HORIZONTAL TRANSFER OF *vanA*-MEDIATED RESISTANCE AMONG COMMENSAL AND STREPTOGRAMIN-RESISTANT ENTEROCOCCI DERIVED FROM MULTI-COMPONENT FOOD SYSTEMS

by

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ABSTRACT OF THE DISSERTATION

Horizontal Transfer of *vanA*-Mediated Resistance Among Commensal and Streptogramin-Resistant Enterococci Derived from Multi-Component Food Systems

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The enterococci are commensal lactic acid bacteria of the gastrointestinal tract of humans and animals, and are ubiquitous in nature. They have a long history of use as starter cultures in European fermented foods and are used commercially as probiotics. Within the past two decades, vancomycin-resistant enterococci (VRE) have emerged as significant nosocomial pathogens, treatable with few antibiotics. In the US, the agricultural use of virginiamycin, a streptogramin growth promoter, has raised concern regarding cross-resistance among VRE to the clinical streptogramin quinupristin-dalfopristin. Horizontal transfer of antibiotic resistance genes is mediated by aggregation substances and sex pheromones that induce conjugation, but the role of these virulence factors in enterococci of food origin is largely unknown. The potential for enterococci isolated from multi-component creamy deli salads (including macaroni, potato, chicken, and seafood) to harbor and disseminate the high-level vancomycin resistance gene *vanA*

among more virulent native enterococci was investigated. Initial filter mating between a clinical *Enterococcus faecalis* isolate carrying *vanA* and a commensal, vancomycin susceptible *E. faecium* salad recipient that harbored no virulence genes was demonstrated at a frequency of 10^{-8} per recipient. The rate of secondary transfer of *vanA* by this transconjugant to both food and animal enterococci carrying the streptogramin resistance genes *vatE* or *vatD* and/or the selected virulence genes *agg, gelE, cpd,* and *efa* increased three log-fold. In primary and secondary matings, multiple antibiotic resistance patterns increased for all transconjugants. Additionally, expression of *vanA* in the absence of selective antibiotic pressure was determined by reverse transcriptase-PCR and found to occur in the primary transconjugant but not in the donor. Results demonstrate that foodborne commensal enterococci from multi-component foods can receive and disseminate *vanA* to food and animal enterococci that harbor streptogramin resistance and/or virulence genes. The potential for transfer events to occur among commensal and virulent enterococci in foods must be considered for the safe use of *Enterococcus* in starter cultures and probiotics.

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DEDICATION

In memory of my grandmother, Nonnie,

and my daughter, Rachel Merry

TABLE OF CONTENTS

Abstract of the Dissertation…….....ii

Acknowledgements……iv

Dedication……v

Table of Contents……vi

List of Tables…….……...x

List of Figures…….…….xi

CHAPTER 1…….…….....1

I. INTRODUCTION…….……1

A. *Use of Enterococci in Foods and Feeds*……..……..…2

B. *Dual Development of Vancomycin-Resistant Enterococci*…….....4

C. *Streptogramin-Resistant Enterococci (SRE): A Mirror of VRE?*.....6

II. RESISTANCE MECHANISMS IN VRE AND SRE……..8

A. *Intrinsic Resistance Mechanisms*…….……..8

B. *Mechanisms of Acquired Resistance*……..……9

III. ETIOLOGY OF VRE AND SRE RESISTANT PHENOTYPES…….….12

A. *Vancomycin Resistance*……..……..13

B. *Streptogramin Resistance*…….....……..15

IV. GENETIC RELATEDNESS & DIVERSITY OF VRE AND SRE……..16

V. VIRULENCE FACTORS ASSOCIATED WITH GENE TRANSFER IN ENTEROCOCCI……19

A. *Pheromone-Responsive Virulence Factors*…….……20

B. *Pheromone Response Factors in Enterococci of Food Origin*……....21

C. *Additional Enterococcal Virulence Determinants*……...23

CHAPTER 2…..43

I. ABSTRACT……..43

II. INTRODUCTION……...44

III. MATERIALS AND METHODS……..…..46

A. *Bacterial Strains*……..46

B. *Enrichment and isolation of enterococci from multi-component salads*…….47

C. *Antimicrobial susceptibility testing*……..48

D. *Multiplex PCR for selected antibiotic resistance genes and virulence determinants*…….48

IV. RESULTS…….……50

A. *Identification of* Enterococcus *from food, clinical, and animal sources*…….50

B. *Minimal inhibitory concentration of antimicrobial agents*…….……50

C. *Distribution of antibiotic resistance genes and selected virulence determinants as detected by PCR*……..52

V. DISCUSSION……..……..54

CHAPTER 3……64

I. ABSTRACT……..64

II. INTRODUCTION……...……65

III. MATERIALS AND METHODS……...…….67

A. *Bacterial strains*……..67

B. *Enrichment and isolation of enterococci from multi-component salads*……68

C. *Antimicrobial susceptibility testing*…….……69

D. *Multiplex PCR for selected antibiotic resistance genes and virulence determinants*.....69

E. *Mating experiments*……71

IV. RESULTS……72

A. *Identification and selection of* Enterococcus *donor and recipient strains from food, clinical, and animal sources*……..……72

B. *Silent* gelE……..…….…….73

C. *Detection of antibiotic resistance genes and virulence determinants by PCR*….…….……73

D. *Generation of primary and secondary transconjugants*……..75

V. DISCUSSION……...….77

CHAPTER 4…….88

I. ABSTRACT……..88

II. INTRODUCTION…….……..89

III. MATERIALS AND METHODS……90

A. *Bacterial strains*…….....90

B. *Preparation of simulated multi-component deli salads*……...……91

C. *Preparation of cells with and without* E. faecium S4 *culture filtrate*……..91

D. *Polymerase chain reaction*…….....92

E. *RNA isolation and reverse transcriptase-PCR*……...……93

IV. RESULTS…….……94

A. *Antibiotic resistance of donor and transconjugant strains*…….94

B. *Detection and expression of* vanA *by RT-PCR*……95

V. DISCUSSION…….……...96

Chapter 5……102

Appendix…….112

Curriculum Vita…....118

LIST OF TABLES

TABLE 2.1 Primer sets for multiplex protocols ……..….49

TABLE 2.2 Percent of enterococcal isolates resistant to vancomycin, streptogramin, and selected antibiotics…….…….51

TABLE 2.3 Percent incidence of antibiotic resistance and virulence genes by PCR and phenotypic screening…….53

TABLE 3.1 Primer sets for multiplex protocols of *Enterococcus* strains used in mating experiments……71

TABLE 3.2 Summary of PCR results characterizing donor and recipient isolates used in mating experiments …….……..….74

TABLE 3.3 Results of primary and secondary mating experiments…….……76

TABLE 3.4 Antimicrobial susceptibility patterns of primary and secondary donors, recipients, and transconjugants …….……77

TABLE 3.5 Selected genes detected by PCR and MIC values for vancomycin and streptogramin in donor, recipient, and transconjugant mating strains…...81

TABLE 4.1 Minimal inhibitory concentrations of selected antibiotics for donor, recipient, and transconjugant strains...…….…….94

TABLE A.1 Source of vancomycin-susceptible *Enterococcus*, coagulase-negative *Staphylococcus* (CNS), and *Bacillus* isolates collected between March, 2005 and November, 2006……...……112

TABLE B.1 Characterization of *Enterococcus* control strains……115

TABLE B.2 Characterization of *Enterococcus* strains isolated from multi-component deli salads…….115

TABLE B.3 Characterization of *Enterococcus* strains isolated from inpatient blood and rectal cultures……...……116

TABLE B.4 Characterization of *Enterococcus* bovine strains…….....117

LIST OF FIGURES

FIGURE 2.1 Summary of total *Enterococcus* isolates demonstrating streptogramin resistance and detection of *agg*, *cpd*, and/or *gelE*……53

FIGURE 4.1 RT-PCR results for *vanA* and *sodA* for *E. faecalis* C2 (donor) and *E. faecium* T2G (transconjugant) cultivated in BHI broth without antibiotics……..95

FIGURE 5.1 Prevalence of vancomycin-resistant *Enterococcus faecalis* and *E. faecium* isolated from RWJUH inpatient samples between January 2005 and December 2006……....104

FIGURE 5.2 Model for horizontal transfer of *vanA* …….....106

CHAPTER 1

INTRODUCTION

I. THE GROUP D ENTEROCOCCI: FRIEND AND FOE

The Group D enterococci are Gram-positive, non-sporeforming, facultative anaerobic lactic acid bacteria that are commensal enteric flora of humans and animals. They have also been isolated from a wide variety of environmental niches, including soil, sewage, grasses, and seawater (Guardabassi and Dalsgaard, 2003; Muller et al., 2001; Giraffa, 2002). Formerly classified by the Lancefield serological typing system in the 1930's under the genus *Streptococcus*, their transfer to the new genus *Enterococcus* was proposed in 1984 when 16S rRNA analysis revealed that *Streptococcus faecium* and *S. faecalis* were unique from other antigenically similar group D streptococci (Schleifer and Kilpper-Balz, 1984). Currently, there are 35 recognized species within the genus *Enterococcus* (http://www.bacterio.cict.fr/e/enterococcus.html), with *E. faecalis and E. faecium* representing the majority of the isolates (Franz *et al.*, 1999; Hancock and Gilmore, 2000). The enterococci have a low (<50 mol%) guanine-plus-cytosine content in DNA, typical of related phylum genera *Clostridium* and *Bacillus* (Schleifer and Kilpper-Balz, 1984) and, based on 16S rRNA analysis, are more closely related to the genera *Vagococcus, Tetragenococcus,* and *Carnobacterium* than *Streptococcus* and *Lactococcus* (Devriese et al., 1993; Facklam et al., 2002) .

The enterococci are isolated from a wide variety of environmental niches, demonstrating that they are well-adapted to survive and thrive in diverse habitats, including foods. Physiologically, they are catalase-negative and oxidase-negative, typically grow in 6.5% NaCl, hydrolyze esculin in the presence of 40% bile, and have a broad pH range (Franz et al., 2003; Giraffa, 2003). Enterococci are more thermophilic than other Gram-positive cocci, growing at both 10^{o}C and 45^{o}C, and surviving temperatures as high as 68^{o}C for 30 minutes (Gordon and Ahmad, 1991). Genes for bacteriocin production, such as those encoding enterocins against pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus* are widely distributed among enterococcal strains isolated from human, animal, and environmental sources (DeVuyst et al., 2003). These unique growth characteristics enable enterococci on the one hand to be beneficial for use as probiotics and food supplements, but also to persist as spoilage contaminants and opportunistic pathogens under appropriate conditions.

A. Use of Enterococci in Foods and Feeds.

In Europe, the enterococci have a long history of use as starter cultures in the production of traditional artisan cheeses such as feta, mozzarella, fontina and Cheddar as well as fermented sausage products (Franz et al., 2003). The thermotolerant and acidophilic nature of enterococci enable their survival in milk throughout pasteurization and cheese fermentation, during which their proteolytic and lipolytic activities contribute significantly to the ripening process and flavor development (Giraffa, 2003). The additional benefit of bacteriocin production makes enterococci useful as protective cultures against spoilage bacteria or pathogens in the cheese (Ennahar and Deschamps,

2000). The predominant enterococcal species isolated from fully ripened cheeses are *E. faecalis, E. faecium* and *E. durans*, at levels between 10^5 and 10^7 CFU/g (Franz et al., 1999). Recently, cheeses containing enterococci have been explored as delivery systems for probiotics to alleviate irritable bowel syndrome and stimulate the immune system in humans (Gardiner et al., 1999). In sausage products, enterococci are isolated from dry fermented sausages (chorizo) and salamis ranging from 10^2 to 10^5 CFU/g (Samelis et al., 1994; Franz et al., 2003).

As probiotic growth promoters, both *E. faecium* and *E. faecalis* are added to animal feeds to minimize numbers of enteric pathogens and to help maintain a balance of normal flora. Enterococcal enterocins have a strong anti-listerial effect as well as general antibacterial activity against indicator strains (Nigutova et al., 2005), and probiotic enterococci have also been added to dry foodstuffs such as dog food to enhance immunity (Benyacoub et al., 2003). In Europe, the Scientific Committee for Animal Nutrition (SCAN) requires demonstration of the positive effects of probiotic use on an animal for provisional approval (Becquet, 2003). The safe use of *Enterococcus* as a probiotic for use in foods and feeds as reported by the FAO/WHO Working Group includes those strains that are "not a significant risk with regard to transferable antibiotic resistance or other opportunistic virulence properties" and places the responsibility of ensuring these properties with the producer (Joint FAO/WHO Working Group Report, 2002).

Since enterococci are part of the normal enteric flora of animals, numbers range from 10^4-10^8 CFU/100 cm^2 in swine and poultry carcasses (Giraffa, 2002). On the retail level in the United States, Hayes et al. (2003) found *Enterococcus* in up to 100% of ground beef and 97% of pork in a survey of over 1,300 samples. The prevalence of

enterococci on retail fruits and vegetables is also high. McGowan et al. (2006) found that of 396 food samples, 47.7% harbored *Enterococcus,* with *Enterococcus casseliflavus* most commonly isolated from produce and *E. faecalis* from chicken, pork, and beef. These studies demonstrate that enterococci are common contaminants of a wide range of raw and ready-to-eat food items sold by retail supermarkets and purchased by the average American consumer.

B. The Dual Development of Vancomycin-Resistant Enterococci.

The development of antibiotic-resistant strains of enterococci over the past two decades has resulted in controversy over the use of these bacteria in animals and foods (Foulquie et al., 2006). In 1956, the glycopeptide vancomycin, which inhibits peptidoglycan synthesis, was isolated from the actinomycte *Amycolatopsis* (formerly *Nocardia*) and sold as Vancocin® until the early 1980's; since then it has been available generically. Concurrently, the glycopeptide growth promoter avoparcin was approved for subtherapeutic use in European animal husbandry in 1974. Despite the recommendations of the 1969 European Swann report that antibiotics used to treat human infections not be used as food additives, widespread use of avoparcin occurred throughout the next two decades, and the incidence of enterococci resistant to human-use glycopeptide antibiotics, particularly vancomycin, began to increase in Europe among enterococci isolated from animals (Bates, 1997; Wegener et al., 1999). In 1986, the first vancomycin-resistant enterococcal (VRE) isolate was reported in humans (Uttley et al., 1988) and, by 1997, sufficient genetic evidence linking VRE and animals that had received avoparcin prompted the European Union to ban the use of avoparcin in animal

husbandry (Bates et al., 1994; Klare et al., 1995a; Bager et al, 1997; Wegener et al., 1999).

In the United States, where avoparcin was never approved for use as a growth promoter, similar glycopeptide-resistance developed among enterococci isolated from hospitalized patients who had received vancomycin therapeutically (Bonten et al 2001). The emergence of penicillin-resistant pathogens in the 1980's, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile,* the spore-forming agent of antibiotic-associated colitis, resulted in the extensive clinical use of vancomycin during that decade (Rice, 2003). Kirst et al. (1998) found that annual use of vancomycin in the U.S. was five to ten times higher than in Europe. In 1989, CDC data indicated that almost all enterococci were susceptible to vancomycin; by 1999, more than 25% of total enterococci from isolated hospitalized patients were vancomycin-resistant, making VRE a leading nosocomial pathogen (CDC/NNIS, 1997, 2004; Hancock and Gilmore, 2000; Bonten, 2001). Worldwide, the SENTRY surveillance program found that of 5000 VRE isolated, 17% were reported by U. S. sentinel hospitals, almost six times the percentage reported from European hospitals, and that in the U.S., resistance among *E. faecium* isolates to vancomycin was 16-fold higher than that of *E. faecalis* (Low et al., 2001).

Due to the dual development of resistance on both continents, today VRE are most prevalent in healthy animal carriers in Europe whereas in the U.S., carriers are primarily healthy humans and hospitalized patients (Kuhn et al., 2005). However, the number of nosocomial VRE isolates is steadily increasing on both continents, and recent genetic linkage evidence demonstrates that an island of pathogenicity exists among enterococci resistant to vancomycin isolated from five continents (Willems et al., 2005).

Today, enterococci account for more than 12% of infections annually in the United States, including 10,000 urinary tract, 25,000 systemic blood, 40,000 wound, and 1,100 heart valve (Huycke et al., 1998). Among clinical isolates, *E. faecalis* is the primary pathogen, although the majority of VRE-type strains are *E. faecium* (DeLisle and Perl, 2003; MacDonald, 2006). Patients infected with VRE are often colonized with multiple pathogens and must be placed in isolation, thus increasing cost of care and length of stay (Pelz et al., 2002). The mortality rate due to VRE infection is estimated at 7-50%; among colonized survivors, a carriage-reinfection cycle often occurs (Giraffa, 2002; Papp et al., 2003).

The persistent nature of enterococci is also evident in their survival on environmental surfaces for up to seven days (Noskin et al., 1995; Cetinkaya et al., 2000; DeLisle and Perl, 2003). In sampling studies, VRE were recovered from 7-30% of healthcare setting environmental surfaces (counters, bedrails, telephones, and stethoscopes) and from 10-43% of health care workers' hands (Noskin et al., 1995; Bonilla et al., 1997). This persistence, along with the carriage rate of antibiotic resistant strains in humans and animals, raises concern over the role of enterococci in transferring antibiotic resistance elements in multiple community settings (Franz et al., 2003; Smith et al., 2003).

C. Streptogramin-Resistant Enterococci (SRE): A Mirror of VRE?

In treating VRE today, physicians resort to bactericidal "last resort" drugs, including streptogramin antibiotics and newer oxazolidinone compounds. Despite a ban on the non-therapeutic use of glycopeptide antibiotics in animal husbandry in the U.S.

and numerous European studies demonstrating a correlation between avoparcin-fed animals and clinical resistance in humans, a 2000 National Animal Health Monitoring System survey revealed that over 70% of U.S. swine farms continue to use antibacterial growth promoters to promote feed efficiency (http://www.aphis.usda.gov). Among these antimicrobial agents are streptogramin compounds, which bind bacterial ribosomes to inhibit elongation during protein synthesis (Bonfiglio and Furneri, 2001). Streptogramins are semisynthetic mixture of polyunsaturated cyclic peptidolide virginiamycin M compounds (streptogramin A) and cyclic hexadepsipeptide virginiamycin S compounds (streptogramin B). The U.S. Food and Drug administration has approved use of virginiamycin (Stafac®) for use in swine, chickens, and turkeys (http://www.phibroah.com/). In humans, quinupristin-dalfopristin (Q/D; Synercid), a 30:70 mixture of streptogramins B and A, was approved for clinical use against vancomycin-resistant *Enterococcus faecium* in 1999 (www.aventispharma-us.com).

