ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 2004, p. 3989–3995 Vol. 48, No. 10
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.10.3989–3995.2004

Tetracycline Resistance in *Chlamydia suis* Mediated by Genomic Islands Inserted into the Chlamydial *inv*-Like Gene

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Received 16 December 2003/Returned for modification 17 February 2004/Accepted 11 June 2004

Many strains of *Chlamydia suis*, a pathogen of pigs, express a stable tetracycline resistance phenotype. We demonstrate that this resistance pattern is associated with a resistance gene, *tet*(C), in the chlamydial chromosome. Four related genomic islands were identified in seven tetracycline-resistant *C. suis* strains. All resistant isolates carry the structural gene *tet*(C) and the tetracycline repressor gene *tetR*(C). The islands share significant nucleotide sequence identity with resistance plasmids carried by a variety of different bacterial species. Three of the four *tet*(C) islands also carry a novel insertion sequence that is homologous to the IS*605* family of insertion sequences. In each strain, the resistance gene and associated sequences are recombined into an identical position in a gene homologous to the *inv* gene of the yersiniae. These genomic islands represent the first examples of horizontally acquired DNA integrated into a natural isolate of chlamydiae or within any other obligate intracellular bacterium.

The chlamydiae are obligate intracellular bacteria that are important pathogens in humans and animals. Following infection of cells by the metabolically inactive elementary bodies (EB), the chlamydiae differentiate to the metabolically active and replication-competent reticulate bodies that multiply within a nonacidified vacuole (the inclusion). In humans, *Chlamydia trachomatis* is the causative agent of diseases of the genital tract and the conjunctiva (32). *Chlamydia pneumoniae* causes pneumonia and has also been implicated in atherosclerosis (6). Many different chlamydiae, including species causing serious diseases in reptiles, birds, and mammals, are important in infections of veterinary significance (21). *Chlamydia suis* is a pathogen that is widespread in farmed pigs and is associated with several chronic diseases, such as conjunctivitis and keratoconjunctivitis (25–27). The type strain of this species, strain S45, was isolated in Europe several decades ago and is tetracycline sensitive (Tc^s) (15).

Both human and veterinary chlamydial infections are often treated with tetracycline and its derivatives (8). While there are reports of human chlamydial infections that do not respond to tetracycline or doxycycline, no human pathogenic chlamydial strains that demonstrate stable tetracycline resistance (Tc^r) have been isolated (14, 19, 31, 35). However, stable Tc^r *C. suis* strains have recently been identified in both diseased and apparently healthy pigs from farms across the midwestern United States. The resistance properties of these strains were confirmed in three laboratories (1, 20, 35), but the mechanism of resistance had not been elucidated.

In this report, we demonstrate that the *C. suis* Tc^r phenotype is manifested through a Tc^r gene, *tet*(C), integrated into the chlamydial chromosome in each of seven Tc^r strains. The Tc^r gene in each strain is contained within one of a family of horizontally acquired genetic elements that share identity with resistance plasmids of gram-negative bacteria and is integrated into a homolog of the invasin gene of the yersiniae (13).

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MATERIALS AND METHODS

Chlamydia and DNA preparation and purification. Tc^r *C. suis* isolates were collected from pigs (*Sus scrufa*) at sites across the midwestern United States (1) (Table 1). The single Tc^s *C. suis* isolate (S45) was collected in the 1960s in Austria (15). Concentrated preparations of chlamydial EBs were prepared from infected McCoy cell monolayers cultured in minimal essential medium with 10% fetal bovine serum, 2 mM L-glutamine, and 10 μg of gentamicin/ml for 40 h at 37°C in 5% CO_2. Infected cells were ruptured by sonication for 3 s at 10 W, followed by centrifugation at 30,500 × *g* for 30 min. The pellet was resuspended in Hanks balanced salt solution and overlaid onto 10 ml of 30% Hypaque-76 (Nycomed, Roskilde, Denmark) diluted in phosphate-buffered saline. This preparation was centrifuged at 30,500 × *g* for 40 min, and the pellet, containing partially purified EBs, was resuspended in SPG (18 mM Na_2HPO_4, 220 mM sucrose, 5 mM L-glutamate). Genomic DNA was prepared using a genomic DNA preparation kit (QIAGEN, Chatsworth, Calif.) according to the manufacturer's recommendations. Five mM dithiothreitol was added to the supplied lysis buffer for complete disruption of the chlamydial EBs.

MIC determination. MICs for each *C. suis* strain were determined by using Vero cells cultured in 96-well plates. Chlamydiae were diluted to approximately 100 inclusion-forming units per well and centrifuged onto the monolayer at 900 × *g* for 1 h. The inocula were then removed, and medium containing 0.5 μg of cycloheximide/ml plus twofold serial dilutions of tetracycline (0.3 to 40 μg/ml) was added to each well. One row of wells was cultured in the absence of tetracycline as a control for chlamydial growth. Four plates were infected for each test, and one plate was fixed with methanol and stained for chlamydiae each day for 4 days. The lowest concentration of tetracycline not showing development of inclusions was recorded as the MIC. All tests were performed at least twice.

