Contribution of Horizontal Gene Transfer to Virulence and Antibiotic Resistance in Pathogens Transmitted in Healthcare Facilities

by

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DEDICATION

To my friends, family and Chris.

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ABSTRACT

Contribution of Horizontal Gene Transfer to Virulence and Antibiotic Resistance in Pathogens Transmitted in Healthcare Facilities

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Healthcare-associated infections (HAIs) are a significant cause of morbidity and mortality in the United States and cost up to $4.5 billion annually. Horizontal gene transfer (HGT) contributes to the evolution and emergence of pathogenic strains causing these infections by allowing DNA to be shared among diverse bacteria. This dissertation identifies and investigates the molecular mechanisms that affect HGT between bacteria and the factors that govern HGT at the patient level.

Catheter-associated urinary tract infection (caUTI), the most common HAI, is often polymicrobial. Using comparative genomics, this study identified ICE*Pm1*, a genomic island shared among *Proteus mirabilis*, *Providencia stuartii*, and *Morganella morganii*, common agents of polymicrobial caUTI. We show that this island is an integrative and conjugative element that is self-transmissible between clinical strains at

a frequency of 1.35 x 10^{-5} and that transfer is dependent on an integrase and a type IV secretion system encoded within the element. ICE*Pm1* also encodes an adhesin and known iron-acquisition system; therefore ICE*Pm1* may provide a fitness advantage during colonization of the catheterized urinary tract. In support of this, I observed that ICE*Pm1* was present in 39/39 *P. mirabilis* urinary isolates screened, while distribution was heterogeneous (15/23) among *P. mirabilis* commensal strains.

Because proximity is necessary for HGT to occur, we investigated risk factors for co-colonization with methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant Enterococci (VRE) among residents of long-term care facilities. Co-colonization with these organisms is of interest because of the emergence of vancomycin-resistant *S. aureus* (VRSA); attributable to HGT of the *vanA* gene cluster from VRE to MRSA. Because MRSA remains susceptible to vancomycin the emergence of vancomycin-resistant *S. aureus* is alarming for healthcare professionals. We show that wounds, indwelling devices and functional disability are risk factors for co-colonization with MRSA and VRE; an event that precedes VRSA emergence.

This dissertation provides insight into the mechanisms of transfer of mobile elements and therefore dissemination of virulence and antibiotic resistance genes. It also describes risk factors for co-colonization with MRSA and VRE within a patient, an event that is necessary for HGT to occur *in vivo*.

Chapter 1

Introduction

HEALTHCARE-ASSOCIATED INFECTIONS

Epidemiology

Healthcare-associated infections (HAIs), those acquired by a patient in a healthcare setting, are a significant cause of morbidity and mortality in acute-care and long-term care facilities (LTCFs) in the United States. HAIs, also referred to as hospital-acquired infections or nosocomial infections, include bloodstream infections, surgical site infections, skin and soft tissue infections, pneumonias and the most common: urinary tract infections (Klevens, Edwards et al. 2007). Due to prolonged incubation periods, heterogeneity of populations in facilities and lack of reporting mandates, the exact number of HAIs that occur each year in the United States is not known (2003; Council 2005; Klevens, Edwards et al. 2007). It is estimated that 1.7 million HAIs occurred in 2002 in acute care facilities and 1.6-3.8 million in LTCFs in 2000 (Strausbaugh and Joseph 2000; Klevens, Edwards et al. 2007). In 2002, the estimated number of deaths resulting from HAIs in the U.S. was 98,987 (Klevens, Edwards et al. 2007).

In addition to causing significant morbidity and mortality, HAIs place a considerable economic and resource burden on healthcare facilities. Treating these

infections is difficult due to underlying patient comorbidities and the increasing rates of antibiotic resistance among organisms that are circulating in healthcare facilities. Total costs attributed to HAIs in the United States vary from $4.5 billion estimated in 1992 to more recently, due to the rising costs in healthcare and rates of HAIs, an estimated $35 billion in 2009 (Jarvis 1996; Scott 2009).

All types of microorganisms can be causative agents of HAIs including viruses (*Influenza A*, Enteroviruses), fungi (*Candida spp.*, *Aspergillus*) and a plethora of bacteria. Both Gram-negative and Gram-positive bacteria are important etiologic agents; *Staphylococcus aureus* is the most common Gram-positive etiologic agent, accounting for 15% of all HAIs and *Escherichia coli* the most common Gram-negative agent, representing 10% of all HAIs (Hidron, Edwards et al. 2008). With *Enterococcus spp* and other members of the Gram-negative *Enterobacteriaceae* family, the previously mentioned pathogens account for more than 75% of all HAIs. Most organisms that cause HAIs are commensal organisms that generally do not cause disease. But, because persons in healthcare facilities can be immunocompromised, have other comorbidities or risks that make them more susceptible to infection, these organisms can be serious pathogens. Moreover, these organisms are often resistant to multiple antibiotics making treatment difficult.

Prevention of HAIs. The Centers for Disease Control and Prevention as well as the Association for Professionals in Infection Control and Epidemiology have published recommendations and guidelines for controlling and preventing HAIs (Strausbaugh, Crossley et al. 1996; Kallen, Hidron et al. 2010). These recommendations include,

among others, use of indwelling devices only when indicated, satisfactory surveillance and infection control programs, proper sterilization and disinfection of patient rooms, contact precautions of healthcare workers and patient decolonization strategies. Although these methods have helped reduce the number of total HAIs, it has been suggested that targeting patients with particular risk factors associated with HAIs may be more cost-effective and have greater success at reducing the rates of HAIs (Mody, Bradley et al. 2011).

Risk factors for HAIs. The use of indwelling devices, such as urinary catheters, feeding tubes and peripherally inserted central catheters, are a major risk factor for colonization and infection with bacterial pathogens. Up to 25% of residents of LTCFs are reported to have indwelling devices (Tsan, Davis et al. 2008). Patients with indwelling devices are more likely to 1) have clinical infections 2) be asymptomatically colonized with antibiotic-resistant pathogens and 3) be colonized by a greater number of different bacterial species than patients without indwelling devices (Mody, Maheshwari et al. 2007; Mody, Bradley et al. 2011).

Indwelling devices provide a surface area for bacterial colonization and biofilm formation at portals of entry into the body and these devices require increased physical contact between patients and healthcare workers. Both of these phenomena could lead to higher rates of bacterial colonization in this population. Additionally, patients with indwelling devices are also more likely to be on an antibiotic regimen, again increasing the risk for colonization and infection with antibiotic-resistant organisms (Mody, Maheshwari et al. 2007).

In addition to the use of indwelling devices, high patient turnover rates as well as outbreaks from introduction of organisms from the community are important causes of HAIs in acute care facilities (Jarvis 1996). In long-term care facilities, or skilled-nursing facilities, the patient population differs. The average resident is 80 years old and these residents often have immune dysfunction, impaired mental status and functional disability which can all contribute to risk for a HAI (High, Bradley et al. 2005; Smith, Bennett et al. 2008). Additionally in this group, high rates of comorbidities and other underlying illnesses are important risk factors for infection (Smith, Bennett et al. 2008).

Antibiotic Resistant Organisms

The rates of infections caused by antimicrobial-resistant pathogens are steadily increasing in both acute-care facilities and long-term care facilities (Pop-Vicas and D'Agata 2005; Crnich, Safdar et al. 2007). Resistance to more than one class of antibiotics is not uncommon and infections caused by multiple-drug resistant organisms increases the morbidity, mortality and cost of treating HAIs (Hawkey and Jones 2009). Infections from these pathogens can be a result of introduction of these pathogens into the healthcare setting and healthcare workers can inadvertently serve as important vectors for transmission of pathogens among patients (Viray, Linkin et al. 2005). This results in outbreaks within restricted units in the hospital or within hospitals themselves. These outbreaks can be limited or controlled, or can result in the organisms becoming endemic in facilities.

A second way infection with antimicrobial-resistant pathogens can occur in healthcare facilities is when resistance develops among bacteria already colonizing a patient. This can occur from selection for *de novo* mutations as a result of high rates of antibiotic use in healthcare facilities. It can also occur by sharing of antibiotic resistance genes between bacteria, known as horizontal gene transfer (HGT). HGT remains the most important mechanism of resistance development and subsequent dissemination of resistance elements among pathogens. Moreover, mobile elements that are horizontally transferred, such as plasmids, consistently encode for resistance to multiple antibiotics, further complicating the problem. A 2005 surveillance study showed that multi-drug resistant pathogens were associated with approximately 16% of all HAIs (Hidron, Edwards et al. 2008). The most common were methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant Enterococci (VRE) and carbapenem- and extended-spectrum cephalosporin- resistant *Enterobacteriaceae* (Hidron, Edwards et al. 2008).

Methicillin-Resistant Staphylococcus aureus. *S. aureus* is a commensal organism that asymptomatically colonizes approximately 30% of individuals in the nares while approximately 1.5% of all individuals are colonized with methicillin-resistant *Staphylococcus aureus* (MRSA) in their nares (Gorwitz, Kruszon-Moran et al. 2008). Although the most common site of MRSA carriage is the nares, individuals can be colonized at other areas on the body including the axilla, groin and gastrointestinal tract. Colonization is generally asymptomatic, yet if MRSA is introduced into a normally

sterile site, serious skin and soft tissue infections, endocarditis or bacteremia can develop (Lindsay and Holden 2004).

Penicillin was first used to treat *S. aureus* infections, until approximately 80% of all strains emerged as resistant in the 1960s (Appelbaum 2007). Semi-synthetic penicillins, such as methicillin, were created as alternative drugs for treatment; resistance quickly developed. The first documented outbreak of MRSA in healthcare facilities occurred in 1976, and now MRSA continues to be an important hospital pathogen (Shanson, Kensit et al. 1976). HAIs caused by *S. aureus* in hospitals are more likely to be methicillin-resistant than sensitive, and these strains often are resistant to multiple additional antibiotics, further complicating treatment of these infections (Boucher, Miller et al. 2010). Vancomycin and linezolid are commonly used to treat MRSA infections, although strains of vancomycin-intermediate *Staphylococcus aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA) have recently emerged as a result of HGT (Appelbaum 2007).

In addition to carrying multiple-drug resistance genes, MRSA may encode other important virulence factors in its genome, several of which have been acquired by HGT. Resistance to methicillin is attributed to acquisition of the staphylcocccal chromosome cassette (*mec*) (Appelbaum 2007). The Panton-Valentine leukocidin cytotoxin which causes necrotizing lesions as well as several other superantigen toxins are encoded in genomic islands that can be mobilized by bacteriophages (Lindsay and Holden 2004; Ubeda, Maiques et al. 2005). HGT of the pathogenicity island encoding two superantigen toxins contributing to virulence in MRSA has specifically been shown to increase transfer in the presence of ciprofloxacin (Ubeda, Maiques et al. 2005).

Vancomycin-resistant Enterococci (VRE). Enterococci are gram-positives that colonize the gastrointestinal tract. The most common species associated with clinical infection are *E. faecalis* and *E. faecium* (Cetinkaya, Falk et al. 2000). Enterococci showing resistance to vancomycin started circulating in hospitals in the 1970s and are now commonly endemic in both acute care and long-term care facilities (Woodford, Johnson et al. 1995; Cetinkaya, Falk et al. 2000). The 2004 National Nosocomial Infection Surveillance System report stated that vancomycin resistance in Enterococci was 28.5% (2004). This represents a 12% increase since 1991 (Benson, Sprague et al. 2007). Resistance to vancomycin can be mediated by several vancomycin resistance phenotypes, *vanA*, *vanB*, *vanC*, *vanD* and *vanE*. *vanA* and *vanB* type resistance are a result of horizontal gene transfer of the vancomycin-resistance mediated genes and the *vanA* phenotype is most described in the healthcare setting (Cetinkaya, Falk et al. 2000). Enterococci are important causes of endocarditis, meningitis, wound infections and urinary tract infections. Additionally, they were reported as the third most common cause of HAI bacteremia in 1991 (Schaberg, Culver et al. 1991).

Multi-drug resistant Gram-negative bacilli. Significant increases have been observed in the prevalence of MDR Gram-negative bacteria (GNB) (D'Agata 2004) highlighted by reports that as high as 53% of GNB are co-resistant to aminoglycosides, third-generation cephalosporins and quinolones (D'Agata 2004; Pop-Vicas and D'Agata 2005). In a retrospective study of 3,314 LTCFs located in 5 states, the incidence rate of antibiotic-resistant infections was 12.7 cases per 1000 residents per year (Rogers, Mody

et al. 2008). The high prevalence of resistance to quinolones and third-generation cephalosporins is alarming because these antimicrobials have broad spectrum activity and are often used empirically (Viray, Linkin et al. 2005). Infections as well as colonization with these organisms are dramatically increasing. Colonization with MDR-GNB is a risk factor for subsequent infection and these organisms have been shown to colonize more persistently and to co-colonize often with different species of MDR-GNB (O'Fallon, Gautam et al. 2009). Infection with MDR-GNB leads to longer hospital stays, increased mortality and higher economic costs (Cosgrove, Kaye et al. 2002).

Catheter-associated urinary tract infections

Urinary tract infections (UTIs) are one of the most common bacterial infections that occur in humans. Half of all women will develop a UTI in their lifetime and many will continue to have recurrent infections (Mobley and Warren 1996). Cystitis (infection of the bladder) and pyelonephritis (infection of the kidneys) are both types of UTI; pyelonephritis is considered more serious and can result in bacteremia (Mobley and Warren 1996). UTIs are a significant problem in healthcare facilities where catheter-associated urinary tract infection (caUTI) accounts for over 80% of all UTIs in these facilities and causes over 40% of all HAIs, making caUTI the most common HAI (Jacobsen, Stickler et al. 2008).

Indwelling urinary catheters are used to manage urinary retention, allow urinary drainage in patients with neurogenic bladder dysfunction, control incontinence, relieve anatomical or physiological obstruction and obtain accurate measurements of urine

output in severely ill patients (Wong and Hooton 1981; Kunin 2006). The Foley catheter is the most common type of urinary catheter used in the United States and is comprised of a tube that is attached to an inflatable balloon that can hold liquid (Jacobsen, Stickler et al. 2008). The tube is inserted into the urethra and the balloon is inflated to hold the catheter in place within the bladder to drain urine. Although theoretically it is an enclosed sterile environment, contamination by healthcare workers or introduction of endogenous microbiota by the catheter into the urinary tract can result in infection. In addition, bacteria can ascend along the catheter surface to reach the bladder, resulting in infection. In the nosocomial and long-term care facility setting, increased measures have been made to prevent UTI, including catheter removal when it is no longer indicated (decreasing the duration of catheterization) and use of alternative and intermittent catheter options (Wong and Hooton 1981).

Catheter-association UTIs can be diagnosed by symptoms and presence of bacteria in the urine, although differences in definitions do exist. Definitions of bacterial colonization load diagnostic of a UTI are not consistent and can range from 10^3 to greater than 10^5 cfu/ml from a patient (Daifuku and Stamm 1984; Foxman 2010). Clinical symptoms may be present and include frequency and urgency of urination, dysuria and flank pain (pyelonephritis), although as many as 50% of catheterized patients with bacteriuria do not have symptoms (asymptomatic bacteriuria) (Ronald 2002; Cope, Cevallos et al. 2009). Empiric antibiotic treatment of UTI has become common without clinical cultures performed in acute uncomplicated cystitis and catheterized cases (Damron, Warren et al. 1986; Foxman 2002). Antibiotic resistance

rates in organisms causing UTI are increasing rapidly and therefore clinical cultures are needed to determine antibiotic susceptibilities profiles (Ronald 2002; 2004).

The risk of developing a UTI increases dramatically by introducing a catheter into the urethral opening (Jacobsen, Stickler et al. 2008). UTIs that occur as the result of a catheter being in place are considered complicated UTIs (Ronald 2002). One in five people entering an acute care facility will receive an indwelling urinary catheter and nearly all that are catheterized for an extended period will go on to develop bacteriuria (Warren, Tenney et al. 1982). Approximately 10% of patients are catheterized in LTCFs, again making UTIs the most common bacterial infection in LTCFs as well (Kunin 2006).

Although bacteriuria can be asymptomatic, over 1 million cases of caUTI occur annually in the US with each episode adding a minimum $676 additional cost to hospital bills (Saint 2000). CaUTI prolongs patient stay by 1 additional hospital day and is a risk factor for bacteremia and other complications resulting from infection. Catheterization in the elderly is of particular concern, considering over 50% of bacteremic episodes in this population originate from the urinary tract (Nicolle, Strausbaugh et al. 1996). Recently (2009) Medicare has ceased to reimburse hospitals for the economic consequences of caUTI, thus providing further incentive for the control and prevention of caUTI (Saint, Meddings et al. 2009).

Causative agents of catheter-associated UTI. Many organisms possess the ability to colonize the urinary tract and the epidemiology differs between caUTI and community-acquired UTI. Although uropathogenic *Escherichia coli* (UPEC) is the most common

etiologic agent of community-acquired UTI and the most common cause of caUTI after short-term catheterization, when catheterization occurs for extended periods (>30 days), it represents only a minor proportion of the causative agents involved (Ronald 2002; Stickler 2008). Infections in catheterized individuals are generally more diverse and of polymicrobial nature (Warren, Tenney et al. 1982; Ronald 2002; Kunin 2006). In a study conducted by Warren et al., more than two strains of bacteria were cultured from 77% of 609 weekly urine specimens. In 16% of specimens 4 and 5 species of organisms were isolated (Warren, Tenney et al. 1982). Organisms besides UPEC that can colonize the urinary tract and are commonly found in caUTI include *Klebsiella* spp., *Enterococci*, *Pseudomonas aeruginosa*, *Staphylococcus spp., Providencia stuartii*, *Proteus mirabilis* and *Morganella morganii*. Specifically, *P. mirabilis*, *P. stuartii* and *M. morganii* are more prevalent due to their persistence over time within the catheterized urinary tract (Warren, Tenney et al. 1982; Mobley and Warren 1996; Ronald 2002).

Model of Pathogenesis. *P. mirabilis*, *P. stuartii*, and *M. morganii* together account for over half of all caUTIs and share a common virulence feature: urease production (M, S et al. 2006). Production of urease confers the ability to hydrolyze urea into ammonia and bicarbonate, which get excreted from the cell. This causes an increase in the pH of the surrounding environment and subsequent precipitation of calcium phosphate and magnesium ammonium phosphate resulting in crystal formation. This pathologic process contributes to significant patient morbidity because of catheter encrustation and

blockage and the formation of bladder and kidney stones that result (Warren, Tenney et al. 1982; Macleod and Stickler 2007).

Urease production also plays a role in the formation of biofilms on these devices. Biofilm formation on the urinary catheter happens regularly by adherent organisms and crystal formation can seed this event. These biofilms are often composed of multiple species and co-colonization by different species has different effects on patient outcomes (Mobley and Warren 1996; Macleod and Stickler 2007). Catheter biofilms are difficult to treat because they can block antibiotics from reaching bacteria residing in the interior of these structures, thus requiring antibiotic concentrations well over the organisms' standard MIC for eradication (Donlan 2001).

In addition to producing urease, these organisms possess other important virulence factors that allows for their colonization of the urinary tract despite the adaptive and innate immune systems of the host. These include toxins that break down host epithelium, iron-acquisition systems aiding in growth, exopolysaccharide production, which benefits biofilm formation and the several motility appendages such as flagella and fimbria which are important in ascension of the urinary tract.

Bacteria isolated from UTIs are generally genetically related to strains endogenously colonizing the patient (Sabbuba, Mahenthiralingam et al. 2003) thus suggesting self-inoculation as a potential cause of UTI. Additionally, bacteria that colonize the urethral meatus can ascend the urinary tract through the catheter lumen or along the catheter-urethral interface, the latter is estimated to be the route of entry 70-80% of the time (Mobley and Warren 1996). Therefore, adhesins that allow for adherence to the catheter and uroepithelium and flagella that provide motility are

important virulence factors of these organisms. In particular, *P. mirabilis* possesses the unique ability to differentiate into multi-flagellated swarming cells, which have been shown remarkably to aid in movement over urinary catheters (Jones, Young et al. 2004).

Vancomycin-resistant *S. aureus* (VRSA)

Documented Cases. In 2002, the first MRSA isolate with high-level resistance to vancomycin was reported. Since then, 10 additional vancomycin-resistant *S. aureus* (VRSA) isolates with an MIC ≥ 16 μg/ml have been identified (Chang, Sievert et al. 2003; Finks, Wells et al. 2009). The VRSA cases have all occurred in individuals with underlying conditions including diabetes, chronic ulcers and end-stage renal disease (Zhu, Clark et al. 2008; Prevention 2011). Many of the isolates were recovered from clinical wound cultures (10/11) and 8/11 isolates were reported in Southeastern Michigan (Table 1-1). The emergence of VRSA is concerning because vancomycin is one of the antibiotics commonly used to treat MRSA infections.

Mechanism of Emergence. Prior to 2002, several isolates of MRSA with intermediate susceptibility to vancomycin (VISA; MIC = 4-8 μg/ml) were reported, first in Japan (Hiramatsu 1997). These organisms have a different mechanism of resistance to vancomycin that makes them intermediately susceptible. In response to vancomycin they thicken their cell walls and thus trap the antibiotic, preventing it from binding to its target site, the C-terminal acyl D-alanyl D-alanine of pentapeptide precursors (Howden,

Davies et al. 2010). In high-level vancomycin resistance (MIC >16 μg/ml), presence of the *vanA* gene cluster results in altered pentapeptide peptidoglycan precursors, D-alanyl-D-lactate, which have reduce affinity for vancomycin (Cetinkaya, Falk et al. 2000). Acquisition of the *vanA* gene cluster occurs by horizontal gene transfer from VRE to MRSA; therefore studies of co-colonization with MRSA and VRE have been of interest.

Transfer of the *vanA* gene from VRE to MRSA has been documented on the skin of hairless mice (Noble, Virani et al. 1992). This suggests that VRSA emergence occurs through a horizontal gene transfer event between VRE and MRSA. In support of this, isolation of VRSA has been coincident with isolation of VRE from the same source in several of the patients (Zhu, Murray et al. 2010). And in several of these cases the *vanA* gene was confirmed to be the same in both the VRE and VRSA isolate (Zhu, Clark et al. 2008). This suggests that co-colonization is a necessary precursor to the transfer event. Several studies have investigated the occurrence of MRSA/VRE co-colonization among several different study populations.

MRSA/VRE co-colonization studies. Prevalence of MRSA and VRE co-colonization has been investigated in several studies. Several of these studies use clinical cultures to look at prevalence and therefore are not actively culturing for MRSA and VRE (Benson, Sprague et al. 2007). The Duke Outreach Infection control network found 0.29% of patients with MRSA to also be infected with VRE and 3.8% of patients with VRE to also have MRSA (McDonald, Engemann et al. 2004). These estimates were based on chart review of clinical cultures from several hospitals, as well as a few

surveillance cultures from a sub-set of the hospitals. The use of clinical cultures can explain why prevalence was so low, and why it differs greatly from a study conducted by Reyes et al. Here, co-colonization was reported as common, yet Reyes et al. restricted their analysis to patients of a tertiary-care facility in Detroit, that had clinical cultures positive for VRE (Reyes, Malik et al. 2010). Among patients with a clinical culture positive for VRE, 19.8% of patients also had a clinical culture positive for MRSA within 14 days of the VRE culture. The prevalence is much higher in this population because the base population is defined as having a positive VRE clinical culture. Few studies have looked at MRSA/VRE co-colonization and used active surveillance to identify patients colonized. Indeed, these studies have limited their surveillance cultures to the nares for MRSA and rectum for VRE (Furuno, Perencevich et al. 2005) or the rectum for both organisms (Ray, Pultz et al. 2003). This does not estimate the true prevalence of co-colonization, as patients can be colonized at many different sites with these organisms.

Table 1-1. Documented VRSA cases 2002-2011, United States.

Characteristics of the 11 confirmed VRSA cases, United States. VRSA cases were identified by clinical culture, therefore culture for VRE was not necessarily done in all cases. No VRSA cases reported from outside of the United States have been confirmed by CDC to have a vancomycin MIC > 32 μg/ml (Tenover 2008). Table modified from (Zhu, Clark et al. 2008; Prevention 2011).

Case #	Isolation date	State	Age	VRSA site of isolation	VRE site of isolation	Diagnosis	Underlying Conditions
1	6/2002	MI	40	foot ulcer, dialysis catheter	foot wound	Plantar soft tissue infection	Diabetes, dialysis
2	9/2002	PA	70	foot ulcer	NA	Osteomyelitis	Obesity
3	3/2004	NY	63	urine from nephrostomy tube	Rectum	No infection	Multiple sclerosis, Diabetes, kidney stones
4	2/2005	MI	78	toe wound	Rectal swab	Gangrene	Diabetes, vascular disease
5	10/2005	MI	58	surgical site wound	SSI	Surgical site infection	Obesity
6	12/2005	MI	48	foot ulcer	Rectum	Osteomyelitis	MVA, chronic ulcers
7	10/2006	MI	43	triceps wound	NA	Necrotizing fasciitis	Diabetes, dialysis, chronic ulcers
8	10/2007	MI	48	foot wound	NA	Osteomyelitis	Diabetes, obesity, chronic ulcers
9	12/2007	MI	54	foot wound	NA	Osteomyelitis	Diabetes, hepatic encephalopathy
10	2009	MI	53	plantar foot wound	NA	Plantar sort tissue infection	Diabetes, obesity, lupus, rheumatoid arthritis
11	2010	DE	64	wound drainage	NA	Prosthetic joint infection	Diabetes, end-stage renal disease

HORIZONTAL GENE TRANSFER AND INTEGRATIVE AND CONJUGATIVE ELEMENTS

Horizontal Gene Transfer (HGT)

Advancing technology has made the sequencing of bacterial genomes faster and more cost effective. As a result, the amount of whole genome sequence data available has increased exponentially in recent years. Comparative genomics yields a detailed look at the sequence divergence between any two genomes of bacteria of the same species (Pallen and Wren 2007). In some cases, when several isolates of a single bacterial species are compared at the sequence level, dramatic differences can be observed in their genetic makeup (Lloyd, Rasko et al. 2007; Tenaillon, Skurnik et al. 2010). Although this dynamic property does not exist for all genomes, comparative genomics has demonstrated how different genomic content can be for any single species of bacteria belonging to the *Enterobacteriaceae* family (Ochman, Lawrence et al. 2000).

The evolution of bacterial genomes occurs in a variety of ways including *de novo* mutations, gene rearrangements and gene gain and loss through acquisition of single genes or larger segments of DNA (Dobrindt, Hochhut et al. 2004). The core genes of a genome, such as metabolism genes necessary for growth, are usually stable and can be supplemented by accessory genes that provide certain properties important for virulence and pathogenesis (Ochman, Lawrence et al. 2000; Bentley 2009). The dynamic composition of these genomes results in phenotypic diversity among species, which allows for adaptation and survival in different environmental niches under

selective pressures (Ambur, Davidsen et al. 2009). The greatest and most rapid changes to a bacterium's genetic composition occurs through HGT (Gal-Mor and Finlay 2006).

Horizontal (or lateral) gene transfer is the process of acquiring DNA from an outside source; that is, not by vertical transmission from a parental cell. It most generally occurs through three different mechanisms: transduction, transformation or conjugation (Koonin, Makarova et al. 2001; Bushman 2002). Transduction, a process mediated by bacteriophages initiates upon phage binding to a receptor on the bacterial cell surface. Bacteriophages then inject their DNA into a host cell and this DNA site-specifically recombines and integrates into the host chromosome (Bushman 2002). Phage DNA encodes proteins that are necessary for the bacteriophage life cycle as well as can carry genes that were taken up from the bacterium it previously infected (Bushman 2002; Chen and Novick 2009). HGT through natural transformation is the uptake of DNA by naturally competent cells (Mazodier and Davies 1991). Plasmids and double-stranded DNA can be introduced through the cell membrane, although specific oligonucleotide sequences or competence factors may be required. Double stranded DNA can be incorporated into the bacterial chromosome using host-encoded recombination mechanisms while plasmids generally are not incorporated and replicate independently within the bacterial cell (Mazodier and Davies 1991).

