Tet(C) gene transfer between Chlamydia suis strains occurs by homologous recombination after co-infection: Implications for spread of tetracycline-resistance among Chlamydiaceae

Hanna Marti*, Hoyon Kim*, Sandeep J. Josephb,c, Stacey Dojiria, Timothy D. Readb,c, Deborah Dean*,#

Center for Immunobiology and Vaccine Development, UCSF Benioff Children's Hospital Oakland Research Institute, Oakland, California, USAa; Department of Medicine, Division of Infectious Diseasesb and Department of Human Genetics, Emory University School of Medicine, Atlanta, Georgia, USAc; Joint Graduate Program in Bioengineering, University of California, San Francisco, California, USA, and University of California, Berkeley, Berkeley, California, USA#.

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*Present address: Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Switzerland

TDR and DD contributed equally to this work

#Address corresponding to Deborah Dean, ddean@chori.org
Abstract

*Chlamydia suis* is a swine pathogen that also causes zoonotic infections. Many strains contain a *tet*(C) cassette conferring tetracycline resistance originally acquired by horizontal gene transfer from a Gram-negative donor. As we and others have described the capacity for *Chlamydiaceae* to exchange DNA by homologous recombination, *tet*(C)-containing *C. suis* strains represent a potential source for antibiotic-resistance spread within and between *Chlamydia* species. Here, we examine the genetics of *tet*(C)-transfer among *C. suis* strains. Tetracycline-sensitive *C. suis* strain S45 was simultaneously or sequentially co-infected with tetracycline-resistant *C. suis* strains in McCoy cells. Potential recombinants were clonally purified by plaque assay. *C. suis* strain Rogers132, which lacks IS200/IS605 transposases, was the most efficient donor, producing three confirmed recombinants of 56 (5.4%) clones with a minimal inhibitory concentration (MIC) of ~8μg/mL. Resistance was stable when recombinants were grown initially in tetracycline at twice the MIC of S45 (0.032 μg/mL). Genomic analysis revealed that *tet*(C) had integrated into the S45 genome by homologous recombination at two sites in different recombinants: a 55kb exchange between *nrqF* and *pckG*, and a 175kb exchange between *kdsA* and *cysQ*. Neither site was associated with repeats or motifs associated with recombination hotspots. Our findings show that cassette transfer into S45 has low frequency, does not require IS200/IS605 transposases, is stable if initially grown in tetracycline, and results in multiple genomic configurations. We provide a model for stable cassette transfer to better understand the capability for cassette acquisition by *Chlamydia* species that infect humans, a matter of public health importance.
INTRODUCTION

Bacteria develop resistance to antibiotics either as a result of mutation in their chromosomal genes or from acquisition of antibiotic resistance transposons by horizontal gene transfer (HGT). Reports of bacterial resistance to antimicrobial agents have occurred almost simultaneously with their first introduction in the late 1930s (1). Resistance through mutation or HGT is promoted by high doses of antibiotics; the use of sub-inhibitory concentrations and broad-spectrum antibiotics; patient non-compliance with the treatment regimens; and the use of antibiotics in lower mammalian and avian species that are bred for human consumption (2). These latter practices have led to an alarming increase in microbial pathogen resistance such as colistin-resistant *Escherichia coli* and multidrug-resistant *Staphylococcus aureus*. Both have been isolated from pigs and zoonotically transmitted to human hosts (3, 4), adding to the current crisis in public health infectious disease control (5, 6).

While there are five families of obligate intracellular bacteria that infect mammals, including *Ehrlichiaeae, Anaplasmataceae, Rickettsiaceae, Coxiellaceae* and *Chlamydia*, *Chlamydia suis* is the only reported obligate intracellular bacterium to have naturally acquired the tetracycline resistance gene *tet(C)*, which occurred by HGT (7, 8). This organism is a pig pathogen associated with conjunctivitis, pneumonia, diarrhea/enteritis and reproductive disorders in addition to unapparent infection (9, 10). *C. suis* has also recently been associated with zoonoses including trachoma (a chronic ocular disease) (11), ocular infection in abattoir workers (12) and asymptomatic nasal, pharyngeal and intestinal infections in farmers (13).

Tetracyclines, including doxycycline, are used to treat a wide variety of bacteria including all species of *Chlamydia*, in particular, complicated infections caused by the human pathogen *Chlamydia trachomatis* (14). Since both *C. suis* and *C. trachomatis* infect the human
conjunctiva and rectum, the *in vivo* opportunity for HGT of the *tet*(C) cassette to *C. trachomatis* is a real concern. Indeed, *C. suis* and *C. trachomatis* co-infections have been reported among trachoma patients (11).

Tetracycline resistance in *C. suis* is conferred by a variable cassette containing the tetracycline class C gene [*tet*(C)], which encodes an efflux pump to export tetracycline from infected cells. Three *tet*(C) cassettes have been identified and described among *C. suis* strains (7, 15). One is comprised of *tet*(C), the tetracycline repressor gene *tet*R(C), replication genes repAC as well as mobilization genes *mob*ABCDE and two insertion sequences, IS605 and IS200 that contain transposases. Another cassette possesses *tet*(C) and *tet*R(C) as well as the two transposases but lacks the replication and mobilization genes. A third cassette is missing only the IS200/IS605 transposases. Dugan *et al.* (16) used an *Escherichia coli*-based mating assay to show that the IS605 transposases are most likely responsible for the integration of the cassette into the *C. suis* genome. In further analyses, Suchland *et al.* (17) was able to develop a co-infection model and successfully generate tetracycline resistant (*tet*<sup>R</sup>) *C. trachomatis* L<sub>2</sub> strains from co-infection with *C. suis* R19, which contains both transposases, and a tetracycline sensitive (*tet*<sup>S</sup>) L<sub>2</sub> strain. However, cassettes lacking the transposases have not been studied for their recombinogenic potential. Here, we tested the requirement of IS200/IS605 transposases for *tet*(C)-containing cassette transfer and developed a model system to study cassette transfer among chlamydiae in the presence and absence of tetracycline.
MATERIALS AND METHODS

Chlamydia strains, tissue culture and tetracycline susceptibility. Table 1 describes the strains used in this study. All strains were individually propagated in McCoy cells prior to density gradient purification as previously described (15, 18). McCoy cells were screened for Mycoplasma contamination before use (Universal Mycoplasma Detection Kit, ATCC® 30-1012K™ Manassas, Virginia). Briefly, McCoy cells were grown in medium consisting of 450 mL Minimal Essential Medium alpha (MEMα, Life technologies, Carlsbad, CA), 10% Fetal Bovine Serum (FBS, JR Scientific, Woodland, CA), 15 mL sodium bicarbonate (2.8%) (Thermo Fisher Scientific, Waltham, MA), 10 mL glucose (45%) (Fisher Scientific), 10 mM HEPES (Life technologies), 100 µg/mL Vancomycin (Fisher Scientific) and 40µg/mL Gentamycin (Fisher Scientific). All infections were grown in propagation medium consisting of growth medium supplemented with 1.4 µg/mL Cycloheximide (Sigma Aldrich, St. Louis, MO) as well as NGV-Amp B solution (29 U/mL Nystatin, 11.85 µg/mL Gentamycin, 29.62 µg/mL Vancomycin and 2.22 µg/mL Amphotericin B (Sigma Aldrich)) at 37°C in 5% CO₂.

