CONJUGATION OF $_{p}$AD1 FROM ENTEROCOCCUS FAECALIS MEDICAL ISOLATES TO ENVIRONMENTALLY OBTAINED STRAINS

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CONJUGATION OF pAD1 FROM *ENTEROCOCCUS FAECALIS* MEDICAL ISOLATES TO ENVIRONMENTALLY OBTAINED STRAINS

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ABSTRACT

Enterococcus faecalis are studied extensively in medical research laboratories due to their constant increase in number of antibiotic resistances and virulence traits. These bacteria are a leading cause of life-threatening infections throughout the world; however, most research regarding this species has focused on medical isolates associated with nosocomial infections. This research has disclosed a possible mechanism for the spread of virulence factors of *E. faecalis* outside the nosocomial setting. In this study, filter mating was used to conjugate the plasmid pAD1 from medical isolates of *E. faecalis* to environmentally obtained strains of *E. faecalis*. The frequency of conjugation was within a 2.256×10^{-6} to 8.893×10^{-4} range. Further experimentation revealed the new transconjugated environmental strains were able to express the virulence factors associated with pAD1, namely β-hemolysis activity. These results suggest environmental *E. faecalis* strains are just as capable as obtaining pAD1 as *E. faecalis* medical isolates; therefore, they may not be as harmless as once thought.

CHAPTER I

INTRODUCTION

Enterococci are recognized as the second leading cause of nosocomial infections by the National Nosocomial Infection Survey in the United States (16). Some life-threatening infections caused by enterococci include urinary tract infections, bacteremia, surgical infections, and endocarditis (18). *Enterococcus* is a relatively new genus of gram-positive cocci. Prior to the 1970's, enterococci were classified as Group D *Streptococcus*. They can survive harsh environments and thrive in soil, water, mammals, birds, insects and on plants (7). In humans, enterococci are normally found in the intestinal tract, oral cavity, and the female reproductive system. They are inherently resistant to β-lactam antibiotics, aminoglycosides and sulfonamides (13), and they have acquired numerous additional antibiotic resistances and virulence traits allowing them to survive the highly competitive gastrointestinal tract and cause disease (17). As a result, physicians are having an increasingly difficult time controlling and confining enterococcal infections.

Enterococci can exchange genetic material through two known plasmid systems—pheromone-responsive and broad host range plasmids. The plasmid pAD1 (58 kb) is pheromone-responsive and was first discovered in a multiple-drug resistant clinical isolate in the late 1970's (23). The plasmid pAD1 is exclusive to *E. faecalis* strains and is only present in one to two copies per cell (23). Despite this low copy number, it can transfer at frequencies approaching 100% under ideal conditions (3). It has been shown that the transfer of pAD1 can facilitate the transfer of unrelated plasmids due to the agglutination phenotype encoded by pAD1 (4); therefore, allowing even more potentially harmful genes to spread among *E. faecalis*.

The transfer success of pAD1 is remarkably high due to this agglutination reaction. Agglutination of *E. faecalis* is mediated by an aggregation substance (AS) encoded by pAD1. The AS is anchored on the surface of *E. faecalis* harboring pAD1. Recipient cells lacking pAD1 excrete a specific sex pheromone cAD1. Donor cells respond to cAD1 by synthesizing AS (3) which will allow donor and recipient cells to clump together. This increases the probability for successful genetic exchange of the plasmid between the donor and recipient. Once the plasmid is successfully transferred, agglutination is terminated (24).

In addition to playing a critical role in plasmid conjugation, research has shown AS assists in binding *E. faecalis* to eukaryotic cells including human neutrophils (PMNs) (19), macrophages (22), and epithelial cells originating from the colon and duodenum (20). Interestingly, when AS-bearing *E. faecalis* were ingested by PMNs and macrophages, the bacteria were resistant to being killed (20, 21).