The process that usually results in incorporation of the most substantial sequences of DNA, is conjugative HGT (Mazodier and Davies 1991; Koonin, Makarova et al. 2001). Conjugative DNA transfer is best characterized in the context of conjugative plasmids where a type IV secretion system (T4SS) is encoded on the plasmid (de la Cruz, Frost et al. 2010). Through the conjugative process, a mating pore

and conjugative pilus are formed which results in cell-to-cell contact between two bacterial cells. Single-stranded plasmid DNA is then transferred through this pilus and is subsequently incorporated into the recipient host chromosome, again by host recombination mechanisms (de la Cruz, Frost et al. 2010). Because single-stranded DNA is transferred, a new double stranded plasmid copy is replicated in the recipient cell and a copy is retained in the donor cell through replication of the template plasmid (de la Cruz, Frost et al. 2010). Plasmids contain their own origin of replication and are thus maintained and propagated independently of the bacterial chromosome within bacterial cells.

Bacterial Type IV secretions systems (T4SSs).

Plasmid-encoded T4SSs mediate conjugal transfer of these self-replicating mobile elements. T4SSs are macromolecular translocation systems consisting of multiple proteins making up a secretion channel and pilus that together span the cellular membrane (Alvarez-Martinez and Christie 2009). These complexes are encoded by multiple genes and are generally organized into a functional unit of DNA (Juhas, Crook et al. 2008). Bacterial T4SSs can be classified into three groups based on the substrates that are translocated; those that release or take up DNA outside the cell, those translocating effectors and those that are conjugation systems that translocate DNA to recipient cells (Alvarez-Martinez and Christie 2009). This third type of T4SS is responsible for translocation of plasmid DNA from cell to cell and thus plays an important role in HGT.

Conjugative T4SSs are well characterized and contribute to the dissemination of plasmids between different bacterial species (Smillie, Garcillan-Barcia et al. 2010). In addition to the T4SS genes or mating pore formation (MPF) genes, in order to be conjugative a plasmid must also encode a set of mobilization genes (MOB). Mobilization genes consist of an origin of transfer (*oriT*) at minimum, and often include a relaxase protein and a type 4 coupling protein (T4CP). The relaxase is important for recognizing *oriT* and with other auxiliary proteins linearizing the plasmid. The T4CP then recruits the relaxasome (relaxase/*oriT*-associated DNA) to the mating pore and possibly supplies the energy for transfer (Smillie, Garcillan-Barcia et al. 2010). The MPF genes form a membrane associated transport channel or the conjugative pilus. Cell to cell contact via the pilus is necessary for successful transfer. All of the proteins necessary for mating pore formation are considered part of the T4SS.

Some plasmids harbor a set of MOB genes, but do not encode their own T4SS. These plasmids are called "mobilizable" plasmids and can be transferred *in trans* by a T4SS of another plasmid. By recognizing similar *oriT* sequences, the T4CP of the T4SS recruits the relaxasome of the mobilizable plasmid to the mating pore formed by the conjugative plasmid. Thus for mobilizable plasmids only a set of MOB genes are necessary for their dissemination (Smillie, Garcillan-Barcia et al. 2010) (Figure 1-1).

Detection of horizontally acquired DNA. Signature properties of horizontally acquired DNA allow for its detection within the host genome (Koonin, Makarova et al. 2001). Horizontally acquired DNA can have GC content distinct from that of the surrounding chromosome. This foreign DNA is often flanked by direct repeats, carries mobile genes

such as transposons and insertion elements, and can carry genes important for survival in different environments (Ochman, Lawrence et al. 2000). Additionally, horizontally acquired DNA is generally found interspersed between genes that are conserved across bacterial species. These regions of DNA that have distinctive properties distinguishing them from the surrounding chromosome are generally referred to as genomic islands (GIs).

GIs can be any region of DNA in the chromosome identified by the properties above, although not all GIs are easily identifiable. GIs are further characterized as symbiosis islands, metabolic islands, or pathogenicity islands (PAIs) (Juhas, van der Meer et al. 2009). The most commonly discussed of these types of islands are PAIs, most notably because of their propensity to confer upon the host pathogenic or virulence properties (Hacker and Kaper 2000). The high pathogenicity island (HPI) of *Yersinia pestis* is a classic example of a PAI which carries the yersiniabactin iron-siderophore operon thus conferring the ability of iron-scavenging within the host on the bacterium (Carniel, Guilvout et al. 1996; Hacker and Kaper 2000). Additionally, the locus of enterocyte effacement (LEE) PAI in enterohemorrhagic *E. coli* encodes a type III secretion system that enhances virulence after attachment to host epithelial cells in the gastrointestinal tract (Perna, Mayhew et al. 1998; Hacker and Kaper 2000). It is widely accepted that these islands, which confer important virulence properties to pathogens, were anciently acquired and have lost their ability to transfer (Boyd, Almagro-Moreno et al. 2009). Yet, recent evidence has suggested that several GIs may have retained their transfer ability; but the detection of this ability proves difficult (Schubert, Darlu et al. 2009).

Factors affecting HGT. Although the complete mechanisms of induction of horizontal gene transfer are not well described, environmental conditions, biofilm formation and antibiotic selective pressure have been shown to play a role in activation (Hausner and Wuertz 1999; Molin and Tolker-Nielsen 2003; Beaber, Hochhut et al. 2004; Minoia, Gaillard et al. 2008). In microbial biofilms, the rate of foreign DNA uptake and transfer of mobile elements by bacteria is increased compared to that of planktonic cells, most likely due to the close proximity of cells in these environments (Ehrlich, Ahmed et al. 2010).

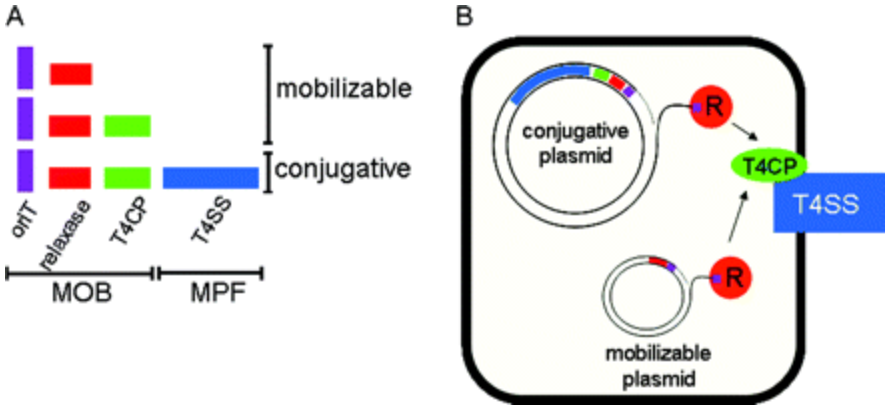
Induction of the SOS response is an important pathway that has been shown to have an effect on transfer of mobile elements (Beaber, Hochhut et al. 2004; Couce and Blazquez 2009; Guerin, Cambray et al. 2009). Although antibiotic treatment leads to cell death in most cells of a population, it may only cause damage in a small sub-set of the population. The generation of single-stranded DNA or other markers of cell damage induces the SOS response: a DNA repair system (Hastings, Rosenberg et al. 2004; Shimoni, Altuvia et al. 2009). Transcriptional regulators important in DNA repair that are activated by the SOS response, concurrently activate transfer of mobile elements (Couce and Blazquez 2009). Therefore, sub-lethal concentrations of some antibiotics fail to kill some bacteria, and as a side effect increase horizontal transfer of mobile elements among the surviving population. Several antibiotics of the quinolone family are known to have this effect on activation of the SOS response. Specifically, ciprofloxacin, a drug commonly used to treat urinary tract infections, increases the rate of horizontal spread of integrative and conjugative elements, of DNA recombination

within the bacterial chromosome and induces integron recombination (Beaber, Hochhut et al. 2004; Lopez, Elez et al. 2007; Guerin, Cambray et al. 2009).

Although we know that antibiotic exposure plays a role in activating HGT, we do not completely understand the environmental conditions and selective pressures present in the niches of commensal that promote the lateral exchange of DNA. Understanding factors that influence HGT is important because these conditions share virulence factors and antibiotic resistance among commensal organisms (Ahmed, Dobrindt et al. 2008). Understanding this process is critical to the control and prevention of emergence of highly virulent and antibiotic resistant pathogens.

Figure 1-1. Depiction of genes necessary for transmissibility of plasmids.

Schematic representation of the genes necessary for a plasmid to be conjugative or mobilizable. A) The *oriT*, relaxase and type 4 coupling protein (T4CP) comprise the mobility (MOB) genes. The mating pore formation (MPF) genes are a type of T4SS. In order to be mobilizable a plasmid must contain at minimum and *oriT*. Plasmids containing MPF genes and thus T4SS are thus conjugative. B) The relaxase nicks the double stranded plasmid DNA and linearizes it. The *oriT*/relaxase DNA component gets recruited to the mating pore by the T4CP. Transfer of DNA proceeds through the transport channel synthesized by the T4SS. Reviewed in (Smillie, Garcillan-Barcia et al. 2010).



Integrative and Conjugative Elements (ICEs)

A specific subest of genomic islands that have recently been identified in many bacteria are integrative and conjugative elements (ICEs) (also referred to as conjugative transposons). ICEs are functionally mobile due to properties similar to bacteriophages and conjugative plasmids (Figure 1-2). ICEs are generally large regions of DNA (20-500 kb) that have all the same properties of genomic islands, but have three functional units that are conserved between all ICEs: one for integration, replication and stability and a T4SS (Ramsay, Sullivan et al. 2006; Wozniak and Waldor 2010). The integration unit encodes an integrase and excisionase and functions in site-specific excision and integration similar to that of bacteriophages. Therefore they can excise from the chromosome, forming a circularized form. In this circularized form stability proteins are important and the ICE can then be replicated and transferred by the T4SS from a donor bacterium carrying the ICE to a recipient cell that is free.

Interspersed within the conserved modules, ICEs often carry strain-specific genes important for survival in its host environments or confer a specific fitness advantage for survival in the niches they colonize. The best characterized ICE is SXT, from *Vibrio cholerae* which encodes genes for resistance to sulfamethoxazole, trimethoprim and chloramphenicol (Waldor, Tschape et al. 1996). As another example, the large, 500 kb ICE found in the *Mesorhizobium loti* genome carries cargo genes important for nitrogen-fixation and is thus also a symbiosis island (Ramsay, Sullivan et al. 2006). Other important ICEs include, but are not limited to PAPI-1 and *clc* both from different *Pseudomonas aeruginosa* strains that carry important genes for virulence and metabolism of chlorocatechols, and YAPI in *Yersinia pseudotuberculosis* that

carries genes for iron-acquisition (Collyn, Billault et al. 2004; Qiu, Gurkar et al. 2006; Gaillard, Pernet et al. 2008).

GI-T4SSs.

T4SS encoded on ICEs have been described as genomic island-like T4SS or GI-T4SSs. As mentioned above, T4SSs are best characterized in the context of conjugative plasmid transfer, but recently are being acknowledged for their role in conjugative transfer of GIs or ICEs. Phylogenetic analyses of the MPF genes of these T4SSs show that they are more similar to one another than T4SSs of conjugative plasmids (Juhas, Crook et al. 2007; Smillie, Garcillan-Barcia et al. 2010). This includes T4SS MPF genes found on the following genomic islands: PAPI-1 and the *clc* element of *P. aeruginosa*, ICE*Hin1056* of *Haemophilus influenzae*, SCRI1043 from the plant pathogen *Erwinia carotovora*, TT01 of the insect pathogen *Photorhabdus luminescens* and SPI-7 in *Salmonella typhimurium* (Duchaud, Rusniok et al. 2003; Bell, Sebaihia et al. 2004; Juhas, Crook et al. 2007; Heermann and Fuchs 2008). These T4SS are encoded on the ICE and contribute the same mobilizable properties to ICEs as they do to conjugative plasmids.

Similar to conjugative plasmids, T4SS on ICEs can mobilize other GIs with similar *oriT* sequences (Daccord, Ceccarelli et al. 2010). Genomic islands that can excise from the chromosome and form circularized intermediates, but do not encode a T4SS, have been reported (Middendorf, Hochhut et al. 2004). These islands contain MOB genes and therefore can be transferred *in trans* by another T4SS, one of an ICE or a conjugative plasmid (Daccord, Ceccarelli et al. 2010). Because of their lack of a

T4SS, these excising genomic islands are called mobilizable genomic islands (MGIs), not ICEs (Daccord, Ceccarelli et al. 2010).

ICE excision and integration. ICE excision from the chromosome is facilitated by an ICE-encoded integrase and excisionase (Lewis and Hatfull 2001; Daccord, Ceccarelli et al. 2010). ICE integrases belong to the tyrosine family of integrases and resemble two types, those of P4 phages and those similar to the XerC/XerD family (Burrus, Pavlovic et al. 2002; Mohd-Zain, Turner et al. 2004). These integrases catalyze the crossover event of the two direct repeats located at each end of the ICE (*attL* and *attR*) (Campbell 1992; Ravatn, Studer et al. 1998; Rajeev, Malanowska et al. 2009). The excisionase is also required for excision of the ICE from the chromosome and plays a regulatory role on the integrase (Lewis and Hatfull 2001; Lesic, Bach et al. 2004). The integrase is also necessary for re-integration back into the chromosome either in the same donor cell or in a new recipient cell (Daccord, Ceccarelli et al. 2010). Integration is again catalyzed by the integrase and the recombination of the identical *attI* and *attB* DNA sequences located in the circularized ICE and the bacterial chromosome, respectively (Burrus, Pavlovic et al. 2002).

ICE maintenance, regulation and exclusion. The excision and circularization of ICEs occur in cells at a low rate (~2%) (Ramsay, Sullivan et al. 2006; Minoia, Gaillard et al. 2008; Wozniak and Waldor 2009). Nevertheless, loss of ICEs from strains has been reported. Considering the number of bacterial cells in any given system and the importance of genes encoded on ICEs, the presence of genes that play a role in

maintenance of these elements is not surprising (Nair, Alokam et al. 2004; Auchtung, Lee et al. 2005). Stability in the extrachromosomal form has been reported to be dependent on genes encoded in the replication and stability modules of the ICE. In PAPI-1, the *soj* gene was necessary for maintenance of the episomal form and subsequent transfer (Qiu, Gurkar et al. 2006). Once circularized, toxin-antitoxin pairs are expressed and ensure segregation into daughter cells and thus maintenance of the ICE (Wozniak and Waldor 2009).

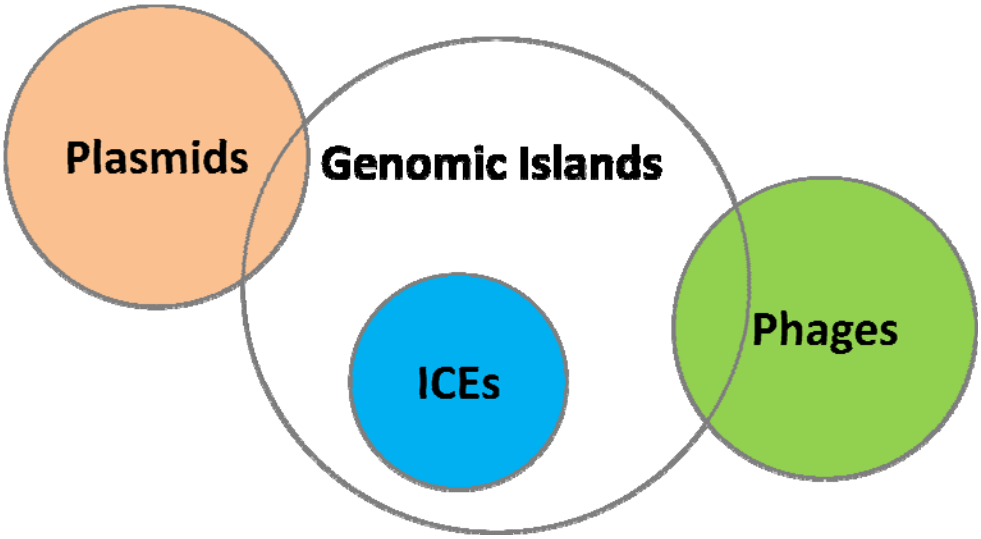
Several conditions promote ICE excision and transfer to new recipient cells. Increased expression of the integrase when the ICE is in its circularized form has been reported for the *clc* element as well as SXT (Burrus and Waldor 2003; Sentchilo, Ravatn et al. 2003). Integrase expression can also be induced in the presence of 3-chlorobenzoate, a carbon source utilized by genes encoded on the *clc* ICE (Sentchilo, Ravatn et al. 2003). DNA damaging agents such as mitomycin C or ciprofloxacin, which induce the SOS response have also been shown to increase excision and transfer rates of ICEs (Auchtung, Lee et al. 2005; Bellanger, Roberts et al. 2009; Daccord, Ceccarelli et al. 2010). These genes are regulated by *setC* and *setD* in the SXT element, homologues which have been discovered in other ICEs (Ramsay, Sullivan et al. 2009; Daccord, Ceccarelli et al. 2010). Lastly, ICE transfer rates have been shown to be responsive to cell density, thus implicating quorum-sensing as a factor in ICE regulation (Auchtung, Lee et al. 2005; Ramsay, Sullivan et al. 2009).

ICE exclusion systems and the total number of ICEs that can reside in any given bacterial cell is not fully understood. In SXT, exclusion of other ICEs of similar exclusion groups is mediated by the Eex and TraG proteins (Marrero and Waldor 2007;

Marrero and Waldor 2007). Other exclusion or immunity systems exist including ImmR of ICE*Bs1* and Pif of pSAM2 (Possoz, Gagnat et al. 2003; Auchtung, Lee et al. 2007). Although these proteins prevent acquisition of other similar ICEs, tandem arrays resulting from integration of multiple of the same ICE (e.g. SXT) have been reported (Garriss, Waldor et al. 2009).

Figure 1-2. Mobile elements contribute to bacterial genome diversity by created genomic islands.

Genomic islands can be introduced into the bacterial core genome through a variety of mobile elements. The figure depicts in color mobile elements (plasmids, phages and ICEs). Genomic islands are not colored, because most genomic islands are not mobile. The few genomic islands that are mobile are those that belong to the family of integrative and conjugative elements, lysogenic bacteriophages and small sub-set of plasmids that integrate into the chromosome.



STATEMENT OF THE PROBLEM

The emergence of virulent and antibiotic resistant bacterial strains continues to represent a significant problem in healthcare facilities. Horizontal gene transfer (HGT) contributes to the evolution and emergence of these pathogenic isolates by allowing large genomic regions of DNA to be shared among diverse bacteria. The molecular events leading to the transfer of DNA between bacteria are poorly understood.

Objective 1: Identify conserved genes among etiologic agents of catheter-associated urinary tract infection (caUTI).

Rationale: Multiple etiologic agents are capable of colonizing the catheterized urinary tract and causing infection and generally caUTI is of polymicrobial etiology. Therefore, we reasoned that there may be important genes shared among these organisms that facilitate colonization. We used a comparative genomic hybridization approach to identify genes conserved among *Proteus mirabilis, Morganella morganii* and *Providencia stuartii*, prevalent agents that cause caUTI, the most common healthcare-associated infection. Furthermore, biofilm formation occurs often on urinary catheters providing the opportunity for HGT between bacteria involved in mixed-species biofilms. In addition to identifying colonization factors, the conserved nature of the identified genes may indicate HGT or represent sharing of these DNA regions between organisms of different genera that colonize a similar niche.

Objective 2: Characterize the molecular mechanisms that govern excision, transfer and integration of the mobile element, ICE*Pm1*.

Rationale: A 93-kb pathogenicity island, ICE*Pm1*, identified using comparative genomic hybridization arrays, is highly conserved and prevalent among common etiologic agents of caUTI. The prevalence and similarity of ICE*Pm1* to other characterized PAIs that are actively mobile lead us to believe that this element also has mobile capabilities. We used a molecular approach, including mutant construction and mating assays to characterize genes important for horizontal transfer of ICE*Pm1* between clinical strains. Because these clinical isolates efficiently colonize catheters and similar devices, and the close proximity of organisms is necessary for gene transfer, it is reasonable to hypothesize that these conditions, common in healthcare facilities, facilitate HGT.

Objective 3: Identify risk factors for patient co-colonization by methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant Enterococci (VRE).

Rationale: Vancomycin-resistant *S. aureus* (VRSA) arises through HGT of the *vanA* gene cluster from VRE to MRSA. Because MRSA/VRE co-colonization is necessary for VRSA emergence, understanding the conditions important for co-colonization and how often it occurs is important. MRSA and VRE are arguably endemic in most healthcare facilities. By identifying residents of skilled nursing facilities, where MRSA

and VRE colonization alone is common, we can assess incidence rates of co-colonization with MRSA and VRE at different body sites, including indwelling devices and wounds, sites consistent with providing the opportunity for HGT to occur. Understanding factors at the host-level that influence optimal conditions for HGT will be informative in reducing the emergence of multiply-antibiotic resistance organisms in healthcare facilities.

Chapter 2

Identification of a Modular Pathogenicity Island that is Widespread among Urease-producing Uropathogens and Shares Features with a Diverse Group of Mobile Elements[1]

ABSTRACT

Pathogenicity islands (PAIs) are a specific group of genomic islands that contribute to genomic variability and virulence of bacterial pathogens. Using a strain-specific comparative genomic hybridization array, we report the identification of a 94-kb PAI, designated ICE*Pm1*, common to *Proteus mirabilis*, *Providencia stuartii* and *Morganella morganii*. These organisms are highly prevalent etiologic agents of catheter-associated urinary tract infections (caUTI), the most common hospital acquired infection. *ICEPm1* carries virulence factors that are important for colonization of the urinary tract, including a known toxin (*Proteus* toxic agglutinin) and the High Pathogenicity Island (HPI) of *Yersinia spp*. Additionally, this PAI shares homology and gene organization similar to PAIs of other bacterial pathogens, several of which have been classified as mobile integrative and conjugative elements. Isolates from this study were cultured from patients with caUTI and show identical sequence similarity at three loci within ICE*Pm1* suggesting its transfer between bacterial genera. Screening for the

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presence of ICE*Pm1* among *P. mirabilis* colonizing isolates showed that ICE*Pm1* is more prevalent in urine isolates compared to *P. mirabilis* strains isolated from other body sites ($p<.0001$) further suggesting it contributes to niche specificity and is positively selected for in the urinary tract.

INTRODUCTION

Genomic variability between bacterial strains of the same species can result from gene gain and loss. Change in genome composition is facilitated by a variety of mechanisms and among these, acquisition of genes by horizontal gene transfer mediates rapid changes in the bacterial genome (Koonin, Makarova et al. 2001; Pallen and Wren 2007; Ahmed, Dobrindt et al. 2008). An assortment of mobile genetic elements, including genomic islands, are acquired horizontally by bacteria thereby contributing to the dynamic genome evolution observed in bacterial species (Koonin, Makarova et al. 2001; Pallen and Wren 2007; Ahmed, Dobrindt et al. 2008). Genomic islands are 30-250 kb segments of DNA that are flanked by direct repeats (DRs), associated with tRNA genes, have a G+C content differing from the surrounding chromosome, and carry genes that confer a fitness advantage to the organism (Hacker and Carniel 2001; Dobrindt, Hochhut et al. 2004). Genomic islands that encode virulence properties important for bacterial survival and pathogenicity within the host are called pathogenicity islands (PAIs) and when under positive selection continue to propagate and evolve within the chromosome (Hacker and Kaper 2000; Hacker and Carniel 2001).

A subset of PAIs has been identified that can excise from the bacterial chromosome following a recombination event at the DRs flanking the island, and actively transfer via a type IV secretion system (T4SS) (Dobrindt, Hochhut et al. 2004; Juhas, van der Meer et al. 2009). The subsequent site-specific integration of the island into a tRNA gene of the new host's chromosome, as well as island excision, is dependent on a functional integrase gene present at either end of the island (Juhas, van der Meer et al. 2009). These mobile genomic islands, identified as integrative and

conjugative elements (ICEs) contain similar genetic composition and structural organization that consist of integration and replication modules as well as a conjugation system which reflects the functional capacity to self-transfer (Burrus and Waldor 2004). These modules are mostly syntenic among ICEs, although interspersed with genes that are specific for that particular bacterial species. These additional, or cargo genes, encode proteins important for survival in the environment of that bacterial host (Juhas, van der Meer et al. 2009; Seth-Smith and Croucher 2009).

Uropathogenic *Escherichia coli*, the most common cause of urinary tract infection, harbors multiple PAIs, but less is known about PAIs in other uropathogens such as *Proteus mirabilis*, *Providencia stuartii* and *Morganella morganii*. Many organisms colonize the urinary tract and create biofilms on urinary catheters (Macleod and Stickler 2007). Of these, *P. mirabilis*, *P. stuartii* and *M. morganii* are of particular interest due to their high prevalence rates among long-term catheterized individuals as well as their propensity to cause catheter obstruction and urolithiasis (Mobley and Warren 1987). Catheter-associated urinary tract infections (caUTIs), the most common hospital-acquired infection, represent up to 40% of nosocomial infections (Tambyah and Maki 2000) and can result in adverse outcomes including sepsis and urinary stones. Once catheterized for ≥30 days, patients inevitably develop bacteriuria (Warren, Tenney et al. 1982) and in up to 77% of bacteriuric urine specimens, more than one bacterial species is isolated (Warren, Tenney et al. 1982; Macleod and Stickler 2007). The polymicrobial etiology of caUTI suggests a common mechanism of colonization that enhances the ability of these organisms to survive in the catheterized urinary tract.

In this study, comparative genomic hybridization (CGH) microarray analysis revealed genes common among three bacterial species isolated from polymicrobial caUTIs and therefore potentially important for urinary tract colonization. Using a strain-specific CGH to detect common genes among different bacterial genera allowed for detection of regions of the genome that were highly conserved. This approach led to the identification of a previously unknown PAI common to *P. mirabilis*, *P. stuartii* and *M. morganii* that possesses the properties consistent with a mobile PAI or ICE. The newly identified PAI carries several virulence factors including an adhesion/protease (*taaP*) and the High Pathogenicity Island (HPI) of *Yersinia spp.* and is found more commonly in *P. mirabilis* urinary isolates than colonizing isolates from different body sites. DNA sequence of the PAI at several loci is identical between *P. mirabilis*, *P. stuartii,* and *M. morganii,* suggesting that its acquisition by these organisms was not only recent, but also that active transfer may be occurring among these bacteria of different genera.

MATERIALS AND METHODS

Bacterial strains.

Proteus mirabilis HI4320, *Providencia stuartii* BE2467 and *Morganella morganii* TA43, as well as the other 87 caUTI isolates, were cultured from the urine of patients catheterized for ≥30 days with bacteriuria (>10^5 CFU/ml) at the time of bacterial culture. These patients were residents of two long-term care facilities (LTCFs) and were not being treated with antibiotics at the time of urine culture. Urine specimens were obtained from the distal end of the urinary catheter, cultured, identified and stored at -80°C as previously described (Warren, Tenney et al. 1982). *P. stuartii* ATCC25827 was obtained from ATCC. As part of a separate study, colonizing strains of patients from 14 LTCFs were isolated from the anterior nares, oropharynx, groin, perianal area, or wound using Culturette rayon-tipped swabs (Becton Dickenson, Inc., Cockeysville, MD) and stored at -80°C (Mody, Maheshwari et al. 2007). A total of 33 strains were used in this study, 14 of which came from patients that had an indwelling catheter or feeding tube. Isolates cultured from the same patient and of the same species, but isolated on different days (from either study population), were excluded from the study.

Comparative genomic hybridizations.

Individual colonies were inoculated into 50 ml Luria-Bertani medium and cultured overnight at 37°C. Bacteria were collected by centrifugation (3 min, 10,000 x g, 25°C) and suspended in one-tenth volume TE (10 mM Tris-HCl, 1 mM EDTA), pH 8.0 containing 10% SDS and proteinase K (20mg/ml). After 1 hour incubation at 37°C,

DNA was purified using CTAB/NaCl and phenol:chloroform extraction (Murray and Thompson 1980).

Genomic DNA was digested with HincII (or HindIII for *P. stuartii* ATCC25827), followed by aminoallyl-labeling (2:1 aminoallyl-dUTP:dTTP). Genomic DNA was labeled with the appropriate cyanine fluorescent molecules (Cy-3 or Cy-5) (GE Healthcare) and dried to completion (Hassemna, Hnath et al. 2006). Cy-dye labeled DNA was hybridized (50% formamide, 5x SSC, 0.1% SDS, 0.6 µg/µL salmon sperm DNA) to pre-hybridized microarray slides at 42°C for 20 hours (Hassemna, Hnath et al. 2006). *P. mirabilis* HI4320 was used as the reference strain in each flip-dye array pair. The *P. mirabilis* microarray consists of 3,719 70-mer oligonucleotide probes printed in triplicate onto UltraGAPSII glass slides (Corning) by Microarrays, Inc. Each oligonucleotide probe is unique to the 3,719 predicted open reading frames (ORFs) of the HI4320 genome and has a target melting temperature of 74°C (probes designed and synthesized by Operon Technologies, Inc.)(M. Pearson and H. Mobley, in preparation).

