PHEROMONE-INTERACTING REPLICATION PROTEIN CONTROLS ENTEROCOCCAL CONJUGATIVE PLASMID HOST RANGE AND STABILITY THROUGH DISULFIDE BONDS

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ABSTRACT

Pheromone-interacting replication protein controls enterococcal conjugative plasmid host-range and stability through disulfide bonds

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Enterococci are found in soil, sewage, food, water, and are commensal to the gastrointestinal tracts of mammals, insects, and birds. Enterococci often become nosocomial pathogens that cause a wide variety of diseases including urinary tract infections, endocarditis, and septicemia. These infections are often difficult to treat with antibiotics because most of the nosocomial strains are multi-drug resistant. Enterococcal plasmids function as reservoirs for resistance genes because they are extremely stable, allow for specific and efficient transfer, and can acquire resistance determinants from the chromosome and other plasmids. Additionally, enterococcal plasmids transfer across species boundaries transferring resistance genes like vancomycin to species like *Staphylococcus aureus*. There are two types of enterococcal plasmids, pheromone-responsive and broad host range. Pheromone-responsive plasmids are extremely stable, have a limited host range, and are primarily found in *Enterococcus faecalis*. Broad host range plasmids of *E. faecalis* and *Enterococcus faecium* are less stable than pheromone-responsive plasmids, but have an expanded host range into other Gram-positive species.

E. faecalis has at least 25 known pheromone-responsive conjugative plasmids. One of the most extensively studied pheromone-responsive conjugative plasmids, pCF10.

Conjugation of pCF10 from donor to recipient cell is induced by pheromone cCF10. cCF10 is contained within n the lipoprotein signal sequence encoded by the *E. faecalis* chromosomal gene *ccfA*. The lipoprotein signal sequence is processed by a series of proteolytic cleavage events to produce mature cCF10. Maturation of pheromone cCF10 produces three peptides: pre-cCF10 ($CcfA_{1-22}$), cCF10 ($CcfA_{13-19}$), and $CcfA_{1-12}$. Cells containing pCF10 continue to produce cell membrane associated precursor pheromone of cCF10 (pre-cCF10), as well as, secreted and cell wall-associated cCF10. The presence of cCF10 does not self-induce conjugation by the donor cell because of two inhibitory molecules, PrgY and iCF10. Transmembrane protein PrgY is encoded by pCF10 and reduces cell wall associated cCF10, iCF10 is a pCF10 encoded inhibitory peptide (AITLIFI) that binds to PrgX, preventing cCF10 binding.

While cCF10 controls pCF10 conjugation, pre-cCF10 controls host range of pCF10 by interacting with pCF10 replication initiation protein PrgW. cCF10 can initiate conjugation and mobilize the transfer of plasmids into other species, including *Lactococcus lactis*, but pCF10 cannot be maintained within the cell. However, if *L. lactis* is engineered to produce pre-cCF10, pCF10 can be maintained. The pre-cCF10 involvement in the establishment of pCF10 into other species might be related to the observation that it binds to the pCF10 replication initiation protein PrgW.

By in vitro affinity chromatography experiments, interaction of cCF10 and pre-cCF10 with PrgW induced changes in PrgW mobility in gel electrophoresis that caused by formation of doublets and formation of aggregates which were thought to be mediated by disulfide bonds. Initial evidence of regulation of PrgW conformation by disulfide bonds was seen in Western blots of *E. faecalis* whole cell lysates where PrgW migration

is sensitive to reduction. Sequence alignment comparisons between PrgW and a group of 54 of 59 known RepA_N superfamily proteins in *E. faecalis* revealed three highly conserved cysteines; these RepA_N proteins had a limited host range to *E. faecalis*.

To study the importance of theses cysteines in pCF10 maintenance and host range limitation, *prgW* single, double, and triple cysteine to alanine (C to A) substitutions were generated. The cysteine mutant *prgW* was cloned into a plasmid functioning as either a contained the *prgW* alone (pORI10), or containing *prgW* with genes necessary for efficient pCF10 maintenance (pMSP6050). While all cysteine mutant plasmids of pORI10 and pMSP6050 were still capable of replicating in *E. faecalis*, the plasmid stability and copy number decreased, providing evidence that the cysteines were important to PrgW function. Additionally, Western blot analysis revealed PrgW C to A substitutions decreased PrgW aggregation.

Mutations of PrgW cysteines reduced pMSP6050 stability and aggregation, but increased host range to *L. lactis*. Both *L. lactis* engineered to produce pre-cCF10 and the mutation of the conserved cysteines of PrgW extended host range of pMSP6050 into *L. lactis.* These data taken together with the observations that pre-cCF10 induced PrgW aggregation suggested that pre-cCF10 regulated the activity of the PrgW replication initiation protein through disulfide bonds. While the conserved cysteines of RepA_N proteins are found only in *E. faecalis*, phylogenetic analysis revealed that RepA_N homologs lacking the three cysteines are also found in *E. faecium* or *S. aureus*, suggesting that the host range of multiple plasmids might be affected by cysteine bond formation.

Phylogenetic analysis also showed that the RepA_N proteins of enterococci and staphylococci appear to have evolved to determine host range based on the presence of two of the three conserved cysteines. Modular evolution of *E. faecalis* plasmids, like pCF10, that contained RepA_N proteins with three conserved cysteines, might have determined the fate of the plasmid as a limited host range, stable reservoir for antibiotic resistance.

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LIST OF ABBREVIATIONS

βME	beta-mercaptoethanol
µg	microgram
µl	microliter
µM	micromolar
AA	amino acid
AS	aggregation substance
ATP	adenosine-5'-triphosphate
bp	base pairs
BSA	bovine serum albumin
cCF10	pCF10 pheromone; $CcfA_{13-19}$
dH_2O	de-ionized water
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNAP	DNA polymerase
dNTP	deoxynucleotide triphosphate
DR	direct repeats
dsDNA	double-stranded DNA
DTT	dithiothreitol
EDTA	ethylenediamine tetra-acetic acid
EB	elution buffer
ELISA	Enzyme-linked immunosorbent assay

EtBr	ethidium bromide
EtOH	ethyl alcohol
GM-17	M-17 with glucose
GTP	guanosine-5'-triphosphate
IRS	inverted repeat structure
kb	kilobase pairs
L	liter
LB	Lysogeny Broth
M	molar
mL	milliliter
mM	millimolar
MOPS	3-(N-Morpholino)propanesulfonic acid
ori	origin of replication
oriC	origin of chromosomal replication
oriR	origin of replication (plasmid)
oriT	origin of transfer
oriV	origin of vegetative replication (plasmid)
PBP	penicillin binding protein
pCF10	pheromone-responsive plasmid from *E. faecalis* SF-7
PCR	polymerase chain reaction
PEG	polyethylene glycol
P_Q	*prgQ* promoter
pre-cCF10	cCF10 precursor; $CcfA_{1-22}$
PSK	post-segregational killing

Q_A	*prgQ* antisense
RGD	arginine-glycine-aspartic acid
RNA	ribonucleic acid
RNAP	RNA polymerase
rRNA	ribosomal ribonucleic acid
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
ssDNA	single-stranded DNA
T4S	type-IV secretion
TAE	tris-acetate-EDTA
TB	Terrific Broth
TBS	tris buffered saline
TH	Todd Hewitt
THG	Todd Hewitt Glycine
THS	Todd Hewitt Sucrose
Tn	Transposon
tris	tris(hydroxymethyl)aminomethane
TRX	thioredoxin
Tween	Polyoxyethylene sorbitan monostearate
VRE	vancomycin-resistant enterococci
VRSA	vancomycin-resistant *Staphylococcus aureus*

CHAPTER 1

INTRODUCTION

Enterococci

Enterococci are Gram-positive cocci, lactic acid producing bacteria of the phylum Firmicutes that often occur in diplococcal pairs and short chains. They are facultative anaerobes that respire in both oxygen-deprived and oxygen rich environments. The hearty nature of enterococci and their ability to tolerate a wide-range of oxygen levels allows enterococci to survive and grow in a variety of environments including: soil, sewage, food, water, and the gastrointestinal tracts of mammals, insects, and birds (Hancock and Gilmore, 2006; Tannock and Cook, 2002).

Enterococci make up less than one percent of the total intestinal flora of the human large bowel (Tannock and Cook, 2002). There are at least 27 *Enterococcus* species (Facklam *et al.*, 2002). *Enterococcus faecalis* and *Enterococcus faecium* are two of the most commonly studied. Enterococci do not form spores. They can grow at high NaCl concentrations (6.5 percent), they grow in a temperature range of 10 to 45^{o}C (tolerate 30 minutes at 60^{o}C), and can survive in a pH range of 4.5-10.0. Enterococci have been identified by their ability to hydrolyze esculin in the presence of 40 percent bile and ability to produce pyrrolidonyl arylamidase (Facklam *et al.*, 1982; Facklam *et al.*, 2002; Fisher and Phillips, 2009; McCormick *et al.*, 2000; Ruoff, 1990; Sherman, 1937). Enterococci were once considered group D streptococci based on the Lancefield classification. However, they are now assigned to their own genus, *Enterococcus,* based on DNA-DNA and DNA-RNA hybridization studies (Schleifer and Kilpper-Bälz, 1984).

Additional oligonucleotide cataloging of the 16s rRNA validated the genus change and further defined the genus (Ludwig *et al.*, 1985). These studies confirmed the original proposal to create a new *Enterococcus* genus by Kalina in 1970 (Kalina, 1970; Ruoff, 1990).

While enterococci are typically considered commensal bacteria, they have emerged over the last three decades as one of the leading causes of hospital acquired infections. A recent study by Hidron showed that of 33,848 pathogenic isolates from hospital patients involving bacteria and fungi, 12 percent were enterococci (Hidron *et al.*, 2008). Nosocomial infections by enterococci include urinary infections, catheter-related infections, wound infections, endocarditis, bacteremia, septicemia, intra-abdominal infections, diverticulitis, meningitis, and pelvic infections (Fisher and Phillips, 2009; Tannock and Cook, 2002). The ability to colonize and form biofilms is an important determinant of infections involving enterococci (Chuang-Smith *et al.*, 2010). In addition, enterococci have intrinsic characteristics that provide a natural-resistance to antibiotics including β-lactam-based antibiotics and many aminoglycosides (Kak and Chow, 2002). Moreover, antibiotic resistance and virulence factors encoded on mobile genetic elements make enterococcal nosocomial infections difficult to treat (Tannock and Cook, 2002).

Pheromone Responsive Conjugative Plasmids

E. faecalis and *E. faecium* are the most common enterococcal isolates from patients with hospital-acquired infections. Some recent enterococcal isolates were resistant to vancomycin, an antibiotic previously considered to be the "last line of defense" in the treatment of multi-resistant enterococcal strains (Hidron *et al.*, 2008).

Enterococcal plasmids and transposons facilitate the spread of resistance genes and virulence factors. There are two categories of plasmids in enterococci, pheromone-response plasmids and broad host range plasmids (Palmer *et al.*, 2010). Broad host range plasmids will be discussed later. Pheromone-responsive plasmids, members of the enterococcal *rep9* plasmid group, are the focus of this study. The best characterized pheromone-responsive conjugative plasmids are pCF10, pAD1, pPD1, pTEF1, pTEF2, pJH2, and pAM373 (Clewell, 2007; Dunny, 2007; Jensen *et al.*, 2010). Pheromone-responsive plasmids are characterized by the ability of the plasmid-containing (donor) cells to form visible mating aggregates when incubated with plasmid-free (recipient) cells in liquid culture (Dunny *et al.*, 1978).

Mating aggregates facilitate the transfer of pheromone-responsive plasmids from donor to recipient cell. Most of the molecular characterization of pheromone-responsive conjugation has been done using pAD1 or pCF10 and their response to their respective pheromones, cAD1 and cCF10 (Clewell, 2007; Dunny, 2007; Jensen *et al.*, 2010). Five major regions of pCF10 (Figure 1) associated with plasmid function have been characterized. Those regions are involved in plasmid maintenance (PM), regulation of conjugation in response to pheromone (PR), formation of mating aggregates and virulence (SP), conjugative transfer/ DNA processing (DP), and the transposon encoding tetracycline resistance (Tn*925*). Genes responding to pheromone induction include *prgX* and *prgQ*, which tightly control the induction of genes downstream of the *prgQ* promoter. Genes responsive to pheromone induction include genes encoding surface proteins (*prgA, prgB,* and *prgC*), components of T4S machinery (*pcfC*), and DNA processing.

The plasmid maintenance region encodes the replication initiation protein of the plasmid (*prgW*), genes involved with plasmid partitioning (*prgO* and *prgP*), pheromone-receptor (*prgZ*), an inactive post-segregation kill system (*par*), and several genes of unknown function important to plasmid maintenance (*prgN*, *uvrA*, *uvaE*, and *uvaF*). Transcription of maintenance genes, *prgN*, *prgO*, *prgP*, *prgW*, *prgZ*, and *prgY* is not affected by pheromone. Interestingly, BLASTP search analysis and conserved domain identification suggests *uvrA* as a putative error prone polymerase. The PM region of pCF10 is a stable replicon, however, it is not known if *prgN*, *urvA*, *uvaE*, or *uvaF* affect plasmid stability (Buttaro *et al.*, 2000; Chandler *et al.*, 2005a; Hedberg *et al.*, 1996; Hirt *et al.*, 2005; Izquierdo, unpublished observation; Leonard *et al.*, 1996; Meloni *et al.*, Submitted).

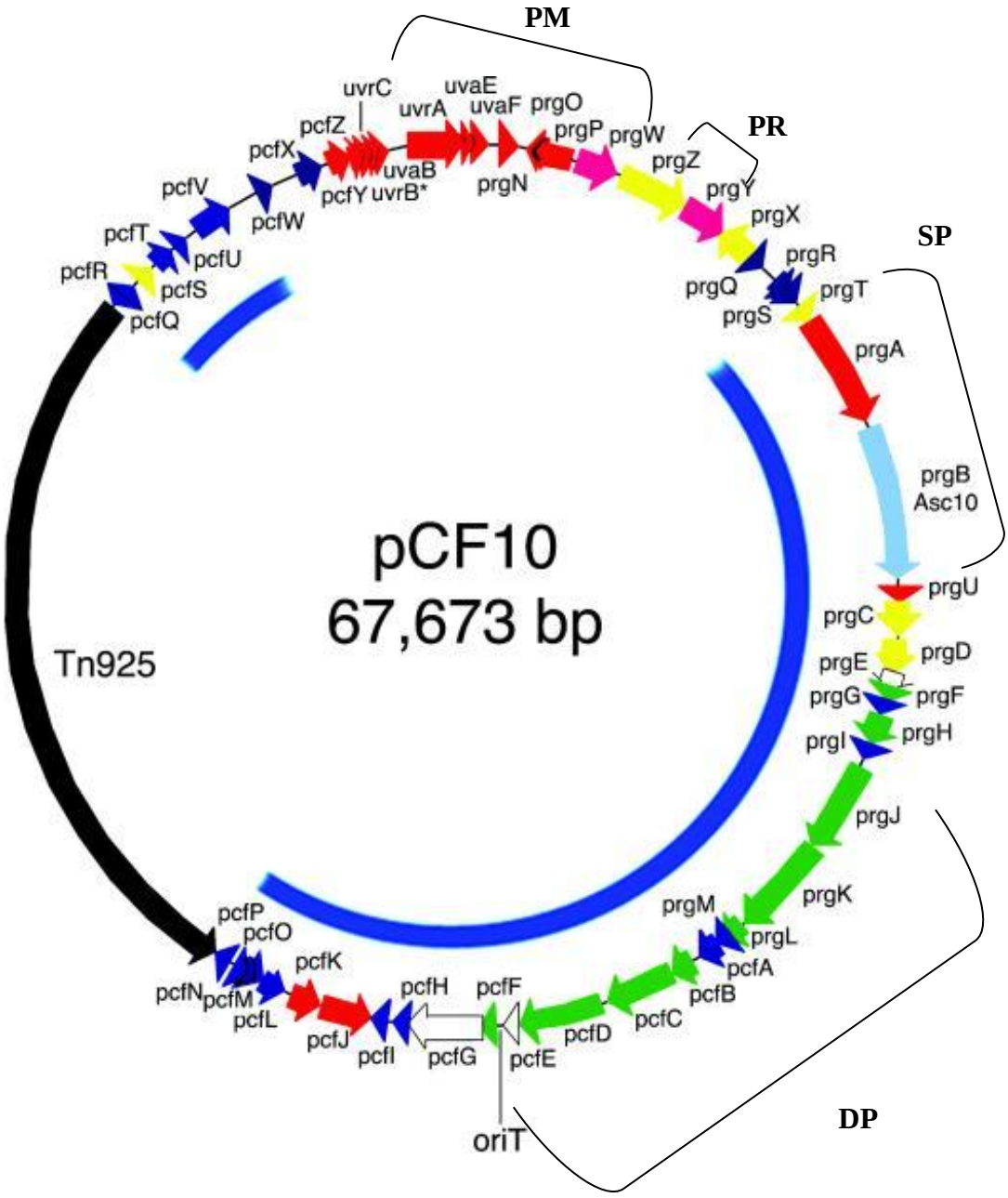


Figure 1: Pheromone-responsive Plasmid pCF10 (Hirt *et al.*, 2005).

pCF10 encodes regions involved with plasmid maintenance (PM), regulating conjugation in response to pheromone (PR), surface proteins involved with conjugation and virulence (SP), conjugation machinery and DNA processing (DP), and a transposon (TN*925*) encoding tetracycline resistance (Tn925).

cCF10 Production

Conjugative transfer of pCF10 from donor to recipient cell is induced by the peptide pheromone cCF10 (Dunny *et al.*, 1981; Figure 2A). cCF10 (LVTLVFV) is contained in the lipoprotein signal sequence encoded by the *E. faecalis* chromosomal gene *ccfA* (Antiporta and Dunny, 2002; Mori *et al.*, 1988). CcfA is a lipoprotein whose function is currently unknown (Antiporta and Dunny, 2002; Urban, 2009). Mutational knockout of *ccfA* or only the signal sequence encoded in *ccfA* is lethal to *E. faecalis* (Izquierdo, unpublished observation). CcfA lipoprotein has homology to *E. coli* YidC, *B. subtilis* SpoIIIJ, and Campylobacteraceae OxaA. YidC, SpoIIIJ, and OxaA are cytoplasmic-membrane translocases (Miller *et al.*, 2007; Samuelson *et al.*, 2000; Tjalsma *et al.*, 2003).

The CcfA lipoprotein signal sequence undergoes a series of proteolytic cleavages by signal peptidase II, Eep endoprotease, and possibly an unknown exoprotease that result in production of mature cCF10. This process produces three peptides pre-cCF10 ($CcfA_{1-22}$), cCF10 ($CcfA_{13-19}$), and $CcfA_{1-12}$ (An *et al.*, 1999; Antiporta and Dunny, 2002). cCF10 is extremely hydrophobic although cells secrete approximately 10^{-11} to 10^{-12} M cCF10 into the supernatant (Nakayama *et al.*, 1994). cCF10 is predominantly found associated with the cell wall of the recipient cells and sequestered at the membrane of the donor cells (Buttaro *et al.*, 2000).

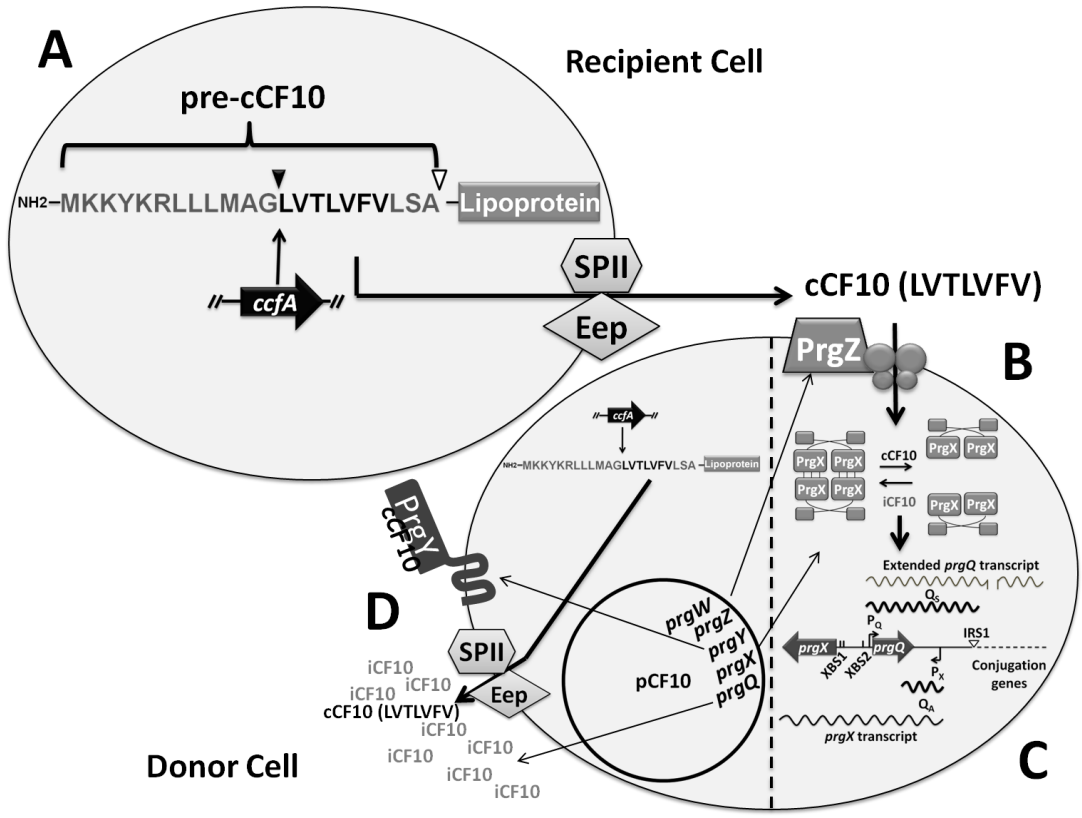


Figure 2: Pheromone-responsive Conjugation of pCF10 in *E. faecalis*.

Diagram depicts the major steps in pheromone inducible conjugation (see text for details). Illustration depicts: (A) pre-cCF10 production and processing which occurs in both plasmid-containing donors and plasmid-free recipient cells; (B) pheromone uptake and role in activation of conjugation; (C) transcription regulation involved with induction of pheromone response; and (D) donor cell mechanisms to prevent self-induction of conjugation.

Pheromone-Responsive Conjugation in pCF10

Figure 2B shows cCF10 produced by a cell lacking pCF10 (recipient cell) being detected by a cell containing pCF10 (recipient cell) through the pCF10 encoded lipoprotein, PrgZ. PrgZ binds to cCF10 and recruits an oligopeptide permease system to facilitate the import of cCF10 into the cell cytoplasm (Leonard *et al.*, 1996; Ruhfel *et al.*, 1993). Inside the donor cell, cCF10 binds to plasmid-encoded transcriptional repressor PrgX, destabilizing the PrgX tetramer. Destabilization of the PrgX tetramer reduces DNA binding and repression at the *prgQ* promoter (Kozlowicz *et al.*, 2006; Shi *et al.*, 2005; Figure 2C). Uninduced *prgQ* transcript (P_Q) is constitutively expressed as a 380 nucleotide transcript (Q_S). Q antisense (Q_A) is derived from *prgX* transcript processing (initiated at Q_A promoter), also inhibits the transcription of conjugation genes by complementary binding to the short *prgQ* transcript (Q_S). Pheromone induction increases P_Q transcript levels and importantly allows for read-through of an inverted repeat structure (IRS1) in transcripts that were not inhibited by Q_A. The extended *prgQ* transcripts (Q_L) encode genes necessary for conjugation of pCF10 (Bae *et al.*, 2000; Bae *et al.*, 2004; Bensing *et al.*, 1996; Bensing and Dunny, 1997; Bensing *et al.*, 1997; Chung and Dunny, 1992, 1995; Johnson *et al.*, 2010; Kozlowicz *et al.*, 2006; Shi *et al.*, 2005).

Aggregation substance (Asc10) is one of the earliest proteins expressed following induction of pCF10 conjugation by cCF10 (Chung and Dunny, 1992). Asc10 is a 137 kDa protein that is encoded on the *prgB* gene of pCF10. Asc10 causes the aggregation mating response involved in conjugation of pCF10 (Hirt *et al.*, 2005; Olmsted *et al.*, 1991). Asc10 expression causes the mating response by forming aggregates of donor and recipient cells that can lead to transfer rates of 10^{-1} transconjugants per donor (Dunny

et al., 1978). *prgB* transcription peaks and returns to uninduced levels after two hours post induction (Hirt *et al.*, 2005).

Transfer of pCF10 is mediated by a Type IV secretion mechanism (Chen *et al.*, 2008: Figure 1, CM). pCF10 genes *pcfE* to *pcfH* are responsible for DNA processing during conjugative transfer (Aakra *et al.*, 2005; Staddon *et al.*, 2004). Sequence homology predicted *pcfG* encodes a putative relaxase, and deletion of *pcfG* dramatically decreases pCF10 transfer. The origin of transfer region, IncP-type *oriT*, is a 40 base pair non-coding region (located between *pcfE* and *pcfF*) that is similar to the *oriT* regions of other Gram-positive conjugative plasmids (Chen *et al.*, 2007; Staddon *et al.*, 2006). Deletion to the *oriT* inhibits plasmid transfer. Additionally, pCF10 mobilized *L. lactis* and *Streptococcus agalactiae* plasmids with cloned *oriT* allowing for intra-species transfer (Staddon *et al.*, 2006). *pcfD* encodes a putative primase (Chen *et al.*, 2008). The genes for pCF10 DNA processing are nearly identical to DNA processing genes of pheromone-responsive plasmid, pTEF2 (Hirt *et al.*, 2005).

The plasmid pCF10 is physically delivered to recipient cell by a Type IV secretion (T4S) mechanism (Chen *et al.*, 2008; Figure 1, DP). T4S conjugation machinery is found in Gram-positive, Gram-negative, and some Archaea bacteria. T4S in Gram-positive species have both an envelope spanning secretion channel and a protein adhesin to mediate attachment (Abajy *et al.*, 2007; Christie and Cascales, 2005; Gomis-Ruth *et al.*, 2004). pCF10 encoded PcfC coordinates DNA processing of pCF10 with TS4 delivery. DNA recruitment to the TS4 secretion channel begins with PcfF mediated binding of PcfG at *oriT* to catalyze pCF10 nicking. PcfF and PcfG bound at *oriT* mediate plasmid DNA docking to PcfC. PcfC is a membrane bound, putative, ATPase. PcfC

initiates pCF10 transfer through the T4S channel by an NTP-dependant mechanism (Chen *et al.*, 2008; Staddon *et al.*, 2006). In addition, *pcfJ* encodes a putative ATPase and *pcfK* a putative cell wall hydrolases that also could be involved with DNA transfer (Chen *et al.*, 2008).

After obtaining the plasmid, the newly formed donor cells continue to produce cCF10, but contain mechanisms to prevent self-induction of conjugation (Figure 2D). pCF10 encodes (1) PrgY transmembrane protein that sequesters cCF10 at the membrane and (2) iCF10 inhibitor peptide that competes with cCF10 for binding to PrgX (Buttaro *et al.*, 2000; Chandler *et al.*, 2005b).

Other Pheromone Binding to Host and Plasmid Encoded Proteins

In addition to PrgZ, PrgX and PrgY, pheromone cCF10 has been shown to interact with other plasmid-encoded proteins and chromosomally-encoded proteins. Ribosomal protein S5, an unknown 43 kDa, and an unknown 39 kDa host protein bind to cCF10 (Leonard *et al.*, 1996). It is not known as to why cCF10 binds to proteins encoded on the host chromosome and whether the binding contributes to conjugation and/or maintenance of the plasmid. Interestingly, cCF10 also binds to the replication initiation protein of pCF10 PrgW, which will be discussed in detail later in the introduction (Leonard *et al.*, 1996; Ruhfel *et al.*, 1993).

Role of Plasmids in Enterococcal Virulence

Pheromone-responsive Plasmids

Pheromone-responsive plasmids are a unique plasmid class almost exclusively found in *E. faecalis* (Clewell and Francia, 2004). Pheromone-responsive conjugative plasmids are ideal reservoirs of genes for virulence factors like aggregation substance and cytolysins, as well as, antibiotic resistance because of: (1) an extreme stability without selection (Meloni *et al.*, Submitted; Wardal *et al.*, 2010; Weaver *et al.*, 2002); (2) spread of plasmid though specific and efficient conjugation (Dunny, 2007) and (3) resistance and virulence factor gene acquisition from the host chromosome or from other plasmids found inside the cell (Manson *et al.*, 2010).

Aggregation Substance

Enterococci are the third leading cause of infectious endocarditis (Moreillon and Que, 2004). The disease is primarily caused by formation of vegetations on the heart valves consisting of a bacterial biofilm together with immune cells, platelets, and fibrin (Chuang-Smith *et al.*, 2010; Moreillon and Que, 2004; Parsek and Singh, 2003). Plasmid encoded aggregation substance and hemolysin contribute to the development of the vegetations (Chow *et al.*, 1993). Aggregation substance is the primary virulence factor involved with infection that is encoded on pCF10 (Asc10). Aggregation substance is also encoded on pAD1, pPD1, pTEF1, pTEF2, pAM373 and other pheromone responsive plasmids. pCF10 aggregation substance is identical to that of pTEF2 although the pTEF2 AS gene is found on a pathogenicity island (Hirt *et al.*, 2005; Shankar *et al.*, 2004). pAD1 and pTEF1 have identical aggregation substance homologs that share 83 percent

homology to Asc10 from pCF10 (Hirt *et al.*, 2005). The aggregation substance of pAM373 (Asc373) has a molecular weight of 75.6 kDa, as opposed to approximately 137 kDa for other aggregations substances, and appears to be distinct from the other pheromone-responsive aggregation substances (Muscholl-Silberhorn, 1999).

In addition to pheromone, AS can be induced in presence of human plasma (Chandler *et al.*, 2005b; Hirt *et al.*, 2002). This is important because AS has been shown to act as a bacterial adhesin to eukaryotic cells in several models (Hirt *et al.*, 2000; Olmsted *et al.*, 1994; Vanek *et al.*, 1999). AS contains two Arg-Gly-Asp (RGD) motifs that facilitates *E. faecalis* adhesion to eukaryotic cells. AS RGD motifs are similar to fibronectin RGD motifs in that they mediate adhesion to host cell surfaces and are also involved in immune system evasion (Gilmore *et al.*, 2002). Asc10 is present on the surface of the cell after eight hours post induction. Hirt *et al.*, (2005) discussed that the presence of AS on the *E. faecalis* cell surface eight hours post-induction could facilitate the role of enterococcal cell binding, adherence to different cell types (Kreft *et al.*, 1992; Sartingen *et al.*, 2000; Wells *et al.*, 2000), or even withstanding an attack from phagocytic cells (Rakita *et al.*, 1999; Sussmuth *et al.*, 2000).

Enterococcal cytolysin

While encoded on many pheromone-responsive plasmids, the secreted virulence factor cytolysin was first identified on pAD1 (Borderon *et al.*, 1982). Cytolysin is encoded in 60 percent of *E. faecalis* isolates associated with outbreaks (Mundy *et al.*, 2000). Virulence was shown in a mouse model where peritonitis was significantly increased because of cytolysin (Ike *et al.*, 1984). The cytolysin encoded on pAD1

functions as both a hemolysin and bacteriocin. Cytolysin causes beta-hemolysis on human and horse blood agar plates and kills bacteria that do not encode the cytolysin operon. When cytolysin has been modified to the functional conformation, it targets and punches holes in target cell membranes eventually leading to cell lysis (Mundy *et al.*, 2000).

Cytolysin is comprised of two small lantibiotic like peptides L_L and L_S. L_L and L_S function together in causing toxic or lytic activity (Coburn and Gilmore, 2003; Coburn *et al.*, 2004; Pag and Sahl, 2002). The cytolysin operon consists of eight genes: *cylR2, cylR1, cylL$_L$, cylL$_S$, cylM cylB, cylA,* and *cylI* (Coburn and Gilmore, 2003). CylL_L and CylL_S undergo translational modification by CylM. CylB transports CylL_L and CylL_S across the membrane and removes their signal sequences. CylA serine protease is also secreted and proteolytically cleaves six amino acids (identical sequences) from both CylL_L and CylL_S (Gilmore *et al.*, 2002). CylI provides immunity to CylL_L and CylL_S toxins. Cytolysin appears to play a role in quorum sensing as well. Increased CylL_S levels inactivate CylR1 repression (Coburn and Gilmore, 2003; Haas *et al.*, 2002).

Gelatinase

The proteolytic enzyme gelatinase is named based on the ability of the enzyme to liquefy gelatin. Gelatinase is a zinc metalloprotease II (Makinen *et al.*, 1989). Gelatinase can hydrolyze gelatin, collagen, casein, lactoglobulin, porcine myofibrillar proteins, porcine sarcoplasmic c-proteins, and other small peptides with biological activity (Gilmore *et al.*, 2002; Makinen *et al.*, 1989). The mature gelatinase protein contains 318 amino acids residues, and is the product of the processing of a 509 amino acid residue

GelE precursor. Byproducts of the processing include a 29 amino acid residue signal sequence and a 162 amino acid residue prosequence (Su *et al.*, 1991).

The gene for gelatinase protease (*gelE*) is located on the same transcript as the gene for serine protease (*sprE*). This dual protease transcript is regulated by the quorum sensing *fsr* locus (Qin *et al.*, 2000). Briefly, the 11 amino acid residue peptide GBAP encoded on *fsrB* interacts with the signal transducer FsrC. FsrC becomes phosphorylated which in turn phosphorylates the response regulator FsrA. Phosphorylated FsrA then upregulates expression of the *fsrB/fsrC* and *gelE/sprE* promoters (Balaban and Novick, 1995; Gilmore *et al.*, 2002).

Gelatinase has been shown to play a number of roles in *E. faecalis*. Gelatinase is essential in the formation of biofilms in *E. faecalis* (Hancock and Perego, 2004) which has been identified as a factor in pathogenesis in a model for endocarditis (Chuang-Smith *et al.*, 2010). Visual comparisons of GelE negative strains show a decrease in bacterial chain length compared to GelE positive strains. Additionally, GelE negative strains have high amounts of autolysis after 24 hours, resulting in a 90 percent decrease in optical density compared to GelE positive strains. Interestingly, a Western blot comparison of *prgB* insertion mutants showed that GelE is involved in degrading misfolded aggregation substance. Furthermore gelatinase is involved with cCF10 signaling (Waters *et al.*, 2003). Gelatinase reduces cCF10 in the supernatant during stationary phase due to cleavage of the peptide pheromone of pCF10 (Makinen *et al.*, 1989; Waters *et al.*, 2003). This causes GelE negative mutants have an approximately 2.3 fold increase in conjugation compared to GelE positive strains (Waters *et al.*, 2003).

Antibiotic Resistance in Enterococci and the Role of Plasmids

Enterococci are resistant to a variety of antibiotics including: β-lactams, aminoglycosides, glycopeptides (vancomycin), macrolides, chloramphenicol, tetracycline, oxazolidinone, evernimicin, and quinolones because of their intrinsic nature and acquired traits. This section focuses on several antibiotics that enterococci have intrinsic-resistance against (β-lactams and aminoglycosides), tetracycline-resistance that is encoded within pCF10, and emerging enterococcal resistance to vancomycin, the only antibiotic to which there has previously been no resistant enterococcal strains.

β-lactam-resistance

Enterococci have a natural, intrinsic-resistance to β-lactams. β-lactams include penicillins, cephalosporins, and carbapenems. β-lactams function as antibiotics by binding to penicillin binding proteins (PBPs) which then inhibit the transpeptidase enzyme function in cell wall synthesis (Kak and Chow, 2002). Enterococci can have between five to nine types of PBP's within once cell that provides PBP variation (Williamson *et al.*, 1986). Enterococcal PBP's have a low affinity to β-lactam antibiotics causing a natural resistance. *E. faecium* clinical isolates are increasingly resistant to β-lactam antibiotics compared to *E. faecalis*. This is because of the over production of low-affinity PBP's, and mutations that are creating PBP's with even less affinity to β-lactams in *E. faecium* (Fontana *et al.*, 1996; Kak and Chow, 2002).

Enterococci also have unique, non-intrinsic, methods for resistance to β-lactam antibiotics. For example, a laboratory mutant of *E. faecium* bypassed the need for the DD-transpeptidation reaction necessary for cell wall synthesis. This DD-transpeptidation

reaction is typically sensitive to β-lactams. There were only LD-transpeptidation crosslinks detected in the *E. faecium* β-lactam-resistant mutants (Kak and Chow, 2002; Marshall *et al.*, 1997).

E. faecalis rarely produces β-lactamases (Gordon *et al.*, 1992). However, there are a few *E. faecalis* isolates that were found that produce β-lactamases. β-lactamases are enzymes that cleave the ring of β-lactam antibiotics. *E. faecalis* β-lactamases are encoded on the *blaZ* gene. *blaZ* has been shown to be identical or highly homologous to a similar staphylococcal type A β-lactamase (Kak and Chow, 2002; Murray, 1992; Tomayko *et al.*, 1996).

Aminoglycoside-resistance

Enterococci also have an intrinsic low level-resistance to aminoglycosides by limiting transport of the antibiotics across the membrane (Kak and Chow, 2002). Aminoglycosides (like gentamicin) typically bind to the 16S rRNA of the 30S ribosomal subunit to interfere with translation (Kotra *et al.*, 2000; Mingeot-Leclercq *et al.*, 1999). Alone, aminoglycosides are not effective in treatment against enterococci. In combination with β-lactams or glycopeptides, aminoglycoside uptake is facilitated and the effectiveness of aminoglycosides in enterococci increases (Moellering and Weinberg, 1971). To combat against synergistic antibiotic treatment, enterococci can acquire genes that encode aminoglycoside-modifying enzymes including: phosphotransferases, acetyltransferases, and nucleotidyltransferases (Kak and Chow, 2002).

Tetracycline-resistance

Tetracyclines function by binding to the ribosome of the bacteria (Hash *et al.*, 1964). The bacterial ribosomal 30S subunit has a high affinity tetracycline binding site. Both 30S and 50S ribosomal subunits also have additional low-affinity tetracycline binding sites (Tritton, 1977). There are three mechanisms for tetracycline-resistance described by Griffin *et al.* (2010): (1) decreased influx or increased efflux of antibiotic; (2) ribosomal protection; or (3) enzymatic inactivation. Enterococci utilize both active efflux of tetracycline and ribosomal protection (Kak and Chow, 2002).

The *tetL* gene is responsible for the most common tetracycline efflux mechanism in enterococci. *tetL* is found on the chromosome or conjugative plasmids of enterococci (Bentorcha *et al.*, 1991; Platteeuw *et al.*, 1995). A common ribosomal protection protein that binds to the ribosome and alters the conformation such that it no longer binds tetracycline is encoded by *tetM*. *tetM* has been widely associated with transposon Tn*916*. *tetM* is found on many conjugative plasmids (Kak and Chow, 2002). pCF10 has *tetM* on Tn*925* (a derivative of Tn*916*) (Hirt *et al.*, 2005). *E. faecalis* strain 62 contains a plasmid (EF62pB) with approximately 95 percent homology to pCF10 in all regions of the plasmid excluding the Tn*925* transposon. The transposon containing the *tetM*-resistance gene is located on the chromosome of *E. faecalis* strain 62 (Brede *et al.*, 2011).

Glycopeptide (Vancomycin)-resistance

Vancomycin had long served as a “last line of defense” in treatment of Gram-positive species because of the lack of vancomycin resistance in these organisms. In 1986 it was first reported that enterococci obtained the ability to become resistant to

vancomycin (Uttley *et al.*, 1988). It was soon discovered that the vancomycin-resistance in *E. faecium* was derived from a plasmid and suggested the problem that the resistance could be rapidly disseminated (Leclercq *et al.*, 1988). Dissemination of the resistance has increased over several decades. A report that analyzed isolated enterococci from hospital patients from North America and Europe by Deshpande in 2007 showed that 30 percent of enterococcal isolates encoded vancomycin-resistance and 90 percent of *E. faecium* isolates had vancomycin-resistance (Deshpande *et al.*, 2007). The Deshpande study showed a ten percent increase from a study in 1998 where 20 percent of enterococci isolates in the US were resistant to vancomycin (Fridkin and Gaynes, 1999). Once in the human gastrointestinal tract, vancomycin-resistant enterococci (VRE) not only persist but can leave the gastrointestinal tract and can colonize other patients (Kak and Chow, 2002). Genetic transfer of the vancomycin resistance determinant between *E. faecalis* and *S. aureus* has been documented (see below).

Glycopeptides like vancomycin were originally predicted never to acquire a resistance against them because they do not work by blocking enzymes involved with cell wall synthesis like β-lactams. Instead, glycopeptides block cell wall synthesis by acting on their cell wall substrates, in particular the peptidoglycan pentapeptide precursor. Specifically, glycopeptides act upon the D-Ala-D-Ala of the peptidoglycan precursor to block cell wall synthesis, reviewed by Jovetic (Jovetic *et al.*, 2010). The method of-resistance against vancomycin involves the modification of the D-Ala-D-Ala pentapeptide precursor to a pair containing a D-Ala-D-Lactate or D-Ala-D-Serine. Because of a loss of hydrogen bond, there is a 1,000-fold decrease in affinity of vancomycin for D-Ala-D-Lac compared to D-Ala-D-Ala. D-Ala-D-Ser has seven-fold

decrease in affinity for vancomycin compared to D-Ala-D-Ala (Billot-Klein *et al.*, 1994; Bugg *et al.*, 1991; Kak and Chow, 2002).

Vancomycin-resistance requires the acquisition of a multi-gene regulon that allows for the correct processing of the cell wall. These multi-genes determinants are classified by their ligase genes (*vanA, vanB, vanD, vanE or vanG*) that allow for the ligation of lactate or serine to the pentapeptide chain. The product of vancomycin-resistance gene, *vanC,* provides an intrinsic-resistance in *Enterococcus casseliflavus, Enterococcus gallinarum* and *Enterococcus flavescens.* Vancomycin-resistant genes *vanA* and *vanB* have been found on both the chromosome and plasmids. This section will focus on the mechanism of resistance enocoded on *vanA* because of its presence on Tn*1546*, a transposon responsible for transferring-resistance from *E. faecalis* to *S. aureus.*

vanA encodes a high-level of inducible-resistance to both vancomycin and teicoplanin and is part of a seven gene operon (Arthur *et al.*, 1993; Brisson-Noel *et al.*, 1990). The presence of glycopeptides in the environment increases transcription of the *vanA*-regulon. VanS, a protein kinase becomes active in the presence of glycopeptides which leads to the autophosphorylation of the cytoplasmic histidine residue of VanS. The cytoplasmic histidine residue of VanS transfers the phosphoryl group to an aspartate residue on the VanR response regulator. VanR binds to the *vanH* promoter and the promoter for *vanR* and *vanS* (Arthur *et al.*, 1992b; Arthur and Quintiliani, 2001; Gholizadeh and Courvalin, 2000; Kak and Chow, 2002).

VanH is an α-ketoreductase that reduces pyruvate to D-Lac (Arthur *et al.*, 1992a; Gholizadeh and Courvalin, 2000). VanA then ligates the newly formed D-Lac to D-Ala. The D-Lac-D-Ala is added to the UDP-*N*-acetyl muramide tripeptide which is then

inserted into the peptidoglycan cell wall. VanX zinc-dependent dipeptidase removes D-Ala-D-Ala precursors being formed (Gholizadeh and Courvalin, 2000; Reynolds *et al.*, 1994). VanY carboxypeptidase breaks down any D-Ala-D-Ala in the cytoplasm (Arthur *et al.*, 1998). VanZ function is unknown but appears to provide low level-resistance to teicoplanin (Arthur *et al.*, 1995; Kak and Chow, 2002).