The plasmid pAD1 also encodes a cytolysin that functions as a bacteriocin and a hemolysin. The hemolytic activity of the cytolysin has been shown to be important in the pathogenicity of *E. faecalis*. Studies using a rabbit endocarditis model revealed that hemolysin and AS from pAD1 significantly increased mortality compared to strains lacking pAD1 (2). Another study revealed that human bacteremia patients infected with *E. faecalis* resistant to gentamicin and capable of hemolysis had a five-fold increased risk of death compared to bacteremia patients with bacterial infections involving non-hemolytic and gentamicin susceptible *E. faecalis* (8). Furthermore, researchers found that hemolytic strains of *E. faecalis* had 10-fold lower lethal dose (LD_{50}) values in systemically infected mice than non-hemolytic strains (15). In this study, mouse mortality was the result of lysed neutrophils and macrophages by the hemolysin rather than by lysis of erythrocytes. Another study, investigating hemolysin and

pathogenicity, found that a significantly higher percentage of hemolysin and bacteriocin-producing *E. faecalis* were more commonly isolated from patients with bacteremia than non-hemolysin and non-bacteriocin-producing strains (14). Furthermore, hemolysin-producing strains of *E. faecalis* caused significantly greater damage in an endophthalmitis model than *E. faecalis* not producing cytolysin (12).

The bacteriocin encoded by pAD1 is active against several gram-positive species including *Streptococcus, Clostridium,* and even plasmid-free strains of *E. faecalis* (11), but it is not active against gram-negative bacteria. This has obvious implications in the human GI tract by allowing *E. faecalis* to compete for nutrients among gram-positive species. It may also have implications in periodontal disease. By altering the gram-positive bacteria content inhabiting the mouth, gram-negative inhabitants continue to thrive and this appears to favor periodontal disease (11).

Rationale and Significance

Nearly all of the research regarding virulence factors and antibiotic resistance genes located on plasmids of *Enterococcus* have focused on medical strains isolated from nosocomial infections. *Enterococcus faecalis* harboring the plasmid pAD1 could thrive in the intestinal tract of humans as well as domesticated and non-domesticated animals with or without symptoms of illness. These potentially harmful bacteria could be excreted and continue to survive in the soil or water. The plasmid pAD1 could then be transferred to environmental *E. faecalis* strains living in the soil or water, increasing the number of *E. faecalis* bacteria containing pAD1. Animals could ingest these potentially harmful bacteria and thus allow for yet another transfer of genetic material among enterococcal populations. In addition, humans could be infected with these

environmental bacteria by participating in recreational sports such as fishing or other water contact. Humans exposed to the water could easily ingest the potentially harmful bacteria.

The goal of this research is to demonstrate that *E. faecalis* containing pAD1 could successfully conjugate with environmentally obtained strains of *E. faecalis*. It is anticipated that pAD1 will successfully transfer to environmentally obtained strains based upon research completed by Eaton and Gasson (6) where pAD1 conjugated to non-medical food starter strains of *E. faecalis*.

If environmentally obtained strains of *E. faecalis* can acquire the plasmid pAD1, which encodes several virulence factors, the environment could serve as a reservoir of virulent strains of *E. faecalis*. This research would disclose a mechanism for the spread of virulence factors among enterococci outside the nosocomial setting. If pathogenic *E. faecalis* were released into the environment, their potentially harmful genes could be transferred to environmental strains of *E. faecalis*. If this were to occur, strains which were once harmless would potentially become harmful and capable of further transfer of the plasmid. Finally, if conjugation of pAD1 to environmental strains is successful, then the boundaries set between a medical strain and an environmental strain would be modified. It is currently believed that medical strains have more potential to be hazardous, while environmental strains are harmless. This study may reveal that environmental strains of *E. faecalis* are just as capable of obtaining plasmids associated with pathogenicity; thus, potentially could cause disease just as easily. Further testing beyond this study using animal models could reveal whether or not the transconjugated environmental strains are indeed pathogenic. The environmental *E. faecalis* strains harboring pAD1 could be studied in a rabbit endocarditis model or systemically infected mice. The degree of damage or mortality rates would be compared to those previously published using medical strains of *E. faecalis*

containing pAD1, as well as comparing environmental strains of *E. faecalis* not harboring pAD1 as controls.