Cy-3 and Cy-5 fluorescence intensities of each spot were acquired using a Perkin-Elmer ScanArray Express microarray scanner and processed by Spotfinder (Saeed, Sharov et al. 2003). Background corrected spot intensities were imported into the Ginkgo comparative genomic hybridization data analysis tool (Pathogen Functional Genomics Resource Center, in preparation). Spots having intensities less than the fifth percentile of the overall intensities in the reference strain were discarded. If intensities in the test strains were undetectable, values were imputed based on the fifth percentile of intensities for the test strain in order to retain spots that did not hybridize in the test

strain for further analysis. Once reference spots were removed and intensities for test spots were imputed, in-slide replicates were merged and flip-dye consistency checking was performed.

GACK analysis (Kim, Joyce et al. 2002) was used to determine dynamic cutoff values of intensity ratios for designating genes present or divergent, which is a method more appropriate for highly divergent strains. Determining a cutoff value in this way allows for a different intensity ratio to be selected based on the same transition point on the curve of the $\log_2$ distributions for each hybridization experiment. Examination of the distribution of $\log_2$ intensity ratios in typical CGH experiments show that the distributions vary between hybridizations, and the distribution is normal with a long left tail representing divergent genes (Kim, Joyce et al. 2002). The distribution of our data is similar, yet because we have a large number of probes that fail to hybridize, we see a distribution with a long right tail. Therefore, the data were transformed by taking the inverse of all $\log_2$ ratios, which yielded a distribution with a long left tail suitable for GACK analysis. A binary analysis was used for determining number of genes present and absent/divergent and a graded analysis was used for construction of the heatmap using the treeview program (Saldanha 2004).

Prevalence screening for PAI.

Isolated colonies were cultured in Luria-Bertani broth to an OD_{600} of 0.8 at 37°C with aeration. DNA was isolated from 1 ml of bacterial suspension using a QIAquick column (QIAGEN) according to the manufacturer's instructions. DNA was RNase-treated and dissolved in 200 µl of the supplied elution buffer (QAIGEN). PCR was

conducted using primers flanking ORFs, PMI2551 (F: CAGAAGATTACATGAATAATG, R: GAGAGTGTGATGAGATGTGAAT), PMI2602 (F: GCGAATGAACTTCACCA, R: GCCACTAATCAGAGGGAGT) and PMI2641 (F: GCACGCTCTGCTCCGCC, R: CGGGAGGTGCGTCCATG) (Invitrogen). Primers for amplification of PMI3255 (F: CGTTGATGCTCTGATGCGTCT,R: GAACTACGTGTTCGCGAAGC), as a positive DNA control, were designed within the sequences of the two flanking ORFs, because PMI3255 did not hybridize in the array experiments. PCRs were carried out in 25 µl reactions containing 30 ng genomic DNA, 1x buffer, 250 µM dNTPs, .75 µl taq DNA polymerase and 0.4 µM forward and reverse primers. Reactions were amplified in a thermocycler at 95°C for 2 min, followed by 30 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 60s. Associations were determined using Fisher's exact test (GraphPad Prism).

DNA sequencing and analysis.

Purified PCR products were sequenced (ABI Model 3730) and aligned using the Wilbur-Lipman Method (Lasergene). Artemis software was used for visualization of direct repeats and calculation of G+C content of the PAI (Rutherford, Parkhill et al. 2000).

RESULTS

Comparative genomic hybridization reveals a small subset of highly conserved genes between Proteus, Providencia and Morganella spp.

Genomic DNA from catheter-associated bacteriuria isolates *P. stuartii* BE2467, *M. morganii* TA43 and fecal strain *P. stuartii* ATCC25827 (test strains) were hybridized to a *P. mirabilis* HI4320 DNA microarray. Very few probes hybridized significantly with genomic DNA from each test strain. GACK analysis classified 365, 116, and 67 genes as conserved between *P. mirabilis* and *P. stuartii* BE2467, *M. morganii,* and *P. stuartii* ATCC25827 respectively, while 3354, 3533 and 3561 genes were determined absent or divergent (Figure 2-1). Among the conserved genes, 110 genes were conserved in *P. stuartii* BE2467 and *M. morganii*, and of those, 20 were also conserved in *P. stuartii* ATCC25827.

The majority of genes classified as conserved in the test strains are located in three contiguous regions within the *P. mirabilis* genome (Figure 2-2*D*). The first region (PMI0456-0538) consists of 81 phage-related genes as well as the previously characterized *uca* genes, important in uroepithelial cell adhesion (Wray, Hull et al. 1986). A second contiguous region PMI2549-PMI2641, found in *P. stuartii* BE2467 and *M. morganii* but not *P. stuartii* ATCC25827 genomic DNA was determined to be a PAI, designated ICE*Pm1*. The third region (PMI3251-3284) represents ORFs encoding 28 ribosomal proteins.

To validate the findings of the comparative genomic hybridizations, genes located in the beginning, middle, and end of ICE*Pm1* were PCR amplified. Sequence and alignment analysis of PMI2551, PMI2602 and PMI2641 revealed each gene to be 100% identical at the nucleotide level over their entire coding length between *P. mirabilis*, *P. stuartii* BE2467 and *M. morganii*. PCR amplification of *P. stuartii*

ATCC25827 genomic DNA for genes PMI2551, PMI2602 and PMI2641 yielded no products. Within the region of ribosomal proteins, PMI3255 was also PCR amplified, sequenced and aligned in each strain used for the microarray experiments. PMI3255 was 90.5% and 90.3% similar at the nucleotide level in *P. stuartii* BE2467 and *M. morganii* respectively, compared to the *P. mirabilis* sequence.

Identification of a highly mosaic, yet highly conserved, 94 kb PAI in *P. mirabilis* HI4320, *P. stuartii* BE2467 and *M. morganii* TA43.

Loci PMI2549-2641 of *P. mirabilis* represent a previously uncharacterized PAI common to *P. mirabilis* HI4320, *P. stuartii* BE2467 and *M. morganii* TA43. This 94 kb region encodes 91 ORFs, has a G+C content of 44.84% which differs substantially from that of the *P. mirabilis* genome (38.88%) (Figure 2-2*C*), is flanked by 52 bp repeats and is integrated into a $tRNA^{Phe}$ gene. This region (ICE*Pm1*) carries several genes involved in DNA mobility, characteristic of PAIs, including an integrase, six transposases, and five plasmid-transfer related proteins (Figure 2-2*A*). All 91 ORFs were absent in *P. stuartii* fecal strain ATCC25827 (Figure 2-2*D*) indicating that this strain lacks ICE*PM1* and suggests its heterogeneous distribution in the population.

Despite being a newly recognized PAI for *P. mirabilis*, *P. stuartii* and *M. morganii*, BlastP comparison (Altschul, Gish et al. 1990) of all 91 ORFs showed similarity to significant portions of six well-known PAIs of other bacterial pathogens (Figure 2-3): SPI-7 of *Salmonella enterica* serovar Typhi CT18 (Parkhill, Dougan et al. 2001), HAI-2 of *Pectobacterium amylovara* SCRI1043 (previously *Erwinia carotovora* subsp. *atroseptica*) (Bell, Sebaihia et al. 2004), $YAPI_{YE}$ of *Yersinia enterocolitica* 8081

(Thomson, Howard et al. 2006), PAPI-1 of *Pseudomonas aeruginosa* PA14 (He, Baldini et al. 2004), ICEHin*1056* of *Haemophilus influenzae* (Juhas, Power et al. 2007) and an unnamed PAI in *Photorhabdus luminescens* TT01 (Duchaud, Rusniok et al. 2003). In addition, several ORFs were highly similar to a contiguous region of DNA of *Serratia proteamaculans* 586 that is flanked by two $tRNA^{Phe}$ genes suggesting that this region is also a PAI. Although most homologous genes were syntenic within the PAIs of these other species, no one organism shared similarity with all 91 ORFs searched, highlighting the highly mosaic structure of the island (Figure 2-3).

We identified a common structure among these PAIs and ICE*Pm1*, consisting of a set of core genes, common to all PAIs discussed, supplemented by genes unique to each PAI, considered the variable region or cargo genes (Figure 2-3). The core genes constitute putative integration, replication, and conjugation modules (Figure 2-2*B*, shaded yellow). DRs flank the ICE*Pm1* and at the left-most end, the 52bp direct repeat is located within the 5' coding end of a $tRNA^{Phe}$ gene (*attL*). The direct repeat at the right-most end (*attR*) is part of the 3' end of a truncated $tRNA^{Phe}$ gene.

The integration module at the left end of ICE*Pm1* (PMI2549-PMI2554) encodes a site-specific integrase (PMI2549). Genes homologous to PMI2549 in the other PAIs encode site-specific recombinases of the tyrosine-like family. The module upstream of *att*R, the rightmost end of ICE*Pm1* (PMI2627-2642), encodes a topoisomerase, DNA helicase and a chromosome-partitioning protein, suggesting its involvement in DNA replication. The chromosome-partitioning related protein is the terminal gene in the island and one of the homologues, *soj* (RL115) of *P. aeruginosa* has been implicated in the stability of the extrachromosomal form of PAPI-1 (Qiu, Gurkar et al. 2006). The

integration and replication modules flanked by the *att* sites highlight the distinct boundaries of ICE*Pm1* from that of the surrounding chromosome.

Another important core segment found in ICE*Pm1* (PMI2569-2592) shows homology to a type IV secretion system (T4SS) that is important for DNA transfer of ICE*Hin1056* (Juhas, Crook et al. 2007). This 26 gene region encodes eight putative exported proteins and nine putative membrane proteins. Additionally, 15/26 genes encode a predicted signal peptide sequence and 14/26 genes encode 1-3 predicted transmembrane domains, common features of proteins in a T4SS (Juhas, Crook et al. 2008).

The cargo genes of ICE*Pm1* (Figure 2-2*B*; shaded grey) are located in three variable regions: one consisting of 13 hypothetical genes, a second region of 19 genes, 13 of which are pseudogenes and lastly, the *nrp* operon (PMI2596-2604). This operon encodes genes for the synthesis, transport, and uptake of the iron siderophore yersiniabactin and is homologous to the region of $YAPI_{YE}$ described to be the HPI common in *Yersinia* spp (Carniel, Guilvout et al. 1996).

ICE*Pm1* is more commonly associated with *P. mirabilis* urinary isolates than *P. mirabilis* colonizing isolates from other body sites.

To establish a correlation between ICE*Pm1* and pathogenicity, we tested 87 urinary isolates from long-term catheterized individuals and 33 colonizing isolates of P. *mirabilis*, P. *stuartii*, and M. *morganii* for the presence of ICEPm1. Colonizing isolates were cultured from patients of LTCFs and isolated from the oropharynx, nasopharynx, wound, groin or perianal area. PCR analysis of PMI2551, PMI2602 and PMI2642

(representing genes located at the beginning, middle, and end of the island, respectively) served as a proxy to estimate ICE*Pm1* presence in colonizing strains. Successful PCR amplification of all three loci was considered positive for the presence of ICE*Pm1*. Of 87 urinary isolates tested, 100% (39/39) of the *P. mirabilis* strains were positive for ICE*Pm1* while prevalence in *P. stuartii* and *M. morganii* was 60% (6/10) and 28.9% (11/38) respectively (Table 2-1). Additionally, *P. mirabilis* urinary isolates were significantly more likely to harbor the island 100% (39/39) compared to *P. mirabilis* colonizing isolates 65.2% (15/23) (P<.0001; OR= 43.32 [1.648, 797.5]). ICE*PM1* was found in 40% (2/5) of *M. morganii* colonizing isolates and 100% (5/5) of *P. stuartii* colonizing isolates, yielding no significant association between ICE*Pm1* and isolate origin for these species (Table 2-1). Using PMI2602 (*nrpT*) as a marker of HPI presence, we identified HPI in 100% (39/39) of *P. mirabilis,* 70% of (7/10) *P. stuartii* and 89.5% of (34/38) *M. morganii* urinary isolates (Table 2-1). The prevalence of HPI in colonizing isolates was 69.6% (16/23), 40% (2/5) and 100% (5/5) in *P. mirabilis*, *M. morganii* and *P. stuartii* respectively. This shows a significant association (P=.0005; OR= 35.9 [1.9, 666.1]) between the prevalence of HPI in urine isolates compared to isolates from other body sites among *P. mirabilis* isolates as well as that HPI is more common among urinary isolates than colonizing isolates (P= .006; OR= 49.7 [1.7, 14.5]) when all species are grouped together (Table 2-1).

DISCUSSION

PAIs are a subgroup of genomic islands that contribute not only to variation in genomic composition within species, but also to the virulence and pathogenesis of microorganisms. Several recently described PAIs have been shown to excise and self-transfer to other bacteria, facilitating bacterial adaptation to surrounding niches (Pickard, Wain et al. 2003; Bueno, Santiviago et al. 2004; Mohd-Zain, Turner et al. 2004; Nair, Alokam et al. 2004; Qiu, Gurkar et al. 2006; Juhas, Crook et al. 2007; Juhas, Power et al. 2007; Minoia, Gaillard et al. 2008). Here, we present the discovery of a highly modular and highly conserved PAI, designated ICE*Pm1*, of *P. mirabilis, P. stuartii* and *M. morganii* strains that has structure similar to these recently described mobile PAIs. The 100% sequence identity at three loci within ICE*Pm1* among the three isolates suggests DNA transfer between these bacteria of different genera has occurred, although further DNA sequencing is required to confirm the identical element in each of these strains. Additionally, we show that the ICE*Pm1* is significantly more likely to be present in *P. mirabilis* urinary isolates than in colonizing isolates from other body sites, suggesting its contribution to a common mechanism of colonization and pathogenicity among agents of caUTI.

Considering the genetic relatedness and common niche inhabited by the strains used in our study, we found it surprising that the majority of *P. mirabilis* microarray probes failed to hybridize significantly with *P. stuartii* and *M. morganii* genomic DNA. Upon sequence investigation, we determined the stringency of our array to be quite high, requiring sequences of approximately >93% identity for hybridization to occur. CGH is rarely applied across different genera of bacteria. In fact, to our knowledge,

CGH has only been used previously to compare the nearly identical *Shigella* and *Escherichia* genomes (Fukiya, Mizoguchi et al. 2004). This study is the first to describe the use of a strain-specific microarray to investigate genome variability across three disparate genera. This is most likely due to the fact that very few genes hybridize under these conditions in divergent strains rendering this technique undesirable for gene discovery applications. Nevertheless, by hybridizing genomic DNA from bacteria of different genera to a strain-specific array, we were able to quickly identify regions of very high sequence similarity between genetically divergent organisms and thus genes of interest conserved among these uropathogens.

Although *P. mirabilis*, *P. stuartii*, and *M. morganii* are common commensal organisms of the human body, they are opportunistic pathogens that cause caUTIs and other diseases (Manos and Belas 2006). The association between the origin of isolate and the presence of ICE*Pm1* in *P. mirabilis* isolates suggests that the island contributes to urinary tract colonization and/or pathogenicity. Organisms causing caUTI are generally of the gut microbiota and self-inoculated by patients (Mathur, Sabbuba et al. 2005). Therefore the island could confer pathogenic potential to commensal or opportunistic organisms once introduced into the urinary tract. The presence of ICE*Pm1* may be a determining factor for whether or not colonization occurs and in this case, we would expect a high prevalence of ICE*Pm1* in the urine isolates, but a heterogenic distribution in strains from other body sites, as was observed for *P. mirabilis* isolates.

The core modules found in ICE*Pm1* show similarity to regions of other genomic islands and in these islands have been shown to be necessary for mobility and transfer.

Precise excision of SPI-7, $YAPI_{YE}$ and PAPI-1 has been observed (Bueno, Santiviago et al. 2004; Nair, Alokam et al. 2004; Qiu, Gurkar et al. 2006; Thomson, Howard et al. 2006) and for PAPI-1, a functional integrase (xerC, RL002) as well as chromosome partitioning protein (*soj,* RL115) (located in the integration and replication modules, respectively) was important for excision and stability of the island (Qiu, Gurkar et al. 2006). Subsequent transfer of the islands into PAI-negative recipient strains is reported for PAPI-1 and ICE*Hin1056* and involvement of a T4SS is shown to be required for transfer of ICE*Hin1056* in *H. influenzae* strains (Qiu, Gurkar et al. 2006; Juhas, Crook et al. 2007). Phylogenetic analysis of T4SS genes in ICE*Hin1056*, SPI-7, PAPI-1, HAI-2 and the PAI from *P. luminescens* TTO1 shows that the T4SS in these organisms are related, yet distinct from traditional T4SSs and have been named Genomic Island (GI)-like T4SSs (Juhas, Crook et al. 2007). Further experiments are required to understand the excision and transfer capabilities of ICE*Pm1*, however, the structural similarity of ICE*Pm1* with other ICEs, including core genes that encode a putative T4SS, suggest that it may be classified as an integrative and conjugative element, ICE (Burrus, Pavlovic et al. 2002; Burrus and Waldor 2004; Juhas, van der Meer et al. 2009; Seth-Smith and Croucher 2009).

The variable regions, containing the cargo genes, of the PAIs described have important functions in their respective organisms (Table 2-2). Therefore, it is not surprising that an important virulence factor such as HPI is located in the variable region of ICE*Pm1*. This cluster of virulence genes is involved in iron acquisition and could enhance fitness during iron-limitation in the host. Consistent with this, we see that

the ICE*Pm1* is significantly more prevalent in urinary isolates, likely because it is well known that the urinary tract is iron-limited.

In our isolates, HPI is associated with ICE*Pm1* as well as present when ICE*Pm1* is not. We attribute this phenomenon to the independent transfer of HPI (Buchrieser, Brosch et al. 1998), and in strains lacking ICE*Pm1* we found that HPI has integrated into another region of the genome. Additionally, the divergent G+C content of the HPI from the flanking regions of ICE*Pm1* (Figure 2-2*C*) suggests that the HPI was mobilized and integrated within ICE*Pm1* as a distinct event. Indeed, ICEs *Ec1* of *E. coli* ECOR31 and *Kp1* of *K. pneumoniae* NTUH-K2044 both harbor HPI and can transfer between bacterial strains, yet do not show similar sequence identity to other regions of ICE*Pm1* outside of the yersiniabactin operon (Schubert, Dufke et al. 2004; Lin, Lee et al. 2008). Either of these elements could be potential elements that introduced HPI into other regions of the chromosome. The significant association observed between HPI and urinary isolates, when all species are grouped together, supports the importance of this virulence factor and thus reflects its widespread distribution within the *Enterobacteriaceae* family (Schubert, Rakin et al. 1998; Bach, de Almeida et al. 2000; Schubert, Cuenca et al. 2000).

The importance of ICE*Pm1* in uropathogenicity has been shown, specifically for PMI2596 (*ton*B-dependent iron receptor) (Nielubowicz, Smith et al. 2008), PMI2575 (*taaP*; adhesin and autoagglutination protein)) (Alamuri and Mobley 2008; Alamuri, Eaton et al. 2009) and PMI2605 (*nrpG*)(Burall, Harro et al. 2004), as mutant constructs of these genes are attenuated in the mouse model of ascending UTI. Furthermore, only three ORFs in the variable region (outside of HPI) share homology to members of the

Enterobacteriaceae, suggesting this PAI may contain novel virulence genes that have not been previously described in this family. Future experiments will focus on the discovery of other virulence phenotypes, including investigation into ICE*Pm1*'s contribution to biofilm formation, an important virulence property in urinary catheter colonization. Characterization of ICE*Pm1*'s ability to mobilize and transfer and conditions affecting these events, will also be undertaken. The presence of known virulence factors carried on ICE*Pm1*, taken together with the high prevalence of ICE*Pm1* and HPI in *P. mirabilis* urinary isolates, suggest positive selection for ICE*Pm1* among uropathogenic strains. Our description of ICE*Pm1* in *P. mirabilis*, *P. stuartii*, and *M. morganii* isolates inhabiting a similar niche, highlights the importance of understanding the selective pressures positively selecting for acquisition and retention of this island as well as those that influence horizontal transfer of this island, thus disseminating virulence factors to a broad range of bacterial hosts.

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Figure 2-1. Comparative genomic hybridization results.

Genomic DNA from *P. stuartii* BE2467, *M. morganii* TA43 and *P. stuartii* ATCC25827 was hybridized to a *P. mirabilis* HI4320 microarray. Genes were categorized as present or absent/divergent by GACK analysis, out of 3719 total ORFs, in each strain hybridized to the array. Genes that were discarded from further analysis because genomic DNA from the reference strain, *P. mirabilis* HI4320, failed to hybridize in that particular hybridization are considered missing.

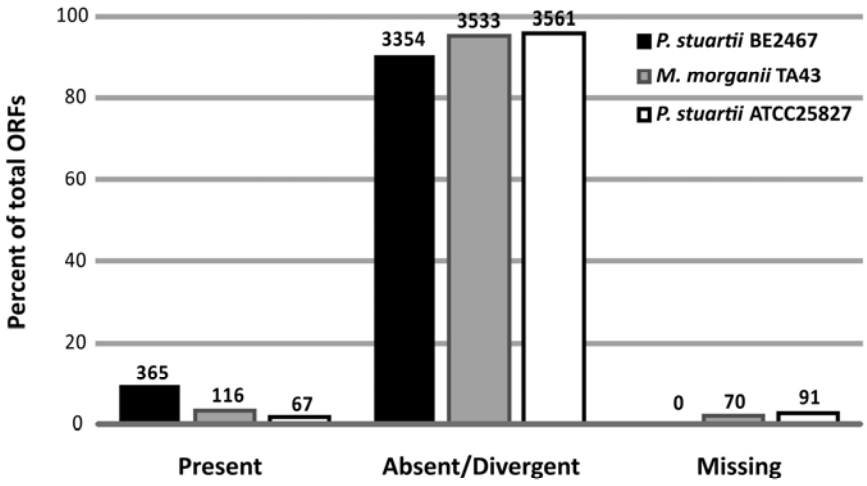

Figure 2-2. Array results and characteristics of ICE*Pm1*.

A) Putative function (by color, see key), coding strand and size of the 91 ORFs of ICE*Pm1*. Patterns correspond to the module to which those genes are located in B. B) Modular structure of ICE*Pm1*. Modules in yellow represent core modules; grey, variable regions. DRs are represented as triangles. C) G + C content of ICE*Pm1* and the flanking chromosome. Boundaries of ICE*Pm1* are denoted by vertical black lines. Horizontal lines represent G + C content, with the middle line representing 39% G + C, that of the *P. mirabilis* HI4320 genome. D) Heatmap representing present (yellow), absent/divergent (blue) and missing genes (grey) in *P. stuartii* BE2467, *M. morganii* TA43, and *P. stuartii* ATCC25827. DRs: direct repeats in *A* and *B*, showing the modularity of the island and suggesting evolutionary history of acquisition of regions of ICE*Pm1*.

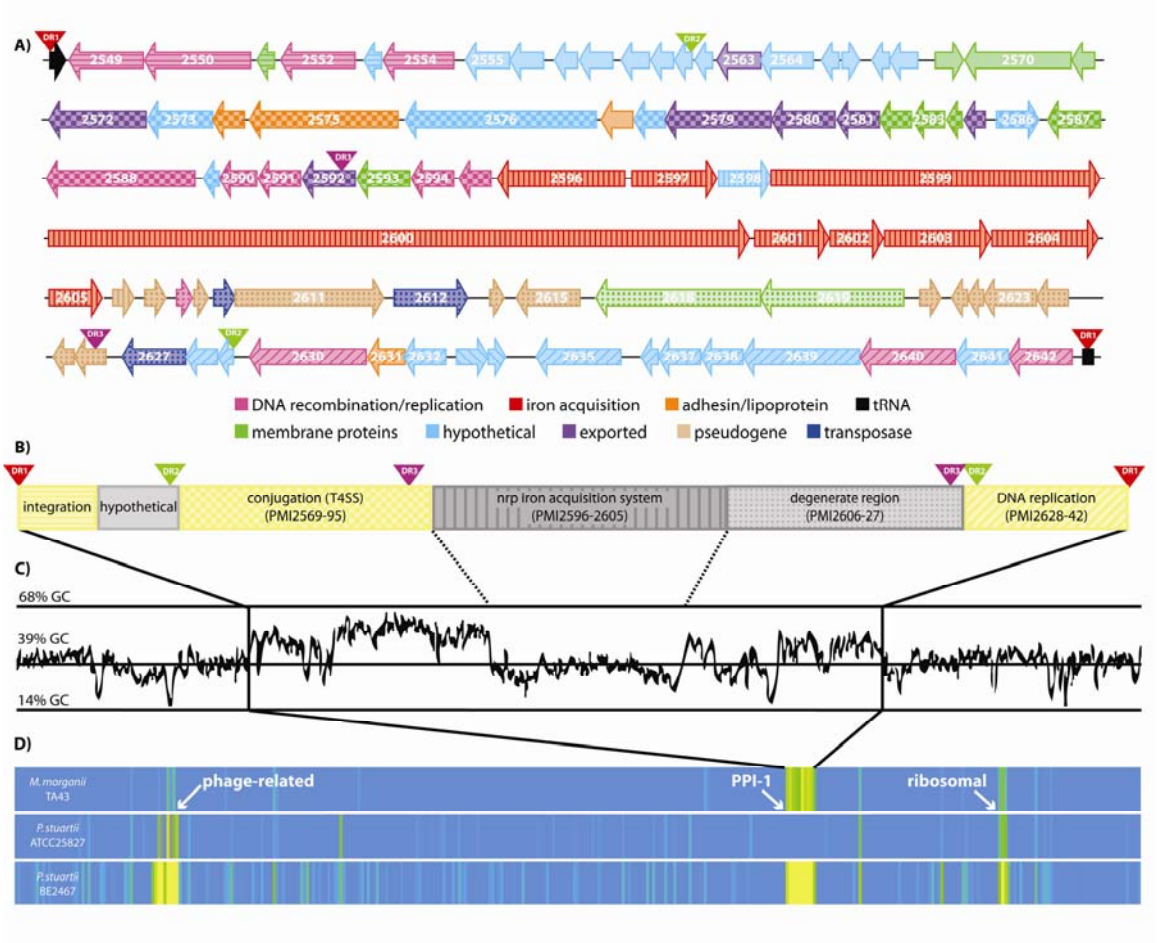

Figure 2-3. BlastP results of all 91ORFs of ICE*Pm1*.

Interrogation of the BlastP database produced proteins similar to ICE*Pm1* proteins most commonly in 7 bacterial strains. Columns represent organisms and are labeled by the loci name: STY: *S. enterica* serovar Typhi CT18 (GenBank accession no. AL513382); plu: *Photorhabdus luminescens* TTO1 (plu0958-plu1166); YE: *Yersinia enterocolitica* 8081 (AM286415); Spro: *Serratia proteamaculans* 586 (CP000826); ECA: *Pectobacterium amylovara* SCRI1043 (BX950851); RL: *Pseudomonas aeruginosa* PA14 (AY273869); p1056.: *Haemophilus influenzae* ICE*Hin1056* (AJ627386). For each ORF showing similarity in the represented organism the locus number is shown and gene name is given when available. Loci with >70% amino acid similarity are shaded in grey. Putative functions of proteins that are bolded represent pseudogenes.

