AN ABSTRACT OF THE DISSERTATION OF

JAE DUGAN for the degree of Doctor of Philosophy in Molecular and Cellular Biology presented on March 15, 2007.

Title: Characterization of the Genomic Islands from Tetracycline-resistant Chlamydia suis.

Abstract approved:

Daniel D. Rockey

Tetracycline (Tet)-resistant strains of Chlamydia suis were isolated from swine farms in the Midwest. The isolation of the resistant strains was significant because Tet is an antibiotic used to treat infections by veterinarians and doctors. One of the tetracycline (Tet)-resistant strains, R19, was able to survive in Tet up to 4 µg/ml. This is in contrast to two Tet-sensitive strains C. suis S45 and C. trachomatis L2, which were sensitive to 0.1 µg/ml. Using sequencing and Southern blot analysis, we were able to identify a set of related genomic islands present in the Tet-resistant strains but not in the Tet-sensitive strain. The genomic islands inserted at the same position, interrupting the invasin gene within the chlamydial chromosome. The islands contained the tet(C) resistance determinant, the Tet repressor, and genes found in plasmids in other bacterial species. In addition, the islands also contained an insertion element, termed IScs605. The IScs605 insertion
element was similar to IS605 insertion elements found in *Helicobacter pylori* and contains 2 phylogenically distinct transposases. This was the first identification of a resistance island and an insertion element in any chlamydiae. The insertion element was further characterized by using a mating assay in *E. coli*. The IS element was placed on a high copy plasmid, pUC18, and a medium copy vector, pBBR1MCS, and transposition activity studied. IScs605 was shown to have high transposition activity compared to the negative control, and had a strong preference to insert adjacent to the pentanucleotide 5’-TTCAA. In addition, the orfA gene was found to be essential for transposition activity while orfB was not essential. The transposition activity in *E. coli* provides strong evidence that this IS element was responsible for the integration of the tet(C) genomic islands into the chlamydial chromosome. Collectively, this work identifies the first genomic island in the chlamydiae and is the first investigation of horizontal resistance in any obligate intracellular bacterium. In addition, it is anticipated that the *C. suis* Tet-resistance islands may be useful in the development of a transformation system for the chlamydiae.
Characterization of the Genomic Islands from Tetracycline-resistant
Chlamydia suis

by
Jae Dugan

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APPROVED:

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

____________________________________
Jae Dugan, Author
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Dr. A. A. Andersen was instrumental in isolating and providing the Tet-resistant *Chlamydia suis* used for this work. Michael Nesson was instrumental in sample preparation and taking pictures with the transmission electron microscope at the Oregon State University Electron Microscope facility. Dr. Dan Rockey was instrumental in reviewing this manuscript.
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Chapter 1: Literature Review

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1.1: Basic Biology: Chlamydia

The Chlamydiae are obligate intracellular bacteria that have a unique biphasic developmental cycle, consisting of alternating developmental forms. The elementary body (EB) is an electron-dense extracellular form, approximately 0.3-0.4 μm in diameter, which is infectious but metabolically inactive (55, Fig. 1.1). After EB attachment and entry, the chlamydiae form an intracellular vacuole termed an inclusion. Within the inclusion, the EB matures into a reticulate body (RB), which is the metabolically active but non-infectious form. The reticulate body is less electron-dense and is 0.5-1 μm in diameter (Fig. 1.1). The RB replicate via binary division and, after approximately 100-200 fold amplification, the RB revert to EB that infect new cells (39).
Chlamydiae are ubiquitous and infect many different animal species. In humans, *Chlamydia trachomatis* causes trachoma, the leading cause of preventable blindness worldwide with 150 million affected, 10 million requiring surgery, and 6 million becoming blind (60). The World Health Organization hopes to eradicate trachoma by the year 2020 (59). *Chlamydia trachomatis* is also responsible for the most common human sexually transmitted infection, with 4 million cases per year in the US (2). The prevalence of Chlamydia is a result of the fact that 70-90% of women and 30-50% of men infected with *Chlamydia trachomatis* report no overt symptoms, and thus, do not get treatment (12). However, serious complications can occur from silent infection. Approximately 20-40% of untreated women get pelvic inflammatory disease (PID), a condition where *C. trachomatis* travels from the cervix and infects the fallopian tubes. Eleven percent of women with PID become infertile while 9% have ectopic pregnancies (12). Complications from *C. trachomatis* infection arise primarily from the inflammatory response to infection and not the infection itself (12). Newborn infants can also be exposed to *C. trachomatis* during delivery, causing trachoma or ophthalmia neonatorum (44). Another serious pathogen in humans is *Chlamydia pneumoniae*, causing up to 43% of all cases of community-acquired pneumonia (69). This pathogen has also been associated with atherosclerosis and peripheral artery disease (58).

*Chlamydia psittaci* is a zoonic pathogen which causes psittacosis, a respiratory disease that used to have high mortality rates. There are 200 reported cases each year in the US, with the actual number far higher as a
result of the difficulty of diagnosing psittacosis (31). With antibiotic therapy, the mortality rates are less than 1% compared to 15-20% if left untreated (31). Different chlamydiae also infect koalas, equine, cattle, sheep, goats, swine, cats, guinea pigs, rabbits, mice, and reptiles (23).

Of particular interest to this work is *Chlamydia suis*. Originally classified as a swine strain of *Chlamydia trachomatis*, this pathogen can cause serious infections such as conjunctivitis, pneumonia, necrotizing enteritis, and abortions (33, 50). This organism has been shown to persist for long periods in the intestinal tract and outbreaks of chlamydiosis in pigs have occurred in Eastern Europe because of infected feces (50). The prevalence of Chlamydia in swine at farms in Europe is between 12-30%.

While chlamydia is prevalent throughout the world, chlamydial infection is highly treatable with extremely low rates of treatment failure. The most common antibiotics for treatment of chlamydial infections in humans are azithromycin, a macrolide, or doxycycline, a tetracycline derivative (44). For pregnant women with chlamydial infection, amoxicillin is recommended. Other antibiotics that are also used to treat chlamydial infection are erythromycin, ofloxacin, and levofloxacin. For veterinary chlamydial infections, however, tetracycline is the most common antibiotic used for treatment.

1.2: Persistence

In addition to the two typical developmental forms, EB and RB, an aberrant, or persistent, form can occur. In the presence of many stressing
agents, the RB is inhibited from dividing and becomes abnormally enlarged (30). In penicillin-treated *C. trachomatis* infections, abnormally large RB contained 16 chromosomal copies each, showing that DNA replication continued while bacterial division was inhibited (39). The persistent form is easily noticeable under EM and fluorescent microscopy because of its large size. The characterization of the aberrant form, however, has been identified only under *in vitro* growth conditions and not *in vivo*.

This is a result of the fact that it has been difficult to differentiate between persistence and reinfection in people with *C. trachomatis* infection. Because there is such a large reservoir of untreated people, reinfection with the same serovar cannot be definitively ruled out. Studies using animal models remain to be performed to completely determine whether persistence occurs *in vivo*.

There is considerable evidence showing that persistence plays an important role *in vivo* (19, 30). One set of evidence is the identification of chlamydial DNA and RNA in culture-negative tissue samples (30). When intervening, culture negative, samples were tested by the Ligase Chain Reaction, it showed a positive result, indicating the presence of persistent or, possibly, dead Chlamydia. There was also evidence for persistence of *C. trachomatis* in patients with recurring infections with the same serovar (19). The identification of treatment failures where patients taking drugs that should have killed the Chlamydia also suggest that persistence might be involved. In these cases, the antibiotic would induce aberrant or persistent form, which
would resist any drug concentration. When the patient stops taking the drug, the pathogen would revert and re-enter the normal developmental cycle (30).

The presence of the aberrant form has important implications for \textit{in vivo} growth. It is possible that the aberrant form is a defense mechanism in which the Chlamydia can evade immune responses or survive antibiotic treatment. In the persistent form, Chlamydia can withstand extremely high concentrations of antibiotic even though it is normally susceptible to very low concentrations (64). However, once the persistent form reverts to the wildtype RB, it is still highly susceptible to antibiotics.

\textbf{1.3: Cell Biology}

Most Chlamydiae infect mucosal epithelial cells in the eye, gastrointestinal tract, or respiratory tract (1). However, some Chlamydia, such as \textit{C. trachomatis} L2, can infect phagocytes and disseminate via trafficking throughout the lymph nodes (21). EB attachment and entry into the cell is crucial as the bacteria cannot survive for long periods outside the cell. Attachment and entry is hypothesized to occur in two stages, a reversible binding to heparin sulfate containing glycosaminoglycans followed by an irreversible binding step via an unknown receptor (1). After attachment and phagocytosis into the cell, a type-three-secreted-protein, Tarp (Translocated Actin Recruiting Protein) is exported into the host cell and modifies the actin cytoskeleton (16). Tarp enables the chlamydia to establish the inclusion near the Golgi apparatus and inhibit phagolysosomal fusion (16).
After an EB infects a cell and establishes an inclusion, it starts to transition to the RB. This entails a number of key steps, the first of which is chromosome decondensation (1). Chlamydial chromosomal DNA is condensed by histone-like proteins HctA and HctB. These histone-like proteins give EB the characteristic dark staining under electron microscopy and have been shown to condense DNA in a heterologous E. coli system (7, 29). Chromosomal decondensation through disruption of the histone-DNA complex occurs following accumulation of a methylerythritol phosphate (MEP) metabolite, 2-C-methylerythritol 2,4-cyclodiphosphate (1). As the DNA becomes decondensed, transcription of early-genes starts and enables the transition from the EB to the RB (1). Even though EB are metabolically inactive, they do contain RNA. This carryover RNA, along with newly transcribed message, allows protein expression to occur very rapidly after infection (55).

After numerous rounds of multiplication, the RB begins the transition into the EB form, a process which is also called secondary differentiation. Transcriptional analysis shows that about 70 genes are considered late genes, two of them being hctA and hctB (1). Secondary differentiation requires chromosomal condensation via the Hc1 and Hc2 proteins which also shuts down transcriptional activity (55). It should be stated that the mechanisms that signal the transition from the EB to RB and the RB to EB remains to be discovered. One hypothesis is that the RB attachment to the inclusion membrane and subsequent detachment signals secondary differentiation (55).
Evidence for this scenario is based on the fact that in a number of strains, RB are concentrated at the inclusion membrane while EB are found in the lumen of the inclusion. By the late stages of infection, the inclusion occupies nearly the entire cell. Inclusion lysis and cell lysis are followed by the subsequent release of infectious progeny. Lysis may result from sheer pressure on the cell membrane from the chlamydia itself or perhaps undiscovered lytic enzymes (55). Both EB and RB forms are released from the cell after lysis, as secondary differentiation is not 100%. This is evident in all mature inclusions which still contain RB.

The inclusion membrane is a vital interface between the chlamydia within the inclusion and the host cytosol. Inclusion modification is, therefore, essential for chlamydia to obtain anabolic precursors and prevent phagolysosomal fusion. Proteins found in the inclusion membrane are normally called Inc proteins, with about 90 Inc proteins identified in C. pneumoniae and 36 in C. trachomatis (1). These Inc proteins contain a signature hydrophobicity domain which is necessary for insertion into the inclusion membrane (6). One inclusion membrane protein, IncA, is involved in homotypic vesicle fusion in C. trachomatis infections (56). Another protein, IncG, interacts with the host protein 14-3-3 (61). Because 14-3-3 has more than 100 ligands and is involved in signal transduction, apoptosis, cell cycle control, and vesicle mediated trafficking, it is unclear what function the host protein has on chlamydial pathogenesis (27, 61).
There are also several proteins that have been identified which lack the signature hydrophobicity domain of Inc proteins but still reside in the inclusion membrane (56). The CT529 protein is a CD8⁺ T-cell target and CopN is a type three secretory substrate (56). Each of these inclusions proteins are introduced into the membrane by a functional type three secretion system (24, 25).

1.4: Immune response and chlamydial evasion of immune response

The immune response to chlamydial infection is complex and multifaceted. Studies in mice have shown that protective immune responses to chlamydial infection require both a Th1-CD4⁺ T cell response and a CD8⁺ T cell response (12, 43). The CD8⁺ T-cells produce IFN-γ, a cytokine that has proven to effectively inhibit chlamydial growth (11, 43). The pro-inflammatory cytokine IFN-γ works by activating indolamine 2,3-dioxygenase, resulting in the depletion of tryptophan, an essential amino acid for Chlamydia (13). IFN-γ also works by upregulating iNOS, increasing the levels of the reactive nitrogen intermediate nitric oxide (43). This cytokine can also inhibit chlamydial infection by reducing transferrin receptor levels, causing an iron shortage within the cell (43). The role of B-cells and antibody levels is unclear but studies show that they might be important in resistance to secondary infections (42, 47).

Chlamydia have evolved effective mechanisms to evade host immune responses. Results have shown that chlamydial infection elicits only a small
protective effect and that reinfection with the same serovar is still possible (43). In addition, chlamydia reside in a vacuole that does not fuse with the phagolysosome and excludes proteins from the trans golgi network (10). Therefore, chlamydial antigens are not broken down and presented on the surface with MHC- class II antigens, inhibiting humoral immune responses. Chlamydia also contains LPS that is 100 times less potent at activating host cells and eliciting immune responses (12). In addition, some strains of C. trachomatis can avoid the inhibitory effects of IFN-γ because they contain functional tryptophan synthase genes and can convert indole into tryptophan (13).

1.5: Antibiotic Resistance

The study of antibiotic resistance is an important field that has wide implications for human health. Because of the prevalence of mobile genetic elements and transposases in many organisms, horizontal transfer plays an important role in transferring antibiotic resistance between organisms. Mobile genetic elements often catalyze the movement of antibiotic resistance genes in an environment of strong antibiotic selection. Thus, bacteria can become multi-drug resistant and have a selective advantage in an antibiotic-contaminated environment.
1.6: Tetracycline

Tetracycline (Tet) is a broad spectrum antibiotic that works to block the attachment of the aminacyl-tRNA to the A site of the ribosome, inhibiting protein synthesis. Tetracyclines are a large class of antibiotics that are distinguished by the presence of 4 aromatic rings, with the main differences being a function of the different side chains. Tet is an antibiotic with a long history and is routinely used to treat both Gram-positive and Gram-negative bacteria in both humans and animal infections. Owing to the large number of bacteria that have become resistant to Tet, there are extensive data on the mechanisms of Tet resistance in a wide family of organisms. One mechanism of resistance is associated with the mutation of the drug target, the ribosome. In *Helicobacter pylori*, many strains have become resistant to Tet because of mutations in the ribosomal RNA gene. This is, however, not a common mechanism of resistance. The most widespread process for acquiring resistance is associated with the acquisition of an antibiotic resistance gene via horizontal transfer. Genes transferred in this way encode proteins that work with 3 main mechanisms: drug efflux, ribosomal protection, and Tet degradation. There are many different Tet resistance proteins that work by efflux (15, 54). These resistance genes encode a membrane-associated pump that uses protons to power the movement of Tet molecules out of the cell, reducing the intracellular drug concentration and enabling protein synthesis.