CHAPTER II

METHODS AND MATERIALS

Donor Enterococcus faecalis

The donor bacteria for the conjugation experiments were *Enterococcus faecalis* strains FA2-2 (5) and OGIX (10) containing the plasmid pAM714 obtained from the laboratory of Dr. Don B. Clewell at the University of Michigan, Ann Arbor, Michigan. The plasmid pAM714 was used because it provides a selectable marker by encoding resistance to erythromycin. This plasmid is a derivative of pAD1 containing the transposon Tn*917* (5, 22). *Enterococcus faecalis* strain FA2-2 is resistant to the following antibiotics at the corresponding concentrations: fusidic acid, 25 μg/ml; rifampin, 25 μg/ml; and erythromycin, 10 μg/ml. *Enterococcus faecalis* strain OG1X is resistant to streptomycin at a concentration of 1000 μg/ml and erythromycin at a concentration of 10 μg/ml.

Recipient Environmental Enterococcus faecalis

The recipient bacteria for the conjugation experiments were environmental *E. faecalis* isolates selected from a collection in Dr. Don Dailey's laboratory at Austin Peay State University, Clarksville, TN. These isolates were obtained from various streams located throughout Robertson County, TN. Since the bacteria needed a selectable marker, each isolate was cultured in the presence of either fusidic acid or streptomycin at concentrations of 25 μg/ml or 1000 μg/ml respectively. All enterococcal strains were grown on Todd Hewitt (TH, Difco

Laboratories) agar plates containing the appropriate antibiotics and incubated at 37°C for 48 h. The bacteria and plasmid used in this study are summarized in Table 1.

Table 1 Enterococcal Strains and Plasmid Used in This Study

Strain or plasmid	Genotype or phenotype[(a)]	Comment and/or reference
E. faecalis strains		
FA2-2 w/pAM714	*ery*, Hly^+/Bac^+, *rif, fus*	Derivative of JH2 (5)
OG1X w/pAM714	*ery*, Hly^+/Bac^+, *str*	Protease-negative mutant of OG1-10 (10)
Environmental Group A	*str*	Austin Peay State University Collection
Environmental Group B	*fus*	Austin Peay State University Collection
Plasmid		
pAM714	*ery*, Hly^+/Bac^+	pAD1::*Tn917*, wild-type transfer (5, 22)

Notes: [(a)]*ery*, erythromycin; Hly+, hemolysin; Bac+, bacteriocin; *str*, streptomycin; *rif*, rifampin; *fus*, fusidic acid.

The environmental isolates of *E. faecalis* that are resistant to streptomycin (1000 µg/ml) formed Group A, while the environmental isolates resistant to fusidic acid (25 µg/ml) comprised Group B. These spontaneous antibiotic-resistant mutants of the environmental isolates were obtained by the following procedure: Seventeen different environmental isolates of *E. faecalis* were cultured overnight in (TH) broth at 37°C. Then, 0.5 ml of each culture was plated on TH agar containing either 25 µg/ml fusidic acid or 1000 µg/ml streptomycin. After 96 h at 37°C, plates were checked for growth. A fusidic acid-resistant mutant and a streptomycin-resistant mutant were subcultured for each of the environmental isolates. The *E. faecalis* environmental isolates in Group A were maintained on TH agar plates containing 1000 µg/ml streptomycin,

while the Group B environmental isolates were maintained on plates containing 25 μg/ml fusidic acid.

Confirmation of Phenotypes

The *E. faecalis* strains FA2-2 and OG1X were checked to verify they contained the plasmid pAM714. If pAM714 is present, β-hemolytic activity should be evident because of the hemolysin gene(s) in pAD1. Resistance to erythromycin (10 μg/ml) was also confirmed for the two strains, because of the presence of Tn*917* in pAM714. The two strains were grown on TH agar supplemented with 10 μg/ml of erythromycin and on 5% horse blood agar plates containing Tryptic Soy (7). Growth on TH agar supplemented with erythromycin and noted β-hemolytic activity, a clear halo around the bacterial colony, confirmed the presence of the plasmid. In addition, *E. faecalis* strain FA2-2 w/pAM714 was checked for susceptibility to streptomycin (1000 μg/ml) and *E. faecalis* strain OG1X w/pAM714 was checked for susceptibility to fusidic acid (25 μg/ml).