The potential for enterococci to become resistant to newer antibiotics designed against VRE may mirror that of vancomycin resistance development either through agricultural use, as in avoparcin-related vancomycin resistance, or through clinical overuse, as was the case with vancomycin use in the U.S. in the past decades. Linezolid (Zyvox®), a synthetic prokaryotic protein synthesis inhibitor that targets peptide linkages in the 50S ribosomal subunit, and daptomycin (Cubicin®), the first commercial lipopeptide for use against Gram-positive organisms, are the latest antimicrobial agents in the battle against VRE (Barry et al., 2001). Linezolid belongs to a new class of antibiotics called oxazolidinones (Livermore, 2003), and is approved for use against VRE, MRSA, and penicillin-resistant pneumococci (http://www.zyvox.com). Similarly,

daptomycin, which disrupts the membrane via formation of transmembrane channels, is currently approved for soft tissue infections (www.cubist.com). Although the mechanism of each of these novel compounds targets prokaryotic structures other than the cell wall, resistant strains have recently been reported (Auckland et al., 2002; Mutnick et al., 2003; Long et al., 2005). This trend may continue in the way resistance to vancomycin occurred despite the more prudent use of antimicrobials clinically and in animal husbandry in the future.

II. RESISTANCE MECHANISMS IN VRE AND SRE

A. Intrinsic Resistance Mechanisms.

Intrinsic glycopeptide resistance is chromosomally mediated and is primarily due to the inefficiency by which this class of antibiotics crosses the Gram-positive cell wall (Tannock and Cook, 2002). In *Enterococcus gallinarum* and *E. casseliflavus/flavescens,* intrinsic resistance is characterized by a minimal inhibitory concentration (MIC) of 2-32 μg/mL of vancomycin due to five constitutive chromosomal *vanC* genes (Leclercq et al., 1992; Arias et al., 2000; Tannock and Cook, 2002). Vancomycin normally binds the peptidoglycan pentapeptide precursor terminus D-Ala- D-Ala, inhibiting glycan linkages and subsequently compromising the integrity of cell wall formation (Klare et al., 2003). Intrinsic resistance of VanC-type enterococci is achieved through a second mechanism whereby the terminal alanine of the normal dipeptide is replaced by serine rather than lactate, forming D-Ala-D-Ser. This results in a six-fold decreased affinity for vancomycin relative to the susceptible strain (Reynolds et al., 1994). Less commonly reported intrinsic vancomycin resistance phenotypes include VanD and VanE, both chromosomal,

which confer low level glycopeptide resistance in *E. faecalis* and *E. faecium* (Perichon et al., 1997; Fines et al., 1999).

Intrinsic streptogramin A resistance is associated only with *E. faecalis* and is mediated by an active efflux ABC transporter-like transmembrane protein encoded by the *lsa* gene (Singh et al., 2002; Dina et al., 2003). The MIC to Q/D for *E. faecalis* is 4-32 μg/mL while intrinsic resistance to Q/D is not associated with *E. faecium*.

Oxazolidinones such as linezolid inhibit translation by binding to the large ribosomal subunit of many multi-drug resistant Gram-positive bacteria, including VRE and MRSA. The binding site of these antibiotics is unique, and so no cross-resistance occurs with other protein-synthesis inhibiting drugs (Fines and LeClercq, 2000). In addition, linezolid is effective at preventing synthesis of certain staphylococcal and streptococcal virulence factors, including hemolysins and coagulases (Gemmell and Ford, 2002). While most Gram-negative bacteria are intrinsically resistant to linezolid, oxazolidinones are one of the only available antibiotic classes that are effective against all species of multi-resistant Gram-positive cocci (Livermore, 2003). Although the intrinsic mechanisms of resistance in Gram-negative bacteria are largely unknown, in emerging Gram-positive strains, resistance is associated with point mutations in genes encoding 23S rRNA (Prystowsky et al., 2001; Bonora, 2006). Recently, a linezolid-resistant, vancomycin-susceptible isolate was reported (Marra et al., 2006).

B. Mechanisms of Acquired Resistance.

Acquired vancomycin resistance proteins in *E. faecalis* and *E. faecium* are most commonly encoded by the inducible genes of two major operons, *vanA* and *vanB* (Arthur

and Courvalin, 1993). The VanA phenotype is characterized by high-level resistance to both vancomycin and the related glycopeptide teichoplanin, while resistance of VanB type is to vancomycin but not teichoplanin (Cetinkaya et al., 2000). Genes of the *vanA* cluster are widely distributed among vancomycin-resistant enterococcal species and also reported in *Corynebacterium, Arcanobacterium, Oerskovia, Lactococcus, Streptococcus, Streptomyces* and *Bacillus* (Power et al., 1995; Ligozzi et al., 1998; Cetinkaya et al., 2000; Patel, 2000) and the *vanB* genes have been reported in *Streptococcus* spp. (Poyart et al., 1997). The MIC ranges of both VanA and VanB phenotypes to vancomycin are 64-1000 μg/mL and 4-1000 μg/mL, respectively (Arthur and Courvalin, 1993).

The high-level resistance operons of VanA and VanB phenotypes are encoded on transposons that can move between enterococci and other Gram-positive bacteria via conjugative plasmid transfer (Quintiliani and Courvalin, 1994; Evers et al., 1996; Guardabassi and Dalsgaard, 2004). In the 10.8-kb transposon Tn*1546*, primarily associated with *vanA* and often carried by self-transferable plasmids, five genes encode inducible vancomycin resistance in *E. faecium* (Leclercq et al., 1988; Arthur et al., 1993). The VanA protein ligase has higher affinity for D-Lactate than D-Alanine, resulting in the formation of abnormal depsipeptide precursors D-Ala- D-Lac. The modified precursor binds vancomycin with 1000-fold reduced affinity than to the normal D-Ala- D-Ala dipeptide precursor (Bugg et al., 1991). In the presence of vancomycin, the membrane protein VanS directs the response regulator VanR, inducing the essential genes for abnormal precursor formation, *vanH, vanA*, and *vanX*. Both VanX and an accessory protein, VanY, cleave the terminal D-Ala from the peptides, and VanH (D-hydroxy acid dehydrogenase) creates a pool of D-lactate, thus ensuring alteration of precursor subunits

(Arthur et al., 1996). Glycopeptide resistant Gram-positive bacteria also have an increase in amount of D-Ala- D-Ala in the outer cell wall layers that may serve to bind vancomycin before reaching the depsipeptide target (Weigel et al., 2003).

VanB resistance is conferred by a similar mechanism via genes that reside on Tn*1547* (Quintiliani and Courvalin, 1996). *vanB* genes may also be incorporated on the chromosome (Arthur et al., 1996). A 70% sequence identity exists between the *HAX* gene sequences of VanA and VanB operons, and the VanB ligase shares 76% identity to VanA (Evers et al., 1994). VanB resistance is reported primarily in *E. faecium* and *E. faecalis* and is common in the U.S. (Zhanel et al., 2003).

Resistance to streptogramin is mediated by several mechanisms, including modification of the antibiotic target site, active antibiotic efflux, and antibiotic inactivation. Genes encoding protein products that mediate streptogramin resistance are found in both staphylococci and enterococci. The MIC breakpoint for resistance is $\geq$ 4 μg/mL, and high-level resistance occurs at an MIC ≥32 μg/mL. Enterococci of the MLS_B (macrolide-lincosamide-streptogramin) phenotype are broadly resistant to all three antibiotic classes due to the presence of a common region of methylation in the 50S ribosomal binding site. The *erm*(B) gene resides on the 5.3 kb transposon Tn917 originally identified on the inducible nonconjugative *E. faecalis* plasmid pAD2 (Shaw and Clewell, 1985). This gene encodes an enzyme that methylates an adenine residue on the 23S rRNA, causing a decrease in streptogramin B affinity (Kak and Chow, 2002). In *E. faecium,* an ATP-binding transporter protein encoded by the *msr* genes confers resistance to both streptogramin B and macrolide antibiotics via active efflux (Portillo et al., 2000; McDermott et al., 2005; Ojo et al., 2006). Type B streptogramin resistance in

enterococci is rarely conferred by the hydrolase-encoding *vgb* genes, initially reported in staphylococci (Jensen et al., 1998; Herschberger et al., 2004).

Resistance to Q/D is mediated by the presence of one or more streptogramin A resistance genes. The synergistic 70:30 ratio of streptogramins B and A in Q/D cause a conformational change in the peptidyltransferase ribosomal domain, increasing antibiotic binding affinity and bactericidal action. The *vatD* and *vatE* genes (formerly designated *sat*) encode acetyltransferases that inactivate streptogramin A. Since streptogramin B action is dependent on the conformation change to the ribosome mediated by streptogramin A, inactivation of streptogramin A alone is sufficient for Q/D resistance (Bozdogan and LeClercq, 1999). The *vat* genes are encoded on various staphylococcal and *E. faecium* plasmids and are known to cross-transfer between animals and human strains; sequence analysis has shown *vatE* to be linked with *ermB* in a majority of isolates (Werner et al., 2000a). In addition, Q/D resistance genes can co-exist with vancomycin resistant genes on the same plasmid (Hammerum et al., 1998; Bozdogan and LeClercq, 1999).

III. ETIOLOGY OF VRE AND SRE RESISTANT PHENOTYPES

In enterococci, glycopeptide resistance is thought to have evolved in other species and been acquired, since the *vanA* nucleotide sequences are not present in *Amycolatopsis* or other Gram-positive bacteria that are intrinsically resistant to vancomycin (Dutka-Malen et al., 1990; Quintiliani et al., 1993; Rice, 2001). The genome sequence of *E. faecalis* strain V583, the first clinical VRE isolated in the United States, reveals that over 25% of the genome consists of mobile or exogenous DNA (Paulsen et al., 2003). The

guanine-cytosine content of the *vanR* and *vanS* regulatory genes as well as the *vanY* and *vanZ* accessory genes in the VanB operon is approximately 10-15% greater than that of typical enterococci (Evers et al., 1993) and appear to be derived from a source other than the essential *vanHAX* genes (Patel, 2003). Further, VanA-type VRE have been isolated from a variety of nonselective habitats that have neither been exposed to vancomycin nor to fecal contamination (Guardabassi and Dalsgaard, 2004).

A. Vancomycin Resistance.

In 1988, the first vancomycin-resistant enterococci were described in the United States (LeClercq et al., 1988). Based on homogeneity studies, there is substantial evidence of community transfer of common vancomycin resistance genetic elements among enterococci isolated from various human and animal reservoirs (Klare et al., 1995b; van den Braak et al., 1998; Willems et al., 1999; Descheemaeker et al., 1999; Aarestrup et al., 2000; Donabedian et al., 2003). Mobile genetic elements that confer high-level vancomycin resistance among enterococci have been identified (LeClercq et al., 1989; Handwerger et al., 1990; Handwerger and Skoble, 1995; Heaton et al., 1996; Tomita et al., 2003). Woodford (1995) first reported the linkage between vancomycin-resistant and high-level aminoglycoside genes on a single transferable plasmid in *E. faecalis*. In mice, the high frequency of resistance gene transfer between porcine and human *E. faecium* indicates that foods could serve as a source of resistance gene dissemination in vivo (Moubareck et al., 2003). Transfer of vancomycin resistance elements among *E. faecalis* during cheese and sausage fermentations demonstrates the potential for resistance gene transfer in the absence of selective antibiotic pressure

(Cocconcelli et al., 2003). Probiotic enterococcal strains may also serve as potential recipients of vancomycin-resistance elements, as Lund and Edlund (2001) demonstrated in the successful filter mating between clinical donor and probiotic recipient *E. faecium.*

Tn*1546* and related elements may exist as a large, mobile chromosome elements, or be transferred via conjugative or nonconjugative plasmids (Arthur et al., 1993; Handwerger and Skoble, 1995). The transfer of the *vanB* gene can also occur within a species via internal transposons or between species by chromosomal translocation (Woodford, 1995; Quintiliani and Courvalin, 1996). The potential for high-level vancomycin resistance to be conferred among multiple reservoirs is not limited to intra-species spread. VRE isolates from human and nonhuman sources demonstrate similar transposable elements that confer resistance, but are otherwise dissimilar, indicating the horizontal transmission of resistance phenotype (Bates et al., 1994; Klare et al., 1995a; Woodford et al., 1998). Noble et al. (1992) successfully transferred vancomycin resistance between *E. faecalis* and *Staphylococcus aureus* in the absence of plasmid DNA both in vitro and in vivo. Moubareck et al. (2003) demonstrated that *vanA* genes as well as genes conferring streptogramin resistance could transfer from porcine to human enterococci in the gut of gnotobiotic mice. In vitro horizontal transmission of the Van phenotype via mobile elements has been shown to occur between *Enterococcus* and *Staphylococcus*, *Corynebacterium*, *Lactococcus, Bacillus, Listeria,* and *Clostridium* in addition to other enterococci and streptococci (Leclercq et al., 1989; Ligozzi et al., 1998; Dahl and Sundsfjord, 2003; DeLisle and Perl, 2003).

B. Streptogramin Resistance.

Streptogramins are naturally isolated from *Streptomyces pristinaspiralis* and consists of several antibiotic classes, including prisinamycin, which is used clinically against staphylococci, as well as virginiamycin. In addition to human isolates, high-level streptogramin-resistant enterococci have been recovered from the farm environment, farm animals, and veterinary samples (Thal and Zervos, 1999; Hayes et al., 2001; Soltani et al., 2000). However, there is limited data regarding the role of virulence genes and horizontal transfer of resistance among enterococci from streptogramin-treated animals (Butaye et al., 2000; Robredo et al., 2000; Klare et al., 2003). Cross-resistance between virginiamycin and Q/D is known to occur (Butaye et al., 2000). As early as 1985, the presence of Tn*917* in human and farm animal MLS_B-type enterococci was reported (Rollins et al., 1985; LeBlanc et al., 1986). It is important to note that the MIC range of resistance between animal and human streptogramin-resistant enterococci varies considerably. In the 2004 Virginiamycin Risk Assessment Draft for Comment, the FDA reports worldwide MICs for poultry were ≥32 μg/mL, while in humans resistant isolates have MICs of 4 μg/mL (http://www.fda.gov/cvm/Documents/SREF_RA_FinalDraft.pdf).

The study of conjugative gene transfer conferring streptogramin resistance has elucidated the mechanism of dissemination of *vatD* and *vatE* genes (Jensen et al., 1998; Hammerum et al., 1998). Werner et al. (2000b) demonstrated conjugative transfer of these genes among Q/D resistant *E. faecium* isolates from animals, sewage, and hospitalized patients. The resistance plasmids were found to be nonhomologous for the various sources, indicating that spread between animals and humans is likely to be via horizontal transfer. Robrero et al. (2000) has reported high-level Q/D resistance in

VanA-type *E. faecium* from humans and poultry, and Borgen et al. (2002) has demonstrated a genetic linkage between *ermB* and *vanA* determinants. Simjee et al. (2002b) detected a high prevalence of low-level streptogramin-resistant among U.S. retail poultry. Further support for horizontal transfer is evidenced by the existence of streptogramin resistance in humans after the introduction of virginiamycin but prior to the clinical introduction of Q/D (Virginiamycin Risk Assessment, www.fda.gov).

The emergence of high-level resistant nosocomial Gram-positive pathogens, such as MRSA and vancomycin-intermediate and -resistant *Staphylococcus aureus* (VISA/VRSA), in the past decade indicates that the spread of antibiotic resistance elements is not limited to enterococci (Hiramatsu et al., 1998; Weigel et al., 2003; Clark et al., 2005). Examining the factors that promote horizontal transfer of glycopeptide resistance elements among enterococci and commensal bacteria is critical to limiting the spread of vancomycin resistance via foods, food handlers, or the environment.

IV. GENETIC RELATEDNESS & DIVERSITY OF VRE AND SRE

Tn*1546* and related elements present in VRE isolated from humans and animals reveals that common DNA polymorphism exists regardless of the host strain (Bates et al., 1994; van den Braak et al., 1998; Descheemaeker et al., 1999; Stobberingh et al., 1999; Willems, et al., 1999; Garnier et al., 2004). This suggests that animal-derived VRE present in foods may transfer resistance to intestinal bacteria carried by humans. The link between avoparcin use and vancomycin resistance in Europe was supported by the presence of genetically identical vanA clusters found in livestock and human clinical isolates and rare occurrence of VanA in nonhuman sources in the United States

(Woodford et al., 1998). Willems et al. (2000) used amplified-fragment length polymorphism (AFLP) analysis to compare the genetic similarities of 255 vancomycin-resistant *Enterococcus faecium* isolates from hospitalized inpatients, healthy persons, domestic animals and farm animals. The results established four major AFLP genogroups among the enterococci and demonstrated the potential for gene exchange between food animals and humans in that 75% of the VRE isolated from healthy persons had common AFLP patterns to that of swine, and 84% of hospitalized patient VRE patterns were common to 100% of cats and dogs and 25% of veal calves. This suggests the community transfer of VRE between pigs and humans, previously associated with high-level glycopeptide resistance due to avoparcin use (van den Bogaard et al., 1997). Additionally, the genetic relatedness of strains may implicate domestic pets as a potential source of VRE in hospitalized patients. Evidence for this type of exchange was recently provided when a high-level vancomycin-resistant *E. faecium* isolate from canine urine, harboring a unique Tn*1546* previously described only in human VRE isolated in the U.S., was successfully transferred to plasmid-free enterococcal recipients (Simjee et al., 2002a).

The diversity of Tn*1546*–like elements has also been demonstrated among enterococci, however, little is known regarding interspecies transfer (Guardabassi and Dalsgaard, 2004). In the Willems *et al.* study (2000), analysis of Tn*1546* showed that VanA variants revealed little random distribution among genogroups and were therefore very host-specific. Guardabassi and Dalsgaard (2000) found that VRE isolated from various ecological niches, including sewage, seawater, and blue mussels carried Tn*1546*-like elements that were identical to those isolated from human VRE. The direct

comparison of resistance traits and virulence factors among donor, recipient, and transconjugant enterococci from human and nonhuman sources will provide evidence for a better understanding of the complex factors involved in the transference of resistance genes between diverse community populations.

