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# Characterization of a thiopurine S-methyltransferase from *Leptospira borgpetersenii* and assessment of pre and posttesting in the middle school classroom

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**Characterization of a thiopurine *S*-methyltransferase from *Leptospira borgpetersenii* and assessment of pre and posttesting in the middle school classroom**

by

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A dissertation submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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2013

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## LIST OF ABBREVIATIONS

<b>Arg 152:</b>	Arginine 152
<b>bTPMT:</b>	Bacterial thiopurine <i>S</i> -methyltransferase
<b>cTPMT:</b>	Cat thiopurine <i>S</i> -methyltransferase
<b>chTPMT:</b>	Chimpanzee thiopurine <i>S</i> -methyltransferase
<b>DAPI:</b>	4',6-diamidino-2-phenylindole
<b>DMHBA:</b>	3,4-dimethoxy-5-hydroxybenzoic acid
<b>DNA:</b>	Deoxyribonucleic acid
<b>dTPMT:</b>	Dog thiopurine <i>S</i> -methyltransferase
<b>ELISA:</b>	Enzyme-linked immuno sorbent assay
<b>EMJH:</b>	Ellinghausen-McCullough/Johnson Harris
<b>ESI:</b>	Electrospray ionization
<b>FBS:</b>	Fetal bovine serum
<b>gTPMT:</b>	Gorilla thiopurine <i>S</i> -methyltransferase
<b>hTPMT:</b>	Human thiopurine <i>S</i> -methyltransferase
<b>IBD:</b>	Inflammatory bowel disease
<b>IPTG:</b>	Isopropyl- $\beta$ -D-thiogalactopyranoside
<b>IS:</b>	Intestinal spirochetes
<b>kDa:</b>	Kilodalton
<b>LB:</b>	Luria broth
<b>LBJ0800:</b>	Putative thiopurine <i>S</i> -methyltransferase from <i>Leptospira borgpetersenii</i>
<b>ljbTPMT:</b>	<i>Leptospira borgpetersenii</i> thiopurine <i>S</i> -methyltransferase
<b>mTPMT:</b>	Mouse thiopurine <i>S</i> -methyltransferase

<b>NSF:</b>	National Science Foundation
<b>OCT:</b>	Optimal cutting temperature
<b>OD:</b>	Optical density
<b>OMPs:</b>	Outer membrane proteins
<b>PBS:</b>	Phosphate buffered saline
<b>PBST:</b>	Phosphate buffered saline containing Tween 20
<b>PCR:</b>	Polymerase chain reaction
<b>psTPMT:</b>	<i>Pseudomonas syringae</i> thiopurine <i>S</i> -methyltransferase
<b>RNA:</b>	Ribonucleic acid
<b>SAH:</b>	S-adenosylhomocysteine
<b>SAM:</b>	S-adenosyl-L-methionine
<b>SDS-PAGE:</b>	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
<b>STEM:</b>	Science, Technology, Engineering, and Mathematics
<b>TGNs:</b>	Thioguanine nucleotides
<b>TPMT:</b>	Thiopurine <i>S</i> -methyltransferase
<b>TWEEN 20:</b>	Polyoxyethylene (20) sorbitan monolaurate

## ABSTRACT

Spirochaete is a diverse phylum of Gram-negative bacteria that is located under the order, Spirochaetales, which is divided into three families: Spirochaetacea, Brachyspiraceae, and Leptospiraceae. Research was focused on the latter, specifically, the genus, *Leptospira*. *Leptospira* consists of many saprophytic, intermediate pathogenic, and pathogenic species, with *L. interrogans* (transmitted via contaminated water) and *L. borgpetersenii* (acquired via host to host transmission) causing the majority of leptospirosis cases. To gain insight into the pathogenesis of *Leptospira*, a putative thiopurine *S*-methyltransferase (LBJ0800) was exploited. Because this gene is not present in any of the sequenced spirochetes, *Brachyspira*, *Borrelia*, *Treponema*, except *Leptospira*, and even then, only in *L. borgpetersenii*, work was concentrated on this novel gene.