The first isolate of *S. aureus* encoding *vanA*-type resistance was obtained from the foot of a diabetic patient (Chang *et al*., 2003). The *vanA* was contained on the Tn*1546* that was part of a conjugative plasmid (Weigel *et al*., 2003). Tn*1546* carries the vancomycin-resistance gene *vanA* as well as the rest of the resistance operon (Arthur *et al*., 1993). The vancomycin-resistance encoded within the *S. aureus* isolate was most likely obtained from a plasmid from an *E. faecalis* isolate. A co-isolate of an *E. faecalis* plasmid carrying Tn*1546* was found from the same diabetic patient foot as the vancomycin-resistant *S. aureus* (Weigel *et al.*, 2003).

Enterococcal Plasmid Replication

Plasmids are extra-chromosomal pieces of DNA that have the ability to replicate independently (autonomously) within a suitable host species. They can range between one and 1000 kilobase pairs. The ability of plasmids to replicate autonomously in a host is dependent upon the ability to initiate and control plasmid replication. This involves the functioning of the replication initiation protein, efficiency of plasmid partitioning functions, and the ability of the plasmid to utilize and interact with host replication machinery. Plasmids are naturally found in bacteria and are abundant in enterococci. For

example, one study showed that 83 of 93 *E. faecium* isolates from clinical, hospital, community, and animal fecal isolates contained one to seven plasmids (Palmer *et al.*, 2010; Rosvoll *et al.*, 2010). There are three types of plasmids in enterococci that are characterized by their replication initiation function: rolling circle, Inc18, and theta-replicating plasmids.

Rolling Circle Plasmids

Rolling circle plasmids are small, high-copy plasmids that can replicate in multiple species by the characteristic rolling circle mechanism. The plasmid-encoded replication initiation protein of rolling circle plasmids initiates replication by ssDNA nick at the dsDNA origin of replication (*dso*). Replication initiation proteins of rolling circle plasmids have two domains that are required for proper replication initiation; (1) one recognizing a specific DNA binding site at the origin and (2) another performing sequence-specific nicking of the DNA. The nicking domain of the initiator protein contains a tyrosine active site that only acts on ssDNA. Binding of the initiator protein to plasmid DNA is essential in order to melt the DNA and present the nick site as ssDNA. After ssDNA nicking, DNA helicase and DNA polymerase III are recruited to the origin of replication where priming of replication occurs at the free 3'OH nick site. Plasmid replication proceeds on the leading strand of the plasmid DNA, while the lagging strand (still containing bound initiator protein) is displaced. Following completion of replication, a nicking and re-ligation action terminates replication. The nicking and re-ligation of the plasmid is catalyzed by still bound replication initiator protein. Replication of the displaced lagging strand is initiated by cleavage of a separate single-

strand origin (*sso*). Host RNA polymerase generates a short RNA primer at *sso* and DNA polymerase I starts DNA synthesis at the primer. DNA polymerase III completes synthesis of the complementary strand following initial DNA synthesis by DNA polymerase I (del Solar *et al.*, 1998; Khan, 1997, 2005).

Theta-replicating Plasmids

pCF10 and other pheromone-responsive plasmids are theta-replicating plasmids. Theta-replicating plasmids are named after a characteristic theta-shaped structure seen on electron micrographs. Theta origins of replication are the minimal *cis* element required for plasmid replication. Theta replication initiation proteins primarily bind to plasmid *oriV* in order to initiate replication. The replication initiation proteins bind to direct or inverted repeats. The repeating regions are located approximately 11 base pairs apart suggesting DNA binding is located on the same side of the DNA helix (del Solar *et al.*, 1998). Theta-replicon origins also have AT rich regions which facilitate DNA melting and DnaA boxes for host DnaA to bind (Chattoraj, 2000; del Solar *et al.*, 1998; Messer, 2002). Pheromone-responsive plasmid theta replication initiation proteins encoded in *prgW* and *repA* function as the pCF10 and pAD1 minimal origins of replication when provided with an exogenous promoter (erythromycin promoter). PrgW and RepA bind to direct repeats encoded within the replication gene (Francia *et al.*, 2004; Meloni *et al.*, Submitted). Replication initiation binding causes plasmid DNA melting to occur. Host DnaA binding further facilitates the DNA melting and leads to recruitment of DNA polymerase III, RNA polymerase primase, and helicase to the replication initiation site (replisome) (del Solar *et al.*, 1998).

Inc18 Plasmids

Plasmids of the Inc18 family can be found throughout erythromycin/lincomycin, vancomycin or methicillin-resistant bacteria. Lioy (Lioy *et al.*, 2010) classified Inc18 plasmids into three groups: (1) plasmids like pSM19035, pSM22095, and pSM10419 that lack a mobilization region thus cannot be mobilized by conjugation to other species, and have replication and stability regions that are duplicated and inverted (Boitsov *et al.*, 1979; Malke, 1974); (2) plasmids like the pVEF series (pVEF1, pVEF2, and pVEF3) and pREW25 that encode self-transmissible elements, and the replication regions contain direct repeat regions separated by long non-repeating segments (Schwarz *et al.*, 2001; Sletvold *et al.*, 2008); and (3) plasmids like pAMβ1 and pIP501 that encode self-transmission elements but do not contain direct repeats in their replication regions (Berryman and Rood, 1995; Dunny and Clewell, 1975; Horodniceanu *et al.*, 1976; Kurenbach *et al.*, 2003).

Broad host range plasmids like pAMβ1 have the ability to replicate in other species. One reason for this is the replication protein, RepE, does not require interaction with the host-encoded DnaA for the replication of the plasmid. For example, pAMβ1 replication requires formation of a DNA-RNA heteroduplex. DNA-RNA heteroduplex formation is mediated by the replication initiation protein RepE to initiate replication. Basically, RepE binds to plasmid dsDNA leading to melting and formation of an open complex at the plasmid *ori*. RNA polymerase transcription starts at the *repE* promoter where it then collides with the RepE at the plasmid *ori*. The collision displaces bound RepE and leads to termination of transcription. RNaseH, RepE, or RNA polymerase

cleaves the transcript creating an RNA primer for the start of replication. DNA polymerase I DNA replication is initiated at the synthesized RNA primer (Bruand and Ehrlich, 1998; Le Chatelier *et al.*, 2001).

Plasmid Replication Initiation Proteins

Conformational Changes and Aggregation

The activity of replication initiation proteins can be controlled by either forming or resolving aggregates. For example, the replication initiation protein of plasmid pPS10, RepA, forms a dimer and acts as a self-repressor of *repA* expression. In the monomer form the pPS10 RepA protein binds to the initiation complex (Garcia de Viedma *et al.*, 1995). On the other hand, the RepA protein of *Salmonella* plasmid R1 increases replication efficiency when dimers form. DnaK regulates the formation of R1 RepA dimers (Giraldo-Suarez *et al.*, 1993). Dimers bind to site 1 of *oriR*, causing the binding of a second dimer to site 2 of *oriR*. This forms a loop in plasmid DNA that is held together by the two dimers (Giraldo and Diaz, 1992). This complex allows for more RepA proteins to fill in the plasmid loop and the nucleoprotein complex recruits DnaA to the DnaA box (Ortega-Jimenez *et al.*, 1992).

R1 is a θ replicating plasmid like pCF10 (del Solar *et al.*, 1998; Meloni, 2005). R1 replication is unidirectional (Nordstrom, 2006). Replication initiation of R1 requires a large aggregation of the replication initiation proteins (possibly 20) to bind to the origin of replication (del Solar *et al.*, 1998; Masai *et al.*, 1983; Masai and Arai, 1987). This is similar to the binding of DnaA to *oriC* of the chromosome where DNA is wrapped around the replication initiation molecule to open the DNA. ssDNA allows for the

recruitment of the replisome in R1 plasmids (Bramhill and Kornberg, 1988; Nordstrom, 2006).

Gram-positive RepA_N Proteins

The replication initiation protein of pCF10 is a member of a superfamily of replication initiation proteins found in low-GC Gram-positive organisms (including enterococci, streptococci, staphylococci, lactococci and lactobacilli) called RepA_N proteins. RepA_N are identified by a conserved 100 AA residue consensus sequence located at the amino terminus. Like other θ replicon proteins, RepA_N proteins also typically bind to direct or inverted repeats located on the chromosome or plasmid DNA that leads to initiation of replication at the origin of replication (Weaver *et al*., 2009a). The binding of the replication initiation proteins to the origin of replication generally leads to melting and DNAP complex (replisome) assembly takes place (del Solar *et al.*, 1998).

RepA_N family protein PrgW initiates replication of the θ replicating pCF10 (Meloni thesis, 2005). PrgW, like RepA of pAD1, functions by binding to the origin of replication direct repeats located within prgW to initiate replication. PrgW binds to ssDNA and dsDNA direct repeats located within the *prgW* gene to initiate replication. When provided with an exogenous promoter, *prgW* and *repA* function as the pCF10 and pAD1 minimal replicon, respectively (Francia *et al.*, 2004; Meloni *et al.*, Submitted).

Copy Number Control

Replication initiation control of a plasmid is important because it maintains the rate of plasmid replication which has an effect on plasmid copy number. The appropriate levels of plasmid copy number are critical to maintain in low-copy plasmids like pCF10. Too few copies and plasmid stability is compromised because there are not enough plasmids to pass onto daughter cells during division. Too many copies and it becomes a metabolic burden (Nordstrom *et al.*, 1984; Uhlin and Nordstrom, 1978; Wagner and Brantl, 1998). Once appropriate levels of a newly established plasmid are achieved, adjustments need to be made to decrease the rate of plasmid replication (del Solar *et al.*, 1998).

Copy number levels are maintained at the necessary levels by inactivating the function of the replication initiation protein when plasmid copy levels become too high. Plasmids encode mechanisms to control rate of replication initiation and negative regulator circuits. For example, in plasmid ColE1, plasmid copy-number is decreased when the *copA* RNA transcript binds to the *repA* mRNA leader region reducing transcription of the plasmid replication initiation protein. Additionally, negative transcriptional regulator proteins (i.e. CopR of pIP501) bind to the upstream promoters and in turn inhibit replication proteins, causing a reduction in copy number. Moreover, theta replicating plasmids utilize the repeating iteron regions to which replication initiation proteins bind to control plasmid copy number. The "handcuffing" model of R6K theorizes that when replication protein levels are low, binding of replication

initiation proteins to iterons allows for plasmid replication. If the copy of the plasmid is too high, replication initiation proteins from separate plasmid origins bind together and this complex inhibits replication of the plasmid (del Solar *et al.*, 1998).

Plasmid Partitioning

Bacterial plasmids have evolved different segregation mechanisms to ensure maintenance of the plasmid throughout the population of cells. Gerdes *et al.* (2010) reviewed these mechanisms and provided detailed descriptions of the three classes of partitioning proteins in prokaryote DNA segregation. Partitioning loci (*par*) have been identified in bacteria to encode: (1) ParM, that pushes plasmids to opposite ends of the cell using unstable actin homolog filaments. (2) Walker A cytoskeletal P loop ATPase (ParA) that pulls the plasmid along the nucleoid; and (3) TubZ, *par* locus that encodes forms cytoskeletal tubulin homologs that form filaments that move rapidly like a treadmill (Gerdes *et al.*, 2000; Larsen *et al.*, 2007). The three partitioning protein systems encode an ATPase or GTPase protein, DNA binding protein, and one or more centromeres where the partitioning proteins bind to the plasmid.

ParM Partitioning

ParM partitioning systems are encoded by three genes; *parMRC*. ParR binds to the centromere site, *parC*. The binding of ParR to *parC* is necessary for the binding of ParM. ParM produces actin-like filaments localizing plasmids to the cell poles by a pushing mechanism caused by the ParM ATP-dependent polymerization. This causes

plasmids to localize at both ends of the cell when the septum forms. ParM filaments have rapid turnover (Moller-Jensen *et al.*, 2002; Moller-Jensen *et al.*, 2003).

TubZ-mediated Partitioning

TubZ is a unique par locus that has recently been discovered in only Gram-positive species. It belongs to the FtsZ superfamily of GTPases. *Bacillus thuringiensis* plasmid pBtoxis and *Bacillus anthracis* plasmids pDSW208 and pXO1 (RepX) encode the TubZ tubulin-like protein (Larsen *et al.*, 2007; Tinsley and Khan, 2006). In vitro studies show that TubZ assembles in double-stranded helical filaments that are similar to microtubule polymers (Chen and Erickson 2008). TubZ "treadmilling" behavior along the bacterial cell membrane most likely is involved in plasmid segregation. TubR appears to be responsible for anchoring TubZ to the plasmids (Larsen *et al.*, 2007).

Walker A Cytoskeletal P Loop ATPase (ParA)

Partitioning protein ParA is an ATPase that utilizes a pulling mechanism to localize the plasmid. ParA-ATP dimers bind to DNA, leading to the formation of ParA filaments because of rapid ParA polymerization (Dunham *et al.*, 2009; Leonard *et al.*, 2005; Pratto *et al.*, 2008). The polymerizing ParA filament binds to ParB-plasmid centromere complex (ParB bound to centromere-site parC), which stimulates ATPase activity of ParA at one end of the ParA filament. This causes the ParA in contact with ParB-plasmid complex to convert ParA-ATP to ParA-ADP and disassociate from the DNA. The plasmid-ParB complex is pulled by the depolymerizing ParA filaments (Ringgaard *et al.*, 2009). pCF10 and pAD1 encode this type of partitioning system (Francia *et al.*, 2007; Izquierdo, unpublished observation).

Partitioning in pAD1 and pCF10

Genes encoding ATPase driven ParA plasmid partitioning have been described in pheromone-response plasmids. RepB ATPase and DNA binding RepC of pAD1 function similar to bacterial plasmid partitioning proteins ParA and ParB. Sequence analysis shows an iteron region of 25 8-base pair direct repeats (TAGTARRR) between the replication initiation protein (RepA) and RepB. Mobility and DNaseI foot printing assays show that RepC interacted with specificity to the iteron region. RepC bound to the iteron region in the absence of RepB, suggesting this was an early step in plasmid partitioning. RepB could not directly bind to the iteron region without RepC. While RepC is essential in stabilizing plasmids with pAD1 centromeres, both RepB and RepC were required for maximum stability of the plasmids. The presence of ATP was essential for RepB-RepC complex formation as RepB was affected by ATP, similar to the RepB ATPase family homologs (Francia *et al.*, 2007).

PrgO and PrgP are partitioning proteins involved in segregation of pCF10. Like PrgC of pAD1, PrgO binds to the *incI* and *incII* regions flanked by the *prgO* and *prgN* and *prgP* and *prgW* regions on pCF10. Similar to PrgB of pAD1, PrgP is a member of the ParA ATPase superfamily that is involved in both plasmid and chromosome partitioning. PrgP binds to the PrgO-*inc* complex and recruits the plasmid to the appropriate location on the cell membrane using ATPase activity. Interestingly, PrgO binding to the *incII* site flanked by *prgP* and *prgW* genes interferes with the read-through transcription of *prgW*, decreasing the levels of *prgW* transcription (Izquierdo, unpublished observations).

Plasmid Host Range

Factors Allowing for Broad Host Ranges

Plasmids can be divided into narrow host range and broad host range. Narrow host range plasmids, like pCF10, generally are found in only one species. Broad host range plasmids can be found in multiple species and even multiple genera. Several factors are known that allows plasmids to have broad host ranges. This has been best characterized for the IncQ (incompatibility group Q) plasmids, and can be summarized as follows. (1) Plasmid replication initiation proteins are DnaA-independent, bypassing the need for direct binding of the replication initiation protein to the DnaA of a given species, since DnaA homologs can have significant divergence in amino acid sequence. (2) Plasmids can be mobilized by a variety of Type IV secretion mechanism, including protein secretors, which allow introduction of the plasmids into a number of different bacterial genera. (3) Plasmid can have high plasmid copy numbers so efficient replication is not essential to maintenance and gene expression modulation of other genes on the plasmid decreases the metabolic burden.

Some broad host range rolling circle replicating plasmids in Gram-positive species can replicate in *E. coli* (Barany *et al.*, 1982; del Solar *et al.*, 1987). Rolling circle plasmids are typically small plasmids with high copy numbers, which is important to extending host range. Rolling circle replication involves the Rep proteins targeting the *nic* specific site at the origin of replication. The nicked DNA is a target of helicase, SSB, and DNA polymerase III recruitment (Goze and Ehrlich, 1980). The ability of the host machinery to interact with newly cut DNA might be essential for replication in new

hosts. Inc18 broad host range plasmids generate their own RNA primer that might also help establish replication in new species. After the Rep protein binds and potentially mediates cleavage of RNA bound at the origin, the RNA acts as a primer recruiting host replication machinery (del Solar *et al.*, 1998). Pheromone-response plasmids do not appear to use a nucleic acid intermediate to interact with the Rep protein.

Replication proteins play a critical role in defining the host range of other plasmids in other species. The ability of the plasmid to establish in a new species is dependent upon the plasmid being initially replicated and then maintained throughout the population. The versatility of the replication protein to interact with host replication machinery like DnaA, a helicase or DNA polymerase might be essential for the plasmid survival. Additionally, the plasmid could encode proteins capable of initiating replisome assembly independent of host DnaA. Furthermore, the presence of two more replication initiation proteins could allow for replication in multiple species. For example, pheromone-response plasmid pRE25 and pTEF1 both encode multiple replication initiation proteins (Toukdarian, 2004).

Enterococcal Narrow and Broad Host Range Plasmids

Enterococcal plasmids are classified into two main groups, narrow and broad host range (Palmer *et al.*, 2010). Although extremely stable, pheromone-responsive plasmids have a limited host range (Weaver *et al.*, 2002). Narrow host range plasmids acquire resistance determinants, virulence factors, and other genes from transposons, broad host range plasmids, the host chromosome and other pheromone-responsive plasmids (Hirt *et al.*, 2005; Manson *et al.*, 2010).

E. faecalis broad host range conjugative plasmids can transfer plasmid DNA to multiple species, usually of *Enterococcus* and *Staphylococcus* (Grohmann *et al.*, 2003). 83 percent of the *E. faecium* from community, clinical, hospital, and animal fecal isolates contained similar replicons to the broad host range plasmids pRE25, pRUM, pIP501, and pHTβ, while pheromone-response replicons were not detected (Rosvoll *et al.*, 2010). Broad host range plasmids such as incompatibility group 18 (Inc18) plasmid pRE25 can have one or multiple antibiotic resistance genes and have the ability to transfer plasmid DNA into other species such as *Listeria innocua* and *L. lactis* (Schwarz *et al.*, 2001).

Transposons contained within the plasmid have long been associated with transfer of antibiotic resistance genes, especially under antibiotic selective pressure (de Niederhausern *et al.*, 2011; Heaton and Handwerger, 1995; Weigel *et al.*, 2003). Both the narrow and broad host range plasmids have been implicated in the transfer of antibiotic resistance to other genera and species through transposons. This includes the transfer of resistance markers between *E. faecalis* and *S. aureus* (Clewell *et al.*, 2002). Resistant *E. faecalis* and *S. aureus* have been observed together in polymicrobial biofilms and in clinical samples from patients (Han *et al.*, 2009; Weigel *et al.*, 2007). The characterized pheromone-inducible conjugative plasmids are restricted in host range to *E. faecalis*. However, the resistance determinants are often encoded by transposons that are carried by these plasmids, so the transient survival of these plasmids within other species might be sufficient to allow, especially under the selection of antibiotic treatment, for the transfer of a transposon carrying the antibiotic resistance marker into the new host (e.g.: the tetracycline-resistance determinant of pCF10 is carried by Tn925) (de Niederhausern *et al.*, 2011; Heaton and Handwerger, 1995; Weigel *et al.*, 2003).

Role of pre-cCF10 and PrgW in Plasmid Host Range

pCF10 and other pheromone responsive plasmids have a limited host range but are extremely stable within *E. faecalis*. In *E. faecalis*, conjugative pheromone precursor pre-cCF10 influences the host range of pCF10 through an autocrine signal. Evidence for pre-cCF10 influencing host range of pCF10 is as follows: (1) *Enterococcus hirae* encodes pre-cCF10 homolog (varying cCF10 region) and maintains pCF10. (2) *E. faecalis* that produces pre-cCF10, but not cCF10, maintains the plasmid. (3) *L. lactis* engineered to produce CcfA (encodes pre-cCF10) maintains pCF10. (4) In vitro affinity chromatograph experiments show PrgW interacts with pre-cCF10.

Pre-cCF10 and cCF10 interaction with PrgW causes motility changes on non-reducing SDS-PAGE gel when compared to purified PrgW. These alternate PrgW conformations are sensitive to reduction, so pre-cCF10 interaction with PrgW might induce conformational changes mediated by disulfide bonds. Sequence alignment *E. faecalis* RepA_N proteins support the idea of a potential role of disulfide bonds in conformation and stability. A basic BLASTP search revealed that PrgW had 59 homologs in the RepA_N family with at least 30 percent identity in amino acid sequence (Figure 3, top). These RepA_N homologs were likely to be replication initiation proteins found on plasmids, however, the plasmids for the corresponding replication initiation proteins have not been identified in all cases according to the Genbank database (Benson *et al.*, 2008). ClustalW2 multiple sequence alignment illustrated that 54 of 59 known RepA_N proteins in *E. faecalis* that were homologous to PrgW contained three highly conserved cysteines (Figure 3, top). Other plasmids including pAM373 and pEJ97-1 contained one conserved cysteine with PrgW (C307) as well as their own set of

conserved cysteines (Figure 3, bottom). Conservation of cysteines within the RepA_N superfamily indicated suggested these cysteines may regulate conformation and function of a group of proteins found within the RepA_N family in *E. faecalis* and may contribute to polyacrylamide gel migration differences seen under non-reducing conditions. The role of these conserved cysteines in pCF10 is the topic of this thesis.

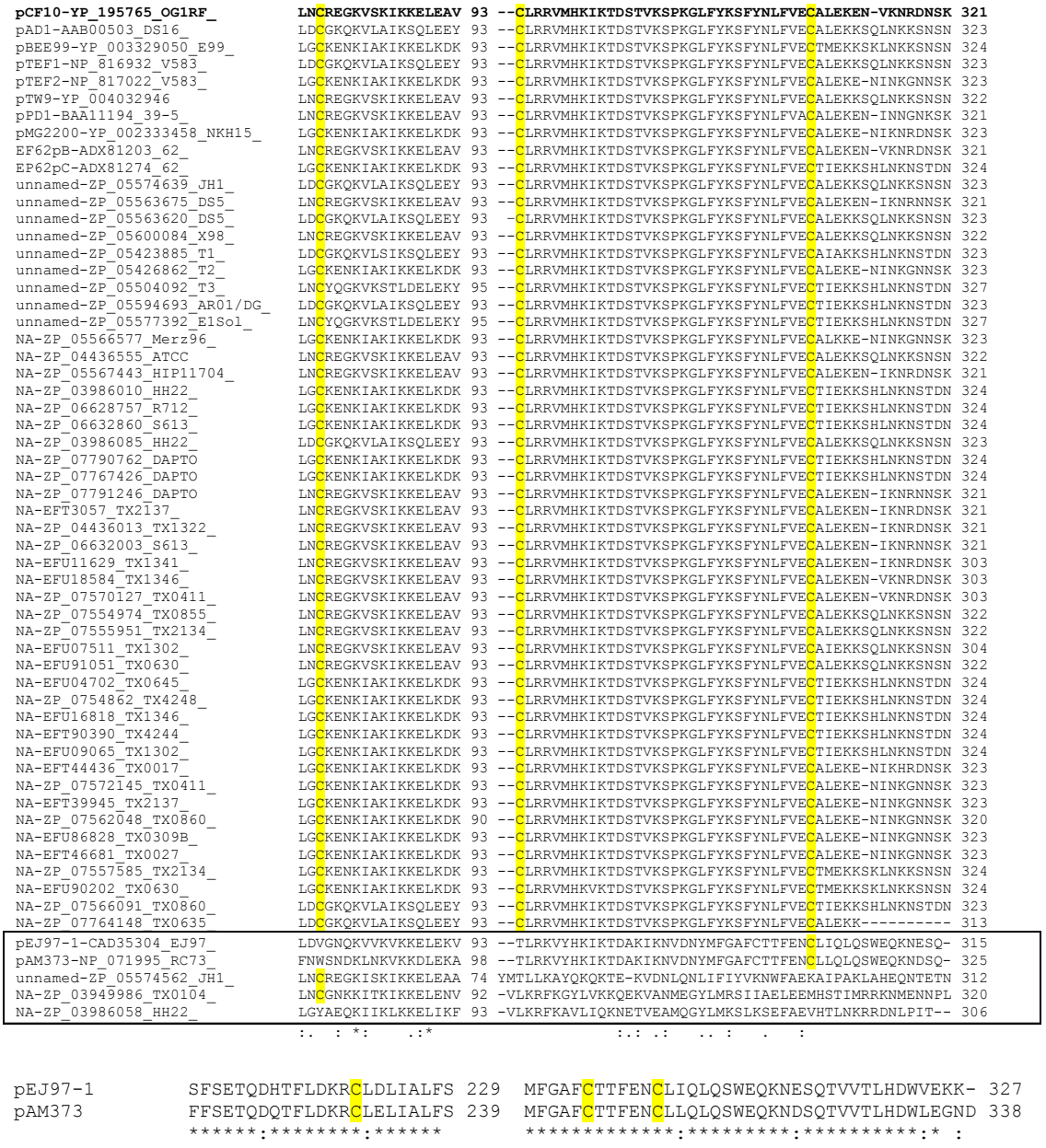


Figure 3. Conserved Cysteines within *E faecalis* RepA_N proteins.

(Top) Sequence comparisons performed using ClustalW2 multiple sequence alignment illustrates that a group of 54 known RepA_N proteins in *E faecalis* also contained three highly conserved cysteines. Sequences located within the box indicate five RepA_N homologs that contain 1 or 0 conserved cysteines. (Bottom) Conserved cysteines of pEJ97-1 and pAM373. Sequence label indicates plasmid name (if available), Genbank ID, and strain.

Disulfide Bond Formation in Bacteria

Disulfide Bonds

Disulfide bonds of proteins are formed by oxidation of two cysteine residues. Disulfide bonds can form within the same protein (intramolecular) or between several proteins (intermolecular) to stabilize the conformation of the protein or proteins. Proteins that form disulfide bonds can be divided into two groups: disulfide bonds that are a stable part of the protein structure and disulfide bond that form and break as the protein(s) alternate between oxidized and reduced states (Kadokura *et al.*, 2003). Disulfide bonds form when an oxidation reaction removes two electrons from each of the two sulfur groups of the cysteine residues. Disulfide bonds can be broken under reducing conditions when two electrons are donated to the two sulfur groups of the cystine residue (Kadokura and Beckwith, 2010).

Disulfide bonds form when the thiol group (S-H) of one cysteine loses an electron causing a nucleophilic attack on another cysteine thiol group. The second thiol group involved with the disulfide bond loses an electron forming a disulfide bridge between the two proteins (Moller and Hederstedt, 2006). Disulfide bonds in proteins can form at random, however at low rates (Anfinsen, 1973). Disulfide bonds forming spontaneously in the presence of an electron acceptor in vitro between two cysteine thiol groups can take hours or days (Kadokura and Beckwith, 2010). In vivo the reaction can occur much faster as oxidoreductases facilitate the formation of the disulfide bond pair (Ito and Inaba, 2008; Kouwen *et al.*, 2007). Prior dogma claimed disulfide bonds were unlikely to occur in the cytoplasm of bacteria because of the low reducing environment, (Kadokura *et al.*, 2003), however, a group of cytoplasmic proteins has been described which form disulfide

bonds in response to stress in both Gram-negative and Gram-positive bacteria. These stress response proteins are capable of mediating protein function based on the formation of intermolecular and intramolecular disulfide bonds, as well as, cysteine modifications.

Thiol-based Redox Switches: OxyR

OxyR is widely conserved in both Gram-positive and Gram-negative bacteria. OxyR orthologs are involved in regulating oxidative stress responses, virulence, biofilm formation, fimbral synthesis, and intestinal or urinary tract colonization (Antelmann and Helmann, 2011). In *E. coli*, OxyR has six cysteine residues. C199 and C209 cysteine residues are conserved, and mutational and structural analysis showed they are necessary for OxyR redox-sensing function (Lee *et al.*, 2004). OxyR disulfide bond formation is induced by the presence of reactive oxygen species which cause C199 oxidation and formation of a sulfenic acid intermediate. The C199 sulfenic acid intermediate forms an intramolecular disulfide bond with C208, forming an OxyR tetramer (Choi *et al.*, 2001; Lee *et al.*, 2004). Debate is ongoing as to whether OxyR is forming intramolecular disulfide bond to mediate function or whether cysteine modifications (sulfenate intermediate, S-nitrosylation, or S-glutathionylation) are causing the conformation and functional changes (Antelmann and Helmann, 2011; Kim *et al.*, 2002).

Thiol-based Redox Switches: Spx

Stress response proteins originally found in *Bacillus subtilis* Spx, OhrR, and YodB use disulfide bonds or cysteine modifications to control the function of proteins (Antelmann and Helmann, 2011). The thiol-redox regulator Spx regulates the *trxA* and

trxB genes based on formation of disulfide bonds between the C10 and C13 residues (Zuber, 2004, 2009). Oxidative stress conditions and thiol-depleting electrophiles induce the disulfide bond formation (Nakano *et al.*, 2003). Oxidized Spx interacts with the RNA polymerase α subunit which increases the transcription of thioredoxin/thioredoxin reductase system encoded by *trxA* and *trxB* (Lamour *et al.*, 2009; Newberry *et al.*, 2005). It has been shown that oxidized Spx and RNAP have increased binding to the *trxB* promoter compared to Spx alone and that this binding is sensitive to DTT (Nakano *et al.*, 2010). This result suggests that when Spx is oxidized, it recruits RNAP to thioredoxin/thioredoxin reductase gene promoters (Antelmann and Helmann, 2011). Under non-stress conditions Spx interacts with Zinc (II) binding adaptor protein YjbH. Spx-YjbH interaction leads to Spx degradation targeted to ClpXP (Garg *et al.*, 2009). It is speculated that ROS or electrophiles may lead to Zn release, causing the intramolecular disulfide bond formation and interaction with RNAP (Antelmann and Helmann, 2011).

Thiol-based Redox Switches: OhrR

OhrR is a MarR family transcription regulator that regulates the expression of the thiol-dependent peroxidase OhrA in response to organic hydroperoxides. Organic hydroperoxides are byproducts of metabolism (e.g. lipid hydroperoxides are derived from peroxyl radicals and unsaturated fatty acids) (Antelmann and Helmann, 2011). OhrR contains one highly conserved cysteine residue (C15) that is essential to the oxidative response in *B. subtilis* (Fuangthong and Helmann, 2002). OhrR dimers repress transcription of *ohrA* under non-oxidizing conditions. Under oxidizing conditions the C15 of the OhrR dimer subunits undergo cysteine modification, releasing binding form

the *ohrA* promoter and relinquishing repression. Depending on the level of oxidation, OhrR cysteine residues can go through various modifications. OhrR oxidized by cumene hydroperoxide forms a sulfenic acid intermediate (Fuangthong and Helmann, 2002) but still binds to the *ohrA* promoter region (Lee *et al.*, 2007). The strong oxidizer linoleic acid hydroperoxide causes OhrR to form irreversible sulfinic and sulonic acid forms (Soonsanga *et al.*, 2008a). There is no evidence for intermolecular disulfide bond formation in OhrR in *B. subtilis* (Antelmann and Helmann, 2011). OhrR in *Xanthomonas campestris* is similar to the one found in *B. subtilis*. However, *X. campestris* OhrR contains three conserved cysteines (C22, C127, and C131). The *X. campestris* OhrR repressor binding to the *ohrA* promoter is inhibited by intermolecular disulfide bonds form between the C22 and C127 residues (Newberry *et al.*, 2007; Panmanee *et al.*, 2006). It is interesting that proteins with similar functions and structures modify cysteine residues in different ways to produce the same regulatory effect in different species. Strikingly, introducing C127 (the conserved cysteine in *Xanthomonas campestris*) into *B. subtilis* allowed for OhrR to function as an intermolecular disulfide bond regulated protein (Soonsanga *et al.*, 2008b).

Thiol-based Redox Switches: YodB

YodB is a reactive electrophilic species (for diamides and quinomes) response regulator that is part MarR/DUF24 subfamily, which is a subfamily of MarR-family to which OhrR belongs. YodB acts as a regulatory factor that controls transcription of genes that provide-resistance to reactive electrophilic species RES (Antelmann *et al.*, 2008). Exposure to RES in vivo and in vitro inactivates YodB transcriptional repression. YodB

contains three conserved cysteine residues at C6, C101, and C108. C6 was determined to be the critical cysteine residue for in vivo DNA binding and redox sensing. It is believed that like OhrR of *X. campestris*, YodB forms intermolecular disulfide bonds between C6 and C101 (Chi *et al.*, 2010; Leelakriangsak *et al.*, 2008). The YodB transcriptional repressor is highly conserved in the low GC bacteria including *Staphylococcus, Enterococcus, Listeria, Bacillus* and *Clostridium* species. Cysteines are conserved among the MerR/DUF24 family that include YkrN, YfcD, YdzF, HxlR, and YvaP (Antelmann and Helmann, 2011).

Cytoplasmic Disulfide Bonds and Cysteine Modification in E. faecalis

Interestingly *E. faecalis* strains contain homologs to several MerR/DUF24 cysteine regulated proteins including more than 60 *E. faecalis* strains have homologs to HxlR (activator of formaldehyde detoxification *hxlAB* regulon) (Yurimoto *et al.*, 2005). Homologs tend to contain three cysteines including the conserved C6 cysteine that has been determined to be critical for function in YodB. BLASTP also shows that homologs of OhrR and Spx are found in Enterococci, giving evidence that proteins in the *E. faecalis* cytoplasm are likely capable of being controlled by disulfide bonds.

Specific Aims

Enterococci contain pheromone-responsive plasmids that are extremely stable, narrow host range plasmids and broad host range plasmids that are less stable but can replicate in multiple species. The broad aim of this project was to identify factors that

determine the stability and host range of enterococcal plasmids. Enterococcal pheromone-responsive plasmid pCF10 has a host range limited to *E. faecalis* and is maintained with a high stability throughout the bacterial population for a hundreds generations without selection. While the mature pheromone cCF10 controls pCF10 conjugation, the precursor pheromone pre-cCF10 is involved in determining plasmid host range. The host range of pCF10 could be extending by engineering a closely-related species such as *L. lactis* to produce pre-cCF10. pre-cCF10 interaction with pCF10 replication initiation protein PrgW appeared to be a possible link between the precursor pheromone and plasmid host range. Pre-cCF10 interaction with PrgW caused conformational changes visible on denaturing, non-reducing acrylamide gels. Initial evidence of regulation of PrgW conformation by disulfide bonds was obtained with Western blots of *E. faecalis* whole cell lysates where PrgW electrophoretic migration was sensitive to reduction. Therefore, it was hypothesized that pre-cCF10 induced conformational changes of PrgW depended on disulfide bond formation.

The major aim of this project was focused on determining if the conformation and function of an enterococcal, cytoplasmic, plasmid-encoded, replication initiation protein could be affected by disulfide bonds. PrgW is part of the RepA_N superfamily of proteins encoded by both plasmids and chromosomes of low-GC, Gram-positive bacteria that typically initiate plasmid replication by binding to repeating DNA sequences at the origin of replication. Sequence alignments were compared and it was found that a group of 54 of 59 known RepA_N proteins contained three highly conserved cysteines, and that these RepA_N proteins had a host range limited to *E. faecalis*. To study the importance of theses cysteines in pCF10, PrgW, single, double, and triple cysteine to alanine

substitutions were introduced in plasmids acting as a minimal origin of replication (pORI10), or as part of the basic stable replicon (pMSP6050). While all cysteine substituted PrgW mutant plasmids of pORI10 and pMSP6050 were still capable of replicating in *E. faecalis*, plasmid stability and copy number decreased, providing evidence that the cysteines were important to PrgW function. Additionally, Western blot analysis revealed PrgW cysteine to alanine substitutions appeared to reduce PrgW aggregation. All cysteine mutants and wild-type PrgW aggregations were resolved to a single band under reducing conditions.

Mutations of PrgW cysteines reduced pMSP6050 stability, but increased host range into *L. lactis.* Mutation of PrgW cysteines expanded pMSP6050 host range into *L. lactis*, a similar effect to engineering production of pre-cCF10. This result in concert with pre-cCF10-inducing PrgW multimerization lead to the idea that a pheromone precursor regulates multimerization through disulfide bonds of a replication protein, which limits the host range of pCF10 to the only natural produce of pre-cCF10, *E. faecalis*. This constitutes a novel mechanism for influencing plasmid stability and host range. Furthermore, in silico analysis showed RepA_N homologs lacking the three cysteines can also be found in other enterococci and staphylococci species, suggesting that the host-range of multiple plasmids may be affected by cysteine bond formation.

A final ongoing aim of this project was to understand how RepA_N plasmids may evolve to either maintain the conserved cysteines and remain restricted in host range to *E. faecalis* or lose the conserved cysteines and exist as broad host range plasmids. In silico studies revealed RepA_N proteins contained a conserved N-terminus domain common among all Gram-positive RepA_N proteins including those from *E. faecium, E. faecalis*,

and *S. aureus*. Phylogenetic analysis of N-terminal amino acid sequences revealed clusters that contained plasmids from multiple species with or without C78. However, phylogenetic analysis of C-terminal sequences revealed that the plasmids tended to cluster into species-specific groups; with the conserved cysteines C275 and C307 in proteins from *E. faecalis*, and proteins from other species lacking the conserved cysteines. This distinction suggested that there may be modular evolution of the proteins, with swapping of N-terminal and C-terminal domains. *E. faecalis* JH1 strain contained two plasmids, pJH1 with an *E. faecium* conserved C-terminus for the RepA_N protein, and pJH2 an *E. faecalis* conserved C-terminus. Ongoing experiments are being done to determine if the host range of pJH1 and pJH2 are dictated by the C-terminus domain. Modular evolution that dictated whether a RepA_N protein contained a C-terminus domain with two of three conserved *E. faecalis* cysteines would then be a source of host range limitation.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Bacterial Strains

Bacterial strains used in this paper are described in

Table 1. *Escherichia coli* DH5α was used in construction of recombinant plasmids. *E. coli* BL21 was used in overexpression of recombinant His_6-PrgW encoded on pET28b vector. *Enterococcus faecalis* OG1RF was used to study recombinant plasmid stability, copy number, PrgW protein aggregation, PrgW localization, and polymicrobial biofilms. *Streptococcus mutants* UA159 was used to study pheromone-induced PrgW aggregation and recombinant plasmid host range. *Lactococcus lactis* LM0230 was used to study recombinant plasmid host range. *Enterococcus hirae 9790* was used to study PrgW aggregation.

Growth Conditions

E. coli DH5α and BL21 were cultured in Luria broth (LB; Appendix) and Terrific broth (TB; Appendix), respectively. The *E. coli* strains were incubated at 37^{o}C, 250 rpm in 50 mL conical tubes. *E. faecalis* OG1RF was cultured in Becton, Dickinson and Company (Sparks, MD, USA) Bacto^{TM} Todd Hewitt (TH) Broth (Catalog # 249230; Appendix) and incubated at 37^{o}C, without shaking; following electroporation, cultures were grown in TH with 0.25 M sucrose (Appendix). *L. lactis* LM0230 strain was

Table 1. Strains used in this study.

Strain	Description	Reference
E. coli **BL21**	Protein expression host; F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm ton*A	Novagen
E. coli **DH5α**	Cloning host; [F^- *endA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1* λ^- *recA1 gyrA96 relA1* Δ(*lacZYA-argF*) *U169* Φ*80dlacZ*Δ*M15*]	Gibco BLR
E. faecalis **OG1RF**	Plasmid-free laboratory reference strain; Gel^+, Spr^+, Rif^R, Fus^R.	(Dunny *et al.*, 1978)
E. hirae **9790**	Laboratory strain	ATCC 9790
L. lactis **LM0230**	Plasmid-free laboratory reference strain Lac^-, Prt^-	(McKay *et al.*, 1976)
S. mutans **UA159**	Plasmid-free laboratory strain.	(Murchison *et al.*, 1986)

cultured in Becton, Dickinson and Company M-17 Broth (Catalog # 218561; Appendix) with 0.5% glucose (GM-17); following electroporation, cultures were grown in GM-17 with 20% sucrose. *L. lactis* cultures were incubated at 30^{o}C, without shaking. *S. mutans* UA159 was cultured in TH; for transformation, it was grown in TH with 10% glucose and MP Biomedicals, Inc. (Solon, Ohio, USA) 10% heat-inactivated horse serum (Appendix). *S. mutans* was incubated at 37^{o}C, 5% CO_2, without shaking.

The final concentrations of antibiotics used for this study were as followed: for *E. coli*, kanamycin at 50 μg ml^{-1}, chloramphenicol at 30 μg ml^{-1} and erythromycin at 300 μg ml^{-1}; for *E. faecalis*, erythromycin at 50 μg ml^{-1} and tetracycline at 15 μg ml^{-1}; for *L. lactis*, erythromycin at 20 μg ml^{-1}; for *S. mutans*, erythromycin at 25 μg ml^{-1}, spectinomycin at 250 μg ml^{-1}, kanamycin at 300 μg ml^{-1}, rifampicin at 0.5 μg ml^{-1}, chloramphenicol at 15 μg ml^{-1}; and for *E. hirae*, erythromycin at 50 μg ml^{-1}. Kanamycin (monosulfate) was obtained from Fisher BioReagents (Fair Lawn, NJ, USA). Erythromycin (98%) was obtained from Acros Organics (Geel, BE). Chloramphenicol was obtained from Acros Organics. Tetracycline hydrochloride was obtained from Acros Organics. Spectinomycin Sulfate was obtained from MP Biochemicals (Solon, OH, USA). For reference, growth conditions are summarized in Table 2.

Table 2. Growth Conditions and Antibiotic Concentrations.

Species and Strain	Media	Conditions	[Antibiotic] When Used (μg/mL)
E. coli BL21	TB	37°C; 250 rpm; atmosphere	Kan 50
E. coli DH5α	LB	37°C; 250 rpm; atmosphere	Erm 300; Kan 50; Cam 30
E. faecalis OG1RF	TH, TH-G, TH-S,	37°C; closed	Erm 50; Tet 10
E. hirae 9790	TH	37°C; closed	Erm 50
L. lactis LM0230	GM-17	30°C; closed	Erm 20
S. mutans UA159	TH	37°C; 5% CO_2	Erm 25; Kan 300; Rif 0.5; Cam 15

Closed = closed lid; atmosphere= loose lid; Kan = Kanamycin; Erm = Erythromycin; Tet = Tetracycline; Cam = chloramphenicol; Rif = Rifampicin.

Plasmid isolation and purification from *Escherichia coli*

Preparation of Cleared Lysate

E. coli DH5α with appropriate antibiotic was inoculated from appropriate selective LB plates. *E. coli* was incubated in 15 to 25 mL LB with appropriate antibiotic in Becton, Dickinson and Company Falcon™ 50 mL conical tube (Catalog # 352098). Volume depended on vector copy number. Cultures were then incubated at 37°C, 250 rpm overnight for 12 to 16 hours; RNA contamination remained low when incubation was for less than 16 hours. According to the Promega Corporation (Madison, WI, USA) Manual # TB173 cultures can be incubated in between 10 to 100 mL to yield 100 to 200 μg of plasmid DNA.