Although there is no data regarding the prevalence of SRE in animal husbandry prior to the use of virginiamycin, genes conferring resistance to both virginiamycin and Q/D have been found in both animals and humans (Hammerum et al., 1998; Jensen et al., 1998). In the U.S., a high prevalence of low-level streptogramin resistance among enterococcal isolates from retail poultry has been reported (Butaye et al., 2000; Simjee et al., 2002c). One study of enterococci from turkeys that were fed virginiamycin found that isolates of *E. faecium* and *E. faecalis* from the oldest flocks were 100% resistant to virginiamycin (Welton et al., 1998). In humans, streptogramin resistance in hospitalized patients with VRE infection treated with Q/D is reported at approximately 4% (Huycke et al., 1998). Continued concerns over the spread of antimicrobial resistance between food animals and humans resulted in a 1999 European ban of several antibiotic growth promoters as feed additives, including virginiamycin, despite mixed criticism (Acar et al., 2000; Courvalin, 2001). Horizontal transfer of streptogramin resistance between *E. faecalis* and *E. faecium* has been demonstrated in vitro, indicating that dissemination of resistance genes for newer antibiotics is possible through enterococcal reservoirs (Simjee et al., 2002a).

Global spread of mobile resistance elements from nosocomial and commensal enterococci to more virulent pathogens is a major public health concern. The recent horizontal transfer of the *vanA* gene from clinical VRE to MRSA has resulted in novel

clinical pathogens with high-level resistance to vancomycin (Chang et al., 2003; Weigel et al., 2003; Tenover et al., 2004). Although fecal carriage of VRE among healthy people in the United States is relatively low, reported at 9%, a relatively large European community reservoir exists both in humans and animals due the agricultural use of avoparcin (Coque et al., 1996; Rice et al., 2003). Evolutionary genetic studies of VRE and vancomycin-susceptible enterococci (VSE) from human and nonhuman sources has identified a genetic lineage of *E. faecium* called complex-17, revealing geographic distribution in 5 continents (Willems et al., 2005). To assess the safe, continued use of enterococci in foods and growth promoters in animals, as well as continued use of streptogramin antimicrobial agents in the U.S. agricultural industry, a thorough understanding of the factors that promote transfer of resistance elements among enterococci from multiple sources is necessary.

V. VIRULENCE FACTORS ASSOCIATED WITH GENE TRANSFER IN ENTEROCOCCI

The virulence of VRE and SRE depends on the presence of genetic factors that promote adhesion, colonization, and transfer of these genes both in vitro and in vivo (Mundy et al., 2000). Proteins that mediate cell clumping and host tissue penetration are encoded by genes carried on conjugative plasmids (Dunny et al., 1991; Clewell and Dunny, 2002). In enterococci, two types of conjugative plasmids have been described: those involved in sex-pheromone response and those that are pheromone-independent. Pheromone-dependent plasmids are generally associated with aggregation substance (AS), a surface protein that promotes cell clumping and facilitates conjugative transfer of genes, while pheromone-independent plasmids generally encode macrolide-lincosamide-

streptogramin B (MLS) resistance over a broad host range (Clewell, 1981). The relative frequency of plasmid transfer between strains may increase depending upon the physical mating environment as well as the presence or absence of pheromones (Ike and Clewell, 1984).

Plasmids that carry sex pheromone genes also harbor genes encoding hemolysins, bacteriocins, and antibiotic resistance (Clewell and Dunny, 2002). In one study, more than half of the *E. faecalis* isolates from hospitalized patients produced gelatinase and AS (Coque et al., 1995), indicating that antibiotic-resistant nosocomial pathogens may be selected for by the presence of particular virulence determinants present on plasmids (Mundy et al., 2000). Although many of these virulence factors have been well-characterized with respect to their role in clinical pathology, their function in the lateral transfer of mobile resistance elements among enterococci of food origin is largely unknown.

A. Pheromone-Responsive Virulence Factors.

The pheromone-induced plasmid response is unique to enterococci and is predominantly associated with *E. faecalis* (Clewell, 1993). In this mechanism, plasmid-free recipient cells produce pheromone proteins that target specific donors carrying particular plasmids. In response to these pheromones, the donor strains produce virulence proteins, such as AS, that promote aggregation for more efficient gene transfer (Clewell et al., 1991).

The most widely-studied enterococcal pheromone-response plasmid is pAD1 of *E. faecalis*, although other pheromone-response plasmids have been described (Clewell,

1993, 1999). Plasmids of this type are generally greater than 45 kb in size and are transferred with high frequency (10^0 to 10^{-3} per donor) in broth mating (Clewell, 1981; Ike et al., 1998). The mating potential of pheromone-induced enterococci has been reported as ≥100,000–fold greater than that between non-pheromone induced cells (Ike and Clewell, 1984).

Dissemination of vancomycin resistance genes via pheromone-responsive plasmids among enterococci is known to occur (Handwerger et al., 1990; Heaton and Handwerger, 1995; Heaton et al., 1996). Huycke et al. (1992) demonstrated that pheromone-responsive plasmid transfer occurs at high frequency between *E. faecalis* strains in the gastrointestinal tract of hamsters. Although the pheromone response system is exclusive to enterococci, Clewell et al. (1985) have reported that the enterococcal sex pheromone cAM373 is produced by virtually all *Staphylococcus aureus* strains. As such, it has been suggested that natural acquisition of vancomycin resistance by non-enterococcal bacteria may occur via the sex-pheromone response system (Showsh et al., 2001; Flanagan et al., 2003). This is conceivable considering that the high-level resistance enterococcal Tn*1546*-like elements isolated from the first clinical VRSA isolates recovered in Michigan and Pennsylvania in 2002 arose from independent genetic occurrences (Clark et al., 2005).

B. Pheromone Response Factors in Enterococci of Food Origin.

Recently, the gene sequences of sex pheromones cAD1, cPD1, cOB1, and cCF10, associated with the pAD1 sex pheromone plasmid involved in intracellular signaling in *E. faecalis*, have been identified (Clewell et al., 2000). Eaton and Gasson (2001)

investigated the incidence of these determinants in *E. faecalis* and *E. faecium* isolates from starter cultures, food, and medical sources. They found that all of the *E. faecalis* isolates harbored these four pheromone genes, while none of the *E. faecium* isolates did, suggesting that the lack of sex pheromone genes in *E. faecium* may be the result of sequence divergence rather than the absence of dissemination since transfer between *E. faecalis* and *E. faecium* is established (Heaton et al., 1996). Additionally, in those *E. faecalis* isolates, the AS-encoding *agg* gene was found in all of the starter strains, 78% of those from food, and 89% of clinical origin and was always associated with sex pheromone genes. The transfer of virulence determinants from clinical to starter strains via pAD1 by filter mating was demonstrated in *E. faecalis* but was unsuccessful in *E. faecium*. However, aggregation substance and cytolysin (hemolysis) *cyl* genes can be co-linked on pheromone response plasmids, and co-expression can result in an eight-fold increase in lethality relative to either factor alone (Chow et al., 1993; Semedo et al., 2003). In another study of enterococci from hospitalized patients and dairy foods, the congruency of *cyl* genes and beta-hemolysis was detected in 79% of clinical, and 96% of food isolates (Semedo et al., 2003). Canzek et al. (2005) also reported that the *agg* gene was almost always associated with sex pheromone determinants in enterococci from artisanal cheese. Further investigation into the incidence and patterns of transfer among enterococci that harbor both types of genes on pheromone-responsive elements is of particular importance to ensure the safety of new enterococci selected to serve as starters or probiotics.

C. Additional Enterococcal Virulence Determinants.

Virulence factors other than aggregation substances are also important mediators of enterococcal pathogenicity of infected hosts. The *gelE* and *esp* determinants encode gelatinase and extracellular surface proteins, respectively, and are not specifically associated with pheromone-induced plasmids (Su et al., 1991; Shankar et al., 1999). Gelatinase is an extracellular enzyme that mediates host tissue invasion by the hydrolysis of gelatin, collagen, and casein. It is regulated by a quorum-sensing response via proteins produced by *fsrABC* genes, and is primarily associated with *E. faecalis* isolates (Qin et al., 2001) but has been detected in other *Enterococcus* species, including nonclinical strains of *E. faecium, E. durans,* and *E. hirae* (Lopes et al., 2006). Extracellular surface protein (Esp) is a high molecular weight cell-surface protein that mediates adhesion of cells to host tissues as well as nonliving surfaces, contributing to biofilm formation (Toledo-Arana et. al., 2001). It is a highly conserved protein among *E. faecalis* and group B streptococci (Shankar et al., 1999), and clinical isolates that express *esp* better colonize and persist on bladder epithelium and may act synergistically with cytolysins during initial infection (Shankar et al., 2001). Both *gelE* and *esp* are prevalent among clinical enterococci, and in one study, more than half of the *E. faecalis* isolates from inpatients produced both proteins (Coque et al., 1995). This indicates that antibiotic-resistant nosocomial pathogens may be selected for by the presence of particular virulence determinants present on plasmids (Mundy et al., 2000). When compared with commensal intestinal *E. faecalis* isolates from patients not given antibiotic therapy, the most frequently isolated virulence factors were *cpd, agg, gelE,* and *esp* (Lempianinen et al., 2005). Although conflicting data exist regarding the correlation between enterococcal

virulence factors and morbidity/mortality rate (Dupont et al., 1998; Vergis et al., 2002), strains that possess virulence determinants are more invasive to host tissues, exhibit greater evasiveness to the host immune response, and may be more resistant to antimicrobial treatment (Mundy et al., 2000; Marra et al., 2007).

In their study among enterococci from clinical, food, and starter sources, Eaton and Gasson (2001) reported the incidence of *gelE* highest in clinical *E. faecalis* (89%), although it was also present in 78% of *E. faecalis* food and 50% of starter isolates. This trend was also evident for silent *gelE* genes, which occurred in *E. faecalis* isolates from all groups. The *esp* determinant, which encodes a surface protein that promotes biofilms formation, was detected among 44% of clinical *E. faecalis* and 33% of food strains, and later found in clinical, commensal, medical and environmental isolates of *E. faecium* (Eaton and Gasson, 2002). Similar results were reported in studies where isolates were also examined for antibiotic resistance. Franz et al. (2001) found only one VRE of 95 food isolates, and that particular isolate possessed none of the virulence factors tested. Canzek et al. (2005) reported none of their food isolates as VRE; however, one *E. faecalis* tested positive for all virulence determinants. MICs for Q/D were not reported in either study.

The presence of virulence factors and pheromone induction mechanisms among enterococci that also harbor antibiotic resistance elements may facilitate the horizontal spread of glycopeptide resistance among enterococci of clinical and food origin in the community. Collectively, these data indicate that examining enterococci from multiple reservoirs for the presence of virulence determinants, particularly those involved in

conjugation and gene exchange, is essential for better understanding the behavior of these bacteria in foods and the complex etiology of glycopeptide-resistant *Enterococcus.*

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CHAPTER 2

STREPTOGRAMIN RESISTANCE AND VIRULENCE DETERMINANTS IN VANCOMYCIN-SUSCEPTIBLE ENTEROCOCCI ISOLATED FROM MULTI-COMPONENT DELI SALADS

The work presented in this chapter was submitted as a full manuscript for publication in the Journal of Applied Microbiology in July, 2007.

I. ABSTRACT

This study examined vancomycin-susceptible *Enterococcus* (VSE) isolated from deli salads for streptogramin resistance and the presence of the conjugative and virulence genes *cpd, agg,* and *gelE*. Fifteen *Enterococcus* isolates from multi-component salads collected from nine major supermarkets were isolated for identification and antimicrobial susceptibility testing by MicroScan and Etest methods. Resistance to virginiamycin was determined by standard agar diffusion testing. Multiplex PCR was used to detect virulence genes for aggregation substance (*agg*), gelatinase (*gelE*), and sex pheromone (*cpd*), the virginiamycin acetyltransferase genes *vatD* and *vatE* that confer streptogramin resistance, and the species-specific superoxide dismutase gene, *sodA.* Results showed that all isolates were VSE type, with 83% of *E. faecium* demonstrating streptogramin resistance. The selected virulence genes were detected in 100% of *E. faecalis* isolates (*cpd,* 40%; *gelE,* 33%; *agg,* 27%). The *vatD* or *vatE* genes were not detected. Experiments demonstrated that streptogramin resistance and the virulence genes *agg,*

cpd, and *gelE* are present in VSE isolated from deli salads. The results of this study will provide useful information regarding streptogramin resistance and virulence determinants in enterococci from foods that are associated with mixed ingredients and multiple handlers.

II. INTRODUCTION

The lactic acid *Enterococcus* bacteria, particularly *E. faecalis* and *E. faecium*, have a long history of use by the food industry as starter cultures in the production of traditional fermented foods and cheeses (Foulquie Moreno et al., 2006). The widespread prevalence of enterococci in nature indicates their potential for adaptation and growth under diverse environmental conditions as well as in foods. As probiotic growth promoters, both *E. faecium* and *E. faecalis* are added to animal feeds to reduce or control the number of enteric pathogenic bacteria and to maintain a normal balance of flora and immunity (Becquet 2003). Despite these benefits, vancomycin-resistant *E. faecalis* and *E. faecium* have emerged as leading nosocomial pathogens, accounting for over 12% of infections annually in the United States (McDonald et al., 2006) and represent more than 28% of intensive care unit infections (CDC, 2004). The multi-drug resistant nature of VRE strains limits the treatment options to the more recent "last resort" antibiotics, including linezolid, the streptogramin quinupristin-dalfopristin (Q/D; Synercid), and, most recently, daptomycin (Rybak et al., 2000).

The emergence of resistance to vancomycin by enterococci is attributed to a dual development that included clinical overuse in the United States and cross-resistance in Europe following the agricultural use of avoparcin, a glycopeptide animal growth

promoter (Klare et al., 1999; van den Bogaard and Stobberingh 2000; Kuhn et al., 2005). As the result of this dual development, the primary reservoirs of VRE in the United States today are healthy human carriers and hospitalized patients, whereas in Europe VRE are most prevalent in healthy animal carriers (Goossens 1998; Kuhn et al., 2005). Among treated individuals, a carriage-reinfection cycle often recurs (Murray 2000). The concern over similar development of resistance to clinical streptogramin antibiotics, including Q/D, resulted in a European ban on the agricultural use of the streptogramin growth promoter virginiamycin (Casewell et al., 2003). Cross-resistance to Q/D is associated with several genes, including *vatD* and *vatE* that encode acetyltransferase action against streptogramin A (Rende-Fournier et al., 1993). However, development of Q/D resistance in animals given virginiamycin is known to occur where *vatD* and *vatE* as well as other streptogramin-resistant genes have not been detected (McDermott et al., 2005). The persistence of enterococci, coupled with the carriage state among colonized humans and animals, has raised questions regarding the associated antibiotic resistance among multiple reservoirs in clinical, agricultural, and community settings (Franz et al., 2003; Smith et al., 2003).

In this study, vancomycin-susceptible enterococci (VSE) isolated from multi-component deli salads were characterized with regard to the presence of the streptogramin resistance genes *vatD* and *vatE* and the presence of selected conjugative virulence factors that could contribute to the horizontal transfer of antibiotic resistance. Multi-component salads were selected because they contain ingredients from multiple sources and have the potential for multiple handlers. VSE isolated from bovine sources and VRE isolated from hospitalized inpatients were also tested during the same period.

Therefore, the overall incidence of streptogramin and virulence determinants in this study was compared from randomly collected VSE isolates from food and animal sources and VRE isolates taken from colonized humans.

III. MATERIALS AND METHODS

A. Bacterial strains.

Food isolates (n=15) were obtained from self-serve salads from nine retail supermarkets in the central New Jersey region (refer to Appendix 1 for specific locations and strains isolated from each salad). Salad types included creamy vegetable and pasta salads (potato, macaroni, coleslaw), meat/poultry (chicken, egg, liver), and fish (tuna, seafood). Clinical isolates were obtained from positive blood (n=16) and rectal (n=16) cultures of hospitalized inpatients at Robert Wood Johnson University Hospital, New Brunswick, New Jersey, over several two- to five-week periods between April, 2004 and November 2006. Animal enterococci (n=17) used in the study were a generous gift from Dr. Joseph Hogan (OARDC Mastitis Laboratory, Ohio State University, Columbus, OH) and included *E. faecium* (n=10) isolated from cows with clinical mastitis and *E. faecalis* (n=7) from various milk samples.

B. Enrichment and isolation of enterococci from multi-component salads.

For selection of *Enterococcus* from food samples, 25 g of each salad was added to 225 mL of buffered peptone water and processed by a stomacher for two minutes. Fifty mL of the supernatant from each sample was added to sterile flasks containing 50 mL of double-strength Enterococcosel broth (BBL Microbiology Systems, Cockeysville, MD) and incubated at 37^{o}C for 24-48 hours in ambient air. Following incubation, flasks were examined for blackening of the broth. Positive broth samples were subcultured to Enterococcosel agar (BBL) and incubated at 35^{o}C for 18-24 hours. The plates were examined for enterococcal-like colonies (surrounded by a blackening of the agar). If enterococcal-like growth was observed, several colonies from each plate were Gram-stained and tested for catalase activity. Isolated colonies containing catalase-negative, Gram-positive cocci were streaked onto Todd-Hewitt agar (BBL) with 3% gelatin to test for gelatinase production. Plates were incubated overnight at 37^{o}C, placed at 4^{o}C for 4 hours, and then examined for an opaque zone around each colony. Identification of presumptive *Enterococcus* isolates was performed on a MicroScan Walk/Away 96 SI system (Dade Behring, Sacramento, CA) using a Microscan Gram-Positive/MIC Combo Panel. All isolates were identified to the species level.

C. Antimicrobial susceptibility testing.

Minimal inhibitory concentrations of clinical antimicrobials for all isolates were determined by MicroScan (see above) and were interpreted according to Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS, 2006) guidelines for broth microdilution methods. *Enterococcus faecalis* ATCC 29212, *E. faecalis* ATTC 51299, *E.*

faecium ATCC 51559 were included as controls. High-level vancomycin resistance for all enterococci and endpoints for quinupristin-dalfopristin resistance for *E. faecium* were determined by Etest (AB Biodisk, Piscataway, NJ) on Mueller-Hinton agar (Difco, Sparks, MD). The MIC for virginiamycin was determined by agar diffusion testing on Mueller-Hinton medium containing 0.125 to 32 μg/mL virginiamycin M1 (Sigma, St. Louis, MO). Virginiamycin-resistant isolates *E. faecium* CVM 3001 and *E. faecium* CVM 3002, kindly provided by Dr. David White (Center for Veterinary Medicine, USFDA, Laurel, MD), were used as controls. Agar diffusion and Etests for MIC determination were performed in duplicate.

