

**THE PROCESSING OF REPLICATION INITIATION
PROTEIN PRGW IN *ENTEROCOCCUS FAECALIS* IS
NECESSARY FOR ACTIVITY AND STABLE
MAINTENANCE OF pCF10**

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ABSTRACT

Enterococcus faecalis are Gram-positive bacteria that colonize the gastrointestinal tracts of mammals, birds and invertebrates and are also found in sewage, soil, food and water. In addition to being commensal organisms, Enterococci can also cause nosocomial infections in humans including urinary tract infections, septicemia and endocarditis. Hospital-acquired infections often present a challenge in treatment due to the emergence of multi-drug resistant strains. Enterococcal plasmids may act as extremely stable reservoirs for resistance genes and other virulence factors. Pheromone responsive plasmids such as pCF10 mediate efficient transfer of genetic material within the species *E. faecalis* but may also be capable of transferring resistance genes across species and genus boundaries. Polymicrobial environments often found in nosocomial infections may expose plasmid-harboring enterococci to pathogenic species, poisoning cells for this type of promiscuous horizontal gene transfer of resistance determinants.

Previous studies showed that *prgW*, which encodes the pCF10 replication initiation protein PrgW, is the minimal origin of replication for this plasmid. The replicon, which is usually limited to Enterococcal spp., can replicate in *Lactococcus lactis* if it is engineered to produce pre-cCF10. Three conserved cysteines (C78/C275/C307) are important for plasmid stability and allow for replication of the pCF10 replicon in *L. lactis* in the absence of pre-cCF10.

PrgW has a predicted molecular weight of 38,635. Four polyclonal antibodies targeting PrgW at the N-terminus (aa 1-20), C-terminus (aa 314-333) and two internal regions (aa 64-80 and aa 250-271) were used in current experiments and retrospective studies. When PrgW was overexpressed in *E. faecalis*, four different apparent

approximate molecular weights were detected by Western blotting (p40*, p36*, p24* and p18*), suggestive of processing. In Enterococci where the replicon is active, p36* was consistently detected by all four antisera; when PrgW was overexpressed in *Streptococcus mutans* where the replicon is non-functional, p49* and p40* were detected but p36* was not observed. PrgW p24* was detected by a mixture of the internally targeting antibodies as well as the C-terminal targeting antibody, but not the N-terminal targeting antibody, suggesting that the N-terminal domain of PrgW has been cleaved off in p24*. The p24* form may play a role in pCF10 stability. Mutations to three cysteines in PrgW (C78/C275/C307), which reduce the stability of pCF10, result in the loss of p24*.

Enterococcal conjugative plasmids have been previously implicated in the transfer of antibiotic resistance genes. The pCF10 plasmid contains the conjugative transposon Tn925, which possesses the *tetM* tetracycline resistance gene. Proximity of donor and recipient cells is a key part of pheromone-responsive conjugation. Aggregation substance allows for formation of clumps of *E. faecalis* in liquid mating experiments. *E. faecalis* forms biofilms; in contrast to filter mating experiments, polymicrobial biofilms provide an in vitro model of a natural scenario during which horizontal gene transfer may occur. Rates of cross-genus genetic transfer of *tetM* between *E. faecalis* OG1RF(pCF10) donor cells and *Staphylococcus aureus* recipient cells growing on glass coverslips as mixed-species biofilm populations were determined to be 10^{-8} after pheromone induction of pCF10 conjugation. This biofilm transfer model also holds potential to test the efficacy of synthetic peptides in the reduction or even prevention of pCF10 transfer, and the consequential dissemination of antibiotic resistance determinants throughout the genus *Enterococcus* and beyond.

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LIST OF ABBREVIATIONS

β ME	beta-mercaptoethanol
μ g	microgram
μ l	microliter
μ m	micrometer
μ M	micromolar
aa	amino acid
AS	aggregation substance
bp	base pairs
BSA	bovine serum albumin
C	Celsius
cCF10	pCF10 pheromone; CcfA ₁₃₋₁₉
CFU	colony forming unit
diH ₂ O	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
DR	direct repeats
dsDNA	double stranded DNA
EDTA	ethylenediamine tetra-acetic acid
EB	elution buffer

EtBr	ethidium bromide
EtOH	ethyl alcohol
kbp	kilobase pairs
L	liter
LB	Lysogeny Broth
M	molar
mg	milligram
min	minutes
ml	milliliter
mm	millimeter
mM	millimolar
NaOAc	sodium acetate
NIH	national institutes of health
nm	nanometer
OD	optical density
<i>ori</i>	origin of replication
<i>oriR</i>	origin of replication (plasmid)
<i>oriT</i>	origin of transfer
<i>oriV</i>	origin of vegetative replication (plasmid)
pCF10	pheromone-responsive plasmid from <i>E. faecalis</i> SF-7
PCR	polymerase chain reaction
PBS	phosphate buffered saline
pre-cCF10	cCF10 precursor; CcfA ₁₋₂₂

RE	restriction enzyme
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	seconds
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
Tween	polyoxyethylene sorbitan monostearate
WCL	whole cell lysate

CHAPTER 1

INTRODUCTION

Enterococci

Enterococci are Gram-positive bacteria that exist as part of the non-pathogenic gastrointestinal flora in mammals as well as birds and invertebrates. These bacteria are classified in the phylum Firmicutes and are lactic acid-producing facultative anaerobes. Previously Enterococci were assigned as Group D streptococci (Lancefield classification) but are now redefined as the separate genus *Enterococcus* (33). *Enterococcus faecalis* is one of the most frequently studied species within this genus and natively possess various plasmids including the pheromone-responsive plasmid pCF10.

Although these bacteria may exist as part of the non-harmful commensal flora in the gut, Enterococci have emerged as important hospital pathogens and can act as reservoirs for pathogenicity genes and antibiotic resistance determinants (28, 34). The transfer of conjugative plasmids results in genetic dissemination and holds dangerous potential to drive emergence of additional multi-drug resistant strains both within the species *Enterococcus* and even more alarmingly, across species and genus boundaries.

Horizontal Gene Transfer and Mobile Genetic Elements

Horizontal gene transfer of virulence factors in bacterial species has become an ever-increasing concern in the biomedical community (18, 28, 34). Enterococci play a major role in the dissemination of Mobile Genetic Elements (MGE) such as conjugative plasmids and transposons (13, 24). Non-conjugative plasmids and chromosomal genes

have also been demonstrated to move laterally throughout bacterial populations with the help of these conjugative elements (23). There are three types of Enterococcal plasmids that mediate horizontal gene transfer: (1) broad host range Rolling Circle Replicating plasmids (2) broad host range Inc18 plasmids, and (3) narrow host range pheromone-responsive plasmids (39). Broad host range plasmids are less stable, but may be easily transferred between multiple Gram-positive species. In contrast, Enterococcal pheromone-responsive plasmids are extremely stable (in more than 98% of cells over 150 generations) but are usually limited to a single native host-species (40).

Pheromone Responsive Conjugative Plasmids

Pheromone-responsive plasmids have previously been shown to transfer at rates as high as 10^{-1} between plasmid containing *E. faecalis* donor cells and *E. faecalis* plasmid-free recipient cells. Transfer of antibiotic resistance to other species has been reported to occur at lower rates. For example, *vanA* can be transferred to *E. faecium* at a rate of 10^{-8} in vitro using filter mating experiments (12). In these experiments, bacterial cells were physically mixed and concentrated on a membrane to ensure that donor and recipient cells were placed in close proximity. Cells grown in polymicrobial biofilms naturally occur in close proximity, and thus biofilm growth may also facilitate conjugative transfer between donor and recipient cells.

Transposons

Transposons such as the well-characterized Tn916 are another important type of MGE in Enterococci (31, 32). Conjugative transposons possess regions encoding excisionase and integrase enzymes that allow for genetic mobility. A Tn916 family member transposon, Tn925 is found on the plasmid pCF10. This transposon possesses a

tetracycline resistance determinant (*tetM*), which can be used to assess genetic transfer between mating bacterial cells such as *E. faecalis* OG1RF(pCF10) donor cells and plasmid-free recipient cells (which could be *E. faecalis* or species of other genera).

Pheromone Responsive Plasmid pCF10

Plasmids are autonomously replicating extra-chromosomal portions of DNA that may be transferrable between cells, and may harbor many different genes including virulence factors. Pheromone responsive plasmids are most commonly found in *E. faecalis*, which is known to harbor 25 described pheromone-responsive conjugative plasmids, including pCF10. The hallmark of a pheromone-responsive conjugative plasmid is that cell-cell signaling induces plasmid transfer. Plasmid-free recipient cells secrete peptide signals that are detected by plasmid-containing donor cells and this induces subsequent transfer of the plasmid to the recipient cell by conjugation. Each enterococcal pheromone-responsive plasmid has a cognate pheromone, which specifically induces its transfer. The plasmid pCF10 is one of the well-studied pheromone-responsive plasmids (15). This plasmid is transferred in response to the sex pheromone signal cCF10 (7, 9, 10). Pheromone is derived from a lipoprotein that is chromosomally encoded by *E. faecalis* by the *ccfA* gene (1). When expressed, the lipoprotein undergoes a series of proteolytic processing events to produce mature pheromone, cCF10 (1). Mature pheromone is recognized by the PrgZ receptor, which is expressed on the surface of donor cells, and pheromone is subsequently internalized via the oligopeptide permease system (7). Once internalized, the pheromone signal cCF10 binds to PrgX and relieves repression of the genes necessary for conjugative transfer. Chromosomally encoded pheromone is produced constitutively by all cells, both donor

and recipient. To prevent auto-induction of conjugation, the plasmid-encoded transmembrane protein PrgY reduces the levels of nascent membrane-associated cCF10 pheromone (8). In addition to this, the plasmid-encoded inhibitory peptide iCF10 competitively inhibits binding of cCF10 to both the PrgZ pheromone receptor and the transcriptional repressor PrgX (2, 15, 27). Due to the highly sensitive nature of this system, plasmid-bearing donor cells are able to prevent auto-induction of conjugation in the absence of plasmid-free recipient cells.

pCF10 Transfer: Type IV Secretion Systems

Bacterial conjugation involves the transfer of genetic material from a donor cell to a recipient cell. Once the transcription of pheromone responsive genes has been induced, (after relief of repression by PrgX) plasmid-harboring donor cells express *prgB*, resulting in production of Asc10, also known as aggregation substance, on the cell surface (6). Aggregation substance binds to “binding substance”, lipoteichoic acid, expressed on the surface of recipient cells leading to the formation of mating pairs. Induction of the conjugation machinery genes results in the expression of the accessory factor PcfF which functions to recruit PcfG to the *oriT* site where a single stranded nick is formed (11, 15). Together, PcfF and PcfG bind to nicked DNA forming the relaxosome, and mediate the mobilization of this DNA-protein complex to the cell membrane where it docks with the putative membrane bound ATPase, PcfC. A transfer mechanism involving PcfC, similar to other known type IV secretion systems, is then proposed to occur: PcfC mediates the transfer of the DNA-protein complex through a channel powered by a NTP-dependent mechanism (25). This effectively transports the plasmid DNA from donor to recipient

cells where DNA polymerase may then create double stranded DNA, producing a replicated copy of the original plasmid (11).

pCF10 Replication, Host Range and Stability

The replication initiation protein PrgW is important in mediating host range of pCF10 and plasmid maintenance after conjugation. When transferred between *E. faecalis* cells, pCF10 is very well maintained in its native host with greater than 95% of the cells maintaining the plasmid in the absence of selection for over 150 generations. However, the plasmid cannot replicate when transferred to other Gram-positive species such as *Lactococcus lactis* or *Streptococcus mutans*. In previous studies, our laboratory has found two novel features of the pCF10 replicon that contribute to replication and stability: (a) the pheromone precursor pre-cCF10 interacts with the pCF10 replication initiation protein PrgW. The host range of pCF10 can be extended to *Lactococcus lactis*, if *L. lactis* is engineered to produce pre-cCF10. (b) Three conserved cysteines (C78, C275 and C307) in PrgW contribute to stability. Substitution of all three cysteines with alanine decreases replicon stability in *E. faecalis*, but extends host range to *L. lactis* independent of pre-cCF10. Investigating the role of the cysteines and pre-cCF10 in plasmid replication may help to elucidate new mechanisms for plasmid replication control and host range restriction.