There are 23 different families of efflux pumps, with some examples being *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H) (54). One important
determinant, tet(C) is an efflux pump that is found in many different bacterial species. The tet(C) resistance determinant has been identified in 12 different bacterial species, with the newest addition being Chlamydia. The discovery of the newest addition is discussed in Chapter 3. This marker is found on a number of common plasmids such as pSC101 and pBR322. The tet(C) resistance gene is present only on low copy plasmids because of the resistance protein’s toxicity at high levels. In efflux pumps, a repressor gene is always adjacent to the resistance gene. In the absence of antibiotic, the repressor protein binds to the tet operator region and shuts down transcription of the efflux pump. In the presence of Tet, the antibiotic binds to the repressor protein and prevents the protein from binding to the tet operator region. This allows transcription of the resistance gene. The meticulous gene regulation is essential for allowing the expression of the resistance only when it is needed and preventing toxic levels of the membrane-associated resistance protein from building up in the bacteria.

Another mechanism of resistance is ribosomal protection where the protein allows the attachment of the aminoacyl-tRNA to bind to the acceptor site in the presence of Tet. Further research is being conducted to determine the precise steps involved in protection by the 11 ribosomal protection genes that have been identified (54). Unlike efflux pumps, ribosomal protection proteins reside in the cytoplasm and appear to have a different type of gene regulation. For ribosomal protection genes, regulation occurs through the 400-
bp region that is upstream of the resistance gene without the presence of any repressor. The mechanism of regulation in this system is currently unclear.

There are also 3 examples of resistance genes with a mechanism involving the enzymatic inactivation of the antibiotic. These proteins use oxygen and NADPH to modify and inactivate the tetracycline. These genes have only just been identified and considerable work remains in understanding their origin and prevalence in bacterial populations (54).

1.7: Antibiotics as feed additives

A common approach used by farmers is to use antibiotics as feed additives for the prevention of disease and for growth promotion. This approach started during the 1930’s when antibiotics were first being used (22). Following their discovery and introduction for treatment of veterinary diseases, farmers noted that one side effect of using antibiotics was that the animals also grew faster. This growth promotion effect was first identified when chickens were fed fermentation waste from tetracycline production (22). Chickens that were fed the fermentation waste grew faster than controls and later studies showed this was a result of residual levels of Tet in the waste. The use of sub-therapeutic levels of antimicrobials was adopted as feed additives over long periods to enhance growth rates, egg production, litter sizes, and milk yields (22). The reason why antibiotics enhance growth may be a consequence of reduced bacterial loads and ability to control enteric diseases (22). This is reinforced by evidence in Sweden where, after
antibiotics were banned as feed additives, piglets had serious problems with weaning diarrhea (70). An increase in antibiotic use was required to help the piglets and reinforced the hypothesis that antibiotics worked by preventing disease (70). There is growing concern that the use of antibiotics as feed additives has increased the number of multi-drug resistant organisms. Since mobile genetic elements are found in many different bacteria, transfer of antibiotic resistance genes would be strongly selected for in this environment. There have been several studies showing high levels of antibiotic resistance and that it continues to increase over the years. When E. coli isolates were obtained from various farm animals, 31% were found to be highly resistant to Tet (3). Salmonella typhimurium isolated from pigs were found to be resistant to multiple antibiotics with streptomycin, sulfadiazine, and Tet resistance being the most prevalent. Resistance to kanamycin, gentamicin, ampicillin, and choramphenicol occurred at a lesser frequency (4). The number of resistant isolates was also greater in 2001 than in 1993 (4). This has ramifications for human health, as there is an increased chance that multi-drug resistant organisms from farm animals can be transferred to humans or the resistance genes can be spread to human pathogens. This makes treatment of infections much harder and increases the chances for treatment failure.

After the ban on the use of antibiotics as feed additives in Europe, there is now a trend to use probiotic microbes such as Enterococcus faecium to help animals fight off infection. Probiotics have been shown to work and would reduce the need for antimicrobial compounds (50).
1.8: Antibiotic Resistance in Chlamydia

There have been several reported cases of apparent drug resistance in both human and reference chlamydial species. *Chlamydia trachomatis* isolated from patients with treatment failures were shown to be resistant to Tet, doxycycline, erythromycin, sulfamethoxazole, clindamycin, and azithromycin during *in vitro* analysis (32, 41, 45, 62). However, resistance was lost when the Chlamydia were grown in the absence of the antibiotic and retested. In the case of azithromycin resistance, mutations were detected in the 23s rRNA gene of the isolates that had the potential for allowing resistance (45). However, the mutations reduced viability and were unable to grow in the absence of drug, leading to questions as to the nature of the resistance (45).

One reason for the identification of treatment failures is a result of “heterotypic” resistance, which is defined by only a small subpopulation being resistant. This “heterotypic” resistance identified in people with treatment failures was not elucidated and still remains to be clarified. Heterotypic resistance and treatment failure suggests the possibility that the persistent Chlamydia could play an important role in resistance to antibiotics and reinfection after the person stops taking the antibiotic. Work by Suchland *et al.* (64) showed that *Chlamydia trachomatis, Chlamydia pneumoniae*, and *Chlamydia psittaci* can, depending on several variables, survive greater than 100 fold MIC when incubated with doxycycline, azithromycin, erythromycin, ofloxacin, and Tet.
However, a retest of the same organisms did not result in an increased MIC for any of the strains. To date, there has been no definitive evidence of homotypic resistance identified in clinical chlamydial isolates from humans. However, resistance can be produced against drugs such as rifampin, ofloxacin, lincomycin, trimethoprim, spectinomycin, and levofloxacin using in vitro culture techniques, leaving the possibility that resistance is possible in vivo (Binet, 2005). In these cases, resistance is acquired by genetic mutation and not via horizontal transfer of a resistance gene. In a recent paper by Demars et al. (20), two C. trachomatis strains that were resistant to either ofloxacin or lincomycin were co-infected. After incubation in the presence of both ofloxacin and lincomycin, progeny that were resistant to both lincomycin and ofloxacin were isolated. Sequencing of the 23s rRNA and gyrA showed that mutations conferring resistance in the double mutants were the same as the single mutants, strongly suggesting that lateral gene transfer had occurred between the two strains. It cannot, however, be completely ruled out that the double mutants resulted from new mutations that were selected for in the presence of the antibiotic.

### 1.9: Mechanisms of Horizontal Transfer

Horizontal transfer is an important mechanism of genomic evolution and pathogenesis, allowing the transfer of antibiotic resistance genes, entire secretion systems, and other virulence factors from one organism to another organism. There are basically three types of horizontal transfer systems:
transformation, conjugation, and transduction. Transformation is the process whereby naked DNA in extracellular solution is uptaken into the bacteria where it is then propagated. The first transformation experiment was conducted by Avery in 1944 in which he transformed unencapsulated pneumococcus with a purified mixture from heat-treated encapsulated pneumococcus and obtained live encapsulated pneumococcus (5). These researchers were also able to prove for the first time that it was DNA that was responsible for the transformation phenomenon. Conjugation is the process where a “donor” bacterium transfers DNA into a recipient bacterium via direct contact. There are many examples of conjugative plasmids in bacteria, one of which is the F-plasmid. The 100 kb F-plasmid was the first identified conjugative plasmid and is one of the most studied. This plasmid contains 37 genes that are involved in conjugation plus IS3 and IS2 insertion elements (26). There are two copies of IS3, IS3a and IS3b, that represent the ends of the Tn1000 transposon. The F-plasmid not only can conjugate and transfer to other bacteria, it can also use the IS elements to mediate integration into the chromosome. When integrated into the chromosome, the F-plasmid can lead to the conjugal transfer of chromosomal genes and these are named "Hfr" or high frequency of recombination strains. A derivative of the F-plasmid lacking the IS elements, pOX38, was used for the mating assays described in chapter 4 of this thesis. This plasmid was useful because it is fully conjugative but cannot facilitate integration events on its own.
Conjugative plasmids can also facilitate the transfer of other plasmids containing a compatible origin of transfer from a donor bacterium to a recipient bacterium. “Mobilizable” plasmids do not contain the transfer genes necessary for conjugation by themselves and require a conjugative plasmid such as the F-plasmid for transfer between bacterium. An example of a mobilizable plasmid that can be transferred by the F-plasmid is pRAS3.2, a plasmid found in the fish pathogen *Aeromonas salmonicida* (38). This plasmid, surprisingly, is the backbone of the genomic islands conferring Tet resistance to *C. suis* that are described in Chapter 3 of this thesis.

Transduction is the process whereby bacteriophages transfer DNA from one bacterium to another bacterium. In the classic example of transduction, a filamentous bacteriophage containing the cholera toxin was able to infect *Vibrio cholera* via the toxin-coregulated pilus (68). The bacteriophage, CTXφ is 7-9.7 kb in length and contains 10 genes, two of which are cholera toxin A and B (68). *Vibrio cholerae* strains containing the bacteriophage with the cholera toxin are fully virulent while strains without the phage are not virulent.

### 1.10: Mobile Genetic Elements

Mobile genetic elements are segments of DNA carrying genes that catalyze the transfer of the DNA segment to another location. Important mobile genetic elements are insertion elements, transposons, genomic islands, and pathogenicity islands. Insertion elements (IS) are segments of DNA encoding only transposases that can catalyze the movement of the IS element to
another location. Transposases usually do not require any sequence homology for DNA integration to occur (18). This is in contrast to recombinases, which are enzymes that mediate DNA insertion at homologous sequences (18). Transposons are segments of DNA that contain insertion elements and other genes, commonly encoding antibiotic resistance or other virulence factors. Typically, transposons contain more than 1 insertion element, one at each end, and it is the transposase within the IS element that is responsible for transposition. Transposons enable the movement of virulence factors between species especially if they are positioned on conjugative plasmids. This is because transposons can only move within the genome of an organism while conjugative plasmids enable movement of transposons between organisms.

Genomic islands are segments of the genome that were horizontally acquired from another organism (28). Typically, genomic islands arise from mobile plasmids or bacteriophages that somehow integrated into the genome (28). Pathogenicity islands are types of genomic islands that contain virulence factors essential for pathogenesis. Genomic islands can be differentiated by differences in GC content and codon usage bias, presence of mobile genetic elements, and phylogenetic analysis (28).

Mobile genetic elements are found in high numbers in many different organisms and have had a profound impact on genomic evolution. These elements allow lateral gene transfer of collections of genes, and therefore facilitate a much faster way to mutate than single nucleotide changes. In
addition, the integration of mobile genetic elements can occur anywhere in the genome, possibly knocking out or altering the function of many chromosomal genes (40).

1.11: Structure and Function of Transposases

There are 5 types of transposases, each of which has a unique method of catalyzing transposition. They are DDE-transposases, reverse-transcriptase/endonucleases, Tyrosine (Y)-transposases, serine (S)-transposases, and rolling circle transposases. The most common and well-understood transposases are the DDE-transposases, which are characterized by the catalytic DDE motif (18).

Of particular interest to this work are the rolling circle transposases, which are phylogenically related to proteins involved in rolling circle replication. Rolling circle transposases are defined by containing the strongly conserved YxxxY motif and a His-hydrophobic-His metal binding domain (18). In plasmids, the insertion elements containing rolling circle transposases are defined by their lack of inverted or direct repeats, differing sequences at each end of the IS element, and a nick site for transposition at conserved target sequences (18). Rolling circle replication begins when one strand is nicked and 3’-end is displaced, being replaced by the extending 5’-chain. As the displaced strand grows, it becomes the template for the complementary strand. This can result in concatemers if replication is not terminated, since
plasmids are circular (18). In Chapter 4 of this thesis, we identified concatemer formation caused by the transposition activity of IScs605.

In viruses, concatemers are cut and packaged into capsid by specific proteins (18). Typically, in other classes of transposases, the insertion elements contain inverted 9-40 bp repeats at its ends, and the transposase generate direct repeats of 2-9 bp at the target site after transposition has occurred (34). For IS91, the most well-known Y2 or rolling circle transposase, the nick site occurs at the conserved tetranucleotide sequence 5’-CTTG or 5’-GTTC.

1.12: The IS605 family of insertion elements

The IS605 family of insertion elements, of which the IS element described in Chapter 3 and 4 is a member, has strong resemblances to the Y2 or rolling circle class of transposases (34-37, 67). The IS605 family of insertion elements is characterized by not containing inverted or direct terminal repeats, the presence of 2 phylogenically different transposases, and a strong preference for sequence specificity at the target site. IS605 was the first insertion element to be identified in Helicobacter pylori, a bacterium that causes gastric ulcers. The two transposases in the IS element share identity with IS200 and IS1341 transposases. Although every member of the IS605 family encode 2 different transposases, all of them apparently require only one of them for transposition. In addition, there have been no reported identification of any IS200 or IS1341 transposase being found independently
in *Helicobacter pylori*, strongly suggesting that there is selective pressure for both transposases to stay together. The Chlamydial insertion element discussed in Chapter 4 is homologous to the IS605 family of IS elements.

There is variation in target sequence for the different members of the IS605 family. The insertion element IS605 has insertion targets to the sequence 5'-TTTAA or 5'-TTTAAC adjacent to the *orfA* (IS200) end (34). The IS606 element has a similar target sequence of (5'-TTTAT). Both IS605 and IS606 show no target specificity at the *orfB* (IS1341) end (34). The insertion element IS607 is slightly different, in that IS607 OrfB had identity at the protein level with the IS605 OrfB while IS607 OrfA had homology to IS1535, a *Mycobacterium tuberculosis* resolvase. The insertion element has an insertion specificity for transposing between or adjacent to GG nucleotides and generate a 0 or 2-bp target site duplication. It was also shown that *orfA* was found to be essential while *orfB* was not necessary for transpositional activity (36).