The in vitro tetracycline susceptibility was determined as the minimal inhibitory concentrations (MIC) according to Suchland et al. (19) with minor changes. Briefly, each chlamydial strain was inoculated onto 20 wells of a 48-well plate (E & K Scientific, Santa Clara, CA) seeded with McCoy cells at a multiplicity of infection (MOI) of 0.5 or 1 depending on the infectivity of the strain. Two wells served as negative controls and were inoculated with 100 µL propagation medium. After inoculation of cells, the plate was centrifuged at 1500 RPM for 1 h at 37°C. A tetracycline (Sigma-Aldrich) stock solution was prepared at a concentration of 10 mg/mL in ddH₂O, and used for a twofold dilution in propagation medium without antibiotics with final concentrations ranging of 0.002 to 256 µg/mL (18 concentrations). After
centrifugation, the chlamydial inocula were aspirated and replaced with the serial tetracycline
dilutions. Two infected and two uninfected wells received propagation medium without any
tetracycline and served as positive and negative controls, respectively. After 24 to 36 hours,
depending on the developmental cycle of the strain, medium was removed and cells were washed
three times with PBS (Gibco, Fisher Scientific) before they were fixed with methanol (-20°C) for
10 min. Chlamydial inclusions were detected by direct immunofluorescence using Chlamydia
Confirmation Pathfinder (Bio-Rad, Hercules, CA) or FITC conjugated anti-chlamydial LPS
monoclonal antibody (ProSci, Poway, CA) at a 1:15 dilution. The MICs of the strains were
evaluated by analyzing size and morphology as well as the number of inclusions at 200 X using a
Nikon Eclipse Microscope and SPOT imaging software (Diagnostic Instruments, Inc. Sterling
Heights, MI). As defined by Suchland et al. (17), we determined the MIC transition point
(MIC_{TP}) to be the tetracycline concentrations where 90% or more of the inclusions displayed
alterations in size and morphology. Furthermore, twofold higher concentrations (two times the
MIC_{TP}) were considered to be the actual MIC.

**Generation and clonal isolation of recombinants.** Two different recombination protocols were
applied in regards to co-infection. For Protocol 1 (recipient-first co-infection), tet^S S45 at a
multiplicity of infection (MOI) of 4 was used to infect 80-100% confluent McCoy cell
monolayers in shell vials (E & K) (without centrifugation) and incubation at 37 °C for 24 hrs
before the addition of tet^R Rogers132, tet^R R19, or tet^R R27 (MOI 0.5). Tetracycline challenge
was at 2 µg/mL (1/2 MIC_{TP} Rogers132) as described below. For protocol 2 (simultaneous co-
infection), tet^S S45 was first grown in McCoy cell monolayers in shell vials to reach 100%
infection; 25-50µL of the infected culture were transferred to a new shell vial with a 80-100%
confluent McCoy cell monolayer and simultaneously or consecutively co-infected with tet$^R$ R19 or tet$^R$ Rogers132 8 to 24 hours post infection (pi) with tet$^S$ S45. Centrifugation was then performed for 1h at 1500 RPM. Tetracycline challenge was with 0.25 µg/mL (8x MIC S45) as described below (Fig. S1).

For each co-infection, three conditions and two controls (single infection with each parental strain) were used. All co-infection experiments were performed in glass shell vials with one shell vial per condition. Condition A did not contain any tetracycline (no tet); Condition B contained sub-inhibitory concentrations of tetracycline (1/2 MIC$_{TP}$ for S45; 0.004 µg/mL); and Condition C contained two times the MIC of S45 (0.032 µg/mL). Co-infected cultures were propagated for 72 hours, sonicated once (20% amplitude, Sonic Dismembrator Ultrasonic Processor, Fisher Scientific), and new shell vials were infected to produce 100% infection. Each condition was either directly challenged with tetracycline (2 or 0.25 µg/mL depending on protocol one or two described above) or passaged once in propagation medium without antibiotics prior to the tetracycline challenge. Following the challenge for 36 to 72 hours depending on the developmental cycle, infected cultures were sonicated, and the inoculum was used to perform a plaque assay, PCR and sequencing of PCR targets to identify putative recombinants (see below, Fig. S1).

The plaque assay was used to isolate clonally pure putative recombinants. The protocol was modified according to Somboonna et al. (20). Briefly, the first well of a 6-well plate with 60% confluent McCoy cell monolayers was infected with 100 to 500 µL of the co-infected shell vial from condition A, B or C, depending on the infection rate; seven serial 10-fold dilutions were used where the 2$^{nd}$ and 7$^{th}$ dilutions were applied to wells 1-6. After 24 h, 2 mL of agarose gel consisting of 0.5% agarose (Lonza, Rockland, ME) in phenol-red free MEM (Gibco), 10%
FBS and 1µg/ml Cycloheximide was added and topped with propagation medium with Gentamycin/Vancomycin or NGV-Amp B.

Following incubation for 4 to 16 hrs at 37 °C, 5% CO₂, the well with detectable but low-level infection was chosen to mark individual inclusions (no neighboring inclusions) at 200 x magnification. Individual inclusions were selected and picked using a sterile transfer pipet (Fisher Scientific) by punching a hole of 1-2 mm in diameter; the plug was transferred to a 1.5 mL Eppendorf vial (Hauppauge, NY) containing 500 µL propagation medium. The vial contents were sonicated once as described above and used to inoculate one shell vial per picked inclusion containing 500 µL propagation medium. Shell vials were centrifuged for 1 h as above and incubated for three days. Putative clones were passaged once into another similarly prepared shell vial. The infected material was collected for PCR and sequencing (see below), either by infection of a T25 flask (Greiner, North Carolina) and subsequent collection as described (21) or direct collection by sonication of the shell vials, centrifugation at 15,000 RPM for 5 min and aspiration of the supernatant. 100-500 µL of sonicated chlamydial suspension (depending on the infection rate) was used for a second passage into a shell vial as above. Once the infection of McCoy cells reached 100%, cultures were sonicated and frozen at -80°C.

**Identification of putative recombinants for genome sequencing.** DNA of the collected clones was extracted using the Roche High Pure PCR Product Purification Kit (Roche, Pleasanton, CA), and PCR was performed as previously described (20). All primers are listed in Table S1. Clones were considered putative recombinants if they had the following characteristics by PCR: Positive for the correct size band for the tet(C) gene; positive for the intergenic region (IGR) between the polymorphic membrane protein gene (pmp)B and pmpC using primers specific for
S45; negative for the *pmpC* region using primers specific for Rogers132; and positive for the major outer membrane protein A gene (*ompA*) with confirmation of the S45 *ompA* genotype by Sanger sequencing.

Putative clonal recombinants were then propagated in 0.063 µg/mL tetracycline (4x MIC of S45) to grow stocks for whole-genome sequencing, MIC determination, and the tet(C) stability assay (see below). After the first passage, a second plaque assay was performed as above to ensure clonal purity. Picked clones were either grown as described above or directly picked and inoculated into 100 µL HBSS (Gibco) prior to DNA extraction, PCR and *ompA* sequencing.