Biofilms

Enterococci form bacterial biofilms, which are populations of cells that exist within a complex matrix. *E. faecalis* matrix material consists of polysaccharides, proteins and eDNA (36, 37). Biofilms may contain heterologous sub-populations that

may differ in gene expression and consequently display varied phenotypes including antibiotic resistance. Biofilms may form on both biotic and abiotic surfaces and may occur in hospital-acquired infections on catheters or other in-dwelling devices (20, 21). Furthermore, the cells in these populations grow in close proximity; this may result in donor and recipient cells perfectly poised for horizontal gene transfer (29). For example, rates of horizontal gene transfer mediated by competence are significantly increased in *S. mutans* biofilms (30). In the gastrointestinal tract *E. faecalis* may be present in mixed species biofilms (22, 41).

Project Aims

Enterococcal plasmids may have broad host ranges with limited stability, or narrow host ranges with high stability. The enterococcal pheromone-responsive plasmid pCF10 is maintained with high stability within its native host *E. faecalis*, but cannot replicate in other genera. Previous studies have demonstrated that PrgW, the replication initiation protein of pCF10, interacts with pre-pheromone (pre-cCF10) and influences plasmid stability and host range. The goal of this project was to further examine PrgW protein and its function both as a replication initiation protein and as a factor influencing host range. Western blotting experiments on various PrgW clones in multiple bacterial species were performed to detect PrgW expressed under different conditions. PrgW protein was detected as multiple sized forms based on migration on SDS-PAGE, therefore a protein processing theory was proposed. To characterize the different processed forms observed, Western blotting and retrospective analysis of blots using multiple polyclonal antibodies was performed to elucidate a processing mechanism.

The other aim of this project was to develop a model to assess the transfer rate of the plasmid pCF10 from *E. faecalis* OG1RF(pCF10) donor cells to *S. aureus* recipient cells. Enterococci form biofilms on both biotic and abiotic surfaces (such as catheters or other in-dwelling devices) and as important nosocomial pathogens these bacteria have been implicated in horizontal gene transfer and dissemination of antibiotic resistance genes. A biofilm transfer model was developed to provide an in vitro model of a natural scenario in which this horizontal gene transfer might occur.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

All bacterial strains used in this study were maintained as freezer stocks in 30% glycerol/70% Todd Hewitt broth (TH) stored at -80°C. All *E. faecalis* clones were derived from the laboratory strain OG1RF. *E. faecalis* strains were grown in Todd Hewitt media (Appendix) at 37°C statically. *E. coli* DH5-alpha and XL1 Blue were used for cloning and BL21 was used for protein expression. *E. coli* strains were grown in Lysogeny Broth (LB) broth or Terrific Broth (TB) at 37°C, shaking at 200 rpm. *S. aureus* strains were grown at 37°C in TH media, statically. *S. mutans* cultures were grown in at 37°C in 5% CO₂. See Table 1 for bacterial strains used in this study.

DNA Isolation and Manipulations

Plasmid Isolation and Purification from E. coli

For plasmids used in this study see Table 2. *E. coli* DH5 α or *E. coli* XL1-Blue cultures were grown in 10 – 20 ml Lysogeny Broth (LB) with appropriate selection in Becton, Dickinson and Company Falcon™ 50 ml conical tubes or sterile 100 ml – 500 ml glass flasks. Cultures were incubated overnight for 12 – 18 hours at 37°C and 250 rpm.

Overnight cultures were centrifuged at 10,000 x g (9,000 rpm; Beckman J2-HS) for 10 min at 4°C to pellet cells. Supernatant was removed and discarded. Pelleted cells were used immediately to extract plasmid DNA or stored at -20°C for up to one week. Plasmid isolation was performed using the using Promega Corporation Wizard® Plus SV

Table 1. Bacterial Strains Used in This Study

Strain	Description	Reference
<i>E. coli</i> BL21	Protein expression host; F ⁻ <i>ompT hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm tonA</i>	Novagen
<i>E. coli</i> DH5α	Cloning host; [F ⁻ <i>endA1 hsdR17</i> (r _k ⁻ m _k ⁺) <i>supE44 thi-1 λ recA1 gyrA96 relA1 Δ(lacZYA-argF) U169 Φ80dlacZΔM15]</i>	Gibco BLR
<i>E. coli</i> XL1-Blue	Cloning host; <i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIq ZD M15 Tn 10(Tet r)]</i>	Agilent
<i>E. faecalis</i> OG1RF	Plasmid-free laboratory reference strain; Gel ⁺ , Spr ⁺ , Rif ^R , Fus ^R .	Dunny <i>et al.</i> , 1978 (16)
<i>E. hirae</i> 9790	Laboratory strain	ATCC 9790
<i>S. aureus</i>	Clinical isolate; Pen ^R	This study
<i>S. mutans</i> UA159	Plasmid-free laboratory strain.	(Murchison <i>et al.</i> , 1986)

Midipreps DNA Purification System kit according to the manufacturers instructions. The plasmid DNA was eluted from the column with 50 – 300 μ l of pre-heated (65°C to 85°C) sterile diH₂O. Elution volume depended on desired concentration of DNA and efficiency of extractions. Columns were incubated for 1 minute with water or Elution Buffer (EB) prior to centrifuging at 10,000x g for 30 seconds to elute DNA. The presence of plasmid DNA was confirmed by gel electrophoresis, ethidium bromide staining and visualization.

Plasmid Isolation and Purification from E. faecalis

Cultures of *E. faecalis* OG1RF were grown in TH plus 3% glycine. Samples were centrifuged for ten minutes at 7,000 x g to pellet cells. Pellets were washed once with 50 mM Tris 1 mM EDTA (TE_{50,1}) (pH 8.0) with 6.7% sucrose, then cells were resuspended in 379 μ l TE_{50,1} (pH 8.0) with 6.7% sucrose, and warmed to 37°C. Lysozyme was added (96.5 μ l of 5 mg/ml in 25 nM Tris pH 8) and samples were incubated at 37°C for five minutes. Next, 48.2 μ l TE_{50,250} (pH 8) was added, followed by 27.6 μ l 20% SDS in TE_{50,20} (pH 8.0) and samples were incubated for 5-10 minutes at 37°C. Samples were vortexed to mix well, and 27.6 μ l 3N NaOH was added; samples were mixed on a rotator for ten minutes at room temperature. Next, 49.6 μ l 2M Tris (pH 7) was added and samples were mixed (rotating) for three additional minutes. Plasmid DNA was extracted once with phenol, once with chloroform, and finally precipitated using 0.1 volume 3M sodium acetate (NaOAc) and 2 volumes ice-cold ethanol. If a visible precipitate formed, samples were incubated at -20°C for 1-2 hours (if no visible precipitate formed samples were incubated overnight) prior to centrifugation for 20 minutes at 4°C to harvest DNA. Supernatant was removed; pellet was washed in 70% ethanol, resuspended in 20 to 50 μ l sterile deionized water (diH₂O) and stored at -20°C prior to gel electrophoresis.

Purification of Chromosomal DNA from S. aureus

Cultures were grown in 20 ml of TH broth overnight at 37°C for 15-20 hours. Cells were pelleted by centrifugation at 10,000 x g for 10 minutes. Supernatant was discarded and cells were resuspended in bead beater lysis buffer (Appendix). Cells were transferred to bead beater tubes containing 1.5 g silica-zirconia beads. Samples were bead beat three times at a setting of 4800 for 1 minute followed by a 1-minute pause on ice. Whole cell lysates were transferred to clean microcentrifuge tubes. DNA was extracted with an equal volume of phenol pH 6.6 (Fischer Scientific). Samples were centrifuged at 16,000 x g for 20 minutes at 4°C to extract DNA. Supernatant was transferred to a fresh tube, avoiding the interface. An equal volume of phenol/chloroform/isoamyl alcohol 50:49:2 (pH 6.7, Fischer Scientific) was used to extract DNA from samples three times, followed by one extraction using chloroform/isoamyl alcohol (Fischer Scientific). Samples were centrifuged to separate the layers. DNA was precipitated with 0.1 volume 3M NaOAc pH 4.6 and 2 volumes of cold ethanol, and precipitated at -20°C for 2 hours to overnight. DNA was harvested by centrifugation at 16,000 x g for 20 minutes at 4°C. The pellet was washed with 70% ethanol and resuspended in sterile water (Nuclease-Free Water; Promega Corporation, Fitchburg, WI). Samples were treated with RNase (2 µl of 10 mg/ml RNase) for 30 minutes at 37°C. DNA was re-precipitated and harvested as outlined above with 0.1 volume 3M NaOAc and 2 volumes of cold ethanol, and centrifugation. Purified DNA was suspended in sterile water (Promega) and stored at -20°C.