ISHp608 also contains two open reading frames with OrfA being identical to IS605 OrfA while OrfB had identity to the Salmonella virulence factor, GipA. ISHp608 has some unusual transpositional properties in that it produces simple insertions, inserted with the *orfA* next to the sequence 5'-GTAA, and required orfA but not orfB (37). In comparison, IS605, IS606, and IS607 produces cointegrates where the insertion element transposed the entire plasmid plus the IS element into the target site. For ISHp608, only the IS element and not the contiguous plasmid sequence, was transposed into the
target site. Like IS605 and IS606, ISHp608 did not result in target site duplication.

ISHp609 is the latest insertion element found in Helicobacter pylori (35). This element contains 4 open reading frames, two of which, OrfA and OrfB, have identity to the OrfA and OrfB of IS605 and IS606. The other two small orfs, jhp960 and jhp961 encode proteins that have no known function. Sequence analysis of the Helicobacter pylori genome showed that ISHp609 has a preference to insert next to the sequence TAT. However, because no work in a heterologous E. coli system was performed to test transposase activity, it remains unclear if there is insertion site specificity, or whether orfA and orfB are necessary for transposition.

All five Helicobacter sp. IS elements had high levels of distribution within Helicobacter pylori, occurring in between 14-43% of strains. In some cases, multiple copies of individual IS elements are present in single strains. It is important to note that all of the functional analyses of the insertion elements were performed in E. coli and not in either Helicobacter pylori or, in our case, Chlamydia suis.

Structural analysis demonstrated that the IS200-like transposase has close similarity to the rolling circle family of transposases. When the IS200-like transposase protein was aligned from 7 different organisms containing IS605-like insertion elements, there was a number of conserved residues and motifs (57) (Fig. 1.2). This included the His-hydrophobic-His motif that is also found in rolling circle transposases. However, only 1 conserved tyrosine was present.
and protein crystallography analysis of the ISHp608 IS200 transposase showed that it is in contact with the His-hydrophobic-His motif. To determine the importance of the conserved tyrosine and the two Histidine amino acids in the His-hydrophobic-His motif, the three amino acids were mutated and mating out assays performed to determine transposition activity. The results showed that the mutations to the conserved tyrosine and the two Histidine residues lead to transposition activity that was similar to negative controls while mutations of other tyrosines had no effect. This showed that the conserved tyrosine and the His-hydrophobic-His were essential for transposition (57). The active site for transposition required the conserved motif that binds a Mg$^{2+}$ ion and the conserved tyrosine (57). The IS200 transposases also contain a Helix-turn-Helix domain, which is found in the DNA binding domain. As shown by Ronning et al., the C. suis orfA encoded transposase is a homolog of these enzymes.
IS200 contains a small transposase that is commonly found in Gram-negative bacteria including Salmonella, *E. coli*, and Shigella (8). In the well-characterized Salmonella IS200, it is approximately 710 bp in length with the transposase gene coding sequence being 453 bp. This IS element has an AT rich insertion specificity and has been known to duplicate 2-bp at the target site (8).

### 1.13: Chlamydial Plasmids and Bacteriophages

Extrachromosomal elements and bacteriophages have been identified in a number of chlamydial species. Almost all *Chlamydiae* carry a 7.3-7.5 kilobase pair cryptic plasmid that encodes 8 highly conserved open reading
frames. Because of the high similarity between plasmids, it was originally thought that the plasmid contained essential genes for chlamydial growth (17). However, clinical strains of *Chlamydia trachomatis*, *C. psittaci*, and *C. pneumoniae* have been identified that lack the cryptic plasmid, throwing into question the role of the encoded proteins (46). Tested plasmid-free strains have the same apparent phenotype *in vivo* and *in vitro* as *C. trachomatis* strains containing the plasmid (46, 49). The plasmid contains 4 repeats of 22 bp that act as the origin of replication, and has a copy number of 7-10 copies/cell (49). Since there is no genetic manipulation system for chlamydia, understanding the plasmid maintenance and replication systems has taken on considerable importance.

It is unclear why chlamydia, which has one of the smallest genomes, would keep a 7.5 kb plasmid that seems to have no essential genes or role in pathogenicity. Analysis of the plasmid open reading frames shows that 4 encode proteins with homology to DNA replication, partition, and integration proteins (Table 1.1). These proteins are most likely involved in plasmid partitioning and replication. The other 4 open reading frames encode proteins that have no homology to proteins in the sequence database. The absence of a genetic system has made the study of the plasmid in pathogenesis, and chlamydial biology in general, extremely difficult.
Table 1.1: C. trachomatis serovar A plasmid open reading frames. Phylogenetic analysis of all 8 open reading frames, with 4 having functional homologues. Phylogenetic analysis of the other 4 proteins was negative and labeled as hypothetical proteins (14).

<table>
<thead>
<tr>
<th>Orf</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGP1-D</td>
<td>DnaB (Replicative DNA helicase)</td>
</tr>
<tr>
<td>pGP2-D</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>pGP3-D</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>pGP4-D</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>pGP5-D</td>
<td>ParA ATPase (Partition Protein)</td>
</tr>
<tr>
<td>pGP6-D</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>pGP7-D</td>
<td>integrase (DNA breaking/rejoining enzyme)</td>
</tr>
<tr>
<td>pGP8-D</td>
<td>integrase (DNA breaking/rejoining enzyme)</td>
</tr>
</tbody>
</table>

In 1982, the first evidence of a bacteriophage in Chlamydia psittaci was reported (63). Subsequently, Chlamydial bacteriophages were identified in Chlamydia abortus and Chlamydia pneumoniae. In Chlamydia psittaci, the Chp1 bacteriophage is a 4.8 kb ssDNA virus that contains 12 open reading frames. The bacteriophage is classified as a member of the microviridae and attaches to the EB developmental form in solution. As the EB infects and develops into the metabolically active RB form, the phage becomes active and begins to replicate, eventually lysing the Chlamydia. Some laboratories have attempted to use the bacteriophages as a tool in genetic transformation of Chlamydia, with no success to date.
In obligate intracellular organisms, the process of lateral gene transfer is greatly reduced compared to facultative or free-living organisms. This is a result of intracellular organisms having little contact with other organisms and therefore, having little chance for DNA exchange. However, there are reported instances of lateral gene transfer in a number of different obligate intracellular organisms (Table 1.2). Based on analysis of genome sequence data, chlamydia has very nearly the lowest number of apparent horizontal transfer events, reinforcing the challenge of developing a genetic transformation system (Table 1.2).
Table 1.2: List of all mobile DNA elements found in both obligate intracellular bacteria and facultative intracellular bacteria (9). In obligate intracellular bacteria, the number of mobile DNA genes are far less than in many facultative bacteria. The only exception is Wolbachia, which contains 123 genes.

<table>
<thead>
<tr>
<th>Species</th>
<th>Total Genes</th>
<th>Mobile-DNA genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Wigglesworthia glossinidia</em></td>
<td>653</td>
<td>0</td>
</tr>
<tr>
<td><em>Chlamydia trachomatis</em> serovar D</td>
<td>935</td>
<td>1</td>
</tr>
<tr>
<td><em>Chlamydia caviae</em></td>
<td>1013</td>
<td>1</td>
</tr>
<tr>
<td><em>Chlamydia pneumoniae</em></td>
<td>1120</td>
<td>1</td>
</tr>
<tr>
<td><em>Rickettsia prowazekii</em></td>
<td>1041</td>
<td>5</td>
</tr>
<tr>
<td><em>Coxiella burnettii</em></td>
<td>2096</td>
<td>37</td>
</tr>
<tr>
<td><em>Wolbachia pipientis</em></td>
<td>1271</td>
<td>123</td>
</tr>
<tr>
<td><em>Salmonella enterica</em></td>
<td>5165</td>
<td>80</td>
</tr>
<tr>
<td><em>Shigella flexneri</em></td>
<td>5155</td>
<td>593</td>
</tr>
</tbody>
</table>

1.15: Published attempts at Transformation

There have been two published papers detailing successful transformations in Chlamydia, although in each case, it was transient in nature. The first published effort was by the laboratory of Priscilla Wyrick (66). The approach was to insert a promoterless chloramphicol resistance gene next to a promoter from the *C. trachomatis* serovar E plasmid and electroporate the construct into *C. trachomatis* EB. The chlamydial plasmid promoter was postulated to drive *cat* gene expression and confer
chloramphenicol resistance. The chlamydial plasmid origin of replication was also present in the plasmid construct for electroporation into Chlamydia. The electroporations were performed with a 0.2 cm cuvette in a BioRad Gene Pulser and the conditions used for transformation were 2 kV, 400 Ω resistance, 25 µF capacitance. Transformants were grown in the presence of 5 µg/ml chloramphenicol and 1 µg/ml DNase. The DNase was used to degrade any free plasmid that might be in solution and prevent false positives when analyzing possible transformations. After electroporation, EB were incubated without chloramphenicol for 18 hours before drug selection for transformants occurred. The results showed that there were chloramphenicol resistant Chlamydia with cat DNA present that did not degrade with DNase. The cat gene was shown to be present by fluorescent microscopy and DNA slot blots plus a biphasic liquid scintillation assay was performed to show that the resistance gene was enzymatically active. However, passaging of the transformants in the presence of chloramphenicol two times resulted in a large reduction in the number of resistant Chlamydia. Only small inclusions were present, signaling an absence of resistance. DNA-based analysis indicated that transformation with the plasmid construct was transient and was lost after 2 passages.

The second published protocol was performed by O'Connell and Maurelli (48). A shuttle plasmid was created containing the R6K origin of replication, a kanamycin resistance gene, ompA1: choramphenicol resistance open reading frame, and the Tn10 transposon (48). The transposon was
designed to allow for transposition of the plasmid in \textit{E. coli} and, eventually, in the \textit{Chlamydia}. This shuttle plasmid was then used to randomly mutagenize a pBluescript plasmid containing the entire \textit{C. trachomatis} L2 plasmid. The pool of random mutants were selected with ampicillin and kanamycin. Plasmid DNA was produced from the pool of random mutants and then digested with an enzyme that would produce a restriction fragment containing the L2 plasmid and the transposed shuttle plasmid, but lacking the pBluescript plasmid. The digests were then religated and transformed back into \textit{E. coli} for propagation and clonal expansion. The L2 plasmid:shuttle plasmid cointegrates were then introduced into \textit{C. psittaci} strain Cal 10, which lacks any plasmid, and \textit{C. trachomatis} serovar L2 through electroporation. The electroporation conditions used were 1.8 KV, 25 µF capacitance, 200Ω resistance in a 0.1 cm cuvette and a BioRad Gene Pulser. After electroporation of the EB, chlamydia were then used to infect McCoy cells and incubated in the absence of antibiotic overnight. Chloramphenicol was added to the media to select for transformants and then incubated for 14 days. In the end, they were able to clonally isolate and expand several transformants. However, the plasmids that were selected tended to be unstable and transformants lost their drug resistance when grown in the absence of drug.

There are a number of important lessons that were learned from the published attempts at transformation. In both efforts, the chlamydial plasmid was utilized and chloramphenicol was the preferred selectable marker. It is unclear whether chloramphenicol is an ideal selectable marker and whether
the chloramphenicol resistance gene can be stably expressed in Chlamydia. In the future, other drug markers should be chosen and tested. Ideally, it will be a drug to which a resistance phenotype is stably expressed and maintained in the chlamydia. The other lesson is that little information is known about the chlamydial plasmid and which open reading frames are essential for plasmid partition and replication. Any insertion of a drug marker into a chlamydial plasmid for propagation in Chlamydia will be complicated by this lack of knowledge. Another important lesson is that there is a big difference between transient and stable transformation. Transient transformation is when the foreign plasmid is either not maintained or deleterious, ultimately resulting in loss of the plasmid and resistance.

1.16: Transformation of Coxiella and Rickettsia

While there have been no successful method to genetically manipulate Chlamydia, there have been successful experiments in other obligate intracellular bacteria. In both Coxiella and Rickettsia, electroporation has been used to successfully transform and confer antibiotic resistance. The first Rickettsiae to be transformed was *Rochalimaea quintana* which causes trench fever (53). Rochalimaea was electroporated at a field strength of 12.5 kV/cm, 400 resistance, 25 µF capacitance with a variety of different plasmids. Successful transformations involved plasmids containing either the RK2 or RSF1010 origin of replication while plasmids containing pMB1, coIE1, F, or p15A origin of replications failed to produce any transformants. *Rickettsia*
*prowazekii* is an obligate intracellular bacterium that causes typhus and resides in the cytoplasm. Stable rifampin and erythromycin resistant transformants were produced by electroporation in a 0.1 cm cuvette and 1.7-2.4 KV, 129 Ω resistance, 50 µF capacitance (51, 52). Successful transformations occurred between 20-33% of electroporation attempts (51, 52).

*Coxiella burnetii* is an obligate intracellular bacterium that causes Q fever and also contains developmental forms similar to chlamydial EB and RB (65). A 5.8 kb Coxiella chromosomal fragment, called the autonomous replicating sequence (ARS), was inserted into pBluescript and electroporated into Coxiella at a field strength of 12.5 – 16 KV/cm for 6, 12, or 24 ms (65). The autonomous replicating sequence (ARS) enabled the stable maintenance of the plasmid as electroporations with plasmids lacking ARS did not produce any transformants (65). The efficiency of transformation was calculated as 1 x 10^6 ifu/ µg of DNA.

### 1.17: Technical challenges of chlamydial transformations

Chlamydia has 2 characteristics that make transformation difficult. It is an obligate intracellular pathogen with 2 different developmental forms and the extracellular form is metabolically inactive. The EB form is much more compact and DNA is tightly bound by histones, making stable transformants much more difficult to achieve. Any introduced DNA may get degraded during the transition from the EB to RB. This is in contrast to Rickettsia which has
only one developmental form. Getting DNA into the RB form is also very
difficult as there are 4 membrane layers to go through. Another limitation is the
isolation of individual transformant clones. Any transformed chlamydia will
have to be isolated and clonally expanded, which may take days or weeks to
perform. Another major challenge is the persistence of chlamydia. Chlamydia
can recover from the persistent form after removal of the agent responsible for
inducing persistence. Thus, there is a possibility of obtaining false positives.
Work by Suchland et al. showed that Chlamydia could be made to appear
antibiotic resistant when they were still susceptible (64).

Of importance is the fact that C. suis can reside in the swine intestine
and spread by the fecal-oral route (50). Since the gastrointestinal tract
contains a large number of bacteria that are potential sources of lateral gene
transfer, it would enable C. suis greater opportunity for obtaining foreign DNA
compared to the conjunctiva or genital tract.