The open reading frame predicted to encode the putative thiopurine methyl *S*-methyltransferase (645 bp) was PCR amplified and directionally cloned into pET101 with a polyhistidine tag at the C terminus to provide plasmid, LBJ0800/pET101. To confirm the presence of a functional recombinant thiopurine *S*-methyltransferase, the expression construct was introduced into *E. coli* BL21 Star (DE3) cells, which were then grown in LB broth for IPTG-initiated induction of the protein. Cells were harvested by centrifugation, resuspended in Lysis Buffer and purified by a Ni-NTA metal-affinity column. Western blot analysis confirmed the purified, recombinant protein with a molecular mass of approximately 28 kDa.

In the second chapter, *in vivo* characterization of the putative thiopurine *S*-methyltransferase was analyzed. It was determined by ELISA experiments and

immunohistochemistry studies that although LBJ0800 is an unique protein, it was not expressed during infection at detectable levels. In chapter 3, research efforts focused on characterizing the gene enzymatically. Demonstrating substrate specificity, LBJ0800 methylated 6-thioguanine, validating its putative function as a thiopurine *S*-methyltransferase.

## CHAPTER 1. INTRODUCTION

### Overview: Research Synopsis

Students have varied perceptions, many of which are misconceptions, of scientific careers. Their knowledge of the field, scientists, and technicians are vague, inaccurate, stereotypic, and often reflects total ignorance of scientific careers, which deters them from desiring to perform well in mandatory science classes and choosing them as electives.<sup>1</sup> While middle school students perceive scientists as working indoors conducting dangerous experiments, high school students perceive scientists as middle to older aged men who wear lab coats and glasses and work in laboratories surrounded by large equipment.<sup>2</sup> As a resident scientist in a middle school classroom, I dispelled this erroneous description of a scientist. In the classroom, I shared my research and personal experiences, as well as determined if students were memorizing or comprehending science material presented on tests, while characterizing a thiopurine *S*-methyltransferase in the laboratory. Describing this novel gene from *Leptospira borgpetersenii*, a pathogenic species of spirochetes, fascinated the students and inspired them to ask questions about my research, bacteria, and possible career choices. By the end of the school year, the effectiveness of identical pre and posttests were determined, an appreciation for science and an understanding of possible scientific professions was gained, and knowledge of spirochetes and bacteria, in general was shared.

### Spirochetes

Spirochaete is a diverse phylum of Gram-negative bacteria that is located under the order, Spirochaetales, which is divided into three families: Spirochaetacea, Brachyspiraceae,

and Leptospiraceae.<sup>3</sup> Amongst these families, there are four genera of spirochetes that contain pathogenic species, *Brachyspira*, *Borrelia*, *Treponema*, and *Leptospira*.<sup>3</sup> Possessing a plasmid membrane surrounded by a peptidoglycan cell wall and an outer membrane sheath, spirochetes are helical-shaped, chemoheterotrophic motile bacteria.<sup>4</sup> Residing between the protoplasmic cell cylinder and the outer membrane sheath is a space often referred to as the periplasm. In this space lies an endoflagellum, resulting in spirochetes being morphologically unique from other prokaryotes.<sup>5</sup> This characteristic allows spirochetes to penetrate tissues and other viscous places that would normally inhibit other bacteria.<sup>6</sup> Virulence factors such as motility and corkscrew shape are critical to pathogenic spirochetes and are contributing virulence factors for leptospirosis, Lyme disease, and syphilis.<sup>7</sup>

Combatting infectious disease began with curing spirochaetal infections with mercury in the wake of the “Spanish disease,” now known as syphilis, in 1493.<sup>8</sup> To date, many pathologically important diseases are caused by a plethora of species of spirochetes and virulence factors are the source of this public health concern.<sup>9</sup> Hideyo Noguchi wrote the following words in response to observing the movement of spirochetes: “These minute filamental organisms dart through the soft medium with great rapidity, first in one direction and then in another, searching for a loose spot which they can pierce through. When encountering an impenetrable obstacle, they reverse their progression and start anew. A striking sight is thus presented by these little vermicular organisms darting in all directions.”<sup>10</sup> Understanding biological factors that are distinctive of each spirochete family such as habitats, metabolic requirements, diseases caused as well as other unique characteristics will assist in overcoming the burden of spirochaetal infections, which affect at least 0.5 million people, as well as animals worldwide.<sup>11</sup>