Overnight cultures of *E. coli* were centrifuged at 10,000 x *g* (9,000 rpm; Beckman J2-HS) for 10 min at 4°C. Supernatant was removed. The pellet was used immediately or stored for up to one week at -20°C. Plasmid isolation from overnight cultures was performed using Promega Corporation Wizard® Plus SV Midipreps DNA Purification System kit (Catalog # A7640; Protocol # TB173). The cell pellet was resuspened in 3 to 5 mL Promega Corporation Resuspension Solution (Appendix; Catalog # A7115). Next, 3 to 5 mL of Promega Corporation Lysis Buffer (Appendix; Catalog # A7125) was added and inverted four to six times (slowly) to thoroughly mix; did not incubate longer than five minutes as alkaline conditions would have denature plasmid DNA. Next, 3 to 5 mL of Promega Corporation Neutralization Solution (Appendix; Catalog # A1485) was added which renatured the plasmid DNA and removed suspension of aggregated chromosomal DNA and debris. Conical tubes were inverted rapidly to mix the Lysis and Neutralization

Solutions. Neutralized cells were centrifuged at 14,000 x *g* (11, 000 rpm; Beckman J2-HS) for 10 minutes.

Plasmid DNA Purification

The supernatant was removed without transferring debris and supernatant was transfered through a cheesecloth placed over 50 mL Falcon™ conical tube to filter. Then, 3 to 5 mL of the Promega Corporation DNA Binding Resin (Catalog # A7701) was added to the filtered plasmid preparation supernatant. Supernatant was transferred to Promega Corporation Wizard® Midicolumns (Catalog #A7651). Promega Corporation Vac-Man® Vacuum Manifold (Catalog # A7231) vacuumed supernatant through midicolumn. 15 mL of Plasmid Column Wash Solution (Appendix) was added two times and vacuum was applied to the columns after each wash. Electroporation grade plasmids were washed in the midi-column with an additional 3 mL of 70 percent Pharmco-AAPER (Brookfield, CT, USA) Ethanol (Catalog # 111ACS200). The washed columns were not allowed to dry over vacuum for more than 30 seconds. Residual ethanol was removed by placing bottom portion of midi-column in centrifuge tube. Midi-column was then centrifuged for two minutes at 10,000 x *g* and again for one minute with centrifuge lid removed at 10,000 x *g*.

Elution

Between 125µl and 300µl of pre-heated dH_2O (65^oC to 85^oC) was added to plasmid DNA on the midi-column; dH_2O on columns incubated for one to five minutes; for plasmids greater than 10 kb, water was pre-heated to 80^oC. The DNA column was

then centrifuged at 10,000 x *g* for 30 seconds. After elution, dH_2O was heated at 65°C with lid off, with Kimberly-Clark® (Chester, PA, USA) Kimwipes™ over top of tubes for 10 to 20 minutes to evaporate residual EtOH and inactivate nucleases. Plasmid DNA was then measured with spectrophotometer or EtBr visualization on agarose gel (see below).

DNA Manipulations

DNA Electrophoresis

Plasmid and PCR amplified DNA was analyzed by separating DNA based on size using agarose gel electrophoresis. Fisher BioReagents Molecular Biology Grade Low EEO/Multipurpose Agarose (Catalog # BP160-500) was dissolved in 1 X TAE Buffer (Appendix); 0.8% agarose for plasmids; 1% agarose general use; and 2% agarose for PCR products. Agarose dissolved within 3 to 5 minutes under high microwave power, which depended on volume and agarose concentration. Dissolved agarose was incubated at 55°C until cool to touch. The appropriate amount of melted agarose gel was poured into FisherBiotech (Fair Lawn, NJ, USA) Mini or Midi-Horizontal Electrophoresis Systems (Catalog # FB-SB-710 and FB-SB-1316) with the respective FisherBiotech UVT gel tray (Catalog # FIS-UVT-710 and FIS-SB-1316). The appropriate comb was immediately placed into slot of UVT gel tray and the gel set.

The gel tray was removed from the gel casting position and the side of the gel with combs was aligned near to the negative (black) electrode which allowed DNA to move to positive (red) electrode. Electrophoresis system was filled with 1X TAE Buffer until top of gel is submerged. The comb was removed from the gel and, in addition,

removed any accompanying air bubbles in well of gel with pipette. Promega Corporation Blue/Orange 6X Loading Dye (Catalog # G190A) was added to DNA sample; each DNA sample was then added to wells of the DNA gel. If amplified PCR DNA was greater than 1000 base pairs or plasmid DNA was being separated, 10 µl of Fisher BioReagents Routine DNA Ladder > 1 kb Full Scale Ladder (Catalog #BP2582-200) was added to one of the gel wells; 10 µl of Promega Corporation 100 bp DNA Ladder (Catalog # G210A) was added to wells if PCR amplified DNA was less than 1000 base pairs. DNA was subjected to 80 to130 V using FisherBiotech Electrophoresis systems power supply (Catalog # FB105) or comparable unit, for 30 to 60 minutes based on size of gel, expected size of the DNA, and desired quality of resolution.

Following electrophoresis, the agarose gel was stained for 10 to 30 minutes in a 1X TAE bath containing 0.5 µg/ml Acros Ethidium Bromide (Catalog # 327220010). Agarose gels were placed in dH_20 bath for 5 to 10 minutes to rinse off residual EtBr. Gels were visualized on an ultraviolet transilluminator and were recorded with a Polaroid camera, digital camera, or Bio-Rad (Hercules, CA, USA) Laboratories GelDoc™ XR Universal Hood II (Catalog # 170-8170) transilluminator capture system.

DNA Extraction from Agarose Gel

Using a razor, the region containing DNA of interest was precisely excised from the agarose gel. DNA excised from agarose gel was purified using Promega Corporation Wizard® SV Gel and PCR Clean-Up System (Protocol # TB308) as followed: 1 µl of Promega Corporation Membrane Binding Solution (Appendix) was added per mg DNA-gel slice. DNA-gel slice in Membrane Binding solution was incubated at 55°C (50 to

65^{o}C) for 10 to 20 minutes (periodically vortexed, unless DNA segment was less than 5 kb) until dissolved. Importantly, gel-DNA mixture was allowed to cool to room temperature as increased temperature affected DNA biding to column. Gel suspension was added to Promega Corporation SV Minicolumn (Catalog # A129A) that had been placed over a Eppendorf (Hauppauge, NY, USA) 1.5 mL centrifuge tube (Catalog # 022363204); did not add more than 700 µl of gel mixture at one time to column. The column was centrifuged at 16,000 x *g* (14,000 rpm; Eppendorf Centrifuge 5415C) for one minute and discarded flow-through liquid.

700 µl of Promega Corporation Membrane Wash Solution (Catalog # A929C; Appendix) was added to DNA column. DNA column was centrifuged at 16,000 x *g* (14,000 rpm) for one minute to wash the column. After the flow through was removed, 700 µl of the Promega Membrane Wash Solution was added to DNA column. The column was then centrifuged at 16,000 x *g* (14,000 rpm; Eppendorf Centrifuge 5415C) for one minute. The flow-through was discarded and the DNA column was again centrifuged at 16,000 x *g* (14,000 rpm; Eppendorf Centrifuge 5415C) for five minutes to help ensure that the residual alcohol in wash column was removed. The column was inserted into a new Eppendorf 1.5 mL centrifuge tube and eluted with 35 to 50 µl Promega Corporation Nuclease-Free Water (Catalog # P1193); water was applied directly to center of column without touching membrane. Water was then incubated at room temperature for one to five minutes on top of the membrane and then centrifuged at 16,000 x *g* (14,000 rpm; Eppendorf Centrifuge 5415C) for one minute. If alcohol remained in sample, the eluted plasmid was incubated at 65^{o}C for 5 to 10 minutes with

the Eppendorf 1.5 mL centrifuge tube top open (Kimwipes™ were placed on top of open tube).

DNA Cleanup

Qiagen (Valencia, CA, USA) QIAquick® PCR Purification Kit (Catalog # 28106; QIAquick Spin Handbook 03/2008) was used to rapidly purify and/or concentrate DNA after several procedures that included: (1) Removal of $O.D._{230}$ contaminants that remained in the DNA eluate resulting from plasmid purification with the Promega Corporation Wizard® Plus SV Midipreps DNA Purification System; (2) Cleanup that followed with a restriction endonuclease digestion; (3) Cleanup that followed DNA ligation and 5' dephosphorylation; and (4) cleanup that followed PCR amplification.

A DNA sample purified and/or concentrated using the QIAquick® PCR Purification Kit was first added to five volumes of Qiagen Buffer PB (Catalog # 19066) and allowed to sit at room temperature for one minute. Next, the Buffer PB-DNA mixture was placed in a QIAquick® spin column (Catalog # 28106) and centrifuged at 16,000 x *g* (14,000 rpm; Eppendorf Centrifuge 5415C) for one minute. 750 µl of Qiagen Buffer PE (Catalog # 19065) was added to the wash column and centrifuged at 16,000 x *g* (14,000 rpm; Eppendorf Centrifuge 5415C) for two minutes. 35 to 50 µl of Qiagen Buffer EB (Appendix) was added to center of membrane, incubated at room temperature for five minutes, and then centrifuged at 16,000 x *g* (14,000 rpm; Eppendorf Centrifuge 5415C) for 30 seconds to elute DNA. The final DNA concentration was measured using spectrophotometer or by EtBr stained agarose gel visualization.

DNA Concentration Determination

The Thermo Scientific® (Wilmington, DE, USA) NanoDrop™ 1000 Spectrophotometer (Catalog #) was used to determine chromosomal and plasmid DNA concentrations. The NanoDrop™ provided rapid measurement of DNA concentration at $O.D._{260}$ with low amounts of sample. Additionally, the NanoDrop™ measured $O.D._{230}$ and $O.D._{280}$ to estimate contaminant levels with the DNA preparations. The NanoDrop was accurate in determining plasmid concentrations greater than 25 ng/µl. Therefore, the NanoDrop™ provided accurate measurements for chromosomal DNA and higher copy number plasmids. If needed, plasmid concentrations were verified by visualization in an EtBr stained agarose gel (see below).

Operation of the NanoDrop™ was performed by first starting the ND-1000 (V3.5.2) computer software. After opening the ND-1000 program, 2 µl dH_2O was added to the NanoDrop™ sensor and then cleaned with Kimwipes™. Then, the 'Nucleic Acid' button on the ND-1000 pop-up screen was selected and 'OK' was pressed. An additional 2 µl of dH_2O was added to the sensor and 'OK' was selected on the initialization prompt. Sample type was 'DNA 50' was selected on measurement screen. dH_2O was removed with Kimwipes™, 2 µl of blank (dH_2O or TE buffer) was added, and 'Blank' button on the prompt was selected. The blank was removed from the sensor with Kimwipes™, 2 µl of chromosomal or plasmid DNA sample was added, and 'Measure' button was pressed. If measurement of additional samples was needed, it was not necessary to recalibrate the blank. The sensor was "cleaned" with the appropriate blank between samples.

If needed, DNA concentrations were determined with DNA agarose gel. Agarose gels were casted, DNA was electrophoresed, and DNA was stained with EtBr as

described in DNA Electrophoresis section. 10 µl of Fisher BioReagents Routine DNA Ladder > 1kb Full Scale Ladder was added to get an accurate comparison of sample band to the DNA ladder standards. Intensity and size of DNA band was estimated and compared to the ladder standards with similar migration on agarose gel. Total DNA weight was calculation depended on volume of DNA loaded onto agarose gel. Standards for BP2582-200 DNA ladder were described in Table 3.

Restriction Digestions

Restriction digestions of plasmids and PCR products were performed using Promega Corporation or New England Biolabs (Ipswich, MA, USA) restriction enzymes. Plasmids were digested for one to two hours depending on quality of DNA and buffer compatibility. PCR products were digested for one to four hours depending on location of digestion site (longer if digestion site was on end of amplification). Restriction digest conditions were performed according to Table 4.

The digested plasmids were cut from 0.8% agarose gel and purified using Wizard® SV Gel and PCR Clean-Up System (Catalog. # A9282) to ensure wild-type DNA was removed. PCR products were generally digested from PCR reaction purified form Qiagen QIAquick PCR Purification Kit (Catalog # 28106). Restriction digestion reactions were stopped by heat inactivation or cleanup, depending on heat inactivation capabilities of restriction digestion enzyme and/or concentration of DNA.

Table 3. DNA Ladder Weight Standards.

kbp	1.0	8.0	6.0	4.0	3.0	2.0	1.55	1.4	1.0	0.75	0.5	0.4	0.3	0.2	0.1	0.05
ng	30	30	45	60	85	150	100	100	120	30	60	20	40	30	20	15

Fisher BioReagents Routine DNA Ladder > 1kb Full Scale Ladder. kb = kilobase paris of DNA; ng = nanograms of DNA

5' DNA Dephosphorylation

To prevent the recircularization and relegation of linearized plasmid DNA, 5' dephosphorylation was performed using Promega Corporation Shrimp Alkaline Phosphatase (SAP; Catalog#. M820A; Protocol # 9PIM820). If possible, prior to the dephosphorylation reaction (DNA concentration could be too low) the restriction digestion buffer was removed using the QIAquick PCR Purification Kit (Catalog # 28106). DNA eluted in dH_2O or TE buffer was incubated with one unit SAP per μg DNA at 37^{o}C for 15 minutes in 1X Promega Corporation SAP Reaction Buffer (Appendix; Catalog # M821A). One unit of SAP was sufficient amount to dephosphorylate blunt and sticky end overhang. Total volume of reaction mixture was typically 30 to 50 μl. SAP was inactivated by heating to 65^{o}C for 15 minutes. Restriction digestion (involving Promega Corporation Restriction Enzymes) and dephosphorylation treatments were at times performed in combination by adding 10 units of SAP to restriction digestion mixture following heat inactivation.

Table 4. Restriction Digestion Enzymes and Conditions.

Restriction Enzyme	Restriction Site	Manufacturer	Catalog #	Buffer	BSA	Temp	Heat inactivation
***Bsr*GI**	**T/GTACA**	NE Biolabs	R0575S	Buffer 4, Buffer 2****	100 µg/mL	37°C	80°C for 20 min
***Hha*I**	**GCG/G**	NE Biolabs	R0139S	Buffer 4	100 µg/mL	37°C	65°C for 20 min
***Stu*I**	**AGG/CCT**	NE Biolabs	R0187S	Buffer 2****	N/A	37°C	65°C for 20 min
***Bam*HI**	**G/GATCC**	Promega	R6021	Buffer E**, Buffer H*	100 µg/mL	37°C	65°C for 20 min
***Pst*I**	**CTGCA/G**	Promega	R6111	Buffer H*	100 µg/mL	37°C	65°C for 20 min
***Xba*I**	**T/CTAGA**	Promega	R6181	Buffer D, Buffer E**	100 µg/mL	37°C	N/A
***Eco*RI**	**G/AATTC**	NE Biolabs	R0101S	EcoR1 Buffer, Buffer 4***	N/A	37°C	65°C for 20 min

(*) Buffer H was most compatible been PstI and BamHI; (**) Buffer E was most compatible between XbaI and BamHI; (***) Buffer 4 was most compatible between EcoRI and NdeI; (****) Buffer 2 was most compatible between BsrGI and StuI. Buffer components are listed in the Appendix.

Ligation

Ligation of DNA was performed using Promega Corporation T4 DNA Ligase (Catalog. # M180A; Protocol # 9PIM822). If concentration was sufficient, DNA was purified using Qiagen QIAquick PCR Purification Kit (Catalog # 28106) prior to ligation (elute in dH_2O or TE buffer). Ligation mixtures of 1:1, 1:2, and 1:4 vector to insert DNA were formulated for ligation. Ratio was based on size of DNA segments as followed:

(ng vector x kb size insert)/(kb size vector) x molar ratio of inset: vector = ng insert

Typically 100 ng of vector was used, 50 ng of vector was sufficient if DNA concentration was dilute. Ligation mixture generally equaled a total volume of 10 to 25 µg which included: 2X Rapid Ligation Buffer (Appendix), 1 µl T4 DNA Ligase (three units) and Nuclease-free water. Reaction was incubated at room temperature for 5 to 15 minutes depending if the reaction involved a cohesive-ended or blunt-ended ligation.

Synthetic Nucleotide Design

Oligonucleotide primers were routinely designated by Clone Manager 5 for Windows 95 (Sci. Ed Central, Cary, NC; Serial No. 374-019-1385; Copyright 1995-1999). Designed primers from the Clone Manger 5 software were routinely evaluated based on melting temperature, GC content, hairpin formation, and primer hetero- and homo-dimer formation using IDT OligoAnalyzer (current version 3.1; Copyright 2011). The IDT OligoAnalyzer software can be accessed on the "analyze" link associated with pending orders on the Integrate DNA Technologies (Coralville, Iowa, USA) website (www.idtdna.com). The primers received from IDT DNA Technologies primers were of

lyophilized form and were resuspended in sterile dH_2O to a concentration of 100 pmol/µl (100µM). Primers were diluted as needed to a working concentration of 10 pmol (10 µM). Oligonucleotides generated are listed in Table 5.

Polymerase Chain Reaction

Genes were amplified for sub-cloning and screening by polymerase chain reaction (PCR). High fidelity Stratagene (Santa Clara, CA, USA) *Pfu* polymerase (Catalog # 600135-81) was used to amplify DNA for sub-cloning to prevent PCR related error. Promega Corporation GoTaq® (Catalog # M300B) was utilized for screening in order to verify appropriate mutant gene that was found in recombinant bacteria. Approximately 100 µg DNA was used for each reaction. Plasmid DNA was purified with QIAquick PCR Purification Kit prior to PCR if difficulties arose with the template plasmid isolated from Wizard® Plus SV Minipreps DNA Purification System. *Pfu* polymerase reactions were formulated as followed: 5 µl of Stratagene 10X Buffer (Catalog # 600135-84); 0.4 µl of 25 mM Promega Corporation dNTP's (Catalog # U151B); 1.25 µl of forward primer (10 µM); 1.25 µl of reverse primer (10 µM); 1 µl of *Pfu* polymerase; q.s. with sterile dH_20 to 50 µl. GoTaq® polymerase reactions were formulated as followed: 10 µl of Promega Corporation GoTaq® 5X Buffer (Catalog # M791B); 2.5 µl of 10 mM

Table 5. Primers Designed in this Study

PCR Template Plasmid	Primer	Sequence 5'→3'	R.D. Site	Plasmid Vector	Plasmid generated
pORI10	bdu01 (Tm58) bdu04 (Tm58) bdu25 bdu26	5'-CACATTCTTGCCCGCCTGAT 5'-ACCGTAACACGCCACATCTTG 5'-CCTTCTCGCGCATTTAGCAGAGTC 5'-GCTAAATGCGCGAGAAGGAAAAG	*Eco*R1	pVA891	pORI-078
pORI10	bdu01 (Tm 60) bdu04 (Tm 60) bdu27b bdu28	5'-CACATTCTTGCCCGCCTGATGAA 5'-ACCGTAACACGCCACATCTTGC 5'-CACGACGCAGCGCTTTGTCTATTTCTT 5'-GACAAAGCGCTGCGTCGTG	*Eco*R1	pVA891	pORI-275
pORI10	bdu01 (Tm 60) bdu04 (Tm 60) bdu29b bdu30	5'-CACATTCTTGCCCGCCTGATGAA 5'-ACCGTAACACGCCACATCTTGC 5'-TTTCTTTTTCTAGCGCCGCTTCAACAAATAAATTAT 5'-TGTTGAAGCGGCGCTAGAAAAAGAA	*Eco*R1	pVA891	pORI-307
pORI-078	bdu01 (Tm 60) bdu04 (Tm 60) bdu27b bdu28	5'-CACATTCTTGCCCGCCTGATGAA 5'-ACCGTAACACGCCACATCTTGC 5'-CACGACGCAGCGCTTTGTCTATTTCTT 5'-GACAAAGCGCTGCGTCGTG	*Eco*R1	pVA891	pORI-078/275
pORI-078	bdu01 (Tm 60) bdu04 (Tm 60) bdu29b bdu30	5'-CACATTCTTGCCCGCCTGATGAA 5'-ACCGTAACACGCCACATCTTGC 5'-TTTCTTTTTCTAGCGCCGCTTCAACAAATAAATTAT 5'-TGTTGAAGCGGCGCTAGAAAAAGAA	EcoR1	pVA891	pORI-078/307
pORI-307	bdu01 (Tm 60) bdu04 (Tm 60) bdu27b bdu28	5'-CACATTCTTGCCCGCCTGATGAA 5'-ACCGTAACACGCCACATCTTGC 5'-CACGACGCAGCGCTTTGTCTATTTCTT 5'-GACAAAGCGCTGCGTCGTG	*Eco*R1	pVA891	pORI-275/307
pORI-78/275	bdu01 (Tm 60) bdu04 (Tm 60) bdu29b bdu30	5'-CACATTCTTGCCCGCCTGATGAA 5'-ACCGTAACACGCCACATCTTGC 5'-TTTCTTTTTCTAGCGCCGCTTCAACAAATAAATTAT 5'-TGTTGAAGCGGCGCTAGAAAAAGAA	*Eco*R1	pVA891	pORI-078/275/307
pORI-078	bdu31 bdu32	5'-ACAAATCTTTATCCAACGACGCAAA 5'-TCAAAGTCGCTACCTGTTCTACTT	*Bsr*GI *Stu*I	pMSP6050	pMSP6-078
pORI-275	bdu31 bdu32	5'-ACAAATCTTTATCCAACGACGCAAA 5'-TCAAAGTCGCTACCTGTTCTACTT	*Bsr*GI *Stu*I	pMSP6050	pMSP6-275
pMSP6050	bdu33 GC-bdu31b bdu31 bdu30 bdu29b bdu34	5'-ACAGGAGGATCAACTTGTACCGGTC 5'-CAATTTTTGCGTCGTTGGATAAAGATTTG 5'-ACAAATCTTTATCCAACGACGCAAA 5'-TGTTGAAGCGGCGCTAGAAAAAGAA 5'-TTTCTTTTTCTAGCGCCGCTTCAACAAATAAATTAT 5'-GGTTGACCAGCTGCAATGGCT	*Pst*I	pMSP6050	pMSP6-307
pORI-078/275	bdu31 bdu32	5'-ACAAATCTTTATCCAACGACGCAAA 5'-TCAAAGTCGCTACCTGTTCTACTT	BsrGI StuI	pMSP6050	pMSP6-078/275
pMSP6-078	bdu33 GC-bdu31b bdu31 bdu30 bdu29b bdu34	5'-ACAGGAGGATCAACTTGTACCGGTC 5'-CAATTTTTGCGTCGTTGGATAAAGATTTG 5'-ACAAATCTTTATCCAACGACGCAAA 5'-TGTTGAAGCGGCGCTAGAAAAAGAA 5'-TTTCTTTTTCTAGCGCCGCTTCAACAAATAAATTAT 5'-GGTTGACCAGCTGCAATGGCT	*Pst*I	pMSP6-307	pMSP6-078/307
pMSP6-275	bdu33 GC-bdu31b bdu31 bdu30 bdu29b bdu34	5'-ACAGGAGGATCAACTTGTACCGGTC 5'-CAATTTTTGCGTCGTTGGATAAAGATTTG 5'-ACAAATCTTTATCCAACGACGCAAA 5'-TGTTGAAGCGGCGCTAGAAAAAGAA 5'-TTTCTTTTTCTAGCGCCGCTTCAACAAATAAATTAT 5'-GGTTGACCAGCTGCAATGGCT	*Pst*I	pMSP6-307	pMSP6-275/307

pMSP6-078/275	bdu33 GC-bdu31b bdu31 bdu30 bdu29b bdu34	5'-ACAGGAGGATCAACTTGTACCGGTC 5'-CAATTTTTGCGTCGTTGGATAAAGATTTG 5'-ACAAATCTTTATCCAACGACGCAAA 5'-TGTTGAAGCGGCGCTAGAAAAAGAA 5'-TTTCTTTTTCTAGCGCCGCTTCAACAAATAAATTAT 5'-GGTTGACCAGCTGCAATGGCT	*Pst*I	pMSP6-307	pMSP6-078/275/307
pORI-078/275	bdu31 bdu32	5'-ACAAATCTTTATCCAACGACGCAAA 5'-TCAAAGTCGCTACCTGTTCTACTT	BsrGI StuI	pMSP6050 Δpar	pMSP6-078/275Δpar

Real-Time PCR

prgW	bdu41	5'-GACAAATGTTTGCGTCGTGTGATGC
	bdu42	5'-AGCCAATTATGCAAAGTGATTTCAGAACCT
rpoA	bdu45	5'-TTCTCAACCATTCCAGGTGTACGAGA
	bdu46	5'-TTAGGATCTCAACATCACTGTCCACGA

Universal eubacterial 16S primers

16s rRNA	EubA	5'-AAGGAGGTGATCCACCCACA
	EubB	5'-AGAGTTTGATCCTGGCTCAG

dNTP's; 2.5 µl of forward primer (10 µM); 2.5 µl of reverse primer (10 µM); 0.25 µl of GoTaq® polymerase; q.s. with dH_20 to 100 µl. MJ Research, Inc. (Waltham, MA, USA) Programmable Thermocycler Controller (PTC-1000™) was used to amplify DNA contained within the reaction. Thermocycler did not have a heated lid so 25 to 50 µl Sigma-Aldrich Corporation (St. Louis, MO, USA) mineral oil (Catalog # M5904) was added to the top of the reaction. Thermocycler reaction was performed according to Table 6.

Final PCR reactions were stored at -20°C until needed (up to one week) or used immediately. GoTaq mixer loading buffer and was ready to use for agarose gel loading. Promega Corporation Blue/Orange 6X Loading Dye was added to *Pfu* mixture prior to gel loading. DNA was separated with one to two percent agarose gel.

Table 6. PCR Conditions Using GoTaq® and *Pfu* Polymerase.

Step #	Temperature	GoTaq®	*Pfu*
1	95°C	2 min	2 min
2	95°C	30 sec	45 sec
3	-5°C below Tm	30 sec	45 sec
4	72°C	1 min per 1000 bp	1 min per 500 bp
5	**Repeat steps 2-4**	**30-40X**	**29 X**
6	72°C	7-10 min	7-10 min
7	4°C	∞	∞

Overlap-extension PCR

Overlap-extension PCR (OL-PCR) was used to generate cysteine to alanine point mutations, translational fusions, and used for generating long amplified inserts. OL-PCR amplified two sections of a gene that complementary base paired: (1) The three prime end of the upstream portion of the gene and; (2) The five prime end of the downstream portion of the gene. Nucleotide substitutions were at times designed in the overlapping region that generated point mutations that are encoded in the recombinant DNA. *Pfu* polymerase was used for this procedure as it has a high fidelity and unlike GoTaq®, would not add a run of adenines to the five prime end of the DNA. The mixture was prepared as followed: 50 ng of upstream amplification and 50 ng of downstream amplification (adjusted if amplification sizes varied); 10 µl of 10X Buffer; 0.8 µl of 25 mM dNTP's; 2 µl of *Pfu* polymerase; q.s. with dH_20 to 100 µl. After first six cycles,

added 2.5 µl of forward primer and 2.5 µl of reverse primer. Thermocycler was programmed as followed for overlap-extension PCR (Table 7).

Table 7. Thermocycler Conditions for Overlap-Extension PCR

Step #	Temperature	Time
1	95°C	5 min
2	95°C	45
3	45°C	45
4	72°C	45
5	**Repeat steps 2-4 (5X)**	
6	95°C	45
7	-5°C below Tm	45
8	72°C	500 bp per min
9	**Repeat steps 6-8 (29X)**	
10	72°C	10 min
11	4°C	∞

Overlap-extension PCR to Generate Cysteine to Alanine Substitutions

Cysteine to alanine substitutions in *prgW* were generated using overlap-extension PCR using oligonucleotides listed in Table 5. Additional silent mutations that encoded a restriction site, HhaI (GCGC), were added to the overlapping PCR product for screening purposes. The *prgW* variants were introduced into the *E. coli* plasmid pVA891 (derivative of p15a), a vector that does not contain a Gram-positive origin of replication

and encodes for erythromycin resistance (Lindler and Macrina, 1986). Overlap-extension PCR amplifications of *prgW* (encoded in pORI10) were generated using Stratagene (Santa Clara, CA, USA) *Pfu* polymerase. pVA891 (or cysteine mutant plasmid derivatives) and mutant *prgW* amplifications were digested with EcoRI and ligated according to Table 4. *prgW* cysteine substitution(s) in the background of pMSP6050 were generated by the amplification of mutant genes from pORI10 using primers containing restriction sites for introduction into pMSP6050. pMSP6050 and the PCR product were digested with appropriate restriction enzymes from the Promega Corporation and New England Biolabs depending on the position of the particular cysteine to alanine substitution(s) (Table 4 and 5). Transformation of plasmids into DH5α was performed using rubidium chloride/heat shock method (Hanahan, 1983). Plasmid size was verified by plasmid isolation, PCR amplification of transformant colonies verified presence of *prgW* genes, and additional HhaI digestion verified presence of cysteine to alanine substitution(s). All *prgW* genes of recombinant plasmids were sequenced to verify that there were no mutations resulting from polymerase errors during PCR.

Chromosomal DNA Isolation from E. faecalis (and Other Gram-positives)

E. faecalis cultures were incubated overnight in TH with appropriate antibiotic (greater than 15 hours). Cultures were diluted in 1:10 in TH with appropriate antibiotic and incubated for two hours. Appropriate culture volume was removed that equaled an adjusted O.D.660 to 3.0 A; this adjusted volume is two-times the approximate stationary phase $O.D._{660}$ for *E. faecalis*. Cultures were then centrifuged at 6000 rpm for 10 minutes

at 4°C and removed supernatant. Cultures were resuspended in 960 µl 50 mM EDTA with 10 mg/mL Sigma-Aldrich Corporation lysozyme (Catalog # L-7651) and then they were incubated at 37°C for 30 to 60 minutes. Next, the treated cultures were centrifuged at 6000 rpm for 10 minutes at 4°C. Following centrifugation, the supernatant of cultures were removed.

600 µl Promega Corporation Nuclei Lysis Solution (Appendix; Catalog # A7941) was added to the treated cells and mixed by pipetting gently. The cells were incubated for five minutes at 80°C in the lysis solution and then cooled to room temperature. The cell-lysis suspension was then bead beated with 0.5 g BioSpec Products, Inc. (Bartlesville, OK, USA) Zirconia/Silica Beads (Catalog # 11079101z) three times at 4800 rpm with Biospec Mini-Beadbeater-1; samples were placed on ice between bead-beating. 600 µl of the sample was removed following bead-beating and the Promega Corporation Wizard® Genomic DNA Purification Kit (Catalog # A1125; Protocol # 9FB022) continued chromosomal DNA isolation as followed: 3 µl of Promega Corporation RNase Solution (Catalog # A797C) was added and the mixture was incubated at 37°C for 15 to 60 minutes, and then cooled to room temperature. 200 µl Promega Corporation Protein Precipitation Solution (Appendix; Catalog # A795A) was added and the mixture was vortexed to mix. Samples were incubated for five minutes on ice to removed protein from suspension. Then, samples were centrifuged at 13,000 to 16000 x g for three minutes.

The supernatant was transferred to a 1.5 mL centrifuge tube that contained 600 µl room temperature isopropanol and mixed by pipetting. The sample was centrifuged at 13,000 to16000 x g for 3 minutes and supernatant was carefully removed. 600 µl room

temperature 70 percent ethanol was added. The sample and alcohol was mixed by pipetting. The sample was centrifuged at 13,000 to16000 x *g* for 3 minutes and following centrifugation, the ethanol was removed. Pellet was air-dried for 10 to 15 minutes to remove any residual ethanol. Samples were placed in a laminar flow biosafety cabinet speed alcohol evaporation. 100 µl Rehydration Solution (Appendix; Catalog # A796A) was added and the sample in rehydration solution was incubated for 1 hour at 65^{o}C or overnight at 4^{o}C. Chromosomal DNA samples were stored at 80^{o}C until chromosomal DNA samples were ready for use.

DNA Sequencing

DNA sequencing was used to verify absence of PCR related errors and verified the correct isolated organisms (e.g. *L. lactis*). Purified PCR amplifications of gene were sent to Genewiz, Inc (South Plainfield, NJ, USA) which typically included one reaction with a forward primer and one with a reverse primer to verify errors that were found in sequence. Genewiz utilized Sanger DNA sequencing technology to sequence DNA. Errors were typically were due to proximity of base to five prime or three prime end of sequenced region. Typically 700 to 800 bp could be sequenced at one time. Samples were prepared as followed with 25 pmol primer to a total volume of 15 µl (Table 8).

Sequences were ordered through and accessed on www.genewiz.com. Samples are stored for 60 days and transferred to Genewiz archives for future access. Sequences were printed and saved in notebook as well as on computer hard drive. Sequences were verified for presence of appropriate mutation and absence PCR related errors with Clustal

W2. Clone manager verified mutations had appropriate encoded amino acid. BLASTN verified 16s rRNA sequenced was appropriate organism.

Table 8. DNA and Primer Formulations for Sequencing with Genewiz, Inc.

	DNA length	Template DNA
Plasmid DNA	< 6 kb	~ 500 ng
	6-10 kb	~ 800 ng
	< 500 kb	~ 500 ng
PCR product	< 500 bp	~ 10 ng
	500-1000 kb	~ 20 ng
	1000-2000	~ 40 ng
	2000-4000	~ 60 ng

Recombinant DNA transformation and electroporation

E. coli Transformation

Transformation of plasmids into DH5α was performed using rubidium chloride/heat shock method (Hanahan, 1983). *E. coli* was inoculated into 150 mL LB and incubated at 37^{o}C, 200 rpm and grown to an O.D.$_{600}$ of 0.4 A. Cells were incubated on ice for 30 minutes and centrifuged at 5,000 rpm for 15 minutes at 4^{o}C. Cell pellets were resuspened in 17 mL of RF1 Solution (Appendix), incubated on ice for 30 minutes, and centrifuged at 5,000 rpm for 15 minutes at 4^{o}C. Supernatant was removed; cells were resuspened with 4 mL of RF2 Solution (Appendix) and incubated on ice for 30 minutes. The competent cells were transferred to 1.5 mL centrifuge tubes in 50 and 150

Table 9. Plasmids Used in this Study

Plasmids	Description	Source
pCF10	Pheromone-responsive to cCF10; Tn*925* encodes *tetM* (Tet^R); contains PrgW RepA_N	(Dunny *et al.*, 1981)
pET28b-*prgW*	pET-28b derivative encoding *prgW* with N-terminal His_6 tag	Dunny Lab
pVA891	*E. coli* plasmid; Erm^r.	(Macrina *et al.*, 1983)
pORI10	Derivative of pVA891. Contains *prgW which* functions as *E. faecalis* origin of replication; Erm^R.	(Meloni *et al.*, Submitted)
pMSP6050	Derivative of pVA891. Contains pORI10 minimal stability replicon consisting of *uvrA, uvaE, uvaF, prgN, prgO, prgP, prgW, prgZ,* and truncated *prgY*; Erm^R.	(Hedberg *et al.*, 1996)
pMSP3535	*E. coli* and *E. faecalis* shuttle vector. Nisin inducible promoter ; Erm^R.	(Bryan *et al.*, 2000)
pORI-078	*prgW* of pORI10 contains C78A substitution; Erm^R. Primers bdu01 (Tm 58) and bdu25 amplified *prgW* of pORI10 upstream of C78 and bdu04 (Tm58) and bdu26 amplified downstream of C78. The two *prgW* amplifications along with bdu01 (Tm 58) and bdu04 (Tm58) generated the overlapping segment. *prgW-C78A* amplification was cloned into pVA891 using *Eco*R1. A *Hha*I restriction site was encoded into the overlapping region.	This study
pORI-275	*prgW* of pORI10 contains C275A substitution; Erm^R. Primers bdu01 (Tm 60) and bdu27b amplified *prgW* of pORI10upstream of C275 and bdu04 (Tm60) and bdu28 amplified downstream of C275. The two *prgW* amplifications along with bdu01(Tm 60) and bdu04 (Tm 60) generated the overlapping segment. *prgW-C275A* amplification was cloned into pVA891 using *Eco*R1. A *Hha*I restriction site was encoded into the overlapping region.	This study

pORI-307	*prgW* of pORI10 contains C307A substitution; Erm^R. Primers bdu01 (Tm 60) and bdu29b amplified *prgW* of pORI10 upstream of C307 and bdu04 (Tm60) and bdu30 amplified downstream of C307. The two *prgW* amplifications along with bdu01(Tm 60) and bdu04 (Tm 60) generated the overlapping segment. *prgW-C307A* amplification was cloned into pVA891 using *Eco*R1. A *Hha*I restriction site was encoded into the overlapping region.	This study
pORI-078/275	*prgW* of pORI10 contains C78A and C275A substitution; Erm^R. Primers bdu01 (Tm 60) and bdu27b amplified *prgW-C78A* pORI-078upstream of C275 and bdu04 (Tm60) and bdu28 amplified downstream of C275. The two *prgW* amplifications along with bdu01(Tm 60) and bdu04 (Tm 60) generated the overlapping segment. *prgW-C78A/C275A* amplification was cloned into pVA891 using *Eco*R1. A *Hha*I restriction site was encoded into the overlapping region.	This study
pORI-078/307	*prgW* of pORI10 contains C78A and C307A substitution; Erm^R. Primers bdu01 (Tm 60) and bdu29b amplified *prgW-C78* pORI-078 upstream of C307 and bdu04 (Tm60) and bdu30 amplified downstream of C307. The two *prgW* amplifications along with bdu01(Tm 60) and bdu04 (Tm 60) generated the overlapping segment. *prgW-C307A* amplification was cloned into pVA891 using *Eco*R1. A *Hha*I restriction site was encoded into the overlapping region.	This study
pORI-275/307	*prgW* of pORI10 contains C78A, C275A, and C307A substitution; Erm^R. Primers bdu01 (Tm 60) and bdu27b amplified *prgW-C307A* of pORI-307 upstream of C275 and bdu04 (Tm60) and bdu28 amplified downstream of C275. The two *prgW* amplifications along with bdu01(Tm 60) and bdu04 (Tm 60) generated the overlapping segment. *prgW-C275A/C307* amplification was cloned into pVA891 using *Eco*R1. A *Hha*I restriction site was encoded into the overlapping region.	This study
pORI-078/275/307	*prgW* of pORI10 contains C78A substitution; Erm^R. Primers bdu01 (Tm 60) and bdu27b amplified *prgW-C78/C307* of pORI-078/307 upstream of C275 and bdu04 (Tm60) and bdu28 amplified downstream of C275. The two *prgW* amplifications along with bdu01(Tm 60) and	This study

	bdu04 (Tm 60) generated the overlapping segment. *prgW-C78/C275A/C307* amplification was cloned into pVA891 using *Eco*R1. A *Hha*I restriction site was encoded into the overlapping region.	
pMSP6-078	*prgW* of pMSP6050 contains C78A substitution; Erm^R. Primers bdu31 and bdu32 amplified *prgW-C78A* of pORI-078 and fragment with was cloned into pMSP6050 using *Bsr*GI (5' of *prgW-C78A*) and *Stu*I (3' of *prgW-C78A*).	This study
pMSP6-275	*prgW* of pMSP6050 contains C275A substitution; Erm^R. Primers bdu31 and bdu32 amplified *prgW-C275A* of pORI-275 and fragment with was cloned into pMSP6050 using *Bsr*GI (5' of *prgW-C275A*) and *Stu*I (3' of *prgW-C275A*).	This study
pMSP6-307	*prgW* of pMSP6050 contains C307A substitution; Erm^R. Primers bdu33 and GC-bdu31b amplified upstream region of insert on pMSP6050, bdu29b and bdu30 amplified region upstream of C307 on pMSP6050, and bdu31 and bdu34 amplified region downstream of C307. The three amplifications along with bdu33 and bdu34 generated the overlapping segment. *prgW-C307A* amplification was cloned into pMSP6050 using *Pst*I. A *Hha*I restriction site was encoded into the overlapping region.	This study
pMSP6-078/275	*prgW* of pMSP6050 contains C78A and C275A substitution; Erm^R. Primers bdu31 and bdu32 amplified *prgW-C275A* of pORI-078/275 and fragment with was cloned into pMSP6050 using *Bsr*GI (5' of *prgW-C275A/C275A*) and *Stu*I (3' of *prgW-C78A/C275A*).	This study
pMSP6-078/307	*prgW* of pMSP6050 contains C78A and 307A substitution; Erm^R. Primers bdu33 and GC-bdu31b amplified upstream region of insert on pMSP6-078, bdu29b and bdu30 amplified region upstream of C307 on pMSP6050, and bdu31 and bdu34 amplified region downstream of C307. The three amplifications along with bdu33 and bdu34 generated the overlapping segment. *prgW-C78A/C307A* amplification was cloned into pMSP6050 using *Pst*I. A *Hha*I restriction site was encoded into the overlapping region.	This study

pMSP6-275/307	*prgW* of pMSP6050 contains C275A and C307 substitution; Erm^R. Primers bdu33 and GC-bdu31b amplified upstream region of insert on pMSP6-275, bdu29b and bdu30 amplified region upstream of C307 on pMSP6050, and bdu31 and bdu34 amplified region downstream of C307. The three amplifications along with bdu33 and bdu34 generated the overlapping segment. *prgW-C275A/C307A* amplification was cloned into pMSP6050 using *Pst*I. A *Hha*I restriction site was encoded into the overlapping region.	This study
pMSP6-078/275/307	*prgW* of pMSP6050 contains C78A, C275A, and C307A substitution; Erm^R. Primers bdu33 and GC-bdu31b amplified upstream region of insert on pMSP6-078/275, bdu29b and bdu30 amplified region upstream of C307 on pMSP6050, and bdu31 and bdu34 amplified region downstream of C307. The three amplifications along with bdu33 and bdu34 generated the overlapping segment. *prgW-C78A/C275A/C307A* amplification was cloned into pMSP6050 using *Pst*I. A *Hha*I restriction site was encoded into the overlapping region.	This study
pPrgW	pMSP3535 derivative. *prgW* controlled by a Nisin inducible promoter; Erm^R.	(Meloni *et al.*, Submitted)
pMSP6050Δpar	pMSP6050 derivative with inactive toxin-antitoxin system deleted; Erm^R.	(Mazeffa thesis, 2008)
pMSP6-078/ 275ΔparΔDR	pMSP6050Δpar derivative with first direct repeat removed from *prgW* unintentionally during sub-cloning; Erm^R.	This study
pMSP6-078/ 275Δpar	*prgW* of pMSP6050Δpar contains C78A and C275A substitution; Erm^R. Primers bdu31 and bdu32 amplified *prgW-C275A* of pORI-078/275 and fragment with was cloned into pMSP6050 using *Bsr*GI (5' of *prgW-C275A/C275A*) and *Stu*I (3' of *prgW-C78A/C275A*).	This study

µl aliquots and then quickly frozen using an ethanol-dry ice bath. Samples were stored at -80^{o}C until ready for use.

When needed competent DH5α *E. coli* were removed from -70^{o}C and thawed on ice. 50 µl of DH5α competent cells were added to plasmid (Table 9 lists plasmids used) or ligation mixture. Typically 5 µl of plasmid DNA and approximately 50 to 100 µg of vector DNA in the ligation mixture were sufficient. Cells were mixed gently via pipette and incubated on ice for 30 minutes. Competent cells were at 42^{o}C for one minute in an H_2O bath and then placed on ice for an additional two minutes. 500 µl LB medium was added into culture heat-shocked culture and incubated at 37^{o}C one hour. 100 µl of transformants was transferred onto LB agar plates with appropriate antibiotic(s) and incubated at 37^{o}C overnight. *E. coli* colonies typically appeared after overnight incubation at 37^{o}C.

Electroporation of Plasmid DNA into E. faecalis

Electrocompetent OG1RF cells were prepared based on the glycine gradient method described by Dunny (Dunny *et al.*, 1991). Cells were incubated overnight in varying concentrations of TH with glycine (Appendix; two to five percent glycine; 0.25 increments). Glycine cultures with a reduced $O.D._{660}$ that was between 60 to 90 percent were centrifuged at 6000 rpm for 10 minutes at 4^{o}C. Cells were washed with one-third volume of Electroporation Buffer (Appendix) and centrifuged at 6000 rpm for 10 minutes at 4^{o}C. Cell pellets were resuspened in 1/50 volume electroporation buffer. If needed, cells were stored at -80^{o}C. Plasmids used for electroporation were isolated in parallel using Wizard® Plus SV Minipreps DNA Purification System kit.