Amplification by thermal cycling. All PCRs were performed with 0.25 mM deoxynucleoside triphosphate, 0.4 nM forward and reverse primers (Table 2), and *Taq* (Promega, Madison, Wis.) or *Pfx* (Invitrogen, Carlsbad, Calif.) DNA polymerase. Reactions were performed in 50-μl volumes using 50 to 100 ng of chlamydial genomic DNA, and each enzyme was used according to the manufacturer's recommendations.

PCR was used to screen *C. suis* strains for Tc^r determinants previously characterized in other resistant bacteria. The PCR was performed using primers specific to 13 different resistance determinants (33), including *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), and *tet*(S).

PCR was also used to link genes known to flank the *inv*-like genes in *Chlamy-*

TABLE 1. *C. suis* Tc^r and Tc^s strains

Strain	Isolated in:		MIC (μg/ml)
	Location	Year	
S45	Austria	1960s	0.6
R19	Nebraska	1992	5
R24	Nebraska	1992	5
R27	Nebraska	1993	5
H5	Iowa	1994	5
H7	Iowa	1994	10
130	Nebraska	1996	5
132	Nebraska	1996	5

dophila caviae (23) and *Chlamydia muridarum* (22). Primers for these reactions were designed from conserved regions within these genes (*dmpP* and 23S rRNA) and used together or with primers from within *repC*, a gene that flanks *tet*(C) in most resistant strains (Table 2). Genomic DNAs from Tc^r strain R19 and Tc^s strain S45 were used as templates for PCRs.

Southern blotting. Genomic DNA was digested with HindIII, electrophoresed through 0.7% agarose, and transferred to a nylon membrane (28). The genomic DNA was then UV cross linked to the membrane and probed with digoxigenin-labeled PCR products. To make digoxigenin-labeled PCR products, digoxigenin-labeled deoxynucleoside triphosphates (Roche Diagnostics, Indianapolis, Ind.) were added to the PCR mixtures. Nested PCR with primers within the target gene was used to confirm that each probe was specific for the gene of interest (data not shown). After incubation with the probe, membranes were washed with 0.1% sodium dodecyl sulfate and 10% 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) before incubation with an anti-digoxigenin antibody conjugated with alkaline phosphatase. The membrane was then developed by incubation with a chemiluminescent peroxidase substrate (Roche). The blots were exposed to film, and the molecular masses of the resulting bands were determined by using a 1-kb DNA ladder (Fermentas, Vilnius, Lithuania).

Cloning and nucleotide sequence analysis. Resistance genes and flanking sequences from *C. suis* strains R19 and R27 were cloned for sequence analysis by using two different methods. The primary method was to create plasmid libraries of size-selected *C. suis* DNA and then carry out PCR analysis of individual clones to identify positives. *C. suis* DNA digested with HindIII was blotted and probed with *tet*(C) and *tetR*(C). A parallel sample of digested DNA was electrophoresed, and a block of agarose that contained a fragment approximately the size of that identified in the Southern blots was excised. DNA was purified from the agarose using a QIAGEN gel extraction purification kit. DNA was then ligated to HindIII-digested pUC18 (Invitrogen) and transformed into *Escherichia coli* DH5α. Transformants were grown on Luria-Bertani agar containing ampicillin (100 μg/ml), and potential clones were screened by PCR for the target gene. Plasmids from positive clones were purified, and the inserts were sequenced at the Oregon State University Center for Gene Research Central Service Laboratory.

TABLE 2. Oligonucleotide primers used for PCR and nucleotide sequence analysis

Primer name	Description	Sequence (5′→3′)
CS43	*tet*(C)	AGCACTGTCCGACCGCTTTG
CS47	*tet*(C)	TCCTCGCCGAAAATGACCC
CS33	*tetR*(C)	CAAGACCGCCGATGATGAGAG
CS38	*tetR*(C)	CCAAGGGATGACGACGACTG
CS09	*orfB*	TTTTGACGCTTCGTTGAGCAC
CS17	*orfB*	AGTCGGTTATTGGTTGATAGCAGC
CS01	3′-*inv*-like	GACTATCGGTAGACAAGACTCGGC
CS08	3′-*inv*-like	GACTCTCAAGGGAAGATTCGCAC
CS02	Intact *inv*-like	CGTTTCAGGAATACCCACTTCG
CS106	Intact *inv*-like	ACACTTCAGGTTTTCGCCGTAG
CS105	5′-*inv*-like	ATCATTCGCAACAGGAGG
CS107	5′-*inv*-like	CTTCGCCTCTCTTCACAAC
CS109	*dmpP*	TGGTCTCTATCCTTCGTGGG
CS111	23S rRNA	TTTGTGGTCCAGCACTTC
CS05	3′-*inv*-like	TGCCGTCAACAACAGATGC
CS108	*dmpP*	TGGTGTGTCGGTTGTTCTG
CS101	*repC*	TGGGAAGAAGCATCAACG
CS68	*mobD*	GAGTTCTTCAAGCGTGCTATTC
CS77	*mobB*	ATCATTGCCAACGCCGACGC
CS81	*mobA*	CGGCATACAGACACTTTTCC
CS84	*mobA*	GCAGCCATCATCAAGCAG
CS86	*mobA*	TGCTCCTGCCGCTTGTCCTG