Table 2. Plasmids Used in This Study

Plasmid	Description
pCF10	Pheromone-responsive to cCF10; Tn925 encodes <i>tetM</i> (Tet ^R); contains PrgW RepA_N (14).
pMSP3535	Gram-positive and Gram-negative shuttle vector for the selective induction of the cloned fragments when they are cloned downstream of the nisin inducible promoter P _{<i>nisA</i>} . It contains the genes encoding the nisin histidine kinase (<i>nisK</i>) and its response regulator (<i>nisR</i>). It also contains a gene that determines resistance to Erythromycin (5).
pORI10	A pCF10 fragment containing the genes <i>prgW</i> was PCR amplified using the primers mm10 and mm11 (Meloni, Dissertation; Table 2). This region was cloned in pGEM-T easy (Promega) and then digested with <i>EcoRI</i> . The digested fragment was then cloned in the <i>EcoRI</i> site of pVA891. The insertion of pCF10 DNA resulted in the inactivation of the Chloramphenicol cassette. The insert was cloned in the same orientation with respect to the CAT gene. (Meloni Dissertation, 2005)
pPRGW	The gene <i>prgW</i> containing its ribosomal binding site was PCR amplified using primers mm1 and mm2 (Meloni, Dissertation; Table 2). The fragment was digested with <i>BamHI</i> (5' of <i>prgW</i>) and <i>PstI</i> (3' of <i>prgW</i>). The insert was then cloned downstream of the nisin inducible promoter P _{<i>nisA</i>} in the <i>BamHI</i> and <i>PstI</i> sites in pMSP3535 (Meloni Dissertation, 2005)
pVA891	<i>E. coli</i> vector derivative of p15A. It contains a gene for Erythromycin resistance functional in both Gram-negative and Gram-positive bacteria. It also contains a gene that encodes a Chloramphenicol resistance determinant. This determinant can be inactivated by insertion of a DNA fragment in the <i>EcoRI</i> site.

DNA Manipulations: Agarose Gel Electrophoresis

Plasmid and PCR products were routinely visualized and size migration was determined by separating DNA using agarose gel electrophoresis. Fisher BioReagents Molecular Grade Low EEO/Multipurpose Agarose was dissolved in 1X TAE Buffer (Appendix). Agarose concentrations were determined by estimated size of DNA samples: 0.8% agarose was used for plasmids and 1.5% - 2% agarose was used for PCR products. Preparative agarose gels (0.7%) were used to separate digested DNA for gel extraction and subsequent cloning. Preparative gels were made with SeaKEM® GTG® Agarose (Lonza; Rockland, ME). Agarose was dissolved fully by microwaving in short intervals and then cooled to touch in a 55°C water bath. All gels were poured using FisherBiotech (Fair Lawn, NJ, USA) Mini or Midi-Horizontal Electrophoresis Systems with the respective FisherBiotech UVT gel tray. The appropriate comb was immediately placed into the appropriate slot of the gel tray and the gel was allowed to set. Once set, the gel and casting tray were removed and positioned so that the wells were closest to the negative (black) electrode. Enough TAE buffer (1X) was added to the electrophoresis system to submerge the top surface of the gel. The comb was removed carefully without tearing the wells. Promega Corporation Blue/Orange 6X Loading Dye (Promega Corporation) was mixed with 10 to 20 µl of sample DNA and wells were loaded with 10 to 20 µl of DNA-dye mixture. Ten microliters of Fisher BioReagents Routine DNA Ladder > 1kb Full Scale Ladder was also loaded onto gels to compare samples to DNA ladder standards. Gels were electrophoresed by applying 70 to 100 volts using a Fisher Biotech Electrophoresis systems power supply (Fisher Scientific) for approximately 45 minutes to 1.5 hours or until the dye front reached the bottom of the gel and the DNA

ladder was well resolved. Following electrophoresis, gels were stained for approximately 20 minutes in a 1X TAE bath containing 0.5 µg/ml Acros Ethidium Bromide and then imaged using UV Ultraviolet transilluminator light box or a Bio-Rad Molecular Imager GelDoc™ XR System according to manufacturer's protocol (Bio-Rad Laboratories; Hercules, CA).

DNA Extraction from Agarose Gel and Cleanup

Gels were minimally exposed to UV light on a light-box to minimize UV damage to DNA samples. Using a clean razor blade, the desired band was excised and transferred to a pre-weighed sterile microcentrifuge tube. The sample was weighed and the manufacturer's protocol for the QIAquick Gel Extraction Kit was followed (Qiagen).

DNA Concentration Determinations

A Spectronic GENESYS 5 spectrophotometer (Thermo Scientific) was used to determine concentrations of purified DNA samples by measuring the absorbance at OD₂₆₀. A NanoDrop™ Spectrophotometer ND-1000 (Thermo Fisher Scientific, Wilmington, DE) was also used to rapidly determine DNA concentrations using low amounts of sample DNA. This was done according to manufacturer's protocols.

Restriction Enzyme Digestions

Restriction enzyme digestions of DNA were performed on purified plasmids using New England Biolabs or Promega restriction endonucleases along with appropriate buffers. Restriction digests were performed according to Table 3 below.

Digested vectors and inserts were excised from agarose gels with clean razor blades and extracted using the Qiagen QIAquick Gel Extraction Kit (Qiagen) as outlined above and purified DNA was used for subsequent ligation and transformation steps.

Ligation

Ligation of purified vector and insert DNA was performed using Promega Corporation T4 DNA Ligase. Ligation mixtures of 1:1, 1:3, 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 insert: vector = ng insert

For most reactions 100 ng of vector was used, 50 ng of vector was sufficient if DNA concentration was low. Ligation mixture generally had a total volume of 15 μ l and included: 2X Rapid Ligation Buffer (Appendix), 1 μ l T4 DNA Ligase and Nuclease-free water. Reactions were performed in a Thermocycler 2400 with heated lid (Perkin-Elmer Corp., Foster City, CA) by incubating samples at 16°C for 16 hours prior to holding reactions at 4° until transformation reactions were performed.

Transformation

Chemically competent cells were thawed slowly on ice. Ligation mixtures were transferred to microcentrifuge tubes, and 50 μ l of thawed cells was transferred to each tube, including negative controls with no insert (vector control) or no DNA. Reactions were incubated on ice for 20 minutes. Cells were submerged in a 42°C water bath to heat shock for 1 minute and then immediately transferred back onto ice for 2 minutes. Cells were rescued by adding 500 μ l broth media to sample tubes. Samples were incubated at

Table 3. Restriction Enzyme Digestion Conditions

Restriction Enzyme	Restriction site	Manufacturer	Catalog #	Buffer	BSA	Temp	Heat Shock
<i>Bam</i> HI	G/GATCC	Promega	R6021	BufferE	100 µg/ml	37°C	65°C
<i>Eco</i> RI	G/AATTC	NE Biolabs	R0101S	Buffer 4	N/A	37°C	65°C
<i>Sal</i> I	G/TCGAC	NE Biolabs	R0138S	Buffer 3	100 µg /ml	37°C	65°C
<i>Sma</i> I	CCC GGG*	NE Biolabs	R0141S	Buffer 4	N/A	25°C	65°C
<i>Sph</i> I	GCATG/C	NE Biolabs	R0182S	Buffer 2	N/A	37°C	65°C
<i>Xba</i> I	T/CTAGA	NE Biolabs	R6181	Buffer 4	100 µg /ml	37°C	65°C
<i>Xma</i> I	C/CCGGG	NE Biolabs	R0180S	Buffer 4	100 µg /ml	37°C	65°C

* *Sma*I is a blunt end cutter. All other restriction endonucleases produce cohesive ends.

37°C for one hour, shaking at 200 rpm. Cells were plated onto selective media and incubated overnight at 37°C. Positive clones were patched onto selective agar plates, and then grown in broth and confirmed by plasmid extraction, gel electrophoresis, PCR and restriction digestion.

Electroporation of Electrocompetent E. faecalis

Electrocompetent *E. faecalis* OG1RF cells were prepared using the glycine gradient method (17) by incubating cultures in TH with varying concentrations of glycine (Appendix). Cells were centrifuged for 10 minutes to form a pellet, and supernatant was discarded. Pelleted cells were washed with one-third volume of Electroporation Buffer (Appendix), and centrifuged for 10 additional minutes. Supernatant was drained and cells were resuspended in 1/50 volume electroporation buffer. Cells were stored at -80°C prior to use. Electrocompetent *E. faecalis* OG1RF cells were thawed slowly on ice. Cells were centrifuged to create a pellet and then washed three times using 100 µl sterile ice cold water. Electrocompetent cells were then resuspended in fresh ice-cold sterile water. Purified DNA (250 to 1000 ng plasmid DNA) was added to samples for a working volume of 100 µl. Bacteria-DNA mixtures were transferred to pre-chilled electroporation cuvettes using the Bio-Rad Gene Pulser electroporator settings of 1,800 V, 25 µF, and 200 Ω. Time constants were recorded (these varied between 2.3 and 3.2). After electroporation, samples were resuspended in TH with 0.25 M sucrose and incubated at 37°C to rescue cells. Electroporated samples (100 µl) were plated onto TH agar with appropriate antibiotic and grown at 37°C overnight. Positive clones were patched onto selective agar plates, and then grown in broth and confirmed by plasmid extraction, gel electrophoresis, PCR and restriction digestion.

Primer Design and Sequencing

Primers were routinely designed using the IDT TechnologiesTM website and SerialCloner 2-5TM. Lyophilized primers were reconstituted to final concentrations of 50 μM – 100 μM to make primer stocks. Working stock solutions (1:10 dilutions) were subsequently created for use in PCR and sequencing reactions. For primers used in this study see Table 4. Sequence data were routinely obtained from Genewiz, Inc. Samples for sequencing were prepared following appropriate Genewiz, Inc. guidelines (Table 5).

Polymerase Chain Reaction

Polymerase Chain Reactions were performed for amplification of target genes. Reactions were set up on ice using 0.25 to 1 μl Promega Corporation GoTaq polymerase; 10 μl Promega Corporation GoTaq 5X colorless Buffer; 2.5 μl of dNPTs (10 mM); 2.5 μl forward primer, 2.5 μl reverse primer. Reactions were run using a Thermocycler 2400 with heated lid (Perkin-Elmer Corp., Foster City, CA) as outlined below (Table 6).

For PCR DNA cleanup, the Qiagen PCR cleanup kit was used according to manufacturer's protocols. Five volumes of Buffer PB was added to 1 volume of PCR sample and mixed by vortexing. To bind DNA, samples were transferred to QIAquick spin columns in 2 ml collection tubes and centrifuged for 30 to 60 seconds at 10,000 x g. Flow-through was discarded and columns were returned to empty collection tubes. To wash, 0.75 ml of Buffer PE was added to each column and samples were spun down for 30-60 seconds at 10,000 x g. Flow-through was again discarded and columns were returned to collection tubes. Columns were centrifuged an additional 1 minute at

Table 4. Primers Used in This Study

Template	Primer	Sequence 5'→3'
<i>tetM</i>	ems001 ems002	5' – CCAGTATCCGGAGAATATCTGTAT – 3' 5' – CCCAGTTTATAATAACTATCTCCTCC – 3'
Universal Eubacterial 16S primers		
16S rRNA	EubA EubB	5'–AAGGAGGTGATCCACCCACA – 3' 5'–AGAGTTTGATCCTGGCTCAG – 3'

Table 5. DNA and Primer Formulations for Sequencing with Genewiz, Inc.