**Genome sequencing.** Stocks of clonally purified putative recombinants were treated with DNase prior to gDNA purification as described previously (21). Libraries for sequencing were prepared from 0.5 to 1 µg of genomic DNA. Illumina MiSeq libraries were constructed using the Nextera kit and sequenced using the 250 bp paired end protocol on a MiSeq instrument. The resulting sequence data was assembled de novo using the SPAdes (22) software. The CONTIGuator web service (23) was used to map assembled contigs against the Rogers132 strain. We visualized the structure of the assemblies aligned against individual regions of the chromosome using the Bandage graph visualization (24) software and its integrated BLAST tool. Querying of short sequences against the raw sequence reads in FASTQ format was performed using the R ShortRead (25) package.

The raw genome data generated for this study are deposited in the SRA database (the accession no. is pending).
Analysis of homologous recombination and tet(C)-containing cassette insertion into C. suis strain S45. We used the parsnp rapid genome alignment tool, which is part of the Harvest suite (26) to identify regions of genome exchange in the recombinants. De novo assembled contigs of the recombinants were mapped against the S45 recipient genome reference and compared to the pattern of SNPs obtained when the Rogers132 donor was mapped against S45. Mosaic regions introduced by homologous recombination events appeared as clusters of ‘Rogers132-like’ SNPs in the background of the S45 genome. The approximate boundaries of recombination events were mapped as the edges of continuous runs of inserted SNPs.

‘SNP painting’ to find regions of homologous recombination in mixed cultures. We noted from preliminary analysis that recombinant DNA preparations in some cases also contained residual DNA from donor and/or recipient strains. To visualize the proportion of reads containing the donor and recipient backbone, we used a technique we called ‘SNP painting’. We extracted two 25mer DNA sequences centered on each SNP identified between the donor and recipient reference sequences that had in their central position either the donor or recipient base. We challenged this reference SNP library against a 25mer database created from the FASTQ of the post-mating mixture using Jellyfish software (27). From the counts of the donor and recipient 25mers at each base, we were able to 1) map recombinant boundaries and 2) ascertain the extent of mixed populations using R software (28).

Phylogenetic analysis. Genes were aligned using PRANK (29). The R package Phangorn (30) was used to calculate maximum likelihood phylogeny using the Symmetric+GI model (chosen based on best fit). Trees were bootstrapped 100 times.
MIC determination (tetracycline susceptibility *In vitro*) and *tet*(C) stability assay. The MIC of all confirmed recombinants (by PCR and *ompA* genotyping) was tested directly from purified clones as described above for the parental strains. To test the stability of the recombinants regarding the presence of *tet*(C), recombinants were used to infect McCoy cell monolayers in shell vials at an MOI of 5. Cultures were passaged five times before they were challenged or not with tetracycline using three different conditions: A) no tetracycline; B) ½ MIC<sub>TP</sub> of the recombinant; and C) 4x MIC of the recombinant. Following challenge, we propagated each recombinant once under each condition and determined the MIC as described above. The recombinant without tetracycline was grown for another five passages (10 total) and was treated similarly as for passage five. The MIC was determined again after one additional passage. Additionally, DNA was extracted from samples of the recombinants obtained after every passage to confirm that the *ompA* genotype was identical to the recombinant prior to the start of the assay and to test for the presence of *tet*(C) using the PCR primers (Table 2).
RESULTS

Co-infection of S45 with the tetracycline resistant strains R19 and R27 does not yield recombinants. Previous studies have shown that co-infection with R19 results in \text{tet}^R C. \text{trachomatis} strains (17, 31). We aimed to obtain \text{tet}(C)-positive \text{C. suis} S45 recombinants by co-infecting S45 with three \text{tet}^R \text{C. suis} strains representing the three \text{tet}(C)-containing cassette types: Cassette I (strain R19, complete cassette with all three segments); Cassette II (strain 27, without Segment 1: replication/mobilization protein genes); and Cassette III (strain Rogers132; without Segment 3: transposases) (Fig. 1). Eight co-infections with R19 were performed using various protocols, including the addition of R19 at 8 h or 24 h pi with S45, and pre-treatment of R19 with high doses of tetracycline prior to co-infection. However, the entire R19/S45 co-infection material tested negative for S45 by PCR using primers specific for the IGR of \text{pmpB/C} and by \text{ompA} genotyping (Table S1) immediately after tetracycline challenge (see Material and Methods). For one protocol, S45 was grown in shell vials prior to co-infection with R19 (MOI 0.5) at 9 h pi with S45 and challenged with only 0.25 µg/mL tetracycline. The initial infections were mixed and contained S45, but picked clones were either initially only positive for R19 by PCR using R19-specific primers for \text{pmpC} and by \text{ompA} genotyping or positive for R19 after two more passages (data not shown). R27 co-infection with S45 was attempted but no recombinants were identified.

\text{tet}(C)-containing cassette transfer to recipient strain under inhibitory and sub-inhibitory tetracycline concentrations.} Recombinants between Rogers132 and S45 were successfully generated by simultaneous co-infection or by infection with Rogers132 after S45 had been grown for 24 h (see Material and Methods). The success rate of acquiring recombinants was
equally low. We obtained seven putative recombinants (Rec2 to Rec8), which were propagated for whole-genome sequencing following a second plaque purification (Table 2). In subsequent genome analysis described below, several of the ‘recombinants’ turned out to be mixtures of strains or only recipients, but we use the original term to describe Rec2 to Rec8 in this manuscript. In detail, for Co-infection 1, 21 clones were Rogers132 (donor strain); 3 were S45 survivors (recipient strain) or mixed infections (including putative recombinant Rec 2); and 2 were true recombinants (Rec 3 and 4). For Co-infection 2, 11 were Rogers132; 18 were S45 or mixed infection (including putative recombinant Rec 6-8); and one was a true recombinant (Rec5). Rec2 and Rec8 were tetracycline sensitive after propagation while Rec6 was positive for both S45 and Rogers132 by ompA genotyping and for Rogers132 by pmpC PCR. Fig. 2 shows the PCR results for tet(C), pmpC and pmpB/C for each of the seven recombinants. Interestingly, we only obtained recombinants from co-infections performed in media containing sub-inhibitory (1/2 MIC<sub>TP</sub>) or inhibitory (4 x MIC) concentrations of tetracycline for S45; no recombinants were obtained without tetracycline. Rec3, Rec4 and Rec5 were confirmed recombinants by genomic analyses (see below).

We evaluated whether the three confirmed recombinants of Rec3, Rec4 and Rec5 remained stable over the course of ten passages in the absence selective pressure, specifically without sub-inhibitory concentrations of tetracycline. After five passages, the recombinants were challenged or not (A) with low (B) and high (C) doses of tetracycline with subsequent analysis of their antibiotic susceptibility (see Materials and Methods). For each recombinant, every passage was collected and tested for the presence of tet(C). At the tenth passage, ompA genotyping was also performed to exclude the possibility of a small population of Rogers132 survivors. As expected, the confirmed Rec3, Rec4 and Rec5 recombinants remained PCR positive for tet(C).
throughout the course of the stability assay as did the \textit{ompA} genotype (data not shown). In addition, all confirmed recombinants continued to be tetracycline resistant after Passages 5 and 10 with relatively high minimal inhibitory concentrations of 8-16 µg/mL (Table 3).