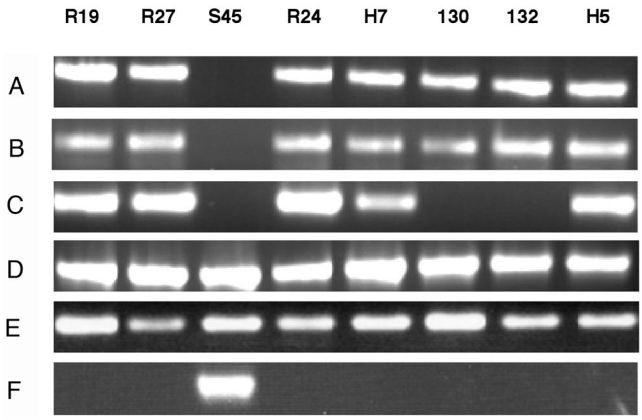

FIG. 1. PCR analysis of eight different *C. suis* strains using primers specific for *tet*(C) (525 bp) (A), *tetR*(C), (400 bp) (B), IScs*605* (500 bp) (C), and the *inv*-like gene (D to F). The PCR products of the *inv*-like gene represent a 3′ fragment (200 bp) (D), a 5′ fragment (700 bp) (E), and a fragment that spans the *inv*-like sequence that is contiguous in the Tc^s strain S45 (900 bp) (F). All primers used in these experiments are listed in Table 2.

A PCR-based primer-walking approach was used to acquire the sequence from one end of the genomic insert from strain R19. Oligonucleotides derived from the accumulating sequence data were used for these experiments (Table 2). Each fragment produced in these PCRs was cloned into pCR2.1:Blunt (Invitrogen), and the nucleotide sequences were determined. Two clones from independent PCRs were sequenced for each region of interest.

RT-PCR. Transcriptional analysis of *tet*(C) was performed using reverse transcriptase PCR (RT-PCR) with template RNA from *C. suis*-infected monolayers (multiplicity of infection, 3) cultured in the presence or absence of tetracycline (1 μg/ml). Infected cells were incubated for 30 h, and RNA was collected by using Trizol (Invitrogen). Lysates were then extracted with chloroform, the RNA was precipitated with isopropanol, and the pellet was washed with 70% ethanol. The concentration of the total RNA was measured using a SmartSpec UV spectrophotometer (Bio-Rad, Hercules, Calif.), and 100 ng of RNA was added to each reaction mixture. The Access system (Promega) was used for all RT-PCRs according to the manufacturer's recommendations. Controls included a genomic DNA positive control for showing the size of PCR products and a reaction mixture lacking RT to confirm that RNA preparations were free of contaminating DNA. A 100-bp DNA ladder (Invitrogen) was used to determine the sizes of the PCR products.

Nucleotide sequence accession numbers. The sequences assembled from strains R19 and R27 were deposited in GenBank under accession numbers AY428550 and AY428551, respectively.

RESULTS

Identification of a Tc^r gene, *tet*(C), in Tc^r *C. suis* strains. PCR analysis of genomic DNA with primers specific for 13 different Tc^r genes (see Materials and Methods), demonstrated that a single gene, *tet*(C), was present in each of the seven resistant strains (Fig. 1). This is one of several Tc^r genes that encode an efflux pump (24). PCR analyses of all other tested *tet* genes were negative (data not shown). In most systems, the structural gene *tet*(C) is adjacent to a gene encoding a repressor of *tet*(C) transcription [*tetR*(C)] (12). This was also true in *C. suis*, as PCR analysis showed that each of the resis-