### *Spirochaetacea*

Four genera comprise the family, Spirochaetacea, with *Borrelia* and *Treponema* being the most well-known. Like most spirochetes, *Borrelia* is a helical-shaped bacterium that resides predominantly as an extracellular pathogen, needing special enriched media and low oxygen tension.<sup>12</sup> Entirely dependent on its environment for nutrition, the only species of Lyme borrelia known to cause human disease in North America, *B. burgdorferi* sensu lato, was isolated and cultured from *Ixodes* ticks and later from Lyme disease patients in the early 1980s.<sup>13,14</sup> *B. afzelii* and *B. garinii* are the infection causing species of most European cases.<sup>15</sup> Lyme disease, transmitted by infected ticks from traveling hosts such as deer, can cause clinical manifestations such as erythema migrans, arthritis, fever, headaches, and heart disorders, all of which are observed in humans.<sup>16</sup> Much research is still focused on host-pathogen interactions associated with lyme borreliosis, as it continues to be an emerging infectious disease.

The genus, *Treponema*, encompasses many nonpathogenic and pathogenic species that contain helical coiled, corkscrew-shaped treponemes.<sup>17</sup> Human pathogenic species that cause chronic treponematoses are *T. pallidum* subsp *pertenue*, *T. pallidum* subsp *endemicum*, *T. carateum*, and *T. pallidum* subsp *pallidum*, which is the causative agent of syphilis, a sexually transmitted disease.<sup>18</sup> The latter is a fastidious organism inactivated by mild heat and most disinfectants. Once thought to be a strict anaerobe, it is now known to be a microaerophilic, relying on its host for required compounds.<sup>19</sup> *T. pallidum* subsp *pallidum*, is the most aggressive of the pathogenic species, creating mild rashes to extremely damaging lesions in a plethora of tissues in the body.<sup>20</sup> As humans are the only reservoirs for this

fragile organism, syphilis is often acquired through sexual activity and across the placenta.<sup>21</sup> Due to the inability to cultivate *T. pallidum* in vitro, it remains difficult to properly and accurately diagnosis syphilis.<sup>22</sup> Observed clinical manifestations, treponemes present in lesions, and PCR techniques are currently being used for diagnosis while new techniques are being developed.<sup>23</sup>

### ***Brachyspiraceae***

The family, Brachyspiraceae, only has one genus, *Brachyspira*, which currently consists of 7 commensal and pathogenic species.<sup>24</sup> These anaerobic spirochetes, often referred to as “gut spirochaetes,” colonize the large intestine of avian and mammalian species.<sup>24</sup> van Leeuwenhoek first observed spiral “animalcules” in 1917 in his stool and Escherich was the first to provide a detailed report on human intestinal spirochetes.<sup>25,26</sup> In 1967, Harland and Lee created the phrase, “intestinal spirochetosis (IS),” to describe spirochetes’ adherence to the colorectal epithelium, which is considered to be the root of human disease caused by this genus.<sup>27</sup> *B. aalborgi* and *B. pilosicoli* are the two best-known species associated with human IS, the former being first identified in 1982.<sup>28</sup> Chronic diarrhea, weight loss, abdominal pain, and blood-stained feces are a few of clinical symptoms caused by IS.<sup>29</sup> *B. pilosicoli* is associated with porcine intestinal spirochetosis and *B. hyodysenteriae* is the best-know causative species of swine dysentery.<sup>30</sup> Malnutrition, lowered food intake, and declining growth rates are a few factors that contribute to extreme economic losses due to IS.<sup>31</sup> Because IS was initially a major concern in veterinary medicine for swine, human intestinal spirochetes’ biology, origin, and clinical significance continues to be datable.<sup>32,33</sup> *Brachyspira spp.* are slow growing organisms, require long incubation time

periods, and very difficult to grow on artificial culture media, making it difficult to isolate, study, and understand IS.<sup>34</sup> Despite these limitations, chemotaxis and motility still remain to be key factors in the colonization process, eventually causing disease.<sup>35,36</sup>