In contrast, tetracycline resistant putative recombinants \textit{Rec7} and \textit{Rec6} did not have any evidence of recombination. The former was \textit{tet(C)} positive by PCR in the first five passages but negative in Passages 6 to 10 with an \textit{ompA} sequence aligning to S45, indicating dominance of the sensitive parental strain, while the latter was \textit{tet(C)} positive throughout the course of the assay and tetracycline resistant both at Passage 5 and 10 but aligned to Rogers132 (Table 3).

All recombinants (putative and confirmed) required growth in 0.064 µg/mL tetracycline (4x MIC S45) to produce stocks for genome sequencing. Attempts to propagate the clones in the absence of tetracycline often led to the loss of the \textit{tet(C)} cassette (\textit{tet(C)} PCR-negative). However, once stocks were produced, the confirmed recombinants remained stable throughout the ten passages performed in the absence of tetracycline for the stability assay.

\textbf{Frequency of recombination.} The frequency of potential recombinants, without selecting for mixed infection or Rogers132 survivors, was low. One co-infection experiment was performed for each of the two protocols under three different tetracycline conditions described in Material and Methods (see also Table 2). For protocol 1 (recipient-first co-infection), a total of 26 clones were picked with six clones per each tetracycline Condition: A (no tetracycline); B (1/2 MIC$_{TP}$ S45); and C (2x MIC S45). The co-infections for the three conditions were used to infect one 6-well plate per condition. Six to 11 clones were picked (26 total) per plate, resulting in two confirmed recombinants, \textit{Rec3} and \textit{Rec4}, from Condition B. For protocol 2 (simultaneous co-infection), 30 clones were picked with ten clones each per Conditions A, B and C, resulting in...
one confirmed recombinant, Rec5, originating from Condition B. Taken together, of the 56 clones, only seven (12.5%) were identified as putative recombinants, and three were confirmed (5.4%). For Condition B, the success rate was 15.0% (3/20).

**Heterogeneity of tet(C) cassette insertion into C. suis strain S45.** The seven putative recombinants described above were genome sequenced. We made a database of 4,864 SNPs between the Rogers donor and S45 recipient using parsnp (26) (Table S2) and used them as markers to distinguish potential recombination junctions in raw sequence data (Fig. 3). Aside from Rec6, which was a mixture of donor and recipient, the recombinants were predominantly comprised of the genetic backbone of S45, evincing our success in screening for the recipient following co-infection. Recombinant regions were determined at the approximate junctions between regions of ‘S45-like’ and ‘Rogers-like’ SNPs (Fig. 3a). While Rec 2 was PCR-positive for tet(C), there was undetectable recombination based on genome sequence analysis. Similarly, Rec7 had no evidence for recombinants. Rec3 and Rec4 were confirmed recombinants with identical tet(C) cassette insertion sites at Rogers132 coordinates of approximately 705,300 to 760,600 (the upstream and downstream cross-over regions varied in length because of sparse SNPs, affecting the ability to precisely determine coordinates – see below) in the S45 backbone. This region constitutes ~55,300 nucleotides (5.3% of the genome) including the rrn operons, consisting of the 16S, 23S and 5S rRNAs. The insertion spans from the Na(+)‐translocating NADH-quinone reductase subunit F gene (nqrF) to the phosphoenolpyruvate carboxykinase [GTP] gene (pckG) in S45.

Rec5 was also a recombinant but with a much larger insertion containing the tet(C) cassette at Rogers132 coordinates of approximately 638,000 to 813,000 (Fig. 3a and 3b, right
plot), spanning the genomic region of Rogers132 2-dehydro-3-deoxyphosphooctonate aldolase gene (*kdsA*) to the 3'(2'), 5'-bisphosphate nucleotidase gene (*cysQ*) in the S45 backbone. This region constitutes 175,000 nucleotides (16.6% of the genome). There were also second and third insertions within Rogers132 coordinates 529,000 to 538,000 in the S45 backbone (Fig. 3b, left plot), including the Rogers 132 putative general secretion pathway protein D gene (*gspD*) to the secretion system effector C gene (*sseC*) family.

Although Rec6 was a mixture of donor and recipients, we identified a small recombinant region of ~40,000 nucleotides between the Rogers132 coordinates of ~856,780 to 884,340. These coordinates are in the *pmpD* and phenylalanine t-RNA ligase (*pheS*) genes (Fig. 3c). Rec8 had no evidence of any recombinants, consistent with the PCR results (Fig. 2), and was considered an S45 survivor.

Figure 4 shows the recombinant locations of the cassette and other insertion sites within the context of the entire circular genome for Rec3 and 4, and for Rec5. While the cassette was confirmed to reside within the invasion gene and within the two *rrn* operons for all 11 previously genome sequenced *C. suis* strains (15), the insertion in Rec3 and Rec4 included the *nqrF* gene extending to the *pckG* gene beyond the downstream *rrn* operon. The upstream crossover region is located within approximately 2.2kb, either in the gene *pckG* or its neighboring gene downstream encoding a hypothetical protein and in proximity to ribosomal bindings sites (RBS), whereas the downstream crossover region is found within *nqrF* (170 bp; Fig. 5A).

For Rec5, the cassette insertion encompassed the span from gene *cysQ* to *kdsA*, which included the *rrn* operons and the *ompA* gene of Rogers132. The upstream crossover region is located within approximately 750 bp between the downstream end of the *kdsA* gene or its neighboring genes tRNA-Arg (*tct*) and a hypothetical protein-encoding gene, while the
downstream crossover region is found in the cysQ gene, spanning 230 bp (Fig. 4, Fig. 5B). Both crossovers are in proximity to RBSs, and palindromes are evident. In addition, we identified two shorter recombinant regions located adjacent to each other between kdsA and pmpBC (Fig. 4). The larger of the two crossover sequences was found upstream within gpsD (~380 bp) and downstream, either in mutL or ipgC, spanning approximately 3kb (Fig. 6A), and in proximity to RBSs with evidence for palindromes where one is at the site of the SNP at nucleotide position 2230 (Fig. 6, A.1.). The complete crossover region located within the sseC-like gene family was found within the span of 1500 bp (Fig. 6B). Figure 6C shows a schematic of the two Rogers132 insertions.

In examining the plasmids for each recombinant, all except Rec7 had the S45 plasmid. Rec6 was a mixture of both S45 and Rogers132 plasmids. None of the plasmids exhibited any genetic exchange.