D. Multiplex PCR for selected antibiotic resistance genes and virulence determinants.

PCR was performed to detect the vancomycin resistance genes *vanA* and *vanB*, the streptogramin resistance genes *vatD* and *vatE*, the virulence genes for aggregation substance (*agg*), gelatinase production (*gelE*) and sex pheromone (*cpd*), and the species-specific superoxide dismutase gene (*sodA*). Primer sets and PCR conditions for all genes are given in Table 2.1 and were selected and based on previously published data (Dutka-Malen et al., 1995, Soltani et al., 2000; Eaton and Gasson, 2001; Elsayed et al., 2001; Jackson et al., 2004). Detection of *vanA, vanB, vatD, vatE* and *agg, gelE, cpd* was done in two multiplex procedures, while PCR for *sodA* was performed using one primer set. PCR assays were performed in a total volume of 25 μL containing 4 μL whole-cell suspension as a template, 0.4 μM (each) primer, and 1.25U *GoTaq* DNA polymerase (Promega, Madison, WI). All reactions were carried out in a Perkin-Elmer GeneAmp PCR system 2400 (Perkin-Elmer, MA) under the following conditions: initial

denaturation (94°C, 5 min); 30 cycles of denaturation (94°C, 30s), annealing (appropriate temperature, 1 min), and elongation (72°C, 1 min) followed by a final extension (72°C, 7 min). Electrophoresis of 10 μL of the PCR product was performed on a 1.5% agarose gel containing 0.5 μL of ethidium bromide and visualized by UV transillumination**.** The following strains were used as positive controls for PCR reactions: *E. faecium* ATCC 51559 (*vanA* and *sodA*), *E. faecalis* ATCC 51299 (*vanB, sodA, agg, cpd,* and *gelE*), and *E. faecium* strains CVM 3001 and 3002 for *vatE* and *vatD*, respectively.

TABLE 2.1 Primer sets for multiplex PCR protocols used in this study.

Gene	Primer Name	Sequence (5'-3')	Product size (bp)	Reference(s)
Antibiotic resistance genes multiplex PCR:				
vanA	vanA1-F	GGGAAAACGACAATTGC	732	Dutka-Malen et al.
	vanA2-R	GTACAATGCGGCCGTTA		(1995)
vanB	vanB1-F	AAGCTATGCAAGAAGCCATG	536	Elsayed et al.
	vanB2-R	CCGACAATCAAATCATCCTC		(2001)
vatE	vatE1-F	ACTATACCTGACGCAAATGC	511	Soltani et al.
	vanE2-R	GGTTCAAATCTTGGTCCG		(2000)
vatD	vatD1-F	GCTCAATAGGACCAGGTGTA	271	Soltani et al.
	vatD2-R	TCCAGCTAACATGTATGGCG		(2000)
Virulence genes multiplex PCR:				
agg	agg1-F	AAGAAAAAGAAGTAGACCAAC	1553	Eaton, Gasson
	agg2-R	AAACGGCAAGACAAGTAAATA		(2001)
cpd	cpd1-F	TGGTGGGTTATTTTTCAATTC	782	Eaton, Gasson
	cpd2-R	TACGGCTCTGGCTTACTA		(2001)
gelE	gelE1-F	ACCCCGTATCATTGGTTT	419	Eaton, Gasson
	gelE-2R	ACGCATTGCTTTTCCATC		(2001)
Species specific genes multiplex PCR:				
sodA	fs1-F	ACTTATGTGACTAACTTAACC	360	Jackson et al.
	fs1-R	TAATGGTGAATCTTGGTTTGG		(2004)
sodA	fm1-F	GAAAAAACAATAGAAGAATTAT	215	Jackson et al.
	fm1-R	TGCTTTTTTGAATTCTTCTTTA		(2004)

IV. RESULTS

A. Identification of Enterococcus *from food, clinical, and animal sources.*

A total of 15 *Enterococcus* isolates (6 *E. faecium* and 9 *E. faecalis*) were obtained from salad samples. Enterococci were distributed among each of the eight salad types with no species isolated from any one particular salad type. Of the clinical enterococci, 29 of the 32 isolates (81% blood, 100% rectal) were identified as *E. faecium,* while 3 isolates (19% blood, 0% rectal) were *E. faecalis.* The genus and species of the 10 *E. faecium* and 7 *E. faecalis* was confirmed by repeat testing. All isolates were identified to the species level by MicroScan with a 99% or greater probability.

B. Minimal inhibitory concentration of antimicrobial agents.

Table 2.2 summarizes the antimicrobial susceptibility data for the 64 enterococcal isolates. All of the food and animal enterococci were susceptible to vancomycin, with MIC values ≤ 2 μg ml^{-1}. The MIC values for vancomycin among all of the clinical enterococci exceeded 16 μg ml^{-1}, with 29 (100%) of *E. faecium* and 2 (67%) of *E. faecalis* demonstrating high-level resistance (≥ 256 μg ml^{-1}). Susceptibility patterns to Q/D and virginiamycin correlated among all isolates. All of the *E. faecalis* were resistant to streptogramin antibiotics, indicative of intrinsic resistance, while a higher level of streptogramin resistance was observed in *E. faecium* isolated from food and animal sources (83% and 40% respectively) than in clinical *E. faecium* (10%). Additionally, *E. faecium* salad isolates were most resistance to streptogramin antimicrobial agents than to any of the additional antibiotics tested. Animal *E. faecium* isolates demonstrated the greatest percentage resistance to erythromycin (80%), followed by rifampin (70%) and

streptogramin (40%). A level of erythromycin resistance was present among animal *E. faecalis* (86%). All isolates were susceptible to linezolid, with the exception of one rectal *E. faecium* with an intermediate MIC of 4 μg ml^{-1}.

TABLE 2.2 Percent of enterococcal isolates resistant to vancomycin, streptogramin, and selected antibiotics

		% Resistant isolates[b]					
		Salad (n=15)		Animal (n=17)		Clinical (n=32)	
Antibiotic	MIC (μg mL^{-1}) [a]	*E. faecium*	*E. faecalis*	*E. faecium*	*E. faecalis*	*E. faecium*	*E. faecalis*
Vancomycin	≤2 –>16	0	0	0	0	100	100
Vanco-HL	0.016– 256	nt	nt	nt	nt	100	67
Synercid	≤0.25–>2	83	100	40	100	10	100
Virginiamycin	0.125– 16	83	100	40	100	10	100
Ampicillin	≤8–≥16	0	0	0	0	100	0
Ciprofloxacin	≤1–≥4	33	0	30	0	100	100
Erythromycin	≤0.5–≥8	67	8	80	86	100	100
Levofloxacin	≤2–≥4	0	0	0	0	100	100
Linezolid	≤2–≥8	0	0	0	0	3	0
Penicillin	≤8–>8	0	0	0	0	100	0
Rifampin	≤1–>2	67	3	70	43	93	33
Tetracycline	≤4–≥16	0	6	30	43	45	33

[a]Range for susceptible and resistant MICs as indicated in Clinical Laboratory Standards Institute Document M100-S16 Vol. 26 No. 3 (2006); [b]Reported for intermediate and resistant MIC values; nt, not tested; HL, high-level resistance

C. Distribution of antibiotic resistance genes and selected virulence determinants as detected by PCR.

All isolates were tested for the presence of the vancomycin resistance genes *vanA* and *vanB* and the streptogramin resistance genes *vatD* and *vatE*. Of the clinical *E. faecium*, 13 (100%) from blood and 15 (94%) from rectal sources carried *vanA* with only one isolate from each source positive for *vanB*. The *vanA* gene was detected in one of the three clinical *E. faecalis* blood VRE and *vanB* in the remaining two isolates. The *vatD* and *vatE* genes were not detected any of the streptogramin-resistant enterococci, regardless of source.

Table 2.3 summarizes the PCR and phenotypic screening results for the selected virulence determinants. Overall, *agg, cpd,* and *gelE* genes were only detected in *E. faecalis*. Among 32 total VSE, eight (25%) of the isolates, five from food and three from animals, possessed all three genes. Additionally, all of the VSE isolated from either food or animal sources had a combination of two of the three determinants. The *gelE* gene was detected in 100% of food VSE; this gene was associated with eight (89%) of the nine VSE that also carried the sex pheromone *cpd* and five (83%) of six food VSE that carried the *agg* determinant. Among the animal VSE, all three genes were detected in three (43%) of the isolates. Six (86%) of the animal isolates carried *cpd* and five (71%) carried *gelE*. The three clinical VRE carried all three virulence determinants. The *gelE* gene was identified but not expressed phenotypically in one salad VSE and one clinical VRE; both of these strains possessed all three virulence genes. Overall, the percentages of streptogramin-resistant enterococci carrying virulence genes were greatest among the food isolates than either the animal or clinical strains (Figure 2.1).

TABLE 2.3 Percent incidence of antibiotic resistance and virulence genes by PCR and phenotypic screening[a]

Gene	Salad		Animal		Clinical	
	E. faecium	*E. faecalis*	*E. faecium*	*E. faecalis*	*E. faecium*	*E. faecalis*
vanA	0	0	0	0	97	33
vanB	0	0	0	0	3	67
vatD	0	0	0	0	0	0
vatE	0	0	0	0	0	0
agg	0	67	0	57	0	100
cpd	0	89	0	86	0	100
*gelE**	0	100 (89)	0	71 (71)	0	100 (67)

[a]Percent of phenotypically positive isolates given in parentheses

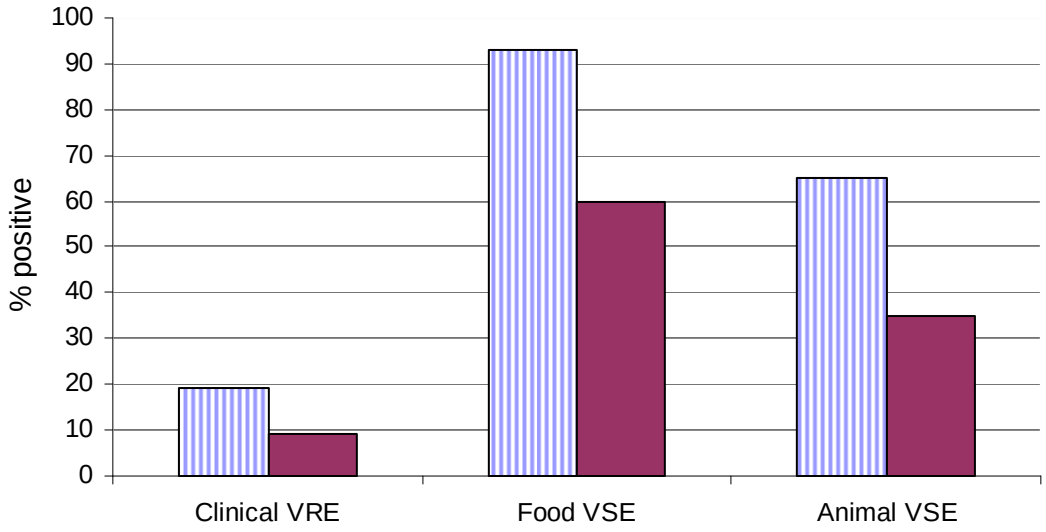

FIGURE 2.1 Summary of total *Enterococcus* isolates demonstrating streptogramin resistance and detection of *agg*, *cpd*, and/or *gelE*. Streptogramin resistance (III) and detection of *agg*, *cpd*, and/or *gelE* (□).

V. DISCUSSION

Multiple pressures contribute to the complex origin of resistant enterococci which differ geographically and genotypically. *Enterococcus* is isolated at a rate of 10^4-10^8 CFU/100 cm^2 in swine and poultry carcasses (Giraffa, 2002), and on the retail level, in up to 100% of ground beef and 97% of pork (Hayes et al., 2003). A high-level of Q/D resistance in VanA-type *E. faecium* from humans and poultry has been reported (Hayes *et al.* 2001), but little is known about the impact of streptogramin use in animal husbandry and the emergence of resistance due to limited data (Butaye et al., 2000; Robredo et al., 2000, Klare et al., 2003). Multi-component salads represent a food vehicle that have the potential to harbor enterococci from multiple sources and consequently may serve as a reservoir of resistant bacteria. Additionally, the nature of self-service salad bars may lead to an increase in bacterial loads due to multiple transfers of product, improper storage, temperature abuse, or contamination through colonized food handlers.

Although *E. faecalis* is more prevalent in clinical isolates, *E. faecium* accounts for between 85-95% of vancomycin-resistant enterococcal strains (McDonald, 2006), and this was reflected in 91% of the species distribution of the clinical VRE isolated in this study. The majority of these isolates were VanA type, and all of the clinical strains exhibited high-level vancomycin resistance whether due to *vanA* or *vanB.* The isolation of relatively equal numbers of both *E. faecium* and *E. faecalis* from salads is important when considering that the potential for high-level vancomycin resistance to be conferred among multiple reservoirs is not limited to intra-species spread, and that VRE isolates from human and nonhuman sources demonstrate similar transposable elements that confer resistance via horizontal transmission (Woodford et al., 1998). The higher

number of salad isolates that were shown to be resistant to Q/D and virginiamycin as compared with the other antibiotics tested suggests that enterococci from animal products or humans treated with streptogramin antimicrobials may persist and thrive in multi-component foods. Among the animal isolates, higher levels of erythromycin resistance in both *E. faecium* and *E. faecalis* may be an indication that streptogramin resistance is due to genes such as *erm* that encodes resistance to streptogramin B. Since 67% of salad isolates were also resistant to erythromycin, further studies are indicated to detect the presence of additional streptogramin resistance determinants.

Streptogramin resistance was demonstrated by all of the *E. faecalis* isolates independent of susceptibility to vancomycin, and this was expected due to intrinsic resistance mechanisms in this species. The *vatD* or *vatE* genes were not detected in any of the streptogramin-resistant *E. faecium,* indicating that an alternative mechanism of streptogramin A resistance is present. In a recent study of *E. faecium* from poultry production environments, Hayes et al. (2005) failed to detect the acetyltransferase genes as well as the streptogramin B hydrolase-encoding gene *vgb(A)* in any of the virginiamycin-resistant isolates tested, indicating that the mechanisms of streptogramin resistance in these populations remain unidentified.

Among the clinical *E. faecium,* the relatively low resistance rate to Q/D was most likely due to the use of synergistic treatment approaches and/or use of linezolid for multi-drug resistant VRE infections. However, it is important to note that few of clinical *E. faecium* were susceptible to any other drugs besides linezolid and Q/D. Recently, resistance to linezolid has emerged in VRE, VSE and methicillin-resistant *Staphylococcus aureus* (Gonzales et al., 2001; Tsiodras et al., 2001; Marra et al., 2006).

Horizontal transfer of streptogramin resistance between *E. faecalis* and *E. faecium* has been demonstrated in vitro, and studies have indicated that dissemination of cross-resistance resistance genes for Q/D is possible via enterococcal reservoirs (Simjee et al., 2002; Kieke et al., 2006). Among our clinical isolates, one vancomycin-resistant, linezolid-intermediate *E. faecium* was identified from a rectal source that was only susceptible to Q/D among all other antibiotics tested, demonstrating the importance of streptogramin susceptibility despite availability of linezolid for VRE infections. Among 193 *E. faecium* isolated from inpatients in our facility in 2006, antibiograms indicate the following cumulative susceptibilities: vancomycin, 25%; Q/D, 90%; linezolid, 97%; and, for four isolates, daptomycin, 75%.

In this study, the high-level of streptogramin resistance exhibited by the majority of salad VSE isolates could be a result of products associated with the agricultural use of virginiamycin, such as the multi-component ingredients (mayonnaise, eggs, chicken cubes) of virginiamycin-fed poultry. The percentage of high-level virginiamycin-resistant VS *E. faecium* from food and animals was greater than the percentage of Q/D-resistant VRE from inpatients while remaining susceptible to most other antibiotics tested with the exception of erythromycin. Although none of the food or animal isolates carried *vatD* or *vatE*, the potential exists for these isolates to serve as conjugative partners with high-level resistant clinical VRE for the horizontal transfer of resistance elements such as *vanA* or *vanB* should contamination of food sources occur. Since the majority of the clinical VRE were also multi-drug resistant, the horizontal transfer of streptogramin resistance into emerging linezolid-intermediate or resistant VRE would leave few to no treatment alternatives.

The virulence of VRE and streptogramin-resistant enterococci depends not only on the presence of resistance genes, but also on factors that promote adherence and colonization (Mundy et al., 2000). In enterococci, virulence factor proteins that are involved in cellular aggregation, invasion of host tissues, and immunoevasion are generally encoded by genes carried on conjugative plasmids, particularly those associated with pheromone response (Clewell 1981). The sex pheromone gene *cpd* was selected based on prior studies indicating that carriage of sex pheromone genes (*cpd, cob, ccf,* and *cad*) in starter, food, and clinical *E. faecalis* was identical (Eaton and Gasson, 2001). Our data show the presence of this gene in all of our *E. faecalis* isolates, both VRE and VSE, with the exception of one animal strain. The particular presence of sex pheromone gene in the VSE from salads is an indicator that enterococci from multi-component foods have the potential to engage in conjugative plasmid transfer. Since the food isolates also included *E. faecium,* it is possible that horizontal transmission of virulence genes could occur via interspecies transfer within the multi-component salads.

Studies have shown that the sex pheromone genes in *E. faecalis* starter strains and a majority of food and clinical strains are also present with *agg* genes (Eaton and Gasson, 2001; Creti et al., 2004). This was the also the case with the majority of our VS food and animal *E. faecalis* isolates as well the three clinical VR *E. faecalis*. The production of aggregation substance and gelatinase by a majority of *E. faecalis* isolates from hospitalized patients indicates that antibiotic-resistant nosocomial pathogens may be selected for by the presence of particular virulence determinants present on plasmids (Mundy et al., 2000). Likewise, our data show that the selected virulence determinants *agg, cpd,* and *gelE* were only present on *E. faecalis* strains. A high percentage of these

VSE isolates carried both the *cpd* and *agg* and/or *gelE* genes, and only one VSE isolate carried a silent *gelE* gene.

Recent evolutionary genetic studies of VRE and VSE from human and nonhuman sources have identified a genetic lineage of *E. faecium* with global distribution (Willems et al., 2005). Our results indicate that multi-component salads contain streptogramin-resistant VSE that may also possess conjugative and virulence genes necessary for conjugative transfer and host tissue invasion. Although the incidence of fecal carriage of VRE is relatively low, the potential exists for horizontal-transmission to occur, particularly in environments such as hospital food service facilities where persistence of enterococci may facilitate transfer events.

The continued use of virginiamycin by U.S. farmers and developing resistance of VRE to alternative drugs, including streptogramin antibiotics, further complicates resistance trends and treatment options. To our knowledge, this is the first study to characterize enterococci from multi-component deli salads. In order to assess the safe, continued use of enterococci in foods and as growth promoters in animals, a thorough understanding of the resistance profiles and virulence traits among enterococci that may coexist in heterogeneous food environs is essential.