E. faecalis cells were washed with 100 µl dH_2O an additional three times prior to electroporation. Electrocompetent cells were resuspended in ice-cold, sterile, dH_2O. 250 to 1000 ng plasmid DNA was added to the dH_2O-*E. faecalis* suspension for a total working volume of 100 µl. The plasmid-bacteria cell mixtures were electroporated in 100 µl Eppendorf electroporation cuvettes (Catalog # 940001005) using the Bio-Rad Gene Pulser electroporator settings of 1,800 V, 25 µF, and 200 Ω. Time constants varied from 2.5 to 4.7 depending on cell and plasmid preparation; time constants of experiments performed in parallel did not vary more than 0.5. The post-electroporation mixture was resuspended with TH with 0.25 M sucrose and incubated for two hours at 37°C. 100 µl of electroporation cells were added to TH agar with 50 µg/mL erythromycin and grown overnight. *E. faecalis* cells typically formed colonies after one day of incubation at 37°C. PCR amplification of transformant colonies verified presence of *prgW* genes and additional HhaI digestion verified presence of cysteine to alanine substitutions.

Electroporation of Plasmid DNA into L. lactis

Electrocompetent *L. lactis* LM0230 were diluted 1:10 in GM-17 (Appendix) and incubated at 30°C overnight. Cells were grown to an $O.D._{600}$ of 0.5 to 0.7 A. 40 ml of cultures was placed on ice for 30 minutes. Cells were harvested at 6000 rpm for 20 minutes at 4°C, washed three times with 20 ml ice-cold, sterile, dH_2O and centrifuged again at 6000 rpm for 10 minutes at 4°C. Cells were resuspended in 40 µl of dH_2O. Plasmids used for electroporation were isolated in parallel using Wizard® Plus SV Minipreps DNA Purification System kit.

L. lactis cells were washed with 100 µl dH_2O an additional three times prior to electroporation. Electrocompetent cells were resuspended in ice-cold, sterile, dH_2O containing 1000 ng plasmid DNA; plasmid DNA was added to a dH_2O-*E. faecalis* suspension for a total working volume of 100 µl. The plasmid-bacteria cell mixtures were electroporated in 100 µl Eppendorf electroporation cuvettes using the Bio-Rad Gene Pulser electroporator settings of 1,800 V, 25 µF, and 200 Ω. The post-electroporation cells were incubated for two hours in 500 µl media as described above and 500 µl of the culture was divided onto two selective agar plates. *L. lactis* formed colonies after two days of incubation at 30°C.

The presence of *prgW* genes was verified using colony PCR and the presence of cysteine to alanine substitutions was verified by additional HhaI digestion. Presumed *L. lactis* colonies were tested by amplifying 16s rRNA using primers for universal eubacterial 16S rRNA primers (Table 5) (Cottrell and Kirchman, 2000). PCR products were sequenced using both EubA and EubB primer and compared using BLAST against NCBI sequence database to verify species identity.

Transformation of Plasmid DNA into S. mutans

Transformation of plasmid DNA into *S. mutants* UA159 was based on a method described by Lindler and Marcina, (Lindler and Macrina, 1986). An overnight 5 mL culture of UA159 was incubated in TH at 37°C at five percent CO_2 and was diluted 1:25 with TH with ten percent heat-inactivated horse serum and 10 percent glucose (Perry and Kuramitsu, 1981). Cultures were incubated at 37°C at five percent CO_2 for 3.5 hours (Lindler and Macrina, 1986). 1 µg/mL of appropriate plasmid DNA was added to *S.*

mutans culture and incubated for an additional two hours at 37°C at five percent CO_2. Transformants were incubated at 37°C at five percent CO_2 for two days on TH agar and selected for using 25 μg/mL erythromycin.

Plasmid Based Assays

Plasmid Maintenance Assay

E. faecalis OG1RF cells with appropriate plasmid were incubated overnight at 37°C and under selection (erythromycin 50 μg/mL) to ensure 100 percent of the population contained a plasmid. Cultures were serial-passaged (diluted 1:100) in non-selective TH broth. Following growth under selection, cultures were serial-passaged over time to allow for plasmid loss. Each culture was passaged every 12 hours until the completion of experiment. Cultures containing pORI10 and pMSP6050 derived plasmids were serial cultured for 142 to 163 generations. Samples of passaged cells were serial diluted (10^{-5} to 10^{-7}) and plated onto TH agar without selection. Samples of passaged cells were plated on TH agar without selection. Formation of a colony required approximately 23 generations of growth (Meloni, 2005). Fifty randomly selected colonies were patched onto non-selective and selective plates. Plasmid maintenance was scored as percent erythromycin-resistant cells.

Plasmid Copy Number Assay

An assay to determine relative copy number was derived from a modified protocol described by Skulj (Skulj *et al.*, 2008). Overnight OG1RF cultures with

appropriate plasmid were diluted 1:100 in TH and grown under selection to an $O.D._{660}$ of 0.6 to 0.8 A. Samples (1 mL) were placed immediately at 95°C for 10 minutes and then stored overnight at -20°C. Samples were thawed and disrupted by bead-beating one time with 1.0 g of 0.1 mm zirconia/silica beads at 4800 rpm for 60 seconds with Biospec Mini-Beadbeater-1. Whole-cell lysate (1 µl) was added to 25 µl of real-time PCR in triplicate. Real time PCR was formulated as followed using real-time 5 PRIME (Hamburg, DE) RealMaster SYBR ROX Mix: 2.5x RealMaterMix SYBR ROX/20X SYBR Solution; 2.5 µM forward primer; 2.5 µM reverse primer. Primers described in Table 5 amplified an internal 150 bp region *prgW* on the plasmid and *rpoA* on the chromosome. Real-time PCR thermocycler (Eppendorf Mastercycler) settings were as follows: 95°C for two minutes; 95°C for 30 seconds; 55°C for 30 seconds; 68°C for 30 seconds (40 cycles). Appropriate SYBR green amplification was verified with the MasterPlex melting curve program. Tm was determined by MasterPlex noiseband threshold default. Samples were normalized by calculating Δ Ct of *prgW* amplification (plasmid) - Δ Ct *rpoA* amplification (chromosomal control). Differences in plasmid copy amounts in mutants were compared relative to wild-type amounts, 2^ (ΔCt mutant-Δ wild-type).

Protein Manipulation

Whole Cell Lysate Preparation for Protein

E. faecalis cultures were inoculated in 20 mL TH with appropriate antibiotic (Table 2) and incubated overnight (no longer than 15 hours) at 37°C. Overnight cultures were diluted in 1:100 in TH with appropriate antibiotic and incubated to mid-exponential

phase (O.D.660 of 0.5 to 0.8A). If necessary, 25 ng/mL of Sigma-Aldrich Nisin (Catalog # N-5764) was added to *E. faecalis* with Nisin inducible promoters on pMSP3535 vectors. Cultures were incubated cultures for a minimum of 30 minutes after nisin induction (120 to 240 minutes for GFP labeled PrgW). Cells were centrifuged at 6000 rpm for 10 minutes at 4^{o}C and supernatant was removed. If needed, cells were stored at -20^{o}C for up to a week. Pellet was resuspended with appropriate dilution of Protein Lysis Buffer (Appendix). Typically pellets were resuspened 1:10 for a total volume of 1.0 mL but samples ranged between 1:1 and 1:100 dilution at a volume of 0.5 to 1.0 mL per sample.

The cell pellet/protein-lysis buffer suspension was then bead-beated with 0.5 g of 0.1 mm zirconia/silica beads five times at 4,800 rpm; samples were placed on ice for one minute between operations. Cells were briefly spun down to reduce froth at top of bead-beating tube and carefully removed supernatant with pipette to avoid removing beads. Whole cell lysate was dispensed into a new 1.5 mL centrifuge tube. Whole cell lysate preparations were stored at -20^{o}C for future use or add immediately to 2X Protein Loading Buffer (Appendix). If needed, 20 percent βME was added to subject samples to reducing conditions. Protein samples were boiled for five minutes to assist in denaturing of protein and then appropriate amount of samples were added to wells (20 to 25 μl for small gel; 50 μl for 0.75 mm thick gel; and 100 μl for 1.5 mm thick gel).

SDS-PAGE Gel Preparation

SDS-PAGE glass gel plates were cleaned with ethanol. Two gel casting spacers (0.75 or 1.5 mm for large gel, 0.5 mm for small gel) were aligned with outside of plates.

Spacers and plates were secured plates with two side clamps (small gel requires outside gasket). Plates were then placed on appropriate casting base. Stacking Buffer was formulated (Appendix) and Resolving Buffer (Appendix) gel using FisherBioReagents® (Fair Lawn, NJ, USA) 40% Acrylamide Solution, Electrophoresis Grade (Catalog # BP1408-1), 10% Acros Ammonium Persulfate (Catalog # 40116-0100) according to Table 10. Thermo Scientific (Rockford, IL, USA) TEMED (Catalog # 17919) was added immediately prior to pouring between glass plates.

A 10 mL pipette with a 100µl pipette tip transferred approximately 13 mL of the resolving gel buffer between the two glass gel plates for 0.75 mm comb gel; or using a 25 mL pipette with 1 mL pipette tip, transferred approximately 23 mL of resolving gel buffer in between the two glass gel plates for 1.5 mm comb gel. 1mL sterile H_2O was placed on top of resolving gel buffer and allowed gel to cast for one hour; sterile H_2O was filled to the top of glass plate if resolving gel is unused overnight. After the gel had polymerized, water was carefully poured of the top of gel. The stacking gel was transferred in between the two glass gel plates and filled to the top. The appropriate gel comb was inserted onto the top of the gel and stacking gel was allowed to polymerize. After gel polymerization the gel comb was removed. Gels were inserted into appropriate electrophoresis unit and the units were filled with 1X running buffer. Samples were added to gel. 100 V was applied to the small gels for two to four hours; four hours is optimal for PrgW doublet resolution. Large gels ran at 100 V through stacking gel and 125V through resolving gel for a total of 14 hours or time needed to allow loading dye front to run through the gel.

Table 10. Resolving and Stacking Gel Formulation.

Acrylamide (%)	40% acrylamide; 0.8 bisacrylamide (mL)	4X Resolving or Stacking buffer (mL)	H_20 (mL)	10% Ammonium Persulfate (μl)	TEMED (μl)
		Stacking			
N/A	**0.562**	**1.25**	**3.08**	**0.05**	**0.005**
		Resolving			
5	1.88	3.75	9.37	0.05	0.01
6	2.25	3.75	9	0.05	0.01
7	2.63	3.75	8.63	0.05	0.01
7.5	2.81	3.75	8.44	0.05	0.01
8	3	3.75	8.25	0.05	0.01
9	3.38	3.75	7.88	0.05	0.01
10	3.75	3.75	7.5	0.05	0.01
12	**4.5**	**3.75**	**6.75**	**0.05**	**0.01**
13	4.88	3.75	6.37	0.05	0.01
15	5.63	3.75	5.62	0.05	0.01
18	6.75	3.75	4.5	0.05	0.01

Coomassie Stain

Following electrophoresis, SDS-PAGE gel was stained with Coomassie Blue Stain (Appendix) for visualization. Depending on size of gel, 50 to 150 mL of Coomassie Blue Stain was added to a PYREX glass tray containing the SDS-PAGE gel that was carefully removed from the glass plates. Gel was incubated in Coomassie blue stain for one to four hours depending on thickness of gel and strength of the stain. Gel could be stained overnight but this increased de-stain time. Coomassie Blue Stain can be reused and until deemed ineffective. Following staining, rinsed gel two times with 100

mL H_2O and then added 50 to100 mL Coomassie De-stain Solution (Appendix) to gel tray. De-stain was removed after approximately one to two hours, or when bands were clearly distinguishable. De-stain can be removed and new de-stain reapplied if needed. Wadded Kimwipes™ were added to corner of gel tray absorbed dye, which decreased the de-stain time. Gel was rinsed with 100 mL dH_20 and wrapped gel in plastic wrap for scanning. If needed, gel could be stored in dH_2O overnight. Gel was scanned onto computer and record file location and name. Printed copy was included into notebook.

Semi-Dry Blot Procedure

SDS-PAGE gel with six Whatman™ (Kent, UK) Chromatography Paper 3MM CHR blot sheets (Catalog #: 3030-153) and one appropriately sized piece of GE Healthcare (Buckinghamshire, UK) Amersham™ Hybond ECL Nitrocellulose Membrane (Catalog# RPN303D) paper were all soaked in approximately 100 mL 1X Towbin Buffer (Appendix). While the gel, membrane, and blot sheets soaked in Towbin buffer (approximately 10 min), the semi-dry blot apparatus was cleaned Fisher Scientific (Fair Lawn, NJ, USA) Semidry Electroblotter (Catalog # FB-SDB-2020) thoroughly by wiping with only Kimwipes™ and dH_2O. The blot sheets were placed on bottom (negative) side of semi-dry blot apparatus. The blot sheets were kept wet (added Towbin Buffer as needed) and the air bubbles within the transfer stack were removed with pipette. Gel was placed on blot sheet with the front of the gel facing down. Nitrocellulose membrane was placed on top of gel. Three wet blot sheets were placed on top of the nitrocellulose membrane. Next, the top of the transfer apparatus was placed on top of transfer stack without moving transfer stack. The lid was secured by tightening the screws

counterclockwise (did not over tighten). Selected "Program 3" on Amersham Biosciences Electrophoresis Power Supply (Cat # EPS-601) and verified voltage was set to 10V, amperage to 400 mA, and wattage to 10 watts (manual recommended a range of 0.8 to 2 mA per cm^2 of gel). Semi-Dry blot procedure time was 30 to 75 min depending on size of gel and wetness of membrane. Following transfer, gel was discarded and blot sheets were carefully removed. The center of the molecular weight bands were marked on the nitrocellulose membrane with a pen tip for future reference. The transfer apparatus was cleaned with only Kimwipes^{TM} and dH_2O and then air dried.

Nitrocellulose Prep/ Antibody Incubation/ Detection

Nitrocellulose membrane was placed in an appropriate sized tray. 100 mL of Milk Wash Buffer (Appendix) was added to the tray and rocked at medium speed for at least 60 minutes. If needed, protein-transferred nitrocellulose was stored in Milk Wash Buffer overnight at 4^oC. The membrane was rocked at medium speed for at least 60 minutes following overnight storage at 4^oC. The membrane was then rinsed with 50 mL 1X TBS Wash Buffer (Appendix) two times. The membrane was then incubated for one hour with Anti-PrgW polyclonal antibody (1:5000) to detect the presence of PrgW (Leonard *et al.*, 1996); Polyclonal antibodies were derived from synthetic peptides (1) YFIYTVAELMTLLNCRE; PrgW amino acids 64 to 80 and (2) RAKTKVEKEYNVVLIGEDYQEE; PrgW amino acids 249 to 272. Antibodies were resuspended in sterile dH_2O 1 mg/mL. After incubation, primary antibody-1X TBS Wash Buffer mixture was removed and the membrane was rinsed twice with 50 mL of 1X PBS Wash Buffer. The nitrocellulose membrane was an additional three times by

rocking at medium speed for five minutes with 50 mL 1X TBS Wash Buffer. The nitrocellulose paper was then incubated with 50 GE Healthcare Amersham™ ECL™ Anti-Rabbit IgG, Horseradish Peroxidase linked whole antibody (from donkey; Catalog # NA934V) Anti-Rabbit IgG Horseradish Peroxidase that was diluted in 1X TBS (1:10,000)Wash Buffer by rocking for 60 minutes. Following secondary antibody incubation, the membrane was rinsed twice with 50 mL 1X TBS Wash Buffer. The membrane was then washed an additional three times at medium speed by rocking for 5 minutes with 50 mL 1X TBS Wash buffer.

The following the final wash, residual 1X TBS Wash Buffer was removed from membrane and membrane was carefully placed on a flat surface avoiding air bubbles. The blot was developed using and GE Healthcare Amersham™ ECL™ Plus Western Blotting Detection System (Catalog # RPN2132). PrgW was visualized by an ECL-substrate reaction catalyzed by the horseradish peroxidase conjugated to the anti-rabbit secondary antibody. Exposure to Pierce® CL-XPosure™ Film (Catalog # 3491) detected the chemiluminescent signal. Exposure times varied typically from one to ten minutes. In a dark room with a red light, film was developed by carefully rocking in a try with Kodak (Rochester, NY, USA) GBX Developer and Replenisher (Catalog # 1900943) until bands were seen. Film was then washed H_2O for 30 seconds to remove residual developer solution. Film was then placed in Kodak GBX Fixer and Replenisher (Catalog # 1901959) fixer for 30 seconds and then rinsed with H_2O prior to air drying. When dry, film was scanned onto a computer using Epson (Long Beach, CA, USA) Perfection 1650 Scanner (Catalog # G850A) with Epson Twain Version 5 scanning software. Brightness and contrast were modified as needed using Adobe® Photoshop® Version 7.0. Files were

stored on laboratory computer located with the "Western Blots" subcategory of the "Bryan" folder.

In vitro Pheromone Induced Aggregation of PrgW

Overnight cultures of *S. mutans* UA159 were diluted 1:1000 and grown to mid-exponential phase (O.D.660 = 0.7-0.8A). PrgW expression was induced from a nisin inducible promoter encoded on vector pMSP3535 (Table 9). 25 nM nisin was added at mid-exponential phase and allowed to incubate 30 minutes. Rifampicin (0.5 μg/mL) and chloramphenicol (15 μg/mL) were then added to inhibit additional PrgW synthesis. 1 mM of synthetic Bachem (King of Prussia, PA, USA) cCF10 (Y-LVTLVFV), pre-cCF10 (MKKYKRLLLMAGLVTLVFVLSA), Pre_{1-12} (MKKYKRLLLMAG,) or FisherChemicals DMSO (Catalog #D128-500) was added every 30 minutes (*S. mutans* does not encode *ccfA*). Whole cell extracts were prepared from cells concentrated to an A.O.D. of approximately 7.0 A and analyzed by SDS-PAGE (12 percent), followed by Western blotting using polyclonal anti-PrgW.

Protein Purification of PrgW

Cell Growth and Protein Expression

PrgW with a His_6 tag was cloned into pET28 (b) vector (Table 9) and expressed with *E. coli* BL21 cells (Table 1). Freezer stocks of BL21 with pPET28-PrgW cultures were inoculated into 25 mL of TB with 30 μg/mL kanamycin and incubated overnight in

Falcon™ 50 mL conical tubes at 37°C, 250 rpm. Overnight cultures were diluted 1:100 for a total volume of 100 mL that was divided into four 50 mL Falcon™ conical tubes. The cultures were incubated at 37°C, 250 rpm. Cultures were induced with 1mM FisherChemicals IPTG (Catalog # BP1755-1) at an $O.D._{600}$ of 0.6 A and grown for an additional two hours. 45 mL of cells divided into two centrifuge tubes and centrifuged at 6,000 rpm (Beckman J2-HS) for 10 minutes at 4°C and stored at -20°C overnight or used immediately.

Cell Lysis and Ni Membrane Binding

Cell pellets (1 centrifuge tube) were removed from freezer and resuspended in 1 mL binding-lysis buffer (Appendix) with 1 mg/mL lysozyme and placed on ice for 30 minutes. After 30 minutes, the resuspended pellets were bead-beated with 0.5 g 0.1 mm zirconia/silica beads at 4800 rpm for 60 seconds (three times). Samples were kept on ice at least one minute between bead-beatings. Samples were centrifuged at 7,200 g (8,800 rpm; Thermo IEC Micromax RF) for 30 minutes (in bead beating vials). Prior to supernatant loading, Qiagen Ni-NTA nickel spin column (Catalog # 31314) were primed with 600 µl Protein Purification Binding Buffer (Appendix). Next, 600 µl whole cell lysate was dispensed onto the pre-equilibrated Ni-NTA spin column and centrifuged at 2,000 rpm for two minutes. Additional whole cell lysate was added to same column in 600 µl increments as needed.

Washing and Elution

Qiagen Ni-NTA nickel spin column was centrifuged for 2 minutes at 2000 rpm and flow through was collected through was collected. The Ni-NTA spin column was washed twice with 600 µl Wash Buffer (Appendix) and centrifuged for minutes at 2,000 rpm and wash flow through samples were collected. The protein was eluted three times three times with 200 µl Elution Buffer (Appendix) that contained 400 mM FisherChemicals Imidazole (Catalog # O3196-500). Each elution was centrifuged for two minutes at 2,000 RPM and samples collected were analyzed by SDS-PAGE and Coomassie staining.

Identification, Alignment, and Consensus Generation

Homolog Identification

Amino acid sequence homologs of pCF10 RepA_N protein, PrgW were identified by BLASTP analysis (http://www.nibi.nih.gov/BLAST; accession number AAA25553.1).

Sequence Alignment

The conserved cysteines of the *E. faecalis* RepA_N homologs were identified using the multiple amino acid sequence alignment feature of ClustalW2 (www.ebi.ac.uk/clustalw) (Chenna *et al.*, 2003).

Phylogenetic Tree Generation

The phylogenetic trees were generated using CLC Combined Workbench 6.0.2 sequence analysis software (UPGMA algorithm; default settings). Protein sequences used in CLC Combined Workbench phylogenetic analysis were acquired form Genbank (Benson *et al.*, 2008).

Consensus Sequence Generation

Homolog consensus sequence was generated using a Web-based analysis program, WebLogo Version 2.8.2 (http://weblogo.berkely.edu) using default parameter settings (Crooks *et al.*, 2004; Schneider and Stephens, 1990).

CHAPTER 3

RESULTS

PrgW Aggregation and Conformation

Pre-cCF10 Induces Replication Initiation Protein PrgW Aggregation

Chromosome-encoded pheromone (cCF10) and precursor (pre-cCF10) bind to the pCF10 encoded replication initiation protein PrgW. Affinity chromatography experiments showed that the binding of cCF10 to PrgW affected PrgW motility on non-reducing gels, suggesting conformational changes of the replication protein (Leonard *et al.*, 1996; Meloni *et al.*, Submitted; Ruhfel *et al.*, 1993). PrgW interaction with $CcfA_{1-12}$ and pre-cCF10 also caused motility changes on a non-reducing gel (Figure 4). Synthetic cCF10, $CcfA_{1-12}$, and pre-cCF10 were added exogenously over time to *S. mutans* cultures that over-expressed *prgW*. *S. mutans* UA159 did not naturally produce cCF10 or pheromone precursors (Mazeffa, 2008) so pheromone levels were controlled by exogenous addition. *prgW* was expressed from an upstream nisin inducible promoter located on pPrgW (Table 9). pPrgW is a derivative of the Streptococcal-*E. coli* shuttle vector pMSP3535 that has a heterologous origin of replication (Bryan *et al.*, 2000); *prgW* does not function as a minimal origin of replication in *S. mutans* (Meloni, 2005).

Over-expressed PrgW in *E. faecalis* formed two protein bands when separated by SDS-PAGE and visualized by Western blot which suggested two conformations of the replication protein (Figure 5-7). Additionally, as cCF10, $CcfA_{1-12}$, and pre-cCF10 (resuspened in DMSO) were added over time, upper molecular weight aggregations

appeared on the Western blot (210 minutes) indicating PrgW conformational changes (Figure 4). Even at 210 minutes, the DSMO control containing no peptides did not induce PrgW aggregation. This provided further evidence that cCF10, $CcfA_{1-12}$, and pre-cCF10, interacted with PrgW and that like cCF10, $CcfA_{1-12}$, and pre-cCF10 induced conformational changes in PrgW. This thesis focused on PrgW interaction with pre-cCF10 because pre-cCF10 is a factor that determines host range of pCF10 (Meloni *et al.*, Submitted).

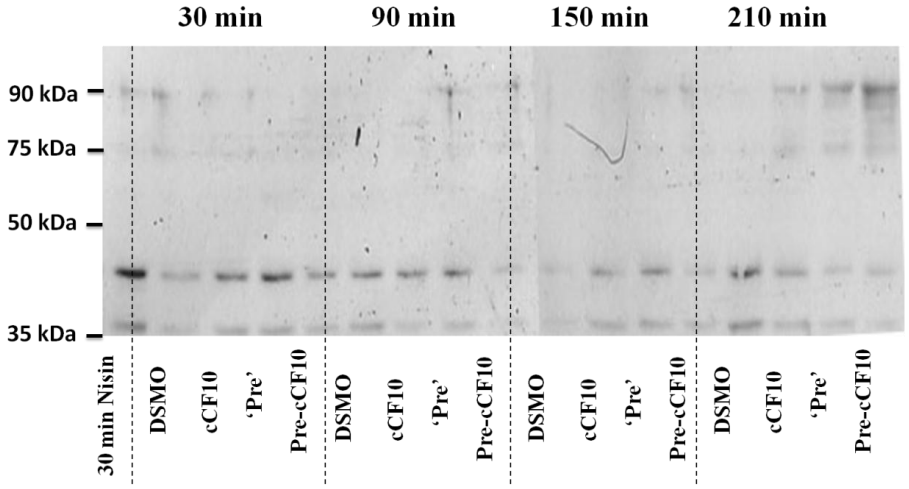


Figure 4. Pheromone Induced PrgW Aggregation

Overnight cultures of *S. mutans* UA159 were diluted 1:1000 and grown to mid-exponential phase ($O.D._{660}$ = 0.7 to 0.8 A). PrgW was expressed from a nisin inducible promoter encoded on vector pMSP3535 for 30 minutes. Rifampicin (0.5 µg/mL) and chloramphenicol (15 µg/mL) were then added to inhibit additional PrgW synthesis. 1 mM of synthetic cCF10, pre-cCF10, Pre_{1-12} or DMSO control was added every 30 minutes (*S. mutans* does not encode *ccfA*). Pheromone was added every 60 minutes following inhibition of PrgW synthesis. Whole cell extracts were prepared from cells concentrated to an $A.O.D._{660}$ of approximately 7.0 and analyzed by SDS-PAGE (12 percent), followed by Western blotting using polyclonal Anti-PrgW.

Aggregation of PrgW in Various Species Backgrounds

The PrgW monomer is predicted to be 38.8 kDa. The strain and expression level caused variation in PrgW electrophoresed migration patterns on non-reducing gels. Two protein bands, described a PrgW migration pattern seen on Western blots when PrgW was over-expressed (Figures 4-7). Two bands were also seen when PrgW was exposed to cCF10 bound to affinity chromatography columns (Meloni *et al.*, Submitted). The two bands of PrgW suggest two different protein conformations. The two PrgW bands were typically not visible in *E. faecalis* whole cell lysates where *prgW* was encoded in pCF10 (Figure 5). PrgW was produced in low levels in *E. faecalis* with pCF10. A 100:1 concentrated whole cell lysate yielded a single migratory band (one arrow) of PrgW while over-expressed PrgW (pPrgW) in *E. faecalis* yielded formation of a two bands (two arrows). Another common migratory feature of PrgW was the formation of a lower molecular weight bands between 25 and 28 kDa. Figure 5 also shows degradation products below the 25 kDa PrgW band which were commonly present when PrgW was highly over-expressed. Like some replication proteins (del Solar *et al.*, 1998), the PrgW cleavage product(s) might be important to the function of the replication protein.

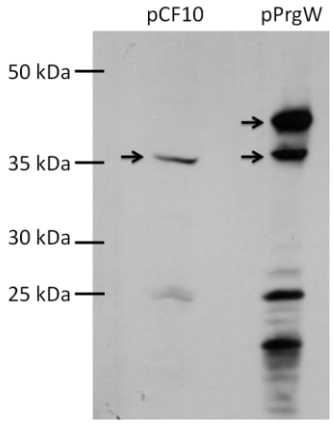


Figure 5. Formation of two Protien Bands when PrgW was Over-expressed

Overnight cultures of *E. faecalis* OG1RF containing either pCF10 or pPrgW were diluted 1:1000 and grown to exponential phase ($O.D._{660}$ = 0.5 to 0.6A). Whole cell extracts were prepared from cells concentrated to an A.O.D. of approximately 60.0 A and analyzed by SDS-PAGE (12 percent), followed by Western blotting using polyclonal Anti-PrgW. Arrows indicated PrgW with one conformation (pCF10) or two different conformations (pPrgW) based on PrgW electrophoresed migration patterns. The result shown was representative of three independent experiments.

PrgW protein migration on SDS-PAGE varied depending on the expression levels and the species producing PrgW. Figures 7 showed when p*rgW* was over-expressed from a constitutive promoter on pORI10, PrgW formed two bands. Sometimes the two PrgW bands cannot be detected (Figure 6). Although PrgW was not functional in *S. mutans* (Meloni, 2005), PrgW was capable of forming two bands when over-expressed from a nisin inducible promoter on shuttle vector pPrgW (Figure 6). Typically, the bands of the PrgW migrated higher in *S. mutans* than the two bands of PrgW in *E. faecalis*. PrgW bands produced from *S. mutans* extracts varied to *E. faecalis* and might be indicative of a misfolded PrgW in *S. mutans*. Interestingly, *E. hirae* did not form a two PrgW bands

(Figure 6 and 7), although PrgW was determined to be a functional replication protein in *E. hirae* (Mazeffa, 2008).

PrgW Conformations were Sensitive to Reduction

Evidence that intramolecular disulfide bonds mediated the formation of two PrgW bands was obtained by subjecting *E. faecalis* whole cell lysate proteins to reducing conditions; two PrgW bands were not observed (Figure 7B EF). In contrast, the two PrgW bands were observed with extracts subjected to denaturing, non-reducing conditions (Figure 7A, EF). The two bands of PrgW were not present in the whole cell lysate of *E. hirae* (Figure 7B, EH) that was prepared in the same Protein Lysis Buffer (Appendix) as the *E. faecalis* extracts. Thus, the two bands of PrgW were not simply an artifact of the conditions of the Protein Lysis Buffer.

Furthermore, a PrgW doublet (Figure 7B) was formed when over-expressed on a pET28 (b) vector. The two bands of PrgW produced in *E. coli* were sensitive to reducing conditions (Figure 7B). Figure 7C showed that PrgW aggregates and cleavage products were sensitive to reduction. PrgW aggregates between102 and 76 kDa were present on denaturing, non-reducing gels and absent on denaturing, reducing gels. Additionally, PrgW cleavage products located between 31 and 38 kDa were present on denaturing, non-reducing gels and absent on denaturing, reducing gels. The differences in SDS-PAGE migration of PrgW under reducing and non-reducing conditions indicated that disulfide bond formation or cysteine modification was regulating PrgW conformation.

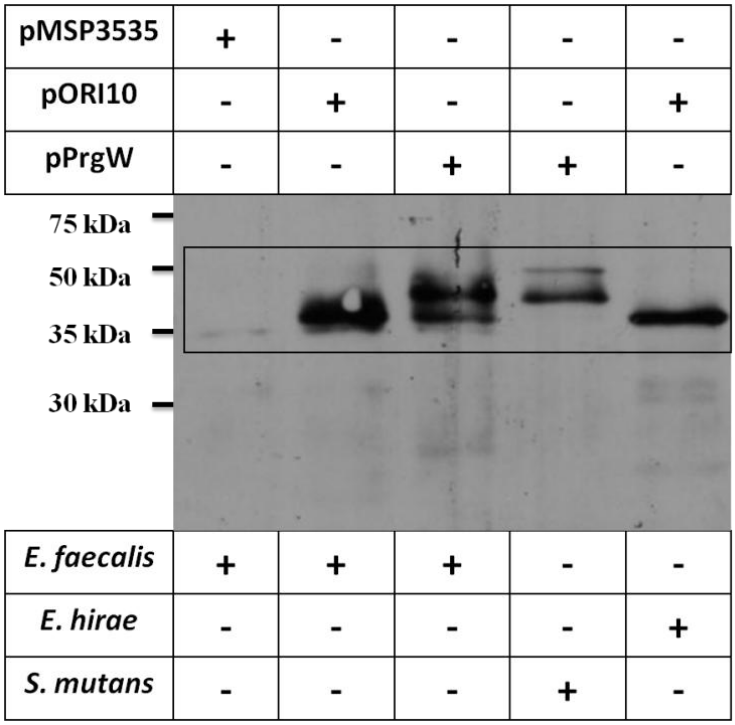


Figure 6. Migration of PrgW Band(s) Varies in Different Species

Overnight cultures of *E. faecalis* OG1RF, *S. mutans* UA159 and *E. hirae* 9790 were diluted 1:1000 and grown to exponential phase ($O.D._{660}$ = 0.5 to 0.6A). Three different plasmids pORI10, pPrgW, and pMSP3535 (negative control) were analyzed. Whole cell extracts were prepared from cells concentrated to an $A.O.D._{660}$ of approximately 6.0 A analyzed by SDS-PAGE (12 percent), followed by Western blotting using polyclonal Anti-PrgW. The result shown was representative of three independent experiments.

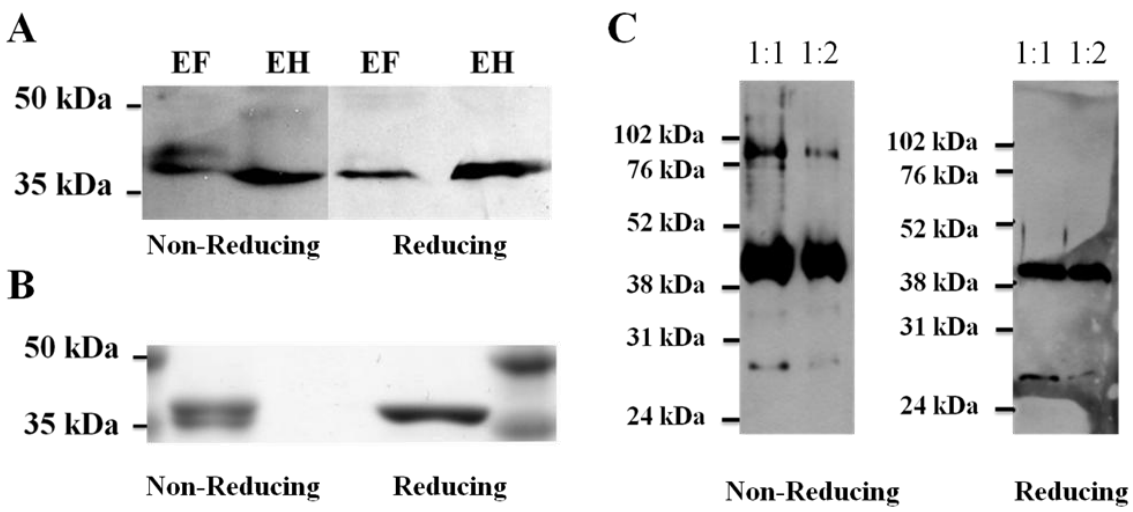


Figure 7. PrgW Conformations were Sensitive to Reduction.

(A) Overnight cultures of *E. faecalis* OG1RF (EF) and *E. hirae* 9790 (EH) were diluted 1:1000 and grown to exponential phase ($O.D._{660}$ = 0.5 to 0.6A). Whole cell extracts were prepared from cells concentrated to an $A.O.D._{660}$ of approximately 6.0 and analyzed by SDS-PAGE (12 percent), followed by Western blotting using polyclonal Anti-PrgW. (B) PrgW was cloned in *E. coli* using pET28b to add a His-tag for nickel column purification. PrgW was analyzed by reducing SDS-PAGE (12 percent) followed by Coomassie staining. (C) PrgW was cloned in *E. coli* using pET28b to add a His-tag. PrgW was analyzed by reducing SDS-PAGE (12 percent) followed by Western blotting using polyclonal Anti-PrgW. 1:1 and 1:2 indicate dilution ratio. Results were representative of at least three independent experiments.

Cysteines Control PrgW Host Range and Function

Conserved Cysteines of E. faecalis RepA_N Proteins

PrgW migration on an acrylamide gel was sensitive to reduction, which suggested disulfide bonds were affecting PrgW conformation (Figure 7). PrgW has been classified as a member of the RepA_N superfamily of replication initiation proteins (Meloni *et al.*, Submitted; Weaver *et al*., 2009a). Sequence alignment *E. faecalis* RepA_N proteins supported the idea of a potential role of disulfide bonds in PrgW conformation and function. ClustalW2 multiple sequence alignment illustrated that 54 of 59 known RepA_N proteins in *E. faecalis* that were homologous to PrgW contained three highly conserved cysteines (Figure 3, top). Table 11 shows the distribution of RepA_N proteins that contained the conserved cysteines. Most *E. faecalis* isolates with a plasmid containing a RepA_N protein with conserved cysteines also contained other plasmids. Plasmids with RepA_N proteins with the three conserved cysteines have been isolated around the world from a variety of samples over a period of almost 80 years.

Conserved RepA_N Cysteines are not Essential to PrgW Function

Cysteine to alanine substitutions (C78A, C275A, C307A, C78A/C275A, C78A/C307A, C275A/C307A, and C78A/C275A/C307A) were generated in *prgW* and were inserted into the *E. coli* plasmid pVA891 (Macrina *et al.*, 1983). *E. coli* plasmid pVA891 (derivative of p15a) did not replicate in *E. faecalis* due to a lack of Gram-positive origin of replication. When provided with an exogenous promoter from the

Table 11. RepA_N Proteins of *E. faecalis* Homologous to PrgW.

RepA_N	Percent Identity (# cysteines)	Strain	Plasmids in isolate	Year isolated	Source	Location
pCF10 PrgW	100 (3)	SF-7	2	1981	urinary tract or wound	New York, USA
ZP_05563675	99 (3)	DS5	3	≤1974	unknown	Ann Arbor, USA
ZP_05567443	99* (3)	HIP11704	1	2002	clinical	Ann Arbor, USA
ZP_06632003	99* (3)	S613	?	2010		
ZP_04436013	99 (3)	TX1322	?	≤2005	fecal	
pPD1 RepA	95 (3)	39-5	6	≤1964	oral	Philadelphia, USA
ZP_05600084	92* (3)	X98	n/a	1934	fecal	
ZP_04436555	92* (3)	ATCC-29200		≤1974	urogenital	Quebec, Canada
pMG2200 RepA	64 (3)	NKH15	2	1999	fecal	Nagano City, Japan
pTEF2 RepA	62 (3)	V583	3	1987	blood	St. Louis, USA
ZP_05566577	62 (3)	Merz96	2	2002	blood	Maryland, U.S.A
ZP_05426862	61 (3)	T2	3	≤1992	urine	Sapporo, Japan
ZP_03986010	59* (3)	HH22	3	≤1982	urine	Houston, USA
ZP_06628757	59* (3)	R712				
ZP_06632860	59* (3)	S613				
pBEE99 PrgW	59 (3)	E99	n/a	≤2005	urine	Little Rock, AR
ZP_05594693	55 (3)	AR01/DG	4	2001	dog wound	New Zealand;
ZP_05504092	55 (3)	T3	n/a	≤1992	urine	Sapporo, Japan

ZP_05577392	55 (3)	E1Sol	2	1960s	fecal	Solomon Islands
ZP_05423885	54 (3)	T1	n/a	≤1950		Sapporo, Japan
ZP_05574639	54 (3)	JH1	2	≤1974	clinical	U.K.
ZP_03986085	54* (3)	HH22	3	≤1982	urine	Houston, U.S.A.
pAD1 RepA	54* (3)	DS16	2	≤1978	clinical	Ann Arbor, U.S.A.
pTEF1 RepA	54* (3)	V583	3	1987	blood	St. Louis, USA
ZP_05563620	54*(3)	DS5	3	≤1974		Ann Arbor, U.S.A.
ZP_05574562	53 (78)	JH1	2	≤1974	clinical	UK
pEJ97-1 RepA	45 (307)	EJ97			waste water	Spain
pAM373 RepA	44 (307)	RC73	5	≤1979	clinical	Ann Arbor, U.S.A.
ZP_03949986	34 (78)	TX-0104				
ZP_03986058	30	HH22	3	≤1982	urine	Houston, U.S.A.

Representative sample of the 59 *E. faecalis* replication initiation proteins homologous to PrgW. Listed under RepA_N are the protein I.D. numbers on Genbank. Homology is scored as percent identity indicated by BLASTP analysis; number in parenthesis next to homology indicates number of cysteines. If one cysteine was present, the conserved cysteine was indicated in parenthesis. Astriks indicated identical replication proteins. Also listed is the strain, number of plasmids in isolate, date, isolate source, and isolate location.

upstream erythromycin gene, *E. faecalis* replication initiation protein genes (*repA* of pAD1 and *prgW* of pCF10) served as minimal origins of replication when cloned into pVA891 (Francia *et al.*, 2004; Meloni *et al.*, Submitted). Transformants were obtained on selective media following electroporation into *E. faecalis* of plasmids encoding all cysteine to alanine substitutions in *prgW* of pORI10. The results indicated that the cysteines of PrgW were not essential to the replication protein function (Table 12). All seven cysteine mutant *prgW* were also cloned into pMSP6050, which is the stable minimum pCF10 replicon, and contained the natural *prgW* promoter located 1214 base pairs upstream of *prgW,* and partitioning genes important to plasmid partitioning and maintenance (Hedberg *et al.*, 1996; Meloni *et al.*, Submitted). All *prgW* cysteine mutants of pMSP6050 were capable of forming electrotransformants on selective media, and therefore replicated in *E. faecalis*.

Conserved RepA_N cysteines of PrgW are Important to pCF10 Stability

Although the PrgW derivatives that contained the cysteine to alanine substitutions were functional, defects were revealed when the stability of their respective plasmids was studied. The rate of plasmid loss was monitored to determine if the PrgW cysteines played any role in the replication protein function(s). A plasmid loss assay detected a defect in replication protein function by monitoring the retention of plasmids throughout the population of cells over time. *E. faecalis* cultures with pORI10, pMSP6050, and their respective triple cysteine mutants plasmids (pORI-78/275/307 and pMSP6-78/275/307) were grown overnight under selection to ensure the entire population of cells contained the appropriate plasmid. Cultures were serially passaged without selection to allow for

Table 12. PrgW Cysteine Mutants Function as Plasmid Replication Initiation Proteins.

Plasmid	Electrotransformants	Plasmid	Electrotransformants
pVA891 (-)	-		
pORI10 (+)	+	pMSP6050 (+)	+
pORI-078	+	pMSP6-078	+
pORI-275	+	pMSP6-275	+
pORI-307	+	pMSP6-307	+
pORI-078/275	+	pMSP6-078/275	+
pORI-078/307	+	pMSP6-078/307	+
pORI-275/307	+	pMSP6-275/307	+
pORI-078/275/307	+	pMSP6-078/275/307	+

Cysteine to alanine mutations were created alone or in combination. *prgW* (pORI10) and mutant *prgW* genes alone or with minimal stable replicon (pMSP6050) were cloned into pVA891, a vector containing only the *E. coli* origin of replication. *prgW* and cysteine substituted *prgW* genes in pORI10 were expressed from an exogenous promoter from the erythromycin gene encoded on the plasmid. In pMSP6050, *prgW* and mutant *prgW* genes were expressed from the natural promoter. Plasmids were purified from DH5α and electroporated into *E. faecalis* OG1RF. Replication function of PrgW was determined by the ability to generate transformants following electroporation into OG1RF. All plasmids used in this study were sequenced to confirm the absence of PCR related errors.

plasmid loss. Samples taken throughout serial-passaged cultures generated colonies on non-selective media which were then patched onto selective and non-selective media. Plasmid maintenance was scored as total resistant colonies divided by total colonies sampled. *E. faecalis* containing pORI-78/275/307 or pMSP6-078/275/307 showed a sustained decrease in stability as compared to the correspondent wild-type (Figure 8B). Cells with pORI-078/275/307 showed a significant (p-value < .01) decrease in plasmid stability compared to pORI10 between generations 37 and 79 (Figure 8A). Cells with pORI-078/275/307 had the greatest difference in stability compared to wild-type of 56 percent at 59 generations. Cells with pORI-78/275/307 showed 99 percent plasmid loss at 79 generations compared to 142 generations for pORI10. A more pronounced stability difference was seen in cells with the triple mutant in the minimal stability background, pMSP6-078/275/307 compared to pMSP6050. Cells with cysteine mutant *prgW* maintained significant decrease in plasmid stability (p-value < 0.013) from generations 58 to 142 compared to pMSP6050. Cells with pMSP6-078/275/307 showed the greatest plasmid loss, 59 percent decrease compared to wild-type at 142 generations. Nonetheless, both vectors showed PrgW cysteines played a role in plasmid stability. It is unclear if the PrgW cysteine effect on stability is due to a role in replication initiation function or an undefined plasmid maintenance function.