PMI Locus	Size (AA)	Putative Function	Organism Locus						
			STY	plu	YE	Spro	ECA	RL	p1056
2549	356	phage integrase	4666 int2	1029	3450	401	614	002 xerC	63 xerC
2550	530	helicase	4665	1028	3451	400	613	003	62 traI
2551	108	membrane							
2552	333	plasmid-related		1027	3458	397			
2553	99	hypothetical	4592	1158	3459	396	597		
2554	324	DNA primase	4592	1158	3460	395	597		56 traC
2555	215	hypothetical							
2556	170	hypothetical							
2557	118	hypothetical	4587			590			
2558	140	hypothetical							
2559	125	hypothetical							
2560	91	hypothetical							
2561	64	hypothetical							
2562	42	hypothetical							
2563	203	exported							
2564	347	hypothetical	4586	1152	3461	390	587		
2565	59	hypothetical							
2566	73	hypothetical							
2567	79	hypothetical							
2568	99	hypothetical							
2569	135	membrane	4580	1091		353	581		49c
2570	504	membrane	4579	1090	3465	352	580	014	48
2571	112	membrane	4578	1089		351	579	015	47
2572	480	exported	4577	1088	3466	350	578	016	51c
2573	324	exported	4576	1087	3467	349	577	017	52c
2574	171	lipoprotein	4575	1086	3468	348	576	018	53c
2575	741	adhesin (pta)							
2576	956	plasmid-related	4573	1085	3482	337		022	45
2577	145	lipoprotein	4571	1084	3483	336	572	023	44
2578	161	hypothetical							
2579	498	exported	4570	1083	3484	335	570	024	43
2580	298	exported	4569	1082	3485	334	569	025	42
2581	214	membrane	4568		3486	333	568	026	41/55
2582	138	membrane		1081	3487	332	567	027	40
2583	137	outer membrane	4566	1080	3488	331	568	028	39
2584	79	membrane	4565	1079	3489	330	565	029	
2585	108	exported	4564	1078	3490	329	564	030	38
2586	202	hypothetical							
2587	202	membrane	4563	1074	3492	327	561	35/46	37
2588	715	plasmid transfer	4562 traG		3493 traG	326	560	047	35
2589	65	ATP synthase subunit							
2590	184	exported	4560	1070	3494	324	557	049	34
2591	212	lytic transgly-cosylase	4559	1069	3494A	323	556	050	
2592	245	exported	4558	1068	3495	322	555	051	32
2593	246	membrane				321		053	
2594	220	conjugal transfer	4539 pilL	1049 pilL	3506 pilL	320	532 pilL	086	31
2595	**117**	**transposase**							
2596	**662**	**siderophore ton-B dep. receptor**		**2316**	**2622 fyuA**				
2597	**409**	**MFS-transporter**		**2317**	**2613 irp8**				
2598	**245**	**hypothetical**							
2599	**2034**	**siderophore synthase**		**2320**	**2617 irp2**				
2600	**3071**	**siderophore synthase (nrpS)**		**2321**	**2618 irp1**				
2601	**362**	**siderophore biosynthesis (nrpU)**		**2322**	**2619 irp3**				
2602	**257**	**siderophore biosynthesis (nrpT)**		**2323**	**2620 irp4**				
2603	**588**	**siderophore ABC transporter (nrpA)**		**2318**	**2615 irp6**				
2604	**575**	**siderophore ABC transporter (nrpA)**		**2319**	**2614 irp7**				
2605	**249**	**transferase (nrpG)**							
2606	**47**	**hypothetical (irpP)**							
2607	**80**	**hypothetical**							
2608	**85**	**prophage regulatory**						**1627**	
2609	**44**	**hypothetical**						**1626**	
2610	**100**	**IS-transposase (insN)**							
2611	**177**	**transposase**							
2612	**354**	**IS-transpoase**							
2614	**31**	**hypothetical**							
2615	**32**	**antirestriction**			**3460**	**395**	**1600**		
2618	**789**	**ton-B dep. receptor**							
2619	**697**	**ton-B dep. receptor**							
2620	**118**	**transposase**							
2621	**51**	**hypothetical**							
2622	**72**	**fimbrial usher**							
2623	**153**	**chaperone**							
2624	**136**	**fimbrial**							
2625	**684**	**ornithine decarboxylase**							
2626	**110**	**exported**	4558	1068	3495	322	555		
2627	307	transposase			3497				
2628	155	hypothetical	4534	1038	3509	311	528	074	12
2629	68	hypothetical							
2630	684	topoisomerase topB	4530 topB	1037	2257 topB	310	525 topB	92 topA	11 topB
2631	178	lipoprotein				309			9
2632	247	hypothetical	4529			308	524	97	6
2633	186	hypothetical		1036	3510				46
2634	53	hypothetical							
2635	435	hypothetical	4528	1035	3511	307	523	100	5
2636	94	membrane				306	522A		
2637	222	hypothetical	4526	1034	3512	305	522	101	4
2638	210	hypothetical							
2639	549	hypothetical	4523	1032	3513	303	250	103	3
2640	450	DNA helicase dnaB2	4522 dnaB	1031	3514 dnaB	302	519		2 dnaB
2641	243	hypothetical					517		
2642	295	chromosome partioning-related	4521 parB	1030	3515	301	516	115 soj	1 parA

Table 2-1. Prevalence of ICE*Pm1* and HPI among urinary and colonizing isolates.

	No. of isolates with ICE*Pm1*[a]/total no. of isolates tested (%)		No. of isolates with HPI[b]/total no. of isolates tested (%)	
Organism	Urinary[c]	Colonizing[d]	Urinary	Colonizing
P. mirabilis	39/39 (100)	15/23 (65.2)***	39/39 (100)	16/23 (69.6)**
P. stuartii	6/10 (60)	5/5 (100)	7/10 (70)	2/5 (40)
M. morganii	11/38 (28.9)	2/5 (40)	34/38 (89.5)	5/5 (100)
Total	56/87 (64.4)	22/33 (66.7)	80/87 (92)	23/33 (69.7)*

Note: The association between origin of isolate and prevalence of ICE*Pm1* and HPI were tested to describe contribution ICE*Pm1* has to pathogenicity. Isolates were considered to harbor ICE*Pm1* if they tested positive for three loci by PCR and for PMI2602 for HPI.

[a] PCR amplification of genomic DNA for PMI2551, 2602 and 2641.

[b] PCR amplification of genomic DNA for PMI2602.

[c] Isolates cultured from the urine of patients with catheter-associated bacteriuria.

[d] Isolates cultured from the anterior nares, oropharynx, groin, perianal area or wound.

*P<.01

**p<.001

***p<.0001

Table 2-2. Characteristics of ICE*Pm1* and PAIs with similar modular structure to ICE*Pm1*

PAI	Organism	Loci	Size (kb)	Integration site[a]	Virulence factors carried in variable region of the PAI
ICE*Pm1*	*P. mirabilis, P. stuartii* and *M. morganii*	PMI2549-2642	94	$tRNA^{Phe}$	HPI, *taaP* toxin
SPI-7	*Salmonella enterica* serovar typhi CT18	STY4521-STY4680[b]	134	$tRNA^{Phe}$	Vi-antigen, *pil* locus
unnamed	*Photorhabdus luminescens* TT01	plu0958-1166	252	$tRNA^{Phe}$	*pil* locus, *tcd* and *tcc* toxin loci
$YAPI_{YE}$	*Yersinia enterocolitica* 8081	YE3450-3515	98	$tRNA^{Phe}$	*pil* locus, HPI, arsenic resistance
unnamed	*Serratia proteamaculans* 586	Spro0301-0401	94	$tRNA^{Phe}$	na
HAI-2	*Pectobacterium amylovara* SCRI1043	ECA0516-0614	97	$tRNA^{Phe}$	polyketide phytotoxin biosynthesis (*cfa*), *pil* locus
PAPI-1	*Pseudomonas aeruginosa* PA14	RL001-115	111	$tRNA^{Lys}$	pyocin, fimbriae, *pil* locus
ICE*Hin1056*	*Haemophilus influenzae* ICE*Hin1056*	p1056.01-.64	59	$tRNA^{Leu}$	tetracylcine, chloramphenicol and ampicillin antibiotic resistance

Note: BlastP comparison yielded 7 PAIs from 7 different species showing similar core gene structure to PPI-1. Each PAI shares core integration, replication and conjugation modules with PPI-1, while the remaining genes in the PAI are part of the variable regions and carry genes important in virulence. Vi-antigen of *S. enterica* is used in typhoid vaccines.

[a] The 52bp repeats flanking all islands associated with $tRNA^{Phe}$ are identical.

[b] SPI-7 has the identical 52bp nucleotide sequence between STY4666 and STY4667 that flanks the island, accounting for the homology seen with STY4666 and PMI2549, the end of the island.

Chapter 3

Self-transmissibility of the Integrative and Conjugative Element ICE*Pm1* Between Clinical Isolates Requires a Functional Integrase, Relaxase and Type IV Secretion System[2]

ABSTRACT

Integrative and conjugative elements (ICEs), chromosomal mobile elements, can conjugatively transfer between bacteria. Recently, we identified a genomic island of *Proteus mirabilis,* a common agent of catheter-associated urinary tract infection (UTI) that possesses all the properties consistent with an ICE. This element, designated ICE*Pm1*, is highly conserved in other causative agents of UTI, suggesting its mobility. We demonstrate that ICE*Pm1* can actively excise from the chromosome in a clonal population of bacteria and that this excision is integrase-dependent. Although in *P. mirabilis* HI4320, ICE*Pm1* is annotated as integrated into the phenylalanine tRNA gene *pheV*, we show that ICE*Pm1* can integrate into either *pheV* or *pheU*. We determined that ICE*Pm1* transfers at a frequency of 1.35 x 10^{-5} transconjugants/donor to ICE*Pm1*-deficient *P. mirabilis* using plate mating assays with clinical isolates. Insertional

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inactivation of a putative integrase gene on ICE*Pm1* decreased transfer frequencies of ICE*Pm1* to below the limit of detection. Mutation of the relaxase of ICE*Pm1* also eliminates transfer and demonstrates that this element is indeed self-transmissible and not transferred *in trans*, as are some mobilizable genomic islands. Together, these findings clearly demonstrate that ICE*Pm1* can actively excise from the chromosome in an integrase-dependent manner, dynamically integrate into both phenylalanine tRNA genes and transfer into clinical strains using its own conjugation machinery.

INTRODUCTION

Comparative genomics and advances in sequencing technology have revealed the diversity in bacterial genomes within a single species. Most organisms require a core set of genes for survival, the core genome; that can further be supplemented by the accessory genome, genes conferring fitness properties for an organism in a specific niche (Dobrindt, Hochhut et al. 2004; Pallen and Wren 2007). Strain-to-strain variability in genetic content is in large part due to the presence or absence of genomic islands (GIs) (Lloyd, Rasko et al. 2007; Juhas, van der Meer et al. 2009). GIs often contain genes that contribute to virulence, antibiotic resistance, metabolism or other fitness properties (Hacker and Carniel 2001; Pallen and Wren 2007). Specific properties of GIs that distinguish them from the surrounding bacterial chromosome include aberrant GC content, association with tRNA genes or other genes conserved between species, and their tendency to encode mobility genes (Hacker and Kaper 2000; Gal-Mor and Finlay 2006).

Genomic islands are the result of horizontal gene transfer (HGT) events, which play a critical role in the evolution of bacterial species (Ochman, Lawrence et al. 2000). Gain and loss of genomic islands result in the greatest and most rapid changes in the pathogenic potential of an organism and contributes to the emergence of pathogens from commensal organisms (Gal-Mor and Finlay 2006). Recently, it has become appreciated that many GIs encode a conjugative type IV secretion system (T4SS) and thus may have the ability to self-transfer (Juhas, Crook et al. 2007; Juhas, Crook et al. 2008; Ambur, Davidsen et al. 2009). The conjugative ability that T4SSs confer onto plasmids is well described, yet the discovery of T4SSs within GIs resulted in the

establishment of a new class of mobile elements known as integrative and conjugative elements (ICEs) (Smillie, Garcillan-Barcia et al. 2010; Wozniak and Waldor 2010).

ICEs, a subset of GIs, have a conserved modular structure composed of three functional units encoding genes important for ICE function: integration, regulation and conjugation (Burrus and Waldor 2004; Wozniak, Fouts et al. 2009). ICEs are flanked by identical direct repeats *attL* and *attR* that can recombine with the aid of an integrase and excisionase to form a circularized form of the ICE (Burrus and Waldor 2003). This circularized form carries the identical direct repeat, *attI* that can then recombine with the reconstituted integration site on the chromosome, *attB*, and result in ICE re-integration. When the ICE-encoded integrase is activated, the ICE can excise from the chromosome, form a circular intermediate and subsequently transfer to a recipient cell via a mating pore formed by the ICE-encoded T4SS (Seth-Smith and Croucher 2009; Wozniak and Waldor 2010). Thus, ICEs site-specifically excise and integrate into the chromosome like that of temperate bacteriophages, yet are self-transmissible, similar to conjugative plasmids. Because HGT contributes to the greatest and most rapid changes in the bacterial chromosome, the discovery of ICEs has new insights into bacterial evolution.

Interspersed among the conserved modules needed for ICE function, are cargo genes that generally encode specific functions that allow for adaptation to the surrounding environment (Burrus and Waldor 2004; Wozniak and Waldor 2010). For instance, the cargo genes of the *clc* element of *Pseudomonas aeruginosa* encode important metabolic properties necessary for growth on 3-chlorobenzoate, while the SXT element of *Vibrio cholerae*, the most extensively described ICE, carries genes

conferring resistance to sulfamethoxazole, trimethoprim and choramphenicol (Waldor, Tschape et al. 1996; Hochhut and Waldor 1999; Gaillard, Pernet et al. 2008; Wozniak and Waldor 2010). While ICEs encode niche-specific functions in their cargo genes, the integration, regulation and conjugation modules of ICEs are fairly conserved.

We previously identified a genomic island, ICE*Pm1*, conserved in *Proteus mirabilis*, *Providencia stuartii* and *Morganella morganii,* using a comparative genomics hybridization array (Flannery, Mody et al. 2009). These organisms are all urease-producing etiologic agents of catheter-associated urinary tract infection, a disease that is typically polymicrobial (Warren, Tenney et al. 1982; Jones and Mobley 1987; Stickler 2008). ICE*Pm1* contains core modules and syntenic structure consistent with protoypical ICEs (Burrus and Waldor 2004; Wozniak and Waldor 2010). It is flanked by identical 52bp direct repeats and integrated into the 3' end of a phenylalanine tRNA gene. A putative tyrosine-like recombinase, a putative helicase that could act as a relaxase and a putative T4SS were all identified in this element by *in silico* analysis (Flannery, Mody et al. 2009). Known cargo genes that are interspersed between the core modules of *ICEPm1* encode a yersiniabactin-related iron-acquisition system and an adhesin/protease that both contribute to virulence in the mouse model of ascending urinary tract infection (Nielubowicz, Smith et al. 2008; Alamuri, Lower et al. 2010; Himpsl, Pearson et al. 2010). ICE*Pm1* was present in all *P. mirabilis* urinary isolates, yet heterogeneously distributed in commensal *P. mirabilis* strains that colonize other body sites (Flannery, Mody et al. 2009). Additionally, several genes within ICE*Pm1* demonstrated 100% sequence identity between *P. mirabilis*, *P. stuartii* and *M. morganii*. Therefore, because of the modular structure of ICE*Pm1* and its widespread

prevalence among multiple bacterial species, we hypothesize that ICE*Pm1* is an active ICE.

The goal of this study was to demonstrate the ability of ICE*Pm1* to excise from the chromosome, form a circularized intermediate and conjugatively transfer to ICE*Pm1*-deficient clinical strains. We demonstrate that ICE*Pm1* can actively excise from the chromosome in an integrase-dependent and site-specific manner and subsequently transfer to clinical *P. mirabilis* isolates. Disruption of the putative relaxase gene, (PMI2550) the integrase gene (*intP)* and gene encoding a putative chromosome partitioning protein (PMI2642) prevented detectable transfer. Mutations in the putative ICE*Pm1*-encoded T4SS highlighted the self-transmissibility of ICE*Pm1*. We demonstrate the dynamic nature of ICE*Pm1* as it can integrate into both *pheU* and *pheV* tRNA genes. Finally, we demonstrate that ICE*Pm1* can be transferred to *Escherichia coli*; however, possessing the identical *attB* sequence is not the only component necessary for this transfer.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. mirabilis* HI4320 was cultured from the urine of a patient catheterized for ≥30 days, presenting with bacteriuria (>10^5 CFU/ml) (Warren, Tenney et al. 1982). *P. mirabilis* 378L was cultured from the groin of a resident from a skilled nursing facility (Mody, Maheshwari et al. 2007). Additional strains with defined mutations are listed in Table 3-1. All strains were cultured in modified Luria-Bertani (LB) broth (per liter, 0.5 g NaCl, 10 g Tryptone, 5 g yeast extract) or LB agar (15 g agar). Overnight broth cultures were inoculated from a single colony and incubated for 18 hours at 37°C with aeration. All strains were maintained at -80°C in 25% glycerol. Antibiotics were used at the following concentrations: kanamaycin (50 μg/ml), ampicillin (100 μg/ml), chloramphenicol (20 μg/ml), trimethoprim (32 μg/ml) and nalidixic acid (50 μg/ml).

Molecular techniques. PCR was used to detect the integrated and excised forms of ICE*Pm1*. Oligonucleotide primer sequences are listed in Table 3-2 and their targeted genomic locations are illustrated in Figure 3-1. Oligonucleotides were specific for the flanking regions of *pheU* and *pheV* and thus could specifically identify ICE*Pm1* attachment sites at these locations. Reactions were performed in a final volume of 50 μl containing 1X PCR buffer, 0.5 μl Taq polymerase and 200 μM dNTPs. PCR conditions were as follows (i) 3 min (10 min for colonies) at 94°C (ii) 30 cycles of 30s at 94°C, 30s at 56°C and 1 min at 72°C and (iii) 7 min at 72°C. For detection of amplification products from clonal populations, colony PCR was performed by inoculating a single colony with a pipet tip. When genomic DNA was used as template, 100 ng total DNA

was used. To isolate genomic DNA, bacteria were collected by centrifugation (13,000 rpm, 10 min) from 500 µl of overnight culture. Genomic DNA was purified over a DNEasy column (Qiagen), treated with RNAse A and eluted in 200 µl elution buffer. For confirmation of transconjugants, multiplex PCR analysis was performed using a Qiagen Multiplex PCR Kit. Primers designed for amplification of PMI2641 (located within ICE*Pm1*) and the *att*Tn*7* site were used together in each reaction. Multiplex colony PCR was performed in a final volume of 50 µl containing 2x Qiagen Multiplex PCR Master Mix and each primer at 2 µM. Primers designed for amplification of PMI2551 and PMI2602 were also used for confirmation of transconjugants. PCR amplification products were purified using a QIAquick column and sequenced on an ABI Model 3730. Sequences were aligned using the Wilbur-Lipman Method in MegAlign software (Lasergene).

Construction of mutants. A kanamycin-resistant marked strain, *P. mirabilis* HI4320 (ICE*Pm1*::kan) was constructed to serve as the donor strain in mating assays using the TargeTron Gene Knockout System (Sigma) following a modified protocol (Pearson and Mobley 2007). Briefly, mutagenic oligonucleotides were synthesized using the TargeTron design site for the intergenic region between PMI2624 and PMI2625 (Table 2) and used to retarget the group II intron using polymerase chain reaction (PCR). This PCR product was ligated into pACD4K-C (Cam^R) harboring a T7 promoter, subcloned into *E. coli* DH5α and confirmed by sequencing. Plasmids of the correct sequence were then introduced by electroporation into *P. mirabilis* already containing the helper plasmid pAR1219 (Amp^R) that expresses T7 polymerase under the control of an IPTG

inducible promoter. Chloramphenicol- and ampicillin-resistant colonies were cultured and expression of the intron was induced by addition of 500 μM IPTG. Kanamycin-resistant colonies were picked and specific insertional mutations were confirmed using PCR primers flanking the expected insertion site (Table 3-2). PCR products were visualized on a 1.0% agarose gel, stained with ethidium bromide. Products appearing ~2 kb greater in size than those of the wild-type were confirmed as mutants. To determine the role of various ICE-encoded genes, insertional mutations were similarly constructed in PMI2549, PMI2550, PMI2594, and PMI2642 (Table 3-1). Additionally, an insertional mutation was constructed in PMI2476, the putative relaxase gene of the SXT-like ICE. The kanamycin cassette was removed from the intron by IPTG induction of the cre recombinase encoded on plasmid pQL123 and a second mutation was made by inserting the kanamycin cassette into the intergenic region between PMI2624 and PMI2625 creating the strain Ω*traI*$_{SXT}$, ICE*Pm1*::kan.

Conjugation assay. Mating assays were performed to determine the frequency of ICE*Pm1* transfer to ICE*Pm1* deficient cells. Overnight cultures of donor and recipient cells were mixed 1:1 and spotted onto the center of an LB agar plate. Plates were dried for 15 min and incubated for 6 hr at 37°C. Bacteria were harvested by flooding the plate with 1 ml LB and cells were suspended using a cell spreader. Serial dilutions were plated onto LB agar supplemented with kanamycin, trimethoprim or dual drug to identify donors, recipients and transconjugants, respectively (Autoplate 4000, Spiral Biotech). Viable colony counts were enumerated and expressed as CFU/ml with a limit of detection of 200 CFU/ml (20 colonies on a plate) (Qcount, Spiral Biotech). The

frequency of transfer was calculated by dividing the number of transconjugants formed by the number of donor bacterial cells present in each mating. The rate of spontaneous resistance of *P. mirabilis* HI4320 to trimethoprim (32 μg/μl) was found to be $< 1 \times 10^{-9}$ and therefore did not interfere with calculating ICE*Pm1* transfer frequencies. For *E. coli* mating assays, nalidixic acid (50 μg/ml) was used to screen for recipients and nalidixic acid and kanamycin to screen for transconjugants.

RESULTS

ICE*Pm1* actively excises from the chromosome in a clonal population of *P. mirabilis* HI4320. In the genome sequence annotation of *P. mirabilis* HI4320, ICE*Pm1* is integrated into the 3' end of a phenylalanine tRNA gene, *pheV*, between loci PMI2548-2549 (genomic location: 2793767-2886300). To demonstrate that ICE*Pm1* can actively excise from the chromosome, we developed a PCR-based assay to identify ICE*Pm1* in both integrated and episomal states (Figure 3-1). We used primers anchored in the chromosome and within the ICE to amplify *attL* (P3 and P4) and *attR* (P5 and P6), 52bp direct repeats that are attachment sites of the ICE. Because primers P3 and P6 bind outside of the ICE, in the chromosome, amplification of *attL* and *attR* is only possible when ICE*Pm1* is integrated into *pheV* (Figure 3-1A). ICE*Pm1* can excise from the chromosome and form a closed circularized, episomal form that can be detected using primers that bind, and face outward, from the outermost ends of ICE*Pm1* (P4 and P5). These primers will only amplify a product when the 93 kb ICE has circularized to form site *attI* (Figure 3-1B). When ICE*Pm1* is in this episomal form, a PCR amplicon for *pheV* can also be detected using primer pairs that bind in the chromosome at regions flanking the ICE (P3 and P6). Amplification product from these primers is only

detectable if the ICE has excised, thus allowing for the shorter PCR amplification of the *attB* site located within *pheV*. All amplification products can be detected from the same chromosomal genomic DNA preparation as well as from colonies obtained from a plating of the parental strain (Figure 3-2). This demonstrates that ICE*Pm1* is dynamically excising, forming a circular intermediate and reintegrating into the chromosome in a clonal population.

Direct sequencing of PCR amplicons resulted in the expected sequence of products for *attL* and *attR* as annotated (Figure 3-3), as well as the expected sequence for *attI* and *attB*, given that ICE*Pm1* has precisely excised from the chromosome (Figure 3-3). Sequencing of the latter two products demonstrates that precise excision from the chromosome of ICE*Pm1* is achieved and that excision results in restoration of the full *pheV* sequence (which contains *attB*). Additionally, the identical 52bp repeat observed at the flanking ends of ICE*Pm1*, is created in the episomal form (*attI*).

P. mirabilis has another tRNA gene that transports phenylalanine, located approximately halfway around the genome from *pheV*, namely *pheU* (genomic location: 375914-375986). The phenylalanine tRNAs PheU and PheV share the same anticodon (GAA) and although ICE*Pm1* is not annotated as being integrated at *pheU*, we attempted to determine whether this integration was possible. An amplification product was obtained using the primer pair P1 and P2, specific to chromosomal DNA flanking *pheU*, demonstrating an empty *attB* site within *pheU*. Amplification products obtained with primer pairs P2 and P4 for *attL-pheU* and P1 and P5 for *attR-pheU* display the integration of ICE*Pm1* into *pheU* (Figure 3-1C and data not shown). Integration into both *pheU* and *pheV* in a clonal population of ICE*Pm1* demonstrates that not only is

ICE*Pm1* actively excising from the chromosome, but that it can excise and reintegrate into either *phe*-tRNA gene. Additionally, we were unable to obtain an amplification product using primer pairs P6 and P4 or P1 and P4 (Figure 3-1 and data not shown), revealing that integration of ICE*Pm1* is directional, a property characteristic of tyrosine-like recombinases (Rajeev, Malanowska et al. 2009).

ICE*Pm1* is transmissible to a clinical ICE*Pm1*-deficient *P. mirabilis* strain and capable of excision in the recipient. Genes encoding the kanamycin resistance cassette were introduced into an intergenic region of ICE*Pm1*, between loci PMI2624 and PMI2625 that encode fimbrial and ornithine decarboxylase pseudogenes, respectively. This serves as a marker for ICE*Pm1* acquisition without disrupting gene function in ICE*Pm1*. *P. mirabilis* 378L, isolated from the groin of a skilled nursing facility resident and already shown to be devoid of the ICE, was used as a recipient in mating assays to demonstrate ICE*Pm1* transfer. Mating assays were performed on LB agar plates or in LB broth with an incubation period of 6 hr at 37°C. ICE*Pm1* transferred at a frequency of 1.35×10^{-5} transconjugants/donors on LB agar plates while transconjugants were undetectable in the broth mating experiments.

To confirm transfer, genomic DNA was extracted and purified from three colonies formed on dual antibiotic-containing plates. Amplification of the *att*Tn*7* site was used to differentiate between *P. mirabilis* strains HI4320 and 378L; the size of the amplification product differs in these strains (Figure 3-4A). Amplification of ICE genes for the beginning, middle and end of the ICE (PMI2551, PMI2602 and PMI2641)

by PCR demonstrated presence of the ICE in the *P. mirabilis* 378L recipient cells indicating transfer had occurred (Figure 3-4A).

To determine whether ICE function was maintained upon formation of the transconjugant, PM378L-ICE*Pm1*::kan, PCR assays for *attL*, *attR*, *attB* and *attI* were conducted and demonstrated that ICE*Pm1* was able to actively excise from and reintegrate into the recipient chromosome, in a manner similar to that observed in the *P. mirabilis* HI4320 host genetic background (Figure 3-4B).

***intP* is required for ICE*Pm1* excision and subsequent transfer.** An insertional inactivation mutation was constructed in the ICE*Pm1* gene adjacent to *attL,* PMI2549, encoding a putative site-specific recombinase of the tyrosine recombinase family. Integrases that are necessary for ICE function are generally encoded near the *attL* or *attR* sites of these elements (Burrus, Pavlovic et al. 2002). Disruption of PMI2549 resulted in an ICE*Pm1* that was no longer capable of excising from the chromosome and forming a circular intermediate. Amplification of *attL* and *attR* were positive, but no product was observed for *attI* or *attB*, indicating that ICE*Pm1* had lost its ability to excise from the chromosome (Figure 3-5A). Based on the loss of this activity, PMI2549 was designated *intP* (integrase ICE*Pm1*).

In addition to 'locking' ICE*Pm1* into the chromosome by disrupting the function of its integrase, mutation of the integrase allowed us to demonstrate that ICE*Pm1* is only present in either *pheU* or *pheV* in a single bacterium. Because the introduction of the kanamycin cassette occurred in one bacterial cell and thus 'locked' ICE*Pm1* in its current genomic state, we only observed amplification products for ICE locked into

pheV. While, amplification of *attB-pheU* was positive in the *intP*::kan mutant, no amplification products were observed for *attL-pheU* or *attR-pheU* indicating ICE*Pm1* is only integrated into p*heV* (data not shown). Furthermore, we created 29 independent *intP*::kan mutations, and in each we were only able to amplify *attL-pheV* products; no *attL-pheU* products were obtained. This verifies that ICE*Pm1* can only be integrated into one *phe* tRNA gene in a single cell and that ICE*Pm1* preferentially integrates into p*heV*, as originally annotated (Pearson, Sebaihia et al. 2008).

Mating assays were performed with *P. mirabilis* HI4320 *intP*::kan as the donor and *P. mirabilis* 378L as the recipient (Figure 3-5B). Transconjugant formation was not observed indicating that disruption of the ICE-encoded integrase is required for excision from the chromosome and without excision subsequent transfer is abolished.

***parA* is required for efficient transfer.** BlastP identified PMI2642 as a putative chromosome partitioning protein. PMI2642 is located near *attR* in ICE*Pm1*, commonly where proteins important for ICE stability are located; its homolog, the Soj protein of *P. aeruginosa* PAPI-1 has been shown to be important in stability of this ICE in its extrachromosomal form (Qiu, Gurkar et al. 2006). Insertional inactivation of PMI2642 did not have an effect on ICE*Pm1* excision, but resulted in no detectable amplification product for the *attI* site (Figure 3-5A). *attL* is not detectable because the primer binding site for P5 is disrupted by insertion of the kanamycin cassette, but can be detected using flanking primers, although these flanking primers fail to amplify *attI*. Furthermore, disruption of PMI2642 resulted in decreased frequency of transfer of ICE*Pm1* into *P. mirabilis* 378L to below the level of detection (Figure 3-5B).