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CHAPTER 3

HORIZONTAL TRANSFER OF HIGH-LEVEL *VANA*-MEDIATED RESISTANCE AMONG COMMENSAL AND STREPTOGRAMIN-RESISTANT *ENTEROCOCCUS* ISOLATES FROM MULTI-COMPONENT DELI SALADS

The work presented in this chapter was submitted as a full manuscript for publication in the International Journal of Food Microbiology in August, 2007.

I. ABSTRACT

The commercial use of *Enterococcus* as a starter culture and probiotic in foods, the agricultural use of the growth promoter virginiamycin in the United States, and the emergence of quinupristin-dalfopristin (Q/D)-resistant nosocomial vancomycin-resistant enterococci (VRE) raise concern over the potential for resistance to be spread via horizontal bacterial transfer in food. The potential for transfer of *vanA* or *vanB* from four Q/D-resistant clinical VRE donors to vancomycin-susceptible enterococci (VSE) recipients isolated from deli salads and animals was investigated. Antibiotic profiles were determined for donors, recipients, and transconjugants by automated microtiter dilution methods and E-test. Isolates were tested by PCR for the presence of virulence genes *agg, cpd,* and *gelE,* and the streptogramin resistance genes *vatD* and *vatE* using appropriate controls. Filter and broth matings were performed using a 1:1 donor to recipient ratio and transconjugants were selected on agar containing vancomycin and the appropriate

selective antibiotic(s). Filter mating experiments resulted in the successful transfer of *vanA* between an *agg*$^{+}$*cpd*$^{+}$*gelE*$^{+}$ *E. faecalis* clinical donor and an *agg*$^{-}$*cpd*$^{-}$*gelE*$^{-}$ streptogramin-susceptible *E. faecium* salad recipient at a frequency of 10^{-8} per recipient. This transconjugant mated with a two- to four-log-fold greater frequency to streptogramin-resistant salad and animal *E. faecalis* and *E. faecium* that contained all three virulence genes and to two streptogramin-resistant animal *E. faecalis* isolates carrying *vatD* and *vatE* but none of the virulence genes. Results showed that primary and secondary transconjugants maintained high-level vancomycin and streptogramin resistance with no transfer of virulence genes. Experiments demonstrated that commensal enterococci from multi-component salads can receive and disseminate *vanA* to streptogramin-resistant enterococcal recipients that carry virulence genes.

II. INTRODUCTION

The enterococci are Gram-positive, non-sporeforming, facultative anaerobic lactic acid bacteria that are commensal enteric flora of humans and animals. They have been isolated from a wide range of environmental niches, including soil, sewage, grasses, and seawater (Muller et al., 2001; Giraffa, 2002; Guardabassi and Dalsgaard, 2004). In foods, the enterococci have a long history of use as starter cultures in the production of traditional European cheeses and fermented sausages (Foulquie Moreno et al., 2006). The thermotolerant and acidophilic nature of enterococci enable their survival in milk throughout pasteurization and cheese fermentation, during which their proteolytic and lipolytic activities contribute significantly to the ripening process and flavor development (Giraffa, 2003). The additional benefit of bacteriocin production makes enterococci

useful as protective cultures against spoilage bacteria or pathogens in the cheese (Ennahar and Deschamps, 2000).

The ability for enterococci to survive and persist in a variety of settings facilitates the horizontal transfer of genetic determinants that lead to antibiotic resistance, virulence in the host, and conjugative processes among enterococci and between enterococci and commensal flora (Franz et al., 2003). Global spread of mobile resistance elements from nosocomial and commensal enterococci to more virulent pathogens is a major public health concern. The emergence of vancomycin-resistant enterococci (VRE) over the past decades leaves few treatment options for patients, resulting in significant increases in length of inpatient stay and cost of patient care (Pelz et al., 2002). The recent horizontal transfer of the vanA resistance gene from clinical VRE to methicillin-resistant *Staphylococcus aureus* (MRSA) has produced novel clinical pathogens with high-level resistance to vancomycin (Chang et al., 2003; Weigel et al., 2003; Tenover et al., 2004). Although fecal carriage of VRE among healthy people in the United States is relatively low, a relatively large European community reservoir exists both in humans and animals due to the former agricultural use of avoparcin. (Coque et al.; 1996 Rice et al., 2003). Concerns that similar resistance to current antibiotics used to treat VRE, including the streptogramin compound quinupristin-dalfopristin (Q/D; Synercid) will emerge due to the continued use of streptogramin growth-promoting antibiotics in the United States such as virginiamycin (Smith et al., 2003). Although streptogramin resistance genes have been identified, development of Q/D resistance in animals given virginiamycin is known to occur where currently known resistance elements have not been detected (McDermott et al., 2005).

Evolutionary genetic studies of VRE and vancomycin-susceptible enterococci (VSE) from human and nonhuman sources have indicated that a common genetic lineage exists among *E. faecium* isolated globally (Willems et al., 2005). To assess the safe, continued use of both enterococci in foods and growth promoters in animals, a thorough understanding of the factors promoting transfer of resistance elements among enterococci from multiple sources is essential. This study examined the potential for enterococci isolated from multi-component deli salads to serve as in intermediate in the dissemination of *vanA* between clinical enterococci and enterococci of food and animal origin. Multi-component salads from self-service bars were selected on the basis that this type of food contains raw ingredients from diverse sources, has the potential to have many handlers, and may become contaminated more quickly than other ready-to-eat foods due to multiple transfers and various holding conditions.

III. MATERIALS AND METHODS

A. Bacterial strains.

Food enterococci were isolated from multi-component deli salads collected from self-service salad bars in nine large retail supermarkets in the central New Jersey region (refer to Appendix A for specific locations and strains). Salad types included creamy vegetable and pasta salads (potato, macaroni, coleslaw), meat/poultry (chicken, egg, liver), and fish (tuna, seafood). All products were collected in store-provided containers and transported on ice to the laboratory for immediate processing. Clinical isolates were obtained from positive blood cultures of hospitalized inpatients at Robert Wood Johnson University Hospital, New Brunswick, New Jersey, from September through October,

2005. Animal enterococci were isolated from cows or milk from cows with clinical mastitis and were a generous gift from Dr. Joseph Hogan (OARDC Mastitis Laboratory, Ohio State University). Strains used in mating experiments were selected based on antimicrobial resistance profiles and presence or absence of selected virulence determinants. The complete list of strains available for the study is given in Appendix B. Control strains for antimicrobial susceptibility and PCR included *E. faecium* ATCC 51559 (*vanA*$^+$*efaA*$_{fm}$$^+$), *E. faecalis* ATTC 51299 (*vanB*$^+$*agg*$^+$*gelE*$^+$*cpd*$^+$), *E. faecium* CVM 3001 (*vatE*$^+$) and *E. faecium* CVM 3002 (*vatD*$^+$). In mating experiments, the rifampin- and fusidic acid-resistant strain *E. faecalis* JH2-2 was included as a reference recipient (Jacob and Hobbs, 1974). *E. faecium* CVM 3001 and CVM 3002 were also used as virginiamycin-resistant recipient strains in secondary mating experiments.

B. Enrichment and isolation of enterococci from multi-component salads.

For selection of *Enterococcus* from food samples, 250 mL of buffered peptone water was added to 25 g of salad in a sterile stomacher bag. Samples were processed in a Lab-Blender 400 stomacher (Seward Medical, London, UK) for two minutes, and fifty mL of the rinsate was added to sterile flasks containing 50 mL of double-strength Enterococcosel broth (BBL Microbiology Systems, Cockeysville, MD). Following incubation at 37^{o}C for 24-48 h, flasks containing blackened broths were subcultured to Enterococcosel agar (BBL) and incubated at 35^{o}C for 18-24 h. Enterococcal-like colonies (characterized by blackening of the agar) from each plate were Gram-stained and tested for catalase activity. Isolated colonies containing catalase-negative, Gram-positive cocci were streaked onto Todd-Hewitt agar (BBL) with 3% gelatin to test for

gelatinase production. Plates were incubated overnight at 37°C, placed at 4°C for 4 h, and then examined for an opaque zone around each colony. Presumptive *Enterococcus* isolates were identified to the species level using a Microscan Gram-Positive/MIC Combo Panel and Walk/Away 96 SI system (Dade Behring, Sacramento, CA).

C. Antimicrobial susceptibility testing.

MicroScan was used to determine antimicrobial susceptibilities according to Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS, 2006) guidelines for broth microdilution methods. High-level vancomycin resistance for all enterococci and endpoints for quinupristin-dalfopristin resistance for *E. faecium* were determined by Etest (AB Biodisk, Piscataway, NJ) on Mueller-Hinton agar (Difco, Sparks, MD). Minimal inhibitory concentration to virginiamycin and fusidic acid was determined by standard agar diffusion testing on Mueller-Hinton medium containing 0.125 to 32 μg/mL virginiamycin M1 (Sigma, St. Louis, MO) or 20 μg mL^{-1} fusidic acid (Axxora LLC, San Diego). Agar diffusion and Etests for MIC determination were performed in duplicate.

D. Multiplex PCR for selected antibiotic resistance genes and virulence determinants.

Primer sets used in PCR reactions to detect the target genes were based on previously published data and are listed in Table 3.1. Detection of *vanA/vanB* and/or *vatD/vatE* was performed on isolates demonstrating vancomycin- and/or streptogramin-resistance. All isolates were tested for the presence of selected determinants for aggregation substance (*agg*), gelatinase (*gelE*), sex pheromone (*cpd*), and the species-specific superoxide dismutase gene (*sodA*). Additionally, *E. faecium* isolates were tested

for *efaA*$_{fm}$ (enterococcal antigen A). Detection of *vanA, vanB, vatD, vatE* and *agg, gelE, cpd* was done in two multiplex procedures, while PCR for *sodA* and *efaA* were performed as single reactions. For all assays, a total volume of 25 μL reaction mix containing 4 μL whole-cell suspension as template, 0.4 μM (each) primer, and 1.25U *GoTaq* DNA polymerase (Promega, Madison, WI) was used. Reactions were carried out in a Perkin-Elmer GeneAmp PCR system 2400 (Perkin-Elmer, MA) under the following conditions: initial denaturation (94^{o}C, 5 min); 30 cycles of denaturation (94^{o}C, 30s), annealing (appropriate temperature, 1 min), and elongation (72^{o}C, 1 min) followed by a final extension (72^{o}C, 7 min). Electrophoresis of 10 μL of the PCR product was performed on a 1.5% agarose gel containing 0.5 μL of ethidium bromide and visualized by UV transillumination**.**

TABLE 3.1 Primer sets for multiplex PCR protocols of *Enterococcus* strains used in mating experiments

Gene	Primer Name	Sequence (5'-3')	Product size (bp)	Reference(s)
Antibiotic resistance genes multiplex PCR:				
vanA	vanA1-F	GGGAAAACGACAATTGC	732	Dutka-Malen et
	vanA2-R	GTACAATGCGGCCGTTA		al. (1995)
vanB	vanB1-F	AAGCTATGCAAGAAGCCATG	536	Elsayed et al.
	vanB2-R	CCGACAATCAAATCATCCTC		(2001)
vatE	vatE1-F	ACTATACCTGACGCAAATGC	511	Soltani et al.
	vanE2-R	GGTTCAAATCTTGGTCCG		(2000)
vatD	vatD1-F	GCTCAATAGGACCAGGTGTA	271	
	vatD2-R	TCCAGCTAACATGTATGGCG		
Virulence genes multiplex PCR:				
agg	agg1-F	AAGAAAAAGAAGTAGACCAAC	1553	Eaton, Gasson
	agg2-R	AAACGGCAAGACAAGTAAATA		(2001)
cpd	cpd1-F	TGGTGGGTTATTTTTCAATTC	782	
	cpd2-R	TACGGCTCTGGCTTACTA		
gelE	gelE1-F	ACCCCGTATCATTGGTTT	419	
	gelE-2R	ACGCATTGCTTTTCCATC		
E. faecalis antigen A:				
$efaA_{fm}$	efaA1-F	AACAGATCCGCATGAATA	735	Eaton, Gasson
	efaA2-R	CATTTCATCATCTGATAGTA		(2001)
Species specific genes multiplex PCR:				
sodA	fs1-F	ACTTATGTGACTAACTTAACC	360	Jackson et al.
	fs1-R	TAATGGTGAATCTTGGTTTGG		(2004)
sodA	fm1-F	GAAAAAACAATAGAAGAATTAT	215	
	fm1-R	TGCTTTTTTGAATTCTTCTTTA		

E. Mating experiments.

Matings were performed in duplicate at 37°C. Secondary mating experiments were carried out at 4°C, 10°C, 22°C, and 37°C. Broth matings were done according to the procedure by Clewell *et al.* (1985). Briefly, overnight cells grown in brain heart infusion (BHI) broth were mixed in a 1:10 donor-recipient ratio (50 μL donor and 500 μL

recipient in 4.5 mL BHI broth) and incubated with agitation at the appropriate temperature for 4 h. Filter matings were performed using a 1:1 donor- recipient ratio diluted in BHI broth and onto a 0.22 μm pore size filter on BHI agar, then incubated overnight at the appropriate temperature. Cells were harvested by washing filters in 1 mL BHI and then spread-plated on media containing 6 μg mL^{-1} vancomycin HCl (Sigma), and the appropriate selective antibiotic: 20 μg mL^{-1} fusidic acid and 20 μg mL^{-1} rifampin (Sigma) and/or 32 μg mL^{-1} nitrofurantoin (Alfa Aesar, Ward Hill, MA) for primary transconjugants or 10 μg mL^{-1} tetracycline HCl (Acros Organics, Morris Plains, NJ) for secondary transconjugants. For additional verification that presumptive transconjugant colonies were recipients rather than donors, 3% gelatin was included in selective agar plates for recipient strains that exhibited the gelatinase phenotype. Plates were incubated at 37^{o}C for 72 h and examined daily. From any plate exhibiting growth, one to six colonies were selected at random and subcultured to BHI agar. Presumptive transconjugants were identified to the species level by MicroScan and tested for gelatinase activity as above.

IV. RESULTS

A. Identification and selection of Enterococcus *donor and recipient strains from food, clinical, and animal sources.*

All isolates were identified by MicroScan to the species level with a 99% or greater probability. Strains used in mating experiments were based on appropriate antimicrobial susceptibility patterns for selection and are listed in Table 3.2. Three *E. faecalis* (C1, C2, and C3) and one *E. faecium* (C9) demonstrating high-level vancomycin

resistance (≥256 μg mL^{-1}) and sensitivity to ≥20 μg mL^{-1} fusidic acid were used as donor cells. Additionally, C1, C2, and C9 were susceptible to ≤1 μg mL^{-1} rifampin and C3 was susceptible to ≤4 μg mL^{-1} tetracycline. All recipient enterococci demonstrated susceptibility to ≤2 μg mL^{-1} vancomycin. For detection of transconjugants, salad and bovine recipients demonstrated resistance to ≥32 μg mL^{-1} nitrofurantoin (all *E. faecium*), >2 μg mL^{-1} rifampin (S1, S4, S8 and all bovine VSE), and/or ≥10 μg mL^{-1} tetracycline (S6, B2, B5, CVM 3001, and CVM 3002). Susceptibility to virginiamycin was the same as to Q/D for all enterococci. Intrinsic resistance was observed in all *E. faecalis*, while three of the salad and two of the bovine *E. faecium* were streptogramin resistant.

B. Silent gelE.

Gelatinase production on Todd-Hewitt agar with 3% gelatin was only observed in *E. faecalis.* Of those isolates in which *gelE* was detected, two (C1 and CVM 3002) were negative for gelatinase production.

C. Detection of antibiotic resistance genes and virulence determinants by PCR.

Results of PCR screening for selected genes are shown in Table 3.2. Species identification was verified for all isolates by detection of *sodA* using species-specific primers. The four clinical VRE strains harbored either *vanA* (C2 and C9) or *vanB* (C1, C3). Virginiamycin acetyltransferase genes *vatD* and *vatE* were detected in none of the isolates other than *E. faecium* CVM 3001 and CVM 3002. Virulence determinants *agg, cpd,* and *gelE* genes were detected together in all but one (B13) *E. faecalis* regardless of source. The enterococcal antigen A determinant $efaA_{fm}$ was detected in all *E. faecium*

isolates. In primary and secondary transconjugants, recipient gene characterization remained the same with the exception of *vanA* or *vanB.*

TABLE 3.2 Summary of PCR results characterizing donor and recipient isolates used in mating experiments

Strain	Source	Species	Genotype[a]	Selective phenotype[b]
Clinical Donors				
C1	Inpatient blood	*E. faecalis*	*vanB*$^+$*agg*$^+$*cpd*$^+$(*gelE*$^+$)	Rf^SFd^S
C2	Inpatient blood	*E. faecalis*	*vanA*$^+$*agg*$^+$*cpd*$^+$*gelE*$^+$	Rf^SFd^S
C3	Inpatient blood	*E. faecalis*	*vanB*$^+$*agg*$^+$*cpd*$^+$*gelE*$^+$	Rf^SFd^S
C9	Inpatient blood	*E. faecium*	*vanA*$^+$*agg*$^+$*cpd*$^+$*gelE*$^+$	Rf^SFd^S
Salad Recipients				
S1	Macaroni salad	*E. faecium*	$efaA_{fm}^+$	Rf^RFd^R
S2	Seafood salad	*E. faecium*	$efaA_{fm}^+$	Rf^R
S4	Macaroni salad	*E. faecium*	$efaA_{fm}^+$	Rf^RFd^R
S6	Egg salad	*E. faecalis*	*agg*$^+$*cpd*$^+$*gelE*$^+$	Rf^RTe^R
S8	Coleslaw	*E. faecium*	$efaA_{fm}^+$	Rf^RFd^R
Animal Recipients				
B2	Bovine mastitis	*E. faecium*	$efaA_{fm}^+$	Rf^RTe^R
B3	Bovine mastitis	*E. faecium*	$efaA_{fm}^+$	Rf^RFd^R
B5	Bovine mastitis	*E. faecium*	$efaA_{fm}^+$	Rf^RTe^R
B7	Milk	*E. faecalis*	*agg*$^+$*cpd*$^+$*gelE*$^+$	Fd^R
B8	Bovine mastitis	*E. faecium*	$efaA_{fm}^+$	Fd^R
B9	Bovine mastitis	*E. faecium*	$efaA_{fm}^+$	Rf^RFd^R
B13	Milk	*E. faecalis*		Rf^R
B16	Milk	*E. faecalis*	*agg*$^+$*cpd*$^+$*gelE*$^+$	Rf^RFd^R
Control Recipients				
JH2-2	Reference strain	*E. faecalis*	*cpd*$^+$(*gelE*$^+$)	Rf^RFs^R
CVM 3001	Reference strain	*E. faecium*	*vatE*$^+$ $efaA_{fm}^+$	Te^R
CVM 3002	Reference strain	*E, faecium*	*vatD*$^+$ $efaA_{fm}^+$	Te^R

[a]Phenotypically negative strains shown in parentheses; [b]Rf, rifampin (20 μg mL^{-1}); Fd, nitrofurantoin (32 μg mL^{-1}); Te, tetracycline (10 μg mL^{-1}); Fs, fusidic acid (20 μg mL^{-1}); S, susceptible; R, resistant

D. Generation of primary and secondary transconjugants.

Results of broth and filter matings are given in Table 3.3. In primary mating experiments, transfer of *vanA* occurred between *E. faecalis* C2 to an *E. faecium* salad recipient (S4) by filter mating at 37^{o}C with an average conjugation frequency of 10^{-8} per recipient. Transconjugants were not observed among any of the other primary conjugative pairs either by broth or filter mating at any of the four incubation temperatures. Upon secondary mating using the primary transconjugant (T2G) as the donor, transfer of *vanA* by filter mating was successful with each of the four recipient enterococci tested (S6, B12, CVM 3001, and CVM 3002) and by broth mating with two of the recipients (CVM 3001 and CVM 3002). Average frequency of transfer increased two- to four-log-fold, with an average frequency of transfer ranging from 10^{-4} to 10^{-6} per recipient. Antimicrobial resistance profiles for transconjugants varied from parent strains in both primary and secondary matings. In all cases, transconjugants showed an increase in number of resistant MIC values than compared with the donors and recipients (Table 3.4). In all cases, high-level resistance to vancomycin was maintained.