One explanation for a decrease of plasmid stability might be a decrease in plasmid copy number for *E. faecalis* cells with the PrgW cysteine mutants. A modified assay described by Skulj *et al*. (2008) was used with real-time PCR to monitor the relative copy number of wild-type plasmids in comparison to plasmids with the triple cysteine substitution. The pORI-078/275/307 and pMSP6-078/275/307 had a greater than 35

percent (p-value < 0.05 and 0.01 respectively) reduction in copy number relative to wild-type (Figure 9A and B). Reduction in copy-number throughout the population could account for observations of reduced stability.

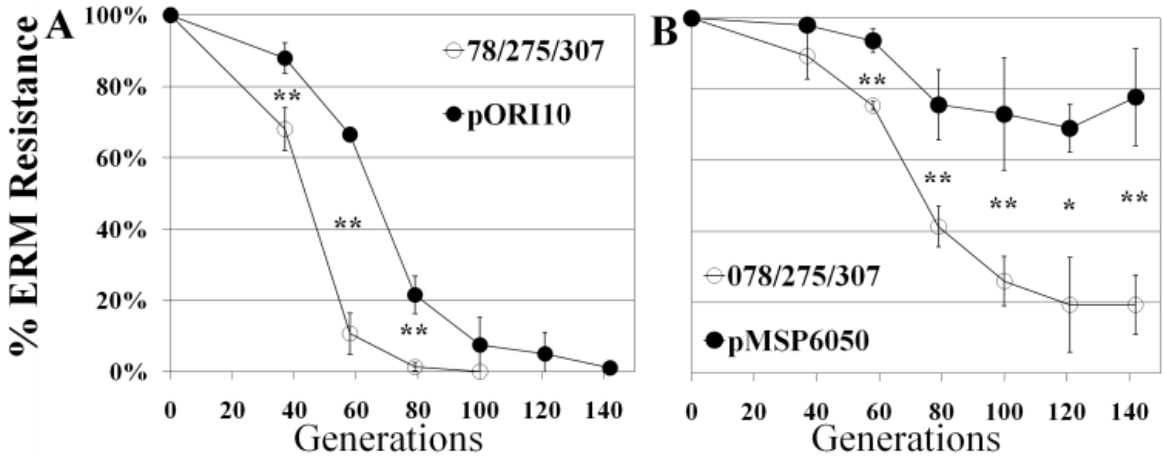


Figure 8. PrgW Cysteines were Important for Plasmid Stability.

Plasmid stability within population (percent erythromycin resistant colonies) after growth without selection (generations). (A) pOR10 and pORI-078/275/307 and (B) pMSP6050 and pMSP6-078/275/307. Closed circles indicate plasmids with wild-type *prgW*; open circles indicate plasmids with triple-cysteine mutant *prgW*. (*) indicated T-test p-value of <0.015; (**) indicated a T-test p-value of < 0.010. Results are the average +/- SD of at least three independent experiments.

PrgW C307 is Critical for Plasmid Stability

PrgW with the triple cysteine substitution was not the only cysteine mutant PrgW that caused a decrease in plasmid stability. Plasmids pMSP6-078, pMSP6-078/275, pMSP6- 078/307, and pMSP6-275/307 all had a stability loss similar to

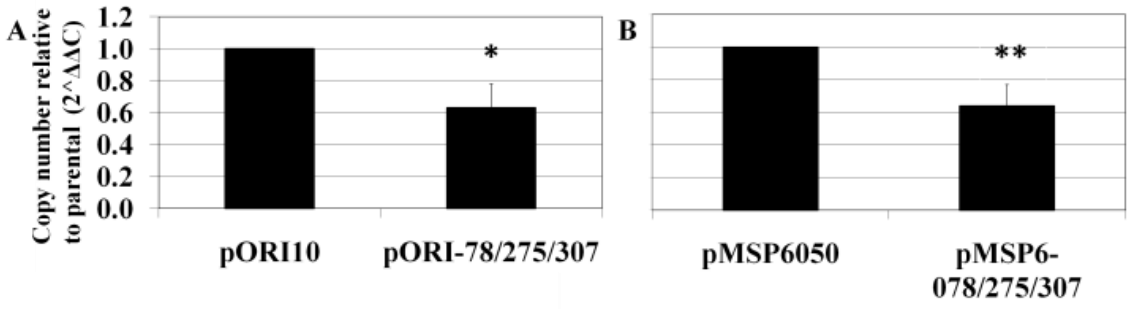


Figure 9. PrgW Cysteine Mutants have a Reduced Copy Number.

Plasmid copy number was determined by RT-qPCR done on whole-cell lysates by a modified protocol from Skulj *et al.* (2008). The plasmid copy of each sample was normalized to the chromosomal control and results reported as the relative number of the mutant to parental plasmid. (A) Comparison of (A) pOR10 and pORI-078/275/307 and (B) pMSP6050 and pMSP6-078/275/307. (*) indicated T-test p-value of <0.05; (**) indicated a T-test p-value of < 0.01. Results are the average +/- SD of three (pORI10) and six (pMSP6050) independent experiments.

pMSP6-078/275/307 (Figure 10A). In contrast, plasmids pMSP6-078 and pMSP6-275 had plasmid loss similar to parental pMSP6050 (Figure 10B**).** The stability results of the cysteine substituted PrgW mutants compared in concert with parental pMSP6050 suggested that all mutants with a decrease in plasmid stability theoretically inhibited intramolecular disulfide bond formation with C307. This observation provided evidence that the C307 of PrgW is the critical cysteine residue in controlling plasmid stability.

Stability decreased in all of the PrgW cysteine substituted pORI10 vectors compared to parental pORI10. All single (Figure 11A) and double (Figure 11B) cysteine to alanine substitutions of PrgW in pORI10 vectors had a similar plasmid stability loss to pORI-078/275/307 (Figure 11C). This difference from pMSP6050 might be attributed to

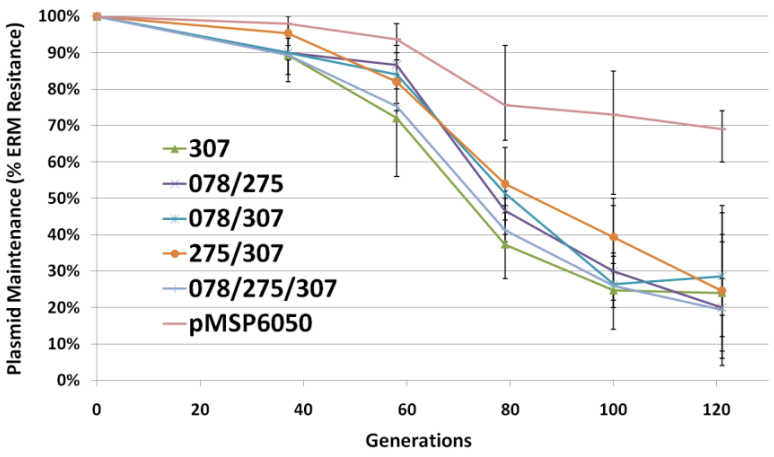
the lack of additional stability factors in pORI10 and to the problem that *prgW* was not expressed from its natural promoter.

Plasmid Host Range Mediated by RepA_N Conserved Cysteiens

Precursor pre-cCF10 and PrgW Triple Cysteine Mutant Extend the pCF10 Host Range

Pre-cCF10 and PrgW directly interact, and the host range of pCF10 was extended to *L. lactis* when *L. lactis* was engineered to produce pre-cCF10 (Meloni *et al*, submitted). One reason why the host range of pCF10 was extended to *L. lactis* might be that pre-cCF10 could mediate proper conformation of PrgW through the cysteines, otherwise, PrgW would fold incorrectly in *L. lactis*. PrgW with alanine substitutions at the three conserved cysteines still functioned in *E. faecalis* but the replicons were less stable. This result suggested the cysteines were important for proper function but not essential. Therefore, it was hypothesized that the triple cysteine to alanine substitution mutant PrgW might replicate in *L. lactis* independent of pre-cCF10. Although the number of transformants was low, pMSP6-078/275/307 electroporated into *L. lactis* yielded an average of 2 transformants per 1000 ng DNA (Table 13). Transformants were not obtained with pMSP6050, pORI10 and pORI-078/275/307, and it was inferred that they did not replicate in *L. lactis.* Transformants were tested for the presence of the mutant *prgW* in *L. lactis* using PCR and restriction digestion patterns specific to the *prgW* triple mutant. The 16S rRNA gene was amplified by PCR, sequenced, and tested against the BLAST database to verify that transformants were *L. lactis.* This evidence showed that both the addition of pre-cCF10 and substitution of PrgW cysteines extended the host

A



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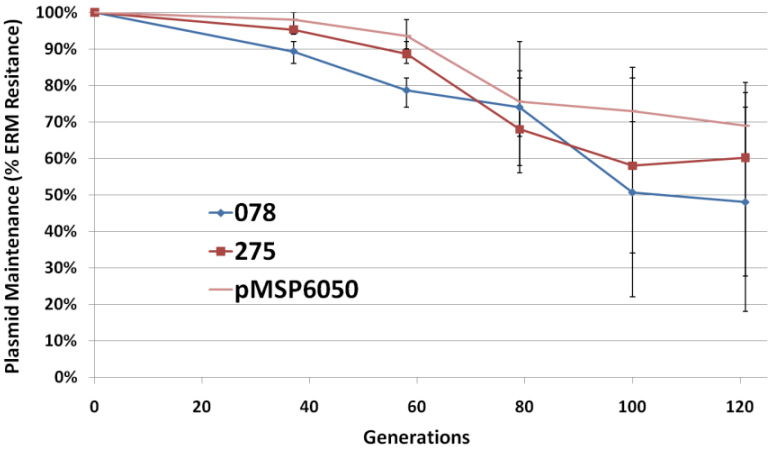


Figure 10. Disruption of Potential Disulfide Bonds with C307 Reduced Plasmid Stability in pMSP6050.

E. faecalis **OG1RF cells containing the indicated plasmid, with a pMSP6050 background, were grown overnight in Todd Hewitt (TH) broth containing erythromycin. Stability of plasmids was determined by serial passage in non-selective TH media. Passaged cultures were plated onto TH agar and 50 randomly selected colonies were replica-plated onto non-selective and selective plates. Plasmid maintenance was scored as percent erythromycin resistant cells. (A) All constructs containing C307A or both C78A/C275A substitutions showed reduced plasmid stability in a pMSP6050 background. (B) Constructs with C78A or C275A substitutions in a pMSP6050 background showed similar plasmid maintenance to wild-type PrgW. Results are the average +/- percent error of three independent experiments.**

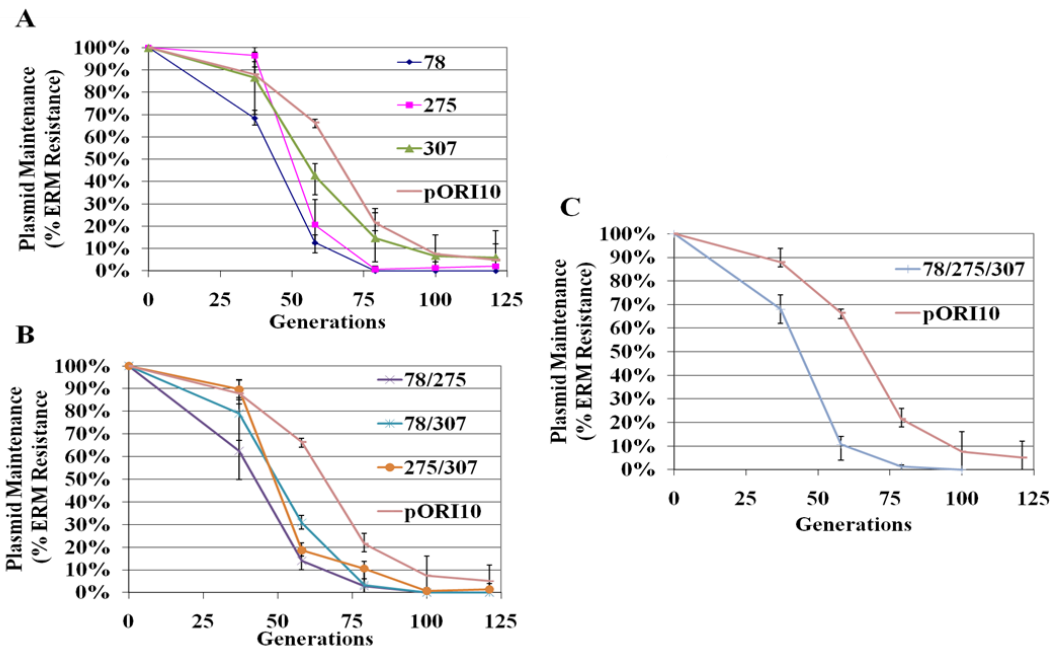


Figure 11. Stability of pORI10 Compared to Plasmids with PrgW Cysteine Substitutions

***E. faecalis* OG1RF cells containing the indicated plasmid were grown overnight in Todd Hewitt (TH) broth containing erythromycin. Stability of plasmids was determined by serial passage in non-selective TH media. Passaged cultures were plated onto TH agar and 50 randomly selected colonies were replica-plated onto non-selective and selective plates. Plasmid maintenance was scored as percent erythromycin resistant cells. (A) Single, (B) double, and (C) triple cysteine to alanine substitutions in *prgW* decreased stability in the *prgW* minimal replicon vector (pORI10). Results are the average +/- percent error of at least three independent experiments.**

Table 13. Extension of pMSP6050 Host Range into *L. lactis*

Strain	Plasmid	Colonies (trial #1)	Colonies (trial #2)	Colonies (trial #3)
LM0230	pMSP6050	0	0	0
LM0230	pMSP6-078/275/307	1	3	2
LM0230	pORI10	0	0	0
LM0230	pORI-078/275/307	0	0	0

Plasmid maintenance in *L. lactis* was scored based on the ability to establish transformants (number of colonies per 1000 ng DNA) on selective media following electroporation. Transformants were tested for the presence of *prgW* using PCR to amplify the gene. The PCR product was digested with HhaI restriction endonuclease to verify the *prgW* triple mutant cleavage pattern. The 16s rRNA gene of *L. lactis* was amplified by PCR, sequenced, and the identity was verified using BLASTN.

range to *L. lactis*, and suggested that a direct or indirect interaction of pre-cCF10 with PrgW could control pCF10 host range.

Additional studies looked at whether pMSP6-078/275/307 could replicate in other species besides *E. faecalis* and *L. lactis*. Unlike *L. lactis, S. mutans* UA159 was not capable of maintain pMSP6050 when engineered to produce pre-cCF10 (Meloni, 2005). This trend held true as pMSP6-078/275/307 could not extend host range to *S. mutans* (Table 14). These results suggested that there are other host factors involved with initiation of the plasmid that are not capable of interaction with PrgW in *S. mutans*.

Table 14. PrgW and PrgW Cysteine Mutant Plasmid did not Establish in *S. mutans*.

Strain	Plasmid	Colonies (trial #1)	Colonies (trial #2)	Colonies (trial #3)	Average
UA159	pMSP6050	0	0	0	0
UA159	pMSP6-078/275/307	0	0	0	0
UA159	pVA891 (-)	0	0	0	0
UA159	pMSP3535 (+)	585	685	260	510

pMSP6050 maintenance in *S. mutans* was scored based on the ability to establish transformants (colonies per 100 ng DNA) on selective media following electroporation.

Conserved RepA_N Proteins Found Exclusively in E. faecalis

The removal of the cysteines of PrgW caused a decrease in plasmid stability and increased host range, suggesting that the PrgW cysteines are important in pCF10 maintenance. Preliminary in silico evidence suggested that the conserved cysteines of RepA_N proteins have also played a role in plasmid host range. CLC Main Workbench tree analysis revealed that RepA_N proteins containing the three conserved cysteines were found exclusively in *E. faecalis* (Figure 12, box). RepA_N homologs containing one or fewer cysteines could be found in other enterococcal (Figure 12, E) and staphylococcal (Figure 12, S) species. pAM373 and pEJ97-1 had their own set of conserved cysteines, which were found in a separate cluster (Figure 12, asterisk). As additional reference sequences become available it may be possible to determine if pAM373 and pEJ97-1 are part of an *E. faecalis* or mixed species cluster. Furthermore,

consensus sequence prediction showed that cysteine conserved RepA_N proteins in *E. faecalis* also contain a conserved C-terminal region that might be related to cysteine mediated protein folding or that could be a factor contributing to plasmid host range (Figure 13).

Cysteines Control PrgW Conformation and Aggregation

Additionally, PrgW might form intermolecular disulfide bonds in *E. faecalis.* PrgW wild-type and triple cysteine substitution mutant whole cell lysate proteins were fractionated on a 4 to 20 percent polyacrylamide SDS-PAGE gel and visualized by Western blot to compare protein migration. In the pMSP6050 background, the most noticeable observation with non-reducing conditions is that even when loading equal quantities of protein, more PrgW monomer was detected in the gel as the number of cysteines was reduced, suggesting cysteine-dependent aggregation of the protein (Figure 14 A). When the same samples were separated under reducing conditions (Figure 14 B) the samples appeared to resolve to the PrgW monomer molecular weight. This provided evidence that PrgW was capable of forming intramolecular (Figure 7A) and intermolecular (Figures 14 A and 14 B) aggregates mediated by the presence of the cysteines. It should be noted that there were aggregations that appeared in the reducing gel (asterisk), but these are attributed to the OG1RF background (Figure 14C and 14D).

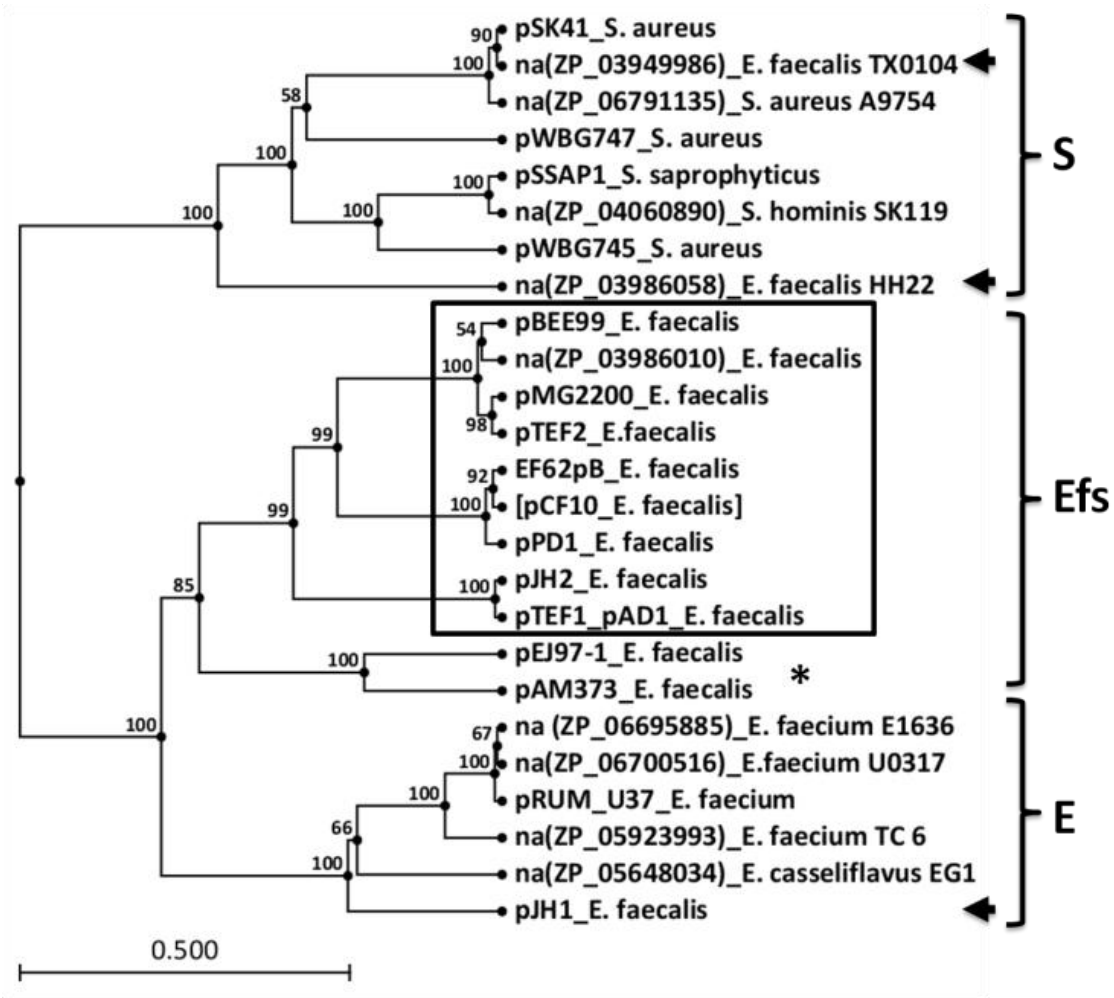


Figure 12. Presence of Conserved Cysteines of RepA_N Proteins in *E. faecalis* and other Species.

The phylogenetic tree was generated using the CLC Combined Workbench 6.0.2 sequence analysis software; bootstrap values are indicated at nodes. RepA_N proteins with conserved cysteines (located within box) are found exclusively in *E. faecalis* cluster (Efs). RepA_N proteins of *E. faecalis* with one or zero conserved cysteines (arrowheads) are found in clusters of other enterococcal species (E) and staphylococcal species (S). pAM373 and pEJ97-1 contain a separate set of conserved cysteines (*).

Figure 13. Consensus sequence of *E. faecalis* RepA_N proteins.

RepA_N proteins of *E. faecalis* containing three conserved cysteines and a species specific C-terminus generated by WebLogo (Version 2.8.2).

Additionally, the effects of cysteine- mediated aggregation were also seen as PrgW remaining sequestered in the well of a 12 percent acrylamide gel. In Figure 15 A, *E. faecalis* cells with pMSP6-078/275/307 appeared to be the only sample where the whole cell lysate contained PrgW. When the same samples were treated under reducing conditions, it was clear that the wild-type, single cysteine substituted, and double cysteine substituted PrgW all contained PrgW in the monomer form. The purified PrgW control (Figure 16, PrgW) also aggregated (Figure 16A) and reducing conditions resolved the aggregates producing increased PrgW in the monomer form. It is important to note that it was assumed that PrgW was trapped in the well of the stacking gel. It is unclear as to why the levels of PrgW appeared to decrease in the reducing gel, compared to the non-reducing gel. Perhaps the reducing conditions caused by βME affected the overall

effectiveness of the ECL chemiluminescence, or could have affected the disulfide bonds of the antibodies used in the Western Blot.

Modular Nature of RepA_N Plasmids

RepA_N plasmids like pCF10 have evolved in distinct modules (Hirt *et al.*, 2005; Weaver *et al*., 2009a). The plasmids have a number of distinct functional modules including plasmid replication (RepA/PrgW), partitioning proteins (PrgO and P/RepB and C), pheromone-response proteins (PrgZ and Y), and proteins of unknown function (PrgN and UvaF). The percent homology between plasmids varied depending on which domains are compared, suggesting modular evolution. Figure 16 shows amino acid identity comparisons between a representative sample of proteins encoded by RepA_N plasmids that contain genes involved in plasmid maintenance; the broad host range plasmid pRE25 was also included. pCF10 was almost identical to EF62pB except for less than 50 percent identity for UvaF. Other RepA_N proteins shared (54 to 93) protein homology to PrgW of pCF10 but lacked homology for the PrgO and PrgP parititioning proteins. For example pMG2200 lacked idientity in the amino acid sequences of PrgO and PrgP compred to PrgO and PrgP of pCF10. However, pMG2200 had a greater than 96% homoloty in UvaF, UvaF, PrgN, PrgZ and PrgY. As previously reported by Hirt *et al.* (2005), broad host range plasmid pRE25 was found to have approximately 80 percent identity to PrgO and PrgP of pCF10, yet no homology to other replicon genes. Homology comparisons also showed that a putative error prone DNA polymerase UvrA, was conserved in almost all RepA_N plasmids (except pTW9; Figure 16). The protein of

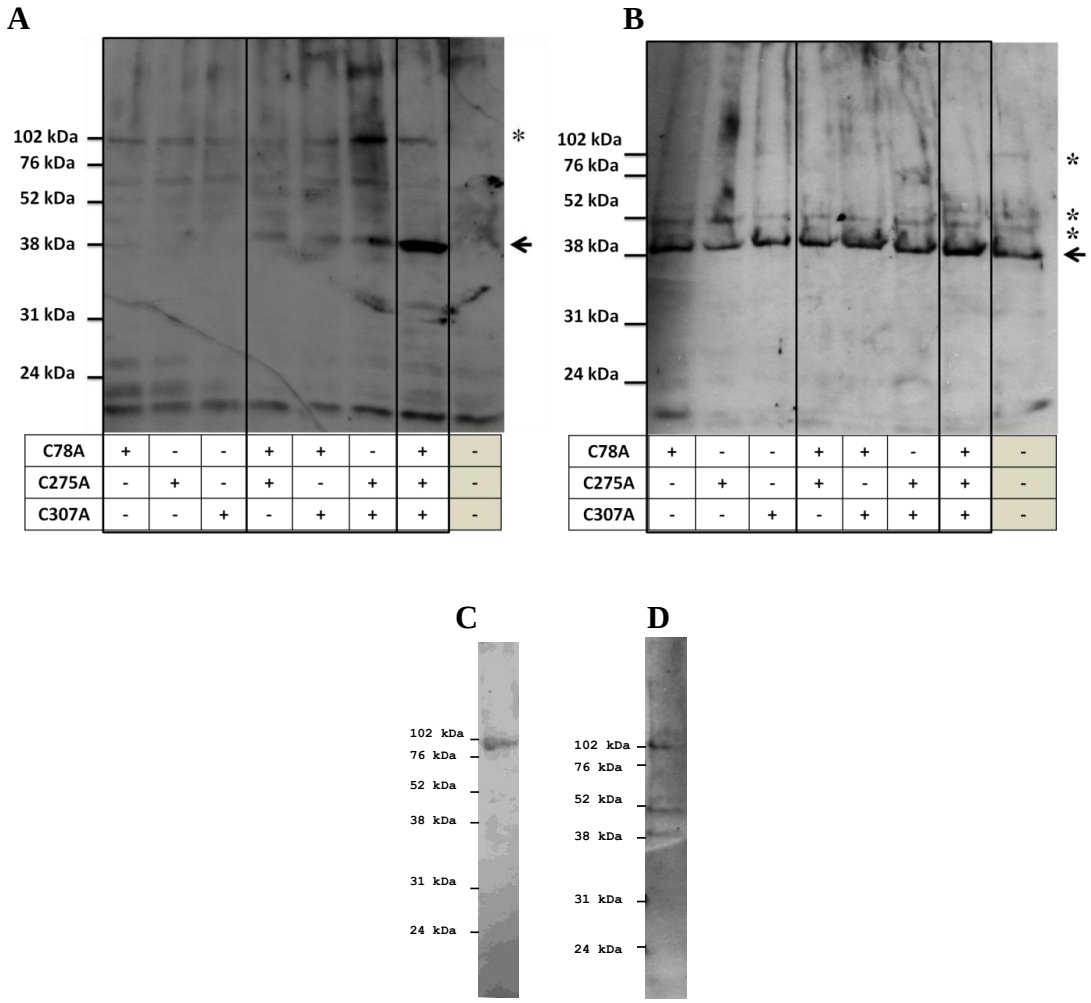


Figure 14. Cysteines Mediate Inter- and Intramolecular Mediated Conformational Changes of PrgW.

Whole cell extracts were concentrated to an A.O.D.$_{660}$ of 14.0 and separated by SDS-PAGE, followed by Western blot using polyclonal Anti-PrgW. (A) Migration and aggregation of PrgW (4-20% gradient PAG) under non-reducing and (B) reducing conditions. Lanes are as follows: (1) pMSP6-078; (2) pMSP6-275; (3) pMSP6-307; (4) pMPS6-078/275; (5) pMPS6-078/307; (6) pMSP6-275/307; (7) pMPS6-078/275/307; and (8) pMSP6050. Arrow indicates the migration of PrgW monomer. Asterisks indicate plasmid-free OG1RF background bands as seen in (C) non-reducing and (D) reducing conditions.

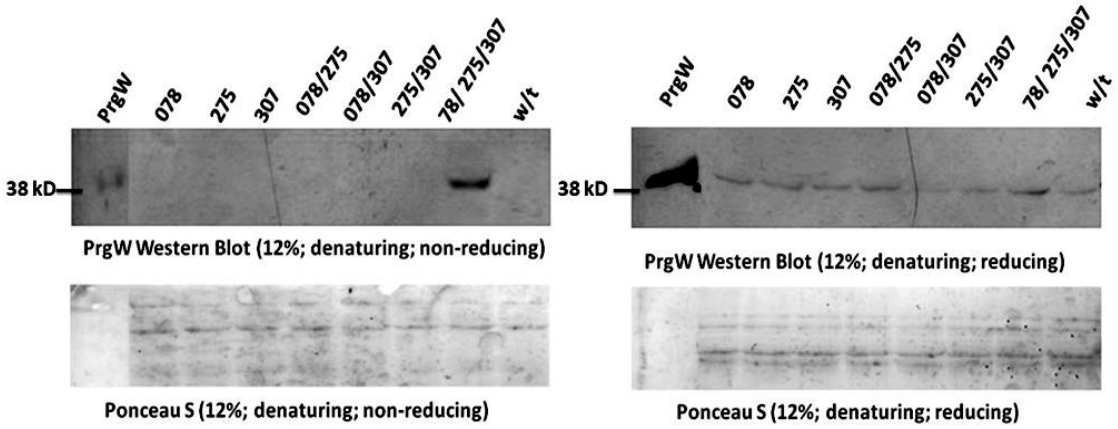


Figure 15. Cysteines Mediate PrgW Aggregation Trapping in PAG Wells

Overnight cultures of *E. faecalis* OG1RF were split 1:1000 and grown to exponential phase ($O.D._{660}$ = 0.6 to 0.7 A). Whole cell extracts were prepared from cells concentrated to an A.O.D. of ~ 14.0 and analyzed by SDS-PAGE (12 percent), followed by Ponceau S stain and Western blotting using polyclonal Anti-PrgW. The results shown were representative of three independent experiments.

unknown function, PrgN, was found in a majority (except pTEF1) of RepA_N plasmids (Figure 16).

These homology differences between different modules described above provided further evidence of the modular evolution of RepA_N plasmids. Prior phylogenetic analysis of Genbank published amino acid sequences suggested that RepA_N replication initiation proteins have evolved within their own genus, while the partitioning proteins like PrgO and PrgP were modularly acquired (Weaver *et al*., 2009a). PrgO and PrgP were found to have high homology to partitioning proteins of the broad host range plasmid pRE25 (Hirt *et al*., 2005). The partitioning proteins of pCF10 were also found to

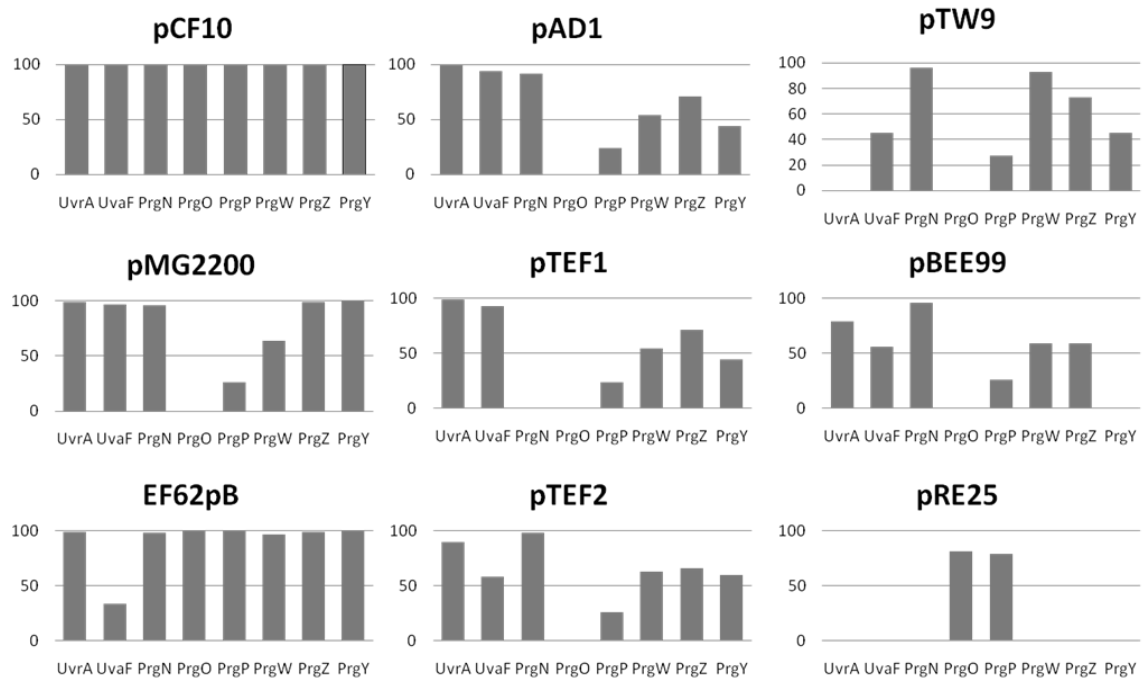


Figure 16. Homology Differences Between Proteins of RepA_N Plasmids.

Representative identity comparisons of as percent of proteins encoded within the pCF10 minimal stable replicon. pCF10, pMG220, pTEF1, pTEF2, pBEE99, pAD1, and EF62pB are pheromone-response plasmids. pRE25 is a broad-host-range plasmid. The pheromone-responsiveness of unnamed plasmid pTW9 is unknown.

plasmids. Figure 17 showed that homologs of the pCF10 centromere binding protein PrgO belonged to a distinct cluster of partitioning proteins. There were very few PrgO homologs found with identity of less than 80 percent. Plasmids either had high amino acid identity to PrgO or had no homology. PrgO partitioning protein provided an excellent example of how proteins encoded on RepA_N plasmids were capable of modular spread within two different species.

Modular Nature of RepA_N Replication Initiation Protein Domains Determine Presence of Cysteine

It had been suggested that, while the partitioning proteins like PrgO and PrgP were modularly acquired, RepA_N replication initiation proteins have evolved within their own genus (Weaver *et al*., 2009a). However, comparison of two recently sequenced unique plasmids suggests RepA_N proteins may evolve modularly across species. *E. faecalis* strain JH1 was isolated in 1974 (Jacob and Hobbs, 1974) and contains two plasmids pJH1 and pJH2 (LeBlanc and Lee, 1982). pJH1 encoded gentamicin, tetracycline, erythromycin and streptomycin resistance. pJH2 encodes the virulence factor hemolysin-cytolysin. pJH1 was determined to be a non-conjugative plasmid. pJH1 contained one conserved cysteine (C78 of PrgW) and had greater homology to RepA_N from other *E. faecium* plasmids than from *E. faecalis* plasmids (Figure 12, E, arrow). pJH2 was determined to be a pheromone-responsive plasmid. pJH2 had the three conserved cysteines of *E. faecalis* RepA_N proteins and was part of an *E. faecalis* cluster (Figure 12, Efs).

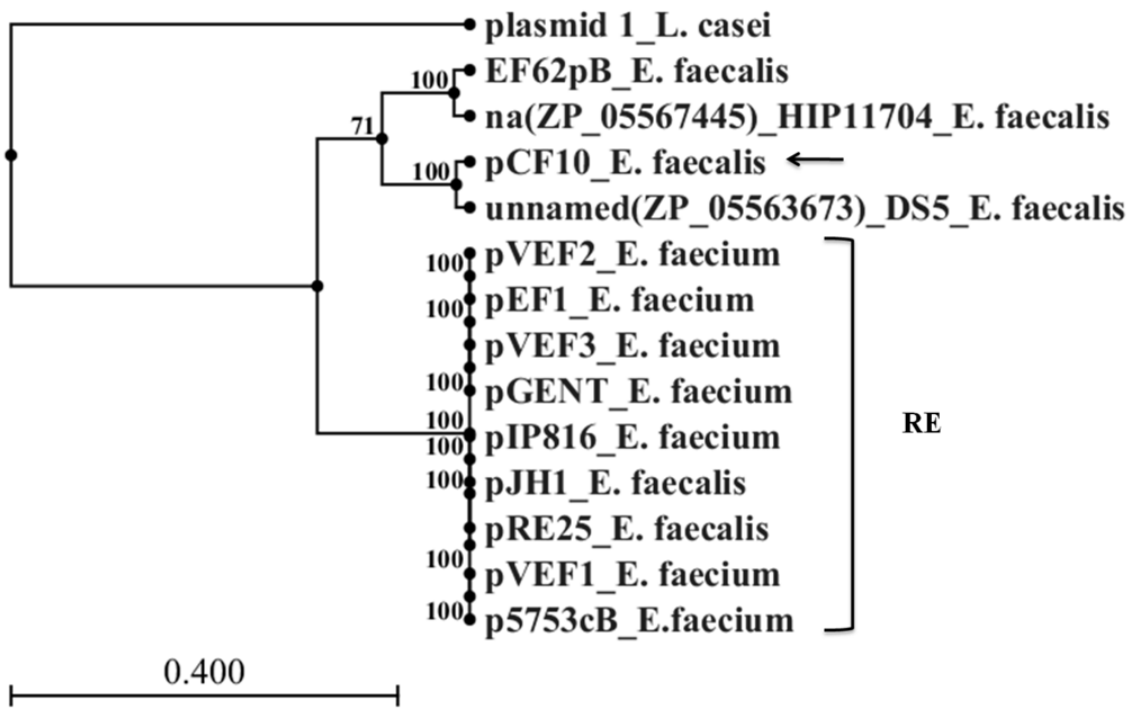


Figure 17. pCF10 Partitioning Protein PrgO Homologous to Broad Host Range Plasmid Partitioning Proteins

The phylogenetic tree of RepA_N homologous to pCF10 PrgO was generated using a CLC Combined Workbench 6.0.2 sequence analysis software; bootstrap values are indicated at nodes. pRE25 (RE) cluster represents 18 *E. faecium* and 11 *E. faecalis* PrgO homologs; EF62pB represents 11 PrgO homologs found in *E. faecalis*. Arrow indicates PrgO of pCF10.

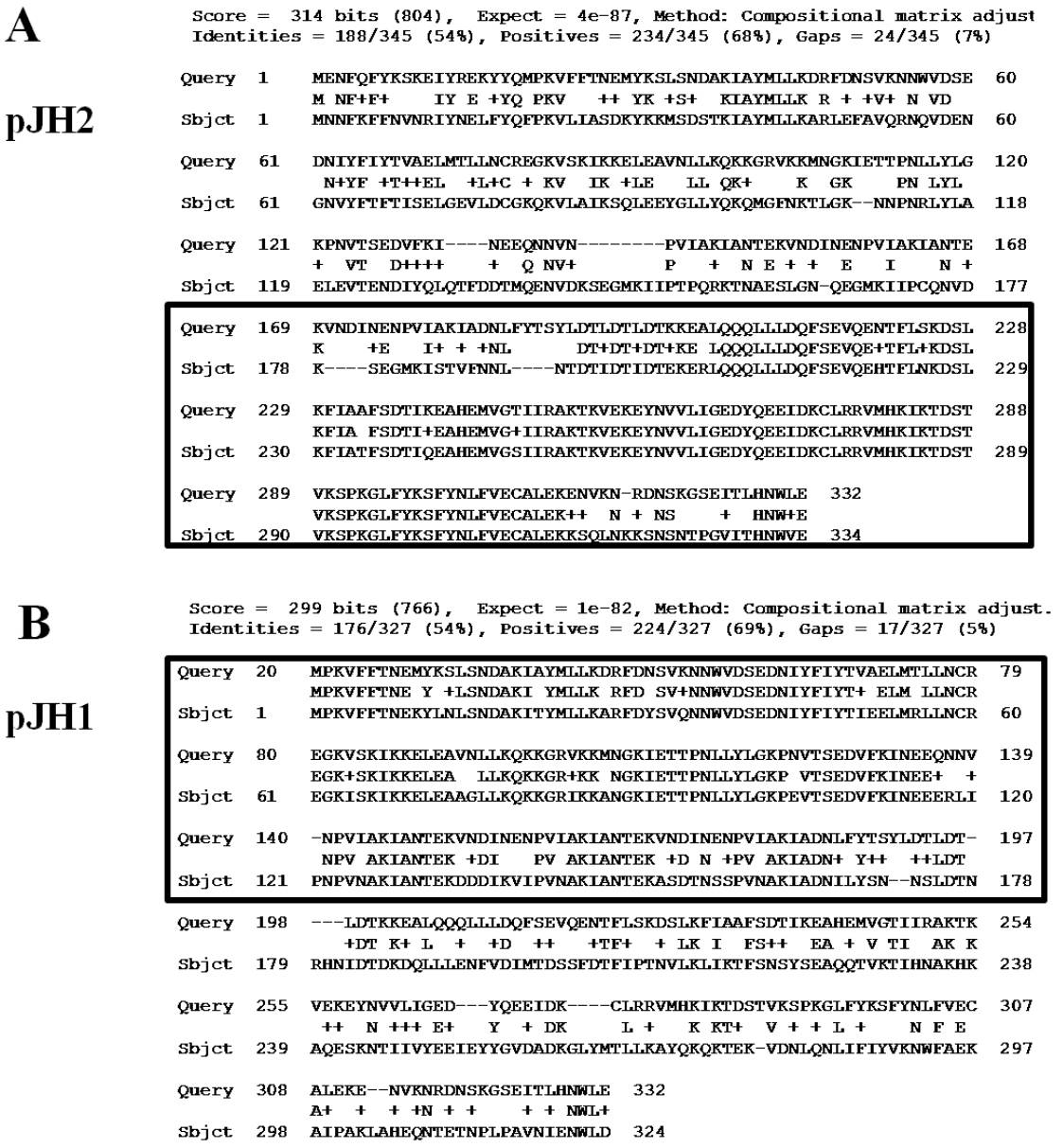


Figure 18. N- and C-terminial Regions of JH1 Plasmids.

BLASTP alignment of RepA_N proteins of (A) pJH2, and (B) pJH1 compared to PrgW of pCF10. Box indicates regions of strong homology to pCF10.

However, when the N-terminal and C-terminal portions of the proteins were analyzed separately by BLASTP homology searches to PrgW from pCF10, a different picture emerged. The N-terminus of pJH1 was found to have a higher homology to the N-terminus of PrgW (Figure 18B). On the other hand, the C-terminus of pJH2 was found highly homologous to the C-terminus of PrgW (Figure 18A). This raised the possibility that domains located at the N- and C-termini of two RepA_N proteins had exchanged as modules.

Consensus sequence alignment showed a high degree of sequence conservation in the C-terminal region if only *E. faecalis* RepA_N proteins (Figure 13) or only *E. faecium* RepA_N proteins (Figure 19) were compared to each other. BLASTP tree analysis comparisons using both these groups revealed species-specific C-terminal domains (Figure 20). The one outlier was pJH1 RepA_N protein, which as discussed above appears to have acquired an *E. faecium*-like C-terminal RepA_N domain. Alternatively, the N-terminus was not species specific (Figure 21). The N-terminus had three distinct *E. faecalis*-*E. faecium* clusters, and one distinct *E. faecalis* cluster. This provided further evidence that the two regions of these RepA_N proteins potentially derived from different sources and new RepA_N proteins were possibly generated in modular evolutionary events.