Since pAM714 encodes resistance to erythromycin and a hemolysin, both Group A and Group B environmental bacteria were tested for these two activities prior to the mating experiments. This was necessary to verify that these traits do not already exist in the environmental strains, excluding the possibility that the plasmid pAD1 was already present. The non-hemolytic environmental strains from both Group A and Group B susceptible to erythromycin were used in the mating experiments. Resistance to erythromycin was later used as the genetic marker to select transconjugates, which were the environmental bacteria harboring the plasmid pAM714. Hemolysin activity was determined on the blood agar plates (7). Each of

the Group A and Group B strains were placed onto the blood agar plates. The plates were placed in a 37°C incubator for 48 h. Hemolysin activity was, again, verified as a clear halo around the bacterial colony. *Enterococcus faecalis* strains FA2-2 and OGIX were used as positive controls as well as two *E. faecalis* medical isolates capable of β-hemolysis activity that were obtained from Gateway Medical Hospital, Clarksville, TN. Resistance to erythromycin was determined by placing the Group A and Group B strains on TH plates supplemented with 10 μg/ml erythromycin. The plates were incubated for 48 h at 37°C and checked for growth. No growth on the plates indicated the strains are susceptible to erythromycin and could be used for the conjugation of pAM714 mating experiments.

Mating Procedures

Filter mating experiments were completed using an approximate 1:10 donor to recipient mating mixture (4). Two sets of mating experiments were completed in triplicate. The first experiment involved the donor *E. faecalis* strain FA2-2 w/pAM714 (*str*$^-$, *ery*$^+$, Hly$^+$) with recipient environmental *E. faecalis* Group A isolates (*str*$^+$, *ery*$^-$, Hly$^-$). The second set of experiments involved donor *E. faecalis* strain OG1X w/pAM714 (*fus*$^-$, *ery*$^+$, Hly$^+$) and recipients environmental *E. faecalis* Group B isolates (*fus*$^+$, *ery*$^-$, Hly$^-$). All of the enterococci were initially cultured in Brain Heart Infusion broth (BHI, Difco Laboratories). Following overnight incubation at 37°C, 0.05 ml of donor and 0.5 ml of recipient cells were combined and filtered through 0.45 μm-pore size filters (6). The filters were then placed on BHI agar plates and incubated overnight at 37°C. The cells were harvested and diluted in 1.0 ml of BHI broth. Two sets of dilutions were then completed, to obtain an approximate 1/20th and 1/200th of the original

donor cells. For the first dilution, 0.5 ml of the cells in the 1.0 ml BHI broth was placed in 5 ml BHI, and 0.5 ml of that solution was placed on BHI plates containing appropriate antibiotics. For the second dilution, 0.5 ml of the cells in the 1.0 ml BHI was placed in 10 ml BHI and 0.1 ml of that solution was placed on BHI plates supplemented with appropriate antibiotics. Plates used in the FA2-2 strain and Group A environmental *E. faecalis* mating experiment contained 10 μg/ml erythromycin and 1000 μg/ml streptomycin, while the BHI plates for the OG1X and Group B mating were supplemented with 10 μg/ml erythromycin and 25 μg/ml fusidic acid. After 48 h incubation at 37 °C, the resulting transconjugates (the surviving colonies)were enumerated.

Conjugation frequency was calculated by dividing the total number of transconjugates by the starting cell density of donor cells. The starting cell density was calculated by completing a dilution series of the 0.05 ml of donor cells. A total of 0.1 ml of each dilution (10^{-1}, 10^{-2}, 10^{-3}) was plated on TH plates supplemented with the appropriate antibiotics. Todd-Hewitt agar supplemented with the appropriate antibiotics was used to help prevent contamination with other colonizing bacteria. The plates were incubated at 37°C for 48 h and the colonies counted. Only plates which contained between 10 and 300 colonies were used to determine the approximate number of donor cells used in the conjugation experiments. This allowed for a more precise estimation of the actual number of donor cells used.

Additional tests were completed investigating whether or not environmental recipient cells are more likely to acquire the plasmid pAM714 if they are exposed to a very low concentration of erythromycin (0.5 μg/ml) prior to the mating experiments (5). To achieve this, 0.5 ml of recipient environmental bacteria from Group A or Group B grown in BHI broth overnight was placed into a 1.5 ml centrifuge tube. The cells were separated from the broth by a

10 second centrifugation at 14,000 x g and resuspended in 0.5 ml BHI broth containing 0.5 μg/ml erythromycin. The cells remained in this solution for 1 or 4 hours at 37°C and then used as recipient cells in the filter mating experiments.