1.18: Concluding Remarks

The purpose of studying the Tet resistant strains of Chlamydia suis was
to determine the mechanism of resistance and horizontal acquisition. The
following chapters demonstrate our success in this area. Determining the
mechanism of resistance enables us to understand what genes are involved,
whether it is on a plasmid or chromosome, and find other associated genes.
Horizontal acquisition is important in determining how the foreign sequence
entered chlamydia. There is evidence to suggest that the genomic islands
were horizontally acquired, because it has not been identified in any other chlamydiae, ruling out the possibility that it is an ancestral sequence that was selected for in swine. Ultimately, understanding resistance and horizontal acquisition gives us a potentially strong tool in establishing a genetic transformation system in chlamydia.

1.19: References


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Chapter 2: Analysis of Tetracycline Resistance of R19, S45, and L2

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2.1: Introduction:

The Chlamydiae are obligate intracellular pathogens with a biphasic lifecycle consisting of the extracellular or elementary body (EB) form and the intracellular or reticulate body (RB) form. *Chlamydia trachomatis* is a serious human pathogen, causing both trachoma and a spectrum of common sexually transmitted infections. *Chlamydia suis* infects swine, causing conjunctivitis, pneumonia, necrotizing enteritis, and abortions.

Previously, results by Andersen et al (1) identified isolates of *Chlamydia suis* from swine that are resistant to tetracycline (Tet) and sulfadiazine. The Tet-resistant strains of *Chlamydia suis* are the only example of stable, homotypic resistance identified in chlamydiae. Since Tet is a leading antibiotic used to treat chlamydial infections in humans and animals, the acquisition of Tet-resistance by chlamydiae has enormous clinical significance.

In the presence of stressing agents, chlamydia can convert to the aberrant or persistent form which is different from the normal EB and RB forms. The differentiation from RB to EB is blocked, creating an enlarged RB (6). *In vitro*, persistence occurs when chlamydia-infected cells are treated with penicillin and IFN-γ, and other stressing agents, and there is evidence to suggest that it is a factor in chlamydial infections in humans. *In vitro* work has shown that persistent chlamydia are resistant to levels of many different antibiotics that are well beyond the MIC used to kill the bacterium. This high level of resistance is hypothesized to result from a persistent, possibly inert, developmental form.
In this chapter, I further characterized the Tet-resistance of *Chlamydia suis* using fluorescent microscopy, electron microscopy, Real-Time PCR, and infectious EB-output experiments. The Tet-resistant R19 was compared to 2 Tet-sensitive strains, *Chlamydia suis* S45 and *Chlamydia trachomatis* L2. R19 grows in the presence of much higher concentrations of Tet than S45 or L2. However, for all strains, as the chlamydia nears the inhibitory concentrations, they become persistent. This persistence is visualized in both fluorescent and electron microscopy images as large aberrant RB. Thus, the Tet-resistant strain R19 responds similarly to the 2 tested Tet-sensitive strains in becoming aberrant, albeit at much higher concentrations of Tet.

### 2.2: Materials and Methods

**Chlamydial Strains.** All studies were conducted with *Chlamydia suis* strains S45 and R19, and *Chlamydia trachomatis* strain L2. Concentrated EB were obtained by mechanical disruption of infected cell lysates and ultracentrifugation of lysate through a Hypaque-76 (Nycomed) gradient. Purified pellets were resuspended in sucrose phosphate glutamine (SPG: 18 mM Na$_2$HPO$_4$, 220 mM sucrose, 5 mM L-glutamate).

**Cell culture and chlamydial infection.** All work was performed using either HeLa 229 or McCoy cells grown at 37 °C in a humidified 5% CO$_2$ environment. Cells were cultured in Minimal Essential Media, 10% FBS, 10 µg/ml gentamicin, and 5 mM L-glutamine (MEM-10). For all infections, EB’s were
thawed, diluted in SPG and added to 6-well or 24-well trays containing monolayers of cells. Inocula were centrifuged at 750 g at room temperature for 1 hour. The supernatant was then discarded and MEM-10 added to the wells. The infected monolayer of cells were incubated for 30 hours prior to analysis.

**Fluorescence microscopy in increasing concentrations of tet: R19, S45, L2**: McCoy cells were inoculated in 24-well trays containing glass coverslips and grown to 100% confluency (4.5 X 10⁵ cells/well). Coverslips were infected with R19, S45, and L2 at a Multiplicity of infection (MOI) = 1. Tet was added 6 hours post infection (hpi) at concentrations between 0-5 µg/ml, as indicated. At 30 hpi, coverslips were fixed with methanol for 20 minutes before washing with PBS and then fluorescent antibody (FA) block (2% BSA, 190 mM Na₂HPO₄, 150 mM NaCl) was added to coverslips. Primary antibody against either Hsp60 or MOMP was added to coverslips and incubated for 1 hour at room temperature. These antibodies label the chlamydial cytoplasm and outer membrane, respectively (2, 3). FA block with primary antibody was removed and coverslips were then washed with 1X PBS 3 times before appropriate secondary antibody was added, conjugated with either Rhodamine (red fluorescence) or FITC (green fluorescence). Coverslips were incubated in secondary antibody for 1 hour at room temperature. The coverslips were then washed 3 times with 1 X PBS. Coverslips were mounted on microscope slides in a drop of 4’,6-diamidino-2-phenylindole (DAPI), a fluorescent dye that binds DNA. All pictures were taken using a Leica DMLB fluorescent microscope.
Output experiments in the presence of tetracycline. McCoy cells were inoculated in 24-well trays at $1.2 \times 10^5$ cells/well. Cells were infected with R19, S45, and L2 at an MOI = 1 by centrifugation at 750 g. At 6 hpi, Tet was added to wells. At 30 hpi, media were removed and 500 µl sterile water was added to each well and cells lysed while on ice for 5 minutes. The solution was then pipetted up and down to further disrupt the cells and then put in 1.5 ml microfuge tubes containing 500 µl Hanks Balanced Salt Solution (HBSS). After mixing the lysate and HBSS, the mixture was added to new 24-well tray containing sterile glass coverslips with a monolayer of $1.2 \times 10^5$ McCoy cells and centrifuged at 750 g for 1 hour. This secondary infection was incubated at 37 °C for 30 hours in the absence of tetracycline. Cells were then fixed with methanol and inclusions were quantified by fluorescent microscopy. Enumeration of inclusions was performed at 40 X magnification by randomly sampling 10 field of views and calculating the average number of inclusions per field of view. The average was multiplied by $10^{19}$ to determine the total number of inclusions on the coverslip (i.e. in the well). Based on the dilution of the inoculum, a titer was calculated for every treatment condition analyzed.

Incubation of chlamydia in cell-free wells. S45 and L2 elementary bodies were added to individual wells in a 24-well tray containing no McCoy cells and centrifuged at 750 x g. Lysate was removed and replaced with MEM-10. Trays were incubated for 30 hours at 37 °C in the presence of various
concentrations of Tet. Wells were then rinsed with HBSS and then 500 µl was
sterile water added to each well. Trays were put on ice for 5 minutes and then
500 µl HBSS added to wells. The lysate was then used to infect glass
coverslips containing 1.2 x 10^5 McCoy cells and enumerated by fluorescent
microscopy.

**Electron microscopy.** McCoy cells were infected with R19, S45, or L2 at an
MOI = 1 and incubated in the presence of Tet at various concentrations (0-5
ug/ml) for 30 hours. At 30 hpi, infected monolayers in 6-well trays were
washed with PBS and then trypsinized for 30 minutes. Trypsinized cells were
then transferred to 1.5-ml microfuge tubes and centrifuged very gently (200 X
g). The trypsin solution was discarded and the pellet washed with 1 x PBS and
centrifuged at 200 X g. The PBS was removed and cell pellets were fixed in
2.5% glutaraldehyde, 1% paraformaldehyde and 0.1 M cacodylate buffer (pH
7.4) for 2 hours at room temperature (7). The cell pellets in fixative were then
incubated at 4°C prior to embedding in resin. The samples were then rinsed
three times in fixative, then stained in 1% osmium tetroxide and 0.1 M
cacodylate buffer for 2 hours at room temperature. The pellets were then
dehydrated in a graded series of acetone and embedded in Spurr’s resin,
which polymerized at 60 °C for 30 hours. Ultrathin sections, 60-80 nm in
thickness, were cut with a diamond knife and stained with Daddow’s double
lead stain technique with lead citrate and uranyle acetate (5). Sections were
examined on a Philips CM12 TEM at 60 kV.
**Real Time PCR.** McCoy cells were added to 6-well trays and infected with R19 or S45 at an MOI = 1. After 6 hpi, tetracycline was added at indicated concentrations. At 24 hpi, infected McCoy cells were treated with Trizol (Invitrogen) and the lysate was pipetted up and down several times to homogenize the solution. The lysate was then added to 1.5 ml microfuge tubes and incubated at room temperature for 5 minutes. Chloroform was added and tubes vortexed at maximum speed for 30 seconds and then incubated at room temperature for 3 minutes. The tubes were centrifuged at 16,100 x g for 15 minutes at 4 °C. After centrifugation, the aqueous phase was added to a new microfuge tube and extracted a second time with chloroform. The aqueous phase was transferred to a new microfuge tube mixed with room temperature isopropanol. The tubes were mixed gently and incubated at room temperature for 10 minutes before spinning at 9,300 x g for 10 minutes at 4 °C. The isopropanol was discarded without disturbing the clear pellet. A 75% ethanol solution was added to remove the isopropanol and centrifuged at 5,200 x g for 5 minutes at 4 °C. The ethanol was discarded and the sample incubated at room temperature for 5 minutes to evaporate any residual ethanol. The pellet was resuspended in DEPC-treated water and treated with DNase for 25 minutes at 37 °C. The DNase-treated sample was added to RNeasy mini columns (Qiagen) and RNA purified according to manufacturers recommendations. RNA quality and concentration were measured using the NanoDrop ND-1000 UV-Vis Spectrophotometer.
After RNA was purified and concentration determined, first strand cDNA synthesis was performed using a gene specific primer \{\text{groEL}, \text{omp2}, \text{tet(C)}\}, Superscript II reverse transcriptase (Invitrogen), dNTP mix, DTT, and RNaseOUT (Invitrogen). First strand cDNA synthesis was performed at 42 °C for 50 minutes and then 70 °C for 15 minutes. The cDNA was then added to PCR reactions containing gene specific primers, Taq polymerase, and Sybr-green. Amplifications were performed at 94 °C for 15 seconds, 60 °C for 15 seconds, and 72 °C for 30 seconds. The change in fluorescence was measured over 38 cycles and cycle thresholds were determined.

2.3: Results

Immunofluorescence analysis of Tet treated Chlamydial infection. In order to visualize the effects of Tet on both resistant and sensitive strains of Chlamydia, immunofluorescent microscopy was performed. The Tet-resistant strain, \textit{Chlamydia suis} strain R19, was compared to the two sensitive strains, \textit{Chlamydia suis} S45 and \textit{Chlamydia trachomatis} L2. The Tet-sensitive strains S45 and L2 were extremely sensitive to very low Tet- concentrations, becoming aberrant at 0.1 µg/ml Tet (Fig. 2.2, 2.3). At 1 µg/ml, there are fewer inclusions seen and they are smaller and contain fewer chlamydia. In contrast, strain R19 exhibited a much higher level of resistance to Tet (Fig. 2.1). At concentrations between 0-1 µg/ml, R19 inclusions appear identical to non-Tet-treated inclusions. Aberrant developmental forms start to appear at 3 µg/ml and chlamydia are 100% aberrant at 5 µg/ml.
Mixed infections were performed to further illustrate the difference in resistance phenotypes of R19 compared to S45 and L2. At low concentrations of Tet (0-0.5 µg/ml), R19 produced normal inclusions while L2 developmental forms became aberrant at all tested Tet concentrations (Fig. 2.3). At concentrations of 3 µg/ml, some R19 chlamydia become aberrant and, at that concentration, no L2 inclusions are present (Fig. 2.4). When S45 was co-infected with L2, only aberrant Chlamydia were detected for all tested concentrations of Tet (0.1-0.5 µg/ml; Fig. 2.5). The results show that both resistant and sensitive strains produced aberrant Chlamydia in the presence of increasing concentrations of Tet, but that R19 resists aberrancy to much higher drug concentrations.
Figure 2.1: Fluorescence microscopic analysis of Tet-resistant *Chlamydia suis* R19. McCoy cells were infected with Chlamydia and incubated in the presence of Tet at the stated concentrations for 30 hours at 37 °C. Red: *Chlamydia suis* strain R19 inclusions. Blue: DNA labeled with DAPI. Scale bar is indicated on the lower left panel.
Figure 2.2: Fluorescent microscopic analysis of Tet-sensitive strains S45 and L2. McCoy cells were infected with Chlamydia and incubated in the presence of Tet at the stated concentrations for 30 hours at 37 °C. Red: *Chlamydia trachomatis* serovar L2 inclusions. Green: *Chlamydia suis* strain S45 inclusions Blue: DNA labeled with DAPI. Scale bar is indicated on the lower left panel.
Figure 2.3: Fluorescent microscopy analysis of Tet-treated (0-0.5 µg/ml) mixed-infection (R19, L2). McCoy cells were co-infected with 2 different strains, R19 and L2, in the presence of Tet at the stated concentrations. Red: *Chlamydia trachomatis* strain L2. Green: *Chlamydia suis* strain R19. Blue: DNA labeled with DAPI. Scale bar is indicated on the lower left panel.
Figure 2.4: Fluorescent microscopy analysis of Tet-treated (1-10 µg/ml) mixed-infection (R19, L2). McCoy cells were co-infected with 2 different strains, R19 and L2, in the presence of Tet at the stated concentrations. Red: *Chlamydia trachomatis* strain L2. Green: *Chlamydia suis* strain R19. Blue: DNA labeled with DAPI. Scale bar is indicated on the lower left panel.
Figure 2.5: Fluorescent microscopy analysis of Tet-treated mixed-infection (S45, L2). McCoy cells were co-infected with two different strains of Chlamydia, S45 and L2, in the presence of Tet at the stated concentrations. Red: *Chlamydia trachomatis* strain L2. Green: *Chlamydia suis* strain S45. Blue: DNA labeled with DAPI.
**Electron microscopy analysis of Tet treated Chlamydial infections.**