**Heterogeneous structure of the tet(C)-containing cassette and phylogeny of tet(C) / tetR(C).**

As indicated previously (7, 15), the tet(C)-containing cassette can be divided into three segments that are nearly identical for each strain though the order of the segments varies, resulting in three distinct cassettes (Fig. 1). Segment 2 is the only segment present in all strains and contains tet(C) as well as the tetracycline repressor gene [tetR(C)]. To better understand the phylogenetic placement of these two genes, a BLASTN search was performed to identify related sequences in other bacterial genera with identity coverage between 98% and 100%. Fig. 7 shows the Maximum Likelihood (ML) phylogeny for tet(C). While there is strong support for two distinct C. suis clades, there is insufficient resolution to determine the order in which the clades branched off and not enough evidence to support multiple tet(C) acquisitions implied by the ML tree. In
contrast, tetR(C) is highly conserved across genera (Fig. S2). The tet(C) and tetR(C) genes are preceded by a gene encoding hypothetical proteins both in *C. suis* and *A. salmonicida* but which was annotated as part of the phage integrase family (*orf*E) on the plasmid pMC2 of an uncultured bacterium isolated from swine manure with 98% identity cover [JN704639.1]. Additionally, that plasmid further identified a mobile element IS101 upstream of *orf*E, which is also present on Segment 2.
DISCUSSION

Mutation, HGT, and genome rearrangement are the forces that shape the evolution of bacterial genomes (32). Most Chlamydiaceae genomes show evidence of intra-species recombination events (15, 18, 33–37) but little to no evidence for the presence of classic HGT acquired pathogenicity islands (38) or other foreign genes except for bacteriophage inserts in Chlamydia pneumoniae (39) and genes possibly acquired in the plasticity zone or invasin locus (40, 41). The most notable exception to this rule is C. suis, which is not only known for the possession of the tet(C)-containing cassette (7), but has also recently been reported to be highly recombinogenic compared to other Chlamydia species (15).

All three classes of tet(C)-containing cassettes described in the 11 tetR C. suis strain genomes sequenced to date (15, 42) were inserted at an identical site within the invasin gene (also referred to as the ilp or inv-like gene), which is flanked upstream by an rrn operon and downstream by nqrF (7, 15). It is likely that they all originated from one ancestral event, although other options are formally possible (15). The tetS strain S45 possesses an intact invasin gene as does C. caviae strain GPIC [AE015925.1], sharing 73% identity and 91% query cover by BLASTN search (40). No other Chlamydia species contain this gene. Similar to the tet(C) and tetR(C), we found that ilp is conserved within the C. suis species with the former two genes displaying high homology to European C. suis strains (Fig. S3) (43). These data suggest that the invasion gene is required for cassette insertion. However, Suchland et al. (17) demonstrated that C. muridarum strain MoPn and C. trachomatis strain L2, both of which lack ilp, are able to acquire the C. suis strain R19 tet(C)-containing cassette in vitro by co-infection, while C. caviae was not receptive.
In a follow-up study, Jeffrey et al. (31) co-infected the tet(C)-positive and -negative L₂ recombinants from the former study with non-LGV C. trachomatis strains such as F and J to produce new tetracycline resistant recombinants. Neither study found specific nucleotide sequences that suggested a mechanism for in vitro recombination, although in the Suchland et al. study (17) the region including the cassette between the rrn operons of R19 was inserted into the downstream rrn operon of L₂ creating a recombinant. In addition to a similar localization of the cassette in the rrn operon, Jeffreys et al. (31) identified what appeared to be non-specific recombination (190 events in 12 recombinant strains) that occurred throughout the genome, unlike the R19/L₂ recombinants, suggesting that the progeny may have contained mixed infections despite selection of clones by limiting dilution.

In contrast to the successfully produced tet<sup>R</sup> C. trachomatis L₂ and C. muridarum recombinants with R19 (17), we were unable to produce any tet(C)-positive C. suis S45 recombinants after co-infection with R19 despite multiple attempts. Similarly, co-infections with R27 were unsuccessful because tet<sup>R</sup> strains outcompeted S45 within two to three passages. This observation was surprising, especially if we consider that resistance-determining accessory resistance genes are thought to generally impair rather than promote biological fitness of bacteria in the absence of antibiotics (44). One possible explanation for this is that S45 was isolated in the 1960s and adapted to cell culture in the laboratory, whereas the tetracycline resistant strain donors have only been cultured since the 1990s. To test this hypothesis, R19 or another strain with a similar cassette would have to be co-infected with a more recently isolated tet<sup>S</sup> C. suis strain in the absence of tetracycline.

Another noteworthy contrast to the previous co-infection studies is that we implemented three different tetracycline conditions. Suchland et al. (17) performed co-infections without any
antibiotics prior to challenge with a high dose of tetracycline. In addition to this, we included sub-inhibitory (0.004 µg/mL) and inhibitory concentrations (0.032 µg/mL) of tetracycline for S45. These conditions were based on the hypothesis that low concentrations of antibiotics promote the selection towards resistant bacteria (45). Indeed, all of the three confirmed recombinants (Rec3, Rec4, and Rec5) were originally grown in sub-inhibitory antibiotic concentrations, suggesting that mating is optimized by a multiplicity of donors. Only one putative recombinant was isolated from cultures that were not initially grown in tetracycline, which was later confirmed as an S45 survivor (Rec2). In order to confirm that sub-inhibitory concentrations of tetracycline promote the transfer of the tet(C) cassette, quantitative analysis is necessary. For example, three independent co-infection experiments with identical protocols could be performed with twenty to thirty clones picked per tetracycline condition instead of six to 10 as in our study. The number of recombinants could then compared among tetracycline conditions.

Dugan et al. (16) previously proposed that one or both transposases within IS605 (termed IScs605 by Dugan et al.) were responsible for the integration of the genomic island into the C. suis chromosome. While the initial HGT event that brought the ancestral tet(C) cassette into C. suis probably involved transposition, we were able to demonstrate that transfer of the cassette between C. suis was through double crossover homologous recombination. Therefore, it is possible that homologous recombination between C. suis strains has been a significant factor in the recent spread of tetracycline resistance.

The recombination boundaries were not near the duplicated rrn operons as observed in the tetR C. trachomatis recombinants described by Suchland et al (17) and one of 12 sequenced recombinants in the study by Jeffrey et al (2013) but rather in conserved, syntenic genome
regions (Fig. 5; Fig. 6). In our previous study, comparative genomics was used to infer that putative ancestral recombination had occurred at high frequency across the *C. suis* genome (15). The recombination boundaries in Rec3 and Rec4 were identical, suggesting they arose from sibling plaques rather than independent events. The Rec3 and Rec4 boundaries overlapped genes that recognized recombinant regions, (CS00632 and CS00678) (15), but the large Rec5 recombination region did not (genes CS00570-572, CS00715). Furthermore, the small insertions of Rec5 were incorporated within one non-recombinant (CS00485) and two recombinant regions (CS00482, CS00488-489). It is unclear, with a limited number of *C. suis* genomes to compare to date, whether the observed patterns reflect selection, recombination hotspots or are purely stochastic. In inspecting the regions, inverted repeats, chi sites or direct target repeats that are typical permissive sites for recombination were not detected. However, the upstream crossover in Rec5 contained a tRNA (Fig. 5B). tRNAs are known to be acquired and involved in recombination for a diversity of bacteria (46, 47). Conserved regions such as RBSs, which were present within or near each cross-over region (Fig. 5 and 6), may also facilitate homologous recombination because gene function would not be altered, allowing new recombinants to be successful (48) as in the present study. It is notable that a similar lack of patterned recombination was noted in a recent study regarding beta-lactam and vancomycin resistance in *Enterococcus faecium*, in which the authors hypothesized that long sequences of highly homologous DNA were targets for recombination (49). Since the majority of *C. suis* strains isolated from farm animals are tet$^R$ (up to 89%) (50, 51) and *C. suis* shares 79.8% average nucleotide identity with *C. trachomatis*, the potential for homologous recombination and cassette transfer are high.