TABLE 3.3 Results of primary and secondary mating experiments

Donor	Recipient(s)	Transconjugant	Frequency[a]	Method
Primary Mating				
C1	S2, S4, S8, B2, B3, B7, B8, B9, B13, B16, JH2-2	None	NA	NA
C2	S1, S2, S4, S8, B2, B3, B7, B8, B9, B13, B16, JH2-2	T2G (C2 x S4)	10^{-8}	Filter, 37°C
C3	S2, S4, S8, B2, B7, B8, B9, JH2-2	None	NA	NA
C9	S1, S4, S8, B2, B3, B13, B16, JH2-2	None	NA	NA
Secondary Mating				
T2G	S6	T7A	10^{-5}	Filter, 37°C
	B12	T7B	10^{-8}	Filter, 37°C
	CVM3001	T7D/T9A	$10^{-5}/10^{-6}$	Filter/broth, 37°C
	CVM3002	T7E/T9A	$10^{-4}/10^{-6}$	Filter/broth, 37°C

[a]Number of transconjugants per recipient; N/A, not applicable

TABLE 3.4 Antimicrobial susceptibility patterns of primary and secondary donors, recipients, and transconjugants[a]

ID	Type[b]	Sp	Va	Am	Cp	E	Lx	Lz	P	Rf	Syn	Te
C2	1° donor	*fs*	R	S	R	R	R	S	S	S	R	R
S4	1° recipient	*fm*	S	S	I	S	S	S	S	R	S	S
T2G	1° transconjugant	*fm*	R	R	R	R	R	S	R	R	S	S
S6	2° recipient	*fs*	S	S	S	I	S	S	S	S	R	R
T7A	2° transconjugant	*fs*	R	S	R	R	R	S	S	S	R	R
B12	2° recipient	*fs*	S	S	S	R	S	S	S	S	R	R
T7B	2° transconjugant	*fs*	R	S	R	R	R	S	S	S	R	R
Q4	2° recipient	*fm*	S	S	S	S	S	S	S	R	R	S
T9A	2° transconjugant	*fm*	R	R	R	R	R	S	R	R	R	R
Q5	2° recipient	*fm*	S	S	S	R	S	S	R	S	R	R
T7E	2° transconjugant	*fm*	R	S	S	R	S	S	R	S	R	R
T9B	2° transconjugant (broth)	*fm*	R	R	R	R	R	S	R	R	S	R

[a]MIC interpretations based on values given in Clinical Laboratory Standards Institute Document M100-S16 Vol. 26 No. 3 (2006); [b]Tranconjugants generated by filter method, except where indicated; *fs, Enterococcus faecalis*; *fm, E. faecium*; R, resistant; I, intermediate; S, susceptible; Va, vancomycin; Am, ampicillin; Cp, ciprofloxacin; E, erythromycin; Lx, levofloxacin; Lz, linezolid; P, penicillin; Rf, rifampin; Syn, synercid; Te, tetracycline.

V. DISCUSSION

Enterococci are regularly isolated from animals and foods due to their commensal nature and ability to thrive in multiple environmental niches and often under adverse conditions. The emergence and prevalence of antibiotic resistance in enterococci as the result of selective antibiotic pressure is an important factor in assessing their continued safe commercial use. Colonization and survival of enterococci on fomites, raw ingredients, and on the fingertips of handlers should be considered in ready-to-eat foods such as salad bar items. Recovery studies indicate the survival and persistence of VRE as

long as seven days post-inoculation and on half of environmental surfaces following cleaning (Noskin et al., 1995; Lankford et al., 2006). On the retail level, a high-level of enterococci are present in meats, fruits, and vegetables (McGowan et al., 2006), shrimp (Duran and Marshall, 2005), and even in ice used to cool foods on display (Nichols et al., 2000). Hayes et al. (2001) has reported a high level of streptogramin-resistant enterococci (SRE) in VanA-type VRE from poultry and humans. The safe selection of starter or probiotic strains is generally dependent upon historical safe use and absence of antibiotic resistance genes. Virulence factors that contribute to horizontal transmission must also be considered when assessing the safety of these strains. This study demonstrates that by using enterococci isolated from multi-component salads in mating experiments with clinical VRE and SRE of animal and food origin, commensal VSE can serve as intermediate disseminators of glycopeptide resistance to more virulent recipient strains.

The clinical, food, and animal strains used for primary mating showed a difference in the incidence of virulence determinants between *E. faecalis* and *E. faecium* consistent with previously published data (Eaton and Gasson, 2001). With the exception of animal isolate B13, all *E. faecalis* recipients carried the selected virulence genes *agg, cpd,* and *gelE*, while *efaA* was detected in all *E. faecium*. There was no other distinguishing characteristic of B13 compared with the other *E. faecalis* isolates. Antimicrobial susceptibility patterns for the four clinical donor VRE were similar in that all were susceptible to nitrofurantoin and fusidic acid. All *E. faecalis* demonstrated characteristic intrinsic streptogramin resistance. Recipient enterococci were selected on the basis of antibiotic resistance patterns for transconjugant selection purposes; hence, all

food and animal *E. faecium* were resistant to nitrofurantoin and/or rifampin. The standard recipient *E. faecalis* JH2-2 was included for mating experiments, and this was the only isolate in the study that demonstrated resistance to fusidic acid.

In primary mating experiments, among 58 donor-recipient pairs, transconjugant generation was only detected between one VanA *E. faecalis* donor, and an *E. faecium* salad recipient, S4 at an average conjugation frequency of 10^{-8} per recipient. This relatively low rate of transfer may be due to the absence of selective antibiotic pressure. In a study of 40 VRE donors from retail poultry carcasses, Novais et al. (2005) reported intraspecies transfer of vancomycin resistance between only two mating pairs, an *E. faecium* donor to *E. faecium* GE1 and an *E. faecalis* donor to *E. faecalis* JH2-2, at a frequency of 10^{-7} and 10^{-8}, respectively, attributing the low transfer frequency to adaptation of transferable elements to particular hosts. The *vanA* gene is carried on transposon Tn*1546* and can be transferred via a conjugative plasmid (Heaton and Handwerger, 1995). However, transconjugants using *E. faecalis* JH2-2, a known pheromone producing recipient, were not detected, indicating that transfer was most likely due to a pheromone-independent process. Alternatively, conservation of *vanA* or *vanB* determinants or differences in binding regions among transposon-like elements in the donors may have accounted for the low frequency of conjugation. Guardabassi and Dalsgaard (2003) reported interspecies transfer of *vanA* among enterococci of environmental origin ranging from 10^{-3} to 10^{-7} per recipient by filter mating when Tn*1546*-like elements were structurally similar among enterococci of differing ecological origin.

The transfer of *vanA* from the primary *E. faecium* transconjugant, T2G, to streptogramin-resistant salad and animal *E. faecalis* and to *E. faecium* CVM 3001 and CVM 3002 that carried *vatE* and *vatD* streptogramin resistance determinants was investigated. Broth and filter matings were carried out in four temperatures (4^{o}C, 10^{o}C, 22^{o}C, and 37^{o}C) to simulate refrigeration, temperature abuse and control conditions. In secondary mating, transconjugants were detected at 37^{o}C by filter mating with all recipients with an increased conjugative frequency ranging from 10^{-4} to 10^{-7} per recipient. This increase in transfer frequency of *vanA* indicates that a native salad recipient that originally carried no glycopeptide or streptogramin resistance genes, or the selected conjugative virulence determinants *agg, cpd,* or *gelE*, could readily disseminate *vanA* to more virulent commensal SRE that may be present in multi-component foods. Additionally, transfer of *vanA* from T2G to CVM 3001 and CVM 3002 in the absence of aggregation substance reveals that SRE could readily acquire glycopeptide resistance in a food matrix that consists of both liquid and solid components as exists in multi-component salads. The pheromone response that promotes conjugation in enterococci is unique, however *S. aureus* is know to produce enterococcal pheromone cAM373 (Clewell et al., 1985). These organisms can coexist as commensal flora in foods, and it has been suggested that natural acquisition of vancomycin resistance by non-enterococcal bacteria may occur via the sex-pheromone response system (Showsh et al., 2001; Flanagan et al., 2003). Further, dissemination of *vanA* via enterococcal probiotic or starter strains has been demonstrated in vitro (Eaton and Gasson, 2001; Lund and Edlund, 2001).

Among the primary and secondary transconjugants, high-level vancomycin resistance of ≥256 μg mL^{-1} was the same as in the clinical donors (Table 3.4). Antimicrobial susceptibility patterns overall resembled the recipient enterococci, with an increase in resistance to four or more additional antibiotics in the transconjugant (data not shown). One notable pattern was the emergence of penicillin resistance in all transconjugants. In *E. faecium*, an increased resistance to β-lactam antibiotics is due to mutations in the intrinsic low-affinity penicillin-binding protein 5 gene *pbp5*. Rice et al. (2005) has recently shown that high-level β-lactam resistance can be conferred among *E. faecium* at low frequency (10^{-10} to 10^{-7}) by mobile transferable elements that also confer resistance to vancomycin. Generation of glycopeptide resistant elements that can lead to multidrug-resistance in a single transfer event in foods can have significant implications should other commensal bacteria, including *S. aureus*, be present.

TABLE 3.5 Selected genes detected by PCR and MIC values[a] for vancomycin and streptogramin in donor, recipient, and transconjugant mating strains

Pair		VA	STR	Transconjugant Type		VA	STR
PRIMARY MATING (DONOR C2)							
C2	*vanA*$^+$*agg*$^+$*cpd*$^+$*gelE*$^+$	≥256	>2				
S4		≤2	4	T2G	*vanA*$^+$	>256	8
SECONDARY MATING (DONOR T2G)							
T2G	*vanA*$^+$	≥256	0.5				
S6	*agg*$^+$*cpd*$^+$*gelE*$^+$	4	≥16	T7A	*vanA*$^+$*agg*$^+$*cpd*$^+$*gelE*$^+$	≥256	≥16
B12	*agg*$^+$*cpd*$^+$*gelE*$^+$	≤2	≥16	T7B	*vanA*$^+$*agg*$^+$*cpd*$^+$*gelE*$^+$	≥256	≥16
3001	*vatE*$^+$	≤2	≥16	T7D	*vanA*$^+$*vatE*$^+$	≥256	≥16
3002	*vatD*$^+$*cpd*$^+$*gelE*$^+$	≤2	≥16	T7E	*vanA*$^+$*vatD*$^+$*cpd*$^+$*gelE*$^+$	≥256	≥16

[a]MIC values expressed in μg/mL; VA, vancomycin; STR, streptogramin

The selective antibiotic pressure created by the use of virginiamycin should also be taken into consideration when evaluating the use of enterococci in animals or foods. Each of the *E. faecium* recipients that acquired *vanA* maintained high level resistance to >2 μg mL^{-1} of Q/D and ≥ 16 μg mL^{-1} of virginiamycin. The prevalence of streptogramin-resistance genes, including *vat D* and *vatE*, among Q/D-resistant *E. faecium* isolated from foods, animals, and humans in the United States is low, suggesting an undetermined mechanism for transfer of streptogramin resistance still exists (Donabedian et al., 2006). The role of conjugative virulence factors has been studied among clinical enterococci; however, the overall contribution of these elements to horizontal transfer of antibiotic resistance determinants in food systems has yet to be determined. Consequently, the benefits of using enterococci in animals and in foods should be weighed against the potential for horizontal transfer of events to occur among enterococci despite the careful selection of avirulent strains. This is particularly true in ready-to-eat food vehicles such as multi-component salads, in which enterococci often coexist with commensal or contaminating organisms that originate from multiple community sources.

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CHAPTER 4

Expression of *vanA* in an *Enterococcus faecium* Transconjugant Derived from a Multi-Component Food *Enterococcus faecium* Isolate

The work presented in this chapter was submitted as a short communication for publication in the International Journal of Food Microbiology in August, 2007.

I. ABSTRACT

The *vanA* gene, encoding high-level resistance to vancomycin, was transferred via interspecies filter mating from clinical *Enterococcus faecalis* to vancomycin-susceptible *Enterococcus faecium* isolated from ready-to-eat macaroni salad collected from a self-service bar at a major supermarket. Presence of the *vanA gene* was confirmed by PCR. High-level resistance to vancomycin (≥256 μg mL^{-1}) was measured in the donor and transconjugant strains by Microscan automated microdilution and Etest. Strains were cultivated in the absence of selective antibiotic pressure in nutrient broth and in simulated salad at 4°C, 10°C, 22°C, and 37°C. Reverse-transcription PCR revealed the presence of *vanA* RNA products in the transconjugant *E. faecium* cultivated in nutrient broth and simulated salad at the four temperatures, while no RNA products were detected in any the *E. faecalis* donor preparations. Results demonstrate the expression of *vanA* in the absence of selective antibiotic pressure under a range of food handling and abuse temperatures.

II. INTRODUCTION

Acquired vancomycin resistance in *Enterococcus*, prevalent among *E. faecalis* and *E. faecium* species, is due to transposable elements encoding high-level resistance via interspecies and intraspecies horizontal transmission (Woodford, 2001; Clewell & Dunny, 2002; Kak and Chow, 2002). Although dissemination of glycopeptide resistance elements has been well-documented, the role of commensal bacteria in the dissemination of antibiotic resistance genes via foods is limited (Wang et al., 2006). Among the enterococci, transposon Tn*1546* encodes VanA-type glycopeptide resistance and is prevalent among clinical strains (Donabedian et al., 2000; Willems et al., 2005). Conjugative plasmids that transfer of Tn*1546* facilitate transfer of *vanA* from *Enterococcus* donors to enterococcal and nonenterococcal recipient cells. In filter mating experiments, Handwerger and Skoble (1995) demonstrated that chromosomal integration of Tn*1546*-like elements carrying *vanA* in both donor and recipient cells occurred, suggesting that conjugal transfer may be directed by the transposon. Transfer of a Tn*1546*–related element carried by a pheromone-responsive plasmid potentially dependent on donor recombination proficiency was reported by Heaton and Handwerger (1995). Thus, the mechanism of intracellular transfer among some strains of enterococci may vary depending on modifications or fitness of donor elements. The genotypic relatedness among strains of food, human, and animal enterococci that carry *vanA* indicate that the mechanisms of gene exchange enable widespread community transfer of glycopeptide resistance genes among donor and recipients of diverse origin (Klare et al., 2003; Donabedian et al., 2003).

Prior mating experiments generated the horizontal transfer of *vanA* between a plasmid-free *E. faecalis* clinical isolate and an *E. faecium* strain isolated from deli macaroni salad (see Chapter 3). The frequency of *vanA* transfer following primary mating (10^{-8} per recipient) was on average three- to four-log-fold lower than that between the transconjugant *E. faecium* and secondary recipient enterococci in the absence of selective antibiotic pressure. High-level vancomycin resistance was demonstrated in primary and secondary transconjugants, with the emergence of multiple antibiotic resistance patterns in all cases. This study reports the expression of *vanA* in the primary and in the transconjugant donor strains in the presence and absence of recipient filtrate when incubated in broth and in simulated multi-component deli salad.

III. MATERIALS AND METHODS

A. Bacterial strains.

Primary donor and transconjugant enterococci were selected based on previously described mating experiments (Chapter 3). Donor *E. faecalis* C2 was isolated from an inpatient blood culture. The transconjugant, *E. faecium* T2G, was derived from filter mating using the recipient *E. faecium* S4, obtained from prepared macaroni salad from the self-service salad bar of a major supermarket. Isolates were identified using a Gram-Positive/MIC Combo Panel on a MicroScan Walk/Away 96 SI system (Dade Behring, Sacramento, CA). Minimal inhibitory concentrations of clinical antimicrobials for donor and transconjugant isolates were interpreted according to Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS, 2006) guidelines for broth microdilution methods. High-level vancomycin resistance was determined by Etest (AB Biodisk,

Piscataway, NJ) on Mueller-Hinton agar (Difco, Sparks, MD). Control strains for identification and antimicrobial susceptibility testing included *E. faecium* ATCC 51559 and *E. faecalis* ATTC 51299. Brain heart infusion agar (BBL Microbiology Systems, Cockeysville, MD) was used to maintain isolates throughout the study.

B. Preparation of simulated multi-component deli salad.

Multi-component salad was prepared using the following commercial products: 60 g Hellmann's Light Mayonnaise (Unilever Corp., Englewood Cliffs, NJ), 120 g DelMonte Diced New Potatoes (DelMonte Corp., San Francisco, CA), and 130 g Swanson White Chicken Breast packed in water (Campbell Soup Co., Camden, NJ). Ingredients were processed in a sterile stomacher bag for 2 minutes, and 1.5 g aliquots were aseptically transferred to sterile 50 mL tubes. Control samples were incubated at 4°C, 10°C, 22°C, and 37°C and examined for sterility following subculture and incubation at 37°C on BHI agar for 24, 48, and 72 hours.

