sites within pOX38, but that the sequence 5'-TTCAA was the predominant target. Sequence analysis also demonstrated that there was no sequence duplication or evidence of repeats at the insertion site (Figs 4 and 5). Southern blotting and PCR demonstrated that IS*Scs605* can also excise from target sequences following integration of entire plasmids (Figs 5 and 6), leaving the sequence 5'-AATTCAA at the excision site. Each of these traits is consistent with the observed organization of the *tet*(C) islands in *C. suis*.

IS*Scs605* is structurally related to IS605, IS606, IS607 and related IS elements from *H. pylori* (Kersulyte *et al.*, 1998, 2000, 2002, 2004). These relationships can be seen in a phylogenetic analysis of *orfA* and *orfB*, which have apparent homologues both within the IS605-like IS elements, and within IS elements containing only single candidate transposases (Dugan *et al.*, 2004). The data from the mating assays demonstrate that there are also functional similarities. *H. pylori* IS605 integrates at chromosomal sites specifically with the *orfA* end adjacent to the sequence 5'-TTTAA or 5'-TTTAAC (Kersulyte *et al.*, 1998). *H. pylori* IS607 requires *orfA* for transposition, but not *orfB*, and inserts adjacent to single G nucleotides (Kersulyte *et al.*, 2000). *H. pylori* IS*Hp608* inserts at the tetranucleotide sequence 5'-TTAC, also in an *orfA*-dependent manner (Kersulyte *et al.*, 2002). In no case does insertion or excision of these islands result in deleted or duplicated chromosomal sequence. Each of these traits is parallel to the insertional properties of IS*Scs605* in *E. coli*, and is consistent with the sequences of IS*Scs605* in the resistant *C. suis* strains.

Analysis of secondary transposition events in the mating assay demonstrated that excision of the IS element was common in this system, and these events resulted in the sequence 5'-AATTCAA remaining at the previous location of IS*Scs605* (Fig. 5b). Such secondary events may help to explain differences in sequence within some of the *tet*(C)

islands. In *C. suis* strains 130 and 132, the *tet*(C) island lacks the IS element, and the sequence surrounding a possible excision site is 5'-AATTCAA (Fig. 1, compare strain R19 with strain 130). A related event may also explain the small size of the R27 and H7 islands. In these strains, the genomic island has a large deletion which occurs at a 5'-AATTCAA sequence found within the other *tet*(C) islands. Therefore, it is likely that IS*Scs605*-mediated excision events are also responsible for production of the smaller *tet*(C) islands, such as those seen in *C. suis* strains R27 and 130.

There were, however, some differences between the behaviour of IS*Scs605* in *E. coli* and the predicted events leading to insertion of the *tet*(C) islands into *C. suis*. First, we observed multiple examples of concatamer formation of plasmids associated with integration of pJD plasmids into pOX38. There are no examples of similar concatamers in any *C. suis* strain (Dugan *et al.*, 2004). Additionally, when the medium-copy-number plasmid pBBR1MCS was used as a source of IS*Scs605* (i.e. pJD7), the predominant insertion event involved an actual transposition of the IS element into the pOX38 target. These results provide evidence that the IS element is also capable of true transposition into target DNA. However, this event has not yet been identified in any *C. suis* strain. The low-copy-number plasmid experiments were conducted with a plasmid containing *aph* inserted into the *orfB* coding sequence, and thus the transposition events were selected for using Kan. The intact IS*Scs605* sequence has no marker, and therefore could not be directly selected for, either in our system or within the original *C. suis* isolates found in farmed pigs. We have previously hypothesized that feeding of Tet was responsible for selection of the Tet^R *C. suis* (Dugan *et al.*, 2004), and that in that model, transposition of IS*Scs605* without the accompanying plasmid sequence would not lead to selection of resistant strains.

Deletion analysis was used to test the role of the individual ORFs within IS*Scs605*. The data suggest that *orfA*, but not *orfB*, is essential for both transposition activity and insertion specificity (Fig. 3). These results parallel the tested IS605-like elements in the *Helicobacter* system, in which the homologous *orfA* sequence appears critical for transposition (Kersulyte *et al.*, 1998, 2000, 2002). The function of the highly conserved *orfB* in the IS605 family of IS elements remains unclear. We have previously hypothesized that *orfB* of *H. pylori* IS605 serves to increase bacterial fitness *in vivo*, and this was supported by BLAST analysis, which showed that IS*Scs605* *orfB* is similar to the putative *E. coli* virulence-factor-encoding gene *ydcM* (Dugan *et al.*, 2004). Another possibility is discussed by Kersulyte *et al.* (2002) who proposed that *ydcM* might serve to complement a deletion in *orfB* in the mating assays. The chlamydiae do not have an apparent homologue of *orfB* within their genomes (not shown), and thus *orfB* within the IS element may have been important for the integration of the *tet*(C) islands in *C. suis*.

The described experiments demonstrate that IS*Scs605* can direct the integration of plasmids into a target sequence with

specificity that is parallel to that seen in the chlamydial *tet*(C) islands. It is therefore likely that IS*cs605* activity led to the integration of the *tet*(C) islands into the *C. suis* chromosome. IS*cs605* is the only known example of an IS element within any chlamydial species, and this work describes the first analyses of the activity of a horizontally acquired element within the chlamydiae. The chlamydial insertion element also may be important in practical aspects of the study of chlamydiae. The *tet*(C) islands have several characteristics that may be useful in the genetic transformation of chlamydiae, a technology that is currently unavailable in these organisms. The exploration of this possibility is currently under way in our laboratory.

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