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

maximum speed to remove residual ethanol. Next, QIAquick columns were transferred to clean, labeled 1.5 ml microcentrifuge tubes. To elute DNA, 50 to 100 μ l of sterile water or Elution Buffer (10mM Tris-Cl, pH 8.5; Qiagen) was placed on the center of the QIAquick membrane and columns were centrifuged for 1 minute at 10,000 x g. Purified PCR product was stored at -20°C prior to use in DNA sequencing or gel electrophoresis.

Protein Manipulations

SDS PAGE Gel Preparation

To prepare SDS-PAGE gels, two glass plates were cleaned carefully with ethanol and Kimwipes (Kimberly-Clark; Irving, TX, USA). Two gel casting spacers (0.75 mm) were cleaned with ethanol and aligned between the glass plates. Spacers and plates were secured evenly with two side clamps (the small gel assembly also required a rubber gasket). Plates were clipped onto the appropriate casting stand. The resolving gel was prepared first (Appendix) using FisherBioReagents[®] (Fair Lawn, NJ, USA) 40% Acrylamide Solution, Electrophoresis Grade, 10% to 20% Acros Ammonium Persulfate according to Table 7. Thermo Scientific (Rockford, IL, USA) TEMED was added immediately prior to pouring between glass plates to allow gel to polymerize.

A 10 ml pipette with a 100 μ l pipette tip was used to transfer approximately 13 ml of the resolving gel mixture between the two glass gel plates for a 0.75 mm comb, leaving an approximately 1 cm space between the top of the resolving gel and the predicted bottom of the stacking gel wells. One milliliter of sterile H₂O was carefully added on top of resolving gel from the sides using a syringe to prevent drying. The gel was allowed to set for 30 minutes to 1 hour. After the gel polymerized, water was

Table 6. Polymerase Chain Reaction Conditions with GoTaq Polymerase

Step #	Temperature	Time
1	95°C	2 min
2	95°C	30 sec
3	5° C below T _m	30 sec
4	72°C	1 min per 1000 bp
5	Repeat steps 2-4	30-40X
6	72°C	7-10 min
7	4°C	∞

carefully drained off the top of the gel. The stacking gel was transferred between the two glass gel plates and filled to the top. The appropriate gel comb (0.75 mm) was inserted onto the top of the gel and the stacking gel was allowed to polymerize. After gel polymerization the gel comb was removed. Gels were inserted into the appropriate electrophoresis units and the units were filled with 1X SDS-PAGE running buffer. Prepared samples were loaded into wells, GE Healthcare Amersham Rainbow Molecular Weight Marker Full Scale (Piscataway, NJ, USA) (10 μ l for Coomassie stains and 5 μ l for Western blotting) was loaded as molecular weight standards, and 20 μ l of 1X SDS-PAGE loading dye was loaded into all empty wells. The Amersham Biosciences Electrophoresis Power Supply was used to apply a current of 100 V to the small gels for two hours or until the dye front reached the bottom of the gel. Large gels were electrophoresed at 50 V for a total of 14 hours or the time needed to allow the loading dye front to reach the bottom of the gel.

Coomassie Staining

Following electrophoresis, SDS-PAGE gels were stained with Coomassie to visualize protein. Gels were added to glass trays with enough Coomassie stain (Appendix) to cover the gel. Gels were stained for approximately 1 hour to overnight, rocking at room temperature. After staining, Coomassie was drained and gels were incubated rocking at room temperature using Coomassie De-stain Solution (Appendix) for approximately 2 hours (or until bands were visible) with a few bunched KimwipesTM added to the corner of the glass tray to absorb excess dye.

Table 7. Resolving and Stacking Gel Formulation

Acrylamide (%)	40% acrylamide; 0.8 bisacrylamide (ml)	4X Resolving or Stacking buffer (ml)	H₂O (ml)	10% Ammonium Persulfate (μl)	TEMED (μl)
Stacking					
N/A	1.125	2.5	6.175	100	10
Resolving					
12	4.5	3.75	6.75	50	10

Semi-Dry Blot Transfer Procedure

Transfer stacks were prepared on a Fisher Scientific (Fair Lawn, NJ, USA) Semidry Electrobloetter. WhatmanTM (Kent, UK) Chromatography Paper 3MM CHR blot sheets were pre-soaked in Towbin Buffer (Appendix) and added to the transfer stack. The gel was flipped horizontally 180° so that the molecular weight standards were on the right side, and placed onto the chromatography paper. Next, a pre-sized GE Healthcare (Buckinghamshire, UK) AmershamTM Hybond ECL Nitrocellulose Membrane was added to the transfer stack, keeping the layers wet with 2 to 3 ml of Towbin buffer (Appendix). A pipette was routinely used to flatten the transfer stack to remove air bubbles. Finally, three sheets of pre-soaked chromatography paper were added to the top. The transfer stack was secured in the semi-dry blot apparatus and transfer was effected using “Program 3” on the Amersham Biosciences Electrophoresis Power Supply. Voltage was set at 10V, current at 400 mA (constant) and wattage to 10 watts; transfers were electrophoresed for 1 hour. Following transfer, the blot was immediately transferred to Blocking Buffer (Appendix) and gel and chromatography papers were discarded. The semi-dry blot apparatus was cleaned with diH₂O and KimwipesTM to remove salt precipitate.

Western Blotting

A nitrocellulose membrane was placed in a clean tray with pre-filtered Blocking Buffer (Appendix). Blocking buffer was rocked at room temperature for a minimum of 30 minutes to remove residual milk aggregates prior to adding blots. Blots were blocked at room temperature for at least 2 hours or if needed blots were stored in Blocking Buffer overnight at 4°C. Membranes were rocked gently at medium speed for all room

temperature incubations. Following blocking, blots were rinsed twice with 50 ml 1X TBST Wash Buffer (Appendix). Primary antibody incubations were performed using anti-PrgW_N terminal and anti-PrgW_C terminal antibodies at ratios of 1:2000 or 1:4000 (Appendix). Blots were incubated in primary antibody statically overnight at 4° for optimal primary antibody binding (specifically for improving sensitivity of anti-PrgW_N). Additionally, polyclonal antibodies directed against synthetic peptides (1) YFIYTVAELMTLLNCRE, PrgW amino acid residues 64 to 80 and (2) RAKTKVEKEYNVVLIGEDYQEE, PrgW amino acid residues 250 to 271 were also used to detect PrgW at ratios of 1:200 and 1:500. Following primary antibody incubations, blots were rinsed twice in 50 ml Wash Buffer and washed rocking at room temperature for 5-minute intervals three times. Secondary antibody solution (Goat anti-Rabbit IgG; Appendix) was prepared in a ratio of 1:15,000 and blots were incubated in PerfectWestern™ dark boxes (GenHunter Corp., Nashville, TN) with secondary antibody for one hour, rocking at room temperature. Blots were rinsed twice in 50 ml 1X TBST Wash Buffer and then washed for 5 minute intervals, three times, rocking at room temperature. Blots were imaged using the Odyssey® Classic Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) according to manufacturer's protocol. Blots were imaged using the 700 nm channel at high quality, with intensity ranging from 3.0 to 5.0 depending on the intensity of standard bands in the preview.

Size Migration Calculations with Polynomial Equations

Polynomial equations of molecular weight standards were calculated using Microsoft Excel™ by plotting distance migrated (in centimeters) divided by total gel length (X) versus molecular sizes (Y) on a marked scatter line. A polynomial trend-line

was added and the equation was shown on the chart by selecting this option under the “trend-line options” menu. The same migration calculations were performed for bands seen on blots by dividing distance migrated (in centimeters) by the total length of the gel. Using the polynomial equation given by the molecular weight standard curve, apparent approximate molecular weights of the different bands detected on blots were calculated. All bands were measured at the apparent vertical center. Minor discrepancies in the sizes may be explained by inconsistencies in the SDS-PAGE runs, including curvature of samples as they migrated through the center wells more rapidly than samples in the exterior wells.

Protein Purifications

Growth Conditions for WCL Preparation

Bacteria were grown under appropriate antibiotic selection overnight in 5-10 ml broth. One or two milliliter portions of overnight culture were used to inoculate 100 ml or 200 ml of fresh broth media containing appropriate antibiotic. Bacteria were incubated and grown to mid-exponential phase (OD of 0.5 to 0.8); this was detected by using OD₆₀₀ for *E. coli* cultures or OD₆₆₀ measurements for *E. faecalis*. For pMSP3535 vectors requiring induction, 25 ng/ml Nisin was added (Sigma-Aldrich Nisin) to cultures and cells were induced for 2 hours.

Whole Cell Lysate Preparations

Cells were harvested by centrifugation and concentrated in 2 ml Lysis Buffer (Appendix) to achieve 20X, 40X or 100X samples; negative controls were concentrated to 100X. Cells were transferred to bead beater tubes containing 1.5 g silica-zirconia

beads and bead beat at a setting of 4800 for 1-minute intervals, followed by 1-minute pauses. This was repeated three times keeping cells on ice during pauses. Whole cell lysates were transferred to new tubes and stored in 60 to 80 μ l volumes to be stored at -20° for up to a week prior to SDS-PAGE electrophoresis. Samples were combined with 20% β ME and 5X Protein Loading dye and boiled for 5 minutes to denature protein immediately prior to loading onto SDS-PAGE. For protein purifications, whole cell lysates were centrifuged, supernatants were filtered with 0.45 μ M filters (Millipore) and samples were used for fast protein liquid chromatography purification by the ÄKTA machine (Amersham Pharmacia Biotech Uppsala, Sweden). Histidine-tagged PrgW was passed through a Ni^{2+} charged HiTrap column (Amersham Pharmacia Biotech, Uppsala Sweden). Recombinant protein was bound by the his-tag to the column and was subsequently washed to remove non-specific proteins from whole cell lysate samples. His-PrgW was finally eluted from columns using a linear gradient of imidazole (10 mM to 500 mM) to obtain purified his-tagged PrgW protein. Sample fractions were stored at -20°C prior to running on SDS-PAGE.

Biofilms

Polymicrobial Biofilm Formation

Biofilms were grown on borosilicate glass coverslips (12 mm diameter) (Fisher Scientific, Pittsburgh, PA;) in sterile polystyrene multi-well plates (Becton, Dickinson and Company Falcon[®]; Franklin Lakes, NJ). Coverslips were soaked in ethanol, flame sterilized and aseptically transferred with forceps to 24-well plates. Two milliliters of TH was added to each well containing one coverslip. Wells were inoculated with 80 μ l of overnight broth culture of *E. faecalis* OG1RF (pCF10) or *S. aureus* (clinical isolate;

Pen^R) or a mixture of both to form both single-species control biofilms and polymicrobial experimental biofilms. The 24-well plate was incubated 24 hours at 37°C. Staggered-growth biofilms were also prepared by growing single species *S. aureus* biofilms as described above for 48 hours to establish recipient cell populations prior to inoculating experimental wells with *E. faecalis* OG1RF(pCF10) plasmid-donor cells. Polymicrobial biofilms were incubated for 24 additional hours after the addition of *E. faecalis* OG1RF(pCF10).