Analysis of Tet resistance was also investigated using electron microscopy of R19, S45, and L2. McCoy cells were infected with R19, S45, or L2 and incubated in the presence of Tet. Electron microscopic images were consistent with the phenotypes seen with fluorescent microscopy (Fig. 2.6, 2.7, 2.8). Strain R19 produced normal Chlamydia RB and EB at 0-1 µg/ml Tet (Fig. 2.6). However, at 3 µg/ml, aberrant developmental forms are found alongside RB and EB. At 5 µg/ml, all Chlamydia become aberrant. In comparison, S45 and L2 become aberrant at 0.1 µg/ml Tet (Fig. 2.7, 2.8). These results are consistent with the immunofluorescent microscopy data, and confirm that the resistant strain can grow at much higher concentrations of Tet than the sensitive strains. The EM results also show that both resistant and sensitive strains develop identical aberrant forms near limiting Tet concentrations.
Figure 2.6: Electron microscopy analysis of *Chlamydia suis* strains R19. McCoy cells were infected with *Chlamydia* and treated with various concentrations of tetracycline before monolayers were trypsinized, pelleted, and fixed for electron microscopy analysis. All pictures were taken at a magnification of 6300 X. Tet concentrations and scale bar are shown.
Figure 2.7: Electron microscopy analysis of *Chlamydia suis* strain S45. Pictures were taken at a magnification of 6300 X. Tet concentrations and scale bar are shown.
Figure 2.8: Electron microscopy analysis of *Chlamydia trachomatis* strain L2. All pictures were taken at a magnification of 6300 X. Tet concentrations and scale bar are shown.
Output experiments. In order to perform a quantitative analysis of Tet resistance of both resistant and sensitive strains, an output experiment was performed. The purpose of the output experiment was to exclude any chlamydia which are non-infectious, excluding RB and aberrant forms, resulting in a more accurate determination of the effect of Tet on the production of infectious chlamydia at each drug concentration. A control experiment demonstrated that C. suis EB survived in empty wells, while C. trachomatis did not, regardless of the antibiotic concentration (Fig. 2.9)

After conducting the control experiment, output experiments were performed with R19, S45, and L2 using tissue culture cells. Results showed that L2 and S45 produced no resistant Chlamydia starting at 1 µg/ml Tet (Fig. 2.10). For R19, there was a gradual reduction in the number of Chlamydia as the concentration of Tet increased (Fig. 2.10).
Figure 2.9: Tet resistance analysis of apparent residual chlamydia. Experiments were performed in which the first round of infection was performed in the absence of McCoy cells and various concentrations of Tet. Chlamydial inclusions were then enumerated using immunofluorescent microscopy with a monoclonal antibody against MOMP. Panel A: S45 incubated without Tet. Panel B: S45 incubated with 1 µg/ml Tet. Panel C: S45 incubated with 5 µg/ml Tet. Panel D: L2 incubated without Tet. Panel E: L2 incubated with 1 µg/ml Tet. Panel F: L2 incubated with 5 µg/ml Tet. L2 bars cannot be seen in any treatment condition since the values are 0. Graph is in logarithmic format. Error values were calculated based on multiple experiments and are shown.
Figure 2.10: Tet-resistance analysis of different Chlamydia strains. McCoy cells were incubated with Tet at various concentrations and then lysed with sterile water. Lysate was then used to infect a new monolayer and fixed after 30 hpi. Chlamydial inclusions were then enumerated using immunofluorescent microscopy with a monoclonal antibody against MOMP. Error values were calculated based on multiple experiments and are shown. The graph is in logarithmic format. Due to previous results shown in Figure 9, the values on the y-axis start from $10^4$ infectious forming units (ifu).
Real-Time analysis of R19 and S45 in the presence of Tet. Real-Time PCR was performed to analyze transcriptional activity in the presence of tetracycline. RNA was isolated from chlamydia-infected McCoy cells incubated in the presence of tetracycline and first strand cDNA was produced. The genes analyzed were groEL, omp2, and tet(C). The groEL gene was chosen because it is a constitutively expressed gene that is also expressed in aberrant Chlamydia, while omp2 encodes an outer membrane protein and is only expressed during late-term infection (4). The tet(C) gene was analyzed because it confers Tet-resistance and previous results indicated that it was constitutively expressed in C. suis. Real-Time analysis was performed with R19-infected and S45-infected cells incubated in 0, 1, 3, and 5 µg/ml tetracycline (Fig. 2.11). The results showed that with R19, the cycle threshold of omp2 increased as the concentration of Tet increased while groEL remained constant (Fig. 2.11). This is indicative of the presence of aberrant Chlamydia, which do not express late genes but continue to express the constitutive groEL. These results are similar to S45 which was tested at 0, 0.1, 1 µg/ml (Fig. 2.12). With S45, omp2 cycle threshold increased as the concentration of Tet increased.
Figure 2.11: Real Time analysis of R19 infected cells. R19 infected cells were incubated with various concentrations of tetracycline and then RNA isolated to determine expression profiles of specific genes. 1: No Tet. 2: 1 µg/ml Tet. 3: 3 µg/ml Tet. 4: 5 µg/ml Tet.
Figure 2.12: Real-Time analysis of S45 infected cells. S45 infected cells were incubated with various concentrations of tetracycline and then RNA isolated to determine expression profiles of specific genes. 1: No Tet. 2: 0.1 µg/ml Tet. 3: 1 µg/ml Tet. Tet(C) was analyzed but had a cycle threshold value greater than 38.
2.4: Discussion:

The results show that there are similarities and differences in the growth of the Tet-resistant strain R19, compared to the Tet-sensitive strains S45 and L2. The Tet-resistant R19 can survive and replicate in Tet concentrations up to 4 µg/ml. In contrast, the two sensitive strains, S45 and L2, become aberrant and do not produce infectious Chlamydia at 0.1 µg/ml. This was demonstrated using both immunofluorescent microscopy and electron microscopy analyses. It is significant that the resistant strain had the same aberrant phenotype as the sensitive strain. At near limiting concentrations of Tet, for either the sensitive or resistant chlamydia, the developmental forms become aberrant. In addition, Real-Time data showed that the level of the late-gene transcript, *omp2*, increased as the concentration of Tet increased. This was found in both R19 and S45 and points to the presence of aberrant Chlamydia.

The immunofluorescent microscopy, electron microscopy, and Real-Time analysis showed that Tet-resistance of the resistant strain was a result of homotypic resistance. This is significant because there had been reports of resistance identified in a number of clinical *C. trachomatis* isolates that were shown to have intermittent, or heterotypic resistance (9, 10). However, the Tet-resistance analysis proved that the resistant strain, R19, was uniformly, or “homotypically” resistant. These results demonstrated that the resistant *C. suis* is the first example of stable, homotypic resistance identified in any chlamydiae. In addition, while persistence has been seen when infected cells
were incubated with other stressing agents (6), this is the first analysis of Tet in the induction of the persistent phenotype.

The output experiment produced an unusual phenotype in S45 and R19. It illustrated that *Chlamydia suis* survives much longer in solution than *Chlamydia trachomatis*. This reinforces the research, which has shown that *Chlamydia suis* can survive in the environment for long periods of time and infect pigs through the fecal-oral route (8). Survivability outside the cell for an obligate intracellular bacterium would be a huge advantage and enable it to spread between pigs more effectively.

This preliminary work was essential for identifying the molecular mechanism of the Tet-resistance phenotype. The subsequent Tet-resistance analysis showed that a set of genomic islands, as discussed in Chapter 3, were present in the resistant strains but not in the sensitive strains. This enabled the correlation of a specific genotype to a Tet-resistance phenotype.
2.5: References


Chapter 3: Tetracycline resistance in *Chlamydia suis* mediated by genomic islands inserted into the chlamydial invasin-like gene

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3.1: Abstract

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 *tet*(C) resistance gene in the chlamydial chromosome. Four related genomic islands were identified in seven tetracycline resistant *C. suis* strains. All resistant isolates carry the *tet*(C) structural gene 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 IS605 family of insertion sequences. In each strain, the resistance gene and associated sequences are recombined into an identical position in a gene homologous to *inv* from 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.

3.2: Introduction

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 (RB) 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 are important in infections of veterinary significance, including species causing serious diseases in reptiles, birds, and mammals (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 organism can also be found in asymptomatic animals. The type strain of this species, strain S45, was isolated in Europe several decades ago and is tetracycline (Tc) sensitive (Tc\textsuperscript{s}; 15).

Both human and veterinary chlamydial infections are often treated with Tc and its derivatives (8). While there are reports of human chlamydial infections that do not respond to Tc or doxycycline, no human pathogenic chlamydial strains have been isolated that demonstrate stable Tc resistance (14, 19, 31, 35). However, stable Tc-resistant (Tc\textsuperscript{r}) *Chlamydia suis* strains have recently been identified in both diseased and apparently healthy pigs from farms across the Midwestern USA. 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, it was demonstrated that the *C. suis* Tc-resistant (Tc\textsuperscript{r}) phenotype is manifested through a *tet(C)* resistance gene integrated into the chlamydial chromosome in each of 7 Tc\textsuperscript{r} strains. The Tc resistance gene in each strain is contained within one of a family of horizontally acquired genetic
elements that share identity to resistance plasmids of gram-negative bacteria, and is integrated into a homolog of the invasin gene of yersinia (13).

3.3: Materials and Methods

Chlamydia and DNA preparation, purification. Tc\(^{r}\) *Chlamydia suis* isolates were collected from pigs (*Sus scrofa*) at sites across the Midwestern USA (Table 3.1; reference 1). The single Tc\(^{s}\) *C. suis* isolate (S45) was collected in the 1960’s 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 (MEM-10), 2 mM L-glutamine, and 10 \(\mu\)g/ml gentamicin for 40 h at 37 °C in 5% CO\(_2\). Infected cells were ruptured by sonication for three seconds at 10 W, followed by centrifugation at 30,500 x g for 30 min. The pellet was resuspended in Hank’s Balanced Salts Solution and overlayed onto 10 ml of 30% Hypaque-76 (Nycomed, Roskilde, Denmark) diluted in phosphate buffered saline. This preparation was centrifuged at 30,500 x g for 40 min and the pellet, containing partially purified EBs, was resuspended in SPG (18 mM Na\(_2\)HPO\(_4\), 220 mM sucrose, 5 mM L-glutamate). Genomic DNA was prepared using a genomic DNA preparation kit (Qiagen, Chatsworth, Cal.) according to the manufacturer’s recommendations. Five mM dithiothreitol was added to the supplied lysis buffer for complete disruption of the chlamydial EBs.
### TABLE 3.1: *Chlamydia suis* Tc<sup>f</sup> and Tc<sup>s</sup> strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Location</th>
<th>Year</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S45</td>
<td>Austria</td>
<td>1960's</td>
<td>0.6</td>
</tr>
<tr>
<td>R19</td>
<td>Nebraska</td>
<td>1992</td>
<td>5</td>
</tr>
<tr>
<td>R24</td>
<td>Nebraska</td>
<td>1992</td>
<td>5</td>
</tr>
<tr>
<td>R27</td>
<td>Nebraska</td>
<td>1993</td>
<td>5</td>
</tr>
<tr>
<td>H5</td>
<td>Iowa</td>
<td>1994</td>
<td>5</td>
</tr>
<tr>
<td>H7</td>
<td>Iowa</td>
<td>1994</td>
<td>10</td>
</tr>
<tr>
<td>130</td>
<td>Nebraska</td>
<td>1996</td>
<td>5</td>
</tr>
<tr>
<td>132</td>
<td>Nebraska</td>
<td>1996</td>
<td>5</td>
</tr>
</tbody>
</table>

**Minimum inhibitory concentration (MIC) determination.** MIC values for each *C. suis* strain was determined in Vero cells cultured in 96-well multiwell plates. Chlamydiae were diluted to approximately 100 inclusion-forming units per well, and centrifuged onto the monolayer at 900 x g for 1 h. The inocula were then removed and medium containing 0.5 µg/ml of cycloheximide plus 2 fold serial dilutions of Tc (0.3 µg/ml to 40 µg/ml) was added to each well. One row of wells was cultured in the absence of Tc as a control for chlamydial growth. Four plates were infected for each test, and one plate was fixed with methanol and stained for chlamydia each day for 4 d. The lowest concentration of Tc not showing development of inclusions was recorded as the MIC. All tests were performed at least twice.
**Amplification by thermal cycling.** All PCR reactions were performed with 0.25 mM dNTP, 0.4 nM forward and reverse primer (Table 3.2), and *Taq* (Promega, Madison, Wisc.) or *Pfx* (Invitrogen, Carlsbad, Cal.) DNA polymerase. Reactions were performed in 50 µl volumes using 50-100 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 gene in *C. caviae* (23) and *C. 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 3.2). Genomic DNA from Tc\(^r\) strain R19 and Tc\(^s\) strain S45 were used as template for PCR reactions.
TABLE 3.2: Oligonucleotide primers used for PCR and nucleotide sequence analysis.

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Sequence (5’ 3’)</th>
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<tr>
<td>CS43</td>
<td>tet(C)</td>
<td>AGCACTGTCCGACCACGTCTTTG</td>
</tr>
<tr>
<td>CS47</td>
<td>tet(C)</td>
<td>TCCTCGCCGAAAATGACCC</td>
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<td>CAAGACCAGCAGATGAGAG</td>
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<tr>
<td>CS38</td>
<td>tetR(C)</td>
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</tr>
<tr>
<td>CS09</td>
<td>orfB</td>
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<td>3'-inv-like</td>
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<td>5'-inv-like</td>
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<td>CS109</td>
<td>dmpP</td>
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<td>CS111</td>
<td>23S rRNA</td>
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<td>CS05</td>
<td>3'-inv-like</td>
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<td>dmpP</td>
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<td>mobD</td>
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<td>mobA</td>
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</tr>
<tr>
<td>CS86</td>
<td>mobA</td>
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**Southern blot.** Genomic DNA was digested with *Hind*III, electrophoresed through 0.7% agarose, and transferred to a nylon membrane (28). The genomic DNA was then UV-crosslinked to the membrane and probed with
Digoxigenin-labeled PCR products. To make Digoxigenin-labeled PCR products, Digoxigenin-labeled dNTPs (Roche Diagnostics, Indianapolis, IN) were added to the PCR reactions. Nested PCR with primers within the target gene was used to confirm that each probe was specific for the gene of interest (not shown). After incubation with probe, membranes were washed with 0.1% SDS and 10% 20 X SSC before incubation with an anti-Digoxigenin-alkaline phosphatase conjugated antibody. The membrane was then developed by incubation with a chemiluminescent peroxidase substrate (Roche). Blots were exposed to film and the molecular masses of the resulting bands were determined using the 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 using two different methods. The primary method was to create plasmid libraries of size-selected *C. suis* DNA, followed by PCR analysis of individual clones to identify positives. *C. suis* DNA digested with *Hind*III was blotted and probed with *tet*(C) and *tetR*(C). A parallel sample of digested DNA was electrophoresed and a block of agarose was excised that contained the approximate size of the fragment identified in the Southern blots. DNA was purified from the agarose using a Qiagen Gel-extraction purification kit. DNA was then ligated to *Hind*III-digested pUC18 (Invitrogen) and transformed into *E. coli* DH5α. Transformants were grown on LB 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 sequenced at the Oregon State University Center for Gene Research Central Service Laboratory.