Figure 19. Consensus Sequence of *E. faecium* RepA_N Proteins.

***E. faecium* RepA_N contain C78 conserved cysteine at the N-terminus and a species specific C-terminus lacking C275 and C307 WebLogo (Version 2.8.2).**

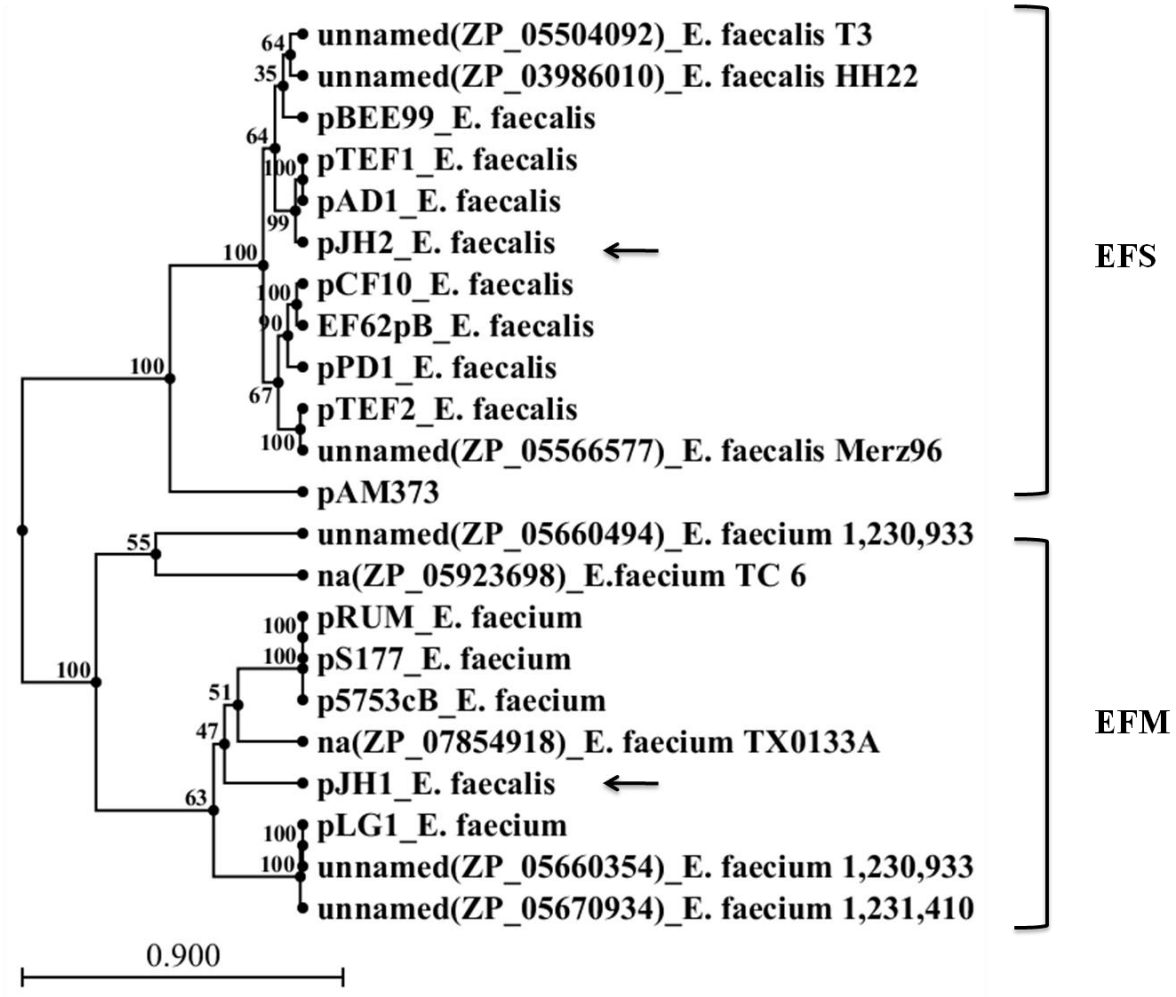


Figure 20. Conserved C- Terminus Alignment of *E. faecalis* RepA_N

The phylogenetic tree of the C-terminus of *E. faecalis* and *E. faecium* RepA_N proteins was generated using the CLC Combined Workbench 6.0.2 sequence analysis software; bootstrap values are indicated at nodes. Brackets indicate *E. faecalis* (EFS) or primarily *E. faecium* (EFM) clusters. Arrows indicate pJH1 and pJH2 plasmids discovered in *E. faecalis* JH1 strain.

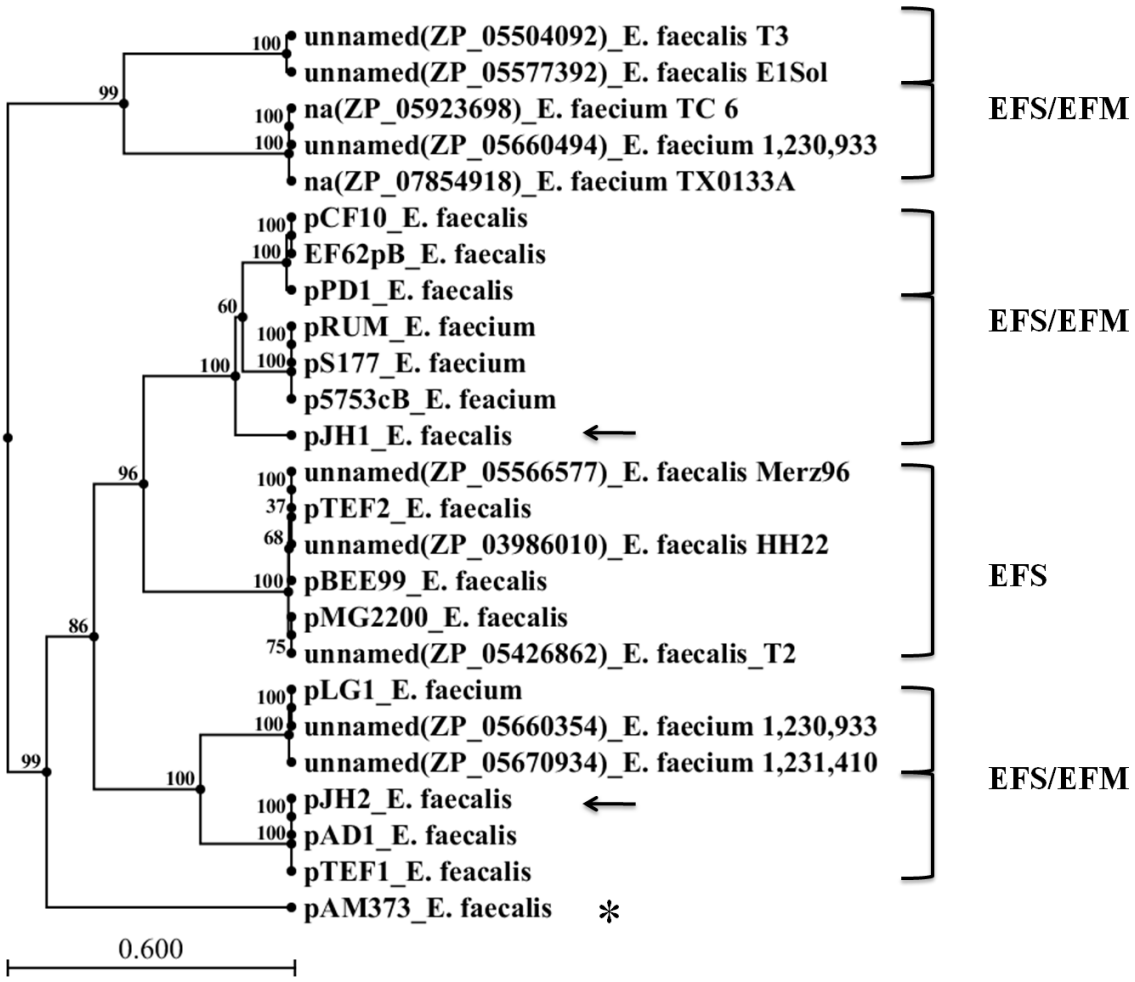


Figure 21. Conserved N- Terminus Alignment of *E .faecalis* RepA_N

The phylogenetic tree of the N-terminus of *E. faecalis* and *E. faecium* RepA_N proteins was generated using the CLC Combined Workbench 6.0.2 sequence analysis software; bootstrap values are indicated at nodes. Boxes indicated four distinct clusters. Clusters with one bracket indicate strictly an *E. faecalis* cluster. Cluster with two brackets indicate *E. faecium* and *E. faecalis* clusters. Arrows indicate *E. faecalis* C-termini that are homologous to *E. faecium* C-termini. pJH2 represents pJH2 RepA_N N-terminus.

Perhaps the modular evolutionary event allowed for adaptation of the RepA_N protein, or modified the replication protein so the plasmid could replicate in other species. In the case of JH1 strain, an *E. faecium*-like plasmid most likely entered *E. faecalis*. Once inside, the RepA_N proteins switched the C- and N-termini making the protein more functional in *E .faecalis* or perhaps more flexible to replicate in other species. Interestingly, the AT rich DR region of each plasmid is located between the N- and C-terminus domains, and could serve as a recombination prone region.

Plasmid Stability Increased After Extended Serial Passage

During extended serial passages in the absence of selection, *E. faecalis* with plasmids containing the cysteine substituted PrgW increased in plasmid stability between generations 121 and 163 (Figure 22). All single and double cysteine substitutions had an increase in stability that started at about generation 121. The stability increase was not seen prior in pMSP6050 stability trials (Mazeffa, 2008; Meloni, 2005). This was most likely because previous stability studies extended only to 100 to 120 generation. Since pMSP6050 did not have conjugation machinery an increase in plasmid stability could have only been attributed to enhanced death of cells without the plasmid or an increased proliferation of cells with the plasmid compared to cells without the plasmid.

Initial studies that examined the stability increase of pMSP6050 ΔCys mutants focused on the *par* stability factor encoded on the plasmid. The *par* or post-segregational kill system (PSK) gene encoded a non-functional toxin and did not have an effect on plasmid stability for up to 100 generations (Mazeffa, 2008). It was hypothesized that the codon encoding the stop signal (at amino acid residue six) was being bypassed because of

to ribosomal ambiguity (Piggot *et al.*, 1972). The translation of a functional toxin because of ribosomal ambiguity might have contributed to the stability increase if cells without the plasmid were selectively killed. Figure 23 compared the plasmid maintenance of pMSP6050Δ *par* to pMSP6-078/275Δ *par*. pMSP6-070/275 was chosen because it had a dramatic increase in stability between generations 121 and 142. In addition, the cysteines of pMSP6-078/275 were located between restriction sites that facilitated the insertion of recombinant DNA (Table 9). Results showed that stability of pMSP6-078/275Δ *par* was similar to the stability of pMSP6-078/275, suggesting that ribosomal ambiguity of the encoded stop codon in *par* did not contribute to the increase in plasmid stability between generations 121 and 163. In addition, Figure 23 showed stability of the Δ*cys* plasmid did not have a difference in stability compared to Δ *par* Δ*cys* plasmid up to 163 generations which expanded upon previous results that showed *par* did not contribute to pCF10 stability past 100 generations (Mazeffa, 2008).

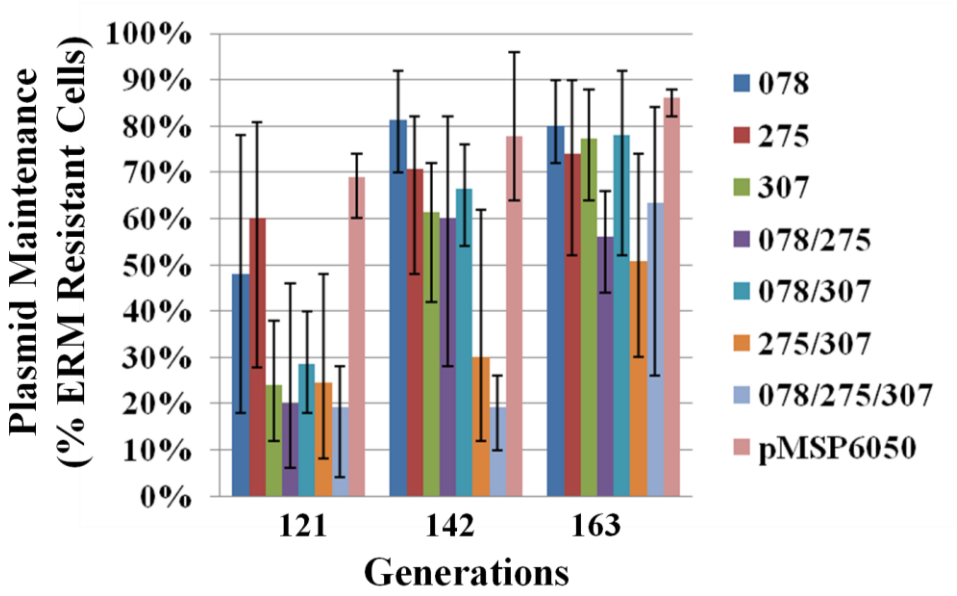


Figure 22. Stability Increase of Cysteine Substituted Minimal Stability Backgrounds

***E. faecalis* OG1RF cells containing the indicated plasmid were grown overnight in Todd Hewitt (TH) broth containing erythromycin. Stability of plasmids was determined by serial passage in non-selective TH media. Passaged cultures were plated onto TH agar and 50 randomly selected colonies were replica-plated onto non-selective and selective plates. Plasmid maintenance was scored as percent erythromycin resistant cells. All cysteine to alanine substitutions in *prgW* plasmid minimal stability replicon (pMSP6050) increased in stability between 121 and 163 generations in the pMSP6050 background. Results are the average +/- percent error of three independent experiments.**

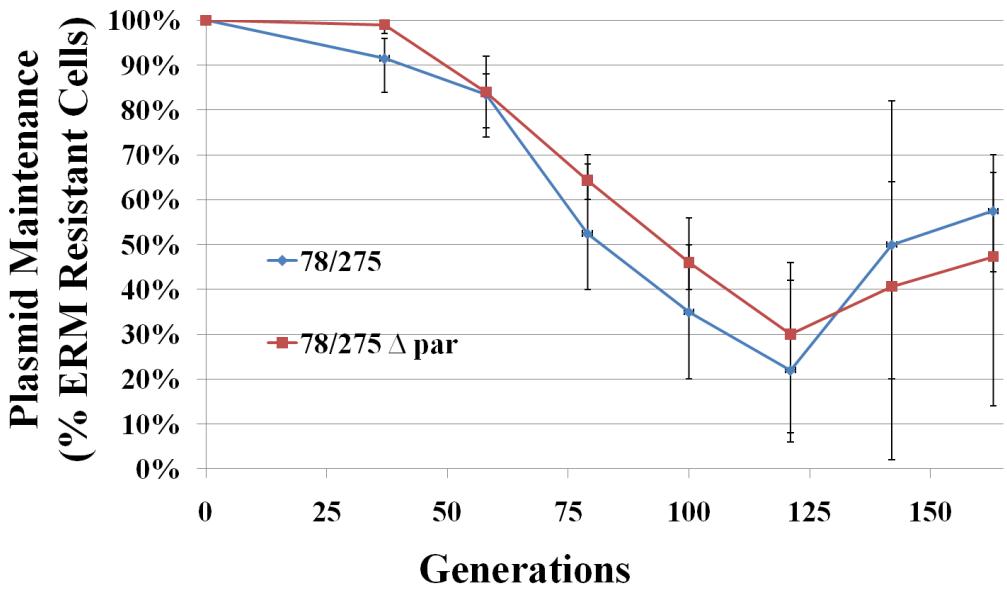


Figure 23. Increased Plasmid Stability of pMSP6050 Derivatives was not Caused by *par*

***E. faecalis* OG1RF cells containing the indicated plasmid were grown overnight in Todd Hewitt (TH) broth containing erythromycin. Stability of plasmids was determined by serial passage in non-selective TH media. Passaged cultures were plated onto TH agar and 50 randomly selected colonies were replica-plated onto non-selective and selective plates. Plasmid maintenance was scored as percent erythromycin resistant cells. Comparing pMSP6050 with *prgW* C78A/C275A (78/275) to *prgW* C78A/C275A (78/275 Δ par). Results are the average +/- percent error of three independent experiments.**

CHAPTER 4

DISCUSSION

PrgW Disulfide Bonds Control Plasmid Stability and Host Range

Pheromone-responsive plasmids are extremely stable plasmids whose host range is restricted to *E. faecalis*. This stability can be accounted for both by intrinsic properties of the replicon and conjugative spread throughout the population. These plasmids can mediate the transfer of chromosomally encoded antibiotic resistance markers and virulence factors between the strains of *E. faecalis.* The replication initiation protein of pCF10, PrgW, interacts with the precursor pheromone pre-cCF10. The pheromone precursor, pre-cCF10 influences PrgW mobility in non-reducing SDS gels and can be used to extend pCF10 host-range to *L. lactis*. The data presented in these studies suggest PrgW interaction with pre-cCF10 dictates plasmid stability and host-range by affecting the conformation of PrgW through its cysteines. This study produced evidence that there is a strong correlation between the cysteines of RepA_N protein PrgW and the stable maintenance of the pheromone-responsive conjugative plasmid, pCF10. There is an expanding list of mechanisms that are important for plasmid maintenance throughout a population. First, essential to plasmid maintenance, is the function of replication initiation proteins (like PrgW of pCF10) that bind to plasmid DNA and interact with host replication machinery allowing for autonomous replication (del Solar *et al.*, 1998; Meloni *et al.*, Submitted). Secondly, copy control is important for low-copy number plasmids like pCF10 as cells must find a balance between having a sufficient amount of plasmid

for dissemination from mother to daughter cell while not over-burdening cellular machinery and processes by generating too many plasmid copies (Nordstrom *et al.,* 1984; Uhlin and Nordstrom, 1978; Wagner and Brantl, 1998). Thirdly, partitioning proteins, like RepB and RepC of pAD1, bind to plasmid DNA and help escort it to the appropriate location at the membrane to ensure transfer will occur from both mother to daughter cells (Francia *et al.*, 2007). Furthermore, toxin-antitoxin systems like *par* of pAD1 cause cell death to cells that lose the plasmid, providing a maintenance mechanism that selects for cells containing the pheromone-responsive plasmid (Weaver *et al* 2009b). Finally, the pheromone-responsive conjugation mechanism itself helps ensure transfer of plasmid through the entire bacterial population (Dunny, 2007).

The pORI10 and pMSP6050 derivatives used in this study did not encode the pCF10 conjugation machinery. pCF10 and pMSP6050 had a point mutation preventing production of the toxic peptide of the partitioning kill system described for pAD1. Additionally, deletion of the region encoding the toxic peptide did not affect pCF10 stability (Mazeffa, unpublished observations). Therefore, PrgW cysteine substitution effects on these stability factors were ruled out. If pCF10 has a copy number of two to four (Weaver *et al.*, 2002) and this number was reduced 35 percent by the cysteine substitution mutations (Figure 9), then it would have resulted in one to three plasmid copies per cell. Any cell with a single copy of the plasmid that would have produced only one daughter cell with a plasmid copy upon division. Thus, it is likely that the decrease in copy number and the decrease in stability are related (Figure 8). The decreased copy number could be caused by various mechanisms such as decreased replication initiation function or effects on plasmid maintenance.

The cysteines affected PrgW migration under non-reducing conditions (Figures 4-7); this suggested cysteines might be important for protein conformation and might explain the decreased plasmid stability and copy number. It has been well established for a number of plasmids that conformational changes and multimerization of replication initiation proteins affect the ability to bind to DNA and initiate replication (del Solar *et al.*, 1998). For example, the R1 plasmid RepA in the dimeric form modulates (using DnaK) replication initiation by changing the DNA conformation to facilitate DnaA binding (Giraldo-Suarez *et al.*, 1993; Giraldo and Diaz, 1992). In the case of pCF10, PrgW, interactions leading multimerization could be important for melting at the origin of replication, especially because the only predicted DnaA boxes (are centered at –29 and 67 base pairs) is approximately 400 base pairs away from the *oriV* (420 to 548 base pairs) and direct repeat binding region of PrgW (Meloni *et al.*, Submitted).

Finally, the binding of PrgW to pre-cCF10 (which is located at the membrane) might recruit plasmid bound to PrgW for passage to the daughter cells, affecting efficient partitioning of the plasmid into the daughter cells. Francia *et al.* (2007) showed the mechanism by which the low-copy plasmid pAD1 (one to four copies per cell) was recruited to the membrane through the plasmid partitioning system. pCF10 also encodes for a putative partitioning system similar to the pAD1 partitioning system. However, the partitioning system is present in pMSP6050-derivatives but not pORI10-derivatives, and both showed decreased stability. It is known that other plasmids can be recruited to the membrane without partitioning systems. For example, the mini-replicon of low-copy RK2 plasmid (five to eight copies) is recruited to the membrane at the poles without a plasmid partitioning system (Kolatka *et al.*, 2008). Therefore, pre-cCF10 interaction with

PrgW might be a factor in the recruitment of pCF10 to the *E. faecalis* membrane for efficient partitioning.

The establishment of pCF10 in species other than *E. faecalis* was affected by PrgW cysteine mutation. pMSP6050 with the triple-cysteine mutant PrgW was capable of establishment in *L. lactis* independent of pre-cCF10 production. Factors for limiting host range in pheromone-responsive conjugative plasmids are generally unknown. However, several factors that determine plasmid host range in the broad-host range incQ plasmids are known and have been recently reviewed by Meyer (2009). These factors are summarized as follows: (1) plasmid replication initiation proteins are DnaA-independent; (2) y a variety of Type IV secretion mechanisms, including protein secretors that can mobilize plasmids; and (3) high plasmid copy number is necessary for maintenance, while gene expression modulation decreases the metabolic burden. The role of Type IV secretion can be ruled out, as the pCF10 Type IV secretion mechanism involved in conjugation is again not encoded in pMSP6050 and pORI10 (Hedberg *et al.*, 1996; Meloni *et al.*, Submitted). Additionally, since plasmid copy number appeared to decrease with the PrgW triple-cysteine mutation, the increased copy number scenario can probably be disregarded. However, it can be speculated that mutation of PrgW cysteines decreases the efficiency of replication initiation because it bypasses host-dependent replication machinery like DnaA. R1 plasmid replication proteins do not need DnaA to replicate; however, stability of plasmids increases with a functioning DnaA (Bernander *et al.*, 1992). In this scenario, plasmid stability could decrease in *E. faecalis* and still avoid the use of DnaA in *L. lactis* to initiate replication, thereby effectively increasing the host range of pCF10. In addition, replication initiation protein conformation affects host

range of the RK2 plasmid replication protein TrfA. The replication proteins encode for both longer and shorter replication conformations using two in frame start codons. The large conformational form TrfA-44 is required for replication in *P. aeruginosa* (Durland and Helinski, 1987; Shingler and Thomas, 1984) while the shorter version TrfA-33 promotes establishment of RK2 in *E. coli, Pseudomonas putida,* and other hosts (Cross *et al.,* 1986; Jovanovic *et al.,* 1994; Nash and Krishnapillai, 1987). Further studies are needed to distinguish between these possibilities.

For *L. lactis* without pre-cCF10, the cysteines of PrgW do not form the correct disulfide bonds and the function of PrgW is inhibited. Mutation of the cysteines result in a less efficient replication initiation protein but one that is now pre-cCF10 independent and is able to support replication in other species. These experiments suggest pre-cCF10 affects cysteine-dependent processes, such as, PrgW conformation and aggregation, which may be important for replication initiation protein function.

Oxidoreductases for Changes in Oxidation State of PrgW

While disulfide bonds can form spontaneously and in response to oxidative stress, PrgW interacts with a membrane peptide (pre-cCF10). Therefore, we hypothesize that pre-cCF10 brings PrgW to the membrane to interact with a membrane-bound oxidoreductase, which can affect disulfide bond formation. Many of the disulfide bonds involved with protein structure are formed and broken through the activity of thiol-disulfide oxidoreductases. Thiol-disulfide oxidoreductases can catalyze disulfide bond formation as well as disulfide bond reduction (Moller and Hederstedt, 2006). Thiol-

disulfide oxidoreductases are part of the thioredoxin (TRX) superfamily. The TRX superfamily is distinguished by the presence of one or more TRX folds that are involved in the disulfide bond formation. A TRX fold contain α-helices juxtaposed to β-strands and a Cys-XX-Cys motif (Carvalho *et al.*, 2006).

TRX family members have specific roles in oxidation, reduction, or isomerization of disulfide bonds. Depending on the redox state of the cell, the enzymes can function in formation or removal of disulfide bonds (Ito and Inaba, 2008). In *E. coli* oxidative folding of extracytoplasmic proteins is mediated by the Dsb system comprised of DsbA, DsbB, DsbC and DsbD. Disulfide bonds are formed within the Dsb system by DsbA, DsbC, and menaquinone. DsbA is the primary inducer of disulfide bonds of the system. It has the highest redox potential among the TRX related proteins. Electrons are removed from the target protein by DsbA causing the formation of a disulfide bond. The electrons are then passed from the transmembrane protein DsbB, reducing DsbB (at a C104-C130 disulfide bond) and re-forming the C30-C33 disulfide bond in DsbA, essentially recycling it (Inaba and Ito, 2002; Inaba *et al.*, 2005; Inaba *et al.*, 2006a). Menaquinone acts as the final electron acceptor reforming the C41-C44 disulfide bond in DsbB (Inaba *et al.*, 2006b; Inaba and Ito, 2008). Reduction (breaking of disulfide bonds) of a target protein is mediated by DsbC, DsbD, and thioredoxin. In this case electrons are transferred from thioredoxin to DsbD (forming C163-C285 disulfide bridge), and from DsbD to DsbC (Berkmen *et al.*, 2005; Cho and Beckwith, 2006; Cho *et al.*, 2007). DsbC acts as the isomerase of disulfide bonds (McCarthy *et al.*, 2000). Reduced DsbC can attack incorrectly formed disulfide bridges with its C98 residue (Porat *et al.*, 2004).

Disulfide bond formation in Gram-positive species is also mediated by thiol-disulfide oxidoreductases. *B. subtilis* contains four extracellular thiol-disulfide oxidoreductases (BdbA, BdbB, BdbC, and BdbD). The biological functions of BdbA are not known. BdbB appears encoded in a prophage region of *B. subtilis* and is responsible for disulfide bond formation in prophage SPβ antibiotic sublancin 168 (Dorenbos *et al.*, 2002; Stein, 2005). BdbD and BdbC function in the formation of disulfides bonds in a manner similar to DsbA and DsbC. BdbD (DsbA) accepts electrons from the target protein causing the formation of disulfide bonds. The electrons from BdbD (DsbA) are passed to BdbC (DsbB) and BdbD is recycled (Bolhuis *et al.*, 1999; Kouwen *et al.*, 2007; Meima *et al.*, 2002; Sarvas *et al.*, 2004).

S. aureus has a DsbA/BdbD homolog that shares 55 percent amino acid residue identity to DsbD of *B. subtilis* (Meima *et al.*, 2002). However, there is no BdbC/DsbB homolog in *S. aureus* (Dumoulin *et al.*, 2005). The *S. aureus* oxidoreductase can function similarly to BdbB, BdbC and BdbD proteins of *B. subtilis* mediating both reduction and oxidation of disulfide bonds (Kouwen *et al.*, 2007).

Basic BLAST amino acid homology search revealed putative thiol-disulfide oxidoreductases in *E. faecalis* and these could play a role in PrgW disulfide bond formation. Interestingly, oxidoreductase EF0770 (NC_004668.1) is a homolog of *B. subtilis* BdbB oxidoreductase that clusters among *Listeria* and *Lactobacillus* oxidoreductases and could be responsible for formation of disulfide bonds (Kouwen *et al.*, 2007). While BdbB is a transmembrane protein, EF0770 is predicted to be a cytoplasmic oxidoreductase (Kouwen *et al.*, 2007) in *E. faecalis*, a good candidate to induce disulfide bonds in PrgW.

Restriction of Host Range by Presence of Two Conserved RepA_N Cysteines

The RepA_N replication initiation protein cysteines may have a greater role in determining host range than just the role in pCF10. In silico evidence supported the hypothesis that the conserved cysteines of RepA_N proteins influence plasmid host-range. CLC Main Workbench phylogenetic analysis revealed that RepA_N proteins containing the three conserved cysteines were found exclusively in *E. faecalis* plasmids (Figure 3, top). RepA_N homologs containing one or fewer cysteines were found in clusters of plasmids from other enterococcal and staphylococcal species. The *E. faecalis*-restricted plasmids pAM373 and pEJ97-1 had their own set of conserved cysteines (Figure 3, bottom), which were found in their own separate cluster (Figure 12). C275 and C307 of PrgW are located in a highly conserved region located on the C-terminal half of the protein that might be related to cysteine mediated protein folding and function (Figure 13). Because RepA_N plasmids are modular in nature (Weaver *et al*., 2009a), the existence of stable narrow host range plasmids and less stable broad host range plasmids in the same cell could make possible the exchange of genetic information between the plasmids and between species. For example, vancomycin-resistant *E. faecalis* strain V583, contains three plasmids; pTEF1, and pTEF2, which contain three conserved cysteines within their RepA_N proteins, and pTEF3, which does not contain the conserved cysteines. pTEF1 and pTEF2 have been shown to mediate horizontal gene transfer within *E. faecalis* (Manson *et al.*, 2010). RepA_N cysteines might provide a stable plasmid reservoir for resistance in *E. faecalis*, while the absence of the conserved

cysteines could extend host-range and contribute to the modular evolution of these plasmids and genetic exchange across species boundaries.

Modularly Evolving RepA_N Proteins Determines Host Range Through Cysteines

Genotypic variation in bacteria is not simply the result of spontaneous mutation. Bacteria can modularly evolve by acquiring genes that have separately evolved involved other organisms. These genes can be acquired through various horizontal gene transfer mechanisms including conjugation, transposition, transformation, and transduction. Genes acquired through modular evolution provide the genotypic content to rapidly drive phenotypic variability. Additionally, modularly evolving genes can be reused to drive variability in various organisms (Kirschner and Gerhart, 1998).

Mutation to antibiotic resistance and acquisition of plasmids can make bacteria less fit (Bouma and Lenski, 1988; Schrag and Perrot, 1996). Lateral gene transfer involved with modular evolution could potentially disrupt genes depending on where the gene is incorporated into the genome. Pleiotropic effects as a result of modular evolution need to be minimized order to prevent bacteria from becoming less fit (Rainey and Cooper, 2004). There might be selection for mechanisms that minimize the negative impact of pleiotropic mutations while maximizing the phenotypic advantage of the genes acquired through modular evolution.

Genes encoded within pheromone response-plasmids, including pCF10, have modularly evolved (Hirt *et al.*, 2005; Weaver *et al*., 2009a). While replication initiation proteins from the RepA_N family of plasmids (containing the conserved cysteines) all

cluster together, the partitioning proteins (RepB and RepC) of the same plasmids are part of multi-species cluster suggesting modular evolution (Weaver *et al*., 2009a). Hirt *et al.* (2005) described that PrgO and PrgP of pCF10 are found more similar to proteins from broad host range plasmids like pRE25 than from the related pheromone-response plasmid partitioning proteins. Sequence analysis of pheromone-responsive plasmids indicated that the DNA processing and DNA transfer modules also originated from many evolutionary sources (Hirt *et al.*, 2005). *prgO* and *prgP* predicted to have been added from different sources other than the source at which *prgW* was derived (Weaver *et al*., 2009a). Interestingly, partitioning proteins PrgO and PrgP modulate the transcript of *prgW* in pCF10 (Izquierdo, unpublished observations).

Similar phylogenetic trends, suggesting modular evolution, can be seen within the domains of the RepA_N protein of RepA_N plasmids. There are four regions contained within *E. faecalis* RepA_N proteins (Figure 24): (1) a conserved 100 amino acid residue N-terminus (N-100); (2) a variable direct repeat (DR) region which was shown to determine replication binding (Francia *et al.*, 2004; Meloni *et al.*, Submitted); (3) a highly conserved 120 amino acid residue near the C-terminus (C-120); and (4) a highly variable approximately 25 amino acid residue region located downstream of the conserved C-120 region at the end (C-END) of the RepA_N protein. There was only an approximately 55 percent identity when the pCF10 N-100 region was compared to a number of other pheromone-response RepA_N proteins (including pAD1). However, a cysteine was conserved in 95percent of the N-100 regions in *E. faecalis*.

Two conserved cysteines were located within the C-120 region of *E. faecalis*. No conserved cysteines were found at that region of *E. faecium* RepA_N proteins. One

conserved cysteine of *E. faecalis* RepA_N proteins is located in the middle of the highly conserved C-120 region. The other cysteine is located at the end of the C-120 region, adjacent to the more variable C-END region. It is unclear why having a variable C-END is important for the protein, unless it is somehow involved in preventing plasmid incompatibility between different RepA_N family plasmids.

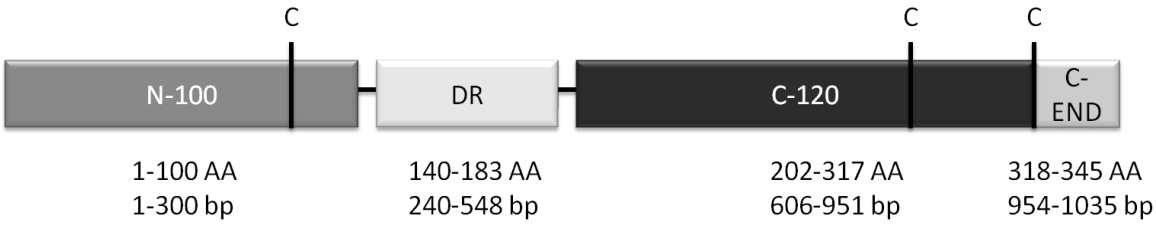


Figure 24. Defined RepA_N Protein Domains of PrgW Homologs of *E. faecalis*.

RepA_N proteins of *E. faecalis* like PrgW can be divided into four distinct domains. The N-terminus previously defined by Weaver (Weaver *et al.*, 2009a) identified the RepA_N by the conserved first 100 amino acids of the N-terminus domain. The N-terminal consensus sequence (N-100) contained one of the conserved cysteines. The variable direct repeat (DR) region (appears at 240 to 548 base pairs of PrgW) has been identified as the region at which the RepA_N protein binds (Francia *et al.*, 2004; Meloni *et al.*, Submitted). Additionally consensus sequence analysis (Figures 13) showed that there is a highly identical 120 amino acid consensus sequence found in the C-terminal half (C-120) of RepA_N proteins that contains the two of the three conserved cysteines. The variable C-terminal end (C-END) is conserved among a few RepA_N proteins, but there is high variability among the RepA_N family.

Extension of Host Range by Modular Evolution

Examination of unusual RepA_N plasmids in *E. faecalis* supports the idea that modular evolution within the RepA_N plasmids might affect plasmid host range.

RepA_N proteins were previously reported to be homologous within the same genus. A recent identity comparison with a larger number of RepA_N proteins showed the trend still held true as the RepA_N proteins primarily remained associated within the same genus and species and therefore most likely evolved within one species. However, as discussed in the results section pJH1 and pJH2 illustrated a possible modular recombination event that overcomes this host range restriction. *E. faecalis* JH1 had two different RepA_N proteins on two different plasmids, pJH1 and pJH2. The RepA_N protein of pJH1 (in *E. faecalis* strain JH1) was found in a cluster with *E. faecium* plasmids, while the sister plasmid pJH2 was found in an *E. faecalis* cluster (Figure 12). Comparison of the N- and C-terminal portions of the RepA_N proteins from JH1 and JH2 suggested that the N-terminus and C-terminus may have evolved as distinct modules (Figure 18).

The high degree of conservation within a genus or species observed by Weaver *et al*., (2009a) was even more striking when comparing the C-terminal portions of the protein. Proteins from staphylococci and enterococci other than *E. faecalis* had C-termini with low amino acid homology to pCF10 and *E. faecalis* RepA_N proteins. The C-120 consensus sequence was strikingly conserved among *E. faecalis* species and within *E. faecium* species (Figures 13 and 19). The exception was the conserved C-120 region of the pJH1 RepA_N protein which was highly homologous to *E. faecium* RepA_N C-120 region. It was possible that the region encoding the C-terminus found on pJH1 RepA_N was acquired from *E. faecium* or it was modified in *E. faecalis* and transferred to *E. faecium.* The region encoding the C-terminus of pJH1 RepA_N is primarily found

homologous to that of other *E. faecium* plasmids, so it could be hypothesized that it originated in *E. faecium*, but this remains untested.

Conversely, the N-terminus of the RepA_N protein of pJH1 had a higher amino acid residue identity than RepA_N N-terminus of pJH2, when compared to PrgW of pCF10. The pJH1 RepA_N protein N-terminus clustered with the *E. faecalis* cluster in which pCF10 was found. The N-terminus of pJH2 clustered between an *E. faecium* and an *E. faecalis* cluster.

Therefore, it appeared that the two plasmids pJH1 and pJH2 may have switched the N- and C termini regions of their RepA_N proteins. One plasmid may have originated from *E. faecium.* When the *E. faecium* plasmid was transferred into an *E. faecalis* cell, a recombination might have taken place. If this was true, is presumably the first known interaction to plasmids from two different species interacting within the *E. faecalis* cell (Weaver, personal communication). The RepA_N proteins might have modularly changed and evolved into a more efficient replication protein, acquire a new function, or broaden the host range.

Modular Evolution within Enterococci

Comparing the species conservation of the C-terminus of RepA_N proteins to the N-terminus provided evidence that the replication proteins themselves evolved modularly within the enterococci genus. Phylogenic tree comparisons performed with just the N-terminus and just the C-terminus of the RepA_N family of proteins yields different groupings. The N-terminus clearly shows that *E. faecalis* RepA_N proteins do not cluster solely within the *E. faecalis* species, whereas, the C-terminus follows strict

species clustering. Therefore, C-terminal homologies likely account for the species specific groupings seen when the whole RepA_N proteins are compared. This has significant implications for the role of C275 and C307 in defining the host range of the plasmids since they are located within C-terminal portions of the RepA_N proteins.

Extension of Host Range by Frameshift Mutation.

The importance of C275 and C307 in restricting host range may be illustrated when comparing pSK41 and pTX0104 from *S. aureus* to pCF10. The replication protein encoded by *S. aureus* pSK41has a 98 percent amino acid residue identity to a replication protein found in TX0104 (pTX0104; Figure 25). Because the RepA_N proteins in TX0104 came from analysis of whole "genome" shotgun sequences, it is unknown if TX0104 really contains plasmids. It is a formal a possibility that the RepA_N proteins might also be incorporated into the chromosome, part of a phage, or located as a second replication protein on the plasmid (Weaver *et al*., 2009a). This question will only be resolved with plasmid isolation and PCR analysis designed to test if the plasmid encodes a RepA_N protein. Therefore, reference to pTX0104 is being made to a putative plasmid in TX0104. The only difference between RepA_N of pTX0104 and pSK41 was the deletion of one a direct repeat region (DR) in pSK41 (Figure 25, arrows).

The RepA_N protein of TX0104 strain and pSK41were only approximately 34 percent identical to PrgW of pCF10, yet the corresponding nucleotide sequence was approximately 59 percent identical. Interestingly, the pSK41 RepA_N N-terminus region was 64 percent identical to PrgW N-terminus, but there was no homology in the C-120 region. The C-terminus appeared to have changed based on a frame shift mutation,

removing the C275 and C307 from that region. In addition, the C-terminus DNA sequence created a number of changes in amino acid sequence resulting in replacement of neutral residues with aspartic acid and lysine residues. These amino acid changes possibly cause a way to affect conformation by hydrostatic changes rather than the disulfide bonds, making the replication initiation protein independent of pre-cCF10 (Figure 25). This change could have allowed for replication in other species. It would have the same effect as modular switching the half that may have occurred with pJH1.

Mechanism

How these plasmids are modularly evolving remains a question. It appears in the case of pCF10 that there are AT rich DNA regions flanking the areas that are prone to evolve as modules. The direct repeat region is located in between the regions encoding the N and C-terminal, and the *inc* region (Izquierdo, unpublished observation) is directly downstream of *prgW*. These AT-rich regions could be prone to recombination events. The AT-rich direct repeats at which PrgW binds to pCF10 DNA themselves have been prone to recombination in *E. coli* (Figure 26A). Sequencing analysis revealed that during the construction of the pMSP6-078 Δ*par* mutant, the DR1 and DR2 were unintentionally removed during cloning procedures to generate pMSP6-078/275 Δ*par* Δ*DR* mutant (region flanked by arrows; Figure 26 A). This mutation created a mutant *prgW* gene that was unable to encode a functional replication initiation protein (Figure 26B). pMSP6050Δpar and pMSP6-078/275 formed *E. faecalis* transformants on selective plates

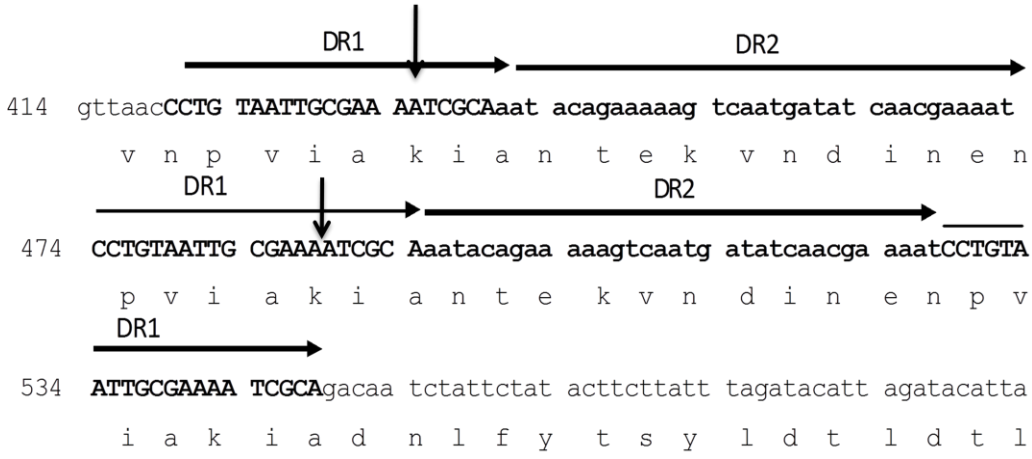
Figure 25. Amino Acid Residue Variability Between the RepA_N Proteins of pTX0104 and pSK41 compared to RepA_N of pCF10.

CLUSTAL 2.0.12 multiple sequence analysis illustrates the amino acid residue variability between the RepA_N proteins of *S. aureus* pSK41, *E. faecalis* pCF10, and *E. faecalis* TX0104. The RepA_N N-100 region of pCF10 has a 64 percent identity to the N-100 regions of the pSK41 and pTX0104RepA_N proteins. In contrast, there is very little identity between the C-120 region of the RepA_N in pCF10 compared to the C-120 region of the RepA_N of pSK41 and pTX0104. RepA_N proteins of pSK41 and pTX0104 contain 98 percent amino acid residue identity. The two percent amino acid residue identity difference was caused by a repeating region found in the RepA_N pTX0104 that was missing in pTX0104 RepA_N (arrows). The repeating region is directly downstream of the direct repeats involved with RepA_N binding .

following electroporation; pMSP6-078/275Δcys/ΔDR only generated one colony in six trials following electroporation. It is unclear if the *E. faecalis* colony that resulted from the electroporation of pMSP6-078/275Δcys/ΔDR still had the same DR deletion in PrgW or if the colony contained a plasmid that replicated. Additionally, the regions flanking the *prgP* and *prgO* homologs of pCF10 found in pRE25 contain genes associated with transposition. It could be possible that transposons are at least partially involved in the addition of these genes.