Harvesting of Biofilms Cells by Sonication

Media supernatants containing planktonic cells were removed from wells and discarded. Coverslips containing biofilms were gently washed three times with sterile PBS and aseptically transferred to 15 ml polypropylene tubes (Becton, Dickinson and Company FalconTM) containing 2 ml sterile 1X PBS and placed on ice. Each tube was vortexed and subsequently sonicated on ice for 20 seconds at 60% amplitude with a Sonic Dismembrator Model 500 (Fisher Scientific, Pittsburgh, PA). Sonicated samples were serially diluted in sterile PBS and plated on selective TH agar. Plates were incubated at 37°C overnight. Colonies were counted and reported as CFU/coverslip, and suspected transconjugants were tested for horizontal gene transfer by chromosomal DNA extraction followed by PCR targeting the *tetM* gene. See Table 4 for Primers used in this study and Table 6 for PCR conditions.

CHAPTER 3

RESULTS

PrgW Processing

PrgW Can Be Detected As Discrete Bands on Western Blots

PrgW is the replication initiation protein of pCF10. This protein contains 333 amino acid residues and has a predicted molecular weight of 38,635. Antibodies directed to PrgW aa 64-80 and aa 250-271 have been used previously in the laboratory for Western Blotting (Figure 1). It appeared from these blots that PrgW might be processed. A retrospective analysis of blots generated by B. Utter and M. Meloni was performed. The apparent approximate molecular weight of each band was determined. The migration distance for each band of stained molecular weight markers was measured and used to generate a standard curve. The best-fit line for the standard curves was a quadratic equation. The apparent approximate molecular weight of each sample band was calculated based on the standard curve generated from the molecular weight markers on the gel. The gels included whole cell lysates of *E. faecalis* OG1RF(pCF10) and *E. faecalis* OG1RF(pPRGW) – *prgW* cloned into pMSP3535 under control of a nisin-inducible promoter (Figure 2). In Western blots of whole cell lysates from *E. faecalis* OG1RF(pCF10) there were two detectable bands: one with an apparent approximate molecular weight ranging from 34,000 – 36,000 which was designated p36*, and one with an apparent approximate molecular weight of 24,000 which was designated p24* (Figure 2). When PrgW was overexpressed in *E. faecalis* OG1RF(pPRGW) by inducing *prgW* with nisin, detectable bands were observed as bands with apparent approximate molecular weights ranging from 38,000 – 43,000, 34,000 – 37,000 and 21,000 – 26,000

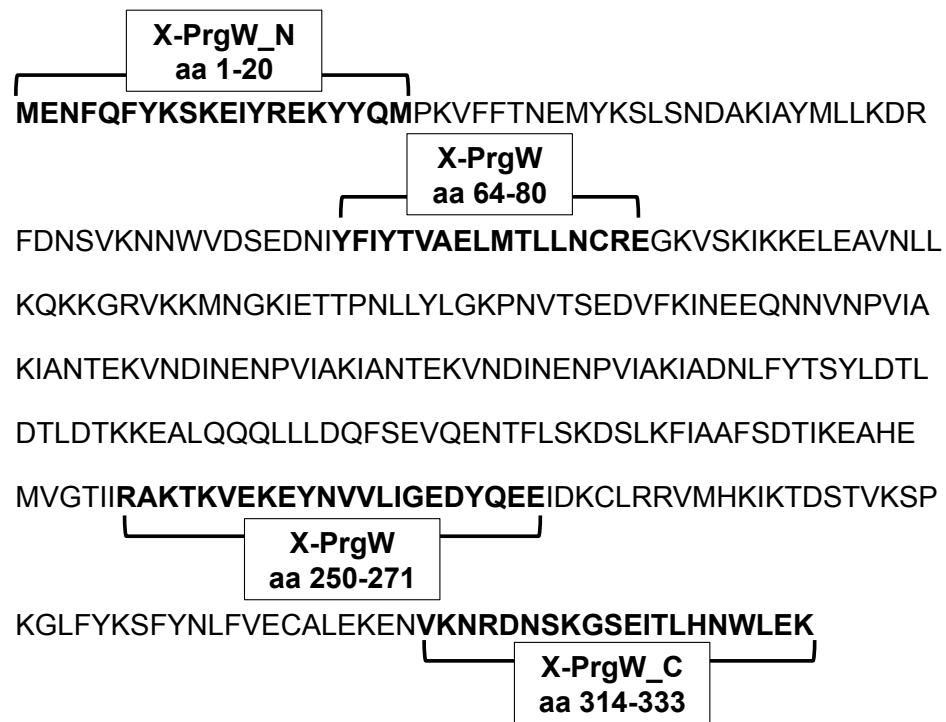


Figure 1. PrgW Protein with Antibody Binding Sites

PrgW contains 333 amino acid residues. Polyclonal antibodies were derived from synthetic peptides to target both the N and C termini (encompassing aa 1-20 and aa 314-333, respectively) as well as two internal fragments spanning aa 64-80 and aa 250-271.

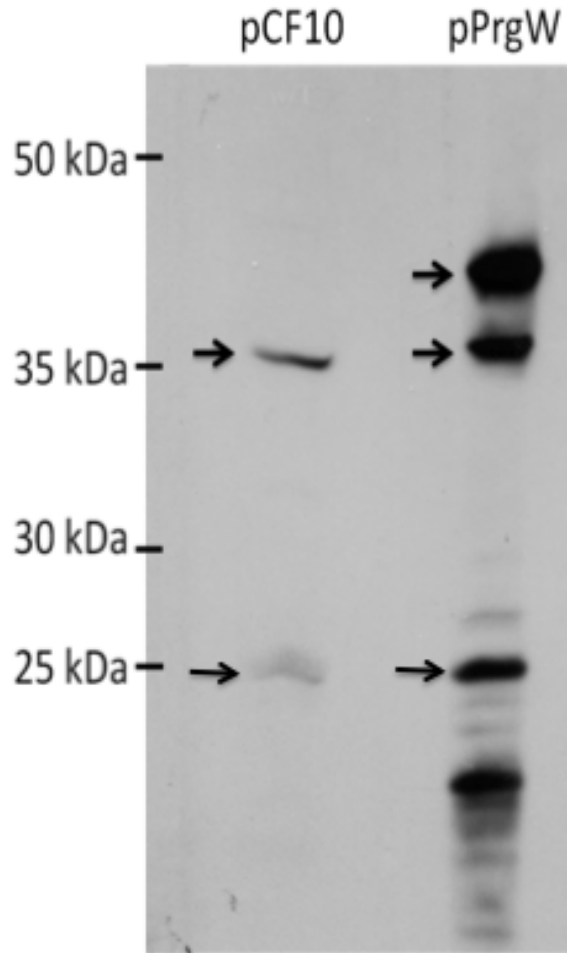


Figure 2. PrgW Can Be Detected as Discrete Bands p40*, p36*, p24* and p18*

Overnight cultures of *E. faecalis* OG1RF containing either pCF10 or pPRGW were diluted 1:1000 and grown to mid-exponential phase (O.D.₆₆₀ = 0.5 to 0.6). Whole cell extracts were prepared from cells concentrated 100X and analyzed by SDS-PAGE (12 percent), followed by Western blotting using polyclonal anti-PrgW antibody targeting aa 64-80 and aa 250-271. Arrows indicated PrgW bands within calculable range. The result shown was representative of three independent experiments. (B. Utter, Dissertation).

which have been designated p40*, p36*, and p24*, respectively. A band with an apparent approximate molecular weight of 18,000 was seen upon visual inspection, but was out of calculable range of the molecular weight markers. This band was designated p18* and approximations of its molecular weight ranged from 18,000 – 22,000.

p36 Correlates with Replicon Activity*

The pCF10 replicon is active in *Enterococcus spp.*, but non-functional in *S. mutans*. Further retrospective analysis was performed on Western blots of multiple PrgW clones to examine trends in replicon activity (Figure 3). Clones where the replicon was active included pORI10 (containing the minimal origin of replication *prgW* under control of the erythromycin promoter) in *E. faecalis* OG1RF, pORI10 in *E. hirae*, and pPRGW in *E. faecalis* OG1RF. In *S. mutans* where the replicon is non-functional, *prgW* was expressed by utilization of the nisin-inducible pMSP3535 expression vector (pPRGW). Retrospective analysis of Western blots demonstrated a correlation between the presence of p36* and an active replicon. In Western blots of whole cell lysates of clones in which the replicon was active, including pORI10 expressed in *E. faecalis* and *E. hirae*, and pPRGW expressed in *E. faecalis*, PrgW was detected with an apparent approximate molecular weight of 37,000 (p36*). When pPRGW was expressed in *S. mutans* where the replicon is non-functional, PrgW was detected with apparent approximate molecular weights of 49,000 (p49*) and 43,000 (p40*); p36* was not detected.

p40 and p36* Contain Both the N-terminus and C-terminus of PrgW*

Previously discussed retrospective analyses suggested that PrgW may be processed. Polyclonal antibodies targeting the N and C termini of PrgW were produced against peptides containing residues 1-20 and 314-333 and used to characterize the

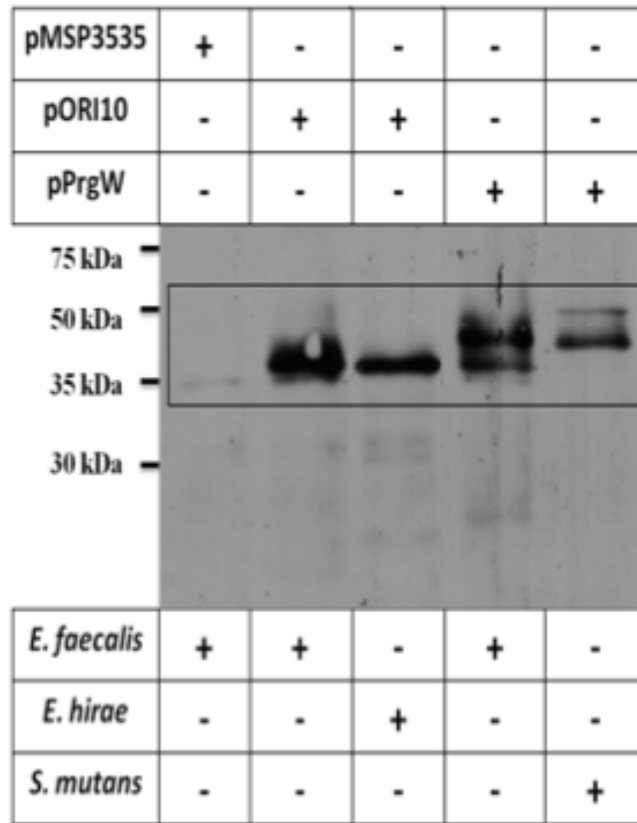


Figure 3. Presence of p36* Correlates with Replicon Activity

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 0.6). Three different plasmids pORI10, pPRGW, and pMSP3535 (negative control) were analyzed. Whole cell extracts were prepared from cells concentrated 10X and analyzed by SDS-PAGE (12 percent), followed by Western blotting using polyclonal anti-PrgW antibody targeting aa 64-80 and aa 250-271. The result shown was representative of three independent experiments. (B. Utter, Dissertation).

presence of the termini in the different forms observed (Figure 1). Expression levels of protein varied in different clones, and thus varying concentrations of whole cell lysates were used for SDS-PAGE and Western blotting. In samples from *E. faecalis* OG1RF(pCF10), whole cell lysates were prepared from cells concentrated 100X to allow for detection of the protein on blots. Lysates of *E. faecalis* OG1RF(pORI10) were concentrated 40X and lysates of *E. faecalis* (pPRGW) were concentrated 20X. Whole cell lysates of *E. faecalis* OG1RF used as negative controls were prepared from cells concentrated 100X.