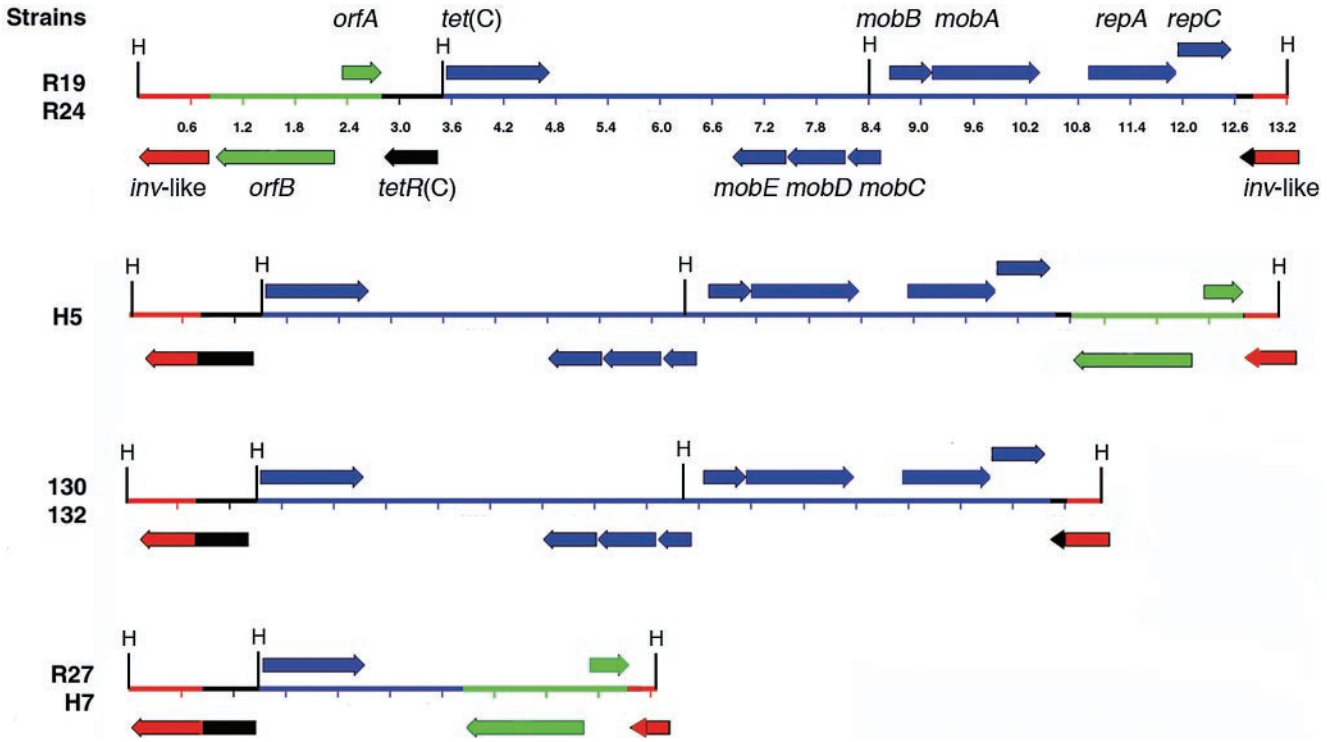

FIG. 2. ORF maps of *tet*(C) and flanking sequences in the Tc^r *C. suis* strains. The *tet*(C) allele and flanking sequences for strains R19 and R27 were cloned and fully sequenced. All other sequences were inferred from overlapping PCR-based gene linkage analysis using the sequencing primers from R19 and R27 to amplify different regions of strains R24, H7, 130, 132, and H5 for sequence comparison. The directions of the arrows represent the coding strands, and all HindIII sites (H) are shown. The scale in kilobases is shown for R19 and R24 and is identical in each map. Note that each island is inserted into the *C. suis inv*-like gene (red), and each contains sequences that share identity with plasmids of gram-negative bacteria (blue and black). The 2,013-bp IScs*605* sequence (green) is located at one or the other end in five of seven strains. The *tetR*(C) sequence (black) is interrupted in each island, with a 5′ fragment remaining adjacent to *tet*(C) and a 3′ fragment found at the opposite end of the integrated sequence in five strains.

tant strains contained *tetR*(C). Neither *tet*(C) nor *tetR*(C) was detected in the Tc^s strain S45 (Fig. 1).

Nucleotide sequence analysis. The complete sequence of the DNA surrounding *tet*(C) was determined for Tc^r strains R19 and R27. A HindIII site between *tetR*(C) and *tet*(C) facilitated the cloning of genomic DNA fragments containing each gene into pUC18. The *tet*(C) and *tetR*(C) genes plus the flanking sequence were not present in the sensitive strain (Fig. 2). The lengths of the genomic inserts in strains R19 and R27 were different (12 and 5 kb, respectively). These two genes share a high sequence identity with homologous genes in plasmids of gram-negative bacteria, including the plasmid pSC101 (9). Sequences within each genomic island also share a high identity with the plasmid pRAS3.2 from the fish pathogenic bacterium *Aeromonas salmonicida* (18). The nucleotide sequence identity between *C. suis* R19 and pRAS3.2 is over 99% throughout the 10.1 kb of shared DNA. An approximately 1.7-kb fragment containing much of a *mobA-repB* hybrid gene is present in pRAS3.2 but is absent in R19 and R27 (18). Most of the differences in the sequences shared between pRAS3.2 and the R19 genomic island can be accounted for by two short deletions within R19. First, there is an eight-nucleotide deletion within the *tet*(C)-*tetR*(C) operator region (4). Second, there is a 44-nucleotide deletion within the pRAS3.2 origin of replication, deleting two of the three iterons (18).

PCR was then used to examine in detail the structure of the inserted islands in each Tc^r strain. These analyses demonstrated that there are four different, yet related, genomic islands in the seven strains (Fig. 2). Three of the islands are represented in two strains each, and one is found only in a single strain. While the Southern blot analysis indicated a different-sized fragment carrying *tet*(C) in strains 130 and 132, careful PCR analysis demonstrated that the gene arrangement in these strains is otherwise identical.

A final difference between the *C. suis* sequences and other similar sequences is a truncation of *tetR*(C) which interrupts the coding sequence 72 bp upstream of the 3′ end of the gene in the resistant strains. This truncation is the result of a recombination event. The 3′ end of *tetR*(C) is located at the opposite end of the island in R19, R24, H5, 130, and 132, but this 72-nucleotide sequence is deleted in R27 (Fig. 2). In five strains (R27, H5, H7, 130, and 132), the 5′ end of *tetR*(C) is fused in frame with the 3′ end of the *inv*-like gene. This truncation site within *tetR*(C) is an apparent recombinatorial hotspot. In each of the *C. suis tetR*(C) genes, and in plasmids pSC101 and pRAS3.2, the nucleotide sequences diverge from near identity to nonhomologous sequence at exactly the same nucleotide position.