Pheromone-responsive plasmids typically have a limited host range and are extremely stable. Modular evolution could increase the efficiency of the RepA_N protein or make the RepA_N protein more functional in other species. This could allow it to replicate or at least allow for a less transient survival in the new host which could lead to incorporation of genes into other plasmids or chromosomes. It has been speculated that the C-terminus could be used for a species specific function while the N-terminus is used for a more conserved function like DNA helicase (Weaver *et al*., 2009a). Perhaps the modular evolution provided an increased chance of the recombination or insertion event to replicate in other species.

A



B

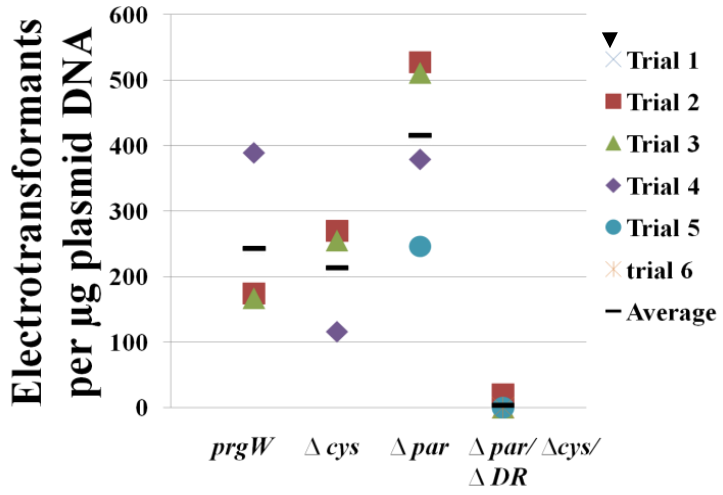


Figure 26. Recombination of RepA_N Direct Repeats in *E. faecalis.*

(A) Sequencing analysis revealed a DR1 and DR2 was unintentionally removed during cloning procedures to generate pMSP6-078/275 Δ*par* mutant (region flanked by arrows). (B) Electroporation of pMSP6050 (*w/t*), pMSP6-078/275 (Δ *cys*) and Δ *par* onto selective plates yielded transformants in three trials while pMSP6-078/275 Δ*par*/Δ*dr* (Δ*par*/Δ*cys*/Δ*DR*) yielded one transformant in six trials.

Future Directions

Role of Cysteines

One important question that remains unanswered is the mechanism by which the cysteines control PrgW. The PrgW cysteines could be causing inter- and intramolecular disulfide bond formation. The resolution of two PrgW bands under reducing conditions (Figure 7) and stability experiments (Figure 10) show intramolecular disulfide bonds play a role in PrgW conformation with C307 being critical in pCF10 stability. Additionally, PrgW aggregation (which could be important to replication protein function) is mediated by the presence of cysteines (Figure 14). This currently is all that is understood about the role of cysteines.

Analyzing the pOR10 and pMSP6050 ΔCys constructs on a Western blot with and without reducing conditions was attempted to understand aggregation and doublet conformational effects controlled by the cysteines of PrgW. However the quality of anti-PrgW primary antibody did not provide good resolution in preliminary experiments. A highly specific monoclonal antibody is needed for further evaluation. Additionally, inserting cysteine mutants into pET28 vectors and over-expressing the cysteine substituted PrgW in *E. coli* might provide some insight as to the cysteines involved in the doublet formation of PrgW. Wild-type PrgW forms two bands under non-reducing conditions so it would be interesting to see which cysteines are responsible for each band of the PrgW doublet

Aggregation could be analyzed by sepharose separation, and PrgW could be measured with a Western dot blot if the antibody is specific enough. Additionally, testing

to see if other cysteine mutant pMSP6050 vectors could replicate in *L. lactis* might help characterize the cysteines important for plasmid host range. Furthermore purified cysteine mutant PrgW could test the importance of cysteines for DNA binding, using a gel shift assay and/or DNase footprinting.

Thiol labeling was also attempted using monobromobimane (mBBr) to determine if the disulfide bonds were forming in *E. faecalis*. The protocol was modified from an *E. coli* mBBr thiol labeling procedure by (Reinhart *et al.*, 2008). This was unsuccessful as the mBBr bound non-specifically to many proteins in the cell. In vivo labeling could provide definitive evidence that disulfide bonds are formed in the cell. Currently cyanylation of potential free thiols and protein cleavage followed by mass spectrophotometric analysis is the method of choice for identifying disulfide bonds. If this is a possibly PrgW disulfide bonds could be identified by comparing mass spectrophotometric cleavage analysis between wild-type PrgW and the PrgW cysteine mutants.

PrgW Domains

RepA_N proteins of *E. faecalis* and *E. faecium* have been shown to have four distinct domains (Figure 23) and it is important to understand the role of each region. The direct repeat regions have been identified as DNA binding regions for the RepA_N proteins in both *prgW* of pCF10, and *repA* of pAD1 (Francia *et al.*, 2004; Meloni *et al.*, Submitted). It remains unclear what regions of RepA_N proteins bind the DNA. DNA binding shift assays with purified PrgW N-terminus and C-terminus truncated proteins could identify the regions of PrgW that bind to direct repeats of pCF10 DNA. Utilizing

purified PrgW and PrgW conserved regions, DNase footprinting could be performed to determine the exact location of PrgW binding. Adding pre-cCF10 to PrgW and using PrgW-ΔCys could help determine the role of pre-pheromone and PrgW cysteines in DNA binding.

PrgW has been shown to regulate *prgZY* transcription, and *prgW* transcription has been shown to decrease because of PrgO centromere binding (Izquierdo, unpublished observation; Meloni thesis, 2005). PrgW must interact with host and possibly plasmid machinery in order to initiate replication, the nature of those interactions remains unclear. PrgW might interact with PrgZ and PrgY, since it binds to the *prgZY* promoter. Additionally, PrgW might interact with PrgO or PrgP since the replication protein is regulated by PrgO binding to the centromere sight. It also remains unclear if an oxidoreductase is involved in the disulfide bond formation of PrgW. A PrgW pull down assay could reveal the interactions of PrgW with other host and pCF10 encoded proteins. Additionally, performing the pull down assay with the C-and N-terminus domains of PrgW would help understand the roles of the conserved regions. Furthermore, adding pre-cCF10 to PrgW and PrgW-ΔCys mutants could determine interactions that are pheromone and cysteine dependant. This could help further reveal the role of pre-cCF10 and the role of the cysteines of PrgW.

Further evaluation of the highly conserved C-120 region must be performed to determine if this is truly a host range determinant region of RepA_N proteins. Plasmids containing the pJH1 and pJH2 minimal origins of replication and the associated hybrid replication proteins were constructed but not tested. Electroporation of these plasmids into *E. faecalis* and *E. faecium* could determine if the cysteines present on the RepA_N

proteins of pJH1 and pJH2 control host range. Additionally, this type of domain swapping experiment could be done for the RepA_N proteins of pTX0104, pSK41, and pCF10, where a frameshift mutation(s) has eliminated C275 and C307. These experiments are only preliminary however, there might be other factors in determining host range besides the C-terminus (and possibly presence of cysteines) of RepA_N proteins in *E. faecalis, E. faecium,* and *S. aureus*. For example, even after the deletion of C78, C275, and C307, pCF10 replicons replicated only in the closely related *L. lactis* and did not replicate in more distantly related bacteria such as *S. mutans* and *B. subtilis* (Meloni, 2005). Therefore, while it is clear the conserved cysteines can affect plasmid host range, they are not the only determinants of host range.

Localization of PrgW and Recruitment by pre-cCF10

To determine if PrgW could interact with membrane bound pre-cCF10 and/or a membrane bound thiol-disulfide oxidoreductase, attempts were made to show localization of PrgW to the membrane. A translation fusion of GFPmut3b* to the C-terminus of PrgW, did not produce an active fusion. A translational fusion of PrgW ΔCys to GFPmut3b* did not fluoresce at all. Therefore, changing the location of the tag, use of a smaller epitope tag like hemagglutinin (HA tag; PYDVPDYA) or immunostaining experiments may be necessary to determine the location of PrgW within the cell.

A possibility for how disulfide bond formation might be occurring in a cytoplasmic protein like PrgW through the action of a thiol-disulfide oxidoreductase. Thiol-disulfide oxidoreductase EF0770 (NP_814517) is a *B. subtilis* BdbD-homologue in

E. faecalis (Kouwen *et al.*, 2007). PCR amplification confirmed the presence of the gene in *E. faecalis* OG1RF. This is an interesting target because it is proposed to be a cytoplasmic oxidoreductase (Kouwen *et al.*, 2007). Knock out of this oxidoreductase might decrease the stability of pMSP6050 and might not have an effect on pMSP6-078/275/307. This would provide evidence of oxidoreductase involvement with PrgW.

Role of PrgW with PrgZ and PrgY

PrgW binds to the *prgZY* promoter and increases the transcription (Meloni thesis, 2005). This was an interesting observation because PrgW, PrgZ, and PrgY all bind to cCF10 (Leonard *et al.*, 1996). This could mean that there is a regulation of PrgZ by PrgW on the transcriptional and the pheromone binding level. Additionally the presence of *prgZ* in pMSP6050 increases plasmid stability (Meloni, 2005). Further work could examine the affects of pre-cCF10 and PrgW ΔCys on cCF10 binding and *prgZY* promoter binding. Analysis of transcript levels could be done by Northern blotting, real-time PCR, and/or the β-galactosidase assay described by Meloni (Meloni, 2005). PrgW ΔCys under the control of a nisin promoter has already been generated for use in the β- galactosidase assay.

Polymicrobial Biofilm

Preliminary evidence showed that *S. aureus* and *E. faecalis* produced a polymicrobial biofilm on a glass coverslip. Polymicrobial biofilms between *S. aureus* and *E. faecalis* have not been studied and further analysis involving horizontal gene

transfer should be pursued. Resistant *E. faecalis* and *S. aureus* have been observed together in polymicrobial biofilms and in clinical samples from patients (Han *et al.*, 2009; Weigel *et al.*, 2007). Although the PNA FISH tagging is not optimized for biofilms, it could help identify the *E. faecalis* and *S. aureus* cells. PNA FISH probes bind to species specific 16s rRNA and fluoresce. Experiment would need to be done separately as there is only green fluorescence color currently available for the two species. Horizontal gene transfer between *E. faecalis* and *S. aureus* could then be studied with the biofilm. Antibiotic resistance genes within conjugative plasmids of *E. faecalis* like pCF10 and conjugative plasmids of *S. aureus* like pSK41 will help determine if resistance is transferred from one species to another. Removal of cysteines from PrgW of pCF10 might increase the transfer rate of antibiotic resistance from *E. faecalis* to *S. aureus*. Filter mating experiments have already shown resistance encoded on plasmids can transfer from *E. faecalis* to *S. aureus* in vitro (Werner *et al.*, 2010). The physical transfer could be visualized by adding a fluorescence marker to pCF10.

Plasmid Stability Increase after Multiple Passages in the Absence of Selection

Figure 23 showed that there was an increase in stability at generations 121 to163 in the pMSP6050 cysteines substitution vectors. This was most likely not seen in previous pMSP6050 maintenance experiments because the plasmid stability was already high. The stability increase was not expected as there is neither conjugation machinery nor genes involved with plasmid DNA processing encoded within pMSP6050. Ribosomal ambiguity of the stop codon found at the seventh amino acid position of the *par* toxin, encoded on pMSP6050, was thought to have been the source of this stability

rise (Mazeffa, 2008; Piggot *et al.*, 1972). However, this was not the case as there was no difference in stability rise at generations 121 to 163 of the cysteine mutant vector pMSP6-078/275 when compared to pMSP6-078/275Δ*par* (Figure 24). Possible ways for an increase in stability would be the death of cells without the plasmid or increased proliferation of cells with the plasmid. The mechanisms could be a novel plasmid maintenance mechanism and should be the subject to further study.

It is unknown whether this potentially unique stability mechanism that increased the proliferation rate of *E. faecalis* cells with the plasmid or caused the death of cells without the plasmid is a plasmid and/or chromosome derived mechanism. To test for mutations, a strain containing a cysteine mutant plasmid that was passaged 163 generations without selection could be added into medium with selection and then again monitored for stability. If the stability of the passaged plasmid is higher than original plasmid stability, a mutation in the plasmid or chromosome would then be predicted. The plasmid should then be isolated, electroporated into unpassaged *E. faecalis* cells, and monitored for plasmid maintenance. If the plasmid stability of passaged plasmid with unpassaged *E. faecalis* cells increases, than a mutation in the plasmid is likely. If not, it could be predicted the chromosome of passaged *E. faecalis* cells contained the mutation that allowed for stability rise at generations 121 to 163.

There might also be additional factors encoded on pMSP6050 cysteine mutant that could be causing an increase in plasmid stability at generations 121 to 163. The effect of deleting specific plasmid genes on stability would be tested. PrgN has been identified as is a potential target because it is important to pCF10 stability, yet the function is undefined. If there is a mutation on the plasmid determined by the experiment

described above, UvrA, putative DNA polymerase IV, might be playing a role. Other possibilities might involve chromosomal encoded stability factors, possibly chromosomal encoded kill systems like the linear pentapeptide extracellular death factor (EDF) of the MazEF system of *E. coli* (Kolodkin-Gal *et al.*, 2007).

Furthermore, the role of cCF10 could be a factor in the increase of plasmid stability during extensive passage of cells without selection. If less cells have plasmids in the population, then less cells would have PrgZ and PrgY. This would leave more pheromone available to cells with the plasmid. Thus, more pheromone would be available for intake by cells with PrgZ, and/or would be sequestered by PrgY. Increased pheromone uptake or pheromone sequestered by PrgY might affect the growth of the cell. Knocking out PrgY and PrgZ under the same vectors could determine if the available levels of cCF10 could be causing a change in cell growth and an increase in cells with plasmid throughout the population. This might be of interest as *prgZ* increases pCF10 stability and PrgW regulates the *prgZY* promoter (Meloni thesis, 2005). Overall, this would be an area of interest but, as yet little is known about what could be causing the stability increase.

Summary

Overall, these studies identify conserved cysteines and disulfide bonds of RepA_N plasmid replication initiation proteins in *E. faecalis* as novel determinants of plasmid host range and stability.

REFERENCES CITED

Aakra, A., Vebo, H., Snipen, L., Hirt, H., Aastveit, A., Kapur, V., Dunny, G., Murray, B.E., and Nes, I.F. (2005) Transcriptional response of *Enterococcus faecalis* V583 to erythromycin. *Antimicrob Agents Chemother.* **49**: 2246-2259.

Abajy, M.Y., Kopec, J., Schiwon, K., Burzynski, M., Doring, M., Bohn, C., and Grohmann, E. (2007) A type IV-secretion-like system is required for conjugative DNA transport of broad-host-range plasmid pIP501 in gram-positive bacteria. *J Bacteriol.* **189**: 2487-2496.

An, F.Y., Sulavik, M.C., and Clewell, D.B. (1999) Identification and characterization of a determinant (eep) on the *Enterococcus faecalis* chromosome that is involved in production of the peptide sex pheromone cAD1. *J Bacteriol.* **181**: 5915-5921.

Anfinsen, C.B. (1973) Principles that govern the folding of protein chains. *Science.* **181**: 223-230.

Antelmann, H., Hecker, M., and Zuber, P. (2008) Proteomic signatures uncover thiol-specific electrophile resistance mechanisms in *Bacillus subtilis*. *Expert Rev Proteomics.* **5**: 77-90.

Antelmann, H., and Helmann, J.D. (2011) Thiol-based redox switches and gene regulation. *Antioxid Redox Signal.* **14**: 1049-1063.

Antiporta, M.H., and Dunny, G.M. (2002) *ccfA*, the genetic determinant for the cCF10 peptide pheromone in *Enterococcus faecalis* OG1RF. *J Bacteriol.* **184**: 1155-1162.

Arthur, M., Molinas, C., Bugg, T.D., Wright, G.D., Walsh, C.T., and Courvalin, P. (1992a) Evidence for in vivo incorporation of D-lactate into peptidoglycan precursors of vancomycin-resistant enterococci. *Antimicrob Agents Chemother.* **36**: 867-869.

Arthur, M., Molinas, C., and Courvalin, P. (1992b) The VanS-VanR two-component regulatory system controls synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. *J Bacteriol.* **174**: 2582-2591.

Arthur, M., Molinas, C., Depardieu, F., and Courvalin, P. (1993) Characterization of Tn1546, a Tn3-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. *J Bacteriol* **175**: 117-127.

Arthur, M., Depardieu, F., Molinas, C., Reynolds, P., and Courvalin, P. (1995) The *vanZ* gene of Tn1546 from *Enterococcus faecium* BM4147 confers resistance to teicoplanin. *Gene* **154**: 87-92.

Arthur, M., Depardieu, F., Cabanie, L., Reynolds, P., and Courvalin, P. (1998) Requirement of the VanY and VanX D,D-peptidases for glycopeptide resistance in enterococci. *Mol Microbiol.* **30**: 819-830.

Arthur, M., and Quintiliani, R., Jr. (2001) Regulation of VanA- and VanB-type glycopeptide resistance in enterococci. *Antimicrob Agents Chemother.* **45**: 375-381.

Bae, T., Clerc-Bardin, S., and Dunny, G.M. (2000) Analysis of expression of *prgX*, a key negative regulator of the transfer of the *Enterococcus faecalis* pheromone-inducible plasmid pCF10. *J Mol Biol.* **297**: 861-875.

Bae, T., Kozlowicz, B.K., and Dunny, G.M. (2004) Characterization of cis-acting prgQ mutants: evidence for two distinct repression mechanisms by Qa RNA and PrgX protein in pheromone-inducible enterococcal plasmid pCF10. *Mol Microbiol.* **51**: 271-281.

Balaban, N., and Novick, R.P. (1995) Autocrine regulation of toxin synthesis by *Staphylococcus aureus*. *Proc Natl Acad Sci USA.* **92**: 1619-1623.

Barany, F., Boeke, J.D., and Tomasz, A. (1982) Staphylococcal plasmids that replicate and express erythromycin resistance in both *Streptococcus pneumoniae* and *Escherichia coli*. *Proc Natl Acad Sci USA.* **79**: 2991-2995.

Bensing, B.A., Meyer, B.J., and Dunny, G.M. (1996) Sensitive detection of bacterial transcription initiation sites and differentiation from RNA processing sites in the pheromone-induced plasmid transfer system of *Enterococcus faecalis*. *Proc Natl Acad Sci USA.* **93**: 7794-7799.

Bensing, B.A., and Dunny, G.M. (1997) Pheromone-inducible expression of an aggregation protein in *Enterococcus faecalis* requires interaction of a plasmid-encoded RNA with components of the ribosome. *Mol Microbiol.* **24**: 295-308.

Bensing, B.A., Manias, D.A., and Dunny, G.M. (1997) Pheromone cCF10 and plasmid pCF10-encoded regulatory molecules act post-transcriptionally to activate expression of downstream conjugation functions. *Mol Microbiol.* **24**: 285-294.

Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., and Wheeler, D.L. (2008) GenBank. *Nucleic Acids Res* **36**: D25-30.

Bentorcha, F., De Cespedes, G., and Horaud, T. (1991) Tetracycline resistance heterogeneity in *Enterococcus faecium*. *Antimicrob Agents Chemother.* **35**: 808-812.

Berkmen, M., Boyd, D., and Beckwith, J. (2005) The nonconsecutive disulfide bond of *Escherichia coli* phytase (AppA) renders it dependent on the protein-disulfide isomerase, DsbC. *J Biol Chem.* **280**: 11387-11394.

Bernander, R., Krabbe, M., and Nordstrom, K. (1992) Mapping of the in vivo start site for leading strand DNA synthesis in plasmid R1. *EMBO J* **11**: 4481-4487.

Berryman, D.I., and Rood, J.I. (1995) The closely related ermB-ermAM genes from *Clostridium perfringens, Enterococcus faecalis* (pAMβ1), and *Streptococcus agalactiae* (pIP501) are flanked by variants of a directly repeated sequence. *Antimicrob Agents Chemother.* **39**: 1830-1834.

Bertani, G. (1951) Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol.* **62**: 293-300.

Bertani, G. (2004) Lysogeny at mid-twentieth century: P1, P2, and other experimental systems. *J Bacteriol.* **186**: 595-600.

Billot-Klein, D., Blanot, D., Gutmann, L., and van Heijenoort, J. (1994) Association constants for the binding of vancomycin and teicoplanin to N-acetyl-D-alanyl-D-alanine and N-acetyl-D-alanyl-D-serine. *Biochem J.* **304 (Pt 3)**: 1021-1022.

Boitsov, A.S., Golubkov, V.I., Iontova, I.M., Zaitsev, E.N., Malke, H., and Totolian, A.A. (1979) Inverted repeats on plasmids determining resistance to MLS antibiotics in group A streptococci. *FEMS Microbiol Lett.* **6**: 11-14.

Bolhuis, A., Venema, G., Quax, W.J., Bron, S., and van Dijl, J.M. (1999) Functional analysis of paralogous thiol-disulfide oxidoreductases in *Bacillus subtilis*. *J Biol Chem* **274**: 24531-24538.

Borderon, E., Bieth, G., and Horodniceanu, T. (1982) Genetic and physical studies of *Streptococcus faecalis* hemolyisn plasmids. *FEMS Microbiol. Lett.* **14**: 51-55.

Bouma, J.E., and Lenski, R.E. (1988) Evolution of a bacteria/plasmid association. *Nature* **335**: 351-352.

Bramhill, D., and Kornberg, A. (1988) A model for initiation at origins of DNA replication. *Cell*. **54**: 915-918.

Brede, D.A., Snipen, L.G., Ussery, D.W., Nederbragt, A.J., and Nes, I.F. (2011) Complete Genome Sequence of the Commensal *Enterococcus faecalis* 62, Isolated from a Healthy Norwegian Infant. *J Bacteriol.* **193**: 2377-2378.

Brisson-Noel, A., Dutka-Malen, S., Molinas, C., Leclercq, R., and Courvalin, P. (1990) Cloning and heterospecific expression of the resistance determinant *vanA* encoding high-level resistance to glycopeptides in *Enterococcus faecium* BM4147. *Antimicrob Agents Chemother.* **34**: 924-927.

Bruand, C., and Ehrlich, S.D. (1998) Transcription-driven DNA replication of plasmid pAMbeta1 in *Bacillus subtilis*. *Mol Microbiol* **30**: 135-145.

Bryan, E.M., Bae, T., Kleerebezem, M., and Dunny, G.M. (2000) Improved vectors for nisin-controlled expression in gram-positive bacteria. *Plasmid.* **44**: 183-190.

Bugg, T.D., Wright, G.D., Dutka-Malen, S., Arthur, M., Courvalin, P., and Walsh, C.T. (1991) Molecular basis for vancomycin resistance in *Enterococcus faecium* BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. *Biochemistry.* **30**: 10408-10415.

Buttaro, B.A., Antiporta, M.H., and Dunny, G.M. (2000) Cell-associated pheromone peptide (cCF10) production and pheromone inhibition in *Enterococcus faecalis*. *J Bacteriol.* **182**: 4926-4933.

Carvalho, A.T., Fernandes, P.A., and Ramos, M.J. (2006) Determination of the DeltapKa between the active site cysteines of thioredoxin and DsbA. *J Comput Chem.* **27**: 966-975.

Chandler, J.R., Flynn, A.R., Bryan, E.M., and Dunny, G.M. (2005a) Specific control of endogenous cCF10 pheromone by a conserved domain of the pCF10-encoded regulatory protein PrgY in *Enterococcus faecalis*. *J Bacteriol.* **187**: 4830-4843.

Chandler, J.R., Hirt, H., and Dunny, G.M. (2005b) A paracrine peptide sex pheromone also acts as an autocrine signal to induce plasmid transfer and virulence factor expression in vivo. *Proc Natl Acad Sci USA.* **102**: 15617-15622.

Chang, S., Sievert, D.M., Hageman, J.C., Boulton, M.L., Tenover, F.C., Downes, F.P., Shah, S., Rudrik, J.T., Pupp, G.R., Brown, W.J., Cardo, D., and Fridkin, S.K. (2003) Infection with vancomycin-resistant *Staphylococcus aureus* containing the vanA resistance gene. *N Engl J Med.* **348**: 1342-1347.

Chattoraj, D.K. (2000) Control of plasmid DNA replication by iterons: no longer paradoxical. *Mol Microbiol.* **37**: 467-476.

Chen, Y., Staddon, J.H., and Dunny, G.M. (2007) Specificity determinants of conjugative DNA processing in the *Enterococcus faecalis* plasmid pCF10 and the *Lactococcus Lactis* plasmid pRS01. *Mol Micorbiol.* **63**: 1549-1564.

Chen, Y., Zhang, X., Manias, D., Yeo, H.J., Dunny, G.M., and Christie, P.J. (2008) *Enterococcus faecalis* PcfC, a spatially localized substrate receptor for type IV secretion of the pCF10 transfer intermediate. *J Bacteriol.* **190**: 3632-3645.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T.J., Higgins, D.G., and Thompson, J.D. (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res.* **31**: 3497-3500.

Chi, B.K., Albrecht, D., Gronau, K., Becher, D., Hecker, M., and Antelmann, H. (2010) The redox-sensing regulator YodB senses quinones and diamide via a thiol-disulfide switch in *Bacillus subtilis*. *Proteomics.* **10**: 3155-3164.

Cho, S.H., and Beckwith, J. (2006) Mutations of the membrane-bound disulfide reductase DsbD that block electron transfer steps from cytoplasm to periplasm in *Escherichia coli*. *J Bacteriol.* **188**: 5066-5076.

Cho, S.H., Porat, A., Ye, J., and Beckwith, J. (2007) Redox-active cysteines of a membrane electron transporter DsbD show dual compartment accessibility. *EMBO J.* **26**: 3509-3520.

Choi, H., Kim, S., Mukhopadhyay, P., Cho, S., Woo, J., Storz, G., and Ryu, S.E. (2001) Structural basis of the redox switch in the OxyR transcription factor. *Cell.* **105**: 103-113.

Chow, J.W., Thal, L.A., Perri, M.B., Vazquez, J.A., Donabedian, S.M., Clewell, D.B., and Zervos, M.J. (1993) Plasmid-associated hemolysin and aggregation substance production contribute to virulence in experimental enterococcal endocarditis. *Antimicrob Agents Chemother.* **37**: 2474-2477.

Christie, P.J., and Cascales, E. (2005) Structural and dynamic properties of bacterial type IV secretion systems (review). *Mol Membr Biol.* **22**: 51-61.

Chuang-Smith, O.N., Wells, C.L., Henry-Stanley, M.J., and Dunny, G.M. (2010) Acceleration of *Enterococcus faecalis* biofilm formation by aggregation substance expression in an ex vivo model of cardiac valve colonization. *PloS one.* **5**: e15798.

Chung, J.W., and Dunny, G.M. (1992) Cis-acting, orientation-dependent, positive control system activates pheromone-inducible conjugation functions at distances greater than 10 kilobases upstream from its target in *Enterococcus faecalis*. *Proc Natl Acad Sci USA.* **89**: 9020-9024.

Chung, J.W., and Dunny, G.M. (1995) Transcriptional analysis of a region of the *Enterococcus faecalis* plasmid pCF10 involved in positive regulation of conjugative transfer functions. *J Bacteriol.* **177**: 2118-2124.

Clewell, D.B., Francia, M.V., Flannagan, S.E., and An, F.Y. (2002) Enterococcal plasmid transfer: sex pheromones, transfer origins, relaxases, and the *Staphylococcus aureus* issue. *Plasmid.* **48**: 193-201.

Clewell, D.B., and Francia, M.V. (2004) *Conjugation in Gram-Positive Bacteria.* Washington, D.C.: ASM Press.

Clewell, D.B. (2007) Properties of *Enterococcus faecalis* plasmid pAD1, a member of a widely disseminated family of pheromone-responding, conjugative, virulence elements encoding cytolysin. *Plasmid.* **58**: 205-227.

Coburn, P.S., and Gilmore, M.S. (2003) The *Enterococcus faecalis* cytolysin: a novel toxin active against eukaryotic and prokaryotic cells. *Cell Microbiol.* **5**: 661-669.

Coburn, P.S., Pillar, C.M., Jett, B.D., Haas, W., and Gilmore, M.S. (2004) *Enterococcus faecalis* senses target cells and in response expresses cytolysin. *Science.* **306**: 2270-2272.

Cottrell, M.T., and Kirchman, D.L. (2000) Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence in situ hybridization. *Appl Environ Microbiol.* **66**: 5116-5122.

Crooks, G.E., Hon, G., Chandonia, J.M., and Brenner, S.E. (2004) WebLogo: a sequence logo generator. *Genome Res.* **14**: 1188-1190.

Cross, M.A., Warne, S.R., and Thomas, C.M. (1986) Analysis of the vegetative replication origin of broad-host-range plasmid RK2 by transposon mutagenesis. *Plasmid.* **15**: 132-146.

de Niederhausern, S., Bondi, M., Messi, P., Iseppi, R., Sabia, C., Manicardi, G., and Anacarso, I. (2011) Vancomycin-resistance Transferability from VanA Enterococci to *Staphylococcus aureus*. *Curr Microbiol.* **62**: 1363-1367.

del Solar, G., Diaz, R., and Espinosa, M. (1987) Replication of the streptococcal plasmid pMV158 and derivatives in cell-free extracts of *Escherichia coli*. *Mol Gen Genet.* **206**: 428-435.

del Solar, G., Giraldo, R., Ruiz-Echevarria, M.J., Espinosa, M., and Diaz-Orejas, R. (1998) Replication and control of circular bacterial plasmids. *Microbiol Mol Biol Rev.* **62**: 434-464.

Deshpande, L.M., Fritsche, T.R., Moet, G.J., Biedenbach, D.J., and Jones, R.N. (2007) Antimicrobial resistance and molecular epidemiology of vancomycin-resistant enterococci from North America and Europe: a report from the SENTRY antimicrobial surveillance program. *Diagn Microbiol Infect Dis.* **58**: 163-170.

Dorenbos, R., Stein, T., Kabel, J., Bruand, C., Bolhuis, A., Bron, S., Quax, W.J., and Van Dijl, J.M. (2002) Thiol-disulfide oxidoreductases are essential for the production of the lantibiotic sublancin 168. *J Biol Chem.* **277**: 16682-16688.

Dumoulin, A., Grauschopf, U., Bischoff, M., Thony-Meyer, L., and Berger-Bachi, B. (2005) *Staphylococcus aureus* DsbA is a membrane-bound lipoprotein with thiol-disulfide oxidoreductase activity. *Arch Microbiol.* **184**: 117-128.

Dunham, T.D., Xu, W., Funnell, B.E., and Schumacher, M.A. (2009) Structural basis for ADP-mediated transcriptional regulation by P1 and P7 ParA. *EMBO J.* **28**: 1792-1802.

Dunny, G., Funk, C., and Adsit, J. (1981) Direct stimulation of the transfer of antibiotic resistance by sex pheromones in *Streptococcus faecalis*. *Plasmid.* **6**: 270-278.

Dunny, G.M., and Clewell, D.B. (1975) Transmissible toxin (hemolysin) plasmid in *Streptococcus faecalis* and its mobilization of a noninfectious drug resistance plasmid. *J Bacteriol.* **124**: 784-790.

Dunny, G.M., Brown, B.L., and Clewell, D.B. (1978) Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. *Proc Natl Acad Sci USA.* **75**: 3479-3483.

Dunny, G.M., Lee, L.N., and LeBlanc, D.J. (1991) Improved electroporation and cloning vector system for gram-positive bacteria. *Appl Environ Microbiol.* **57**: 1194-1201.

Dunny, G.M. (2007) The peptide pheromone-inducible conjugation system of *Enterococcus faecalis* plasmid pCF10: cell-cell signalling, gene transfer, complexity and evolution. *Philos Trans R Soc Lond B Biol Sci* **362**: 1185-1193.

Durland, R.H., and Helinski, D.R. (1987) The sequence encoding the 43-kilodalton trfA protein is required for efficient replication or maintenance of minimal RK2 replicons in Pseudomonas aeruginosa. *Plasmid.* **18**: 164-169.

Facklam, R.R., Thacker, L.G., Fox, B., and Eriquez, L. (1982) Presumptive identification of streptococci with a new test system. *J Clin Microbiol* **15**: 987-990.

Facklam, R.R., Carvalho, M.S., and Teixeira, L.M. (2002) *History, Taxonomy, Biochemical Characteristics, and Antibiotic Suseptablity Testing of Enteroocci.* Washington, D.C.: ASM Press.

Fisher, K., and Phillips, C. (2009) The ecology, epidemiology and virulence of Enterococcus. *Microbiology* **155**: 1749-1757.

Fontana, R., Ligozzi, M., Pittaluga, F., and Satta, G. (1996) Intrinsic penicillin resistance in enterococci. *Microb Drug Resist.* **2**: 209-213.

Francia, M.V., Fujimoto, S., Tille, P., Weaver, K.E., and Clewell, D.B. (2004) Replication of *Enterococcus faecalis* pheromone-responding plasmid pAD1: location of the minimal replicon and *oriV* site and RepA involvement in initiation of replication. *J Bacteriol.* **186**: 5003-5016.

Francia, M.V., Weaver, K.E., Goicoechea, P., Tille, P., and Clewell, D.B. (2007) Characterization of an active partition system for the *Enterococcus faecalis* pheromone-responding plasmid pAD1. *J Bacteriol.* **189**: 8546-8555.

Fridkin, S.K., and Gaynes, R.P. (1999) Antimicrobial resistance in intensive care units. *Clin Chest Med.* **20**: 303-316, viii.

Fuangthong, M., and Helmann, J.D. (2002) The OhrR repressor senses organic hydroperoxides by reversible formation of a cysteine-sulfenic acid derivative. *Proc Natl Acad Sci USA.* **99**: 6690-6695.

Garcia de Viedma, D., Giraldo, R., Ruiz-Echevarria, M.J., Lurz, R., and Diaz-Orejas, R. (1995) Transcription of repA, the gene of the initiation protein of the Pseudomonas plasmid pPS10, is autoregulated by interactions of the RepA protein at a symmetrical operator. *J Mol Biol.* **247**: 211-223.

Garg, S.K., Kommineni, S., Henslee, L., Zhang, Y., and Zuber, P. (2009) The YjbH protein of *Bacillus subtilis* enhances ClpXP-catalyzed proteolysis of Spx. *J Bacteriol* **191**: 1268-1277.

Gerdes, K., Moller-Jensen, J., and Bugge Jensen, R. (2000) Plasmid and chromosome partitioning: surprises from phylogeny. *Mol Microbiol.* **37**: 455-466.

Gerdes, K., Howard, M., and Szardenings, F. (2010) Pushing and pulling in prokaryotic DNA segregation. *Cell.* **141**: 927-942.

Gholizadeh, Y., and Courvalin, P. (2000) Acquired and intrinsic glycopeptide resistance in enterococci. *Int J Antimicrob Agents.* **16 Suppl 1**: S11-17.

Gilmore, M.S., Coburn, P.S., Nallapareddy, S.R., and Murray, B.E. (2002) *Enterococcal Virulence.* Washington, D.C.: ASM Press.

Giraldo-Suarez, R., Fernandez-Tresguerres, E., Diaz-Orejas, R., Malki, A., and Kohiyama, M. (1993) The heat-shock DnaK protein is required for plasmid R1 replication and it is dispensable for plasmid ColE1 replication. *Nucleic Acids Res.* **21**: 5495-5499.

Giraldo, R., and Diaz, R. (1992) Differential binding of wild-type and a mutant RepA protein to oriR sequence suggests a model for the initiation of plasmid R1 replication. *J Mol Biol.* **228**: 787-802.

Gomis-Ruth, F.X., Sola, M., de la Cruz, F., and Coll, M. (2004) Coupling factors in macromolecular type-IV secretion machineries. *Curr Pharm Des.* **10**: 1551-1565.

Gordon, S., Swenson, J.M., Hill, B.C., Pigott, N.E., Facklam, R.R., Cooksey, R.C., Thornsberry, C., Jarvis, W.R., and Tenover, F.C. (1992) Antimicrobial susceptibility patterns of common and unusual species of enterococci causing infections in the United States. Enterococcal Study Group. *J Clin Microbiol.* **30**: 2373-2378.

Goze, A., and Ehrlich, S.D. (1980) Replication of plasmids from *Staphylococcus aureus* in *Escherichia coli*. *Proc Natl Acad Sci USA.* **77**: 7333-7337.

Griffin, M.O., Fricovsky, E., Ceballos, G., and Villarreal, F. (2010) Tetracyclines: a pleitropic family of compounds with promising therapeutic properties. Review of the literature. *Am J Physiol Cell Physiol.* **299**: C539-548.

Grishin, N.V. (1995) Estimation of the number of amino acid substitutions per site when the substitution rate varies among sites. *J Mol Evol.* **41**: 675-679.

Grohmann, E., Muth, G., and Espinosa, M. (2003) Conjugative plasmid transfer in gram-positive bacteria. *Microbiol Mol Biol Rev.* **67**: 277-301.

Haas, W., Shepard, B.D., and Gilmore, M.S. (2002) Two-component regulator of *Enterococcus faecalis* cytolysin responds to quorum-sensing autoinduction. *Nature.* **415**: 84-87.

Han, S.H., Chin, B.S., Lee, H.S., Jeong, S.J., Choi, H.K., Kim, C.K., Kim, C.O., Yong, D., Choi, J.Y., Song, Y.G., Lee, K., and Kim, J.M. (2009) Recovery of both vancomycin-resistant enterococci and methicillin-resistant *Staphylococcus aureus* from culture of a single clinical specimen from colonized or infected patients. *Infect Control Hosp Epidemiol.* **30**: 130-138.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol.* **166**: 557-580.

Hancock, L.E., and Perego, M. (2004) The *Enterococcus faecalis* fsr two-component system controls biofilm development through production of gelatinase. *J Bacteriol.* **186**: 5629-5639.

Hancock, L.E., and Gilmore, M.S. (2006) *Pathogenicity of Enterococci.* Washington, D.C.: ASM Press.

Hash, J.H., Wishnick, M., and Miller, P.A. (1964) On the Mode of Action of the Tetracycline Antibiotics in *Staphylococcus aureus*. *J Biol Chem.* **239**: 2070-2078.

Heaton, M.P., and Handwerger, S. (1995) Conjugative mobilization of a vancomycin resistance plasmid by a putative *Enterococcus faecium* sex pheromone response plasmid. *Microb Drug Resist.* **1**: 177-183.

Hedberg, P.J., Leonard, B.A., Ruhfel, R.E., and Dunny, G.M. (1996) Identification and characterization of the genes of *Enterococcus faecalis* plasmid pCF10 involved in replication and in negative control of pheromone-inducible conjugation. *Plasmid.* **35**: 46-57.

Hidron, A.I., Edwards, J.R., Patel, J., Horan, T.C., Sievert, D.M., Pollock, D.A., and Fridkin, S.K. (2008) NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. *Infect Control Hosp Epidemiol.* **29**: 996-1011.

Hirt, H., Erlandsen, S.L., and Dunny, G.M. (2000) Heterologous inducible expression of *Enterococcus faecalis* pCF10 aggregation substance *asc10* in *Lactococcus Lactis* and *Streptococcus gordonii* contributes to cell hydrophobicity and adhesion to fibrin. *J Bacteriol.* **182**: 2299-2306.

Hirt, H., Schlievert, P.M., and Dunny, G.M. (2002) In vivo induction of virulence and antibiotic resistance transfer in *Enterococcus faecalis* mediated by the sex pheromone-sensing system of pCF10. *Infect Immun.* **70**: 716-723.

Hirt, H., Manias, D.A., Bryan, E.M., Klein, J.R., Marklund, J.K., Staddon, J.H., Paustian, M.L., Kapur, V., and Dunny, G.M. (2005) Characterization of the pheromone response of the *Enterococcus faecalis* conjugative plasmid pCF10: complete sequence and comparative analysis of the transcriptional and phenotypic responses of pCF10-containing cells to pheromone induction. *J Bacteriol.* **187**: 1044-1054.

Horodniceanu, T., Bouanchaud, D.H., Bieth, G., and Chabbert, Y.A. (1976) R plasmids in *Streptococcus agalactiae* (group B). *Antimicrob Agents Chemother.* **10**: 795-801.

Ike, Y., Hashimoto, H., and Clewell, D.B. (1984) Hemolysin of *Streptococcus faecalis* subspecies zymogenes contributes to virulence in mice. *Infect Immun.* **45**: 528-530.

Inaba, K., and Ito, K. (2002) Paradoxical redox properties of DsbB and DsbA in the protein disulfide-introducing reaction cascade. *EMBO J.* **21**: 2646-2654.

Inaba, K., Takahashi, Y.H., and Ito, K. (2005) Reactivities of quinone-free DsbB from *Escherichia coli*. *J Biol Chem.* **280**: 33035-33044.

Inaba, K., Murakami, S., Suzuki, M., Nakagawa, A., Yamashita, E., Okada, K., and Ito, K. (2006a) Crystal structure of the DsbB-DsbA complex reveals a mechanism of disulfide bond generation. *Cell.* **127**: 789-801.

Inaba, K., Takahashi, Y.H., Ito, K., and Hayashi, S. (2006b) Critical role of a thiolate-quinone charge transfer complex and its adduct form in de novo disulfide bond generation by DsbB. *Proc Natl Acad Sci USA.* **103**: 287-292.

Inaba, K., and Ito, K. (2008) Structure and mechanisms of the DsbB-DsbA disulfide bond generation machine. *Biochim Biophys Acta.* **1783**: 520-529.

Ito, K., and Inaba, K. (2008) The disulfide bond formation (Dsb) system. *Curr Opin Struct Biol.* **18**: 450-458.

Jacob, A.E., and Hobbs, S.J. (1974) Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. zymogenes. *J Bacteriol* .**117**: 360-372.

Jensen, L.B., Garcia-Migura, L., Valenzuela, A.J., Lohr, M., Hasman, H., and Aarestrup, F.M. (2010) A classification system for plasmids from enterococci and other Gram-positive bacteria. *J Microbiol Methods.* **80**: 25-43.

Johnson, C.M., Manias, D.A., Haemig, H.A., Shokeen, S., Weaver, K.E., Henkin, T.M., and Dunny, G.M. (2010) Direct evidence for control of the pheromone-inducible *prgQ* operon of *Enterococcus faecalis* plasmid pCF10 by a countertranscript-driven attenuation mechanism. *J Bacteriol.* **192**: 1634-1642.

Jovanovic, O.S., Ayres, E.K., and Figurski, D.H. (1994) Host-inhibitory functions encoded by promiscuous plasmids. Transient arrest of *Escherichia coli* segregants that fail to inherit plasmid RK2. *J Mol Biol.* **237**: 52-64.

Jovetic, S., Zhu, Y., Marcone, G.L., Marinelli, F., and Tramper, J. (2010) beta-Lactam and glycopeptide antibiotics: first and last line of defense? *Trends Biotechnol* **28**: 596-604.

Kadokura, H., Katzen, F., and Beckwith, J. (2003) Protein disulfide bond formation in prokaryotes. *Annu Rev Biochem.* **72**: 111-135.

Kadokura, H., and Beckwith, J. (2010) Mechanisms of oxidative protein folding in the bacterial cell envelope. *Antioxid Redox Signal.* **13**: 1231-1246.

Kak, V., and Chow, J.W. (2002) *Aquired Antibiotic Resistance in Enterococci.* Washington, D.C.: ASM Press.

Kalina, P. (1970) The taxonomy and nomenclature of enterococci. *Int J Syst Bacteriol.* **20**: 185-189.

Khan, S.A. (1997) Rolling-circle replication of bacterial plasmids. *Microbiol Mol Biol Rev*. **61**: 442-455.

Khan, S.A. (2005) Plasmid rolling-circle replication: highlights of two decades of research. *Plasmid*. **53**: 126-136.