The anti-PrgW_N antibodies recognized p40* and p36* with apparent approximate molecular weights ranging from 38,000 – 39,000 and 34,000 – 35,000 respectively (Figure 4a). The anti-PrgW_C antibodies recognized p40*, p36* and p24* (Figure 4b). These bands ranged from 38,000 – 39,000, 34,000 – 35,000 and 21,000 – 22,000 respectively. Consistent with previous observations, p40* was only detected when PrgW was overexpressed. PrgW p24* was not detected in *E. faecalis* OG1RF(pORI10) by either antibody, probably because of low abundance, but was detected in *E. faecalis* OG1RF(pPRGW) by the C-terminally targeting antibody. PrgW p18* was not recognized by either terminally targeting antibody. The N-terminal antibody also recognized a host protein in whole cell lysates of *E. faecalis* OG1RF negative controls with an apparent, approximate molecular weight ranging from 35,000 – 37,000. This protein was designated H36*.

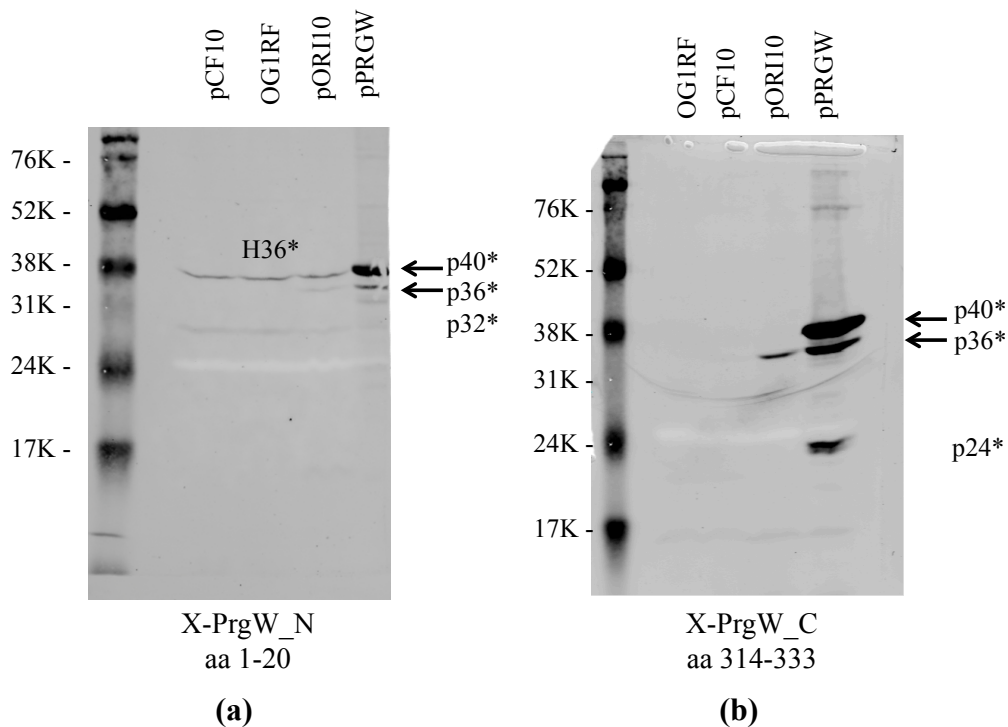


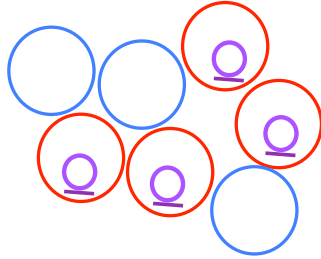
Figure 4. p40* and p36* Contain Both the N- and C- termini of PrgW

Western blots were performed on whole cell lysates of *E. faecalis* OG1RF, *E. faecalis* OG1RF(pCF10), *E. faecalis* OG1RF(pORI10), and *E. faecalis* OG1RF(pPRGW). Lysates were concentrated 100X for both *E. faecalis* OG1RF and *E. faecalis* OG1RF(pCF10), 40X for *E. faecalis* OG1RF(pORI10), and 20X for *E. faecalis* OG1RF(pPRGW). The binding of the primary antibodies was visualized by use of UV-600 nm fluorescently labeled secondary rabbit antisera and Odyssey scanning. (a) Primary antibody was directed against the N terminus of PrgW (aa 1-20); (b) Primary antibody was directed against the C terminus of PrgW (aa 314-333). Results are representative of two independent experiments.

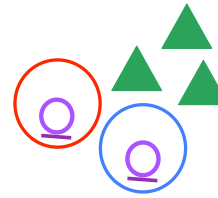
Tetracycline Resistance Determinant (*tetM*)
Transfer from *E. faecalis* to *S. aureus* in Biofilms

The biofilm transfer model was designed to examine horizontal gene transfer of the tetracycline resistance marker *tetM* from *E. faecalis* OG1RF(pCF10) donor cells to a clinical isolate of *S. aureus* (Figure 5). Multispecies biofilms were established by co-inoculation of static biofilms with a penicillin-resistant *S. aureus* clinical isolate and *E. faecalis* OG1RF(pCF10) in a ratio of 100:1. After 1 day of co-incubation, conjugation in biofilms was induced with synthetic cCF10 for 4 hours, and cells were harvested by sonication. Samples were plated on TH_{pen} and TH_{rif} for enumeration of *S. aureus* and *E. faecalis*, respectively. The remaining biofilm culture was plated on TH_{pen/tet} for selection of putative transconjugants. *S. aureus* and *E. faecalis* were present in approximately equal numbers (2.8×10^7 and 2.7×10^7 , respectively). In 2 out of 18 experiments, 6 putative transconjugants were isolated. Chromosomal DNA was extracted from putative transconjugants and *tetM* targeting primers (ems001, ems002) were used for PCR reactions to amplify the *tetM*. The *tetM* gene (2.2 kbp) was detected in four samples (Figure 6). Chromosomal DNA from biofilm transconjugant samples was also used as template for sequencing of 16S ribosomal RNA (1550 bp) and PCR products were sequenced (Figure 7). Biofilm Transconjugant 1, which was positive for *tetM* (Figure 6) was confirmed to be *S. aureus* by sequencing of the resulting PCR product generated using universal 16S eubacterial ribosomal RNA primers EubA and EubB (Table 8). The transfer frequency was calculated to be 10^{-8} .

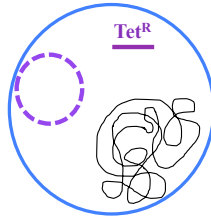
1. Grow mixed-species biofilms *E. faecalis* OG1RF (Rif^R) containing pCF10 (Tet^R) with *S. aureus* (Pen^R).



2. Synthetic cCF10 is added to induce conjugation of pCF10



3. pCF10 cannot replicate in *S. aureus* so Tet^R determinant *tetM* moves to *S. aureus* chromosome



4. Movement of *tetM* to *S. aureus* can be detected by plating on Penicillin (*S. aureus*) and Tetracycline and PCR

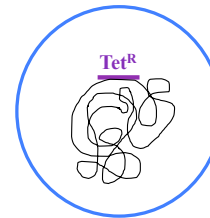


Figure 5: Biofilm Transfer Model Overview

An in vitro model was designed to examine horizontal gene transfer rates between *E. faecalis* OG1RF(pCF10) donor cells and *S. aureus* recipient cells grown in polymicrobial biofilms. Multi-species biofilms were grown on glass coverslips in 24-well plates. Biofilms were induced with synthetic cCF10 pheromone for four hours prior to sonicating and selective plating. Potential transconjugants were screened for the *tetM* gene by PCR.

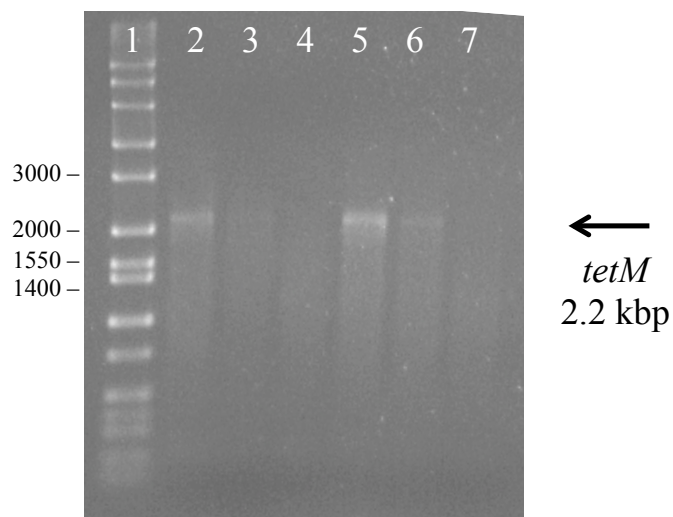


Figure 6. Polymerase Chain Reaction Detects *tetM* Gene in Transconjugants

Polymerase chain reaction (PCR) using *tetM* directed primers *ems001* and *ems002* detected the *tetM* gene in transconjugants (Lanes 2-7) from multi-species biofilms. Agarose gel (2%) electrophoresis of PCR product demonstrated bands at approximately 2.2 kbp, consistent with the size of *tetM*.

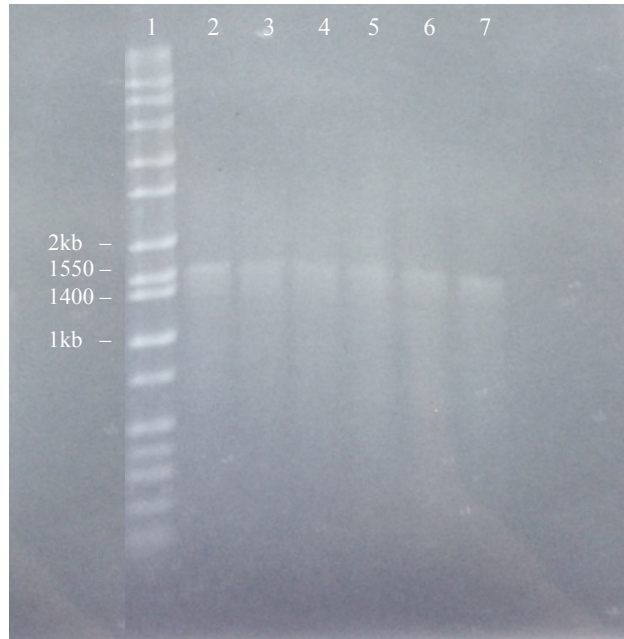


Figure 7. Polymerase Chain Reaction Detects 16S Eubacterial Ribosomal RNA in Biofilm Transconjugants

Universal EubA and EubB primers were used to amplify the 16S ribosomal RNA gene in six biofilm transconjugants. Agarose (2%) gel electrophoresis demonstrated PCR product as a band at approximately 1550 bp, consistent with the size of the 16S ribosomal RNA gene (Lanes 2-7). Samples were used for DNA sequencing.