### ***Leptospiraceae***

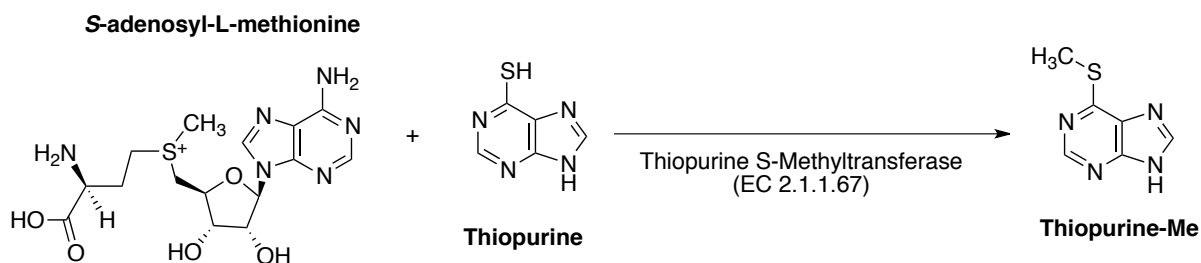
Leptospiraceae consists of long, thin, tightly coiled, helical-shaped spirochetes that have characteristics of both Gram-positive and Gram-negative bacteria in addition to periplasmic flagella, which make them highly motile.<sup>37,38</sup> Possessing translational or nontranslational movement, their cells have pointed ends and a hook at either or both ends, a leptospire hallmark.<sup>39</sup> Acquiring energy from the  $\beta$ -oxidation pathway, leptospire are obligate aerobes that can grow at an optimum growth temperature between 28-30°C in simple media, consisting of required ammonia as the nitrogen source and long chain fatty acids as the fuel and carbon sources.<sup>40,41</sup> Containing oleic acid, bovine serum-albumin, and polysorbate, Ellinghausen-McCullough/Johnson Harris (EMJH) medium is the most commonly used culture medium.<sup>41</sup> Because leptospire growth is slow on primary isolation, cultures are retained for approximately 26 weeks before being discarded.<sup>42,43</sup> Cultures are usually maintained by repeated subculture or in liquid nitrogen.<sup>43</sup>

*Leptonema* and *Leptospira* are the two genera in the family, *Leptospiraceae*.<sup>3</sup> Initially classified as *Leptospira* sp. serovar *illini*, after studying this organism by electron microscopy, it was determined that these strains were morphologically different than the cells of leptospire, thus needing a new genus, *Leptonema*.<sup>44</sup> *Leptospira* consists of many saprophytic, intermediate pathogenic, and pathogenic species, with *L. interrogans* (transmitted via contaminated water) and *L. borpetersenii* (acquired via host to host

transmission) causing the majority of leptospirosis cases.<sup>45,46</sup> Leptospirosis is an emerging infectious zoonosis that is a worldwide public health concern and toxin production, alteration of host immune responses, and motility appear to be the main pathogenic mechanisms in leptospirosis.<sup>47,48</sup> Reservoirs of the disease include rodents, dogs, and livestock.<sup>49</sup> *Leptospira spp.* colonize in the renal tubules of infected hosts and shed leptospire from its urine during its life span.<sup>45,50</sup> Leptospirosis is maintained via continued exposure to reservoirs.<sup>45</sup> Humans are accidental hosts, acquiring it directly, via interaction with infected animals or indirectly, via contaminated water or soil with infected urine.<sup>45,51</sup> Leptospire infiltrate broken skin or mucous membranes, disseminate via the bloodstream into tissues, and preferentially colonize the liver and kidney where they can obtain a great lipid supply, which is a necessary requirement for leptospiral growth.<sup>45,52a,52b</sup> Human infection can range from acute to chronic illnesses such as hepatic dysfunction and jaundice, acute renal failure, pulmonary haemorrhage syndrome, myocarditis and meningoencephalitis.<sup>45</sup> In animals, chronic infections with renal and hepatic failure as well as abortion and infertility in cattle can occur.<sup>53</sup> Even after the pathogen is eliminated from the host, leptospire may linger in immunoprivileged sites and cause uveitis months after exposure and urinary shedding weeks after the infection is cleared.<sup>45</sup> Although *L. interrogans* and *L. borgpetersenii* create similar clinical manifestations and share 2708 genes between them, there are several genes that are present in one species and not the other, namely, a thiopurine transferase that possesses a putative function of methylating thiopurines, selenium derivatives, and tellurite.<sup>45,46</sup>

