

April 21<sup>st</sup>, 2016

To the reviewers of the Baker-Koob Endowment proposals,

As the faculty advisor of the project **“Effect of Hormonal Contraceptives on the Selection of Antibiotic Resistant Bacteria”**, I wholeheartedly support Rianna Flores and Chase Bowen’s application for the Baker-Koob endowment grants.

This project addresses a crucial issue in the field of microbiology, which is the alarming increase in antibiotic resistance. Overuse and abuse of antibiotics on a global scale has selected for bacteria that can resist many commonly used antibiotics leading to a severe public health crisis for this generation. Certainly, the antibiotics themselves provide the strongest selection for resistance, but other drugs that affect bacterial growth, like steroid hormones which have been shown to slow bacterial growth under *in vitro* test conditions, may also contribute to the selection. A common response of the gut bacterium *Escherichia coli* to antibiotics and to hormones is to remove them from the cell via transmembrane efflux pumps. One of the **merits of this project** is that it proposes to determine if the use of a very common drug (hormonal contraceptives) selects for intestinal *E. coli* with increased antibiotic resistance, by either 1) selecting for *E. coli* with an increased ability to efflux the antibiotic, or 2) inducing expression of efflux pumps in the presence of hormones simultaneously increasing antibiotic resistance. **The project has broad implications for public health, and specifically women’s health.**

Another **merit of this project is that it involves many Cal Poly students**, both as the study population, and as student researchers. Target populations (hormonal contraceptive users and non-users) right here at Cal Poly have contributed samples and information to the project, and five undergraduates, including the applicants Rianna Flores and Chase Bowen, have experienced the Cal Poly Learn by Doing credo as they have participated in preliminary aspects of this research.

Chase Bowen and Rianna Flores have been working on aspects of this project since Fall quarter, 2015. The initial stages of this research required collecting 30 *E. coli* isolates from the fecal samples of hormonal contraceptive users and non-users. They exhibited a professional attitude toward this unsavory aspect of the project and each of them processed several samples. In addition, they have both shown enthusiasm for the project by working on new and related experiments. For example, one analysis of the *E. coli* isolates is to test for the presence of genes correlated with pathogenicity in the bladder. They performed several experiments to modify a published multiplex PCR assay, and have written up the new protocol for use by their team members.

Both students are involved or plan to be involved in activities helping their fellow students. This quarter, Rianna applied to be a peer mentor, and Chase runs a study session in Biology. They are both smart, responsible, and dependable. They also exhibit excellent time management; they have initiated this grant’s writing process on their own, meeting on a regular schedule together, and then with their supervisor, Dr. Jennifer VanderKelen. I have every confidence that **they have the ability and the motivation to meet the objectives listed in the proposed narrative.**

The proposed experiments will be performed in the Center for Applications in Biotechnology (CAB) under the supervision of my collaborator, Dr. Jennifer VanderKelen. The CAB lab is fully equipped for the research planned with incubators, a spectrophotometer, pipettors, and autoclave, and ample lab space. This project falls within a larger project (involving my lab and my collaborators Dr. Chris Kitts and Dr. Jennifer VanderKelen) that looks at changes in the intestinal microbiome in response to hormonal contraceptives use.

# CAL POLY

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We will meet regularly with Rianna and Chase, lead discussions and literature reviews, analyze data, generate conclusions, and publish results as appropriate. Additionally, if the project is funded, I will supervise budget allocation, serve as signature authority, and ensure final report submission.

Please do not hesitate to contact me if you have any questions.

Sincerely,

Alejandra Yep, PhD

Assistant Professor  
Biological Sciences Department  
California Polytechnic State University  
Phone (805) 756-5355  
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## Warren J. Baker Endowment

*for Excellence in Project-Based Learning*

## Robert D. Koob Endowment for Student Success

### PROPOSAL NARRATIVE

#### I. Project Title

The Effect of Hormonal Contraceptives on Antibiotic Resistance of *Escherichia coli*

#### II. Abstract

The prevalence of antibiotic resistance continues to rise and it is known that efflux pumps are a determinant of multiple drug resistance in *Escherichia coli*. Additionally, studies have shown that efflux pump activity increases in the presence of steroid hormones. The effect of hormonal contraceptives on antibiotic resistance has not been fully elucidated. This study proposes to analyze the effect of antibiotic resistance of *E. coli* in the presence of hormones and hormonal contraceptives and determine the mechanism by which any observed resistance might happen. The ultimate goal of this project is the completion of two senior projects, poster presentations at the Spring Symposium and CSUPERB, and industry publication.

#### III. Introduction

Resistance to antibiotics is growing rapidly due to over-prescription and improper use of the drugs; nearly four out of five individuals in the United States are prescribed antibiotics, annually<sup>1</sup>, and compliance with recommended dosing is not always perfect. Additionally, commercial and agricultural use of antibiotics has contributed to what is becoming a global public health crisis. Other drugs might select for bacteria with increased antibiotic resistance through activating common resistance components; however, this has not been fully elucidated.