Table 8. 16S ribosomal RNA Sequencing Confirms Transconjugant is *S. aureus*

	EubA sequence BLAST	EubB sequence BLAST
Biofilm transconjugant 1	<i>S. aureus</i>	<i>S. aureus</i>
Biofilm transconjugant 2	<i>E. faecalis</i>	<i>E. faecalis</i>
Biofilm transconjugant 3	<i>E. faecalis</i>	<i>E. faecalis</i>
Biofilm transconjugant 4	<i>E. faecalis</i>	<i>E. faecalis</i>
Biofilm transconjugant 5	<i>E. faecalis</i>	<i>E. faecalis</i>
Biofilm transconjugant 6	<i>E. faecalis</i>	<i>E. faecalis</i>

EubA/EubB PCR product was sequenced and sequences were analyzed using the BLAST feature from NCBI to determine bacterial species based on 16S rRNA.

CHAPTER 4

DISCUSSION

PrgW Is Processed in *E. faecalis*

PrgW protein was detected by polyclonal antibodies at multiple apparent approximate molecular weights; the various forms are characterized into designations p49*, p40*, p36*, p32*, p24* and p18* (Table 9). The various banding patterns were repeatable and were detected in at least three blots done by three independent investigators over the course of six years using mixed antibodies directed to internal epitopes of PrgW (aa 64-80 and aa 250-271) and all except p49* and p18* were detected in the current studies using newly generated antibodies directed to the N- and C- termini (aa 1-20 and aa 314-333).

Expression Levels of PrgW Influence Number of Bands Detected

PrgW expression levels varied in different clones, influencing the number of bands that were detectable by Western blotting (Table 10). When the native plasmid pCF10 was expressed in its natural host *E. faecalis*, the major stable bands observed were p36* and p24* and lysates had to be concentrated 100X to detect the protein. In pCF10, *prgW* transcription starts at a promoter 1023 bp upstream of the transcription start site. Transcription of *prgW* is down regulated by the binding of the partitioning protein PrgO to *par*, which lies between the promoter and *prgW* coding sequence (Izquierdo, unpublished observations). In pORI10, *prgW* is transcribed from the erythromycin promoter and, while its expression is stronger, p36* and p24* were still the only detectable bands. If PrgW was expressed in *S. mutans*, p49* was observed; this form was

Table 9: Apparent Approximate Molecular Weights of PrgW Expressed in Different Clones Calculated from Multiple Western Blots.

PrgW Form	X-PrgW aa 64-80 X-PrgW aa 250-271					X-PrgW_N aa 1-20		X-PrgW_C aa 314-333		X-PrgW_N X-PrgW_C	
p49*					49	ND		ND			
p40*		40		43	43	39	38	39	38		
p36*	36	37	37	37	37	35	33 34	34 34 35	34 34 34	37	36
p32*						33	31				
p24*	26	26						22	21		
p18*		X									
H36*						35	37				

Apparent approximate molecular weights of multiple PrgW clones were grouped into different size designations based on calculations with standard curves and polynomial equations. Each number represents the approximate apparent molecular weight of each band detected on a blot. The colors represent the different strains that gave rise to the bands: Orange represents *E. faecalis* OG1RF negative control, green represents *E. faecalis* OG1RF(pCF10), blue represents *E. faecalis* OG1RF(pPRGW), red represents *E. faecalis* OG1RF(pORI10) and pink represents *S. mutans* UA159(pPRGW). The numbers appearing in a single column denoted by the grey line were obtained from the same blot. ND = results not determined.

never observed in *E. faecalis* and thus is likely rapidly processed to p40* in the natural host. PrgW p40* was only observed in the native host when protein was overexpressed in pPRGW clones. This observation is likely due to a bottleneck effect in the processing mechanism that occurs when protein is highly overexpressed due to nisin-induction of the pMSP3535 vector. The presence of p32* and p18* was variable and can be observed with retrospective analysis of blots of pPRGW whole cell lysate. These blots showed evidence of degradation products suggesting that these forms may be unstable. Time courses of PrgW processing post-induction could be used to further elucidate the steps in processing.

Processing Depends on the Host

Complete processing of PrgW appeared to be specific to Enterococci. When protein was expressed in the native host genus, *Enterococcus spp.*, PrgW forms p40*, p36*, and p24* were detected by Western blotting. Alternatively, if the protein was expressed in *Streptococcus mutans* forms p49* and p40* were observed but p36* was not detected. This result suggests that the protein cannot be processed in this genus in the same way that it is processed in its native host.

Generation of p36 and p24* Correlates with Replicon Activity*

Detection of the p36* form correlated with the presence of a functional replicon (Table 11). When p49* and p40* were seen in the absence of p36* and p24*, such as in clones where protein was overexpressed in *S. mutans*, the replicon was non-functional; this observation indicates that the generation of p36* is critical for replicon activity. For example, processing could be necessary to expose residues that allow PrgW to bind

Table 10: Expression Levels of PrgW Influence the Number of Bands Detected

	OG1RF	pCF10	pORI10	pPRGW
PrgW Form				
p49*				++
p40*				++++
p36*		++	+++	++++
p32*				+/-
p24*		+	+	++++
p18*				++++
H36*	+			

+ **faint**
 ++ **visible**
 +++ **strong**
 ++++ **very strong**

PrgW was expressed in different clones and detected by Western blotting using antibodies directed against aa 1-20, 64-80, 250-271 and 314-333. Variation in expression levels influenced the number of observable bands. When overexpressed in pPRGW four very strong bands are observed, and designated p40*, p36*, p24* and p18*. PrgW form p18* is implicated in brackets because the band was outside the range of the molecular weight markers and its apparent approximate molecular mass could not be accurately calculated using the standard curve.

the origin of replication. In addition, retrospective examination of blots from B. Utter suggest the generation of p24* depends on the presence of three conserved cysteines (C78, C275, and C307), but this observation requires verification. Mutation of these cysteines destabilizes the plasmid, suggesting the cysteines may allow for processing to p24* which is required for stable maintenance of pCF10. This further processing of PrgW could increase the avidity of PrgW for the origin of replication or allow for more efficient multimerization of PrgW leading to melting of the DNA at the origin. Either of these mechanisms would allow for increased replicon activity and more stable maintenance of the plasmid. Conversely, processing could be important for controlling the amount of active PrgW in the cell. The plasmid pCF10 is a low copy number, large, 67.8 kbp plasmid. Cleavage to p24* may inactivate PrgW thus preventing runaway replication, which could result in plasmid loss from the cell. Finally, cleavage to p24* could release PrgW from the membrane after its putative recruitment to the membrane by pre-cCF10. This release may be important for proper plasmid partitioning.

p40 and p36* Contain Both the N and C termini; p24* Only Contains the C terminus;
p18* is Recognized Only by Anti-PrgW 64-80/250-271 Antibodies*

p40* and p36* are both detectable by the anti-PrgW_C and anti-PrgW_N antibodies, suggesting that both the N and C terminus were present in these forms of the protein (Table 12). The presence of both termini may be explained by three options: processing may involve (1) cleavage from the center while the termini are associated because of formation of stable intermolecular bonds (2) modification without cleavage, or (3) a change in conformation. All of these possibilities would yield a form possessing both termini that migrates on SDS-PAGE as p36*. Future experiments characterizing the

Table 11. Generation of p36* and p24* Correlates with Replicon Activity

	Active Replicon	Inactive Replicon
PrgW Form		
p49*		X
p40*		X
p36*	X	
p32*		
p24*	X	
p18*		

The presence of PrgW forms p36* and p24* seen in *E. faecalis* clones pCF10, pORI10, and pPRGW correlated with an active replicon. In contrast, p36* and p24* are not observed in samples where the replicon is non-functional, such as when pPRGW is expressed in *S. mutans*.

different suspected processing products by N-terminal sequencing and MS/MS would help to elucidate the mechanism of this processing step.

Two additional proteins p32* and a host protein H36* were recognized by the anti-N-terminal antibody. Host protein H36* migrates at the same apparent approximate molecular weight as p36*, however, unlike, p36*, it is a much weaker band and it does not bind the anti-C-terminal antibody. Interestingly, a host protein of approximately the same size binds to cCF10 and pre-cCF10 and appears to be essential in *E. faecalis* (unpublished observations). Purification and identification of the host band could yield further insight into these observations. The detection of p32* by the anti-N-terminal antibody was variable this band was detected only in Western blots of pPRGW lysates. It is likely that the processing step that transforms p36* to p32* is rapid, and p32* is quickly turned over to yield p24*. PrgW processing time courses could be used to verify this hypothesis.

PrgW p18* does not bind to either the anti-N-terminal or anti-C-terminal PrgW antibodies. It does however, bind to a mix of anti-PrgW aa 64-80 and anti-PrgW aa 250-271 antibodies and to pre-cCF10. Blots using individual anti-PrgW internal antibodies and time course experiments could be used to further refine this hypothesis.

Taken together, these data can be used to formulate a processing model (Figure 8). When PrgW is overexpressed in *S. mutans*, both form p49* and form p40* were observed, but form p36* was not detected. This evidence is suggestive that *S. mutans* lacks the mechanism responsible for the processing form p40* to form p36*; this subsequent processing step may be limited to Enterococci. When PrgW is overexpressed

Table 12. Band Intensity Varies with Different Antibody Recognition Epitopes

PrgW Form	X-PrgW aa 64-80 X-PrgW aa 250-271	X-PrgW_N aa 1-20	X-PrgW_C aa 314-333
p49*	++		
p40*	++++	++++	++++
p36*	++ to ++++	++ to +++	+ to ++++
p32*		+/-	
p24*	+ to ++++		++++
p18*	++++		
H36*		+	

+ **faint**
 ++ **visible**
 +++ **strong**
 ++++ **very strong**

PrgW protein was detected by four different polyclonal antibodies with varying intensity levels in different clones. PrgW antibodies directed at the N and C termini both detected PrgW as forms p40* and p36* with strong intensity.

in pPRGW clones in both *E. faecalis* and *S. mutans*, form p40* is observed with apparent approximate molecular weights ranging from 40,000 to 43,000; preliminary evidence suggests this form binds pre-cCF10 (Cutrera, unpublished observations). Since pre-cCF10 is a signal sequence likely located in the membrane, it could recruit PrgW to the membrane for subsequent processing. Since pre-cCF10 is only present in *E. faecalis*, it may be the step missing in bacteria, such as *S. mutans*, that lack pre-cCF10. The processing of form p40* to yield form p36* is only observed in *E. faecalis* and *E. hirae*. Since the replicon is only active in Enterococci where form p36* is observed this form is likely required for replicon function. The processing step that transforms form p36* to form p32* is likely rapid and form p32* is quickly turned over to yield form p24*. Comparison of blots on wild type PrgW and PrgW- Δ C78A/C275A/C307A demonstrated an absence of form p24* in cysteine mutants. This implies that the processing of form p36* to yield form p24* requires these cysteines which are also known to influence plasmid stability. PrgW p18* may be an unstable form of PrgW that becomes degraded quickly; this form also binds pre-cCF10.