ICE*Pm1* carries a T4SS that is necessary for conjugative transfer. The PMI2569-PMI2592 gene cluster encodes a series of proteins with predicted signal peptide sequences and transmembrane domains that share homology with the T4SS present in ICE*Hin1056* of *Haemophilus influenzae* (Juhas, Crook et al. 2007; Flannery, Mody et al. 2009). To verify that ICE*Pm1* carries a functional T4SS, necessary for its self transmissibility, we disrupted gene loci of two proteins predicted as important in DNA mobility, PMI2550 and PMI2594. PMI2550 is a predicted helicase and, based on its genomic location adjacent to *intP*, is hypothesized as the relaxase of ICE*Pm1*. Relaxases recognize the origin of transfer (*oriT*) of plasmids and ICEs and are thus necessary for mobility (Lee and Grossman 2007; Smillie, Garcillan-Barcia et al. 2010). PMI2594 is part of the conj_PilL superfamily of proteins (TIGR03748) and is 52% similar to tfc2 (p1056.31), a protein encoded on ICE*Hin1056*. Disruption of this gene in *H. influenzae* resulted in a 100,000-fold reduction in transfer frequency of the ICE and abolished pilus formation (Juhas, Crook et al. 2007).

When we disrupted PMI2594 (*traP*), expected amplification products for all attachments sites were observed (Figure 3-6A). Additionally, the frequency of ICE*Pm1* transfer was reduced 1000-fold to 2.8×10^{-8} transconjugants/donor (Figure 3-6B). Similarly, disruption of PMI2550 (*traI*) had no effect on ICE excision (Figure 3-6A) yet decreased ICE*Pm1* transfer significantly to 4.2×10^{-8} transconjugants/donor. This confirms the roles of *traI* (PMI2550) and *traP* (PMI2594) in conjugative transfer of ICE*Pm1*.

Another ICE showing significant homology to the SXT ICE of *V. cholerae* and thus a member of the R391/SXT family of ICEs has been reported in *P. mirabilis* HI4320 (genomic location: 2651089-2730732) (Wozniak, Fouts et al. 2009). The SXT-like ICE, named ICE*Pmi*Usa1, is located approximately 100kb downstream from ICE*Pm1* and was identified by *in silico* analysis. Therefore, we were interested in determining whether this element, like ICE*Pm1*, is actively excising from the chromosome. We demonstrate excision of the SXT-like ICE from the chromosome using a PCR-based assay similar to that used to identify ICE*Pm1* active excision (Figure 3-7). Similar to conjugative plasmids, ICE T4SSs have been shown to be able to transfer other mobile genomic islands (MGIs) *in trans* (Daccord, Ceccarelli et al. 2010; Smillie, Garcillan-Barcia et al. 2010). To confirm that the T4SS encoded on ICE*Pm1* was responsible for its self transmissibility, we first demonstrated that the T4SS of the SXT-like ICE is not involved in the conjugative transfer of ICE*Pm1*.

We insertionally inactivated the relaxase gene of the SXT-like ICE (*traI*$_{SXT}$), excised the kanamycin cassette, and re-marked ICE*Pm1* with the kanamycin resistance cassette in the same intergenic region as before. The *traI*$_{SXT}$ gene was recently demonstrated to be necessary for transfer of a nearby MGI *in trans* in *V. cholerae* (Daccord, Ceccarelli et al. 2010). This mutant in *P. mirabilis* HI4320 maintained excision properties of ICE*Pm1* (Figure 3-6A) as well as wild-type levels of conjugative transfer of the ICE*Pm1* mobile element at 2.2 x 10^{-5} transconjugants/donor (Figure 3-6B). This suggests that ICE*Pm1* is self-transmissible and is not being transferred by the T4SS of the SXT-like ICE.

ICE*Pm1* transfer to *Escherichia coli* is strain-dependent. The DNA sequence of p*heU* and *pheV* of *P. mirabilis* HI4320 is conserved in *E. coli* strains CFT073 and MG1655 (K12). *In silico* sequence analysis of *E. coli* CFT073 shows two known genomic islands integrated into the *pheU* and *pheV* sites (Figure 3-8). By comparison, the genes surrounding the *pheU* and *pheV attB* sites in a K12 strain are genes that are adjacent to the terminal ends of the GIs encoded by *E. coli* CFT073. This suggests that the CFT073 *attB* integration sites already carry genomic islands, but that the K12 *attB* sites are empty and could thus potentially receive ICE*Pm1*.

We performed mating assays between *P. mirabilis* HI4320-ICE*Pm1*::kan and the *E. coli* strains CFT073, K12 and *C* to determine the specificity of ICE*Pm1* transfer. No transconjugants were observed in the mating assays with *E. coli* K12 or CFT073 as recipients, yet transfer frequencies with a restriction modification-negative strain, *E. coli C,* as the recipient yielded transfer frequencies of 6.6×10^{-6}, similar to *Proteus* to *Proteus* intraspecies matings (Figure 3-8). Mating assays with another *P. mirabilis* commensal isolate, *P. mirabilis* 523L, as the recipient also showed similar transfer frequencies to those matings with *P. mirabilis* 378L as the recipient, further confirming transfer into clinical strains (Figure 3-8).

DISCUSSION

ICE*Pm1* was previously predicted to belong to a class of genomic islands known as integrative and conjugative elements based on *in silico* and comparative genomics analyses (Flannery, Mody et al. 2009). In this study we show that ICE*Pm1* is a functional integrative and conjugative element that can excise from the chromosome, form a detectable circularized intermediate, and subsequently transfer to clinical ICE*Pm1*-deficient strains. Mutation of the ICE-encoded integrase, *intP* renders ICE*Pm1* unable to excise from the chromosome and decreases transfer to undetectable frequencies. ICE*Pm1* directionally integrates into either *pheU* or *pheV*, although it is predominately integrated at *pheV*. Insertional inactivation of the relaxase and a mating pore formation gene resulted in a 1000-fold decrease in transfer efficiency, verifying that ICE*Pm1* is indeed self-transmissible.

Many tRNA genes can serve as integration sites for genomic islands, yet phenylalanine tRNA genes are especially recognized as hot spots for foreign DNA integration (Rumer, Jores et al. 2003; Chen, Ou et al. 2010). Several bacteriophages and PAIs are known to be integrated at these sites, yet we believe that ICE*Pm1* and ICE*MI*Sym^{R7A} are the first functionally characterized ICEs integrating at these loci (Lawrence and Ochman 1998; Ramsay, Sullivan et al. 2006). One reason integration into phenylalanine tRNAs occurs frequently may be related to their conserved sequence across species. Yet mating assays failed using two *E. coli* strains, CFT073 and MG1655, that contain both *pheU* and *pheV* genes identical to *P. mirabilis*. This suggests that additional factors besides an identical *attB* site are required for ICE transfer and integration into a recipient strain.

After screening multiple separate mutants in the integrase gene, which eliminates ICE*Pm1* excision from the chromosome, we never observed ICE*Pm1* locked into *pheU*, despite identifying integration at *pheU* in the parental strain. This observation, together with the observation that ICE*Pm1* is annotated as integrated at *pheV* in HI4320, suggests that the conformation in which ICE*Pm1* is integrated at *pheV* likely dominates over integration at *pheU* or existing as an episome (Pearson, Sebaihia et al. 2008). Further studies are needed to determine why preferential integration occurs at *pheV*, as *pheU* and *pheV* are identical.

We did not observe any transconjugants when mating assays were performed in broth, while we saw frequent transfer on agar plates, suggesting that a solid surface may be necessary for mating. Conjugative type IV pili that lack the ability to mate in broth are generally more rigid and thick than pili that allow for broth mating (Bradley, Taylor et al. 1980). The requirement of a solid surface for transfer is also interesting in the context of the pathogens that harbor ICE*Pm1*. We previously showed that *P. mirabilis*, *P. stuartii* and *M. morganii* all harbor ICE*Pm1* and that the ICE is predominant in *P. mirabilis* urinary isolates. Biofilms facilitate horizontal gene transfer between bacteria (Ehrlich, Ahmed et al. 2010). All of these organisms commonly colonize the catheterized urinary tract and are also known for forming biofilms on Foley catheters (Stickler 2008). Additionally, these isolates were cultured from catheterized patients with urinary tract infection (Flannery, Mody et al. 2009). Thus, the catheter could serve multiple functions in the wide dissemination of ICE*Pm1* among these organisms as a solid-surface to bring bacteria in close proximity, promoting cell to cell contact (necessary for conjugal transfer), and facilitating biofilm formation which protect the

bacteria from the surrounding environment (Stickler 2008). In support of this notion, we show transfer of ICE*Pm1* between an organism that was isolated from the catheterized urinary tract and an organism that was colonizing the groin of a patient. This suggests that if these organisms can come into close contact within a patient, HGT could occur, thus disseminating important virulence factors, such as iron acquisition, to the commensal organism. Further studies are required to understand what induces ICE*Pm1* transfer in these conditions.

Similar to plasmids, ICEs have the ability to transfer other mobile elements *in trans* (Hochhut, Marrero et al. 2000; Daccord, Ceccarelli et al. 2010). The relaxase produced by an ICE can recognize similar *oriT* sequences located within mobilizable genomic islands (MGI) or a plasmid and therefore recruit these other mobile genetic elements to the mating pore (Daccord, Ceccarelli et al. 2010; Smillie, Garcillan-Barcia et al. 2010). *In silico* analysis of *P. mirabilis* HI4320 identified an SXT-like ICE located approximately 100 kb 5' to *pheV* where ICE*Pm1* is annotated (Wozniak, Fouts et al. 2009). We show that the SXT-like ICE cannot transfer ICE*Pm1* because the ICE*Pm1* encoded relaxase, *traI*, and a mating pore formation gene, *traP*, are necessary for transfer. To confirm that the SXT-like ICE is incapable of transferring ICE*Pm1 in trans* we constructed insertional mutations in the relaxase gene of the SXT-like ICE, $traI_{SXT}$. Mating assays performed with this strain as a donor demonstrate wild-type frequencies of ICE*Pm1* transfer thus confirming that ICE*Pm1* is self-transmissible. Further studies are needed to investigate transfer of the SXT-like ICE to determine whether transfer occurs at the same frequency, and under the same conditions as

ICE*Pm1* as well as to understand the role these elements play in lateral transfer of other genomic components in *P. mirabilis*.

Exclusion mechanisms have been reported for both SXT in *V. cholerae* and ICE*Bs1* in *Bacillus subtilis* that can limit ICE acquisition when an ICE is already present (Auchtung, Lee et al. 2007; Marrero and Waldor 2007; Wozniak and Waldor 2009). Therefore, it is interesting that *P. mirabilis* HI4320 harbors two ICEs, both of which can actively integrate and excise from the chromosome. The T4SS genes of ICE*Pm1* are distinct from the SXT/R391 family and share homology with genes from the *P. aeruginosa* PAPI island, *H. influenzae* ICE*Hin*1056 island and *Salmonella typhi* SPI-7 islands (Flannery, Mody et al. 2009). In a phylogenetic analysis of the T4SS genes, these islands were evolutionarily distinct from P-like, F-like and I-like T4SSs and denoted GI-like T4SSs for genomic island-like type IV secretions systems (Juhas, Crook et al. 2007). The T4SSs from the SXT/R391 family of ICEs cluster with the F-like systems. Furthermore, *intP* of ICE*Pm1* is an integrase of the XerC/D family of tyrosine-like recombinases while the integrases encoded on ICEs of the SXT/R391 family are more similar to the P4-type lineage of tyrosine-like recombinases. This suggests that these two ICEs segregate into two different ICE families and therefore could explain why both exist in the same cell. Additionally, although ICE*Pm1* shows similarity to ICE*Hin*1056 and PAPI-1, amino acid similarity is rarely greater than 70%. This limited homology differs dramatically from homology among SXT/R391 ICEs, where most of the integrases are 99% identical. Therefore, ICE*Pm1* may fall into a third, yet to be described, family of ICEs.

To our knowledge, this is the first report of a single bacterium, harboring two self-transmissible ICEs. The active integration and excision as well as transfer of these elements implies that the chromosome of *P. mirabilis* HI4320 is quite dynamic. Additionally, understanding how genome location and ICE conformation affect expression of the genes encoded on the ICE as well as the genes surrounding *pheU* or *pheV* will provide insight into the effect the dynamic nature of ICEs has on gene expression. *P. mirabilis* HI4320 also carries a 36kb plasmid, thus suggesting that mobile genetic elements are common in its genome, which is relatively small at 4.1 Mb compared to other enterobacterial pathogens. It would not be surprising to find other MGIs that are transferred *in trans* by either of these ICEs or the plasmid, suggesting that the mobilome of *P. mirabilis* is substantial. This study demonstrates the activity of ICEs and thus the potential these mobile elements have for disseminating virulence determinants and antibiotic resistance genes among clinical isolates.

ACKNOWLEDGEMENTS

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Additionally, EF was supported by a Rackham Dissertation award.

Figure 3-1. Location of primers used in this study to detect ICE*Pm1* integration and excision.

Oligonucleotide sequences are listed in Table 2. *pheU* and *pheV* are located on opposite DNA strands, share identical nucleotide sequence and are 73 bp in length. Boxes represent the 52 bp repeat (black), phenylalanine tRNA genes (dark grey) and ICEPm1 (light grey). The 3 possible conformations of ICEPm1 are: A) integrated into *pheV*, B) excised from the chromosome and not integrated into either *phe* tRNA, C) integrated into *pheU*. Figure not to scale.

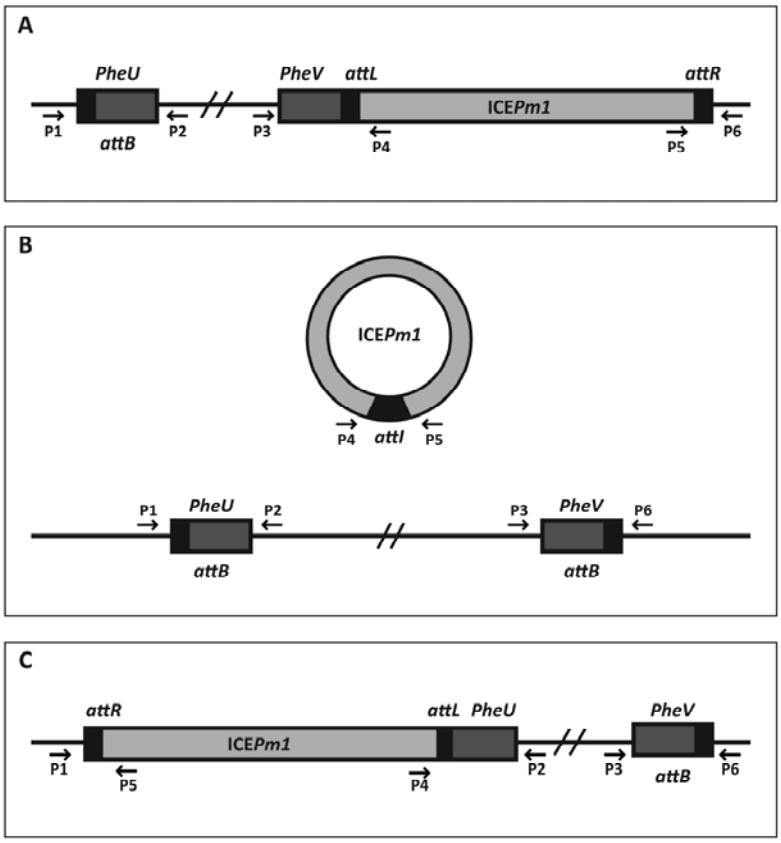

Figure 3-2. ICE*Pm1* actively excises from the chromosome.

The following primer pairs were used for amplification of attachment sites: *attL* (P3 and P4); *attR* (P5 and P6); *attI* (P4 and P5); *attB* (P3 and P6). Lane 1: HI4320 genomic DNA, Lane 2: NTC, no-template control. Colony PCR is shown for 5 isolated colonies obtained from a plating of the parental strain, *P. mirabilis* HI4320.

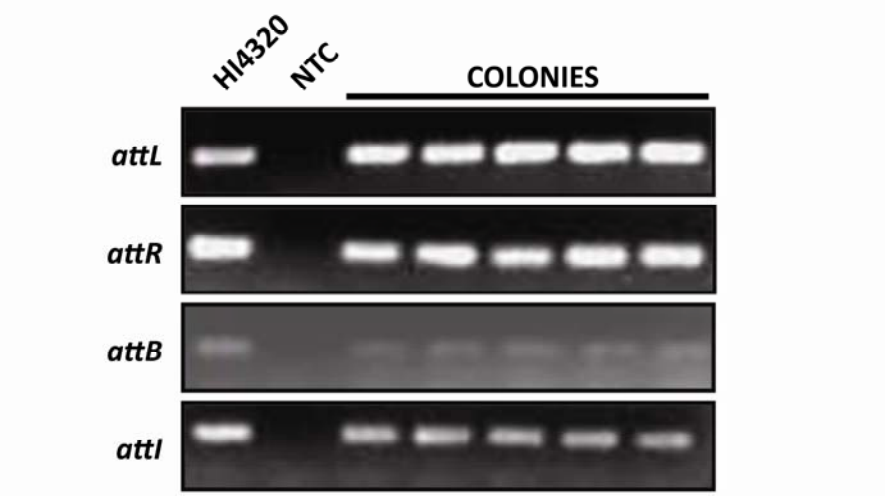

Figure 3-3. ICE*Pm1* precisely excises from the chromosome at the PheV-tRNA.

Direct sequencing of PCR products for *attL*, *attB, attI,* and *attR* are aligned below. All four products share the same 52 bp direct repeat (DR, shaded in black). Alignments also show the intact *pheV*-tRNA sequence maintained when ICE*Pm1* is integrated into the chromosome (*attL*) and when excised (*attB*).

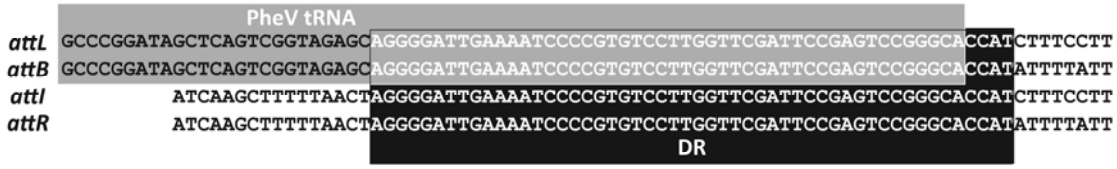

Figure 3-4. Confirmation of ICE*Pm1* transfer to a clinical *P. mirabilis* commensal isolate and its retained function.

A) Mating assays were performed and three transconjugants were selected for isolation and extraction of genomic DNA to confirm transfer of ICE*Pm1* by PCR. Amplification products were obtained for the beginning (PMI2551), middle (PMI2602) and end (PMI2641) of ICE*Pm1* to demonstrate complete transfer of the ICE. The *att*Tn*7* site, which produces different size products in the donor (PM HI4320 ICE::kan) and the recipient (PM 378L) strain, was used to differentiate between the donor and recipient genetic backgrounds. B) Amplification products for *attI* and *attB* in the transconjugant (378L ICE*Pm1*) demonstrates that ICE*Pm1* is capable of excising in the recipient host background. NTC, no template control.

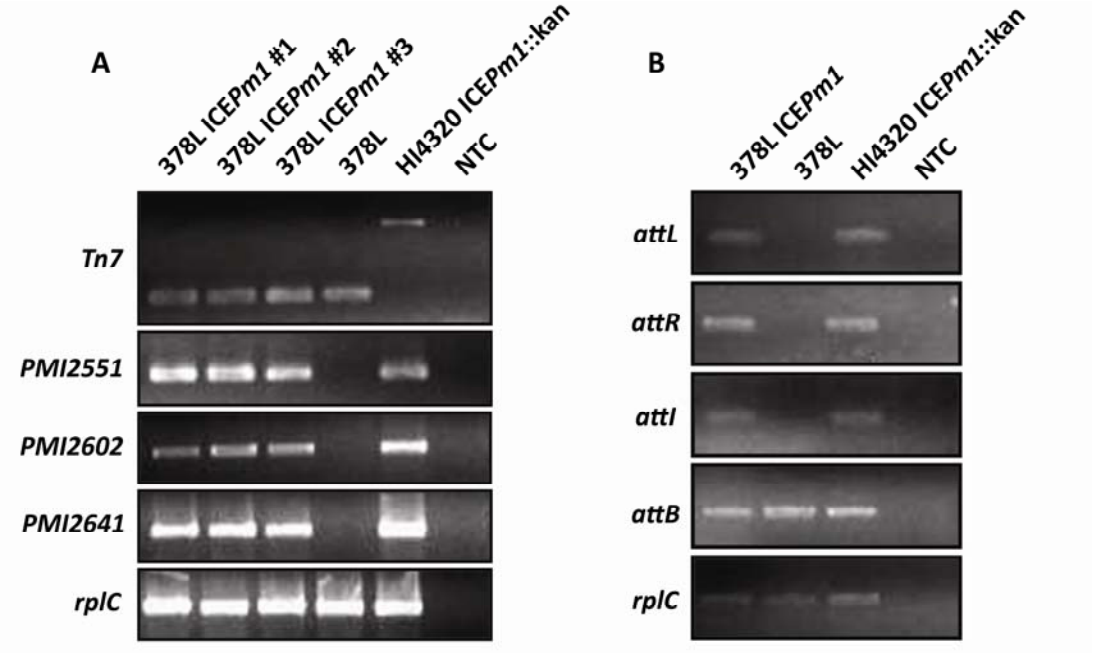

Figure 3-5. *intP* is necessary for ICE*Pm1* excision and transfer.

A) Amplification products for *attL*, *attR*, *attB* and *attI* from ICE*Pm1*::kan, *int*::kan (PMI2550::kan) and *parA*::kan (PMI2642::kan). No amplification products were observed for *attB* or *attI* in the *int::*kan mutant because ICE*Pm1* is unable to excise from the chromosome. B) Transfer frequencies (donors/transconjugants) of ICE*Pm1* transfer obtained with PM 378L as the recipient and with ICE*Pm1*::kan, *int::kan,* and *par::*kan as donors.

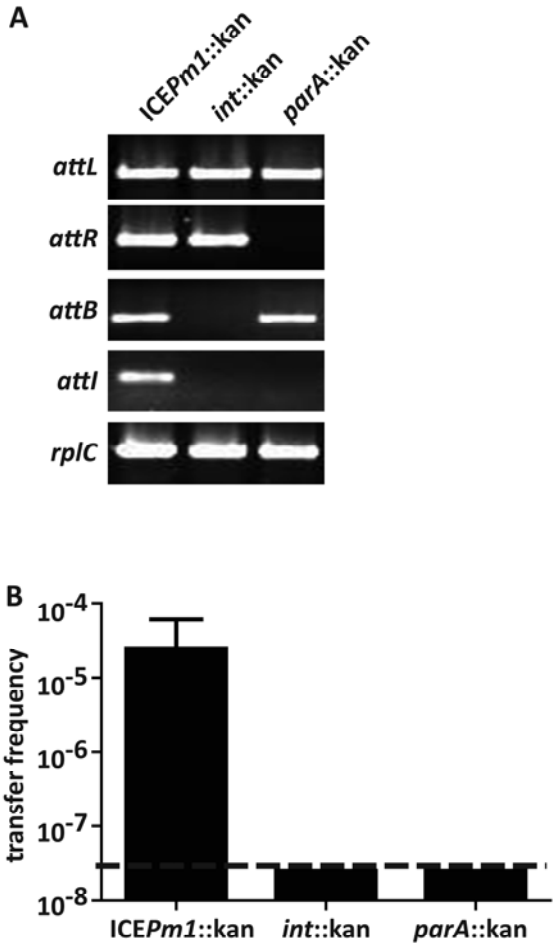

Figure 3-6. ICE*Pm1*- encoded T4SS genes are important for conjugative transfer.

A) Disruption of the ICE*Pm1* relaxase (*traI*) gene and conjugative pore forming gene (trap) no effect on the ability of ICE*Pm1* to excise from the chromosome as all amplification products are obtained that represent excised and integrated forms. Additionally, insertional inactivation of the relaxase of the SXT-like ICE ($traI_{SXT}$) also did not affect excision. B) Disruption of the ICE*Pm1* relaxase and conjugative pore forming genes decreased transfer frequency of ICE*Pm1* by almost 3-fold. The transfer frequency of ICE*Pm1* when the relaxase of the SXT-like ICE was disrupted was similar to wild-type levels suggesting the SXT-like ICE cannot transfer ICE*Pm1 in trans.*

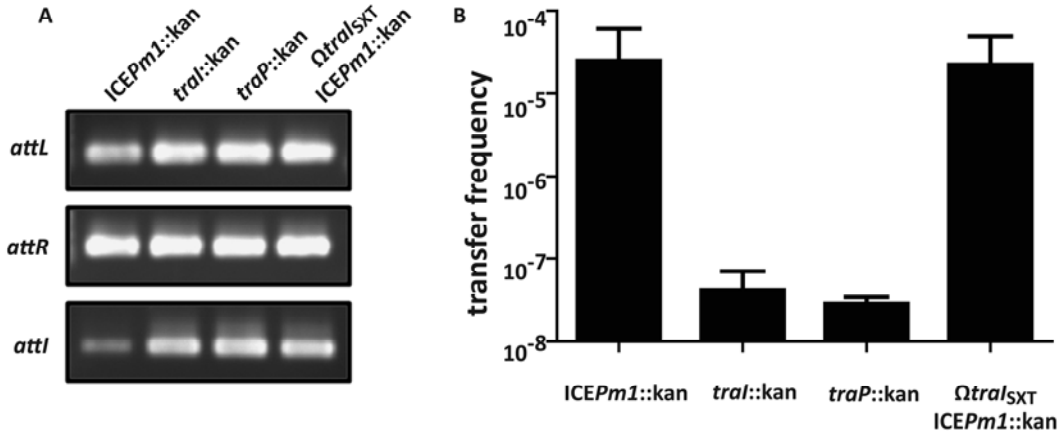

Figure 3-7. Active excision of the SXT-like ICE from *P. mirabilis* HI4320.

Active excision of the SXT-like ICE from the chromosome of *P. mirabilis* HI4320. Primers were designed in a similar way as the PCR assay for detection of ICE*Pm1* excision. Primer pair SXT-P1 and SXT-P2 was used to amplify SXT-*attL*. Primer pair SXT-P3 and SXT-P4 was used to amplify SXT-*attR*. And primer pairs SXT-P1 with SXT-P4 and SXT-P2 with SXT-P3 were used to amplify the *attB* and *attI* sites respectively.

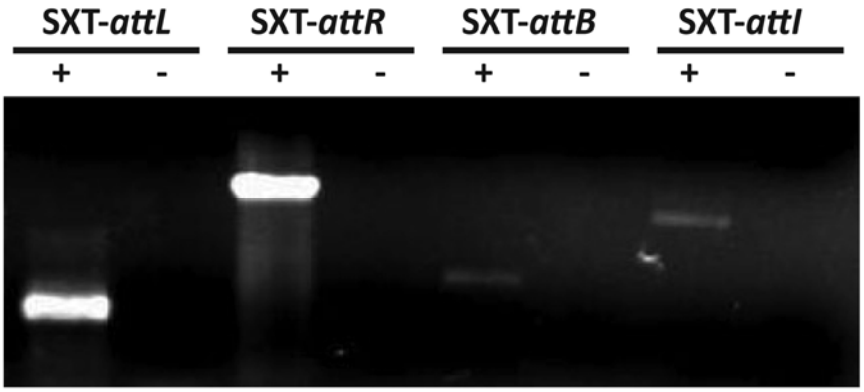

Figure 3-8. Genetic organization of *pheU* and *pheV* sites in *Escherichia coli*.

The annotated genomic sequence of A) *P. mirabilis* HI4320, B) *E. coli* CFT073 and C) *E. coli* MG1655 (K12) shows conserved DNA sequence for *pheU* and *pheV* tRNAs, yet variability in whether a pathogenicity island (PAI) is integrated into these tRNAs. The PheU site in *P. mirabilis* is empty as previously described. Both *pheU* and *pheV* carry genetically distinct PAIs in *E. coli* CFT073 that carry important virulence factors for adhesion and red blood cell lysis (Hacker, Bender et al. 1990; Dobrindt, Blum-Oehler et al. 2002). No reported PAIs have been described in the *pheU* and *pheV* sites of K12. Each tRNA is surrounded by those genes flanking the respective PAIs in CFT073, thus suggesting that these *attB* sites are "empty".