The G+C content in *tet*(C) within the 10.1 kb of shared sequence is approximately 54%. This is in contrast to each

sequenced chlamydial genome, where the G+C content is approximately 40% (22, 23).

Identification of a chlamydial insertion element, IScs*605*. While the sequence analysis demonstrated that DNA flanking *tet*(C) in both R27 and R19 contained regions with high identity to known resistance plasmids, there were also sequences that shared no identity with these plasmids. Five of seven strains carried a novel insertion element that is homologous to the IS*605* family of insertion sequences. These insertion sequences were identical at the nucleotide level in strains R19 and R27 and were located at opposite termini of the inserted islands in each strain. The 2,013-bp chlamydial IS*605*-like element, designated IScs*605*, shares 39% nucleotide sequence identity with IS*605* from *Helicobacter pylori* (16). Similar IS*605* insertion sequences are common in *Helicobacter* spp. that are commensals or pathogens in pigs and other animals (7). As with other IS*605* insertion sequences, IScs*605* is composed of divergently oriented members of the IS*200* and IS*1341* families of insertion sequences, which are individually found in many different bacteria (17). The smaller open reading frame (ORF), *orfA*, encodes a 151-amino-acid protein that shares 52% identity with the IS*200* protein from *Streptomyces avermitilis* (gi 29604461). The larger ORF, *orfB*, encodes a 459-amino-acid protein that shares 29% identity with a transposase from *Thermobifida fusca* (gi 23018063).

Chromosomal localization of *tet*(C). Pulsed-field gel electrophoresis of intact *C. suis* genomic DNA, followed by Southern blotting with *tet*(C), suggested that the resistance gene is located on the chromosome (data not shown). These results were confirmed through the sequencing analysis. The *tet*(C) gene and flanking sequences in R19 and R27 are integrated into a gene that is homologous to the invasin gene of the yersiniae (13). Genome sequence analyses demonstrate that at least two other chlamydial pathogens of animal species, *C. caviae* and *C. muridarum*, have a full-length *inv*-like gene or gene fragment, while the human pathogenic chlamydiae do not (22, 23, 34).

PCR was used to show that the *inv*-like gene in *C. suis* strains S45 and R19 was located between the genes encoding NADH: ubiquinone oxidoreductase (*dmpP*) and the 23S rRNA gene, consistent with the location of the *inv*-like gene in *C. caviae* and *C. muridarum*. PCR analysis using primers specific for the 23S rRNA gene and *repC*, a gene adjacent to *tet*(C) in the R19 genomic island, confirmed that this sequence was located between *dmpP* and 23S rRNA (Fig. 3).

Mapping regions flanking *tet*(C) and *tetR*(C) in other strains. Using the nucleotide sequence data generated from analysis of strains R19 and R27, primers were designed to amplify *tet*(C), *tetR*(C), IScs*605 orfB*, and different regions of the *inv*-like gene. Using these primers, each additional Tc^r strain (R24, H5, H7, 130, 132) and the Tc^s strain S45 were analyzed to determine whether they contain sequences similar to those found in R19 and R27. The results demonstrated that in all of the resistant strains, the *inv*-like gene was interrupted by the genomic island, while in S45, the gene was intact (Fig. 1). All resistant strains were positive for *tet*(C) and *tetR*(C), and all but two strains contained IScs*605*.

Southern hybridization with probes for *tet*(C), the *inv*-like gene, and IScs*605 orfB* confirmed that each *C. suis* strain has the *inv*-like gene and that this gene is interrupted in all Tc^r strains but not in the Tc^s strain S45 (Fig. 4). These blots showed that single copies of *tet*(C) were present in all seven Tc^r strains, while single copies of IScs*605* were detected in five of seven resistant strains. Neither *tet*(C) nor IScs*605* was present in the Tc^s strain S45.