The mechanism of antibiotic resistance varies depending on the drug class and the mode of action of the antibiotic, but several studies have shown that Multi Drug Resistant efflux pumps export many different substances that can affect bacterial cell growth, including antibiotics<sup>2</sup>. A certain class of efflux pumps in the gram-negative bacteria *Escherichia coli*, composed of three proteins, AcrA (the membrane fusion protein), AcrB (the efflux transporter) and TolC (the transmembrane pore), has been shown to export toxins, such as antibiotics, out of the cell, thus allowing for their further survival<sup>2</sup>. Also, mammalian steroid hormones are substrates for efflux pumps in *E. coli*<sup>3</sup>. Sequestration of steroid hormones like estradiol and progesterone in *E. coli* mutants lacking AcrAB-TolC efflux pump activity causes slowed growth. Use of hormonal contraceptives, synthetic derivatives of the natural hormones, may expose cells to an abnormally high level of hormones that could stress the cell, making those with more active efflux activity grow faster. Finally, studies have shown that synthetic estrogens can induce genes that encode protein components of the chemical efflux pumps in gram-negative bacteria<sup>4</sup>. Based on these observations, it can be hypothesized that hormones, such as those in hormonal contraceptives, may either 1) induce the genes that encode for these efflux pumps, or 2) select for *E. coli* with a higher number of efflux pumps. Increased efflux activity may be observed as an increase in antibiotic resistance. In this particular study, we propose to analyze the effect of hormones and hormonal contraceptives on antibiotic resistance of human gut *E. coli* and determine the mechanism by which any observed resistance might happen.

Two separate experiments will be conducted to determine the mechanism of this form of antibiotic resistance; whether hormones **induce** the overall efflux pump expression in the

cell or if hormonal contraceptives **select** for *E. coli* with more effective efflux pumps.

Under the Learn by Doing credo, student researchers in the Center for Applications in Biotechnology have obtained 100 *E. coli* isolates from hormonal-contraceptive users and 100 *E. coli* isolates obtained from non-hormonal contraceptive within the female student body of Cal Poly. These isolates will be characterized as described in Objective 2 below.

#### IV. Objective(s)

1. To determine if there is increased antibiotic resistance in *E. coli* in the presence of estradiol-based steroid hormones. *Hypothesis 1*: Hormones **induce** efflux pump activity in *E. coli*. *Prediction 1*: If Hypothesis 1 is true, then *E. coli* strains should have higher antibiotic resistance as measured using the standard assay for antibiotic susceptibility, the Minimal Inhibitory Concentration Assay (MIC) in the presence of the steroid hormone. Additionally, this induction of antibiotic resistance in the presence of hormones should not be observed in the loss of efflux activity mutant ( $\Delta tolC$  mutant).
2. To determine if there is increased antibiotic resistance in *E. coli* isolated from hormonal contraceptive users. *Hypothesis 2*: Hormonal contraceptive use **selects** for *E. coli* with either a higher number of efflux pumps or for more effective efflux pumps overall. *Prediction 2*: If Hypothesis 2 is true, then hormonal-contraceptive users would have *E. coli* with higher MICs demonstrating an increase in antibiotic resistance.

These **project objectives are measurable and attainable given the time and financial constraints**, and they are **project based, Learn by Doing** experiments.

#### V. Methodology

To determine if steroid hormone, estradiol or ethinyl estradiol, induces *E. coli* efflux pump expression or activity in a way that increases antibiotic resistance, the antibiotic susceptibility profiles to four different antibiotics will be tested in the presence and absence of these two steroid hormones. Three different *E. coli* strains will be assayed, a wild-type strain standardly used as a control strain in MIC assays<sup>5</sup>, a  $\Delta tolC$  mutant, and the isogenic non-mutant strain. Two estradiol-derived hormones will serve as the experimental treatments: 1) A natural  $\beta$ -estradiol [either  $\beta$ -estradiol-17-( $\beta$ -D-Glucuronide) or  $\beta$ -estradiol-water soluble], and 2) ethinyl estradiol. Since estradiol is the prevalent form of estrogen in young women and ethinyl estradiol is the synthetic form of estrogen used in hormonal contraceptives, these chosen hormones are the most suitable for this study.

Four different antibiotics, ciprofloxacin, nitrofurantoin, bactrim (sulfamethoxazole and trimethoprim), and ceftriaxone, will serve as the second experimental treatment. These antibiotics were chosen because they represent different classes of antibiotics with different resistance mechanisms. There will be four groups of study: 1. (-)Hormone (-)Antibiotic, 2. (+) Hormone (-)Antibiotic, 3. (-) Hormone (+) Antibiotic, 4. (+) Hormone (+) Antibiotic. The effects of the different treatments on growth will be determined by the Minimum Inhibitory Concentration (MIC) Assay on microtiter plates, and growth curves will be performed to observe any specific effects on growth kinetics. If these steroid hormones do induce *E. coli* efflux pump expression or activity in a way that increases antibiotic resistance, a higher MIC will be observed in the presence of hormone. The  $\Delta tolC$  mutant, lacking the AcrAB-TolC efflux pumps should not show increased antibiotic resistance.