C. Preparation of cells with and without* E. faecium S4 *culture filtrate.

Culture filtrate from *E. faecium* S4 was prepared from cells grown at 37°C in BHI broth CFU/mL. At OD_{600} =1, cultures were centrifuged for 10 min at 5400 rpm, supernatant was collected by filtration through a 0.22 μm-pore Millipore membrane and diluted 50% with BHI broth. Donor and transconjugant cells were grown in BHI broth without antibiotics overnight and standardized to approx. 10^9 CFU/mL (OD_{600}=0.3-0.4). Five hundred microliters of the cell suspension or 250 μL cell suspension plus 250 μL *E. faecium* S4 filtrate was added to 4.5 mL sterile BHI broth and 1.5 g simulated salad,

briefly vortexed, and incubated at 4°C, 10°C, 22°C, and 37°C for 4 hours without agitation. Following incubation, 2 mL of BHI broth was added to tubes containing salad samples. Broth and salad tubes were vortexed, centrifuged (5 min; 10,000 rpm) and supernatant decanted. The pellet was suspended in 100 μL of RNAlater (Ambion, Austin, TX) and stored at -20°C.

D. Polymerase chain reaction.

PCR was used to detect the presence of *vanA* in donor and transconjugant isolates using the method described by Dutka-Malen et al. (1995). The sequences of the forward and reverse primers were as follows: 5'-GGGAAAACGACAATTGC-3' and 5'-GTACAATGCGGCCGTTA-3'. PCR assays were performed in a total volume of 25 μL containing 4 μL whole-cell suspension as a template, 0.4 μM (each) primer, and 1.25U *GoTaq* DNA polymerase (Promega, Madison, WI). All reactions were carried out in a Perkin-Elmer GeneAmp PCR system 2400 (Perkin-Elmer, MA) under the following conditions: initial denaturation (94°C, 5 min); 30 cycles of denaturation (94°C, 30s), annealing (54°C, 30s), and elongation (72°C, 30s) followed by a final extension (72°C, 10 min). Electrophoresis of 10 μL of the PCR product was performed on a 1.5% agarose gel containing 0.5 μL of ethidium bromide and visualized by UV transillumination.

E. RNA isolation and reverse transcriptase-PCR.

Cells were grown to log phase in BHI broth and disrupted by incubation in 15 mg/L TE-lysozyme (Sigma, St. Louis, MO) for 10 minutes, vortexing every 2 minutes. Total RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). Samples were eluted from the column using 50 mL RNase/DNase-free water (Fermentas, Hanover, MD) and DNase-treated by adding 5 μL RQ1 10× DNase reaction buffer (Promega, Madison, WI), 5 μL RQ1 RNase-free DNase (Promega) and incubated 30 minutes at 37^{o}C. Total RNA was quantified using spectrophotometric analysis at OD260. DNase-treated RNA samples (100-50 ng) were amplified with 0.5 mL of ImProm-II reverse transcriptase (Promega) in ImProm-II 5X reaction buffer (Promega) with 3mM MgCl2, 300 mM dNTPs, and 400 mM each forward and reverse primers in a total reaction volume of 30 mL. cDNA was synthesized at 48°C for 45 min, heated at 94°C for 2 min and then amplified for 35 cycles (denaturing at 94°C for 30 s and annealing at 55°C for 45 s), with a final extension step of 7 min at 72°C. Reactions were carried out in a GeneAmp PCR system 2400 (Perkin-Elmer). Forward and reverse primer sequences for the species-specific superoxide dismutase gene *sodA*, included as a housekeeping control, were based on previously published data (Jackson et al., 2004). Electrophoresis of 10 μL of RT-PCR product was performed on a 1.5% agarose gel containing 0.5 μL of ethidium bromide and visualized by UV transillumination**.** *Enterococcus faecium* ATCC 51559 (*vanA*$^{+}$) was used as the control strain.

IV. Results

A. Antibiotic resistance of donor and transconjugant strains.

High-level resistance to vancomycin (>256 μg mL^{-1}) was demonstrated by both the donor *E. faecalis* C2 and the recipient *E. faecium* T2G. *E. faecalis* C2 was also resistant to ciprofloxacin, erythromycin, gentamicin, levofloxacin, synercid, and tetracycline. The susceptibility pattern of *E. faecium* T2G matched that of the original recipient *E. faecium* S4 with additional resistance to ampicillin, ciprofloxacin, erythromycin, levofloxacin, and penicillin. Minimal inhibitory concentrations are given in Table 4.1.

TABLE 4.1 Minimal inhibitory concentrations of selected antibiotics for donor, recipient, and transconjugant strains

Antimicrobial Agent	Breakpoint[a] μg mL^{-1}	*E. faecalis* C2 Donor	*E. faecium* S4 Recipient	*E. faecium* T2G Transconjugant
Ampicillin	≥16	2	≤0.25	>8
Ciprofloxacin	≥2	>2	2	>2
Erythromycin	≥8	>4	≤0.5	>4
Levofloxacin	≥4	>4	≤2	>4
Linezolid	≥8	1	2	2
Penicillin	≥8	8	0.25	>8
Rifampin	≥2	≤1	>2	>2
Synercid (Q/D)	≥2	>2	≤0.25	0.5
Tetracycline	≥8	>8	≤4	≤4
Vancomycin	≥8	>256	≤2	>256

[a]Interpreted from Clinical Laboratory Standards Institute Document M100-S16 Vol. 26 No. 3 (2006)

B. Detection and expression of vanA *by RT-PCR*

Amplification products for *vanA* were detected in *E. faecalis* C2 and *E. faecium* T2G by PCR in all samples. Reverse transcriptase-PCR detected *vanA* RNA in *E. faecium* T2G grown in broth for cells grown with and without filtrate at all temperatures (Figure 4.1). No *vanA* RNA products were detected in *E. faecalis* C2. Results were similar for cells grown in simulated salad (data not shown). Species-specific *sodA* mRNA was detected in isolates from all samples.

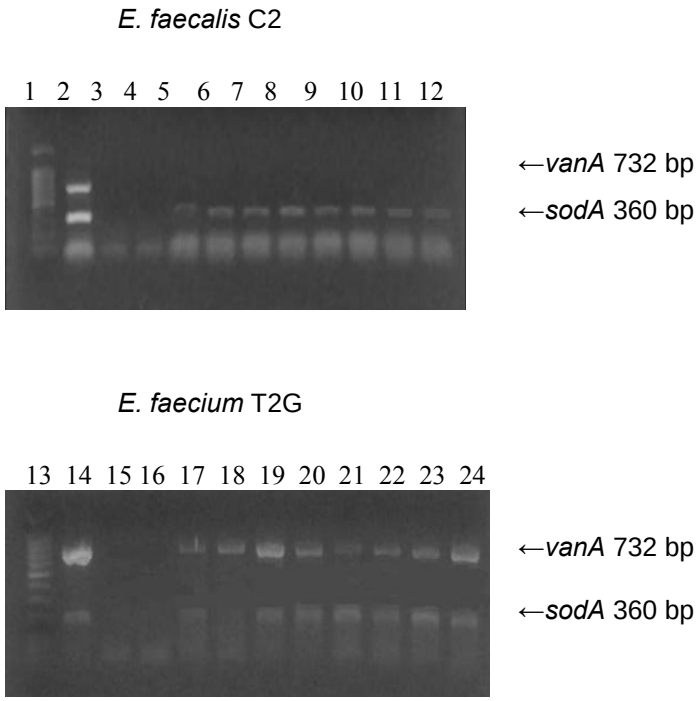

FIGURE 4.1 RT-PCR results for *vanA* and *sodA* for *E. faecalis* C2 (donor) and *E. faecium* T2G (transconjugant) cultivated in BHI broth without antibiotics. Lane 1, molecular marker (100 bp); lane 2, *E. faecium* ATCC 51559 (positive PCR control for *vanA* and *sodA*); lanes 3/15, negative control (no template); lanes 4/16, negative control (no reverse transcriptase); lanes 5/17, 4°C without filtrate; lanes 6/18, 10°C without filtrate; 7/19, 22°C without filtrate; 8/20, 37°C without filtrate; 9/21, 4°C with filtrate; 10/22, 10°C with filtrate; 11/23, 22°C with filtrate; 12/24, 37°C with filtrate

V. Discussion

Van-A-type *Enterococcus* are characterized by high-level resistance to glycopeptide antibiotics, particularly vancomycin (MIC, 64-1,000 μg mL^{-1}) and teichoplanin (MIC, 16-512 μg mL^{-1}). Resistance is due to a gene cluster located on transposon Tn1546, which often resides on a plasmid but can integrate into the chromosome (Arthur et al., 1993; Handwerger and Skoble, 1995). Induction of resistance genes is controlled by a dual-component regulatory system, mediated by proteins VanR and VanS that control transcription of resistance genes *vanHAXYZ* in the presence of glycopeptide antibiotics vancomycin or teichoplanin (Wright et al., 1993; Baptista et al., 1996). Hydrolysis of normal dipeptide peptidoglycan precursors D-Ala-D-Ala and synthesis of depsipeptide precursors D-Ala-D-Lac are associated with significantly decreased vancomycin affinity (Arthur et al., 1992). Activation of VanR is also inducible by nonglycopeptide compounds, including the antibiotics bacitracin and polymyxin B as well as robenidine, an anti-coccidial agent used in poultry. (Allen and Hobbs, 1995; Lai and Kirsch, 1996).

Vancomycin-resistant enterococci of the VanA type are established nosocomial pathogens (Willems et al., 2005), but are also isolated from areas where no exposure to vancomycin or feces has occurred, including foods and environmental surfaces (Guardabassi and Dalsgaard, 2004). Horizontal transmission of *vanA* via Tn*1546*-related elements can spread glycopeptide resistance to other enterococci as well as nonenterococcal species, including *Staphylococcus aureus* (Chang et al., 2003). In this study, a transconjugant VanA-type *E. faecium* that was previously generated by filter mating a high-level resistant clinical *E. faecalis* with vancomycin-susceptible recipient

strain isolated from deli macaroni salad. was examined. The level of vancomycin resistance in the transconjugant (MIC, ≥256 μg mL^{-1}) was equivalent to that of the donor, demonstrating that high-level glycopeptide resistance was maintained following *vanA* transfer. The increased number of antibiotics to which *E. faecium* T2G demonstrated resistance following *vanA* transfer indicates the potential for vancomycin-susceptible enterococci from multi-component foods to acquire multi-drug resistance due to horizontal gene exchange. Cotransfer of additional resistance genes, including those that originate or disseminate within livestock pools, may broaden the resistance profiles of commensal bacteria within foods (Garcia-Migura et al., 2007).

The presence or absence of *vanA* transcription products differed between the donor and the transconjugant, but was not dependent on temperature or the presence of pheromone (filtrate). All cells were cultivated without exposure to vancomycin, therefore no detection of *vanA* RT-PCR products in RNA preparations from *E. faecalis* C2 was most likely due to lack of an inducing agent. However, in transconjugant cells, *vanA* mRNA was detected in the presence and absence of filtrate regardless of temperature. Since growth conditions for RNA isolation were the same for both strains, it is possible that lack of induction was due to accumulated depsipeptide peptidoglycan precursors in donor cells. Alternatively, since the transconjugant resulted from interspecies mating, inherent responses to environmental or biological cues could account for differences in gene expression (Shepard and Gilmore, 2002).

In this study, the interspecies horizontal transfer of *vanA* between clinically-derived and food-borne *Enterococcus* resulted in a transconjugant that demonstrated active *vanA* gene expression. Detection of RNA products in transconjugant cultures

incubated at 4°C, 10°C, and 22°C indicates that *vanA* expression could occur over a range of temperatures and food storage conditions. The spread of glycopeptide resistance among enterococci in self-serve foods such as deli salads, often having multiple ingredients and numerous handlers, is of particular concern in settings where vancomycin-resistance enterococci may persist, such as hospital food service facilities (Buccheri et al., 2007). Additionally, the role of commensal antibiotic-resistant bacteria from raw materials in multi-component foods, particularly from treated animals, and the induction of glycopeptide gene expression warrant further investigation. This study demonstrates that in transconjugant commensal *E. faecium* from a multi-component ready-to-eat salad, *vanA* gene expression can be induced in the absence of selective antibiotic pressure, thus increasing the number of actively transcribing antibiotic-resistant flora that is passed on to the consumer via food.

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CHAPTER 5

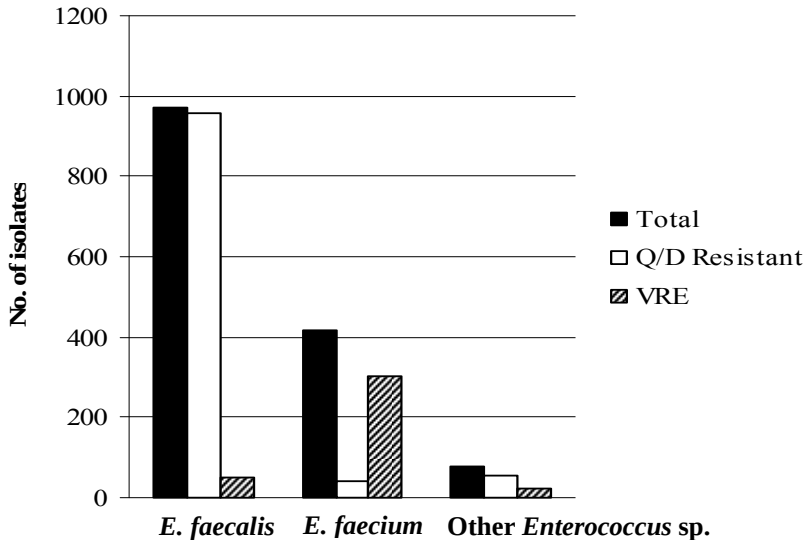
These collective studies provided insight into the potential spread of vancomycin resistance among commensal enterococci from multi-component foods that harbor streptogramin resistance and/or conjugative virulence determinants. To our knowledge, it is the first study of horizontal *vanA* gene exchange among enterococci isolated from deli bar salads. Previous studies have shown that horizontal transfer of vancomycin resistance genes occurs among enterococci and nonenterococci in foods, but the role of virulence genes in mediating the dissemination of resistance is not well understood (Giraffa, 2002; Franz et al., 2003; Foulquie-Moreno et al., 2006). Much of the work in horizontal gene exchange among enterococci has either been in the clinical arena or has focused on dissemination among common sources, i.e., the farmer and the animal product. Multi-component salads represent a unique microenvironment because the potential for bacteria from multiple sources to coexist is great. The factors promoting resistance gene exchange among enterococci of animal, human, and food origin are co-mingling and may be exposed to a range of holding temperatures. Starter cultures and probiotics that contain enterococci are deemed safe for use if antimicrobial resistance genes and certain virulence factors are absent (Joint FAO/WHO Working Group Report, 2002). These studies demonstrated that even in the absence of these specific factors, commensal enterococci can acquire and spread *vanA* to more virulent enterococci in a food system. Additionally, modeling data indicate that the continued use of virginiamycin in U.S. agricultural practice coupled with the clinical use of quinupristin-dalfopristin has led to an emergence of streptogramin-resistant enterococci (Smith et al.,

2003). The European bans on avoparcin and streptogramin have demonstrated that limiting the use of these antibiotics agriculturally has led to a reduction in the numbers of antibiotic resistant bacteria in animals, food, and humans without major consequences to animal health and productivity (Wegener, 2003). Although the carriage rate of VRE in the general U.S. population is low, the incidence of nosocomial VRE continues to be problematic worldwide and global dissemination of Van-type pathogenicity islands is documented (Willems et al., 2005). Nonetheless, resistance spread via hospital food services in the U.S. is a serious concern due to the persistence of VRE on hands and environmental surfaces (Lankford et al., 2006)

In vitro horizontal transfer of *van* genes among enterococci is generally demonstrated using a pheromone-responsive donor and plasmid-free recipient. *Enterococcus faecalis* JH2-2, originally derived from *Streptococcus faecalis* var. *zymogenes*, a plasmid-free, high-level multidrug-resistant strain (Jacob and Hobbs, 1974), is routinely used as a recipient strain due to its resistance to rifampin and fusidic acid, an antimicrobial agent to which many VRE are susceptible. A goal of this project was to examine the transfer behavior between clinical VRE and native enterococci, i.e., those organisms from natural food environments. As such, complete characterization of strains was necessary in order to determine the antibiotic resistance patterns for use in selection of transconjugant strains. During the period from January 2005 through December 2006, a total of 1460 *Enterococcus* were isolated from blood, urine, wound, and rectal samples collected from inpatients at Robert Wood University Hospital in New Brunswick, NJ (Figure 5.1). Although the major species (969; 66%) was *E. faecalis,* only 49 (5%) of those were vancomycin-resistant, while 303 (73%) of the 415 *E. faecium* isolated were

VRE type. The overall level of streptogramin resistant among the clinical *E. faecium* was relatively low (9.8%).