Phenotypic Verification of pAM714 in Transconjugates

A total of 44 transconjugate colonies were randomly selected and tested for β-hemolytic activity. This displayed phenotypically that the transconjugates contain pAM714 because of the plasmid's ability to lyse red blood cells. To accomplish this, a colony was grown overnight at 37°C in BHI broth supplemented with the appropriate antibiotics. Colonies from the FA2-2/Group A mating experiments were placed in BHI containing streptomycin and erythromycin, while the colonies from the OG1X/Group B mating experiments were propagated in broth supplemented with fusidic acid and erythromycin. Using a sterile inoculating needle, the bacteria will be transferred from broth to 5% horse blood agar plates. After an incubation period of 48 hours at 37°C, the plates were checked for β-hemolysis activity.

Chapter III

RESULTS

Confirmation of Phenotypes

Table 2 is a compilation of the phenotypic tests completed prior to the conjugation of pAD1 to environmental *E. faecalis* strains via filter mating. The environmental strains were first tested for susceptibility to erythromycin (10 μg/ml) and hemolytic activity on blood agar plates. These tests helped decipher whether or not pAD1 was already present in these environmental strains. After a 48 h incubation period at 37°C, TH plates containing 10 μg/ml erythromycin were inspected for colonies. No colonies were present on the plates; therefore, none of the environmental strains were resistant to erythromycin. After a 48 h incubation period at 37°C, 5% horse blood agar plates were examined for β-hemolysis activity present in the environmental *E. faecalis* strains. None of the environmental strains demonstrated β-hemolysis activity. The donor strains,OGIX and FA2-2, on the other hand, were both resistant to erythromycin and capable of β-hemolysis indicating pAM714 was present.

Table 2 Results of Phenotypic Tests for Environmental *E. faecalis* Strains and Donor *E. faecalis* Strains

Environmental Strain	Erythromycin[a]	β -Hemolysis[b]
IV-19	S	-
8--14	S	-
10--16	S	-
10--3	S	-
5--4	S	-
IV-20	S	-
10--12	S	-
V-14	S	-
IV-18	S	-
5--19	S	-
Donor Strain		
OGIX	R	+
FA2-2	R	+

Notes: [a] Erythromycin at a concentration of 10 μg/ml on TH plates. "S" indicates strain was susceptible, while "R" indicates resistance.
[b] The symbol "-" indicates β-hemolysis activity was absent, while "+" indicates β-hemolysis was present on 5% horse blood agar plates.

Specific colonies from the strains of environmental bacteria used for receiving pAM714 were selected based upon growth on plates containing either 1000 μg/ml of streptomycin or 25 μg/ml fusidic acid. Four of the 10 environmental strains plated on TH agar with 1000 μg/ml streptomycin produced mutant colonies. The total number of colonies and corresponding strains are listed in Table 3. These surviving colonies consist of bacteria which underwent a spontaneous mutation, and are resistant to streptomycin at the concentration of 1000 μg/ml. A total of six of these seventeen colonies were used as recipient strains for the conjugation of pAM714 in the mating experiments with donor FA2-2 cells. In addition, these bacterial colonies resistant to streptomycin were categorized as Group A Environmental strains.

All 10 of the environmental strains plated on TH agar containing 25 μg/ml

fusidic acid produced at least 1 colony, as shown in Table 3. Again, a total of six of these colonies (out of a total 69) were used as recipients for the conjugation of pAM714 using donor OG1X cells. These colonies resistant to fusidic acid were identified as Group B Environmental strains.

Table 3 Number of Resistant Bacterial Colonies Formed on TH Plates Supplemented with Streptomycin or Fusidic Acid and Corresponding Environmental Strain

Environmental Strain	Streptomycin[a]	Fusidic Acid[b]
IV-19	0	5
8--14	7	15
10--16	6	12
10--3	0	11
5--4	0	3
IV-20	2	1
10--12	0	8
V-14	2	3
IV-18	0	3
5--19	0	8

Notes: [a] The number indicates the total number of colonies formed which were resistant to streptomycin (1000 μg/ml).
[b] The number indicates the total number of colonies formed which were resistant to fusidic acid (25 μg/ml).

Table 4 lists which strain the six colonies came from for each of the Group A and Group B Environmental strains.