To determine if hormonal contraceptives select for *E. coli* with either a higher number of efflux pumps or for more effective efflux pumps overall, the antibiotic resistance profile of *E. coli* collected from either hormonal contraceptive users or non-users will be assayed. This



experiment will utilize *E.coli* strains collected from a prior study conducted in the Center for Applications in Biotechnology lab. Fecal samples were collected from ten hormonal contraceptive users and ten non-users, and 20 *E. coli* isolates were identified from each sample. These 200 isolates--100 from non-hormonal contraceptive users and 100 from hormonal contraceptive users will be subject to the MIC assay using the four antibiotics listed above. This antibiotic effect will be measured by observing the MIC on microtiter plates and supplemented with growth curves. If hormonal contraceptives select for *E.coli* with either a higher number of efflux pumps or for more effective efflux pumps overall, a higher MIC will be observed for the *E.coli* of Hormonal Contraceptive Users.

## VI. Timeline



## VII. Final Products and Dissemination

Upon completion of this project, **our plan for dissemination of the results** includes a College of Science and Math poster for the Spring Symposium, multiple Senior Projects, participation in CSUPERB, attending Biotechnology meetings, and an ultimate goal of industry publication.

## VIII. Budget Justification

The cost for Travel In State to the CSUPERB meeting in Long Beach was estimated to be about **\$385.00 total**. Car travel to Long Beach is 250 miles; standard reimbursement of 50 cents/mile is \$125 for transportation. One room for two nights is estimated to be about \$260.

Non-computer supplies and materials were estimated to cost about **\$3950**, including tax. Expected purchases include three hormone preparations,  $\beta$ -estradiol-17-( $\beta$ -D-Glucuronide) (\$93), Ethinyl Estradiol (\$102) and  $\beta$ -estradiol-water soluble (\$143), and materials for the MIC assays of 200 isolates with 4 antibiotics, and assays with and without hormone/antibiotic: approximately 300 microtiter plates (\$507), 400 boxes of pipette tips (\$2712), and one case of troughs (\$133). Because of the complexity of the MIC assay, setting up one assay plate uses one box of pipette tips. However, tips will also be utilized for preparatory purposes, justifying the need for an additional 100 boxes.

Printing and Duplication were estimated to cost about **\$150**. A 3x4 foot poster will be needed for CSUPERB and Spring Symposium. The printing of a poster costs about a \$10 per square foot. Registration for CSUPERB is about **\$25**. Journal Publication costs will cost about **\$400**. Journal publication can range from \$50-\$150 per page. Our goal is to produce a short communication publication. This will range from 1-3 pages. In summary, the **proposed budget is appropriate to attain the project objectives**.

<sup>1</sup> Center for Disease Control and Prevention, "Antibiotic Resistance Threats in the United States" <http://www.cdc.gov/features/antibioticresistancethreats/>

<sup>2</sup> Piddock, Laura JV (2006). "Multidrug-resistance efflux pumps? Not just for resistance." *Nature Reviews Microbiology*. 4(8): 629-636.

<sup>3</sup> Christopher Elkins, Lisa Mullis (2006). "Mammalian Steroid Hormones are Substrates for the Major RND- and MFS-Type Tripartite Multidrug Efflux Pumps of *E.coli*." *Journal of Bacteriology*. 188 (3): 1191-1195.

<sup>4</sup> Xinhua Li, Sondra Teske, Otakuya Conroy-Ben (2015). "Estrogen mimics induce genes encoding chemical efflux proteins in gram-negative bacteria." *Chemosphere*. 128: 327-331.

<sup>5</sup> Wiegand, I., Hilpert, K., & Hancock, R. E. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols*. 3(2): 163-175.

# Warren J. Baker Endowment

*for Excellence in Project-Based Learning*

## Robert D. Koob Endowment *for Student Success*

CAL POLY

### PROPOSAL BUDGET

<b>Student Applicant(s):</b>	Chase Bowen Rianna Flores
<b>Faculty Advisor:</b>	Alejandra Yep
<b>Project Title:</b>	<b>Requested Endowment Funding</b>
<b>Travel</b> <i>subtotal</i>	<b>\$ 385.00</b>
Travel: In-state	<b>\$385.00</b>
Travel: Out-of-state	<b>\$ 0</b>
Travel: International	<b>\$ 0</b>
<b>Operating Expenses</b> <i>subtotal</i>	<b>\$ 4525.00</b>
Non-computer Supplies & Materials	<b>\$3950.00</b>
Computer Supplies & Materials	<b>\$0</b>
Software/Software Licenses	<b>\$0</b>
Printing/Duplication	<b>\$150.00</b>
Postage/Shipping	<b>\$0</b>
Registration	<b>\$25.00</b>
Membership Dues & Subscriptions	<b>\$0</b>
Multimedia Services	<b>\$0</b>
Advertising	<b>\$0</b>
Journal Publication Costs	<b>\$400.00</b>
<b>Contractual Services</b> <i>subtotal</i>	<b>\$0</b>
Contracted Services	<b>\$0</b>
Equipment Rental/Lease Agreements	<b>\$0</b>
Service/Maintenance Agreements	<b>\$0</b>
<b>TOTAL</b>	<b>\$ 4910.00</b>