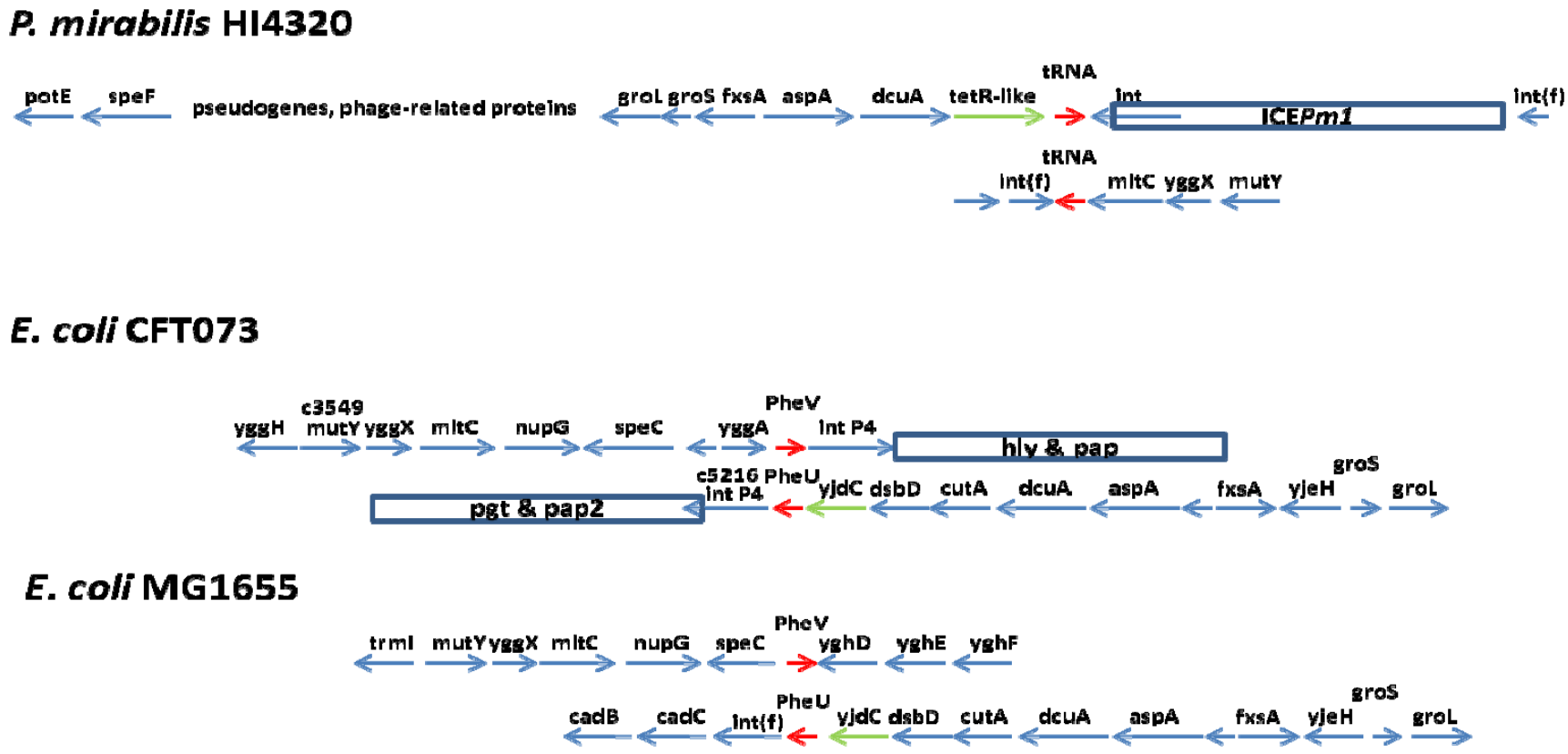

Figure 3-9. Transfer of ICE*Pm1* to select recipients.

Transfer frequency (transconjugants/donors) of mating experiments with ICE*Pm1*::kan as the donor and *P. mirabilis* (378L, 523L) or *E. coli* (K12, CFT073 or C) as recipients. All *E. coli* strains contain the identical *attB* sequence as *P. mirabilis*. Plate matings were conducted with a 1:1 ratio of donor to recipient, at 37°C for 6 hours. The limit of detection is 2 x 10^{-8} transconjugants/donor.

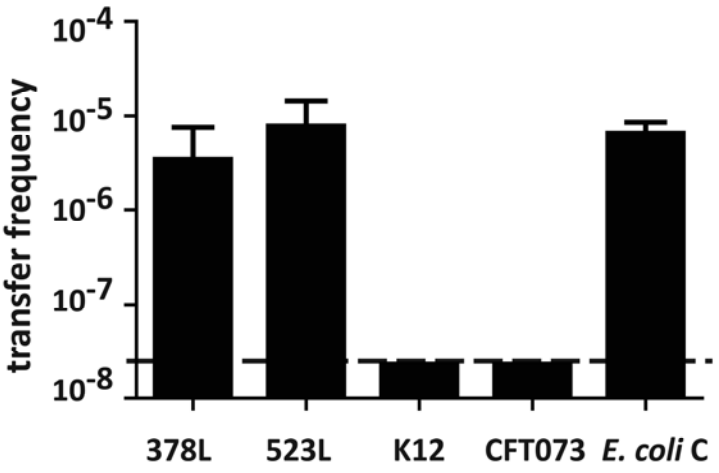

Table 3-1. Bacterial strains and plasmids used in the study of ICE*Pm1* function

Strain/plasmid	Description (Phenotype or Genotype)	Reference
Proteus mirabilis		
HI4320	urine isolate from catheterized patient	Warren, Tenney et al., 1982
ICE*Pm1*::kan	HI4320, ICE*Pm1* marked Kan^R	this study
int::kan	HI4320, PMI2549::kan insertion mutant	this study
rlxS::kan	HI4320, PMI2550::kan insertion mutant	this study
traP::kan	HI4320,PMI2594::kan insertion mutant	this study
parA::kan	HI4320, PMI2642::kan insertion mutant	this study
2476Ω, ICE*Pm1*::kan	HI4320, PMI2476 (*traI* of SXT) insertion; ICE*Pm1* marked kan^R	this study
378L	*P. mirabilis* groin isolate, Tri^R	Mody et al., 2007
378L ICE*Pm1*	ICE*Pm1* containing transconjugant	this study
Escherichia coli		
E. coli C	restriction modification negative strain	Bertani et al., 1952
CFT073	uropathogenic pyelonephritis isolate	Mobley et al., 1990
K12	MG1655	Tatum et al, 1947
Plasmids		
pACD4K-C	Targetron vector containing group II intron and kanamycin resistance cassette; Cam^R	Sigma
pACD4K-C-*loxP*	Targetron vector with loxP sites flanking the kanamycin resistance cassette; Cam^R	Sigma
pAR1219	IPTG inducible T7 polymerase; Amp^R	Sigma
pQL123	IPTG inducible cre recombinase; Amp^R	Sigma

Table 3-2. DNA sequence of oligonucleotides used in this study of ICE*Pm1* function.

ICE identification primers	
P1	CAACTCTTGTTTGCCCTCTCAG
P2	TGAGATCGGGTTTAATACGC
P3	CGTTGACGCATCACGCTGAATA
P4	CCGAGCGTTGGAGATCTCACTTCC
P5	GATATAATTGGAACAATTCTGGTC
P6	CTCAGTGACTTAACTCACTGACG

SXT identification primers	
SXT-P1	GTTCCTGCACGTTGGATAGCTT
SXT-P2	CGACAAGCTATCATCGAT
SXT-P3	GCCACAGCTTGTTTCGTGTA
SXT-P4	CGCAATGCTCGTTCATTATCT

Transconjugant confirmation primers	
Tn*7*F	AGATGCTGGCTTTGAAGAAAGTG
Tn*7*R	CACAGCATAACTGGACTGATTTC
2551F	CAGAAGATTACATGAATAATG
2551R	GAGAGTGTGATGAGATGTGAAT
2602F	GCGAATGAACTTCACCA
2602R	GCCACTAATCAGAGGGAGT
2641F	GCACGCTCTGCTCCGCC
2641R	CGGGAGGTGCGTCCATG
*rplC*F	CGTTGATGCTCTGATGCGTCT
*rplC*R	CGACTACTTGATGCACAAGCGC

Chapter 4

Wounds and Functional Disability are Associated with Co-Colonization by Methicillin-resistant *Staphylococcus aureus* and Vancomycin-resistant Enterococci in Southeast Michigan[3]

ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) remains sensitive to vancomycin so the emergence of vancomycin-resistant *S. aureus* (VRSA) is of great concern. VRSA Emergence is attributed to conjugative transfer of the vancomycin-resistance gene cluster from vancomycin-resistant Enterococci (VRE) to MRSA. Because co-colonization with MRSA and VRE precedes VRSA development, we investigated the epidemiology of co-colonization, with a focus on residents with indwelling devices. A prospective surveillance study of residents with indwelling devices (urinary catheters and feeding tubes) was conducted among 15 skilled nursing facilities in SE MI. Residents were cultured monthly for MRSA and VRE and clinical data were recorded. 178 residents were enrolled and followed for a total of 907 resident-months. The incidence rate of MRSA/VRE co-colonization was 6.5/100 resident-months in the device group and 0.8/100 resident-months in the non-device group (rate ratio = 8.1, 95% CI: 0.8-82.9). MRSA/VRE co-colonization in the device

[3] This study will be published with the following authors: Flannery EL, Wang L, Zöllner Z, Foxman B, Mobley HLT, Mody L.

group occurred most frequently at wound sites (4.1/100 resident-months). Among residents with devices, functional disability was significantly associated with co-colonization (rate ratio = 1.3, 95% CI: 1.1-1.4). Colonization with VRE predicted co-colonization better than colonization with MRSA. Indwelling devices, functional disability and wounds are significantly associated with co-colonization with MRSA and VRE. These individuals should be monitored for the emergence of VRSA.

INTRODUCTION

Since the first vancomycin-resistant *Staphylococcus aureus* (VRSA) isolate was cultured in 2002, there have been 10 additional cases reported, the most recent occurring in April, 2010 (Zhu, Murray et al. 2010). Interestingly, 8 of the 11 cases occurred in Michigan. Because vancomycin is commonly used to treat methicillin-resistant *S. aureus* (MRSA) infections, the emergence of VRSA is a major concern (Arias and Murray 2009).

High level vancomycin-resistance in *S. aureus* (MIC ≥ 32 mg/ml) develops from acquisition of the vancomycin-resistance gene (*vanA*) from a vancomycin-resistant Enterococci (VRE), a bacterium that commonly colonizes the gastrointestinal tract (Noble, Virani et al. 1992; Perichon and Courvalin 2009). Acquisition of the *vanA* gene cluster occurs by direct conjugal transfer of DNA from VRE to MRSA via a pilus (frequently mediated by the Inc18-family of plasmids) (Perichon and Courvalin 2009), making proximity requisite for acquisition. Thus, co-colonization with MRSA and VRE is necessary for *vanA* transfer and subsequent emergence of VRSA. Indeed, several of the VRSA cases were also co-infected with VRE at the time of VRSA isolation (Finks, Wells et al. 2009; Zhu, Murray et al. 2010). Therefore, because MRSA/VRE co-colonization precedes VRSA emergence, we chose to investigate the incidence of co-colonization by these two bacteria in a long-stay population. By using such a population we can observe incidence rates of MRSA/VRE co-colonization as well colonization patterns over time.

Previous studies have investigated the prevalence rates of MRSA/VRE co-colonization, but have been limited to clinical cultures or surveillance cultures of

specific body sites such as the nares (for MRSA surveillance) or rectum (for VRE surveillance) (Warren, Nitin et al. 2004; Furuno, Perencevich et al. 2005). For nearly all documented VRSA cases (10/11), the VRSA isolates were recovered from wounds; additionally 8 of the VRSA infected patients had diabetes. Prior data from our group have shown that residents with indwelling devices are at greater risk for multi-anatomic site colonization with antibiotic resistant organisms (Mody, Maheshwari et al. 2007). Therefore, in this study we sought to understand not only the overall incidence of MRSA/VRE co-colonization, but also how incidence rates vary at different body sites, especially wounds. We were also interested if known risk factors for VRE and MRSA colonization were predictive of co-colonization. Specifically, we were interested in potentially mediating effects of diabetes, use of indwelling devices and functional disability on risk of co-colonization.

The prevalence of healthcare-associated infections caused by antibiotic resistant organisms is increasing, especially in skilled nursing facilities (SNFs) (Crnich, Safdar et al. 2007). Residents of SNFs are at high risk for infection with antibiotic resistant organisms because of their many comorbidities, and high antibiotic usage rates in these facilities (Strausbaugh and Joseph 2000; Mody, Maheshwari et al. 2007; Tacconelli, De Angelis et al. 2009; Chen, Anderson et al. 2010). To achieve our objectives, we used a prospective active surveillance approach to identify risk factors for and incidence rates of co-colonization with MRSA and VRE in residents in SNFs in Southeastern Michigan.

MATERIALS AND METHODS

Study design and population.

This prospective observational study, involving 15 SNFs in southeast Michigan, was conducted from October 2005 to January 2010. All residents with a new indwelling device (enteral feeding tube or urinary catheter) were identified and asked to enroll. Upon identification and enrollment of a resident with an indwelling device, a device-free resident was randomly selected using a random number generator and asked to enroll. All residents were followed for up to 12 months. Loss to follow-up was due to device removal, transfer, death or culture refusal. Demographic data were recorded at enrollment and clinical and microbiologic data were obtained at monthly study visits. This study was approved by the University of Michigan and Veterans Affairs Ann Arbor Health Care System Institutional Review Boards. Written informed consent was obtained from all enrolled residents or their durable power of attorney.

Data collection and variables.

Upon enrollment, resident's age, sex and length of stay at the facility were recorded. Charlson Comorbidity Index was used to assign a comorbidity score ranging from 0-11 (Charlson, Pompei et al. 1987). Clinical data were obtained from all enrolled residents at each study visit by chart review. Antibiotic use, presence of wounds (including pressure ulcers) and hospitalization, all in the previous 30 days, were recorded for each resident. The Lawton and Brody physical self-maintenance scale (PSMS) was used to assess functional status at each study visit (Lawton and Brody 1969).

Microbiologic and molecular methods.

At each monthly study visit, culture samples were obtained from the anterior nares, oropharynx, groin and peri-anal area using culturette rayon-tipped swabs (Becton Dickenson, Inc., Cockeysville MD). In addition, enteral feeding tubes, suprapubic catheters and wounds or pressure ulcers were cultured when present. One participant refused to be cultured, so one resident-month is missing in the nares and oropharynx in the device-group.

For isolation and identification of *S. aureus*, swabs were streaked on mannitol salt agar and incubated at 35°C for 24 hours. Colonies presumptive for S. *aureus* based on phenotype were further confirmed by positive catalase and Accustaph (*vendor*) reactions. Growth on Mueller-Hinton agar (MHA) (BD) containing oxacillin (6 μg/ml) identified MRSA. All MRSA were subsequently tested for vancomycin resistance on MHA containing vancomycin (6 μg/ml) (Prevention 2010).

Culture swabs were also streaked on bile-esculin-azide agar containing vancomycin (6 μg/ml) for identification of vancomycin-resistant Enterococci. VRE isolates were confirmed by plating on tryptic soy agar containing 5% sheep's blood and positive pyroglutamate aminopeptidase tests (pyrrolidonyl-beta-naphthylamide).

Polymerase Chain Reaction (PCR) using primer pairs: E1: atcaagtacagttagtct and E2: acgattcaaagctaactg and F1: tagagacattgaatatgcc and F2: tcgaatgtgctacaatc identified *E. faecalis* and *E. faecium* respectively (Dutka-Malen, Evers et al. 1995). For identification of Inc18-like plasmids, PCR was performed with primer pairs: *vanA* F: catgaatagaataaaagttgctgcaata and *vanA* R: ccccttttaacgctaatacgatcaa; *traA* F: taatcgcaatggcttcttatc and *traA* R: tctgcccaatctttacgaat; and *repR* F: gcttcatgacggcttgtta

and *repR* R: ttggctgctttgacagattta (Zhu, Clark et al. 2008). PCR was performed in 50 µl reactions with 1 µl colony re-suspended in sterile dH_2O as template. PCR conditions were as follows (i) 10 min at 94°C; (ii) 30 cycles of 30 sec at 94°C, 30 sec at 56°C and 1 min at 72°C; and (iii) 7 min at 72°C.

Outcome measures.

Our primary outcome was the overall incidence rate of co-colonization with MRSA and VRE at the resident-level defined as colonization with MRSA and VRE on the same visit from any combination of sites. A resident could be colonized with both VRE and MRSA at the same anatomic site (e.g. wounds) or at different anatomic sites (e.g. wound and groin). Similarly, colonization at the resident-level with MRSA only or VRE only was defined as being colonized at any anatomic site. To determine anatomic site-specific incidence rates, simultaneous isolation of MRSA and VRE from the same culture swab was considered MRSA/VRE co-colonization.

Statistical Analyses.

We identified differences between the device and non-device groups at baseline. A chi-square test was used to detect differences in categorical variables and a Student's *t* test for differences in continuous variables, both with a two-sided significance level of $\alpha = 0.05$.

We used logistic regression to conduct three different risk factor analyses, only in the device group. For each analysis, co-colonization at the resident-level was the outcome group. We calculated the rate ratios for co-colonization among 3 different

sub-populations to highlight differences in outcomes conditional on the base population used for these analyses. The three sub-populations used for the analyses were 1) all residents (these residents could be singly colonized with VRE alone or MRSA alone), 2) residents colonized with MRSA and 3) residents colonized with VRE. We used generalized estimating equations (GEE) to describe the associations between MRSA/VRE co-colonization and clinical characteristics (Twisk, Smidt et al. 2005). GEE was used to account for the repeated measures design of the study. A log link function with a Poisson distribution was used to calculate the MRSA/VRE co-colonization rate ratios with robust error variances (Lindquist). For the multivariate analysis, all variables significantly associated with co-colonization (α=0.01) in the univariate analysis were included in the full regression model. Variables not significantly associated with the outcome in the full model were removed in a stepwise manner. The final model only includes variables significantly associated with the outcome (α=0.05). Data were analyzed using SAS, version 9.2.

RESULTS

Study population characteristics.

A total of 178 SNF residents, 90 residents with indwelling devices and 88 residents without devices, were enrolled in this prospective observational study. Participants were followed for a total of 907 resident-months; 263 in the device group and 644 in the non-device group. On average, residents were followed for 5.1 months. SNF residents with devices differed significantly in all characteristics from non-device residents, except for the proportion of residents with diabetes (26% vs. 30%, respectively) (Table 4-1). Because residents in the device and non-device groups differed significantly on most characteristics, and because indwelling devices are a substantial risk factor for colonization with these antibiotic resistant organisms (Mody, Maheshwari et al. 2007), we stratified by device group for all subsequent analyses.

14 residents were co-colonized with MRSA and VRE.

At the resident-level, 14 residents (10 device residents and 4 non-device residents) were co-colonized with MRSA and VRE on at least one study visit during the study period (outlined in black in Figure 4-1). Co-colonization with MRSA and VRE was dynamic within each resident over time and occurred a total of 22 times at the resident-level (Figure 4-1). Residents were colonized with MRSA at 204 study visits; on 10.8% of these visits, they were also co-colonized with VRE. Although VRE colonization was lower- occurring only at 44 visits, in 50% of these visits residents were also colonized with MRSA (i.e. MRSA/VRE co-colonized). Using a logistic regression model, MRSA and VRE each predicted colonization with each other. That

is, residents colonized with VRE were more likely to be colonized with MRSA than residents not colonized with VRE (rate ratio = 2.4, 95% CI = 1.3-4.2) and residents colonized with MRSA were more likely to also be colonized with VRE than residents not colonized with MRSA (rate ratio= 1.8, 95% CI = 1.3-2.5); although at a lower rate ratio.

Risk of colonization at each study visit.

Among residents with indwelling devices, the risk of MRSA/VRE co-colonization at each study visit (number of residents colonized/number of residents cultured) was either higher or the same as the risk for colonization with VRE only (Figure 4-2). MRSA colonization was much higher than VRE or co-colonization at all study visits. This same observation was made in residents with no-device; MRSA colonization risk was higher at every study visit. In the no-device group, the risk of MRSA/VRE co-colonization is only greater than zero at the first two study visits and the risk of VRE colonization is also low, yet higher than MRSA/VRE co-colonization.

Associations of clinical and demographic characteristics with MRSA/VRE co-colonization.

Risk factors for independent colonization with MRSA or VRE are well-known (Boyce 1989; Zirakzadeh and Patel 2005). Therefore, we calculated the rate ratios for co-colonization among residents either colonized with MRSA or colonized with VRE to determine the added risk for co-colonization within these two colonization groups. We also calculated the rate ratios for co-colonization among all device residents for

comparison with other studies. We used a logistic regression model to determine the associations between MRSA/VRE co-colonization and several demographic and time-varying clinical characteristics. We restricted our analysis to residents with indwelling devices because of the low incidence rate of co-colonization in the non-device group.

Functional status and wounds were significant predictors of MRSA/VRE co-colonization among those with devices. For every unit increase in functional status score (increasing dependency) there is a 30% increase in the rate of MRSA/VRE co-colonization (95% CI = 1.1-1.4) (Table 4-2).

Among residents with devices, predictors of co-colonization compared to residents with MRSA alone or VRE alone differed. Among all residents colonized with MRSA, residents who received antibiotics in the previous 30 days had an almost 4-fold increase in MRSA/VRE co-colonization compared to residents who did not receive antibiotics (RR= 3.7, 95% CI: 1.3-10.3) (Table 4-2). Functional status (RR= 1.2, 95% CI: 1.0-1.4), male sex (RR= 3.3, 95% CI: 1.1-10.2), and wounds (RR= 2.6 (1.3-5.3) were also significantly associated with co-colonization among MRSA colonized residents.

On the other hand, in the VRE colonized group use residents were half as likely to be co-colonized with MRSA and VRE if they received antibiotics in the previous 30 days (RR = 0.5, 95% CI: .3-.9). Among the residents colonized with VRE, again increasing PSMS score (functional disability) was significantly associated with co-colonization (RR= 1.1, 95% CI: 1.03-1.19) as well as follow-up months (RR= 1.1, 95% CI: 1.0-1.2). Diabetes was not associated with MRSA/VRE co-colonization in any of our analyses.

Using a multivariate logistic regression model, functional status and male sex were both independent predictors of co-colonization among all device residents and among residents with MRSA colonization. Among residents with VRE colonization, functional status and prior hospitalization were independent predictors of MRSA/VRE co-colonization.

Incidence rate of co-colonization by anatomic site.

Incidence rates for co-colonization with MRSA and VRE were determined for six different anatomical sites and compared to the rate of colonization with MRSA alone and VRE alone. For the site-level analysis, co-colonization was defined as simultaneous colonization with MRSA and VRE at the same site on the same study visit (blue boxes in Figure 4-1). Among residents with indwelling devices, the incidence rate for co-colonization with MRSA and VRE was highest in wounds and the rectum (41/1000 and 38/1000 resident-months respectively) (Table 4-3). Of note, MRSA/VRE co-colonization was never observed in the nares or oropharynx. Additionally, in the device group, when the device sites and wounds were colonized with VRE, they were always colonized with MRSA. Importantly, MRSA/VRE co-colonization only occurred in the rectum in non-device residents, at a rate of 6/1000 resident-months (Table 4-3).

Because residents were cultured at up to 6 anatomical sites, we were able to determine the percentage of sites colonized (number of sites colonized/number of sites cultured) at a single visit. The average MRSA/VRE co-colonization percentage (17.1%) was lower than that observed for MRSA colonization alone (35.6%) and VRE colonization alone (28.3%) in the device group. There was no significant difference in

the percentage of MRSA/VRE co-colonized sites between residents with and without indwelling devices.

Molecular analysis of VRE isolates.

We used PCR of Enterococci-specific genes (*ddl*) to identify all VRE isolates to the species-level (Dutka-Malen, Evers et al. 1995). *E. faecalis* was isolated more commonly in both the device and non-device groups and accounted for over half (52%) of the total VRE isolates. *E. faecium* was isolated more commonly from co-colonized residents (41%) compared to residents that were not co-colonized (19%), although this difference was not statistically significant (p=.0627). VRE isolates that were neither *E. faecalis* nor *E. faecium* were more commonly found in the no-device group (p= 0.0004).

Plasmids of the Inc18 family have been associated with several of the VRSA cases because of successful conjugation with MRSA. Therefore, we determined the prevalence of Inc18-like plasmids in our study population. PCR for the *repR*, *traA* and *vanA* genes was used to identify Inc18-like plasmids carried by the Enterococcal isolates. Only 2/66 VRE isolates were positive for the presence of Inc18-like plasmids (3%). These VRE isolates were cultured from the same resident on the same day from the groin and the device sites. Interestingly, the isolate from the device sites was *E. faecalis* and the groin isolate was VRE, but not positive for *E. faecalis* or *E. faecium ddl* genes. This suggests these isolates are not clonal.

DISCUSSION

In this prospective study of 178 residents of SNFs in southeast MI, the incidence of MRSA/VRE co-colonization was 6.5 per 100 person-months (95% CI: 3.9-10.1/100 person-months). Wounds were an important co-colonization site for MRSA and VRE, and functional status an independent predictor of co-colonization in the resident-level analysis.

Prevalence estimates of MRSA/VRE co-colonization in the literature vary dramatically, from 0.27% to 34% (Ray, Pultz et al. 2003; McDonald, Engemann et al. 2004; Reyes, Malik et al. 2010). This variation may be explained by variability in study population, the use of either clinical or surveillance cultures, and whether screening was conditional on the prior existence of MRSA or VRE (McDonald, Engemann et al. 2004; Warren, Nitin et al. 2004; Furuno, Perencevich et al. 2005; Kurup, Wong et al. 2008; Reyes, Malik et al. 2010). Because of the different study designs, it is difficult to compare with our results, but the cross-sectional prevalence of co-colonization at baseline in our study was 7.9%.

In 10/11 VRSA cases, VRSA isolates were cultured from a wound of an infected patient (Prevention 2010). This is consistent with our finding that wounds had the highest incidence rate of MRSA/VRE co-colonization when compared to other sites cultured. The emergence of VRSA in these cases could simply reflect that co-colonization is more common in wounds. Alternatively, or in addition, proximity of organisms is necessary for horizontal gene transfer to occur. Rates of horizontal gene transfer are increased in biofilms, which commonly form in wounds (Ehrlich, Ahmed et al. 2010; Siddiqui and Bernstein 2010). Thus, wounds could be important for

colonization in close proximity by these organisms and an environment that stimulates HGT, and thus horizontal transfer of vancomycin resistance from VRE to MRSA.

Screening for MRSA in wounds has already been suggested (Boyce 2008); our data support the active screening of wounds additionally for MRSA/VRE co-colonization. Interestingly, wounds were predictive of resident-level co-colonization, even when co-colonization events that occurred in wounds were excluded from the analysis (RR= 2.6, 95% CI: 1.3-5.3). This suggests that not only are wounds a prevalent site of MRSA/VRE co-colonization, but also that the presence of a wound is a risk factor for co-colonization at other sites within the resident.

Residents with indwelling devices have been identified as a high-risk group for colonization with antibiotic-resistant organisms and are therefore suggested as a targeted group for reducing bacterial colonization rates (Mody, Bradley et al. 2011). Surveillance screening of high-risk individuals is a more effective way to allocate resources to limit colonization. We show that the risk of co-colonization is higher in individuals with indwelling devices. Residents without a device were only co-colonized in the peri-anal area, even though wounds were also present. Because functional disability (increasing PSMS) was an important predictor of co-colonization in the devices group, we would suggest this group be additionally considered high-risk for MRSA/VRE co-colonization, especially in the presence of wounds.

Interestingly, diabetes was not found to be a significant predictor of co-colonization in any of our analyses, even though the majority of the reported VRSA case patients were diabetic (Zhu, Murray et al. 2010). One hypothesized reason that the majority of the VRSA cases are from Michigan is the high rates of diabetes observed in

Michigan (Tenover 2008). Despite our lack of association between diabetes and co-colonization, it is well-known that wounds and pressure ulcers are common in diabetics. Therefore, it remains possible that wounds, not diabetes, may be the common risk factor these case patients shared.