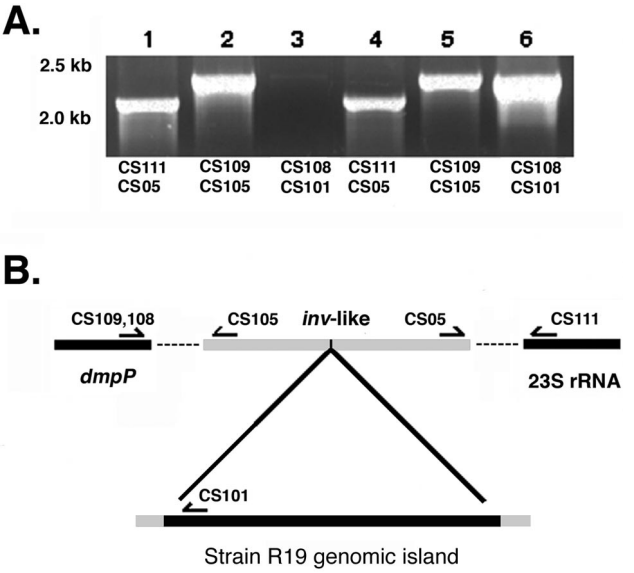

FIG. 3. Chromosomal location of *tet*(C) in the resistant strains. (A) PCR results using S45 genomic DNA (lanes 1 to 3) or R19 genomic DNA (lanes 4 to 6) as template. Lanes 1 and 4 show a product amplified using primers that link the *inv*-like gene to the 23S rRNA. Lanes 2 and 5 show amplified products linking the *inv*-like gene to *dmpP*. Lanes 3 and 6 are products amplified from *repC*, a gene within the genomic island, to *dmpP*. Note that a product is generated from R19 template when primers for *repC* and *dmpP* are used (lane 6), but no such product is produced in a parallel reaction using strain S45 as a template (lane 3). Molecular size standards are indicated (in kilobases) to the left of panel A. (B) Linkage map showing how *tet*(C) and flanking sequences are positioned between *dmpP* and the 23S rRNA gene in R19. The dashed lines indicate the genomic sequence between genes targeted by the amplification.

Site of insertion within the *inv*-like gene. Sequence analysis was used to examine the site within the *inv*-like gene that was targeted for integration by *tet*(C) and the flanking sequences. Each of the seven strains showed evidence of an integration event at an identical position within the *inv*-like gene, and the donor DNA recombined at a precise nucleotide position within *tetR*(C) (Fig. 5). The sequence 5′-TTCAA-3′ is found in both the *inv*-like gene and *tetR*(C), and this sequence is the only region of identity at the recombination site (Fig. 5). The pentanucleotide TTCAA is also found at the 3′ end of all apparent IScs*605* integration events, but this appears to be the result of directed targeting of the insertion and not a function of duplication of any sequence during integration. This is consistent with IS*605* in *H. pylori*, where insertion is not associated with the generation of sequence repeats in the target (16). The importance of the TTCAA sequence is reinforced by analysis of the truncated island found in strains R27 and H7. This apparent truncation occurred at another TTCAA site in the R19 island.

RT-PCR. The nucleotide sequencing showed that *tetR*(C) in the resistant *C. suis* strains is truncated and that the operator region has an octanucleotide deletion relative to homologous

