Research Grants for Graduate Students

Departmental Evaluation Sheet

Please fill out an evaluation form for each RGGS application submitted by your department. The completed evaluation forms and RGGS proposals are due in the Graduate School by 4:30 PM, October 1, 2008, or February 4, 2009. Proposals should be evaluated according to the three primary criteria for the RGGS program:

1. The originality/creativity and significance of the student's proposed research.
2. The clarity and appropriateness of the student's research design and procedure.
3. The feasibility of the student's proposed research.

Also note that the RGGS research projects should be for work that is to be conducted. Proposals that describe projects where significant work has already been completed are ordinarily not funded. Please pay particular attention to the timeline of the proposal to see that it accurately reflects the status of the project. Please note that RGGS funds cannot be used to reimburse money spent prior to the award. If you have questions about the evaluation of proposals, please contact the Graduate School.

Student Name: _____

Project Title: Biofilm formation and survival of capsule deficient mutants of Enterococcus faecalis in a root canal infe

This proposal was ranked ____ out of ____ proposals submitted by the department

In the space below, please provide your departmental evaluation of this proposal. If more than one proposal is submitted from your department, clearly explain the reasons for the relative ranking of this proposal. Attach additional sheet, if necessary.

Department: Biological Sciences

Signature of Chair: _____

Print Form

RESEARCH GRANTS FOR GRADUATE STUDENTS
Application Cover Sheet

2009-2010
Deadlines 10/5/2009 OR 2/8/2010

NAME	Student Number	Date:	Email Address
Home Town	Mailing Address		
Department Name		Dept. Campus Box	Requested Amount
Biological Sciences			$500.00

Project Title

Biofilm Formation and Survival of Capsule Deficient Mutants of Enterococcus faecalis in a Root Canal Infection Model

Nature of Project (check one)	Is this a resubmission? (Check one)
☑ Thesis	☐ Yes. If yes, previous app: _____
☐ Other Research Project	☑ No
Expected Date of Graduation:	Student's Signature:
7/10	

Compliances (Please check if your project involves any of the following):

☐ Animal Care	☑ Biosafety	☑ Hazardous Waste	☐ Human Subjects	☐ Radiological Safety

Project Summary (No more than 300 words)

This study will examine several E. faecalis factors important in the failure of endodontically treated teeth in an in vitro model. We will study the importance of the capsule in the penetration and colonization of E. faecalis in root canals using confocal and scanning electron microscopy. We predict that encapsulated E. faecalis will penetrate further into dentinal tubules and survive more effectively than capsule-deficient strains. In addition, we propose to identify E. faecalis factors relevant in this model. We will determine expression of virulence factors identified in other studies by RT-PCR. We expect to identity genes involved in root canal failure that could be used as targets in future therapeutic procedures.

APPROVALS

Major Advisor (Printed Name)	Major Advisor Signature	Date
		9-29-09
Department Chair (Printed Name)	Department Chair Signature	Date
		10/5/09

FOR GRADUATE SCHOOL USE

GPA: _____ **Earned Hours:** _____ **Reviewed** _____
Approved: _____ **Not Recommended:** _____

RPAB Chair Signature: _____ **Date:** _____

Project Begin Date: _____ **Final Report Due:** _____

2. Background and Significance

a. Problem to be solved. This study will test whether the capsule of *Enterococcus faecalis* facilitates the ability of the bacterium to penetrate the tooth root canal system after endodontic therapy, a procedure commonly referred to as a root canal. *E. faecalis* is the most common bacterium isolated after endodontic therapy failure. In this proposal, two aspects of *E. faecalis* will be addressed: 1) Previous studies have shown the bacterium can penetrate teeth root canal systems after endodontic therapy. This study will test if the capsule of *E. faecalis* facilitates the organism's ability to penetrate and colonize the root canal system post-treatment. 2) Various virulence-related genes of *E. faecalis* have been previously identified. This study will not only compare the expression of these genes between wild-type and capsule-deficient mutants, but also the temporal expression of these genes from biofilm isolates.