Kim, S.O., Merchant, K., Nudelman, R., Beyer, W.F., Jr., Keng, T., DeAngelo, J., Hausladen, A., and Stamler, J.S. (2002) OxyR: a molecular code for redox-related signaling. *Cell*. **109**: 383-396.

Kirschner, M., and Gerhart, J. (1998) Evolvability. *Proc Natl Acad Sci USA*. **95**: 8420-8427.

Kolatka, K., Witosinska, M., Pierechod, M., and Konieczny, I. (2008) Bacterial partitioning proteins affect the subcellular location of broad-host-range plasmid RK2. *Microbiology*. **154**: 2847-2856.

Kolodkin-Gal, I., Hazan, R., Gaathon, A., Carmeli, S., and Engelberg-Kulka, H. (2007) A linear pentapeptide is a quorum-sensing factor required for *mazEF*-mediated cell death in *Escherichia coli*. *Science*. **318**: 652-655.

Kotra, L.P., Haddad, J., and Mobashery, S. (2000) Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance. *Antimicrob Agents. Chemother*. **44**: 3249-3256.

Kouwen, T.R., van der Goot, A., Dorenbos, R., Winter, T., Antelmann, H., Plaisier, M.C., Quax, W.J., van Dijl, J.M., and Dubois, J.Y. (2007) Thiol-disulphide oxidoreductase modules in the low-GC Gram-positive bacteria. *Mol Microbiol*. **64**: 984-999.

Kozlowicz, B.K., Dworkin, M., and Dunny, G.M. (2006) Pheromone-inducible conjugation in *Enterococcus faecalis*: a model for the evolution of biological complexity? *Int J MedMicrobiol. IJMM*. **296**: 141-147.

Kreft, B., Marre, R., Schramm, U., and Wirth, R. (1992) Aggregation substance of *Enterococcus faecalis* mediates adhesion to cultured renal tubular cells. *Infect Immun*. **60**: 25-30.

Kurenbach, B., Bohn, C., Prabhu, J., Abudukerim, M., Szewzyk, U., and Grohmann, E. (2003) Intergeneric transfer of the *Enterococcus faecalis* plasmid pIP501 to *Escherichia coli* and Streptomyces lividans and sequence analysis of its tra region. *Plasmid*. **50**: 86-93.

Kwong, S.M., Skurray, R.A., and Firth, N. (2004) *Staphylococcus aureus* multiresistance plasmid pSK41: analysis of the replication region, initiator protein binding and antisense RNA regulation. *Mol Microbiol* **51**: 497-509.

Lamour, V., Westblade, L.F., Campbell, E.A., and Darst, S.A. (2009) Crystal structure of the in vivo-assembled *Bacillus subtilis* Spx/RNA polymerase alpha subunit C-terminal domain complex. *J Struct Biol.* **168**: 352-356.

Larsen, R.A., Cusumano, C., Fujioka, A., Lim-Fong, G., Patterson, P., and Pogliano, J. (2007) Treadmilling of a prokaryotic tubulin-like protein, TubZ, required for plasmid stability in *Bacillus thuringiensis*. *Genes Dev.* **21**: 1340-1352.

Le Chatelier, E., Janniere, L., Ehrlich, S.D., and Canceill, D. (2001) The RepE initiator is a double-stranded and single-stranded DNA-binding protein that forms an atypical open complex at the onset of replication of plasmid pAMβ1 from Gram-positive bacteria. *J Biol Chem.* **276**: 10234-10246.

LeBlanc, D.J., and Lee, L.N. (1982) Characterization of two tetracycline resistance determinants in *Streptococcus faecalis* JH1. *J Bacteriol* **150**: 835-843.

Leclercq, R., Derlot, E., Duval, J., and Courvalin, P. (1988) Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. *N Engl J Med* .**319**: 157-161.

Lee, C., Lee, S.M., Mukhopadhyay, P., Kim, S.J., Lee, S.C., Ahn, W.S., Yu, M.H., Storz, G., and Ryu, S.E. (2004) Redox regulation of OxyR requires specific disulfide bond formation involving a rapid kinetic reaction path. *Nat Struct Mol Bio.l* **11**: 1179-1185.

Lee, J.W., Soonsanga, S., and Helmann, J.D. (2007) A complex thiolate switch regulates the *Bacillus subtilis* organic peroxide sensor OhrR. *Proc Natl Acad Sci USA.* **104**: 8743-8748.

Leelakriangsak, M., Huyen, N.T., Towe, S., van Duy, N., Becher, D., Hecker, M., Antelmann, H., and Zuber, P. (2008) Regulation of quinone detoxification by the thiol stress sensing DUF24/MarR-like repressor, YodB in *Bacillus subtilis*. *Mol Microbiol*. **67**: 1108-1124.

Leonard, B.A., Podbielski, A., Hedberg, P.J., and Dunny, G.M. (1996) *Enterococcus faecalis* pheromone binding protein, PrgZ, recruits a chromosomal oligopeptide permease system to import sex pheromone cCF10 for induction of conjugation. *Proc Natl Acad Sci USA.* **93**: 260-264.

Leonard, T.A., Butler, P.J., and Lowe, J. (2005) Bacterial chromosome segregation: structure and DNA binding of the Soj dimer--a conserved biological switch. *EMBO J.* **24**: 270-282.

Lindler, L.E., and Macrina, F.L. (1986) Characterization of genetic transformation in *Streptococcus mutans* by using a novel high-efficiency plasmid marker rescue system. *J Bacteriol.* **166**: 658-665.

Lioy, V.S., Pratto, F., de la Hoz, A.B., Ayora, S., and Alonso, J.C. (2010) Plasmid pSM19035, a model to study stable maintenance in Firmicutes. *Plasmid.* **64**: 1-17.

Ludwig, W., Seewaldt, E., Kilpper-Balz, R., Schleifer, K.H., Magrum, L., Woese, C.R., Fox, G.E., and Stackebrandt, E. (1985) The phylogenetic position of *Streptococcus* and *Enterococcus*. *J Gen Microbiol.* **131**: 543-551.

Macrina, F.L., Evans, R.P., Tobian, J.A., Hartley, D.L., Clewell, D.B., and Jones, K.R. (1983) Novel shuttle plasmid vehicles for *Escherichia-Streptococcus* transgeneric cloning. *Gene.* **25**: 145-150.

Makinen, P.L., Clewell, D.B., An, F., and Makinen, K.K. (1989) Purification and substrate specificity of a strongly hydrophobic extracellular metalloendopeptidase ("gelatinase") from *Streptococcus faecalis* (strain 0G1-10). *J Biol Chem* **264**: 3325-3334.

Malke, H. (1974) Genetics of resistance to macrolide antibiotics and lincomycin in natural isolates of *Streptococcus pyogenes*. *Mol Gen Genet.* **135**: 349-367.

Manson, J.M., Hancock, L.E., and Gilmore, M.S. (2010) Mechanism of chromosomal transfer of *Enterococcus faecalis* pathogenicity island, capsule, antimicrobial resistance, and other traits. *Proc Natl Acad Sci USA.* **107**: 12269-12274.

Marshall, C.G., Broadhead, G., Leskiw, B.K., and Wright, G.D. (1997) D-Ala-D-Ala ligases from glycopeptide antibiotic-producing organisms are highly homologous to the enterococcal vancomycin-resistance ligases VanA and VanB. *Proc Natl Acad Sci USA.* **94**: 6480-6483.

Masai, H., Kaziro, Y., and Arai, K. (1983) Definition of *oriR*, the minimum DNA segment essential for initiation of R1 plasmid replication in vitro. *Proc Natl Acad Sci USA.* **80**: 6814-6818.

Masai, H., and Arai, K. (1987) RepA and DnaA proteins are required for initiation of R1 plasmid replication in vitro and interact with the oriR sequence. *Proc Natl Acad Sci USA.* **84**: 4781-4785.

Mazeffa, V.J. (2008) Thesis.

McCarthy, A.A., Haebel, P.W., Torronen, A., Rybin, V., Baker, E.N., and Metcalf, P. (2000) Crystal structure of the protein disulfide bond isomerase, DsbC, from *Escherichia coli*. *Nat Struct Biol.* **7**: 196-199.

McCormick, J.K., Hirt, H., Dunny, G.M., and Schlievert, P.M. (2000) Pathogenic Mechanisms of Enterococcal Endocarditis. *Curr Infectious Dis Rep.* **2**: 315-321.

McKay, L.L., Baldwin, K.A., and Efstathiou, J.D. (1976) Transductional evidence for plasmid linkage of lactose metabolism in *Streptococcus lactis* C2. *Appl Environ Microbiol.* **32**: 45-52.

Meima, R., Eschevins, C., Fillinger, S., Bolhuis, A., Hamoen, L.W., Dorenbos, R., Quax, W.J., van Dijl, J.M., Provvedi, R., Chen, I., Dubnau, D., and Bron, S. (2002) The bdbDC operon of *Bacillus subtilis* encodes thiol-disulfide oxidoreductases required for competence development. *J Biol Chem.* **277**: 6994-7001.

Meloni, M. (2005) Thesis.

Meloni, M., Mazeffa, V.J., Manias, D., Dunny, G.M., and Buttaro, B.A. (Submitted) Pheromone precursor pre-cCF10 is a host range determinant of the *Enterococcus faecalis* conjugative plasmid pCF10. *J. Bacteriol.*

Messer, W. (2002) The bacterial replication initiator DnaA. DnaA and oriC, the bacterial mode to initiate DNA replication. *FEMS Microbiol Rev.* **26**: 355-374.

Meyer, R. (2009) Replication and conjugative mobilization of broad host-range IncQ plasmids. *Plasmid.* **62**: 57-70.

Miller, W.G., Parker, C.T., Rubenfield, M., Mendz, G.L., Wosten, M.M., Ussery, D.W., Stolz, J.F., Binnewies, T.T., Hallin, P.F., Wang, G., Malek, J.A., Rogosin, A., Stanker, L.H., and Mandrell, R.E. (2007) The complete genome sequence and analysis of the epsilonproteobacterium *Arcobacter butzleri*. *PLoS One.* **2**: e1358.

Mingeot-Leclercq, M.P., Glupczynski, Y., and Tulkens, P.M. (1999) Aminoglycosides: activity and resistance. *Antimicrob Agents Chemother.* **43**: 727-737.

Moellering, R.C., Jr., and Weinberg, A.N. (1971) Studies on antibiotic syngerism against enterococci. II. Effect of various antibiotics on the uptake of 14 C-labeled streptomycin by enterococci. *J Clin Invest.* **50**: 2580-2584.

Moller-Jensen, J., Jensen, R.B., Lowe, J., and Gerdes, K. (2002) Prokaryotic DNA segregation by an actin-like filament. *EMBO J* **21**: 3119-3127.

Moller-Jensen, J., Borch, J., Dam, M., Jensen, R.B., Roepstorff, P., and Gerdes, K. (2003) Bacterial mitosis: ParM of plasmid R1 moves plasmid DNA by an actin-like insertional polymerization mechanism. *Mol Cell.* **12**: 1477-1487.

Moller, M., and Hederstedt, L. (2006) Role of membrane-bound thiol-disulfide oxidoreductases in endospore-forming bacteria. *Antioxid Redox Signal.* **8**: 823-833.

Moreillon, P., and Que, Y.A. (2004) Infective endocarditis. *Lancet.* **363**: 139-149.

Mori, M., Sakagami, Y., Ishii, Y., Isogai, A., Kitada, C., Fujino, M., Adsit, J.C., Dunny, G.M., and Suzuki, A. (1988) Structure of cCF10, a peptide sex pheromone which induces conjugative transfer of the *Streptococcus faecalis* tetracycline resistance plasmid, pCF10. *J Biol Chem.* **263**: 14574-14578.

Mundy, L.M., Sahm, D.F., and Gilmore, M. (2000) Relationships between enterococcal virulence and antimicrobial resistance. *Clin Microbiol Rev.* **13**: 513-522.

Murchison, H.H., Barrett, J.F., Cardineau, G.A., and Curtiss, R., 3rd (1986) Transformation of *Streptococcus mutans* with chromosomal and shuttle plasmid (pYA629) DNAs. *Infect Immun* **54**: 273-282.

Murray, B.E. (1992) Beta-lactamase-producing enterococci. *Antimicrob Agents Chemother.* **36**: 2355-2359.

Nakano, M.M., Lin, A., Zuber, C.S., Newberry, K.J., Brennan, R.G., and Zuber, P. (2010) Promoter recognition by a complex of Spx and the C-terminal domain of the RNA polymerase alpha subunit. *PLoS One.* **5**: e8664.

Nakano, S., Kuster-Schock, E., Grossman, A.D., and Zuber, P. (2003) Spx-dependent global transcriptional control is induced by thiol-specific oxidative stress in *Bacillus subtilis*. *Proc Natl Acad Sci USA.* **100**: 13603-13608.

Nakayama, J., Ruhfel, R.E., Dunny, G.M., Isogai, A., and Suzuki, A. (1994) The *prgQ* gene of the *Enterococcus faecalis* tetracycline resistance plasmid pCF10 encodes a peptide inhibitor, iCF10. *J Bacteriol.* **176**: 7405-7408.

Nash, J., and Krishnapillai, V. (1987) DNA sequence analysis of host range mutants of the promiscuous IncP-1 plasmids R18 and R68 with Tn*7* insertions in *oriV*. *Plasmid.* **18**: 35-45.

Newberry, K.J., Nakano, S., Zuber, P., and Brennan, R.G. (2005) Crystal structure of the *Bacillus subtilis* anti-alpha, global transcriptional regulator, Spx, in complex with the alpha C-terminal domain of RNA polymerase. *Proc Natl Acad Sci USA.* **102**: 15839-15844.

Newberry, K.J., Fuangthong, M., Panmanee, W., Mongkolsuk, S., and Brennan, R.G. (2007) Structural mechanism of organic hydroperoxide induction of the transcription regulator OhrR. *Mol Cell.* **28**: 652-664.

Nordstrom, K., Molin, S., and Light, J. (1984) Control of replication of bacterial plasmids: genetics, molecular biology, and physiology of the plasmid R1 system. *Plasmid.* **12**: 71-90.

Nordstrom, K. (2006) Plasmid R1--replication and its control. *Plasmid.* **55**: 1-26.

Olmsted, S.B., Kao, S.M., van Putte, L.J., Gallo, J.C., and Dunny, G.M. (1991) Role of the pheromone-inducible surface protein Asc10 in mating aggregate formation and conjugal transfer of the *Enterococcus faecalis* plasmid pCF10. *J Bacteriol.* **173**: 7665-7672.

Olmsted, S.B., Dunny, G.M., Erlandsen, S.L., and Wells, C.L. (1994) A plasmid-encoded surface protein on *Enterococcus faecalis* augments its internalization by cultured intestinal epithelial cells. *J Infect Dis.* **170**: 1549-1556.

Ortega-Jimenez, S., Giraldo-Suarez, R., Fernandez-Tresguerres, M.E., Berzal-Herranz, A., and Diaz-Orejas, R. (1992) DnaA dependent replication of plasmid R1 occurs in the presence of point mutations that disrupt the dnaA box of oriR. *Nucleic Acids Res.* **20**: 2547-2551.

Oshima, T., Aiba, H., Masuda, Y., Kanaya, S., Sugiura, M., Wanner, B.L., Mori, H., and Mizuno, T. (2002) Transcriptome analysis of all two-component regulatory system mutants of *Escherichia coli* K-12. *Mol Microbiol.* **46**: 281-291.

Pag, U., and Sahl, H.G. (2002) Multiple activities in lantibiotics--models for the design of novel antibiotics? *Curr Pharm Des.* **8**: 815-833.

Palmer, K.L., Kos, V.N., and Gilmore, M.S. (2010) Horizontal gene transfer and the genomics of enterococcal antibiotic resistance. *Curr Opin Microbiol* **13**: 632-639.

Panmanee, W., Vattanaviboon, P., Poole, L.B., and Mongkolsuk, S. (2006) Novel organic hydroperoxide-sensing and responding mechanisms for OhrR, a major bacterial sensor and regulator of organic hydroperoxide stress. *J Bacteriol.* **188**: 1389-1395.

Parsek, M.R., and Singh, P.K. (2003) Bacterial biofilms: an emerging link to disease pathogenesis. *Annu Rev Microbiol.* **57**: 677-701.

Perry, D., and Kuramitsu, H.K. (1981) Genetic transformation of *Streptococcus mutans*. *Infect Immun.* **32**: 1295-1297.

Piggot, P.J., Sklar, M.D., and Gorini, L. (1972) Ribosomal alterations controlling alkaline phosphatase isozymes in *Escherichia coli*. *J Bacteriol.* **110**: 291-299.

Platteeuw, C., Michiels, F., Joos, H., Seurinck, J., and de Vos, W.M. (1995) Characterization and heterologous expression of the tetL gene and identification of iso-ISS1 elements from *Enterococcus faecalis* plasmid pJH1. *Gene.* **160**: 89-93.

Porat, A., Cho, S.H., and Beckwith, J. (2004) The unusual transmembrane electron transporter DsbD and its homologues: a bacterial family of disulfide reductases. *Res Microbiol*.**155**: 617-622.

Pratto, F., Cicek, A., Weihofen, W.A., Lurz, R., Saenger, W., and Alonso, J.C. (2008) *Streptococcus pyogenes* pSM19035 requires dynamic assembly of ATP-bound ParA and ParB on parS DNA during plasmid segregation. *Nucleic Acids Res.* **36**: 3676-3689.

Qin, X., Singh, K.V., Weinstock, G.M., and Murray, B.E. (2000) Effects of *Enterococcus faecalis* fsr genes on production of gelatinase and a serine protease and virulence. *Infect Immun.* **68**: 2579-2586.

Rainey, P.B., and Cooper, T.F. (2004) Evolution of bacterial diversity and the origins of modularity. *Res Microbiol.* **155**: 370-375.

Rakita, R.M., Vanek, N.N., Jacques-Palaz, K., Mee, M., Mariscalco, M.M., Dunny, G.M., Snuggs, M., Van Winkle, W.B., and Simon, S.I. (1999) *Enterococcus faecalis* bearing aggregation substance is resistant to killing by human neutrophils despite phagocytosis and neutrophil activation. *Infect Immun.* **67**: 6067-6075.

Reinhart, F., Achebach, S., Koch, T., and Unden, G. (2008) Reduced apo-fumarate nitrate reductase regulator (apoFNR) as the major form of FNR in aerobically growing *Escherichia coli*. *J Bacteriol.* **190**: 879-886.

Reynolds, P.E., Depardieu, F., Dutka-Malen, S., Arthur, M., and Courvalin, P. (1994) Glycopeptide resistance mediated by enterococcal transposon Tn*1546* requires production of VanX for hydrolysis of D-alanyl-D-alanine. *Mol Microbiol.* **13**: 1065-1070.

Ringgaard, S., van Zon, J., Howard, M., and Gerdes, K. (2009) Movement and equipositioning of plasmids by ParA filament disassembly. *Proc Natl Acad Sci USA.* **106**: 19369-19374.

Rosvoll, T.C., Pedersen, T., Sletvold, H., Johnsen, P.J., Sollid, J.E., Simonsen, G.S., Jensen, L.B., Nielsen, K.M., and Sundsfjord, A. (2010) PCR-based plasmid typing in *Enterococcus faecium* strains reveals widely distributed pRE25-, pRUM-, pIP501- and pHTbeta-related replicons associated with glycopeptide resistance and stabilizing toxin-antitoxin systems. *FEMS Immunol Med Microbiol*. **58**: 254-268.

Ruhfel, R.E., Manias, D.A., and Dunny, G.M. (1993) Cloning and characterization of a region of the *Enterococcus faecalis* conjugative plasmid, pCF10, encoding a sex pheromone-binding function. *J Bacteriol.* **175**: 5253-5259.

Ruoff, K.L. (1990) Recent taxonomic changes in the genus Enterococcus. *Eur J Clin Microbiol. Infect Dis* **9**: 75-79.

Saitou, N., and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Bio Evol.* **4**: 406-425.

Samuelson, J.C., Chen, M., Jiang, F., Moller, I., Wiedmann, M., Kuhn, A., Phillips, G.J., and Dalbey, R.E. (2000) YidC mediates membrane protein insertion in bacteria. *Nature.* **406**: 637-641.

Sartingen, S., Rozdzinski, E., Muscholl-Silberhorn, A., and Marre, R. (2000) Aggregation substance increases adherence and internalization, but not translocation, of *Enterococcus faecalis* through different intestinal epithelial cells in vitro. *Infect Immun.* **68**: 6044-6047.

Sarvas, M., Harwood, C.R., Bron, S., and van Dijl, J.M. (2004) Post-translocational folding of secretory proteins in Gram-positive bacteria. *Biochim Biophys Acta.* **1694**: 311-327.

Schleifer, K.H., and Kilpper-Bälz, R. (1984) Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the Genus *Enterococcus* norn. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. *Int J Syst Bacteriol.* **34**: 31-34.

Schneider, T.D., and Stephens, R.M. (1990) Sequence logos: a new way to display consensus sequences. *Nucleic Acids Res.* **18**: 6097-6100.

Schrag, S.J., and Perrot, V. (1996) Reducing antibiotic resistance. *Nature* **381**: 120-121.

Schwarz, F.V., Perreten, V., and Teuber, M. (2001) Sequence of the 50-kb conjugative multiresistance plasmid pRE25 from *Enterococcus faecalis* RE25. *Plasmid.* **46**: 170-187.

Shankar, N., Coburn, P., Pillar, C., Haas, W., and Gilmore, M. (2004) Enterococcal cytolysin: activities and association with other virulence traits in a pathogenicity island. *Int J Med Microbiol.* **293**: 609-618.

Sherman, J.M. (1937) The Streptococci. *Bacteriol Rev.* **1**: 3-97.

Shi, K., Brown, C.K., Gu, Z.Y., Kozlowicz, B.K., Dunny, G.M., Ohlendorf, D.H., and Earhart, C.A. (2005) Structure of peptide sex pheromone receptor PrgX and PrgX/pheromone complexes and regulation of conjugation in *Enterococcus faecalis*. *Proc Natl Acad Sci USA.* **102**: 18596-18601.

Shingler, V., and Thomas, C.M. (1984) Analysis of the *trfA* region of broad host-range plasmid RK2 by transposon mutagenesis and identification of polypeptide products. *J Mol Biol.* **175**: 229-249.

Skulj, M., Okrslar, V., Jalen, S., Jevsevar, S., Slanc, P., Strukelj, B., and Menart, V. (2008) Improved determination of plasmid copy number using quantitative real-time PCR for monitoring fermentation processes. *Microb Cell Fact.* **7**: 6.

Sletvold, H., Johnsen, P.J., Hamre, I., Simonsen, G.S., Sundsfjord, A., and Nielsen, K.M. (2008) Complete sequence of *Enterococcus faecium* pVEF3 and the detection of an omega-epsilon-zeta toxin-antitoxin module and an ABC transporter. *Plasmid.* **60**: 75-85.

Soonsanga, S., Lee, J.W., and Helmann, J.D. (2008a) Oxidant-dependent switching between reversible and sacrificial oxidation pathways for *Bacillus subtilis* OhrR. *Mol Microbiol*. **68**: 978-986.

Soonsanga, S., Lee, J.W., and Helmann, J.D. (2008b) Conversion of *Bacillus subtilis* OhrR from a 1-Cys to a 2-Cys peroxide sensor. *J Bacteriol.* **190**: 5738-5745.

Staddon, J.H., Bryan, E.M., Manias, D.A., and Dunny, G.M. (2004) Conserved target for group II intron insertion in relaxase genes of conjugative elements of gram-positive bacteria. *J Bacteriol.* **186**: 2393-2401.

Staddon, J.H., Bryan, E.M., Manias, D.A., Chen, Y., and Dunny, G.M. (2006) Genetic characterization of the conjugative DNA processing system of enterococcal plasmid pCF10. *Plasmid.* **56**: 102-111.

Stein, T. (2005) *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Mol Microbiol.* **56**: 845-857.

Su, Y.A., Sulavik, M.C., He, P., Makinen, K.K., Makinen, P.L., Fiedler, S., Wirth, R., and Clewell, D.B. (1991) Nucleotide sequence of the gelatinase gene (*gelE*) from *Enterococcus faecalis* subsp. liquefaciens. *Infect Immun.* **59**: 415-420.

Sussmuth, S.D., Muscholl-Silberhorn, A., Wirth, R., Susa, M., Marre, R., and Rozdzinski, E. (2000) Aggregation substance promotes adherence, phagocytosis, and intracellular survival of *Enterococcus faecalis* within human macrophages and suppresses respiratory burst. *Infect Immun.* **68**: 4900-4906.

Tannock, G.W., and Cook, G. (2002) *Enterococci as Members of the Intestinal Microflora of Humans.* Washington, D.C.: ASM Press.

Tartof, K.D., and Hobbs, C.A. (1987) Improved media for growing plasmid and cosmid clones. *Focus.* **9**: 12.

Tinsley, E., and Khan, S.A. (2006) A novel FtsZ-like protein is involved in replication of the anthrax toxin-encoding pXO1 plasmid in Bacillus anthracis. *J Bacteriol.* **188**: 2829-2835.

Tjalsma, H., Bron, S., and van Dijl, J.M. (2003) Complementary impact of paralogous Oxa1-like proteins of *Bacillus subtilis* on post-translocational stages in protein secretion. *J Biol Chem.* **278**: 15622-15632.

Tomayko, J.F., Zscheck, K.K., Singh, K.V., and Murray, B.E. (1996) Comparison of the beta-lactamase gene cluster in clonally distinct strains of *Enterococcus faecalis*. *Antimicrob Agents Chemother.* **40**: 1170-1174.

Toukdarian, A. (2004) *Plasmid Srategies for Broad-Host-Range Replication in Gram-Negative Bacteria.* Washington, D.C.: ASM Press.

Tritton, T.R. (1977) Ribosome-tetracycline interactions. *Biochemistry.* **16**: 4133-4138.

Uhlin, B.E., and Nordstrom, K. (1978) A runaway-replication mutant of plasmid R1drd-19: temperature-dependent loss of copy number control. *Mol Gen Genet.* **165**: 167-179.

Urban, S. (2009) Making the cut: central roles of intramembrane proteolysis in pathogenic microorganisms. *Nat Rev Microbiol.* **7**: 411-423.

Uttley, A.H., Collins, C.H., Naidoo, J., and George, R.C. (1988) Vancomycin-resistant enterococci. *Lancet.* **1**: 57-58.

Vanek, N.N., Simon, S.I., Jacques-Palaz, K., Mariscalco, M.M., Dunny, G.M., and Rakita, R.M. (1999) *Enterococcus faecalis* aggregation substance promotes opsonin-independent binding to human neutrophils via a complement receptor type 3-mediated mechanism. *FEMS Immunol Med Microbiol*. **26**: 49-60.

Wagner, E.G., and Brantl, S. (1998) Kissing and RNA stability in antisense control of plasmid replication. *Trends Biochem Sci.* **23**: 451-454.

Wardal, E., Sadowy, E., and Hryniewicz, W. (2010) Complex nature of enterococcal pheromone-responsive plasmids. *Pol J Microbiol.* **59**: 79-87.

Waters, C.M., Antiporta, M.H., Murray, B.E., and Dunny, G.M. (2003) Role of the *Enterococcus faecalis* GelE protease in determination of cellular chain length, supernatant pheromone levels, and degradation of fibrin and misfolded surface proteins. *J Bacteriol.* **185**: 3613-3623.

Weaver, K.E., Rice, L.B., and Churchward, G. (2002) *Plasmids and transposons.* Washington, D.C.: ASM Press.

Weaver, K.E., Kwong, S.M., Firth, N., and Francia, M.V. (2009) The RepA_N replicons of Gram-positive bacteria: a family of broadly distributed but narrow host range plasmids. *Plasmid.* **61**: 94-109.

Weigel, L.M., Clewell, D.B., Gill, S.R., Clark, N.C., McDougal, L.K., Flannagan, S.E., Kolonay, J.F., Shetty, J., Killgore, G.E., and Tenover, F.C. (2003) Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. *Science.* **302**: 1569-1571.

Weigel, L.M., Donlan, R.M., Shin, D.H., Jensen, B., Clark, N.C., McDougal, L.K., Zhu, W., Musser, K.A., Thompson, J., Kohlerschmidt, D., Dumas, N., Limberger, R.J., and Patel, J.B. (2007) High-level vancomycin-resistant *Staphylococcus aureus* isolates associated with a polymicrobial biofilm. *Antimicrob Agents Chemother.* **51**: 231-238.

Wells, C.L., Moore, E.A., Hoag, J.A., Hirt, H., Dunny, G.M., and Erlandsen, S.L. (2000) Inducible expression of *Enterococcus faecalis* aggregation substance surface protein facilitates bacterial internalization by cultured enterocytes. *Infect Immun.* **68**: 7190-7194.

Werner, G., Freitas, A.R., Coque, T.M., Sollid, J.E., Lester, C., Hammerum, A.M., Garcia-Migura, L., Jensen, L.B., Francia, M.V., Witte, W., Willems, R.J., and Sundsfjord, A. (2010) Host range of enterococcal *vanA* plasmids among Gram-positive intestinal bacteria. *Antimicrob Agents Chemother.* **66**: 273-282.

Williamson, R., Gutmann, L., Horaud, T., Delbos, F., and Acar, J.F. (1986) Use of penicillin-binding proteins for the identification of enterococci. *J Gen Microbiol.* **132**: 1929-1937.

Yurimoto, H., Hirai, R., Matsuno, N., Yasueda, H., Kato, N., and Sakai, Y. (2005) HxlR, a member of the DUF24 protein family, is a DNA-binding protein that acts as a positive regulator of the formaldehyde-inducible *hxlAB* operon in *Bacillus subtilis*. *Mol Microbiol.* **57**: 511-519.

Zhou, L., Lei, X.H., Bochner, B.R., and Wanner, B.L. (2003) Phenotype microarray analysis of *Escherichia coli* K-12 mutants with deletions of all two-component systems. *J Bacteriol.* **185**: 4956-4972.

Zuber, P. (2004) Spx-RNA polymerase interaction and global transcriptional control during oxidative stress. *J Bacteriol.* **186**: 1911-1918.

Zuber, P. (2009) Management of oxidative stress in *Bacillus*. *Annu Rev Microbiol.* **63**: 575-597.

APPENDIX A

Growth Media

GM-17 (M-17 with Glucose)

37.25 g Difco™ M-17 powder (Catalog # 218561)

q.s. to 1 L with dH_2O

Autoclaved for 35 minutes at 121°C.

10 mL 100X Filter Sterilized Glucose (0.5%)

GM-17 Agar

37.25 g Difco™ M-17 powder (Catalog # 218561)

q.s. to 1 L with dH_2O

15 g (1.5%) Difco™ Agar, Granulated (Catalog # 214530)

Autoclave for 35 minutes at 121°C.

10 mL 50% Filter Sterilized Glucose (0.5%)

Lysogeny Broth (LB) (Bertani, 1951, 2004)

10 g Bacto™ Tryptone (Catalog # 211705)

5 g Bacto™ Yeast Extract (Catalog # 212750)

10 g FisherChemicals Sodium Chloride (Catalog # S271-3)

q.s. to 1 L with dH_2O.

Autoclave for 35 minutes at 121°C.

LB Agar

10 g Bacto™ Tryptone (Catalog # 211705)

5 g Bacto™ Yeast Extract (Catalog # 212750)

10 g FisherChemicals Sodium Chloride (Catalog # S271-3)

15 g (1.5%) Difco™ Agar, Granulated (Catalog # 214530)

q.s. to 1 L with dH_2O.

Autoclave for 35 minutes at 121°C.

Terrific Broth (Tartof and Hobbs, 1987)

12 g Bacto™ Tryptone (Catalog # 211705)

24 g Bacto™ Yeast Extract (Catalog # 212750)

4 mL FisherChemicals Glycerol (G33-1)

q.s. to 900 mL with dH_2O.

Autoclave for 35 minutes at 121°C.

Mix Potassium Phosphate solution (below) when both solutions are below 60°C.

0.17 M Sigma® KH_2PO_4 (Catalog # P-5379)

0.7 M FisherChemicals K_2HPO_4 (Catalog # P288-500)

q.s. to 100 mL with dH_2O.

Autoclave for 20 minutes at 121°C.

Todd Hewitt (TH) Broth

30 g Bacto™ TH powder (Catalog # 249240)

q.s. to 1 L with dH_2O

Autoclave for 35 minutes at 121°C.

TH Agar

30 g Bacto™ TH powder (Catalog # 249240)

15 g (1.5%) Difco™ Agar, Granulated (Catalog # 214530)

q.s. to 1 L with dH_2O

Autoclave for 35 minutes at 121°C.

TH Glycine (THG)

30 g Bacto™ TH powder (Catalog # 249240)

100 g (10 %) Fisher BioReagents® Glycine (Catalog # BP381-1)

q.s. to 1 L with dH_2O.

Autoclave for 35 minutes at 121°C.

TH Sucrose (THS)

30 g Bacto™ TH powder (Catalog # 249240)

0.25 M FisherChemicals Sucrose (S5-500)

q.s. to 1 L with dH_2O

Autoclave for 35 minutes at 121°C.

Electroporation and Transformation Solutions

E. faecalis Electroporation

EB buffer

0.625 M FisherChemicals Sucrose (S5-500)

1 M FisherChemicals Magnesium Chloride (Catalog # M33-500)

Adjust to a final pH 4.0 with HCl and sterilize by filtration

E. coli Transformation

RF1

100 mM Acros Organics Rubidium Chloride (Catalog # 7791-11-9)

50 mM FisherChemicals Manganese Chloride (Catalog # M87-500)

30 mM FisherChemicals Potassium Acetate (P171-500)

10 mM FisherChemicals Calcium Chloride Dihydrate (C-79)

15% (w/v) FisherChemicals Glycerol (G33-1)

Adjust to a final pH 5.8 with Acetic acid. Sterilize by filtration.

RF2

10 mM MOPS (see below)

10 mM Acros Organics Rubidium Chloride (Catalog # 7791-11-9)

75 mM FisherChemicals Calcium Chloride Dihydrate (C-79)

15% (w/v) FisherChemicals Glycerol (G33-1)

Adjust to a final pH 6.8 with NaOH. Sterilize by filtration.

10X MOPS

DEPC-treated water

200 mM Ambion® MOPS (Catalog # AM9570)

50 mM FisherChemicals Sodium Acetate (Catalog # S210-500)

10 mM FisherChemicals BP120-1 EDTA (Catalog # BP120-1)

Adjust to a final pH 7.0 with Acetic acid.

50X TAE Buffer

242.2 g FisherChemicals Tris-Base (Cat #BP152-1)

57.1 mL FisherChemicals Glacial Acetic Acid (Catalog # A38-212)

100 mL of 0.5 M FisherChemicals EDTA (Catalog # BP120-1) – pH 8.0

q.s. to 1 L with dH_2O.

Protein Purification Solutions

Binding buffer

500 mM FisherChemicals Sodium Chloride (Catalog # S271-3)

200 mM (pH 7.9) FisherChemicals Tris-HCl (Catalog # BP153-1)

80 mM FisherChemicals Imidazole (Catalog # O3196-500)

Wash Buffer

500 mM FisherChemicals Sodium Chloride (Catalog # S271-3)

200 mM (pH 7.9) FisherChemicals Tris-HCl (Catalog # BP153-1)

160 mM FisherChemicals Imidazole (Catalog # O3196-500)

Elution Buffer

500 mM FisherChemicals Sodium Chloride (Catalog # S271-3)

200 mM (pH 7.9) FisherChemicals Tris-HCl (Catalog # BP153-1)

400 mM FisherChemicals Imidazole (Catalog # O3196-500)

Protein Lysis Buffer

1.95 mL (0.5 M) Sigma® KH_2PO_4 (Catalog # P-5379)

3.05 mL (0.5 M) FisherChemicals K_2HPO_4 (Catalog # P288-500)

50 μl (1 M) FisherChemicals Manganese Chloride (Catalog # M87-500)

50 mg (1mg/ml) Sigma® Lysozyme (Catalog # L-7651)

25 mg (500 μg/ml) Calbiochem® DNase (Catalog # 260913)

12.5 mg (250 μg/ml) Calbiochem® RNaseA (Catalog # 556746)

45 mL Baxter Sterile H_2O (Catalog # 2F7114)

SDS-PAGE Solutions

Stacking Buffer

28 g FisherChemicals Tris-HCl (Catalog # BP153-1)

2.2 g FisherChemicals Tris-Base (Cat #BP152-1)

4 g FisherChemicals SDS (Catalog # BP166-500)

q.s. to 1 L with dH_2O.

Adjust to final pH of 6.8 with HCl.

Resolving Buffer

60 g FisherChemicals Tris-HCl (Catalog # BP153-1)

30 g FisherChemicals Tris-Base (Cat #BP152-1)

4 g FisherChemicals SDS (Catalog # BP166-500)

q.s. to 1 L with dH_2O.

Adjust to pH 8.8 with HCl.

10X SDS Page Running Buffer

30.3 g (25 mM) FisherChemicals Tris-Base (Cat #BP152-1)

144.1 g (192 mM) Fisher BioReagents® Glycine (Catalog # BP381-1)

10 g (0.1%) FisherChemicals SDS (Catalog # BP166-500)

q.s. to 1 L with dH_2O.

Adjust to a final pH of 8.3 with HCl.

2X Loading Buffer

757 mg FisherChemicals Tris-HCl (Catalog # BP153-1)

10 mL 10% FisherChemicals SDS (Catalog # BP166-500)

10 mL FisherChemicals Glycerol (Catalog #G33-1)

0.25 mL FisherBiotech Bromophenol Blue (Catalog # BP115-25)

Adjust to a final pH of 6.8 with NaOH.

Coomassie Blue Stain

500 mL (50%) FisherChemicals Methanol (Catalog # A411-4)

100 mL (10%) FisherChemicals Glacial Acetic Acid (Catalog # A38-212)

600 mL dH_2O

0.05% Bio-Rad Coomassie® Brilliant Blue R-250 (Catalog # 161-0400)

Coomassie De-stain Solution

300 mL (30%) FisherChemicals Methanol (Catalog # A411-4)

100 mL (10%) FisherChemicals Glacial Acetic Acid (Catalog # A38-212)

600 mL dH_2O

Western Blot Solutions

Towbin Buffer

1 g FisherChemicals Tris-HCl (Catalog # BP153-1)

2 g FisherChemicals Tris-Base (Cat #BP152-1)

14.4 g Fisher BioReagents® Glycine (Catalog # BP381-1)

200 mL FisherChemicals Methanol (Catalog # A411-4)

q.s. to 1 L with dH_2O

Adjust to a final pH of 8.3 with NaOH.

1X TBS Wash buffer (1 X TBS with 0.1% Tween)

100 mL 10X TBS

12.1 g (20 mM) FisherChemicals Tris-Base (Cat #BP152-1)

40 g FisherChemicals Sodium Chloride (Catalog # S271-3)

19 mL (1N) FisherChemicals Hydrochloric Acid (Catalog # A144-212)

q.s. to 500 mL with dH_2O

Adjust to a final pH of 7.6 using HCl.

q.s. to 1 L with dH_2O

Mix 1 mL FisherChemicals Tween (Catalog #BP337-500) while stirring.

Milk Wash Buffer

100 mL 1X TBS Wash Buffer (see above)

5.0 g Nestle® Carnation® Instant Nonfat Dry Milk (Catalog # 01-4280-00)

Commercial Buffers

Promega Corporation Wizard® Genomic DNA Purification Kit Reagents

Chromosomal Lysis buffer

50 mM FisherChemicals EDTA (Catalog # BP120-1)

10 mg/mL Sigma® Lysozyme (Catalog # L-7651)

Nuclei Lysis Solution (Catalog # A7941)

Proprietary

RNaseA Solution, 4 mg/mL (Catalog #A797C)

Proprietary

Protein Precipitation Solution (Catalog # A795A)

Proprietary

DNA Rehydration Solution (Catalog # A796A)

10 mM Tris (pH 7.4)

1 mM EDTA (pH 8.0)

Promega Corporation Wizard® Plus Midipreps DNA Purification System

Cell Resuspension Solution (Catalog # A7115)

50 mM Tris-HCL

10 mM EDTA

100 µg/mL RNaseA

Cell Lysis Solution (Catalog # A7125)

0.2 M NaOH

1% SDS

Neutralization solution (Catalog # A1485)

1.32M potassium acetate, pH 4.8

Plasmid Column Wash Solution

5.845g (200 mM) NaCl

10 mL of 1M Tris (20 mM), pH 7.5

5 mL of 0.5M EDTA (5 mM)

275 mL 55% Pharmco-AAPER EtOH (Catalog # 111ACS200)

q.s. to 500 mL with dH_2O.

Promega Corporation Wizard® SV Gel and PCR Clean-Up System Reagents

Promega Corporation Membrane Binding Solution (Catalog # A9303)

4.5 M guanidine isothiocyanate

0.5 M potassium acetate (pH 5.0)

Promega Corporation Membrane Wash Solution (Catalog # A929C)

80% EtOH, 10 mM potassium acetate (pH 5.0)

16.7 µM EDTA (pH 8.0)

Qiagen QIAquick® PCR Purification Kit Reagents

Buffer PB Binding Buffer (Catalog # 1015089)

Proprietary

Contains guanidine hydrochloride

Buffer PE Wash Buffer (Catalog # 1015210)

275 mL Buffer PE (Proprietary)

220 mL ethanol

Buffer EB Elution Buffer (Catalog # 1014612)

10 mM Tris-HCl (pH 8.5)

DNA Modification Enzyme Buffers

Promega Corporation SAP Enzyme Storage Buffer (Catalog # M820A)

25mM Tris-HCl (pH 7.6 at 4°C),

1mM $MgCl_2$

0.1mM ZnCl

50% glycerol.

Promega Corporation SAP (1X) Reaction Buffer (Catalog # M821A)

50mM Tris-HCl (pH 9.0 at 37°C)

10mM $MgCl_2$

Promega Corporation T4 DNA Ligase Storage Buffer (Catalog # M180A)

10mM Tris-HCl (pH 7.0)

50mM KCl

1mM DTT

0.1mM EDTA

50% glycerol.

Promega Corporation 2X Rapid Ligation Buffer (Catalog # C671B)

60mM Tris-HCl (pH 7.8)

20mM $MgCl_2$

20mM DTT

2mM ATP

10% PEG

Restriction Digestion Buffers

Promega Corporation (1X) Buffer D (Catalog #R004A)

pH 7.9 at 37^{o}C

6 mM Tris-HCl

6 mM $MgCl_2$

150 mM NaCl

1 mM DTT

Promega Corporation (1X) Buffer E (Catalog # R005A)

pH 7.5 at 37^{o}C

6 mM Tris-HCl

6 mM $MgCl_2$

100 mM NaCl

1 mM DTT

Promega Corporation (1X) Buffer H (Catalog # R008A)

pH 7.5 at 37^{o}C

90 mM Tris-HCl

10 mM $MgCl_2$

50 mM NaCl

New England Biolabs (1X) NEBuffer 2 (Catalog # B7002S)

pH 7.9 at 25^{o}C

50 mM NaCl

10 mM Tris-HCl

10 mM $MgCl_2$

1 mM DTT

New England Biolabs (1X) NEBuffer 4 (Catalog # B7004S)

pH 7.9 at 25^{o}C

50 mM potassium acetate

20 mM Tris-acetate

10 mM Magnesium Acetate

1 mM DTT

New England Biolabs (1X) NEBuffer EcoRI (Catalog # B0101S)

pH 7.5 at 25°C

100 mM Tris-HCl

50 mM NaCl

10 mM $MgCl_2$

0.025 % Triton X-100

DNA Polymerase Buffers

Promega Corporation GoTaq® 5X Buffer (Catalog # M791B)

Proprietary

Stratagene 10X Buffer (Catalog # 600135-84)

Proprietary

5 PRIME RealMaster SYBR ROX/20X SYBR Solution

Proprietary