A PCR-based, primer walking approach was used to acquire sequence from one end of the genomic insert from strain R19. Oligonucleotides derived from the accumulating sequence data were used for these experiments (Table 3.2). Each fragment produced in these PCRs was cloned into pCR2.1:Blunt (Invitrogen) and the nucleotide sequence was determined. Two clones from independent PCR reactions were bidirectionally sequenced for each region of interest.

The assembled sequences collected from strains R19 and R27 were deposited in Genbank with accession numbers AY428550 and AY428551, respectively.

**Reverse-transcriptase PCR (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 of 3) cultured in the presence or absence of Tc (1 µg/ml). Infected cells were incubated 30 h and RNA was collected 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 (Biorad, Hercules, CA), and 100 ng RNA was added to each reaction. The Access system (Promega) was used for
all RT-PCR according to the manufacturer’s recommendations. Controls included a genomic DNA positive control for showing the size of PCR products, and a reaction lacking RT to confirm that RNA preparations were free of contaminating DNA. A 100 bp DNA ladder was used to determine the sizes of the PCR products (Invitrogen).

3.4: Results

Identification of a tet(C) resistance gene in Tc\textsuperscript{r} C. suis strains. PCR analysis of genomic DNA with primers specific for 13 different Tc resistance genes (see Materials and Methods), demonstrated that a single gene, tet(C), was present in each of the 7 resistant strains (Fig. 3.1). This is one of several Tc resistance genes that encode an efflux pump (24). PCR analyses of all other tested tet genes were negative (not shown). In most systems, the tet(C) structural gene 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 resistant strains contained tetR(C). Neither tet(C) nor tetR(C) were detected in the Tc\textsuperscript{s} strain S45 (Fig. 3.1).
Fig. 3.1: PCR analysis of 8 different \textit{C. suis} strains using primers specific for \textit{tet}(C) (525 bp; row A), \textit{tetR}(C), (400 bp; row B), IScs605 (500 bp; row C), and the \textit{inv}-like gene (rows D-F). The PCR products of the \textit{inv}-like gene represent a 3' fragment (200 bp; row D), a 5' fragment (700 bp; row E), and a fragment that spans the \textit{inv}-like sequence that is contiguous in the \textit{Tc}^\text{S} S45 (900 bp; row F). All primers used in these experiments are listed in Table 2.
**Nucleotide sequence analysis.** The complete sequence of the DNA surrounding \textit{tet}(C) was determined for Tc\textsuperscript{T} strains R19 and R27. A \textit{Hind}III site between \textit{tet}R(C) and \textit{tet}(C) facilitated the cloning of genomic DNA fragments containing each gene into pUC18. The \textit{tet}(C) and \textit{tet}R(C) genes were flanked by DNA not found in the sensitive strain (Fig. 3.2). The length of the genomic inserts in strains R19 and R27 were different (12 and 5 kb, respectively), but each carried \textit{tet}(C) and \textit{tet}R(C). These two genes share high sequence identity with homologous genes in plasmids of gram-negative bacteria, including the plasmid pSC101 (9). Sequences within each genomic island also share high identity with the plasmid pRAS3.2 from the fish pathogenic bacterium \textit{Aeromonas salmonicida} (18). The nucleotide sequence identity between R19 \textit{C. suis} and pRAS 3.2 is over 99% throughout the 10.1 kb of shared DNA. An approximately 1.7 kb fragment containing much of a \textit{mobA}-\textit{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 in two short deletions within R19. First, there is an 8 nucleotide deletion within the \textit{tet}(C)-\textit{tet}R(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\textsuperscript{T} strain (Fig. 3.7). These analyses demonstrated that there are four different, yet related, genomic islands in the seven strains (Fig. 3.2). Three of the islands are represented in two strains each, and one is found only
Fig. 3.7: PCR-based mapping analysis of the Tc\textsuperscript{f} *C. suis* strains. The genomic islands within strains R19 and R27 were fully sequenced, and PCR was used to determine gene order in each of the other Tc\textsuperscript{f} strains. Primers generated for the sequencing were utilized to amplify overlapping PCR products from each strain. Arrows show the location and orientation of the primers used in the analysis. Pluses (+) and minuses (-) indicated whether the PCR using each listed primer pair produced a PCR product, with template DNA from each indicated Tc\textsuperscript{f} strain.

<table>
<thead>
<tr>
<th>Primers</th>
<th>R19</th>
<th>R24</th>
<th>R27</th>
<th>H7</th>
<th>H5</th>
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<th>132</th>
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<tr>
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Regions sequenced:
- H5: CS75-CS106
- 130,132: CS84-CS106 CS84-CS106

ISca605 primers: CS09, CS12, CS15, CS17, CS18, CS22, CS27, CS29

Note that an important difference between strains is due to the location of the IS element.
Fig. 3.2: Open reading frame maps of tet(C) and flanking sequences in the Tc<sup>f</sup> 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 R24, H7, 130, 132, and H5 for sequence comparison. The direction of arrows represents the coding strand 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/black). The 2013 bp IS<sub>Cs</sub>605 sequence (green) is located at one or the other ends in 5 of 7 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 5 strains.
in a single strain. While the Southern blotting 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 a function of a recombination event, as the 3’ end of tetR(C) is located at the opposite end of the island in both R19 and R27 (Fig. 3.2). In R27 this leads to an in-frame genetic fusion between tetR(C) and 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), and in plasmids pSC101 and pRAS3.2, the nucleotide sequences diverge from near identity to nonhomologous sequence at exactly the same nucleotide position.

The percent G + C 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 percentage is approximately 40% (22, 23).

**Identification of a chlamydial insertion element, IScs605.** 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
IS605 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 2013 bp chlamydial IS605-like element, designated IScs605, shares 39% nucleotide sequence identity with IS605 from *Helicobacter pylori* (16). Similar IS605 insertion sequences are common in *Helicobacter* spp. that are commensals or pathogens in pigs and other animals (7). As with other IS605 insertion sequences, IScs605 is composed of divergently oriented members of the IS200 and IS1341 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 to the IS200 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 the resistance gene is located on the chromosome (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 yersiniae (13). Genome sequence analyses demonstrate that at least two other chlamydial pathogens of animal species, *Chlamydiophila caviae* and *Chlamydia muridarum*, have a full length
inv-like gene or a gene fragment while the human pathogenic chlamydiae do not (22, 23, 34).

PCR was used to demonstrate 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. 3.3).
Fig. 3.3: Chromosomal location of tet(C) in the resistant strains. Panel A: PCR results using S45 genomic DNA (lanes 1-3) or R19 genomic DNA (lanes 4-6) as template. Lanes 1 and 4 show an amplified product when 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 amplified products 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 template (lane 3). Molecular size standards are indicated (in kb) at the left of panel A. Panel B: A linkage map showing how tet(C) and flanking sequences are positioned between dmpP and the 23S rRNA gene in R19. The dashed lines indicate genomic sequence between genes targeted by the amplification.
Mapping regions flanking \textit{tet}(C) and \textit{tetR}(C) in other strains. Using the nucleotide sequence data generated from analysis of strains R19 and R27, primers were designed to amplify \textit{tet}(C), \textit{tetR}(C), IScs605 \textit{orfB}, and different regions of the \textit{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 \textit{inv}-like gene was interrupted by the genomic island, while in S45, the gene was intact (Fig. 3.1). All resistant strains were positive for \textit{tet}(C) and \textit{tetR}(C) and all but two strains contained IScs605.

Southern hybridization with probes for \textit{tet}(C), the \textit{inv}-like gene, and IScs605 \textit{orfB} confirmed that each \textit{C. suis} strain has the \textit{inv}-like gene, and that this gene is interrupted in all Tc$^R$ strains, but not in the Tc$^S$ strain S45 (Fig. 3.4). These blots demonstrated that single copies of \textit{tet}(C) were present in all seven Tc$^R$ strains, while single copies of IScs605 were detected in five of seven resistant strains. Neither \textit{tet}(C) nor IScs605 was present in the Tc$^S$ strain S45.
Fig. 3.4: Southern blots of *C. suis* genomic DNA digested with *Hind*III and probed with sequences from the *inv*-like gene of S45 (panel A), *tet*(C) (panel B), and a fragment of IScs605 (panel C). The individual strains are indicated at the top of each panel. Molecular size standards are indicated in kilobase pairs.
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 within *tetR*(C) (Fig. 3.5). 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. 3.5). The pentanucleotide TTCAA is also found at the 3’ end of all apparent IScs605 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 IS605 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.
Fig. 3.5: Nucleotide sequences surrounding the recombination sites at the junction of the integrated \textit{tet}(C) island and the \textit{C. suis} \textit{inv}-like gene (boxed sequences). Only the nine terminal nucleotides are represented for the left and right end of each island. Each island shown in Figure 2 is represented in this figure, with the representative strain indicated to the right of the sequences. Nucleotide sequences in outlined text are \textit{tetR}(C) while sequences in italics are \textit{IScs605}. The TTCAA sequences within \textit{tetR}(C) and the interrupted \textit{inv}-like gene are underscored. The dotted lines represent the internal sequence of the genomic island.
Reverse-transcriptase 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 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 Tc (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 Tc, tet(C) transcript was found in C. suis R19 cultured in the presence and the absence of Tc (Fig. 3.6).
Fig. 3.6: Analysis of *tet(C)* transcription in R19 and *E. coli* (pSC101). Reverse-transcriptase PCR was conducted on each bacteria cultured in the presence or absence of Tc. Lanes 1-5 represent RT-PCR products *C. suis* RNA and lanes 6-10 are from *E. coli* (pSC101). Lanes 1 and 6: *tet(C)* transcripts detected in bacteria cultured in the presence of 1 µg/ml Tc. Lanes 2 and 7: *tet(C)* transcript detected in the absence of Tc. Lanes 3 and 8: Negative control—RT-PCR products without using reverse transcriptase (RT). RNA from bacteria cultured in the presence of 1 µg/ml Tc. Lanes 4 and 9: Negative control—RT-PCR products without RT using RNA from bacteria cultured in the absence of Tc. Lanes 5 and 10: Positive control using bacterial genomic DNA as template. Molecular mass standards are indicated in base pairs.
3.5: Discussion

These studies demonstrate that recent Tc\textsuperscript{r} isolates of \textit{C. suis} carry a \textit{tet}(C) gene that is located in one of a highly related set of apparent plasmids that has integrated into the chromosome. In each case, the resistance determinant is adjacent to \textit{tetR}(C), and each is flanked by sequences common to known resistance plasmids from Gram-negative bacteria. Five of the Tc\textsuperscript{r} strains also carry a novel insertion element that is related to the IS605 family of insertion sequences common in \textit{Helicobacter} species. \textit{C. suis} IScs605 is the first insertion sequence identified in any chlamydiae. The \textit{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 \textit{Yersinia} spp. invasin gene. The \textit{tetR}(C) gene is the target of recombination within the donor DNA and is interrupted at an identical site in each strain. The percent G + C in each genomic island was approximately 54%. In contrast, the G + C percentage is approximately 40% within sequenced chlamydial genomes, and the 885 nucleotides of the \textit{inv}-like gene that were sequenced in this study have a G + C ratio 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 \textit{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, and thus it is not likely 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 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 IS element could have happened sequentially or co-temporally with the integration of the plasmid sequences. We are currently 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 includes all regions of each tet(C) island with the exception of the IScs605 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 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 Tc. 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$^f$ 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 suggest that the inv-like gene may be a target for experiments designed to introduce genes into the chlamydiae.

The occurrence of stable Tc resistance in $C. suis$ is in contrast to the absence of Tc resistance in the human chlamydial strains. This may be a function of the feeding of large amounts of Tc 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 \textit{tet}(C) islands within \textit{C. suis} is the first example of horizontal acquisition of resistance by a strain of an obligate intracellular bacterium. 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 \textit{Rickettsia} spp., \textit{Coxiella burnetii}, \textit{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 \textit{tet}(C) islands by these pathogens and by searching for possible donor bacteria responsible for transmission of \textit{tet}(C) to \textit{C. suis}. We are also examining the possible utility of this system for introducing genes into the chlamydiae, a process currently unavailable to researchers in this field of study.

\section*{3.6: ACKNOWLEDGEMENTS}

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and S. Humphrey (NADC, Ames IA) for providing the PCR primers to the various TcR determinants.

3.7: References


Chapter 4: Functional activity and insertion specificity of IScs605, a novel insertion element from tetracycline-resistant *Chlamydia suis*

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4.1: ABSTRACT

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 encode several plasmid-specific genes, the *tet*(C) resistance gene, and, in most cases, a novel insertion element (IScs605) which encodes two predicted transposases. The hypothesis that IScs605 mediated the integration of the *tet*(C) resistance islands into the *C. suis* genome was tested in a plasmid-based recombination system in *Escherichia coli*. Both high copy (pUC18) and medium copy (pBBR1MCS) plasmids containing the IScs605 element 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 each integration event adjacent to a 5’-TTCAA sequence. Deletion studies demonstrated that only one of the two candidate transposases encoded within IScs605 was necessary for the observed recombination activity and target specificity in the *E. coli* system. IScs605-dependent excision was also detected, and, in each case, the sequence 5’-AATTCAA remained at the site of excision. Collectively, these results support the hypothesis that IScs605 encodes a transposase responsible for integration of the *tet*(C) islands into the *C. suis* chromosome.
4.2: Introduction

Chlamydiae are obligate intracellular bacteria that cause serious disease in humans and many other animal species (2, 17, 23). One chlamydiae of veterinary concern is *Chlamydia suis*, a pathogen that can cause disease at many mucosal sites in swine (17). Recent work in our laboratory has identified and characterized the nature of a set of tetracycline (Tet) resistant (TetR) *C. suis* strains isolated from pigs in the Midwestern USA (Fig. 4.1). These isolates are stably resistant to Tet and, in some cases, sulfadiazine (1, 15, 22). This resistance phenotype was conferred by genomic islands (*tet*(C) islands) that were apparently acquired through horizontal transfer (5).