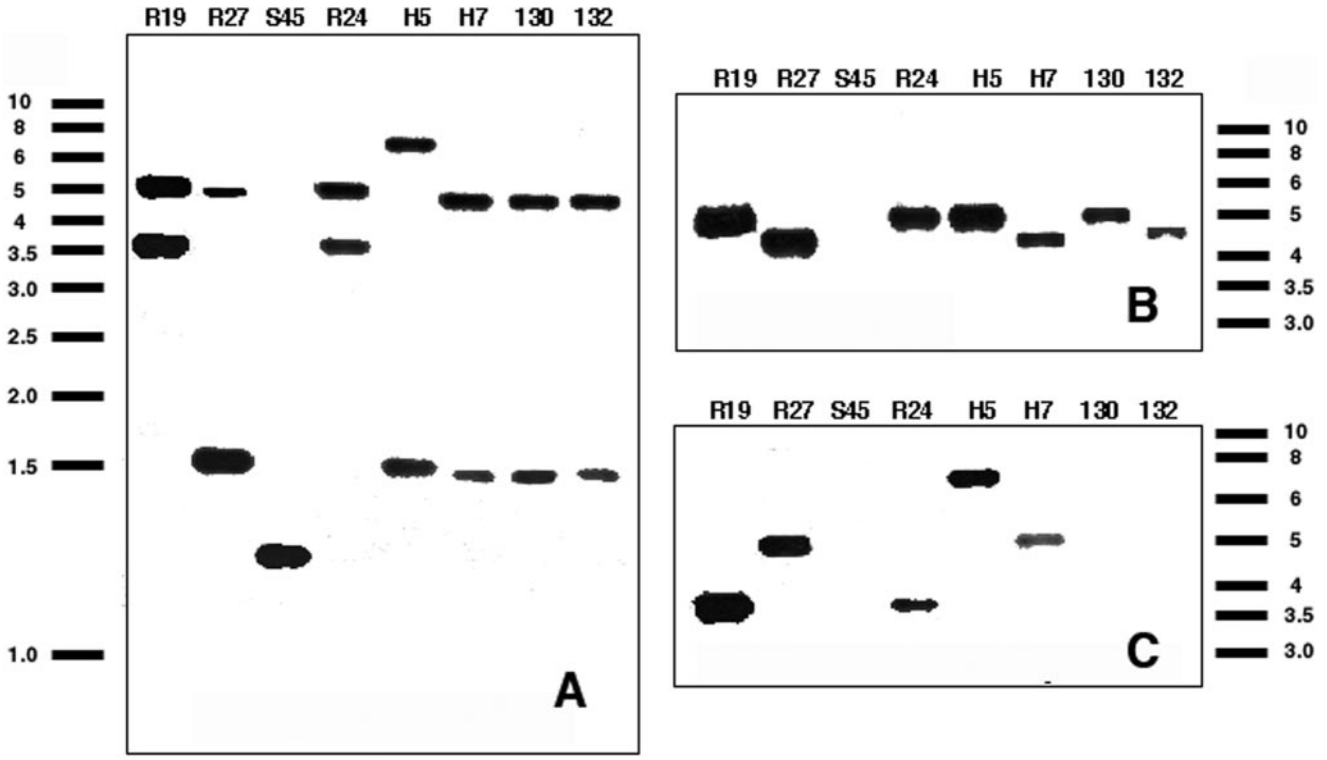

FIG. 4. Southern blots of *C. suis* genomic DNA digested with HindIII and probed with sequences from the *inv*-like gene of S45 (A), *tet*(C) (B), and a fragment of IScs*605* (C). The individual strains are indicated at the top of each panel. Molecular size standards are indicated in kilobase pairs.

sequences in pSC101 and pRAS3.2. It was hypothesized that these differences might eliminate the tight control placed on *tet*(C) expression in the absence of tetracycline (12). Analysis of transcription of *tet*(C) in Tc^r *C. suis* demonstrated that this was the case. While *tet*(C) transcript was not detected in *E. coli*(pSC101) in medium lacking tetracycline, *tet*(C) transcript was found in *C. suis* R19 cultured in the presence and absence of tetracycline (Fig. 6).

DISCUSSION

These studies demonstrate that recently isolated Tc^r strains of *C. suis* carry a *tet*(C) gene that is located in one of a set of

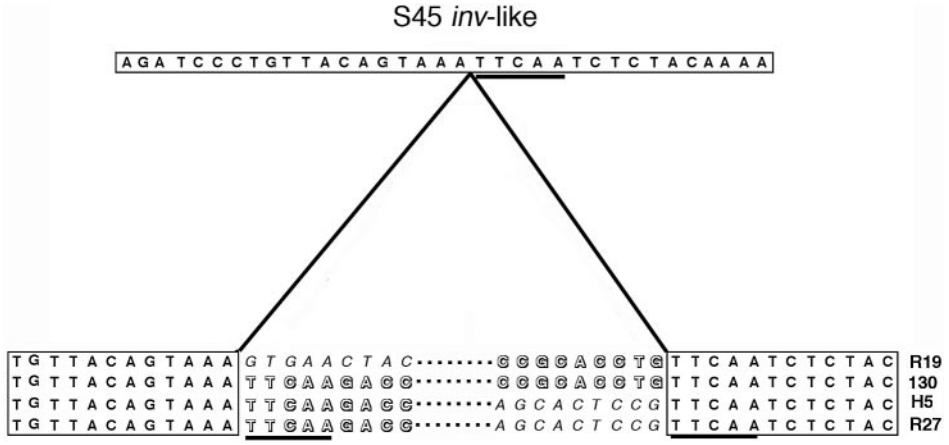

FIG. 5. Nucleotide sequences surrounding the recombination sites at the junction of the integrated *tet*(C) island and the *C. suis inv*-like gene (boxed sequences). Only the nine terminal nucleotides are represented for the left and right ends of each island. Each island shown in Fig. 2 is represented in this figure, with the representative strain indicated to the right of the sequences. Nucleotide sequences in block letters are *tetR*(C), while sequences in italics are IScs*605*. The TTCAA sequences within *tetR*(C) and the interrupted *inv*-like gene are underscored. The dotted lines represent the internal sequences of the genomic islands.

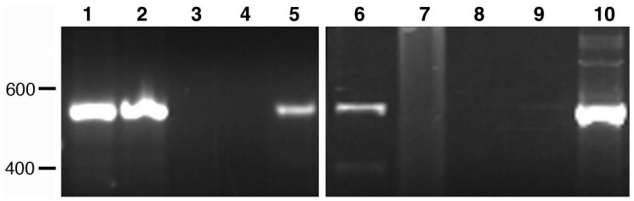

FIG. 6. Analysis of *tet*(C) transcription in R19 and *E. coli*(pSC101). RT-PCR was conducted on each bacterium cultured in the presence or absence of tetracycline. Lanes 1 to 5 represent RT-PCR products from *C. suis* RNA, and lanes 6 to 10 represent RT-PCR products from *E. coli*(pSC101). Lanes 1 and 6 show *tet*(C) transcripts detected in bacteria cultured in the presence of 1 μg of tetracycline/ml. Lanes 2 and 7 show *tet*(C) transcript detected in the absence of tetracycline. Lanes 3 and 8 show negative controls—RT-PCR products without using RT. RNA from bacteria were cultured in the presence of 1 μg of tetracycline/ml. Lanes 4 and 9 show negative controls—RT-PCR products without RT using RNA from bacteria cultured in the absence of tetracycline. Lanes 5 and 10 show a positive control using bacterial genomic DNA as a template. Molecular mass standards are indicated in base pairs.