Table 4 Colonies Used and Labeled With a New Identification From Corresponding Parent Environmental Strains.

Parent Environmental Strain	Number of Colonies Used
Resistance to Streptomycin	
8--14	1
10--16	2
IV-20	2
V-14	1
Resistance to Fusidic Acid	
10--16	1
10--3	1
IV-20	1
V-14	2
IV-18	1

Notes: The six colonies resistant to streptomycin comprise Group A Environmental Strains, while the six colonies resistant to fusidic acid comprise Group B Environmental Strains.

Donor strain FA2-2 was tested for resistance to 1000 µg/ml streptomycin. This was necessary, because Group A Environmental *E. faecalis* were resistant to streptomycin and FA2-2 cannot be if the mating experiments were to be completed successfully. The same was true for the donor OGIX. This donor was tested for resistance to 25 µg/ml fusidic acid, because Group B Environmental *E. faecalis* were resistant to fusidic acid. Both donor strains were found to be susceptible to the corresponding antibiotic tested.

Filter matings could now be completed by using Group A Environmental strains (str^+, ery^-, Hly^-) with FA2-2 (str^-, ery^+, Hly). The successful transfer of pAM714 from FA2-2 to

Group A Environmental bacteria would result in transconjugate colony growth on BHI plates supplemented with both erythromycin (indicating Tn*917* was present, which is in pAM714) and streptomycin (indicating the colonies were Group A Environmental *E. faecalis*.) Group B Environmental strains (*fus*$^+$, *ery*$^-$, Hly) could be mated with OGIX (*fus*$^-$, *ery*$^+$, Hly$^+$), and transconjugates would survive on BHI plates containing erythromycin (again, Tn*917* was present) and fusidic acid (indicating the colonies were Group B Environmental *E. faecalis*).

Mating Procedures

The filter mating experiments were successful. In the first set of filter matings, no dilutions were performed. As a result, transconjugate plates contained more than 300 colonies and dilutions were necessary for the remaining sets of filter matings. A total of four additional trials were completed. Each trial consisted of two dilutions—one used approximately 1/20th and the other 1/200th of the original donor and recipient cells. The rates of transfer were calculated for transconjugate plates containing <300 colonies. Only plates with less than 300 colonies were used for obtaining an accurate count of transconjugates. The data in Table 5 reports the calculated frequencies of the conjugation of pAM714 from donor cells to recipient cells. The trials not listed in Table 5 had several, if not all, transconjugate plates containing >300 colonies; therefore, accurate measurements could not be made regarding the transfer rate of pAM714.

Table 5 Frequency of Transfer of pAM714 from FA2-2 to Group A Environmental *E. faecalis* and from OGIX to Group B Environmental *E. faecalis* Per Donor

# of Donors (FA2-2)	222,640	22,264	1,330,000
Group A[a] (Recipient)	Trial 1 (dilution 1)	Trial 1 (dilution 2)	Trial 2 (dilution 1)
10-16C	2.066×10^{-4}	8.983×10^{-4}	2.256×10^{-6}
10-16D	1.123×10^{-4}	2.021×10^{-4}	7.520×10^{-6}
V-14C	1.168×10^{-4}	3.593×10^{-4}	2.256×10^{-6}
IV-20C	2.236×10^{-4}	4.716×10^{-4}	0
IV-20D	3.490×10^{-5}	3.818×10^{-4}	0
8-14C	1.392×10^{-4}	3.818×10^{-4}	2.256×10^{-6}

# of Donors (OGIX)	1,370,000	125,000	950,000	95,000
Group B[b] (Recipient)	Trial 1 (dilution 1)	Trial 2 (dilution 2)	Trial 3 (dilution 1)	Trial 3 (dilution 2)
V-14A	too many[c]	5.578×10^{-5}	too many	7.368×10^{-5}
IV-20	3.650×10^{-6}	6.534×10^{-4}	4.105×10^{-5}	2.105×10^{-5}
IV-18B	1.095×10^{-5}	3.187×10^{-4}	1.284×10^{-5}	3.158×10^{-5}
V-14B	3.650×10^{-6}	0	too many	2.105×10^{-5}
10--3	7.230×10^{-7}	8.765×10^{-4}	1.474×10^{-5}	0
10-16B	0	0	4.000×10^{-5}	8.421×10^{-5}

Notes: [a] Group A Environmental *E. faecalis* which are resistant to 1000 µg/ml streptomycin.
[b] Group B Environmental *E. faecalis* which are resistant to 25 µg/ml fusidic acid.
[c] The term "too many" indicates there were more than 300 transconjugate colonies on the plates and accurate counts could not be completed.