A major limitation in developing an understanding of the chlamydial host:pathogen interaction process is the lack of a genetic system. The challenge of transforming Chlamydia in the laboratory is perhaps a biological and not a technical problem, as genome sequencing data supports the conclusion that these organisms are generally not receptive to foreign DNA (21). In fact, the *tet*(C) islands represent the first example of the recent horizontal acquisition of DNA in any chlamydial strain and, to our knowledge, in any obligate intracellular bacterium (18). It is anticipated that an understanding of the mechanisms associated with acquisition of the *tet*(C)
Fig. 4.1. Genomic islands present in Tet-resistant C. suis strains (2). The 5'-AATTCAA and 5'-TTCAA nucleotide sequence found at sites involved in apparent IScs605 recombination and excisions are shown. The individual strains carrying a particular island are indicated at 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 kilobase pairs is shown in the top island.
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, plus *tet(C)* and *tetR(C)*, which encode an efflux pump for Tet resistance and the cognate repressor protein, respectively (4). Most of these islands also carry a novel insertion element (IS), IScs605 (5, Fig. 3.2). IScs605 is homologous to the IS605 family of insertion sequence elements, most commonly found in *Helicobacter pylori* (9-12, Fig. 4.2). This family of IS elements consist of two divergently oriented open reading frames. One of these (*orfA*) is homologous to IS200 transposases while the second (*orfB*) is homologous to an apparent transposase found in IS1341 (3, 5, 16). There is 100% sequence identity in IScs605 from the different *C. suis* strains. A pentanucleotide sequence, 5'-TTCAA, is always present adjacent to the *orfA* end of IScs605, suggesting a target specificity of the element (Fig. 3.2).

Nucleotide sequence analysis suggested that IScs605 may have had a role in the acquisition of the *tet(C)* islands by *C. suis*. The experiments in this manuscript examined this possibility. The results demonstrated that IScs605 can direct recombination in an *E. coli* based mating assay, yielding recombination products consistent with those observed in the TetR *C. suis* strains.
Fig. 4.2. Phylogenic analysis of *C. suis* IScs605 as it relates to similar IS elements. The accession numbers and references for the individual sequences used for these trees are indicated in the Material and Methods section. Panel A: Phylogenic tree of IScs605 orfA homologs with IScs605 orfB included as an outlier. B: Phylogenic tree of IScs605 orfB homologs with IScs605 orfA included as an outlier.
4.3: MATERIALS AND METHODS

*Escherichia coli* strains and culture conditions. *E. coli* RZ212 is a Gentamicin (Gent)-resistant strain containing a deletion derivative of the F plasmid, pOX38, that serves as a recombination host and a mating donor strain (8). *E. coli* SF800 is resistant to nalidixic acid (Nal) and has a deletion in polA, which prevents the strain from supporting ColE1-based origin of replications (7, 9, 19). *E. coli* TOP10 (Invitrogen, Carlsbad, CA) was used for plasmid rescue and for constructing the pUC18 and pBBR1MCS based vectors. All *E. coli* were grown in Luria-Bertani (LB) broth or on LB agar at 37 °C. When indicated, particular antibiotics were added to the medium at the following concentrations: 10 µg/ml Nal; 50 µg/ml Kanamycin (Kan); 100 µg/ml Ampicillin (Amp); 100 µg/ml Chloramphenicol (Cm); and 10 µg/ml Gent. All antibiotics were purchased from Sigma (St. Louis, MO).

Phylogenetic analysis of IScs605. Phylogenetic trees were produced using the ClustalW function within the MacVector Sequence analysis program (Accelrys, San Diego, CA). The following parameters were used to produce the trees: Open Gap Penalty = 10; Extend Gap Penalty = 0.1, Delay Divergent = 40%; Gap Distance = 8; Similarity Matrix = blosum. Accession numbers (gi) for all the protein sequences are as follows: Iscs605(OrfB): 40850632; Iscs605(OrfA): 40850633; IS605(TnpA): 1752704; IS605(TnpB): 63350380; IS606(TnpA): 2114470; IS606(TnpB): 2114471; IS607(OrfA): 6176523; IS607(OrfB): 6176524; ISHp608(OrfA): 13926104; ISHp608(OrfB):
General polymerase chain reaction (PCR) conditions. PCR reactions were performed with 0.25 mM dNTP (Fermentas, Hanover, MD), 0.4 nM forward and reverse primers (Table 1), and Taq (NEB, Beverly, MA) or Pfx polymerase (Invitrogen) as indicated. PCR was performed with the following parameters: denaturation for 45 s at 94 °C, annealing for 45 s at 52 °C, extension for 90 s at 72 °C. Amplification was performed for 30 cycles. Digoxigenin-labeled PCR products were made using previously described methods (5). The Digoxigenin-PCR probe of aph was produced using Taq polymerase and the primers IS11, IS12 with the template pCR-Blunt (Invitrogen). The Digoxigenin-PCR probe of bla was produced using Taq polymerase and the primers IS13 and IS14, with pUC18 as template. The Digoxigenin-PCR probe of cat was produced using Taq polymerase and the primers IS15 and IS16, with pBBR1MCS as template. The Digoxigenin-PCR probe of pOX38 was produced using Taq polymerase and the primers IS9 and IS10 with pOX38 as template.
Table 4.1. Oligonucleotide primers used in this study.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS1: 5'-AGCAAGCTTAAGTGAACTACTCGCTGCTA-3'</td>
<td>orfB</td>
</tr>
<tr>
<td>IS2: 5'-AGCAGAATTCTTTAAGCGGAGTGCTTGC-3'</td>
<td>orfA</td>
</tr>
<tr>
<td>IS3: 5'-AGCAGAATTCCGGAGTGCTTGCAGCT-3'</td>
<td>orfA</td>
</tr>
<tr>
<td>IS4: 5'-TCGCAAACACAAGCCCTG-3'</td>
<td>249 bp from orfB</td>
</tr>
<tr>
<td>IS5: 5'-AACAAACCAGAGAAACCCGGCC-3'</td>
<td>71 bp from orfA</td>
</tr>
<tr>
<td>IS6: 5'-TTGCGTTTTCCCTTGCC-3'</td>
<td>aph</td>
</tr>
<tr>
<td>IS7: 5'-TCCTGGTTTTGCCTCCGGC-3'</td>
<td>IScs605</td>
</tr>
<tr>
<td>IS8: 5'-GCTCACAATCCCATCTGCAGCTCAAG-3'</td>
<td>IScs605</td>
</tr>
<tr>
<td>IS9: 5'-CACACCCCCACGCAAAACAGC-3'</td>
<td>pOX38</td>
</tr>
<tr>
<td>IS10: 5'-CCATCGGATACATAGGAACCTCC-3'</td>
<td>pOX38</td>
</tr>
<tr>
<td>IS11: 5'-ACTGGGCTATCTGGACAAGG-3'</td>
<td>aph</td>
</tr>
<tr>
<td>IS12: 5'-TATCTACACGACGGAAGCTC-3'</td>
<td>aph</td>
</tr>
<tr>
<td>IS13: 5'-CATCCATAGTTGCTGACTCC-3'</td>
<td>bla</td>
</tr>
<tr>
<td>IS14: 5'-GTATTATCCCGTGATTAGGCC-3'</td>
<td>bla</td>
</tr>
<tr>
<td>IS15: 5'-TTAATGAATCGGCAACAGC-3'</td>
<td>cat</td>
</tr>
<tr>
<td>IS16: 5'-CAATGGCAGCGTTGAAGAACA-3'</td>
<td>cat</td>
</tr>
<tr>
<td>IS17: 5'-CGGTGATGACGGGTGAAAACCTC-3'</td>
<td>pUC18</td>
</tr>
<tr>
<td>IS18: 5'-CCTGCCTATCCCCTGATTCTG-3'</td>
<td>pUC18</td>
</tr>
</tbody>
</table>
Fig. 4.3. Plasmid constructs used in this study. The details of the constructs can be found in the Materials and Methods. Plasmids pJD1-pJD6 were constructed within pUC18 while pJD7 was built within the medium-copy plasmid pBBR1MCS (14). The plasmid pJD1 contains an intact IScs605 adjacent to a kan-resistance gene (aph) which is flanked by the ends of IScs605. The plasmid pJD2 contains aph with the IS ends, but which lacks the intact IScs605. Plasmid pJD3 is pJD1, but with a deletion introduced into the orfA open reading frame. Plasmid pJD4 is identical to pJD1, but carries a deletion that inactivates the orfB reading frame. 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 IScs605. Plasmid pJD7 consists of pBBR1MCS containing the pJD5 insert.
**Plasmid constructs.** A collection of pUC18-based and pBBR1MCS plasmids were produced to test the recombinational activity of Iscs605. All plasmids are shown schematically in Figure 4.3, 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-resistant *Chlamydia suis* strain R19 using Pfx polymerase PCR (Invitrogen) with the primers IS1 and IS2 (Table 1). The PCR product was cloned into pUC18 and transformed into Top10 cells. Since there is no antibiotic resistance marker on Iscs605, a kanamycin resistance gene (*aph*) was added to these plasmids, flanked by the ends of Iscs605. Each end of Iscs605 was amplified using the plasmid pUC18:Iscs605 as template with the primers IS4, IS5 in an outward-PCR protocol. For this construct, the PCR was designed to amplify the entire pUC18:Iscs605 lacking the orfsA and B. This produced a linear product with Iscs605 ends flanking the entire pUC18 molecule (pUC18:Iscs605*). The ends of this construct were engineered to contain the enzyme sites for NotI and EcoRV. The *aph* sequence was then amplified (from the pCR blunt vector: Invitrogen) using Pfx PCR with primers IS11 and IS12, which resulted in an *aph* sequence containing NotI and EcoRV restriction enzyme sites. This product was then ligated to the pUC:Iscs605*amplification product. The resulting plasmid (pJD2) was transformed into *E. coli* and selected by growth on Kan. The Iscs605/aph fragment from pJD2 was then excised with EcoRI and ligated into the full length pUC:Iscs605.
to create a plasmid (pJD1) carrying the entire intact Iscs605 adjacent to the orfA and orfB deleted, aph-containing, Iscs605 fragment.

orfA and orfB knockouts. Each of the two orfs in Iscs605 were independently inactivated using a restriction enzyme-based approach. A 200 bp fragment of orfA was deleted by digesting pJD1 with PsiI and PflMI, enzymes which each cut only once within the orfA open reading frame. The restricted plasmid was electrophoresed through 1% agarose and the vector-containing fragment 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-resistance marker aph. The orfB sequence within pJD1 was replaced with the Kan-resistance 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 open reading frame was amplified with the primers IS11 and IS12 (Table 1). Transformed clones were identified by plating on LB agar plus Kan, and resistant colonies 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 IS1 and IS3 using pJD5 as the template. This amplification produced an IS element identical to that found in pJD5, but which lacks the 5’-TTCAA sequence. This PCR product was inserted into pUC18, creating pJD6.

**Iscs605 insertions into pBBR1MCS.** A second plasmid (pBBR1MCS; (14)) was chosen to analyze Iscs605 recombination activity. The entire Iscs605 from pJD5 (containing the aph substitution for orfB) was PCR amplified using the primers IS1 and IS2. 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. 4.3) 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 + 0.5% glucose and grown 2-4 hours to an $A_{600}$ 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 hours 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 on Kan and Nal, to determine the number of recipient bacteria that carried pOX38 + Iscs605 recombinant plasmids. The values reported represent the number of transconjugants ($\text{Kan}^R, \text{Nal}^R)/ 10^9$ recipient bacteria ($\text{Nal}^R$), 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 3,000 x 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 hours and extracted 3 times with 1:1 phenol:chloroform, followed by a single chloroform extraction. The DNA was precipitated with ethanol/acetate and resuspended in water to a concentration of 1 µg/µl.

**Plasmid rescue.** Genomic DNAs from individual transconjugants clones were restricted with the enzymes *MluI* and *BssHII* for 4 hours. These restriction enzymes are isoschizomers that 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, Valencia, CA), self-ligated overnight, and then transformed into TOP10 cells. Individual Amp<sup>R</sup> Kan<sup>R</sup> colonies were transferred to LB and cultured overnight. Plasmids were purified from these cultures using a commercial kit (Qiagen), and the nucleotide sequence of DNA flanking the likely insertion site was determined using primers IS6, IS7, and IS8.

**Southern analysis.** Purified genomic DNA of transconjugants were analyzed by Southern blots as described previously (3). Genomic DNA was digested with *MluI* + *BssHII*, *PstI*, or *PstI*, followed by electrophoresis through a 1% agarose gel and then passively blotted to nitrocellulose. The enzymes *MluI* + *BssHII* do not cut within any of the *pJD* plasmids, and thus *MluI* + *BssHII* restriction fragments analyzed by Southern blot represent DNA not digested
within the original plasmid source of Iscs605. In contrast, the enzymes PstI or PsI cut one time within pJD1 through pJD6 or pJD7, respectively, and were useful for identifying multimers of donor plasmids within pOX38. The blots were analyzed using probes for aph, bla, Iscs605 orfB, cat, and pOX38.

**Excision analysis.** Genomic DNA of transconjugants were used as template for PCR reactions to look for evidence of excision of Iscs605 from pOX38 recombinants. Primers for these experiments (IS17-IS18) were designed to amplify across sites that would be very closely linked 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 determined.

**Transposition of Iscs605 vs integration of entire plasmids.** Transconjugants from both pUC18-based (pJD5) and pBBR1MCS-based (pJD7) constructs were analyzed for the ability of Iscs605 to independently transpose following insertion into pOX38 and conjugation to the recipient bacterial strains. For these experiments, constructs containing aph sequence inserted into orfB were used. Standard mating assays were conducted and colonies selected using Kan and Nal. Transconjugants from each mating were then individually transferred to plates containing Nal and either Amp (for pJD5) or Cm (for pJD7). For each donor plasmid, the ratio of clones that grew on Kan and Nal, but not on either Amp or Cm, versus those that grew in the presence of Amp or Cm, was determined.

Southern blotting was also conducted on clones from pJD1 mating assays. Genomic DNAs digested with PsI from Cm-resistant (Cm^R) and Cm-
sensitive (Cm<sup>S</sup>) transconjugants were used in these blots, and probed with \textit{aph}.