Among the device group, MRSA/VRE co-colonization was more common than VRE colonization alone at any study visit. Further, the rate ratio for co-colonization given VRE colonization was greater than the rate ratio for co-colonization given MRSA colonization. This suggests that colonization with VRE is more predictive of co-colonization than colonization with MRSA. This may result from MRSA endemicity in these facilities and thus the risk of being colonized with VRE is driving co-colonization risk. Preventing VRE colonization among residents with indwelling devices might limit MRSA/VRE co-colonization (Zhu, Murray et al. 2010).

Because our study was conducted in a similar population in SE Michigan, where the majority of documented VRSA emergence occurred, we were able to identify risk factors for the development of VRSA in an important population. Most VRSA cases occurred in older patients with much comorbidity, similar to the residents of SNFs in our study. In addition, we found the prevalence rate of Inc18-like plasmids to be 3%, which is similar to the findings reported by Zhu et al., and was shown to be higher in Michigan in comparison to other states (Zhu, Murray et al. 2010). Because Inc18-like plasmids can mediate transfer of the vancomycin-resistance gene cluster, this may be an additional factor to consider when developing measures to prevent VRSA emergence.

Clinical cultures do not identify the true prevalence rates of bacterial colonization in healthcare facilities. By actively culturing 4-6 anatomical sites in every

resident we are able to more accurately describe the rates of colonization in SNFs. Although a clear limitation of this study is the sensitivity of culture samples, because we sampled multiple sites at multiple time points, we were able to better estimate true resident-level colonization than sampling specific sites. In addition, because we sampled all residents and did not rely on previous identification of MRSA or VRE colonization, we believe our results estimate the true reservoir of MRSA/VRE co-colonization among patients with indwelling devices. Additionally, at the site-level, decreased sensitivity of culture methods would suggest we are underestimating the incidence rate of co-colonization in wounds, a rate we found to be substantial.

Although there has been no documented person-to-person transmission of VRSA, it would be naïve to assume this will not happen. Because all of the VRSA cases have arisen independently, the prevalence of MRSA/VRE co-colonization is important (Zhu, Clark et al. 2008). The greater the prevalence, the greater the possibility of gene transfer subsequent evolution of VRSA strains that transmit well between persons or vancomycin-resistance to other MRSA strains. Like the burden VRE and MRSA endemicity imposes on healthcare facilities, one would imagine VRSA would as well if it were common. In conclusion, our study highlights the need to conduct further studies in different geographic locations to compare risk factors for co-colonization with MRSA and VRE.

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Figure 4-1. Colonization patterns of 14 residents co-colonized a minimum of one visit during the study period.

VRE and MRSA Colonization patterns from 14 residents of skilled-nursing facilities in Michigan co-colonized a minimum of one visit during the study in a prospective surveillance study of MRSA and VRE conducted in skilled nursing facilities in Michigan, October 2005 to January 2010 (n=178). Each row represents the follow-up time for a single study participant. The top horizontal axis denotes the number of days post enrollment. Each vertical rectangle represents a study visit for a particular resident; the six anatomical sites eligible for culture: N= nares, O=oropharynx, G= groin, R= rectum, D= device and W= wound or pressure ulcer. Colored boxes indicate MRSA (green) was cultured at that site, VRE (yellow), and both MRSA and VRE (blue), no organism present (black outline) or not cultured (grey). Residents were defined as co-colonized for the resident-level analysis if they were colonized with MRSA and VRE on the same study visit at any combination of sites (black outlined vertical rectangles). Residents were considered co-colonized for the site-level analysis if MRSA and VRE were both isolated from the same anatomical site at a study visit. Whether or not residents had indwelling devices is indicated on the y-axis.

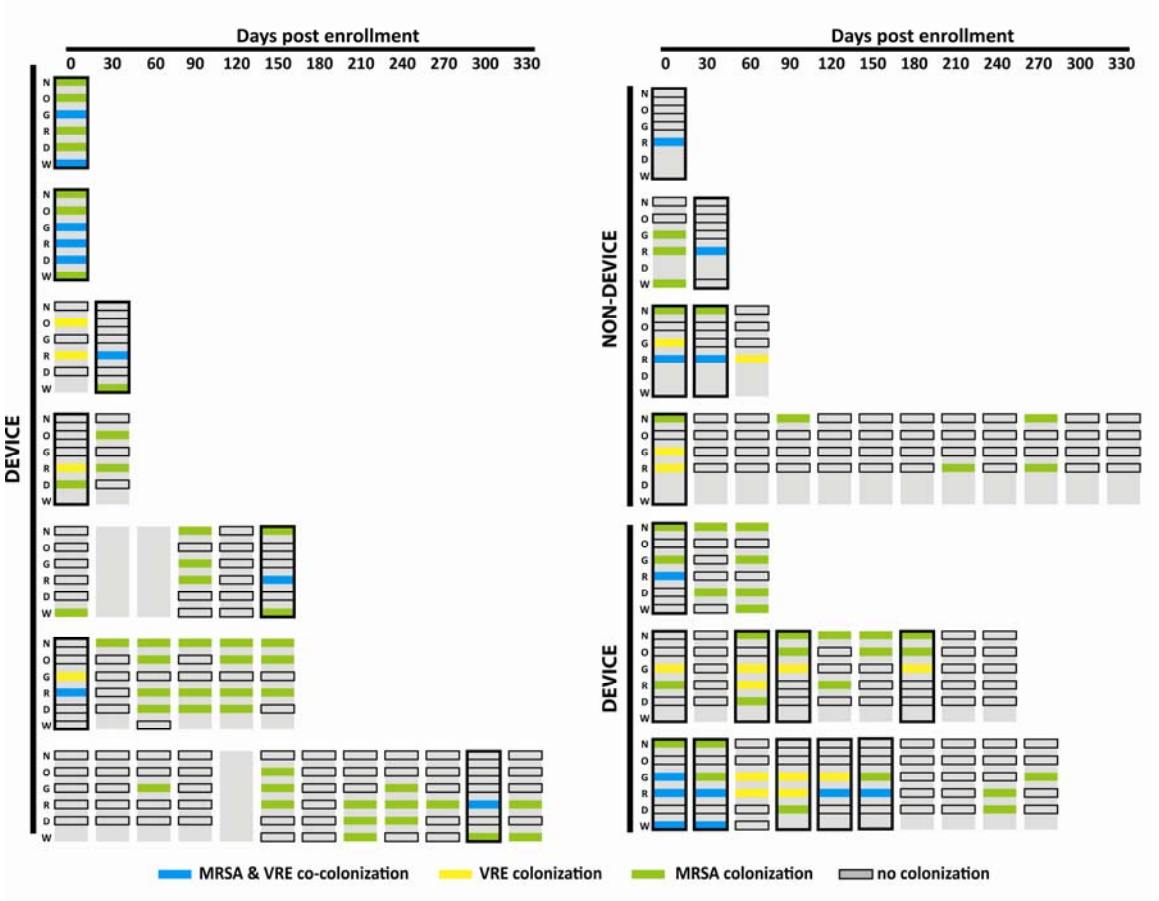


Figure 4-2. Risk of co-colonization with MRSA and VRE, colonization with MRSA and colonization with VRE at each follow-up visit.

Risk of co-colonization with MRSA and VRE, colonization with MRSA and colonization with VRE at each follow-up visit in a prospective surveillance study of MRSA and VRE conducted in skilled nursing facilities in Michigan, October 2005 to January 2010 (n=178). Colonization risk in residents with indwelling devices (A) and device-free residents (B) was determined by dividing the number of residents that were colonized by the total number of residents cultured at that study visit. The number of days post enrollment and the number of residents that were cultured at that study visit are indicated on the x-axis. Colonization groups are mutually exclusive.

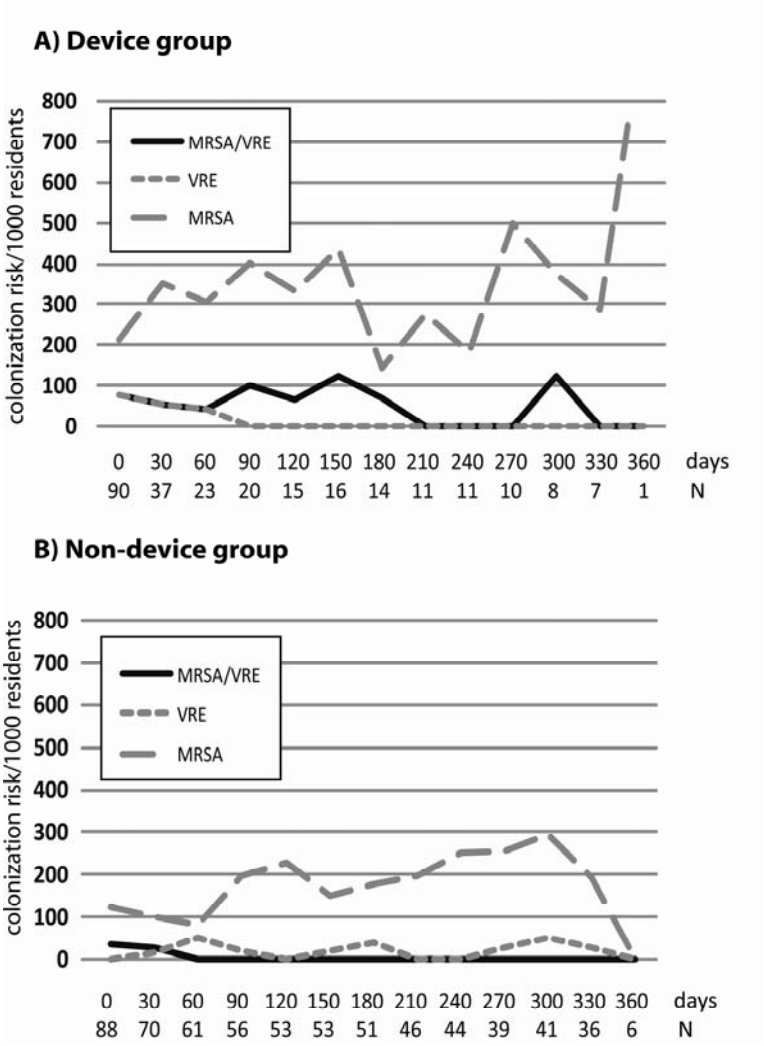


Table 4-1. Baseline characteristics of 178 residents participating in a prospective surveillance study.

Baseline characteristics of 178 residents participating in a prospective surveillance study of MRSA and VRE conducted in skilled nursing facilities in Michigan, October 2005 to January 2010.

	Device	Non-device	
Characteristic	n (%)	n (%)	*p*
Overall	90 (51)	88 (49)	
Mean ± SD			
Age (years)	78.9 (11.2)	82.2 (10.7)	0.049
Charlson Comorbidity Score	3.1 (2.0)	2.2 (1.6)	0.0012
Follow-up (months)	2.9 (3.3)	7.3 (4.5)	<.0001
PSMS score	21.1 (5.4)	18.2 (5.7)	0.0006
No. (%) of residents			
Male	41 (46)	21 (24)	0.0024
Diabetes	23 (26)	26 (30)	0.58
Wound presence	29 (32)	9 (10)	0.0003
Antibiotic use	61 (68)	28 (32)	<.0001
Hospitalization	66 (73)	13 (15)	<.0001
Long stayers (>90 days)	24 (27)	70 (80)	<.0001
Device type			
urinary catheter	48 (53)	-	
feeding tube	30 (33)	-	
both	12 (13)	-	

NOTE. PSMS, physical and self-maintenance score. Lawton and Brody physical and self-maintenance scale score was used to assess functional status. Scores range from 6 to 30 indicating increasing functional disability. A score of 6 represents independence in all activities of daily living while 30 represents complete dependence.

Table 4-2. Associations between MRSA/VRE co-colonization and clinical and demographic characteristics.

Associations between MRSA/VRE co-colonization and clinical and demographic characterisitics.[a] Co-colonized participants are compared to all other participants, to those colonized only with MRSA and with those colonized only with VRE. Prospective surveillance study of MRSA and VRE conducted in skilled nursing facilities in Michigan, October 2005 to January 2010 (n=178).

	MRSA/VRE co-colonization[b]	No co-colonization[c]		MRSA only[d]		VRE only[e]	
Characteristic	(n = 17)	(n = 246)	rate ratio (95% CI)	(n = 77)	rate ratio (95% CI)	(n = 10)	rate ratio (95% CI)
Mean							
Age (years)	75.9	77.9	1.0 (1.0-1.1)	78.7	1.0 (1.0-1.0)	79.4	1.0 (1.0-1.0)
CCS	3.2	3.2	.9 (.8-1.1)	3.1	0.9 (.7-1.2)	3.5	0.9 (.7-1.2)
Follow-up (months)	6.8	6.6	1.0 (.9-1.2)	7.3	1.0 (.8-1.1)	2.9	1.1 (1.0-1.2)*
PSMS	26.3	22	1.3 (1.1-1.4)*	23.5	1.2 (1.0-1.4)*	21.7	1.1 (1.0-1.3)*
No. (%) of residents							
Male	13 (76)	103 (42)	3.1 (.9-10.4)	30 (39)	3.3 (1.1-10.2)*	7 (70)	1.0 (.5-2.0)
Diabetes	7 (41)	63 (27)	1.3 (.3-4.8)	19 (25)	1.1 (.3-3.9)	4 (40)	0.9 (.4-2.1)
Wound presence	8 (53)	62 (27)	3.4 (1.4-8.6)*	24 (35)	2.6 (1.3-5.3)*	4 (40)	1.2 (.48-2.84)
Antibiotic use	9 (53)	82 (33)	3.0 (1.0-9.1)	18 (23)	3.7 (1.3-10.3)*	9 (90)	0.5 (.3-.9)*
Hospitalization	6 (38)	69 (30)	1.6 (.3-9.9)	14 (21)	10.6 (.7-151.4)	8 (80)	0.5 (.3-1.1)
Long stay (>90 days)	7 (41)	97 (39)	1.8 (.5-6.3)	29 (38)	1.3 (.4-4.3)	1 (10)	1.9 (1.0-3.7)

NOTE: [a]Adjusted for repeated measures using generalized estimating equations. [b]MRSA/VRE co-colonization is the outcome in each analysis. Sub-populations that the logistic regression analyses were conducted in are [c]all device residents (included residents singly colonized with VRE alone or MRSA alone), [d]MRSA only colonization and [e]VRE only colonization. n, number of resident-months colonized; CCS, Charlson comorbidity score was used to assess comorbidities, ranges from 0-6, with increasing CCS is associated with increasing 1-yr mortality rates; PSMS, Physical and self-maintenance scale score assesses functional status and Scores range from 6 to 30 indicating increasing functional disability. A score of 6 represents independence in all activities of daily living while 30 represents complete dependence.

*p<.05

Table 4-3. Incidence rates of co-colonization by anatomical site.

Incidence rates per 100 person months of co-colonization with MRSA and VRE, colonization with VRE only and colonization with MRSA only at 6 different anatomical sites stratified by device group.[a] Prospective surveillance study of MRSA and VRE conducted in skilled nursing facilities in Michigan, October 2005 to January 2010 (n=178).

	Incidence rate[b] (95% confidence intervals)					
	Device			Non-device		
	MRSA/VRE	VRE only	MRSA only	MRSA/VRE	VRE only	MRSA only
Nares	0	0	15.3 (11-21)	0	0	11.0 (8.7-14)
Oropharynx	0	3.8 (.2-19)	8.0 (5.1-12)	0	0	0.9 (.4-1.9)
Groin	1.1 (.3-3.1)	5.3 (3.0-8.7)	7.6 (4.8-12)	0	1.7 (.9-3.0)	3.1 (2.0-4.7)
Rectum	3.8 (1.9-6.8)	3.8 (1.9-6.8)	12.9 (10-18)	0.6 (.2-1.5)	1.4 (.7-2.6)	5.3 (3.7-7.3)
Device	0.4 (.02-1.9)	0	14.5 (10-20)	-	-	-
Wound	4.1 (1.0-11)	0	14.9 (7.8-26)	0	0	13.1 (6.1-15)
Overall[c]	6.5 (3.9-10.1)	10.3 (6.9-14.7)	35.7 (29.0-43.5)	0.8 (.3-1.7)	2.6 (1.6-4.1)	18.2 (15.1-21.7)

NOTE: [a]There were 263 and 644 resident-months exposed in the device and non-device, groups respectively. There were 74 and 61 resident-months exposed to wounds in the device and non-device groups respectively. [b]colonization/100 resident-months [c]Overall colonization rates are for resident-level colonization.

Chapter 5

Conclusions and Future Directions

SUMMARY OF RESULTS

The findings presented in this dissertation address the role of horizontal gene transfer (HGT) in the dissemination of virulence determinants and antibiotic resistance genes in pathogens transmitted in healthcare facilities. We found mechanisms affecting HGT at the microbe-level as well as factors that govern this process at the patient-level.

The major findings of this work are:

- A 93-kb genomic island is conserved between the uropathogens *Proteus mirabilis, Providencia stuartii* and *Morganella morganii*.
- This genomic island is a functional integrative and conjugative element (ICE*Pm1*) that can excise from the chromosome, form a circular intermediate and self- transfer to ICE-deficient recipient cells at a frequency of 1.35×10^{-5} transconjugants per donor cell.
- ICE*Pm1* excision is integrase-dependent.
- The ICE-encoded type IV secretion system (T4SS) is necessary and sufficient for self-transmissibility.

- ICE*Pm1* can integrate into either phenylalanine transfer RNA gene (*pheU* or *pheV*) encoded in the genome.
- ICE*Pm1* is more prevalent in urinary disease isolates of *P. mirabilis, P. stuartii* and *M. morganii* than their commensal counterparts.
- Co-colonization by methicillin-resistant *S. aureus* and vancomycin-resistant Enterococci occurs in the rectum and wounds of residents of skilled-nursing facilities (SNFs) with indwelling devices.
- Functional status, indwelling device use and the presence of wounds are risk factors for co-colonization among residents of SNFs.

CONCLUSIONS AND PERSPECTIVES

Conservation of a genomic island in three uropathogens

In our initial study, we were interested in determining genes conserved among three organisms that are common etiologic agents of caUTI. We reasoned that genes conserved among these organisms may be important virulence factors associated with UTI as well as establishment of colonization in the bladder (and/or kidneys). We found few genes overall that were conserved between these organisms (at >93% nucleotide identity), yet a 93kb genomic island was found intact in all three species. Furthermore, this island was prevalent in all *P. mirabilis* urinary isolates screened, but heterogeneously distributed among *P. mirabilis* colonizing strains isolated from other anatomical sites.

That ICE*Pm1* is present in all urinary isolates of *P. mirabilis* is intriguing. Because we saw a heterogenic distribution of the ICE in strains colonizing other body sites, it suggests that this genomic island may be necessary for colonization of the urinary tract. Genomic islands can be classified into numerous types of islands including symbiosis islands, metabolic islands, fitness islands and pathogenicity islands, all contributing advantageous properties to organisms (Hacker and Carniel 2001). It is possible that this island carries elements important for colonization and thus fitness or metabolism within the host, or contains virulence factors required for pathogenicity or virulence during disease processes.

Bacteria isolated from patients presenting with UTI have been shown by molecular analyses to be genetically related to strains that colonize the rectum and groin of the same patients (Sabbuba, Mahenthiralingam et al. 2003). The colonizing commensal strains could serve as a reservoir for strains that are able to subsequently colonize a catheter once inserted, and cause disease. The presence of this genomic island could be the factor that differentiates strains that can and cannot colonize and ascend catheters and subsequently colonize the bladder. Thus, this island could provide pathogenic potential for commensal colonizing strains to cause disease in the catheterized urinary tract.

One potential reason that we see these differences in island distribution among the two study collections (urinary and colonizing isolates) could be due to the different populations from which the isolates were obtained. The patients in each study are both geographically and temporally distinct. The urinary isolates were cultured from patients residing in LTCFs in Maryland from 1981-1982 while the colonizing isolates were obtained from patients residing in Michigan in 2007 (Warren, Tenney et al. 1982; Mody, Maheshwari et al. 2007). Nevertheless, because they are both long-term care populations and we observed that every *P. mirabilis* urinary isolate (n = 39) carried ICE*Pm1*, we believe this observation to be significant.

If we assume that all urinary isolates harbor ICE*Pm1* because of its contribution to colonization of the catheterized urinary tract, we would assume there are proteins encoded for on the ICE that, in turn, contribute to virulence and/or colonization. In fact, other members of the Mobley lab have investigated genes on the ICE that show its importance in virulence in the murine model of ascending UTI. An isogenic mutant of

PMI2575 (*taaP*, a gene important in adhesion and autoagglutination of *P. mirabilis*) showed a fitness defect compared to *P. mirabilis* HI4320 in co-challenge studies in the mouse model of ascending urinary tract infection (Himpsl, Lockatell et al. 2008; Alamuri, Lower et al. 2010). Additionally, PMI2596 was shown to be antigenic and is an important iron-receptor in *P. mirabilis* HI4320 (Nielubowicz, Smith et al. 2008; Himpsl, Pearson et al. 2010). A mutant of this gene displayed a fitness defect in kidney colonization in the murine model. These are only a few of the genes carried on ICE*Pm1*, however, that have been studied.

ICE*Pm1* is 93kb in length and carries 94 ORFs. Twenty-four of these genes are annotated as hypothetical proteins. Thirteen of these hypotheticals do not reside in any of the three conserved modules suggested to be important for ICE-function (PMI2555-PMI2568). They also do not show any homology to the other genomic islands that ICE*Pm1* is similar to, suggesting they are *P. mirabilis*-specific. This region (PMI2555-PMI2568) could encode for an important virulence system or collection of genes specific to *P. mirabilis* that contributes to virulence phenotypes.

The two regions of the ICE that appear unique to ICE*Pm1* and therefore consist of its cargo genes are the region described above (PMI2555-PMI2568) and a region carrying 20 genes, 13 of which are annotated as pseudogenes due to frame shift mutations (PMI2606-PMI2636). This region encodes interesting genes including a degenerate fimbrial operon, an anti-restriction protein and an ornithine decarboxylase, yet all are pseudogenes. Situated in the middle of these pseudogenes are two ton-B dependent receptors, both of which appear to be intact ORFs. These may also play a role in iron-acquisition, an important virulence factor that has been demonstrated for

colonization of the urinary tract (Alteri, Hagan et al. 2009; Himpsl, Pearson et al. 2010; Garcia, Brumbaugh et al. 2011).

Interestingly, the ICE-specific modules of the island show similarities to other PAIs found in human pathogens that have not been reported as ICEs (Figure 5-1). One of these islands, SPI-7 of Salmonella enterica serovar Typhi CT18, has been reported to spontaneously and precisely be lost from the chromosome. This not only suggests this PAI is also an ICE, but also has major implications for efficacy of the typhoid vaccine as the target of the vaccine, the Vi-antigen, is encoded on this island.

The *nrp* iron-acquisition operon, located on ICE*Pm1*, is important for virulence and is similar, but by no means identical to the yersiniabactin iron-acquisition system which is encoded for on the high-pathogenicity island (HPI), well described in *Yersinia spp*. The modules important in ICE-function are most similar to the genomic island YAPI of *Yersinia pseudotuberculosis* 8081. Additionally, the *nrp* operon of ICE*Pm1* shows high similarity to the HPI of *Y. pseudotuberculosis*, yet the synteny is not conserved in *Y. pseudotuberculosis.* This means that the HPI is integrated elsewhere in the *Y. pseudotuberculosis* chromosome, outside of YAPI, while the HPI in *P. mirabilis* has integrated into ICE*Pm1* of *P. mirabilis*. In fact, we found *P. mirabilis* strains that did not harbor the ICE, but were positive for HPI. This is not surprising considering the high frequency of dissemination of the HPI in the *Enterobacteriaceae*. Although its specific integration site is not obvious, it is flanked by two transposases (PMI2595 and PMI2610-2611) suggesting delineation of HPI within ICE*Pm1*. In addition, we identified PMI2608 as the excisionase of the HPI, although it does not show identical similarity, suggesting that the integration of HPI into ICE*Pm1* is an ancient event and

the DNA sequence has evolved to become more similar to that of the surrounding chromosome.

The genes of ICE*Pm1* that we chose to sequence in *P. mirabilis, P. stuartii* and *M. morganii* (PMI2551, PMI2602 and PMI2641) were 100% identical at the nucleotide level between species. This is striking and strongly implicates horizontal gene transfer of this element. Ancient acquisition of these elements tends to result in the DNA being altered and changed to resemble that of their surrounding genome. Comparisons with other integrative and conjugative elements and the modular structure of ICE*Pm1* suggested that this element was indeed an ICE and actively able to excise from the genome and transfer to recipient cells. Therefore we sought to show active ICE*Pm1* excision from the chromosome and subsequent transfer.

ICE*Pm1* is an active ICE that is self-transmissible.

Because of the homology we observed of ICE*Pm1* with other integrative and conjugative elements, in our second study we sought to determine if ICE*Pm1* was able to actively excise and thus transfer to a recipient cell. Our PCR-based assay demonstrated active excision of ICE*Pm1* from the chromosome. We screened 23 *P. mirabilis* isolates to determine a suitable recipient for mating experiments to observe transfer of ICE*Pm1* into ICE-deficient cells. We found ICE*Pm1* to mate most efficiently on LB agar plates. We did not observe any transconjugants formed when matings were conducted in broth cultures despite the knowledge that other ICEs have been shown to mate in broth. This suggests that the mating channel formed by the T4SS of ICE*Pm1* is more rigid and necessitates a solid surface for mating. This is

consistent with the phenotypes observed for other ICE T4SSs (Klockgether, Reva et al. 2004).

The fact that transconjugants are formed only when mating is conducted on a solid surface is interesting in light of the ability of *P. mirabilis* to adhere to and colonize urethral catheters. As described previously, caUTI is often polymicrobial and catheter insertion could provide a solid surface for gene transfer between multiple different species.

Transfer of ICE*Pm1* into clinical strains is novel. In much of the ICE literature, specific lab strains are used as recipients to determine transfer frequencies of these mobile elements. After extensive literature searching through back citations, often, the recipient strain can be unveiled as a restriction modification negative strain among many other mutations that may be in these strains. These strains are classically known for accepting foreign DNA and further recombination into the host chromosome. In fact, this is what we observed with the *E. coli C* strain, which was a kind gift from David Friedman, in our department. He suggested this as "the strain you have to use" to get transfer to occur. In fact, we cannot observe in our *E. coli* C transconjugants whether ICE*Pm1* has integrated into a *phe*-tRNA. Because the sequences flanking the *phe*-tRNAs are divergent between *P. mirabilis* and *E. coli*, our primers will not hybridize. It may be possible to use the annotated K12 sequence to design primers to answer where ICE*Pm1* has integrated into *E. coli* C.

We attempted transfer of ICE*Pm1* into *M. morganii*, *P. stuartii* and *Proteus vulgaris* ICE*Pm1* deficient strains and were not successful. We hypothesize that these strains have conserved *phe*-tRNAs as many other species do, but we do not know for

certain as we do not have annotated sequences of these strains. That *P. stuartii* ATCC25827 is annotated and contains only one *phe*-tRNA, could explain the inability to transfer ICE*Pm1* into this strain.

Additionally, *in silico* analyses of organisms that have PAIs integrated into *phe*-tRNAs commonly show integrase fragments around the tRNAs. This raises the question of whether a chromosomally-encoded integrase in the recipient is important for integration into the chromosome. This has been demonstrated as necessary for the integration for some bacteriophages.

ICE*Pm1* excision is integrase dependent

Excision of ICE*Pm1* from the chromosome is dependent on an integrase encoded on the ICE. Other groups have shown that mitomycin C induces (MC) expression of the integrase and thus induces subsequent transfer frequencies. We did not observe increases in transfer efficiency of ICE*Pm1* after treatment with MC. This could suggest that our ICE is regulated in a different way than the R391/SXT family of ICEs that has been reported. In fact, we found no conditions that affected ICE transfer frequency including mating on high NaCl plates, or temperature. Yet this does not mean that conditions do not exist that can induce transfer frequency of ICE*Pm1*.

The integrases encoded on ICEs fall into two phylogenetic groups. The one encoded on the SXT-like ICE of *P. mirabilis* is more similar to that of the P4 family of integrases. The integrase encoded on ICE*Pm1* is more like that of the XerC/D family of integrases. This may be another mechanism that further divides ICEs into

incompatibility groups/families and therefore they may be regulated in different ways, as we see for the MC result.