Additional trials were completed identifying whether or not a brief exposure (1 h or 4 h) of the environmental strains to a small quantity of erythromycin (0.5 µg/ml) enhanced the ability of these cells to acquire the plasmid pAM714. The results from the first trial (both dilutions) utilizing FA2-2 with Group A Environmental strains indicated pAM714 transferred at the same or similar frequencies as it did in the previous trials (see Table 6) when the recipient bacteria were not exposed to erythromycin. No additional trials were completed, because transfer of pAM714 from donors to recipients was successful without prior exposure to erythromycin.

Table 6 Comparing the Rate of Transfer of pAM714 to Group A Environmental *E. faecalis* Strains from FA2-2 in an Erythromycin-Induced Environment

# of Donors (FA2-2) Group A Strain (Recipient)	222,640 0.0 μg/ml Erythromycin	222,640 0.5 μg/ml Erythromycin for 1 h	222,640 0.5 μg/ml Erythromycin for 4 h
10-16C	2.066×10^{-4}	1.842×10^{-4}	1.931×10^{-4}
10-16D	1.123×10^{-4}	1.886×10^{-4}	1.662×10^{-4}
V-14C	1.168×10^{-4}	3.234×10^{-4}	1.842×10^{-4}
IV20-C	2.236×10^{-4}	1.168×10^{-4}	2.156×10^{-4}
IV-20D	3.490×10^{-5}	1.482×10^{-4}	1.258×10^{-4}
8-14C	1.392×10^{-4}	1.392×10^{-4}	5.890×10^{-5}

# of Donors (FA2-2) Group A Strain (Recipient)	22,264 0.0 μg/ml Erythromycin	22,264 0.5 μg/ml Erythromycin for 1 h	22,264 0.5 μg/ml Erythromycin for 4 h
10-16C	8.983×10^{-4}	3.818×10^{-4}	2.470×10^{-4}
10-16D	2.021×10^{-4}	2.290×10^{-4}	2.246×10^{-4}
V-14C	3.593×10^{-4}	2.695×10^{-4}	3.144×10^{-4}
IV20-C	4.716×10^{-4}	2.470×10^{-4}	2.020×10^{-4}
IV-20D	3.818×10^{-4}	1.123×10^{-4}	4.267×10^{-4}
8-14C	3.818×10^{-4}	1.123×10^{-4}	7.860×10^{-4}

β-Hemolysis Activity

A total of 44 transconjugate colonies were randomly selected from the transconjugated plates and further tested for their ability to undergo β-hemolysis activity. All of the transconjugate colonies had clear halos around them when plated on Tryptic Soy 5% horse blood agar plates for 48 h at 37° C; thus, each colony tested positive for β-hemolysis activity.

Chapter IV

DISCUSSION

The plasmid, pAD1, found exclusively in *Enterococcus faecalis,* is of great concern to medical doctors and researchers. This plasmid encodes several virulence factors, including a cytolysin which is capable of hemolysin and bacteriocin activities. It can spread very quickly among patients in a nosocomial setting causing numerous infections including urinary tract infections, bacteremia, surgical infections and endocarditis (18). The plasmid pAD1 can also spread from bacterium to bacterium with extremely high efficiency due to an agglutination mechanism for conjugation within the pAD1 genome. Previous research regarding pAD1 has been primarily limited to medical isolates of *E. faecalis.* In this study, the possible conjugation of the plasmid pAD1 from medical isolates to environmentally obtained *E. faecalis* strains was investigated.