**4.4: RESULTS**

The IScs605 mobile genetic element was analyzed for functional activity and insertion specificity in an \textit{E. coli}-based mating assay (10, 12). This assay involves transforming a donor strain with IScs605-containing plasmids (Fig. 4.3) and selecting for recombination 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 approximately 4000 transconjugants/10<sup>9</sup> recipient bacteria (Fig. 4.4). Control experiments using pJD2 resulted in less than 10 transconjugants/10<sup>9</sup> recipient bacteria. These data demonstrate that the presence of intact IScs605 resulted in an approximately 800-fold increase in recombination efficiency in this system.
Fig. 4.4. Logarithmic representation of the number of transconjugants per $10^9$ recipient bacteria resulting from mating assays using the pUC18-based pJD plasmids as a source of IScs605 or its derivatives. Error bars show the standard deviation based on at least three replicate experiments for each plasmid construct.
The nature of the pJD1:pOX38 recombination was then examined. Initial experiments showed that all transconjugants were resistant to both Kan (Kan$^R$) and Amp (Amp$^R$). These data suggest the IS element integrated into pOX38 within its pUC framework, resulting in Amp$^R$ colonies. This possibility was examined using Southern hybridizations of genomic DNA from independent transconjugants. Blots of MluI and BssHII-digested Genomic DNA from 36 transconjugants were probed with the Kan resistance gene (aph), the ampicillin resistance gene (bla), orfA from IScs605, and a fragment from pOX38. All transconjugants were positive for each gene, and probes for aph, bla and IScs605 all reacted with an identical band in each individual transconjugant (Fig. 4.5). These data support the conclusion that the entire pJD1 plasmid was integrated into pOX38 following pJD1-mediated recombination in all examined clones.
Fig. 4.5. Southern blots of *Mlu*I and *BssH*I-digested genomic DNA from transconjugants. The plasmid pOX38 was used as a control. The different probes for the blots are listed on the right. The two dashes to the left of each blot represent DNA standards of 8,000 and 10,000 base pairs for each panel.
To determine the 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 affect on the formation of AmpR/NalR transconjugants compared to wildtype IScs605 (Fig. 4.4). These results were expanded by mating experiments using pJD5, which has the orfB open reading frame replaced by aph. This construct was also capable of facilitating integration of DNA into pOX38 (Fig. 4.4). These experiments supported the conclusion that orfA was essential for recombination while inactivation of orfB had no detectable effect on recombination frequency.
Fig. 4.6. 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 or right, respectively. The filled arrows represent selected genes or collections of genes within pOX38. An asterisk indicates sites of insertion in mating assays based on pBBR1MCS transconjugants. See next page.
Nucleotide sequence analysis was performed to determine the site of insertion of the IScs605 plasmids into pOX38. These data demonstrated that integration of pJD1 was relatively random throughout pOX38 and that insertions occurred in both orientations (Fig. 4.6). 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* (6). These data also demonstrated that each insertion event was adjacent to a 5'-TTCAA sequence present within pOX38 (Fig. 4.7, panel A). This insertion site specificity was identical when using the orfB-deletion plasmid, pJD5. There was no evidence of insertion specificity at the orfB end of IScs605 (Fig. 4.7, panel A). 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, pJD5 and pJD7 each integrated with the orfA end adjacent to a 5'-TTCAA site (Fig. 4.7). Sequencing of orfB deletion transconjugants showed no difference in terms of insertion specificity (Fig. 4.7). Thus, the presence of orfA within the IS element is responsible for both the transpositional activity and insertion specificity of IScs605.
Fig. 4.7. Sequence analysis of the pOX38 insertion site and excision events. Panel A: Sequences adjacent to the sites of insertion within pOX38 showing association with the pentanucleotide 5'-TTCAA (underlined). Panel B: Sequences at the margins of IScs605 before and after apparent excision in transconjugants. Note the sequence 5'-AATTCAA remaining at the site of excision. This sequence was identical in 8 independent transconjugants containing the plasmid pJD5.
The nucleotide sequencing was often complicated by the presence of apparent overlapping electropherogram peak profiles. In these cases, the sequences were easily readable until the sequence 5’- TTCAA was encountered, and then two different profiles were evident. The clones that yielded overlapping sequence reads were further analyzed using Southern blotting and PCR. Southern blots of PstI-digested DNA from transconjugants were probed with bla, aph, and IScs605 orfB. These data demonstrated that there were multiple copies of each of these genes within genomic DNA from clones that yielded overlapping nucleotide sequence data (Fig. 4.8). These experiments suggested several different scenarios; it was possible that more than one pOX38 recombinant was present in a single cell, that secondary recombination was occurring inside either the donor or recipient cell, or that concatamers of pJD1 were inserted into single pOX38 within the donor. A second set of Southern blots (not shown) were performed using the enzymes MluI and BssHII which do not have recognition sites within pJD1. These blots yielded results consistent with those shown in Figure 4.5, and demonstrated that a single bla-positive restriction fragments was identified in the clones. Collectively, these data are consistent with complete or partial concatamers of two pJD1 molecules within products of the mating assay, followed by secondary excision events. The presence of concatamers help to explain the “multiple peak” phenomenon within the sequencing electropherograms. The sequencing primer used in these experiments was within aph, and, therefore, clones that had two copies of aph (Fig. 4.8, Lanes 1-4, 6) yielded multiple
peaks in the sequencing electropherograms. In contrast, those DNAs that yielded a single profile within the nucleotide sequence electropherograms have only a single copy of *aph* within pOX38 (Fig. 4.8, Lanes 5, 7). These data indicate that, in *E. coli*, the IScs605 element commonly facilitates 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 recombination following the original integration event (Fig. 4.8, 4.9). This was supported by detailed analysis of several products recovered for nucleotide sequence analysis (not shown). These secondary events led us to hypothesize that IScs605 may have excised itself in some of these recombinants. This was tested by PCR-based analysis of selected genomic DNAs. Oligonucleotide primers flanking IScs605 in pJD1 and pJD5 and in apparent 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 site of cloning of the IS element into pUC18 (Fig. 4.7, panel B). Evidence of such excision events was found in both pJD1- and pJD5-based transconjugants, suggesting that the apparent excision event was also orfA-mediated. Therefore, IScs605 can excise from primary recombination events in this *E. coli* system.
Fig. 4.8. Southern blots of transconjugants. Genomic DNA from transconjugants were restriction digested with PstI. Results showed the presence of multiple copies of the bla, IScs605, aph elements in the transconjugants. Panel A: Southern blot probed for bla. Panel B: aph. Panel C: IScs605. Predicted MW standards are listed to the right (Fermentas).
In each sequenced pOX38:IScs605 recombinant, the pentanucleotide 5’-TTCAA was used as a target for integration. The importance of this sequence in IScs605-mediated recombination was examined by conducting mating assays with a plasmid construct that had the 5’-TTCAA sequence deleted from the IS element (pJD6, Fig. 4.3). This deleted plasmid was not able to facilitate integration into pOX38 (Fig. 4.4). Therefore, the 5’-TTCAA sequence of IScs605 is essential for recombination in the E. coli system.

To examine the possible role of the vector in defining integration events in the mating assay, parallel experiments were conducted with plasmid pJD7, in which the medium copy plasmid pBBR1MCS is the vector for delivery of IScs605 into the mating assay. Analysis of the transconjugants using pJD7 as the source of IScs605 showed both similarities and differences to experiments with the pUC-based plasmids. Sequencing of the transconjugants showed that pJD7 inserted adjacent to 5’-TTCAA (Fig. 4.6), and there was evidence of concatamers in the inserted products (Fig. 4.9, panel B). The transconjugants from the pBBR1MCS matings routinely resulted in only the IS element – and not the vector– to be incorporated into pOX38 (Fig. 4.9, panel A). This is in contrast to the pUC-based delivery of the IS element which universally resulted in the integration of the entire plasmid (Fig. 4.9, panel A).
Fig. 4.9. Analysis of recombination or transposition in mating assays conducted with pJD7, the donor plasmid based on pBBR1MCS. Panel A: Percentage of transconjugants that contained the resistance marker in the pBBR1MCS or pUC18 vectors used in pJD7 and pJD5, respectively. In these assays, transconjugants were initially selected for kan resistance and then pJD5 transconjugants were tested for Amp resistance while pJD7 transconjugants were tested for Cm resistance. The data represent the percentage of Kan\textsuperscript{R} colonies that were also resistant to the second antibiotic. The error bars represent the standard deviation of three independent assays, with a total of 100 colonies examined for each replicate. Panel B: Southern blot for \textit{aph} of \textit{Psil} digested genomic DNA from Cm\textsuperscript{R} and Cm\textsuperscript{S} colonies resulting from pJD7-based mating assays. Lanes 1, 2, 3, 4 are DNA from Cm\textsuperscript{R} colonies. Lanes 5, 6, 7, 8 are DNA from Cm\textsuperscript{S} colonies. Molecular standards in kilodaltons are indicated to the left of the blot (Fermentas).
4.5: DISCUSSION

The results of the described experiments support the hypothesis that IScs605 was 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. 4.5, 4.8). Both low and high copy plasmids were successfully used in these assays (Fig. 4.9). Nucleotide sequencing showed that, while recombination was essentially random across the pOX38 target, all integration events occurred adjacent to the pentanucleotide sequence 5’-TTCAA. Sequence analysis demonstrated that there was no sequence duplication or evidence of repeats at the insertion site (Figs. 4.6, 4.7). IScs605 can also excise from target sequences following integration of entire plasmids (Figs. 4.7, 4.8). Each of these traits is consistent with the observed organization of the tet(C) islands in C. suis.

IScs605 is structurally related to IS605, IS606, IS607 and related IS elements from Helicobacter pylori (10-13). These relationships can be seen in a phylogenetic analysis of orfA and orfB, which have apparent homologs both within the IS605-like IS elements, and within IS elements containing only single candidate transposases (Fig. 4.2). 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 of the IS adjacent to the sequence 5’-TTTAA or 5’-TTTAAC. H. pylori IS607 requires orfA for transposition, but not orfB, and inserts adjacent to single G nucleotides. H. pylori ISHp608 inserts at the tetranucleotide sequence 5’-
TTAC, also in an orfA-dependent manner. 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 activity of IScs605 in *E. coli*.

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 IScs605 (Fig. 4.7, panel B). In some instances (strains 130 and 132) the tet(C) island lacks the IS element, and the sequence surrounding a possible excision site is 5'- AATTCAA (Fig. 4.1: compare strain R19 versus strain 130). An excision event might also explain the small size of the R27 and H7 islands. In these strains, the genomic island has a large deletion relative to homologous larger islands (i.e. strain R19 and R24; Fig. 4.1) and the deletion occurs at a 5'- AATTCAA sequence within the larger tet(C) islands. Therefore, it is likely that IScs605-mediated excision events were also responsible for production of the smaller tet(C) island such as those seen in *C. suis* strains R27 and 130.

There were, however, some differences between the behavior of IScs605 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 (5). Additionally, when the medium copy plasmid pBBRMCS was used as a source
of IScs605 (i.e. pJD7), the predominant insertion event involved an actual transposition of the IS element into the pOX38 target. These results provided 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 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 IScs605 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 TetR C. suis (4), and in that model, only integration of the entire tet(C) island into the chlamydial genome would lead to selection of natural recombinants.

Deletion analysis was used to test the role of the individual orfs within IScs605. The data suggested that orfA, but not orfB, is essential for both recombination activity and insertion specificity (Fig. 4.4). These results parallel the tested IS605-like elements in the Helicobacter system, in which the homologous orfA sequence appears critical for transposition in IS605-related elements (10, 12, 13). The function of the highly conserved orfB in the IS605 family of IS elements remains unclear. It was hypothesized that orfB of H. pylori IS605 serves to increase bacterial fitness, and this is supported by BLAST analysis which shows that IScs605 orfB is similar to the putative E. coli virulence factor, ydcM (4). Another possibility is discussed by Kersulyte et al. (12) who noted that the product of orfB shares similarity with a predicted E.
coli protein – encoded by gene B1432 (13) – which might serve to complement a deletion in orfB in the mating assays. The chlamydiae do not have an apparent homolog of orfB within their genomes (not shown) and thus the gene may be important for integration in this system.

The described experiments demonstrate that IScs605 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 IScs605 activity led to the integration of the tet(C) islands into the C. suis chromosome. IScs605 is the only example of an IS element within any chlamydial species, and this work describes the first analyses of the activity of any 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 Chlamydia, a technology that is currently unavailable in this system. The exploration of this possibility is currently underway in our laboratory.

4.6: ACKNOWLEDGMENTS

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4.7: REFERENCES


5: General Conclusion

The central theme of this thesis was the analysis and characterization of Tet-resistant *Chlamydia suis* that were isolated from swine farms in the Midwest. These isolates were shown to be resistant to high concentrations of Tet. One of these Tet-resistant strains, R19, was shown to produce infectious chlamydia in Tet-concentrations up to 4 g/ml, while the two Tet-sensitive strains, S45 and L2, were inhibited by less than 0.1 g/ml Tet. The Tet-resistant *C. suis* represented the first example of stable, homotypic resistance in any chlamydiae. Further analysis of the Tet-resistant *C. suis* strains found a set of related genomic islands that were not found in S45. The genomic islands contained the *tet*(C) resistance gene, *mob* and *rep* genes, and the first example of an insertion element, IScs605, in any chlamydiae. The *tet*(C) gene is an efflux pump while the *mob* and *rep* genes contain homologs that are found in a number of mobilizable plasmids. The IScs605 insertion element had homology to the IS605 family of IS elements and encoded 2 phylogenically distinct transposases. The IScs605 element was studied in *E. coli* and was shown to have high transposition activity and insertion specificity. The insertion specificity provides evidence that the insertion of the genomic islands into the chlamydial chromosome of the Tet-resistant strains was mediated by the IScs605 insertion element.

The rationale of this research was to understand the mechanism of horizontal transfer and to use that information to produce a genetic transformation system for chlamydiae. There is currently no genetic
transformation system in chlamydiae and the Tet-resistant C. suis represent the first example of horizontal transfer. In the case of the Tet-resistant strains, genomic islands were transferred into C. suis from a donor organism and then inserted into the chlamydial chromosome where it was stably propagated. Analysis of the Tet-resistant strains showed that the genomic islands had high homology to the mobilizable pRAS3 plasmids that are found in Aeromonas salmonicida. It is doubtful that chlamydia interacted with Aeromonas so the identity of the responsible donor organisms continue to be a mystery. It is hypothesized that because of the mob and rep genes, a mobilizable plasmid was transferred into chlamydia by conjugation.

The genomic islands should provide valuable tools in establishing a genetic transformation system. They show that the chlamydia can become resistant to Tet so this should be a viable selectable marker. The genomic islands also contain an insertion element that has been shown to be functionally active in E. coli and were involved in the integration of the island into the chlamydial chromosome. Our laboratory is establishing a genetic transformation system using the insertion element and the Tet marker.