b. Significance of the research. This study will further the understanding of the role of polysaccharides in endodontic infections, specifically endodontic colonization by *E. faecalis*. With the increase in bacterial resistance, results from this study can provide a potential target for drug therapy. Also, this will be the first report using an *in vitro* root canal infection model to study the capsule of *E. faecalis* as well as the temporal expression of *E. faecalis* virulence-related genes within the model.

c. Hypothesis and objectives. We will test two hypotheses: 1) polysaccharide capsule contributes to *E. faecalis* invasion of medicated root canals; 2) genes encoding virulence factors will be upregulated in biofilms formed by a wild-type strain of *E. faecalis* in the *in vitro* model. For this experiment, a previously developed *in vitro* model will be used to compare the ability of wild-type and capsule-deficient *E. faecalis* cells to invade root canal systems. We will test biofilms formed as a result of infection for the temporal expression of genes encoding known virulence factors.

The following experiments will test our hypothesis:

1. *In vitro* root canal infection models will be infected with *E. faecalis* TX4002 (wild-type) and mutants TX5179 and TX5180, which are deficient in glycoproteins involved in capsule formation. Models will be infected and observed for the ingress into the root canal (as described under *Test Model*). The days to failure will be recorded, and survival as well as colonization of the wild-type and mutant strains will be determined.
2. *E. faecalis* cells that have invaded the root canal will be tested for the level of expression of genes encoding virulence factors by reverse transcription RT-PCR.

d. Discussion of related literature. *E. faecalis* is a gram-positive cocci found in the gastrointestinal tract, but is not indigenous to the oral cavity. Due to its ability to survive harsh environments, including extreme pH, salt, and temperature ranges, enterococci species have emerged as some of the most common pathogens in nosocomial infections. In fact, the *E. faecalis* is most frequently isolated among other pathogens after endodontic therapy. Previous studies have proposed that the biofilms formed as a result of infection allows the bacterium to resist antibacterial intracanal medicaments (1). It is believed biofilms play an important role in horizontal transfer of virulence-related genes from pathogenic to non-pathogenic strains of *E. faecalis* (2). Thus biofilm formation is essential to not only the survival, but penetration and colonization within the root canal system.

Polysaccharides consist of repeating oligonucleotide units and play an important role in the bacterial pathogenesis. This study will compare virulence-related genes in the wild-type OG1RF *E. faecalis* and mutant strains, TX5179 and TX5180. These mutants of *E. faecalis* contain an insertional disruption in the *epa* gene cluster, which is involved in polysaccharide biosynthesis (3). TX5179, named *epaB* mutant, and TX5180, *epaE* mutant, both produce *E. faecalis* with a deficient polysaccharide capsule (4). In addition to *epa* genes, this study will focus on the expression of other *E. faecalis* virulence-related genes including *efA* (endocarditis antigen), *esp* (*e*nterococcal *s*urface protein involved in adhesion), *asa* (aggregation substance), *gls24* (a stress protein related to virulence), *Ace* (collagen-binding adhesion) and *gelE* (a metalloprotease which targets biomolecules) (5). The expression of these genes within the biofilm differs from that of planktonic cells growing in a free suspension (6).

3. Procedure/ Methodology

a. Design of the study. This study will harvest cells that have infected the root canal system and test them for gene expression. A total of 80 roots will be tested: 10 control roots with uninoculated Brain Heart Infusion (BHI) broth, 20 will be inoculated with the wild-type, 40 will be inoculate with mutants (20 with TX5179, 20 with TX5180), and the last 10 roots will serve as models to study temporal gene expression in biofilm formation.