highly related apparent plasmids that has integrated into the chromosome. In each case, the resistance determinant is adjacent to *tetR*(C), and each is flanked by sequences common to known resistance plasmids from gram-negative bacteria. Five of the Tc^r strains also carry a novel insertion element that is related to the IS*605* family of insertion sequences common in *Helicobacter* species. *C. suis* ISc*s605* is the first insertion sequence identified in any chlamydia. The *tet*(C) genes and associated sequences are integrated as single copies into the chromosome at the same nucleotide position within each strain. It is not likely that this was a single integration event that has been expanded and altered through pig populations, as the major outer membrane protein sequences of each resistant strain are different (5). The target of integration is a gene encoding a chlamydial homolog of the invasin gene of *Yersinia* spp. The *tetR*(C) gene is the target of recombination within the donor DNA and is interrupted at an identical site in each strain. The G+C content in each genomic island was approximately 54%. In contrast, the G+C content is approximately 40% within sequenced chlamydial genomes, and the 885 nucleotides of the *inv*-like gene that were sequenced in this study have a G+C content of 40%. These properties demonstrate that the integrated DNAs have the characteristics of genomic islands (10, 11), and we have therefore labeled them as the *tet*(C) islands.

The nucleotide sequencing demonstrated a high degree of identity between the *tet*(C) islands and pRAS3.2, a resistance plasmid from *A. salmonicida* (18). This organism is found in salmon and trout populations worldwide and has an optimal growth temperature of below 20°C; thus, it is not likely that this bacterium was directly involved in genetic transfer to *C. suis*. It is most likely that the sequences are also carried on a mobilizable element in an organism within the pig microflora and were transferred to *C. suis* in that environment. The mechanism of transfer is also unresolved. Models can be developed that assume that the IS element was integrated into progenitor plasmid sequences prior to acquisition of the island by the chlamydiae. Alternatively, the integration of the plasmid and the IS element could have happened sequentially or simultaneously with the integration of the plasmid sequences. We are presently examining porcine tissue for evidence of the IS element in additional *C. suis* samples or in other bacteria, with a goal of further characterizing the source of the *tet*(C) islands and the integration mechanisms.

The sequences shared between pRAS3.2 and the *tet*(C) islands include all regions of each *tet*(C) island with the exception of the ISc*s605* element. The most significant differences in the shared sequences include a deletion at the plasmid origin of replication (44 nucleotides) and a deletion upstream of the *tet*(C) start site (8 nucleotides). It is likely that the deletion in the origin of replication blocks the independent initiation of replication within the integrated island. The deletion within the region upstream of *tet*(C), as well as the truncation of *tetR*(C), may affect the regulation of *tet*(C). Transcriptional analysis confirmed that this was the case, as *tet*(C) transcript was detected in *C. suis*-infected cells cultured in the presence or absence of tetracycline. We are examining the regulation of the chlamydial *tet*(C) in a heterologous system to determine which of these changes is responsible for the lack of regulatory control by the chlamydial *tetR*(C).

In each Tc^r strain, the *tet*(C) island is recombined into a precise location within the *C. suis inv*-like gene. Sequence analysis of these and other chlamydial strains demonstrate that several veterinary chlamydial pathogens carry an *inv* homolog, but this gene is commonly truncated or otherwise inactivated (22, 23). The integration of the *tet*(C) island at the *inv*-like gene in these clinical *C. suis* isolates shows that this gene is not required in the *C. suis* system in vivo or in vitro and suggests that the *inv*-like gene may be a target for experiments designed to introduce genes into the chlamydiae.

The occurrence of stable Tc^r in *C. suis* is in contrast to the absence of Tc^r in the human chlamydial strains. This may be a function of the feeding of large amounts of tetracycline and other antibiotics as growth promoters to poultry, swine, and cattle (8, 24). This practice has created an antibiotic gradient that begins with the feed or water source, proceeds through the animal, and is deposited in the soil beneath the facilities (29). It is likely that this practice established an environment where the *tet*(C) islands could be acquired and maintained by *C. suis*.

The identification of the *tet*(C) islands within *C. suis* is the first example of horizontal acquisition of resistance by a strain of obligate intracellular bacteria. In contrast to the many examples of antibiotic exchange in free-living and facultative intracellular pathogens, horizontal acquisition of an antibiotic resistance marker by obligate intracellular bacterial organisms has never been demonstrated. This includes the obligate intracellular pathogens *Coxiella burnetii*, members of the genera *Rickettsia* and *Ehrlichia* (2, 3, 30), and bacteria that are commensals in insects (36). We are working to expand our understanding of this system by investigating the mechanisms associated with the acquisition of *tet*(C) islands by these pathogens and by searching for possible donor bacteria responsible for transmission of *tet*(C) to *C. suis*. We are also examining the possible utility of this system for introducing genes into the chlamydiae, a process presently unavailable to researchers in this field of study.

ACKNOWLEDGMENTS

This work was supported by PHS awards AI42869, AI48769, and NIEHS P30 ES00210 and through USDA ORE00047. Jae Dugan received support through the N. L. Tartar Fund at Oregon State University.

We are grateful to M. C. Roberts (University of Washington) for her evaluation of our data and for editing the manuscript and D. Berg (Washington University in St. Louis) for helpful discussions about IS*cs605*. We also thank T. Stanton and S. Humphrey (NADC, Ames, Iowa) for providing the PCR primers for the various Tc^r determinants.

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