ICE*Pm1* encodes a T4SS necessary for self-transmissibility

We were aware of another ICE that was very similar to the prototypical SXT ICE also found within the *P. mirabilis* genome, located about 100 kb upstream from ICE*Pm1*. This ICE also encodes its own T4SS. Therefore, it was questionable whether the T4SS system of the SXT-like ICE could transfer ICE*Pm1 in trans*. We answered this question by not only making mutations in the T4SS of ICE*Pm1*, but also by mutating the relaxase in the T4SS of the SXT-like ICE. Mutation of T4SS genes in ICE*Pm1* decreased transfer frequency showing that the T4SS is necessary for transfer. We also showed wild-type levels of transfer in the SXT relaxase mutant further confirming that the T4SS of SXT is not transferring ICE*Pm1 in trans*.

We are unaware of another organism in the literature that also carries two true ICEs; that is, self-transmissible genomic islands each possessing their own T4SSs. Because ICEs are similar to that of plasmids in some ways, we expect that incompatibility groups and methods for exclusion exist for these ICEs much in the same way that they do for plasmids. It would be interesting to know not only how prevalent SXT is in all of the other *P. mirabilis* strains, but also if it transfers at the same rate and under the same conditions as ICE*Pm1*.

ICE*Pm1* can integrate into either *phe* tRNA gene

That ICE*Pm1* can integrate into either of *phe*-tRNA of *P. mirabilis* is interesting due to the fact that the annotated genome for *P. mirabilis* HI4320 only shows the ICE integrated into *pheV*-tRNA. This has broad implications to all annotated genomes in which mobile elements (specifically ICEs) may be actively moving in and out of the chromosome and forming episomal structures.

The method by which this integration occurs is also interesting because we believe it does not disrupt function of the Phe-tRNA. Thus, these mobile elements have the propensity to integrate into most genomes because of the conserved integration site, yet without disturbing function. Nevertheless, we did not assay for function of this tRNA. Although the conserved tRNA DNA sequence is reconstituted upon integration, we do not know if function is retained for this transfer RNA.

Co-colonization with MRSA and VRE occurs most commonly in wound and rectal sites

We sampled six anatomical sites (nares, orpharynx, groin, rectum and devices and wounds if applicable and found co-colonization to occur most frequently in wounds and the rectum. Wounds are known to be colonized by many species of bacteria, especially upon biofilm formation (Ehrlich, Ahmed et al. 2010). Table 4-3 shows that colonization of the rectum with MRSA occurs at similar rates to nares colonization, the most well described site of MRSA colonization. Co-colonization may occur at this site just by chance, the combined probabilities of MRSA alone or VRE alone being present. On the other hand, in the device and wound sites, co-colonization occurred every time

VRE colonized one of these sites, that is VRE was never present without MRSA also being present in these sites. This suggests that there may be specific risk factors for acquisition of both MRSA and VRE at these sites.

Functional disability is a risk-factor for MRSA/VRE co-colonization

We found functional disability to be associated at the patient-level with MRSA/VRE co-colonization. Functional status has recently become recognized as an important risk factor for acquisition of antibiotic resistant organisms, this is most likely due to decreased immunity and increased healthcare worker contact in these patients (Mody, Bradley et al. 2011). Because of the elevated risk for co-colonization in these patients, it may be important to target patients with functional disability to prevent co-colonization of their wounds and devices with MRSA and VRE.

FUTURE DIRECTIONS

ICE*Pm1* gene knockouts and phenotypic studies

To determine the contribution of the island to virulence and/or colonization a series of knockout mutants could be made in a combination of hypothetical and putative genes in ICE*Pm1*. The region from PMI2555-PMI2568 encodes *P. mirabilis*-specific proteins and thus could contain novel genes important in pathogenesis or colonization. Additionally, genes in this region could be important for metabolism and subsequent fitness within the urinary tract. A closer *in silico* examination of many of these ORFs may identify potential candidates for characterization that were beyond the scope of this study. The region between PMI2606-PMI2636 also should be targeted and particularly mutagenesis of the putative anti-restriction, ornithine decarboxylase and tonB-dependent receptor genes. Phenotypic screening for swarming and virulence attenuation in the mouse model could thus be conducted with these mutants.

Furthermore, one should consider that, although the dogma exists that foreign acquired genes are generally regulated by the surrounding genes that were also horizontally acquired, it may be possible that many of the genes within ICE*Pm1* could be interacting with genes elsewhere in the chromosome.

Model catheter studies

P. mirabilis is a common etiologic agent of catheter-associated UTI. Although transurethral inoculation of *P. mirabilis* into the bladder of mice is successful in our murine model of urinary tract infection, this model does not adequately explain the role

colonization of the catheter by *P. mirabilis* may play in urinary tract infection. Because long-term catheterization of mice in a stable manner is extremely difficult (H. Mobley, personal communication), using catheter colonization studies *ex vivo* may be a useful tool for examining adherence and swarming properties on the catheter. Studies examining adherence abilities to silicon catheters and the ability of *P. mirabilis* to swarm across catheters with and without ICE*Pm1* could be conducted. These studies may show important phenotypes for the contribution ICE*Pm1* has to catheter colonization.

Isogenic deletion mutant of ICE*Pm1* in the *P. mirabilis* HI4320 background

An isogenic deletion of ICE*Pm1* would be desirable for conducting phenotypic studies. We have attempted many different conditions to cure *P. mirabilis* HI4320 of ICE*Pm1* including passage over several days (39), passage in the presence of ethidium bromide, acridine orange, a plasmid over-expressing the integrase, a plasmid over-expressing the potential excisionase, and using an ICE*Pm1* strain with a mutation in the toxin of the toxin-antitoxin system. Many of these conditions have resulted in curing of ICEs from other species, yet we have not observed spontaneous loss of the ICE under any of these conditions.

I have also constructed an ICE*Pm1* mutant that contains *loxP* sites at either end of ICE*Pm1*. Introduction of a plasmid encoding the cre-recombinase and subsequent IPTG-induced expression has also not resulted in artificial deletion of ICE*Pm1*. This technique had been successful for another large region of DNA in *P. mirabilis* HI4320 conducted by another lab member (M Pearson, personal communication). The

difficulty of eliminating this genomic island from the chromosome further strengthens our hypothesis that ICE*Pm1* provides a selective advantage, although unknown, to *P. mirabilis.*

Activating conditions of *in vitro* transfer of ICE*Pm1*

Because ICE*Pm1* carries an iron-acquisition system, transfer under iron-limiting conditions may be a plausible study condition to attempt for increased rates of transfer. Additionally, studies already performed have suggested that *P. mirabilis* has different phenotypes under anaerobic conditions (Alteri and Himpsl, unpublished), thus this may also be another condition to try transfer.

I have created a pLux-*int* plasmid that will luminesce when transcription of the integrase of ICE*Pm1* is expressed. We could therefore use *P. mirabilis* containing this plasmid to screen for other compounds to see if there are other potential compounds that increase expression. Previous studies have shown that antibiotics such as ciprofloxacin induce integrase expression and subsequent transfer of ICEs.

Additionally, because MC does not induce transfer of ICE*Pm1*, it would be interesting to know whether it has an effect on SXT-like ICE. This would tell us whether the SOS response induction observed for the SXT/R391 family is specific to this family of ICEs, or if MC induction is not effective in *P. mirabilis*. It is also possible that *P. mirabilis* has a higher tolerance for MC as it does for other compounds due to the efflux pumps it carries. Perhaps a higher concentration than that reported in the literature is necessary for proper induction. Although we did do a MIC against MC

and attempted to use sub-lethal concentrations of this compound, this could be further characterized.

Furthermore, understanding the rates of transfer of the SXT-like ICE would be important. These studies could be conducted in the same manner as the mating assays for ICE*Pm1*, although it is known that our *P. mirabilis* recipient strain 378L and 523L already carry SXT-like ICE. It will be interesting to know if these ICEs transfer at the same frequencies and under the same conditions.

Transfer of ICE*Pm1* to other species

Although our attempted mating experiments between *P. mirabilis* and *M. morganii*, *P. stuartii* and *Proteus vulgaris* were unsuccessful, we believe these transfer events should be possible. We were limited in our studies to very few strains that had proper innate antibiotic susceptibility and resistance patterns that were compatible with our mating assay. For instance, we only found two strains of *P. stuartii* that were ICE-deficient, susceptible to kanamycin and resistant to an antibiotic that *P. mirabilis* HI4320 is susceptible to. It is possible that we could screen other isolates to determine additional possible recipient strains.

Furthermore, we are not certain that the recipient strains possess the correct *attB* sequence needed for ICE*Pm1* integration. We expect that these organisms will possess Phe-tRNA with the same anticodons as *P. mirabilis* HI4320, but it is difficult to know without genome sequences of these organisms. For one of the *P. stuartii* recipient strains which mating was attempted, we do have the annotated genome sequence (ATCC25827). In this isolate, there is only one *phe*-tRNA gene present. This may be

why transfer was unsuccessful in this isolate. It would be interesting to know if the other recipients only possess one *phe*-tRNA gene and if this is a barrier to transfer. Southern hybridizations could be conducted to determine if the same *phe*-tRNA sequence is present in these organisms as well as if they possess more than one *phe*-tRNA.

In vivo transfer of ICE*Pm1* in the mouse bladder.

The mobile element ICE*Pm1* was present in several different species of bacteria that cause caUTI, which is often polymicrobial. We were interested in demonstrating *in vivo* transfer of this element in the murine model of ascending UTI. We conducted co-colonization experiments where both ICE*Pm1*::kan and the recipient strain 378L were inoculated into the bladders of CBAJ mice. After 48hours of incubation, we did not observe any transconjugants that had formed.

Failure to observe transconjugants could have happened for numerous reasons. Population density is an important factor in activating the transfer of ICEs (Auchtung, Lee et al. 2007; Wozniak and Waldor 2010). We do not see bacterial loads as high as 1 $x10^8$ cfu/ml in mouse bladders as we can obtain with mating experiments on agar. We also know that proximity is important for HGT to occur. It would be interesting to mark both strains with immunofluorescence, inoculate into the bladder, and then look at cryogenic cross-sections to determine where these organisms are colonizing. If each strain occupies its own niche and mixing of the two species in the bladder does not occur, it would be impossible to demonstrate transfer.

Identification of other ICEs

The PCR assay we used to identify active excision of ICE*Pm1* is simple to develop and conduct. I would hypothesize that many other classical PAIs are actually active ICEs that are moving in and out of the chromosome. For example, the PAP PAIs of CFT073, which are integrated into the *phe*-tRNA genes, both harbor P4-type integrases and are flanked by DRs. Therefore I would suggest that these genomic islands are also ICEs that can dynamically move in and out of the chromosome and be transferred to other cells.

***pheU* and *pheV* are hotspots for foreign DNA integration**

It is unclear why *phe* tRNAs are hotspots of DNA integration. Many identified PAIs and other mobile elements are integrated into *phe* tRNAs. Further studies to understand why these genes are preferred for integration could be conducted. It would first be important to determine if function of these tRNAs is maintained after integration although their sequence is maintained. Secondly, disruption of one of the two *phe* tRNAs within a genome may give clues to if both tRNAs are necessary for cell viability.

The annotated genome sequence *P. mirabilis* HI4320 does not show integration of ICE*Pm1* into the *pheU* tRNA. It would be interesting to contact Sanger Center, which performed the sequencing and alignment of the genome to see if they observed sequencing reads where this conformation of the ICE was present. Because it would be a small portion of the total number of reads, it may have been disregarded when

aligning the contigs. This may have implications for the direct-sequencing of subsequent full genomes.

Genomic location as a level gene regulation

It would be interesting to know if the level of transcription of genes on ICEs is the same regardless of the conformation of the ICE. Nearby co-factors could be important for gene regulation depending on which *phe*-tRNA gene the ICE is integrated into. Additionally, circularization of the ICE results in activation of genes at the opposite ends of the ICE because promoters have been brought into proximity (Qiu, Gurkar et al. 2006). It would be interesting to do transcriptional microarray experiments with the island "locked in" strain as well as if we could find a strain with the island only integrated into the *pheU* tRNA. This may reveal that genome location could be another level of regulation of genes.

Co-colonization with MSSA and VRE.

In the prospective study that sampled 6 anatomical sites, we never saw co-colonization with methicillin-sensitive *S. aureus* and VRE. VRE is known to have a fitness disadvantage compared to that of vancomycin-susceptible Enterococci (Foucault, Depardieu et al. 2010). It is very interesting in the study that we conducted that we did not ever see co-colonization with MSSA and VRE. This has similar implications because it has also been reported that MRSA is less fit than MSSA. Thus, colonization with MSSA may actually be protective against co-colonization with VRE.

In vitro transfer studies of *vanA* from VRE to MRSA.

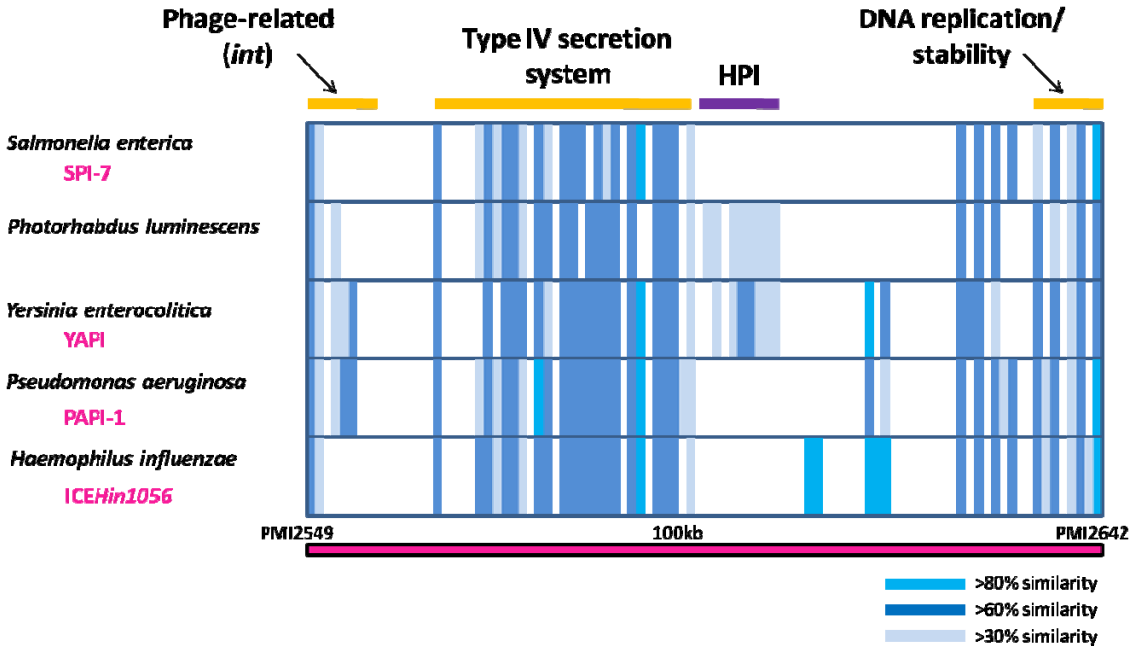
Much like the ICE transfer studies that were conducted, we could monitor the transfer of vancomycin resistance from VRE to MRSA. Mating assays are established and could be performed in the same way as the ICE transfer assays. We could then study different environmental conditions and genotypes that make transfer between these organisms possible. By screening several strains we could look at other important genes encoded in the genomes of the MRSA recipients, such as restriction modification systems, which can be barriers to transfer. This may partially explain the emergence of only 11 VRSA isolates in the past 9 years. Additionally, transfer frequencies with VRE strains possessing the Inc18-like plasmids could be assayed. These plasmids are suggested to transfer to MRSA at higher frequencies than other types of plasmids and this could be determined experimentally.

Will VRSA become endemic in healthcare facilities?

No VRSA secondary transmission events from case patients have been reported. I think it would be naïve to assume that this will not happen. Failure to transmit could be a result of the fitness of the organism or good infection control could limit its spread. Regardless, considering our history with containing the emergence of antibiotic resistant organisms I would not be surprised if VRSA did become endemic in healthcare facilities. The endemicity observed today with MRSA resulted from the introduction and subsequent outbreaks of a few specific MRSA clones. That is why it is crucial to understand mechanisms leading to VRSA emergence to try and prevent dissemination of the *vanA* gene cluster to MRSA strains.

Figure 5-1. Conserved modular structure of all ICE*Pm1* related PAIs.

Conserved modular structure of all ICE*Pm1* related PAIs. Percent similarity of each other pathogenicity island ORF to the ICE*Pm1* ORF is shown in blue shading. Modular structure of the PAIs is labeled on the x-axis above the figure.



APPENDICES

APPENDIX A

MRSA/VRE Co-colonization study codebooks

Table A-1. Clinical Data codebook

Clinical data codebook for patient-level data analysis. Data file = "clinical data 1.2.11.xls"

Label	Variable
Study_ID	Resident ID number
Day	days since enrollment, multiples of 30. (range from 0-360)
month	calendar month of the visit
year	calendar year of the visit
Visits	total visits for that resident (range 1- 12)
Device	0 = control, 1 = urinary catheter, 2 = feeding tube, 3 = both
age	in years
sex	0 = male, 1 = female
admission	days of admission in the facility
total admit	day of admission + days in the study
chco	charlson's comorbidity score
PSMS	Physical and self-maintenance score
Hosp	Previous hospitalization in the last 30 days. 1 = yes, 0 = no
Wound	Previous wound in the last 30 days. 1 = yes, 0= no
Pressure	Previous pressure ulcer in the last 30 days. 1 = yes, 0= no
woundpress	Previous wound OR pressure ulcer in the last 30 days. 1 = yes, 0= no
Infection	Previous infection in the last 30 days. 1 = yes, 0 = no.
Inf_type	Type of infection. Infection codes match the original study form.
sitecc	Simultaneous co-colonization at the same site. 1 = yes, 0 = no.
daycc	Co-colonization within the patient at that visit (at any combination of sites). 1 = yes, = no.
COCOL_EVER	Resident was ever co-colonized, over their entire enrollment. 1 = yes, 0 = no.
MRSA	Resident was colonized with MRSA at that study visit (at any site). 1 = yes, 0 = no.
MRSA_EVER	Resident was ever colonized with MRSA, over their entire enrollment. 1 = yes, 0 = no.
MSSA	Resident was colonized with MSSA at that study visit (at any site). 1 = yes, 0 = no.
MSSA_EVER	Resident was ever colonized with MSSA, over their entire enrollment. 1 = yes, 0 = no.

VRE	Resident was colonized with VRE at that study visit (at any site). 1 = yes, 0 = no.
VRE_EVER	Resident was ever colonized with VRE, over their entire enrollment. 1 = yes, 0 = no.

Table A-2. Organism log code book.

Codebook for organism log. Data file "devices SAS file 11.9.10.xls".

Label	Variable
ID	resident ID #
device	0 = control, 1 = urinary catheter, 2 = feeding tube, 3 = both UC and FT
Day	day visit was conducted (range 0-360)
site	1 = nares, 2 = oropharynx, 3 = groin, 4 = rectum, 5 = device, 6 = wound
cultured	1 = this site was cultured, 0 = this site was not cultured, although there was a visit, 88 = this site does not exist (i.e. resident does not have a device or wound), '.' = missing, we do not know if this resident has a wound because the data is missing
abx1	was the resident on antibiotics at this visit? There is no order to the abx listings, 1 = yes, 0 = no.
abx2	was the resident on antibiotics at this visit? There is no order to the abx listings, 1 = yes, 0 = no.
abx3	was the resident on antibiotics at this visit? There is no order to the abx listings, 1 = yes, 0 = no.
abx4	was the resident on antibiotics at this visit? There is no order to the abx listings, 1 = yes, 0 = no.
gnb1	0 = no organism cultured, 1-43 = organism cultured, see species code, 88 = not applicable (was not cultured)
gnb1ID	freezer code ID for the organism
gnb1CeftR	0 = organism was ceftazidine sensitive, 1 = organism was ceftazidine resistant, 88 = no organism cultured, '.' = missing data
gnb1CipR	0 = organism was ciprofloxacin sensitive, 1 = organism was ciprofloxacin resistant, 88 = no organism cultured, '.' = missing data
	*gnb2, gnb3 and gnb4 follow same as for gnb1. There is no order to gnb1-4.
sa1	0 = no staphylococcus aureus cultured, 1 = staphylococcus aureus cultured, 88 = site not cultured
sa1ID	freezer code ID for the organism
sa1R	0 = s. aureus sensitive to methicillin, 1 = organism methicillin resistant
VRE	0 = VRE not cultured, 1 = VRE cultured from site, 88 = site not cultured
VREID	freezer code ID for the organism

APPENDIX B

SAS Code For MRSA/VRE Co-Colonization Study

Data management for patient-level data analysis with dataset “clinical data 1.2.11.xls”

```
proc import out = work.clinical
      DATAFILE= "M:\Dissertation\clinical data 1.2.11.xls"
      DBMS= XLS REPLACE;
      GETNAMES = YES;
      RUN;


DATA  RECODE;
SET CLINICAL;

/*MAKE THESE VARIABLES NUMERIC BECAUSE THEIR FIRST OBS IS MISSING AND
SAS MAKES THEM CHARACTER*/
      ADMIT = ADMISSION *1;
      HOSPITAL = HOSP *1;
      IF HOSPITAL = 0 THEN HOSPITAL = 2;
      INFECT = INFECTION *1;
      PSMSCONT= PSMS*1;
      WOUNDGRP = WOUNDPRESS*1;
      BEADIES = DIABETES*1;

/*Recode of device status into cohorts*/
IF device = 0 THEN cohort = 'control';
            ELSE IF device = 1 THEN cohort = 'device';
            ELSE IF device = 2 THEN cohort = 'device';
            ELSE IF device = 3 THEN cohort = 'device';

/*Recode of age into Age groups*/
/*Dichotomize by median*/
IF AGE <= 84 THEN AGEGRPMED = 1;
IF AGE >84 THEN AGEGRPMED= 2;

/*Dichotomize by 65*/
IF AGE <= 65 THEN AGEGRP65 = 1;
IF AGE >65 THEN AGEGRP65 = 2;

/*4 categories*/
IF AGE <= 65 THEN AGECAT = 1;
IF AGE > 65 AND AGE <=75 THEN AGECAT = 2;
IF AGE > 75 AND AGE <=85 THEN AGECAT = 3;
IF AGE > 85 THEN AGECAT = 4;
```

```
/*Recode of admission time into categories*/
ADMITMOS = ADMIT/30;
IF ADMITMOS NOT = . THEN DO;
      IF ADMITMOS <= 4 THEN ADMITGRP = 1;
      IF ADMITMOS >4 AND ADMITMOS <=18.6 THEN ADMITGRP = 2;
      IF ADMITMOS >18.6 AND ADMITMOS <= 41.5 THEN ADMITGRP = 3;
      IF ADMITMOS >41.5 THEN ADMITGRP = 4;
END;

IF ADMITMOS NOT = . THEN DO;
      IF ADMITMOS <=18.6 THEN ADMITGRP2 = 1;
      IF ADMITMOS >18.6 THEN ADMITGRP2 = 2;
END;

IF ADMITMOS NOT = . THEN DO;
      IF ADMITMOS <= 3 THEN ADMITGRP3 = 1;
      IF ADMITMOS >3 THEN ADMITGRP3 = 2;
END;

/*Recode CCS into categories*/

IF CHCO <= 3 THEN CCSGRP = 1;
IF CHCO >3 THEN CCSGRP = 2;

IF CHCO <= 2 THEN CCSMED = 1;
IF CHCO >2 THEN CCSMED = 2;

/*Recode of follow-up time into categories*/

IF VISITS <=3 THEN VISITGRP =1;
IF VISITS >3 THEN VISITGRP = 2;


/*Recoding PSMS into categorical variable*/
If PSMS not = . then do;
      If PSMS <= 12 then PSMSgroup = 1;
      If PSMS >12 and PSMS <= 17 then PSMSgroup = 2;
      If PSMS >17 and PSMS <= 23 then PSMSgroup = 3;
      If PSMS > 23 and PSMS <= 30 then PSMSgroup =4;
      END;

IF PSMS NOT = . THEN DO;
      IF PSMS <= 21 THEN PSMSMED = 1;
      IF PSMS >21 THEN PSMSMED = 2;
      END;

IF PSMS NOT = . THEN DO;
      IF PSMS <=23 THEN PSMSGRP2 = 1;
      IF PSMS >23 THEN PSMSGRP2 = 2;
      END;

/*Creating variable for prior antibiotic use in the previous 30 days
(ABX)*/
IF ceftriaxone OR ampsul OR aug OR trisulf OR nitro OR
cefuroxime_axetil OR azith OR piper
      OR sulf OR dexato OR cefpodoxime OR amoxclav OR levo OR
cephalexin OR moxi OR amox OR
```

```
      cipro OR tobra OR doxy OR line OR clind OR cefaclor OR tetra OR
imipen OR norflox OR
      claforan OR ceftazidine OR cefotaxime OR cefazolin OR pipertaz
OR amp OR gent OR tige OR
      clarith OR metro OR vanco OR cefepime OR erythr OR tri OR flu OR
oflox OR erta OR trolea
      OR cefuroxine_sodium OR baci OR cefditoren NOT = .  THEN DO;
IF ceftriaxone = 0 and ampsul = 0 and aug = 0 and trisulf = 0 and
nitro = 0 and
      cefuroxime_axetil = 0 and azith = 0 and piper = 0 and sulf = 0
and dexato = 0 and
      cefpodoxime = 0 and amoxclav = 0 and levo = 0 and cephalexin = 0
and moxi = 0 and
      amox = 0 and cipro = tobra = 0 and moxi = 0 and line = 0 and
clind = 0 and cefaclor = 0
      and tetra = 0 and imipen = 0 and norflox = 0 and claforan = 0
and ceftazidine = 0 and
      cefotaxime = 0 and cefazolin = 0 and pipertaz = 0 and amp = 0
and gent = 0 and tige = 0
      and clarith = 0 and metro = 0 and vanco = 0 and cefepime = 0 and
erythr = 0 and tri = 0
      and flu = 0 and oflox = 0 and erta = 0 and trolea = 0 and
cefuroxine_sodium = 0 and
      baci = 0 and cefditoren= 0  THEN ABX = 0;
            ELSE ABX = 1;
END;

IF MRSA = 1 AND VRE = 1 THEN NOCOL = 1;
IF MRSA = 0 AND VRE = 0 THEN NOCOL = 0;
IF MRSA = 1 AND VRE = 0 THEN NOCOL = 2;
IF MRSA = 0 AND VRE = 1 THEN NOCOL = 2;

RUN;
```

The following code was used for the univariate analysis of associations between MRSA/VRE co-colonization and clinical and demographic variables. We accounted for the repeated measures design using Generalized Estimating Equations and estimated the rate ratios of colonization with robust error variances. One example is given for each analyses. The variables were altered to give the associations for each covariate of interest. The same model was used for the multivariate analysis adding covariates in a step-wise manner.

Among all residents in the study (Table 4-2 (No co-colonization)).

```
PROC GENMOD DATA = RECODE DESCENDING;
      WHERE COHORT = 'device';
      CLASS STUDY_ID SEX (REF = '1') / PARAM = REFERENCE;
      MODEL DAYCC = SEX/ DIST = POISSON LINK = LOG;
      REPEATED SUBJECT = STUDY_ID / TYPE = CS CORRW;
      ESTIMATE 'MALE V. FEMALE' SEX 1/EXP;
      RUN;
```

Among all residents colonized with MRSA (Table 4-2 (MRSA only).

```
PROC GENMOD DATA = RECODE DESCENDING;
      WHERE MRSA = 1 AND COHORT = 'device';
      CLASS STUDY_ID SEX (REF = '1') / PARAM = REFERENCE;
      MODEL DAYCC = SEX/ DIST = POISSON LINK = LOG;
      REPEATED SUBJECT = STUDY_ID / TYPE = CS CORRW;
      ESTIMATE 'MALE V. FEMALE' SEX 1/EXP;
      RUN;
```

Among all residents colonized with VRE (Table 4-2 (VRE only).

```
PROC GENMOD DATA = RECODE DESCENDING;
      WHERE VRE = 1 AND COHORT = 'device';
      CLASS STUDY_ID SEX (REF = '1') / PARAM = REFERENCE;
      MODEL DAYCC = SEX/ DIST = POISSON LINK = LOG;
      REPEATED SUBJECT = STUDY_ID / TYPE = CS CORRW;
      ESTIMATE 'MALE V. FEMALE' SEX 1/EXP;
      RUN;
```

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