Test Model: This study will use a previously designed *in vitro* model system developed in Dr. Gillespie's laboratory. Before models are assembled, single-rooted human teeth are collected and disinfected in sodium hypochlorite. The teeth are prepared by removing the nerve of the tooth by instrumentation, known as endodontic therapy, and the crown at the top is removed so only the root remains. The model itself consists of a 5-mL Wheaton serum vial affixed by a hole-punched rubber stopper. The

prepared roots are inserted into the rubber stopper such that the top of the root remains exposed outside the model and the bottom tip of the root is oriented inside the vial. The test vial contains BHI broth to ensure immersion of the bottom tip of the root portion. The rubber stopper holding the tooth root is affixed to the serum vial by parafilm. All model components are sterile and assembled in a laminar flow hood using aseptic technique.

The model is inoculated with between 5-10 µL, depending on OD readings (see *Inoculum*) of log phase cells of *E. faecalis*. Cells are suspended in 50% concentrated BHI to limit available nutrients for the bacteria fostering ingress into the root canal system. After initial infection, the roots are sealed with a zinc oxide filling material, Cavit (3M ESPE, St. Paul, MN), which is normally used during endodontic therapy and has antibacterial properties. As the bacteria move down the root canal, they form biofilms and penetrate the dentinal tubules, which are microscopic canals that connect the inside of the tooth to the outside. At the bottom tip of the root, the bacteria that have penetrated the root canal system fall into the BHI broth creating turbidity, which indicates failure of the endodontic therapy.

Inoculum: *E. faecalis* cultures will be adjusted to an optical density at 600 nm (OD_{600}) of between 0.5 and 0.8. At this OD_{600}, 5 to 10 µL is sufficient to deliver between 1×10^6 and 2×10^6 *E. faecalis* cells (1). After initial inoculation, the models will be incubated at 37°C in an aerobic incubator for a 21-day incubation period and checked daily for failure as indicated by turbidity in the broth. A subset of 10 models will be used to look for temporal expression of genes within the biofilm, 5 early (24 hr-incubation) and 5 late (21 day-incubation). After model disassembly, all samples are stored in -80°C freezer.

Cell preparation: Teeth roots will be sonicated and/or milled in liquid nitrogen to isolate bacteria from the root canal system. These cells will be resuspended in a lysis buffer and then placed in a bead-beater to lyse the bacterial cells. The Qiagen RNA Bacterial Protect Reagent will be used to stabilize bacterial RNA before it is isolated on the miniprep column.

RNA isolation and RT-PCR: Qiagen miniprep column will used to isolate RNA from *E. faecalis* cells that have infected the root canal system. cDNA will be synthesized from RNA using Qiagen QuantiTect Reverse Transcription. RT-PCR will be conducted to quantitate specific mRNA levels using Qiagen SYBR Green Kit.

Confocal and Scanning Electron Microscopy (SEM): Live/dead bacterial staining using BacLight Bacterial Viability Kits will facilitate visual quantification of the surviving bacteria residing in the root canal system using confocal microscopy. Sputter coating

will be used for SEM imagery to visualize the colonization of *E. faecalis* in the dentinal tubules.

Data analysis: Statistical software will be used to process data collected from confocal microscopic images to quantify penetration depth into the dentinal tubules of the teeth roots.

Facilities and equipment. The research will be performed in two laboratories located on two separate campuses. Molecular analysis will be done at Dr. Vance McCracken's laboratory located on the third floor of the SIU science building in Edwardsville. Model infections and microscopy will be performed in Dr. M. Jane Gillespie's laboratory in the Science Building at SIU School of Dental Medicine in Alton. Dr. McCracken's lab is equipped with a bead-beater, and a Stratagene RT-PCR machine as well as gel electrophoresis equipment. Dr. Gillespie's lab is equipped with an Olympus light and phase microscope, a fume hood, a sonicator, and a Biotech Spiral Autoplater. In the building, there is a Varian-Carey spectrophotometer, a Nikon light and phase microscope with fluorescent and photographic capabilities, a scanning electron microscope (Jeol Model JSM35CF), a liquid-nitrogen freezer mill, 2 autoclaves, and a -80°C freezer. Also, there is a cell culture room with a laminar flow hood, where models are assembled. Both labs have a microcentrifuge, an incubator, 2 refrigerators, -20°C freezer, and a microbalance.