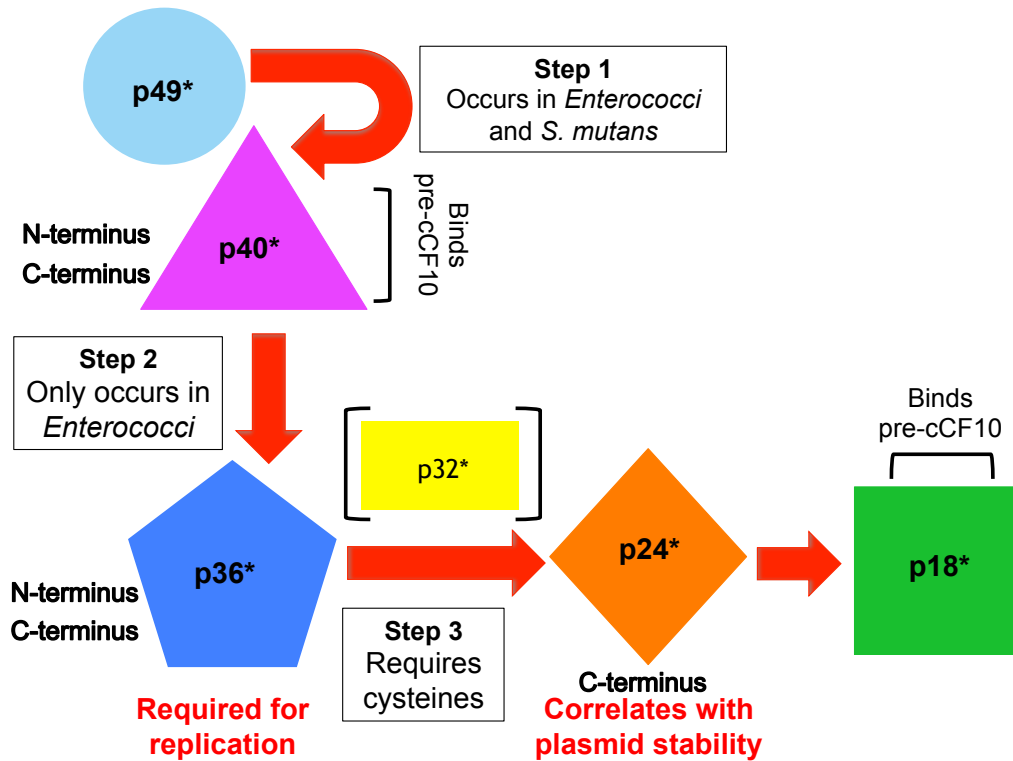


Figure 8. PrgW Processing Model

A processing model for PrgW is proposed as follows: Initially, p49* is processed to p40* which possesses both termini and binds pre-cCF10; this processing step occurs in both *Enterococci* and *S. mutans*. Next, form p40* gives rise to form p36*, which also contains both the N and C termini; this step only occurs in *Enterococci*. PrgW p36* is processed to form p32* and rapidly turned over to yield form p24*, which contains only the C terminus. PrgW p24* may play a role in plasmid stability and is further processed to form p18* which binds pre-cCF10 and may be an unstable form that is subsequently degraded.

Tetracycline Resistance Determinant (*tetM*) Transfer from *E. faecalis* to *S. aureus* in Biofilms

Transfer of Conjugative Transposon Tn925 versus Conjugative Plasmid pCF10

Enterococcal conjugative plasmids such as pCF10 have been implicated as stable genetic reservoirs that contribute to the dissemination of antibiotic resistance genes. The plasmid pCF10 is a narrow host range plasmid that cannot replicate in genera other than the native host *Enterococcus spp.*, however horizontal gene transfer may still be mediated by the pheromone responsive conjugative mechanism.

The conjugative plasmid pCF10 carries the transposon Tn925. This transposon possesses a tetracycline resistance gene (*tetM*). The plasmid pCF10 can be transferred between *E. faecalis* donor cells and plasmid-free recipient cells of multiple species by a Type IV secretion system. Since pCF10 only replicates in Enterococcal species, after transfer the plasmid would not be maintained in other species. However the conjugative transposon Tn925 may integrate into the bacterial chromosome of the new host cell. In biofilm transfer model experiments (Figure 5) *tetM* was detected in one potential transconjugant (Figure 6), which was confirmed to be *S. aureus* by 16S ribosomal sequencing (Figure 7; Table 8). The genetic transfer rate was determined to be 10^{-8} . In these experiments the *S. aureus* was a penicillin resistant clinical isolate and selection for *S. aureus*, which obtained the *tetM* gene, was done on penicillin tetracycline plates. While one of the isolates was *S. aureus*, which obtained the *tetM* gene, five of the isolates were penicillin resistant *E. faecalis*. The penicillin resistance phenotype of the *E. faecalis* would suggest either *E. faecalis* mutated to resistance or was able to obtain the penicillin

resistance from *S. aureus*. Conjugative plasmids have been shown to mediate the exchange of chromosomal markers between enterococci, so it would be interesting to determine whether the penicillin resistance arose spontaneously or was obtained from *S. aureus*.

The conjugative transposon Tn925 has been shown to mediate the transfer of *tetM* in *E. faecalis* in the presence of tetracycline suggesting that “conjugal” transfer of transposons can occur in the absence of conjugative plasmids (19, 38). Furthermore, Tn916 and Tn916-like transposons such as Tn925 are promiscuous conjugative transposons capable of dissemination of the *tetM* gene (26). This transfer occurs at rates of $\sim 10^{-8}$. In these experiments, the pheromone cCF10 was added to induce the transfer of pCF10. To determine the transfer rate of *tetM* mediated by the conjugative transposon alone, experiments can be repeated in the presence and absence of cCF10. In addition, the conjugation experiments were performed in the absence of selection for tetracycline. Inclusion of sub-lethal doses of tetracycline during the induction of conjugative transfer may increase the apparent rate of *tetM* transfer by applying a selective advantage for tetracycline resistant bacteria. Regardless, these experiments clearly demonstrate horizontal gene transfer of an antibiotic resistance marker from *E. faecalis* to *S. aureus* in mixed species biofilms.

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APPENDIX

Growth Media

Lysogeny Broth (LB) (3, 4)

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 diH₂O.

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 diH₂O.

Autoclave for 35 minutes at 121°C.

Terrific Broth (35)

12 g Bac^{to}™ Tryptone (Catalog # 211705)

24 g Bacto™ Yeast Extract (Catalog # 212750)

4 ml FisherChemicals Glycerol (G33-1)

q.s. to 900 ml with diH₂O.

Autoclave for 35 minutes at 121°C.

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

0.17 M Sigma[®] KH₂PO₄ (Catalog # P-5379)

0.7 M FisherChemicals K₂HPO₄ (Catalog # P288-500)

q.s. to 100 ml with dH₂O.

Autoclave for 20 minutes at 121°C.

Todd Hewitt (TH) Broth

30 g Bacto[™] TH powder (Catalog # 249240)

q.s. to 1 L with dH₂O

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 dH₂O

Autoclave for 35 minutes at 121°C.

TH Glycine 10% (THG)

30 g Bacto[™] TH powder (Catalog # 249240)

100 g Fisher BioReagents[®] Glycine (Catalog # BP381-1)

q.s. to 1 L with dH₂O.

Autoclave for 35 minutes at 121°C.

TH Glycine Gradient

	TH	TH 10% Glycine
2%	8 ml	2 ml
2.25%	7.75 ml	2.25 ml
2.5%	7.5 ml	2.5 ml
2.75%	7.25 ml	2.75 ml

3%	7 ml	3 ml
3.25%	6.75 ml	3.25 ml
3.5%	6.5 ml	3.5 ml
3.75%	6.25 ml	3.75 ml
4%	6 ml	4 ml
4.25%	5.75 ml	4.25 ml
4.5%	5.5 ml	4.5 ml
4.75%	5.25 ml	4.75 ml
5%	5 ml	5 ml

TH Sucrose (THS)

30 g Bacto™ TH powder (Catalog # 249240)

0.25 M FisherChemicals Sucrose (S5-500)

q.s. to 1 L with dH₂O

Autoclave for 35 minutes at 121°C.

Electroporation and Transformation Solutions

***E. faecalis* Electroporation**

EB Buffer (Electroporation 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

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 dH₂O.

Protein Whole Cell Lysate and 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 (ÄKTA)

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[®] KH₂PO₄ (Catalog # P-5379)
3.05 ml (0.5 M) FisherChemicals K₂HPO₄ (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 H₂O (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 dH₂O.

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 dH₂O.

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 dH₂O.

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 dH₂O

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 dH₂O

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 dH₂O

Adjust to a final pH of 8.3 with NaOH

1X TBST 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 dH₂O

Adjust to a final pH of 7.6 using HCl.

q.s. to 1 L with dH₂O

Add 1 ml FisherChemicals Tween (Catalog #BP337-500) while stirring.

Blocking Buffer (Milk Buffer 5%)

100 ml 1X TBS Wash Buffer (see above)

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

Milk buffer (3%)

100 ml 1X TBS Wash Buffer (see above)

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

Primary Antibody

50 ml Milk buffer (3%)

12.5 µl anti-PrgW_N terminus and/or anti-PrgW_C terminus (1:4000)

OR 1:1000 dilution for internally targeting anti-PrgW antibodies:

50 ml Milk buffer (3%)

50 µl anti-PrgW_aa 64-80 and/or anti-PrgW_aa 250-271

Secondary Antibody Solution (1:15,000 dilution)

25 ml Milk buffer (3%)

1.67 μ l Goat anti-Rabbit IgG IRDye® 680 Polyclonal (Thermo Scientific Pierce
Odyssey Li-Cor Biosciences; Catalog # 926-32221)

Phosphate Buffered Saline (0.1 M)

0.2 M K_2HPO_4

0.2 M KH_2PO_4

Deionized Water

Autoclave for 35 minutes at 121°C and store at room temperature.