In summary, we present a co-infection model that produces recombinants, demonstrating for the first time that the tet(C)-containing cassette is transferred between *C. suis* strains by
homologous recombination without the need for Segment 3 transposases. We also discovered that, while the frequency of recombination is low, sub-inhibitory concentrations of tetracycline may promote transfer and that, rather than targeting highly polymorphic regions, recombination occurs in long homologous sequences and genomic regions with tRNAs. Our model will serve as a template for determining the mechanisms and frequency of cassette transfer among \textit{Chlamydia} species such as \textit{C. trachomatis} that may co-infect humans with \textit{tet}^R \textit{C. suis} zoonotic strains. Cassette transfer would have major implications for public health approaches to treatment for humans and domesticated animals alike.
ACKNOWLEDGMENTS

We are indebted to Dr. Art Andersen for providing his collection of Chlamydiaceae species and strains for this research. This collection is now housed, maintained and curated in Dr. Dean’s laboratory. We also thank Tyler Morgan for excellent technical assistance.

FUNDING INFORMATION

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FIG. 1. The structure of the tet(C)-containing cassette is comprised of three unique segments. Shown is the tet(C)-containing cassette and its neighboring genes, depending on the C. suis strain. The structure of the cassette comprises three diversely arranged segments (purple, red and orange) consisting of different genes. Four cassette types, termed Class I to IV, have been identified in ten C. suis strains: (I) contains all three segments in the listed segment order (R19 and R24); (II) the cassette of Rogers132 is lacking segment 3, which contains transposases IS200 and IS605; (III) the cassette of H5 is similar to that of cassette I but occurs in reverse segment order; (IV) is missing segment 1 (replication/mobilization protein genes). fisK, DNA translocase gene; rrn operon, consisting of 16S, 23S and 5S rRNA; nqrF, Na(+) -translocating NADH-quinone reductase subunit F gene; yajC, preprotein translocase subunit gene; methyltrans., putative RNA methyltransferase gene; repC, replication protein C gene; repA, replication protein A gene; H, hypothetical protein gene; T, Toxin mazF gene; relaxase, conjugal transfer relaxase gene; CO, CO dehydrogenase maturation factor gene; ME, mobile element IS101; tetC, tetracycline resistant gene class C; tetR, tetracycline repressor gene; tetracycline resistance protein class A from transposon 1721; tra., transposase (IS200); put. trans., putative transposase (IS605).

FIG. 2. Strain-specific PCR primers identify putative recombinants. Shown are the PCR results of each putative recombinant Rec2 to Rec8. Rec6, a mixed population of S45 and Rogers132, displays a faint positive band for Rogers132-specific pmpC (middle lane). All putative recombinants were PCR positive for the S45-specific IGR between pmpB and pmpC (top lane) and for tet(C).
FIG. 3. Identification of Rogers132 insertion(s) and mixed infections in putative recombinant genomes. Plots recognizing recombinant regions were created by distinguishing the two parental strains Rogers132 (red) and S45 (black) (see Materials and Methods). a) Schematic of the putative recombinants Rec2, Rec3, Rec5, Rec6, Rec7 and Rec8 clockwise from top left exemplifying crossover regions in Rec3 and Rec5; b) Enlarged region of the two recombinant regions found in Rec5, and c) Schematic representation of recombinant regions within Rec6, a putative recombinant, which was a mixed infection.

FIG. 4. Tet(C)-cassette Insertions in the S45 Genome are Heterogeneous. Shown is the site of insertion for the three confirmed recombinants, Rec3/4 (top) and Rec5 (bottom), in the circular S45 genome. PZ, plasticity zone; cysQ, adenosine-3'(2'),5'-bisphosphate nucleotidase; rrn, rrn operon (16S rRNA, 23S rRNA, 5S rRNA); nqrF, Na(+)‐translocating NADH‐quinone reductase subunit F; pckG, Phosphoenolpyruvate carboxykinase [GTP]; ompA, major outer membrane protein A; kdsA, 2‐dehydro‐3‐deoxyphosphooctonate aldolase; gspD, Putative general secretion pathway protein D; hp, hypothetical protein.

FIG. 5 Recombinant crossover regions in Rec3 and Rec4, and Rec5 based on sequence alignments with S45 and Rogers132. Shown are the nucleotide sequences of the crossovers up- and downstream for Rec3 and Rec4 (A) and the large insertion of Rec5 (B). Numbers on top of the sequences represent the positions relative to the respective gene. Gene IDs (e.g., CS00631) refer to the annotation of Rogers132. Crossover regions are highlighted in grey with SNPs indicated in magenta if the recombinants aligned to the S45 backbone and in green if they
aligned to Rogers132. Palindromes are indicated by blue letters, while tRNAs are highlighted in light grey with white letters. The tRNA associated inverted repeats are highlighted in blue with white letters. Putative ribosomal binding sites (RBS) are highlighted in red and further indicated by “-RBS-” on top of the nucleotide sequence.

FIG. 6 Recombinant crossover regions for Rec5 based on sequence alignments with S45 and Rogers132. Shown are the nucleotide sequences of the small insertion of Rec5. A) represents the slightly longer region upstream, while B) shows the shorter crossover region downstream. Numbers on top of the sequences represent the positions relative to the respective gene. Gene IDs (e.g., CS00631) refer to the annotation of Rogers132. Crossover regions are highlighted in grey with SNPs indicated in magenta if the recombinants aligned to the S45 backbone and in green if they aligned to Rogers132. Blue letters indicate palindromes while putative ribosomal binding sites (RBS) are highlighted in red and further indicated by “-RBS-” on top of the nucleotide sequence.

FIG. 7. Tet(C) reveals strong support for two clades. Shown is the unrooted Baysian phylogenetic tree of tet(C) for a number of tetracycline resistant bacteria (NCBI, BLASTN search, identity cover: 98%) and ten C. suis strains, which correspond to two separate clades (Clade 1: red; Clade 2: green) and H7 (blue).
TABLE 1: Characteristics of *C. suis* strains used in this study.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Site/disease</th>
<th>Location</th>
<th>Isolation Date</th>
<th>MIC (µg/mL)</th>
<th>Cassette Class</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>S45</td>
<td>Feces</td>
<td>Austria</td>
<td>1960’s</td>
<td>0.016</td>
<td>none</td>
<td>(52)(^a)</td>
</tr>
<tr>
<td>Rogers132</td>
<td>Intestine, lung, conjunctiva</td>
<td>Nebraska, USA</td>
<td>1996</td>
<td>8</td>
<td>II</td>
<td>(7)(^a)</td>
</tr>
<tr>
<td>R19</td>
<td>Enteritis</td>
<td>Nebraska, USA</td>
<td>1992</td>
<td>16</td>
<td>I</td>
<td>(7)(^a)</td>
</tr>
<tr>
<td>R27</td>
<td>Enteritis</td>
<td>Nebraska, USA</td>
<td>1993</td>
<td>8</td>
<td>IV</td>
<td>(7)(^a)</td>
</tr>
</tbody>
</table>

\(^a\) Samples obtained from Dr. Art Andersen’s collection (maintained and curated in Dr. Deborah Dean’s lab)
**TABLE 2.** List of potential recombinants and their antibiotic susceptibility profiles.