Materials. Model infections for the 21-day incubation period are currently in progress. To proceed, funding is needed for further molecular analysis. Supplies for RNA isolation and RT-PCR reagents are needed, including Qiagen RNA miniprep spin columns, Qiagen cDNA synthesis kits, and Qiagen SYBR Green kits.

b. Timeline. Preliminary infections and samples for this experiment have been collected. The second round of infections has commenced and the 21-day incubation period will end on October 9th, after which samples will need to be processed. The remaining molecular and microscopy work will take place over the next 5-8 months, if necessary, with the majority of the work completed by the end of 2009.

c. Anticipated Results. We predict capsular *E. faecalis* will penetrate further and survive more effectively within the root canal system than capsule deficient strains. We still expect penetration and survival of mutant *E. faecalis* strains as other factors besides the polysaccharide capsule contribute to its virulence and pathogenicity. Regarding gene expression, several genes encoding known virulence factors will be evaluated. Strains TX5179 and TX5180 are insertional *epa* mutants; therefore, expression of the capsular polysaccharide is impaired in these mutants.

We also predict other products of virulence-related genes including *efA*, *esp*, *asa*, *gls24*, *Ace*, and *gelE* will be more abundant in the wild-type strain than its capsule deficient counterparts.

References

1. Distel J, Hatton J, Gillespie MJ. Biofilm Formation in Medicated Root Canals. J Endod 2001. 28: 689-693.
2. Coburn PS, Baghdayan AS, Dolan GT, and Shankar N. Horizontal transfer of virulence genes encoded on Enterococcus faecalis pathogenicity island. Mol Microbiol 2007. 63: 530-544.
3. Teng F, Jaques-Palaz KD, Weinstock GM, Murray B. Evidence that the Enterococcal Polysaccharide Antigen Gene (*epa*) Cluster is Widespread in *Enterococcus faecalis* and Influences Resistance to Phagocytic Killing of *E. faecalis*. Infect Immun 2002. 2010-2015.
4. Teng F, Singh KV, Bourgogne A, Zeng J, Murray B. Further Characterization of the *epa* Gene Cluster and Epa Polysaccharides of Enterococcus faecalis. Infect Immun 2009. 77: 3759-3767.
5. Shepard BD and Gilmore MS. Differential Expression of Virulence-Related Genes in Enterococcus faecalis in Response to Biological Cues in Serum and Urine. Infect Immun 2002. 70: 4344-4352.
6. Donlan, RM. Biofilms: Microbial Life on Surfaces. Emerg Infect Dis 2002. 8:881-890.

4. Budget Narrative

$386 Qiagen RNA miniprep spin columns. Due to the low magnitude of cells that will be isolated from teeth root fragments, high-yield kits, such as Qiagen RNA miniprep spin columns, are required for efficient RNA isolation. Preliminary isolation experiments using existing Trizol reagents located in the lab failed to yield sufficient amounts of RNA from bacterial cells.

$114 RT-PCR kits and supplies including plastics, consumables. Additional reagents including Qiagen cDNA synthesis and SYBR Green kits will be provided by Dr. McCracken.

RESEARCH GRANTS FOR GRADUATE STUDENTS (RGGS)
BUDGET REQUEST

		Requested Amount	Department Recommendation
COMMODITIES *(Supplies, etc.):*			
1.	Qiagen RNA miniprep spin columns	$386.00	
2.	RT-PCR kits, supplies (plastics, consumables)	$114.00	
3.			
4.			
5.			
	Commodities Sub-Total:	$500.00	
TRAVEL:			
1.			
2.			
3.			
4.			
	Travel Sub-Total:	$0.00	
CONTRACTUAL SERVICES *(Postage, photocopying, etc.)*			
1.			
2.			
3.			
4.			
	Contractual Services Sub-Total:	$0.00	
EQUIPMENT:			
	Equipment Sub-Total:	$0.00	
TOTAL REQUEST:		$500.00	