FIGURE 5.1 Prevalence of vancomycin-resistant *Enterococcus faecalis* and *E. faecium* isolated from RWJUH inpatient samples between January 2005 and December 2006

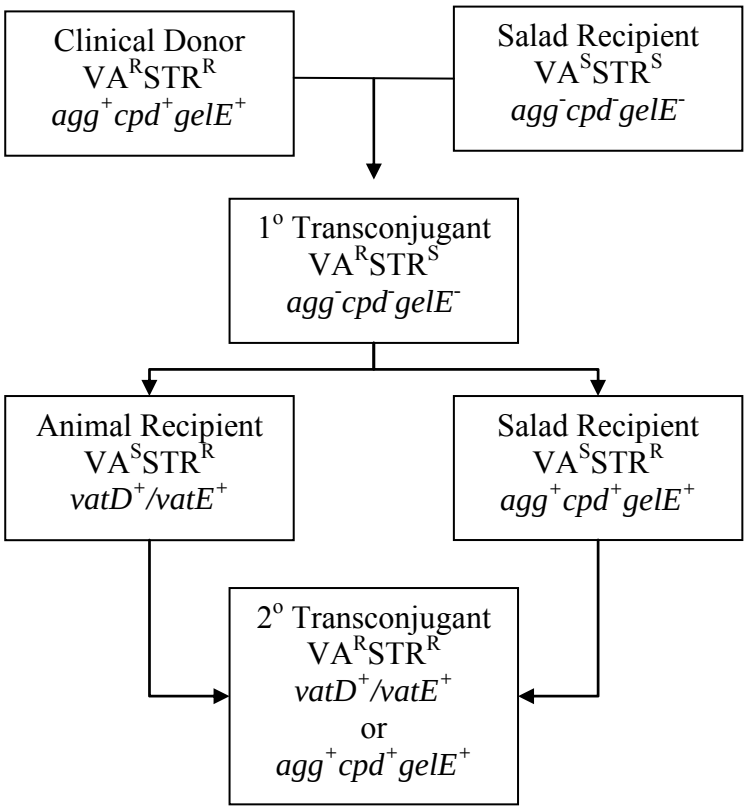


In contrast, none of the enterococcal salad or bovine isolates were vancomycin resistant; however, a higher level of streptogramin resistance was demonstrated among *E. faecium* from animals (40%) and salads (83%). Since streptogramin resistance is not intrinsic in *E. faecium*, the presence of these bacteria in foods or food products indicates that a reservoir of antibiotic-resistant enterococci exists and that these organisms have the potential to acquire additional resistance genes. Carriage of VRE among healthy persons outside the hospital setting in the United States compared with Europe is rare (Coque et

al., 1996), although some epidemiological studies support the existence of community carriers (McDonald et al., 1997). The persistence of VRE on skin and fomites, coupled with the presence of receptive streptogramin-resistant commensal bacteria in vehicles such as foods, could potentially increase the incidence of multidrug resistant enterococci in the community via horizontal exchange of mobile glycopeptide resistance elements. The model of transfer in this study was created to exemplify this type of exchange (Figure 5.2). Through filter mating, it was demonstrated that a streptogramin-susceptible, vancomycin-susceptible *E. faecium* strain isolated from multi-component deli salad, that did not harbor the conjugation or virulence determinants *agg, cpd,* or *gelE*, could acquire and disseminate *vanA* to more virulent or resistant enterococcal strains also isolated from foods or animals. This model is unique in that all strains were collected from native habitats without genetic modification or manipulation, and in particular, from a food system that contains multiple ingredients, has the potential for multiple handlers, and in some instances, varying storage temperatures. Results of the transfer of *vanA* in these studies demonstrated that the frequency of conjugation between the primary donor and recipient was low (10^{-8} per recipient) and that transfer occurred with only one in twelve recipients among all donors. These data indicate that *E. faecalis* JH2-2, when used as a recipient strain, may not reflect actual transfer events in situ. The conjugative frequency of *vanA* increased three- to four-log-fold (10^{-4} to 10^{-6} per recipient) in secondary inter- and intra-species matings using the primary transconjugant as the donor. In all cases, *vanA* was successfully transferred into streptogramin-resistant strains that carried *vatD, vatE, agg, cpd, gelE,* and/or $efaA_{fm}$. In each of the transconjugants, overall MIC profiles showed an increase in resistance compared with the donor or recipient. Therefore, the

safe selection of starter enterococci based on the absence of antibiotic resistance or virulence genes may be compromised by commensal recipients existing in or introduced into foods if transfer of *vanA* should occur.

FIGURE 5.2 Model for horizontal transfer of *vanA*



Expression of antibiotic resistance proteins is another important consideration in enterococci that may acquire glycopeptide resistance. In our study, the presence of RNA products in the primary transconjugant rather than the donor demonstrated active transcription of *vanA* in the absence of selective antibiotic pressure at varied temperatures. This indicates that changes in expression of antibiotic resistance genes can occur when commensal enterococci from food sources acquire *vanA*, and could occur regardless of controlled storage temperatures. In environments where VRE may exist, such as health-care food service facilities, the potential for vancomycin resistance to spread via nosocomial transmission may result in food-borne enterococci that actively express VanA, further compromising infection control measures. A recent European study conducted among 401 nurses indicated that the majority of respondents lacked knowledge regarding food pathogens, vehicles associated with the spread of foodborne disease, and proper food handling procedures; additionally, over 80% of the respondents had never attended any type of course regarding food hygiene (Buccheri et al., 2007). There exist limited data regarding horizontal gene exchange and resistance rates among bacteria isolated from foods, inpatients, and workers in health care food service facilities, and greater research is needed in this area.

This work provided a basis for examining native commensal isolates from multi-component food systems and their potential to harbor, disseminate, and actively express genes conferring VanA-type resistance. The model can be applied to future studies examining the transfer of virulence determinants among streptogramin-resistant *E. faecium*. Co-transfer of *van*, *vat*, or novel streptogramin resistance determinants with conjugative or immunoevasive virulence genes among enterococci from foods is poorly

understood and can be further studied using the transconjugant donors generated in this study. Recently, Paoletti et al. (2007) reported the co-transfer of *vanA* and aggregation substance genes using *E. faecalis* donors from humans and food. Other virulence determinants, including the extracellular surface protein encoded by the *esp* gene, are highly conserved among certain strains of enterococci and may play a significant role in infection-derived strains (Shankar et al., 1999).

Identifying the role and prevalence of virulence determinants that promote the transfer of antibiotic resistance elements is also important in multi-component foods where commensal nonenterococcal Gram-positive flora such as *Staphylococcus*, *Corynebacterium,* or *Bacillus,* or even probiotic *Lactobacillus,* could act as recipients for vancomycin-resistant and/or streptogramin-resistant enterococcal donors. The emergence of new glycopeptide-resistant pathogens, including vancomycin-resistant *Staphylococcus aureus,* the rise in aged and immunocompromised populations, use of streptogramin antibiotics agriculturally and clinically in the United States, and the continued incidence of nosocomial enterococci among hospitalized patients warrant a closer monitoring of the conservation and dissemination of virulence traits associated with VRE and SRE. Multi-component foods contain ingredients from multiple sources which may serve as a reservoir of resistant bacteria, and are an optimal vehicle for horizontal gene transfer among various species. Contamination may occur by the introduction of these bacteria through colonized food handlers, and bacterial loads may increase due to temperature abuse or improper food storage. This work evaluated the potential for vancomycin resistance to spread via "safe"-type enterococci, *i.e.*, suitable for starter culture as the current definition suggests and the role in which commensal streptogramin-resistant and

virulent enterococci serve as intermediate carriers of transferable vancomycin resistance elements. Collectively, these studies contribute to a greater understanding of the nature of horizontal transfer among enterococci. This information is essential in understanding the mechanisms for the spread of resistance that could affect newer antibiotics designed against VRE, such as linezolid, daptomycin, and the streptogramin Q/D. Knowledge of the horizontal spread of transferable resistance elements is especially significant in health-care settings where food handlers may come into regular contact with patients or workers that are colonized by resistant enterococci. Finally, understanding the nature and mechanisms of virulence factors associated with the transfer of resistant elements among enterococci of various origins and the corresponding susceptibility profiles of transconjugant strains is critical to the safe selection of enterococcal strains for starter cultures or probiotics, and to prevent the global spread of antibiotic resistances via foods.

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APPENDIX A

TABLE A.1 Source of vancomycin-susceptible *Enterococcus*, coagulase-negative *Staphylococcus* (CNS), and *Bacillus* isolates collected between March, 2005 and November, 2006

Supermarket	Date	Salad type[1]	Isolate type	Primary reference no.	Rutgers reference no.
Store A, E. Brunswick, NJ	4/05	Chicken Egg Macaroni Seafood Tuna Lobster Pasta Bean			
Store B, Spotswood, NJ	5/05	Chicken Cole Slaw Macaroni Tuna Egg Potato	CNS CNS CNS, *Bacillus* CNS, *Bacillus* *Bacillus* *Bacillus*	052-C1 052-S1 052-M1, M2 052-T1, T2 052-E1 052-P1	
Store A, E. Brunswick, NJ	5/05	Chicken Coleslaw Egg Macaroni Potato Tuna	 CNS *Bacillus* CNS, *Bacillus* CNS	 051-S1 051-M1 051-P1, P2 051-T1	
Store C, Milltown, NJ	6/05	Chicken Coleslaw Egg Macaroni Potato Tuna	 *Bacillus* *E. faecium* *Bacillus*	 063-E1 063-M1 063-T1	 S1
Store D, S. Brunswick, NJ	6/05	Chicken Coleslaw Egg Macaroni Potato Tuna	 CNS *Bacillus* *Bacillus*	 064-S1 064-M1 064-T1	
Store E, E. Brunswick, NJ	6/05	Chicken Coleslaw Egg Macaroni Potato Tuna	CNS *Bacillus* *Bacillus*	065-C1 065-P2 065-T2	

Supermarket	Date	Salad type[1]	Isolate type	Primary reference no.	Rutgers reference no.
Store F, Old Bridge, NJ	6/05	Chicken Coleslaw Egg Macaroni Potato Tuna	 *Bacillus* *Bacillus* *Bacillus* *Bacillus* *Bacillus*	 066-E1 066-M1 066-P1 066-T1	
Store A, E. Brunswick, NJ	7/05	Chicken Coleslaw Egg Macaroni Potato Tuna	 *E. faecium* CNS *Bacillus* *Bacillus* *Bacillus*	 071-S1	 S2
Store G, Perth Amboy, NJ	8/05	Egg Tuna	 *Bacillus*		
Store H, Woodbridge, NJ	8/05	Egg Tuna Macaroni	 *E. durans* *E. faecium*	 088-T1 088-M1	 S3 S4
Store I, Kenilworth, NJ	8/05	Carrot Chicken Egg Fish Potato Rotini Seafood Tortellini Tuna	*Bacillus* *Bacillus* *Bacillus* *Bacillus* *Bacillus* *Bacillus* *Bacillus* *Bacillus*		
Store B, Spotswood, NJ	6/06	Greener Selections[2] Classic Iceberg[2] Double Carrots[2] Spring Mix[2] Italian Blend[2] Classic Romaine[2]			
Store A, E. Brunswick, NJ	6/06	Baby Arugula[3] Green Leaf Blend[3] Escarole Blend[3]			
Store A, E. Brunswick, NJ	7/06	Liver Egg Macaroni Potato	*E. faecalis* *E. faecalis*	171-L1 171-E1	S5 S6
Store G, Perth Amboy, NJ	7/06	Coleslaw Macaroni	*E. casseliflavus*	177-S1	S7
Store A, E. Brunswick, NJ	7/06	Chicken Egg			

Supermarket	Date	Salad type[1]	Isolate type	Primary reference no.	Rutgers reference no.
Store I, Kenilworth, NJ	7/06	Chicken Egg Seafood	*E. faecium* *E. faecium*	179-C1 179-F1	S8 S9
Store B, Spotswood, NJ	10/06	Chicken Coleslaw Egg Macaroni Potato Tuna	 *E. faecalis* *E. faecalis*	 192-E1 192-M1	 S10 S11
Store B, Spotswood, NJ	10/06	Potato Coleslaw Tuna Egg Macaroni Seafood	 *E. faecalis* *E. faecalis*	 1102-E1 1102-M1	 S13 S14
Store A, E. Brunswick, NJ	10/06	Coleslaw Macaroni Seafood Egg Tuna Chicken	 *E .faecalis* *E. faecium*	 1101-F1 1101-T1	 S16 S18
Store A, E. Brunswick, NJ	11/06	Chicken Coleslaw Egg Macaroni Potato Tuna	*E .faecalis* *E. faecalis*	1111-C1 1111-P1	S19 S20

[1]Collected from self-serve deli bars, except where noted; [2]bagged leafy salad (Dole, Salinas, CA); [3]bagged leafy salad (Master Choice Brand, The Great Atlantic & Pacific Tea Co., Montvale, NJ)

APPENDIX B

TABLE B.1 Characterization of *Enterococcus* control strains

Strain		MIC[a]			PCR Profile							
No.	Sp.	Va[b]	Q/D	Vm	*vanA*	*vanB*	*vatD*	*vatE*	*agg*	*cpd*	*gelE*[c]	*efaA*$_{fm}$
29212	fs	≤2	>2	≥16	-	-	-	-	-	+	(+)	-
51299	fs	≥256	>2	≥16	-	+	-	-	+	+	+	-
51559	fm	≥256	1	1	+	-	-	-	-	-	-	+
3001	fm	≤2	>2	≥16	-	-	-	+	-	-	-	+
3002	fm	≤2	>2	≥16	-	-	+	-	-	-	-	+
JH2-2	fs	≤2	>2	≥16	-	-	-	-	-	+	(+)	-

[a]expressed in μg/mL; [b]high-level vancomycin resistance determined by Etest; [c]phenotypically negative indicated by parentheses; Va, vancomycin; Q/D, quinuspristin/dalfopristin; Vm, virginiamycin; *fm*, *E. faecium*; *fs, E. faecalis*

TABLE B.2 Characterization of *Enterococcus* strains isolated from multi-component deli salads

Strain		MIC[a]			PCR Profile							
No.	Sp.	Va	Q/D	Vm	*vanA*	*vanB*	*vatD*	*vatE*	*agg*	*cpd*	*gelE*[b]	*efaA*$_{fm}$
S1	*fm*	≤2	2	≥16	-	-	-	-	-	-	-	+
S2	*fm*	≤2	2	≥16	-	-	-	-	-	-	-	+
S4	*fm*	≤2	0.5	4	-	-	-	-	-	-	-	+
S5	*fs*	≤2	>2	≥16	-	-	-	-	+	-	+	-
S6	*fs*	4	>2	≥16	-	-	-	-	+	+	+	-
S8	*fm*	≤2	2	≥16	-	-	-	-	-	-	-	+
S9	*fm*	≤2	2	≥16	-	-	-	-	-	-	-	+
S10	*fs*	≤2	>2	≥16	-	-	-	-	-	+	+	-
S11	*fs*	≤2	>2	≥16	-	-	-	-	-	+	+	-
S13	*fs*	≤2	>2	≥16	-	-	-	-	-	+	+	-
S14	*fs*	≤2	>2	≥16	-	-	-	-	+	+	(+)	-
S16	*fs*	≤2	>2	≥16	-	-	-	-	+	+	+	-
S18	*fm*	≤2	2	≥16	-	-	-	-	-	-	-	+
S19	*fs*	≤2	>2	≥16	-	-	-	-	+	+	+	-
S20	*fs*	≤2	>2	≥16	-	-	-	-	+	+	+	-

[a]expressed in μg/mL; [b]phenotypically negative indicated by parentheses; Va, vancomycin; Q/D, quinupristin-dalfopristin; Vm, virginiamycin; *fm*, *E. faecium*; *fs, E. faecalis*

TABLE B.3 Characterization of *Enterococcus* strains isolated from inpatient blood and rectal cultures

Strain		MIC[a]			PCR Profile							
No.	Sp.	Va[b]	Q/D	Vm	*vanA*	*vanB*	*vatD*	*vatE*	*agg*	*cpd*	*gelE*[c]	*efaA*$_{fm}$
Blood												
C1	fs	≥256	>2	≥16	-	+	-	-	+	+	(+)	-
C2	fs	≥256	>2	≥16	+	-	-	-	+	+	+	-
C3	fs	16	>2	≥16	-	+	-	-	+	+	+	-
C5	fm	≥256	0.5	4	+	-	-	-	-	-	-	+
C6	fm	≥256	0.5	4	+	-	-	-	-	-	-	+
C7	fm	≥256	0.5	4	+	-	-	-	-	-	-	+
C8	fm	≥256	0.5	4	+	-	-	-	-	-	-	+
C9	fm	≥256	<0.25	4	+	-	-	-	-	-	-	+
C10	fm	≥256	0.5	2	+	-	-	-	-	-	-	+
C11	fm	≥256	0.5	4	+	-	-	-	-	-	-	+
C12	fm	≥256	0.5	4	+	-	-	-	-	-	-	+
C13	fm	≥256	0.5	4	+	-	-	-	-	-	-	+
C14	fm	≥256	>2	≥16	+	-	-	-	-	-	-	+
C15	fm	≥256	2	≥16	+	-	-	-	-	-	-	+
C16	fm	≥256	0.5	4	+	-	-	-	-	-	-	+
C17	fm	≥256	>2	≥16	+	-	-	-	-	-	-	+
Rectal												
R1	fm	≥256	0.5	4	+	-	-	-	-	-	-	+
R2	fm	≥256	0.5	4	+	-	-	-	-	-	-	+
R3	fm	≥256	0.5	4	+	-	-	-	-	-	-	+
R4	fm	≥256	0.5	4	+	-	-	-	-	-	-	+
R5	fm	≥256	0.5	4	+	-	-	-	-	-	-	+
R6	fm	≥256	0.5	4	-	+	-	-	-	-	-	+
R7	fm	≥256	0.38	4	+	-	-	-	-	-	-	+
R8	fm	≥256	0.75	4	+	-	-	-	-	-	-	+
R9	fm	≥256	0.5	4	+	+	-	-	-	-	-	+
R10	fm	≥256	0.38	4	+	-	-	-	-	-	-	+
R11	fm	≥256	0.75	4	+	-	-	-	-	-	-	+
R12	fm	≥256	0.5	4	+	-	-	-	-	-	-	+
R13	fm	≥256	0.5	4	+	-	-	-	-	-	-	+
R14	fm	≥256	0.5	4	+	-	-	-	-	-	-	+
R15	fm	≥256	0.75	4	+	-	-	-	-	-	-	+
R16	fm	≥256	0.75	4	+	-	-	-	-	-	-	+

[a]expressed in μg/mL; [b]high-level vancomycin resistance determined by Etest; [c]phenotypically negative indicated by parentheses; Va, vancomycin; Q/D, quinuspristin/dalfopristin; *fm*, *E. faecium*; *fs, E. faecalis*

TABLE B.4 Characterization of *Enterococcus* bovine strains

Strain		MIC[a]			PCR Profile							
No.	Sp.	Va	Q/D	Vm	*vanA*	*vanB*	*vatD*	*vatE*	*agg*	*cpd*	*gelE*	$efaA_{fm}$
B1	*fm*	≤2	<0.25	≤0.5	-	-	-	-	-	-	-	+
B2	*fm*	4	<0.25	8	-	-	-	-	-	-	-	+
B3	*fm*	≤2	2	≥16	-	-	-	-	-	-	-	+
B4	*fm*	≤2	<0.25	4	-	-	-	-	-	-	-	+
B5	*fm*	≤2	2	≥16	-	-	-	-	-	-	-	+
B6	*fm*	≤2	<0.25	4	-	-	-	-	-	-	-	+
B7	*fm*	≤2	<0.25	8	-	-	-	-	-	-	-	+
B8	*fm*	≤2	<0.25	4	-	-	-	-	-	-	-	+
B9	*fm*	≤2	2	≥16	-	-	-	-	-	-	-	+
B10	*fm*	≤2	2	≥16	-	-	-	-	-	-	-	+
B11	*fs*	≤2	>2	≥16	-	-	-	-	+	+	-	-
B12	*fs*	≤2	>2	≥16	-	-	-	-	+	+	+	-
B13	*fs*	≤2	2	≥16	-	-	-	-	-	-	-	-
B14	*fs*	≤2	>2	≥16	-	-	-	-	-	+	+	-
B15	*fs*	≤2	>2	≥16	-	-	-	-	+	+	+	-
B16	*fs*	≤2	>2	≥16	-	-	-	-	+	+	+	-
B17	*fs*	≤2	>2	≥16	-	-	-	-	-	+	+	-

[a]μg/mL; Va, vancomycin; Q/D, quinupristin-dalfopristin; Vm, virginiamycin; *fm*, *E. faecium*; *fs, E. faecalis*

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