<table>
<thead>
<tr>
<th>Name</th>
<th>Parental Strains</th>
<th>Protocol 1 or 2</th>
<th>Tetracycline Condition A, B or C</th>
<th><em>ompA</em> genotype / 132 <em>pmpC</em> PCR / S45 <em>pmpB/C</em> IGR PCR</th>
<th>tet(C) PCR</th>
<th>MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rec2</td>
<td>S45 / 132&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1. 24 hpi, 2µg/mL</td>
<td>A. no tetracycline</td>
<td>S45 / - / S45</td>
<td>positive</td>
<td>0.125</td>
</tr>
<tr>
<td>Rec3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>S45 / 132</td>
<td>1. 24 hpi, 2µg/mL</td>
<td>B. ½ MIC&lt;sub&gt;Tp&lt;/sub&gt; S45</td>
<td>S45 / - / S45</td>
<td>positive</td>
<td>8</td>
</tr>
<tr>
<td>Rec4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>S45 / 132</td>
<td>1. 24 hpi, 2µg/mL</td>
<td>B. ½ MIC&lt;sub&gt;Tp&lt;/sub&gt; S45</td>
<td>S45 / - / S45</td>
<td>positive</td>
<td>8</td>
</tr>
<tr>
<td>Rec5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>S45 / 132</td>
<td>2. Co-inf., 0.25 µg/mL</td>
<td>B. ½ MIC&lt;sub&gt;Tp&lt;/sub&gt; S45</td>
<td>132 / - / S45</td>
<td>positive</td>
<td>4</td>
</tr>
<tr>
<td>Rec6</td>
<td>S45 / 132</td>
<td>2. Co-inf., 0.25 µg/mL</td>
<td>C. 2x MIC S45</td>
<td>132-S45 / 132 / -</td>
<td>positive</td>
<td>8</td>
</tr>
<tr>
<td>Rec7</td>
<td>S45 / 132</td>
<td>2. Co-inf., 0.25 µg/mL</td>
<td>C. 2x MIC S45</td>
<td>S45 / - / S45</td>
<td>positive</td>
<td>4</td>
</tr>
<tr>
<td>Rec8</td>
<td>S45 / 132</td>
<td>2. Co-inf., 0.25 µg/mL</td>
<td>C. 2x MIC S45</td>
<td>S45 / - / S45</td>
<td>negative</td>
<td>0.064</td>
</tr>
</tbody>
</table>

<sup>a</sup>Rogers132; <sup>b</sup>true recombinant; (-), negative PCR
### TABLE 3. Stability Assay for five recombinants, showing results for passage 5 (P5) and passage 10 (P10).

<table>
<thead>
<tr>
<th>Name</th>
<th>(tet[C] PCR) Start to P5 (A)</th>
<th>(tet[C] PCR) P5 (B / C)</th>
<th>(tet[C] PCR) P6 to P10</th>
<th>MIC (µg/mL) P5</th>
<th>MIC (µg/mL) P10</th>
<th>ompA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rec3</td>
<td>+ / + / + / + / +</td>
<td>+ / +</td>
<td>+ / + / + / + / +</td>
<td>8</td>
<td>16</td>
<td>S45</td>
</tr>
<tr>
<td>Rec4</td>
<td>+ / + / + / + / +</td>
<td>+ / +</td>
<td>+ / + / + / + / +</td>
<td>8</td>
<td>16</td>
<td>S45</td>
</tr>
<tr>
<td>Rec5</td>
<td>+ / + / + / + / +</td>
<td>+ / +</td>
<td>+ / + / + / + / +</td>
<td>16</td>
<td>16</td>
<td>132a</td>
</tr>
<tr>
<td>Rec6</td>
<td>+ / + / + / + / +</td>
<td>+ / +</td>
<td>+ / + / + / + / +</td>
<td>16</td>
<td>16</td>
<td>132a</td>
</tr>
<tr>
<td>Rec7</td>
<td>+ / + / + / + / +</td>
<td>+ / -</td>
<td>- / - / - / - / -</td>
<td>0.25</td>
<td>ND</td>
<td>S45</td>
</tr>
</tbody>
</table>

*Denotes Rogers132

ND, Not done
Supplementary Material

Figure S1. Co-infection Protocols and Conditions. 1) Shown are the two co-infection protocols, which consisted of a) staggered infection of the donor (strains R19, R27 and Rogers132) 24 hours post infection with the recipient strain S45 (Protocol 1) and b) simultaneous co-infection of recipient and donor after the recipient was first grown to 100% infection in shell vials (Protocol 2). 2) Shown are the culturing conditions at the time of co-infection without tetracycline (Condition A), subinhibitory concentrations of tetracycline (Condition B) and inhibitory concentration of tetracycline (Condition C) for the recipient strain.

Figure S2. The tetracycline repressor gene tetR(C) is highly conserved across genera. Shown is the unrooted Maximum Likelihood (ML) phylogenetic tree of tetR(C) for a number of tetracycline resistant bacteria (NCBI, BLASTN search, identity cover: 98%) and ten C. suis strains, which correspond to two separate clades (Clade 1: red; Clade 2: green) and H7 (blue).

Figure S3. Phylogeny of invasin gene of C. suis and C. caviae. Shown is the unrooted Baysian phylogenetic tree of the chlamydial invasin gene that is found only in C. suis and C. caviae.

Table S1: Primers used to identify putative recombinants.