Through the experiments completed in this study, it was shown that environmentally obtained strains of *E. faecalis* could serve as a recipient for pAD1. The calculated frequency of conjugation was very similar to those previously published involving the conjugation of pAD1 among laboratory strains (5) as well as from laboratory strains to food starter strains (6). Table 7 compares the frequencies of transfer from these two studies to the study completed here. All six strains tested from Group A Environmental *E. faecalis* and all six strains from Group B Environmental *E. faecalis* were capable of acquiring pAD1. These environmental strains were first shown to be non-hemolytic and susceptible to erythromycin at a concentration of 10 μg/ml. After the mating experiments, these environmental *E. faecalis* strains, now referred to as transconjugates, could grow on TH plates supplemented with 10 μg/ml erythromycin; therefore, they were resistant to the antibiotic at that concentration. Resistance to erythromycin indicated

Table 7 Frequency of Transfer of pAM714 from FA2-2 to Group A Environmental *E. faecalis* (This Study) Compared to Previously Published Frequencies

Donor Strain	Recipient Strain			
	Group A (Recipient)	Trial 1 (dilution 1)	Trial 1 (dilution 2)	Trial 2 (dilution 1)
FA2-2	10-16C	2.066×10^{-4}	8.983×10^{-4}	2.256×10^{-6}
FA2-2	10-16D	1.123×10^{-4}	2.021×10^{-4}	7.520×10^{-6}
FA2-2	V-14C	1.168×10^{-4}	3.593×10^{-4}	2.256×10^{-6}
FA2-2	IV-20C	2.236×10^{-4}	4.716×10^{-4}	0
FA2-2	IV-20D	3.490×10^{-5}	3.818×10^{-4}	0
FA2-2	8-14C	1.392×10^{-4}	3.818×10^{-4}	2.256×10^{-6}

Donor Strain	Recipient Strain	Frequency of Transfer
FA3714[(a)]	FA2-2	4.1×10^{-4}
F19530[(b)]	FA2-2	3.6×10^{-5}
FA2-2	F41[(c)]	2.1×10^{-6}

Notes: [(a)] Strain FA3714 is a laboratory strain of *E. faecalis* (5). This frequency was obtained via filter mating.
[(b)] Strain F19530 is a transconjugate food starter strain of *E. faecalis*. This frequency was obtained via broth mating experiments not filter mating (8).
[(c)] Strain F41 is a food starter strain used as a commercial milk product starter (8). This frequency was obtained via filter mating.

the transposon Tn*917,* which harbors the erythromycin resistant marker, was present in the transconjugate strains. Since the plasmid pAM714 is pAD1 with Tn*917* inserted into it, pAD1 was now present in the transconjugates. The data clearly demonstrates that pAM714 was transferred from donor strain FA2-2 to Group A Environmental *E. faecalis* and from donor strain OGIX to Group B Environmental *E. faecalis*.

The transconjugates were not only shown to be resistant to erythromycin, but also to have β-hemolytic activity. All of the environmental *E. faecalis* strains were originally shown not to have β-hemolytic activity. Once pAD1 was transferred, the recipients demonstrated β-hemolysis. This is quite significant, because it reveals the new transconjugates were not only

able to receive pAD1, but also able to express the virulence-associated genes encoded by the plasmid.

Research completed beyond this study could reveal whether or not the transconjugate environmental *E. faecalis* strains are pathogenic compared to parent environmental strains. As mentioned in the Introduction, the environmental *E. faecalis* strains harboring pAD1 could be studied in a rabbit endocarditis model (2) or systemically infected mice (15). The degree of damage or mortality rates would be compared to those previously published using medical strains of *E. faecalis* containing pAD1, as well as comparing the parent environmental strains of *E. faecalis,* which do not contain pAD1, as controls.

Bacteriocin capabilities could also be tested on several gram positive species of bacteria, including *Streptococcus* and *Clostridium,* and compared to parent environmental strains. This would reveal whether or not the transconjugates are capable of utilizing both virulence factors attributed to the cytolysin of pAD1, namely the hemolysin and bacteriocin. Another interesting study would investigate whether or not these transconjugate environmental strains can conjugate pAD1 to other environmental *E. faecalis* strains. If environmental *E. faecalis* strains can obtain pAD1 and could pass it onto other strains, the virulent factors associated with this plasmid could be spread in an environmental setting as well. The environment, then, could serve as a reservoir for virulent strains of *E. feacalis.*

This research has disclosed a possible mechanism for the spread of virulence factors within *E. faecalis* strains outside the nosocomial setting. Once harmless environmental strains could now potentially be lethal to a particular host, especially for those who are immune-compromised.

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