Table S2. Database of 4,864 SNPs identified between the Rogers132 donor and S45 recipient.
FIG. 1. The structure of the \textit{tet(C)-containing cassette} is comprised of three unique segments. Shown is the \textit{tet(C)-containing cassette} and its neighboring genes, depending on the \textit{C. suis} strain. The structure of the cassette comprises three diversely arranged segments (purple, red and orange) consisting of different genes. Three cassette types have been identified in ten \textit{C. suis} strains: (I) contains all three segments, though the cassette of H5 is arranged differently from R19 and R24; (II) the cassette of Rogers132 is lacking segment 3, which contains transposases IS200 and IS605; (III) is missing segment 1 (replication/mobilization protein genes). \textit{ftsK}, DNA translocase FtsK; \textit{rrn operon}, 16S, 23S and 5S rRNA; \textit{nqrF}, Na(+) translocating NADH-quinone reductase subunit F; YajC, preprotein translocase subunit YajC; methyltrans., putative RNA methyltransferase; \textit{repC}, replication protein C; \textit{repA}, replication protein A; H, hypothetical protein; T, Toxin \textit{MazF}; relaxase, conjugal transfer relaxase; CO, CO dehydrogenase maturation factor; ME, mobile element IS101; \textit{tetC}, tetracycline resistant gene class C; \textit{tetR}, tetracycline repressor gene; (tetracycline resistance protein class A from transposon 1721); \textit{tra+}, transposase (IS200); \textit{put. trans.}, putative transposase (IS605).
**FIG. 2.** Strain-specific PCR primers identify putative recombinants. Shown are the PCR results of each putative recombinant Rec2 to Rec8. Rec6, a mixed population of S45 and Rogers132, displays a faint positive band for Rogers132-specific *pmpC* (middle lane). All putative recombinants were PCR positive for the S45-specific IGR between *pmpB* and *pmpC* (top lane) and for *tet(C)*.
FIG. 3. Identification of Rogers132 insertion(s) and mixed infections in putative recombinant genomes. Plots recognizing recombinant regions were created by distinguishing the two parental strains Rogers132 (red) and S45 (black) (see Materials and Methods). a) Schematic of the putative recombinants Rec2, Rec3, Rec5, Rec6, Rec7 and Rec8 clockwise from top left exemplifying crossover regions in Rec3 and Rec5; b) Enlarged region of the two recombinant regions found in Rec5, and c) Schematic representation of recombinant regions within Rec6, a putative recombinant, which was a mixed infection.
FIG. 4. Tet(C)-cassette Insertions in the S45 Genome are Heterogeneous. Shown is the site of insertion for the three confirmed recombinants, Rec3/4 (top) and Rec5 (bottom), in the circular S45 genome. PZ, plasticity zone; cysQ, adenosine-3'(2'), 5'-bisphosphate nucleotidase; rrn, rrn operon (16S rRNA, 23S rRNA, 5S rRNA); nqrF, Na(+)-translocating NADII-quinone reductase subunit F; pckG, Phosphoenolpyruvate carboxykinase (GTP); ompA, major outer membrane protein A; kdsA, 2-dehydro-3-deoxyphosphooctonate aldolase; gspD, Putative general secretion pathway protein D; hp, hypothetical protein.
A. Insertion Rec3 and Rec4

1. Upstream Crossover between phosphoenolpyruvate carboxykinase (pckG) to hypothetical protein gene (CS00631, CS00632)

<table>
<thead>
<tr>
<th>Rec3</th>
<th>Rec4</th>
</tr>
</thead>
<tbody>
<tr>
<td>132</td>
<td>132</td>
</tr>
<tr>
<td>pckG</td>
<td>pckG</td>
</tr>
<tr>
<td>RBS-</td>
<td>RBS-</td>
</tr>
<tr>
<td>470</td>
<td>470</td>
</tr>
</tbody>
</table>

2. Downstream Crossover in Na⁺-translocating NADH-quinate reductase subunit F (nqrF) (CS00679)

B. Insertion Rec5

1. Upstream Crossover between 2-dehydro-3-deoxypentose-phosphate aldolase (kdaA); IRNA-Arg (tct) and a hypothetical protein gene (CS00570, CS00571, CS00572)

<table>
<thead>
<tr>
<th>Rec5</th>
<th>kdaA (570)</th>
</tr>
</thead>
<tbody>
<tr>
<td>132</td>
<td>763</td>
</tr>
<tr>
<td>790</td>
<td>810/108 kdaA/tct</td>
</tr>
</tbody>
</table>

2. Downstream Crossover in adenine-sulfate (Ⅲ), β-hydroxylase nucleosidase (cruQ) (CS00715)

FIG. 5 Recombinant crossover regions in Rec3 and Rec4, and Rec5 based on sequence alignments with S45 and Rogers132. Shown are the nucleotide sequences of the crossovers up- and downstream for Rec3 and Rec4 (A) and the large insertion of Rec5 (B). Numbers on top of the sequences represent the positions relative to the respective gene. Gene IDs (e.g., CS00631) refer to the annotation of Rogers132. Crossover regions are highlighted in grey with SNPs indicated in magenta if the recombinants aligned to the S45 backbone and in green if they aligned to Rogers132. Palindromes are indicated by blue letters, while tRNAs are highlighted in light grey with white letters. The tRNA associated inverted repeats are highlighted in blue with white letters. Putative ribosomal binding sites (RBS) are highlighted in red and further indicated by "-RBS-" on top of the nucleotide sequence.
A 1. Crossover in *gsuD* (CS0482)

<table>
<thead>
<tr>
<th>Rec5</th>
<th>132</th>
</tr>
</thead>
<tbody>
<tr>
<td>S45</td>
<td></td>
</tr>
<tr>
<td>AAGCCCAATTTGTCAACCAAGGCTTTATATCCCAATAGCCGGCTAGCTACGAGATGGAAACAGTCAACAAAGTGATGCTCGGAGTGAC</td>
<td>132</td>
</tr>
<tr>
<td>Rec5</td>
<td></td>
</tr>
<tr>
<td>S45</td>
<td></td>
</tr>
<tr>
<td>AAGCCCAATTTGTCAACCAAGGCTTTATATCCCAATAGCCGGCTAGCTACGAGATGGAAACAGTCAACAAAGTGATGCTCGGAGTGAC</td>
<td>132</td>
</tr>
</tbody>
</table>

B 2. Crossover in *mutL* and *ipgC* (CS0485 and CS0486)

**Putative peptidase**

<table>
<thead>
<tr>
<th>Rec5</th>
<th>132</th>
</tr>
</thead>
<tbody>
<tr>
<td>S45</td>
<td></td>
</tr>
<tr>
<td>ATATCCGAACAATACCCCTCATAGCAAACTCCAATATCAATTAAGACAA</td>
<td>132</td>
</tr>
<tr>
<td>Rec5</td>
<td></td>
</tr>
<tr>
<td>S45</td>
<td></td>
</tr>
<tr>
<td>ATATCCGAACAATACCCCTCATAGCAAACTCCAATATCAATTAAGACAA</td>
<td>132</td>
</tr>
</tbody>
</table>

C Schematic of the two small cross over regions

<table>
<thead>
<tr>
<th>Rec5</th>
<th>132</th>
</tr>
</thead>
<tbody>
<tr>
<td>S45</td>
<td></td>
</tr>
</tbody>
</table>

**FIG. 6** Recombinant crossover regions for Rec5 based on sequence alignments with S45 and Rogers132. Shown are the nucleotide sequences of the small insertion of Rec5. A) represents the slightly longer region upstream, while B) shows the shorter crossover region downstream. Numbers on top of the sequences represent the positions relative to the respective gene. Gene IDs (e.g., CS00631) refer to the annotation of Rogers132. Crossover regions are highlighted in grey with SNPs indicated in magenta if the recombinants aligned to the S45 backbone and in green if they aligned to Rogers132. Blue letters indicate palindromes while putative ribosomal binding sites (RBS) are highlighted in red and further indicated by “*”-RBS-“ on top of the nucleotide sequence.
**FIG. 7.** *Tet*(C) reveals strong support for two clades. Shown is the unrooted Bayesian phylogenetic tree of *tet*(C) for a number of tetracycline resistant bacteria (NCBI, BLASTN search, identity cover: 98%) and ten *C. suis* strains, which correspond to two separate clades (Clade 1: red; Clade 2: green) and H7 (blue).