## Thiopurine Methyltransferase

Thiopurine metabolism is very complex, involving numerous enzymes, thiopurine metabolites, and other compounds.<sup>54</sup> Thiopurine methyltransferase (TPMT; EC 2.1.1.6.7) is an extremely important enzyme to study because it is the only one tested routinely in patients.<sup>55</sup> TPMT is an *S*-adenosyl methionine-dependent methyltransferase that modulates cytotoxic effects of aromatic and heterocyclic sulfhydryl compounds by utilizing them as methyl acceptor substrates, resulting in inactivation (Scheme 1.1.).<sup>56,57</sup> These compounds, which are thiopurines such as 6-thioguanine, 6-mercaptopurine, azathioprine are cytotoxic, immunosuppressant compounds used for the treatment of organ transplants, neoplasias, autoimmune disorders, and inflammatory bowel disease.<sup>58,59,60</sup> In vivo, these prodrugs are converted to thioguanine nucleotides (TGNs), incorporate themselves into DNA, and trigger programmed cell death.<sup>61</sup> By using the compounds as methyl acceptor substrates and *S*-adenosyl-L-methionine (SAM) as a methyl donor, TPMT regulates their efficiency by methylation, efficiently inactivating a percentage of the administered dosage.<sup>62</sup>



**Scheme 1.1.** Basic enzymatic reaction catalyzed by thiopurine *S*-methyltransferase

Although the rodent thiopurine methyltransferase and the genetic polymorphism in humans was identified and characterized beginning in the early 1960s and 1980s,

respectively, the bacterial thiopurine methyltransferases research is relatively in the early stages.<sup>63,64,65</sup> In 1998, originally identified as a genetic determinant of tellurite resistance, a bacterial thiopurine methyltransferase was isolated from the plant pathogen, *Pseudomonas syringae* pathovar *pisi*, possessing 45% similarity with the aforementioned TPMTs.<sup>63,66</sup> Five years later, its tertiary structure was determined.<sup>67</sup> In addition to TPMT using thiopurine analogues as substrates, it has been reported that the bacterial TPMT (bTPMT) can metabolize the methylated selenium derivatives, dimethyl selenide and dimethyl diselenide, from inorganic and organic selenium.<sup>68,69</sup> Freshwater bacteria have demonstrated the same capability.<sup>70</sup> With the recent report of bTPMT being able to methylate tellurite, it is obvious that these enzymes are multitalented.<sup>71</sup> Identifying and characterizing other bTPMTs will give insight into additional functions and roles in bacterial pathogenesis. In an effort to identify and characterized other bTPMTs, LBJ0800, a putative thiopurine S-methyltransferase from *L. borgpetersenii* was cloned, expressed, and studied. In *Leptospira* metabolism, purines and pyrimidines do not inhibit growth; however, purine analogues such as 6-mercaptopurine does.<sup>72</sup> Determining LBJ0800's enzymatic function and substrate specificity will contribute to its role in *Leptospira*, as well as cell metabolism.

### **Initiatives to Increase the Representation of Students in Science, Technology, Engineering, and Mathematics (STEM)**

The National Science Foundation (NSF) has funded many outreach programs to ensure students are afforded a thorough understanding of information taught in STEM courses, through experimental learning.<sup>73</sup> Sympi, Iowa's GK-12 program is one of the initiatives to incite interest in STEM among middle, junior, and high school students.

Graduate students have the opportunity to increase their communication and skill sets by explaining their research to a nonscientific audience while providing students with hands-on activities and everyday life examples of the science and math topics that are located in their books.<sup>73</sup> As a Symbi, GK-12 fellow, a year of my graduate research was allocated to science education so that I could integrate a knowledge of bench science with teaching in a middle school classroom.

In middle school (grades 6 through 8), students learn basic math and science skills that can be utilized to answer fundamental scientific questions in preparation for answering the many questions researchers ask themselves daily.<sup>74</sup> During this delicate time period, students can gain an interest in science and math or lose interest if their curiosities remain untouched. Declining test scores and the opting out of advanced math and science courses are the undesired consequences of students not being exposed to the various applications of these fields.<sup>74</sup> Oftentimes elementary teachers lack an in-depth knowledge of STEM and their applications.<sup>75</sup> Mandating that they acquire such detailed knowledge while staying abreast of their field of expertise is impractical. Being the “resident scientist” in the classroom afforded me the opportunity to connect with middle school students and share my love for research. This experience allowed me to gain an even deeper appreciation for teaching science and the skillful craft involved as well as explore pedagogical approaches.

The effectiveness of various pedagogical approaches such as hands on experiential activities, lectures, case study experiences, problem-based learning, impacts and influences how students perform on the standardized state-wide exam. Along with the implementation of these approaches, identical pre and posttests are commonly utilized as a gauge to determine how much a student has comprehended over a period of time. Although testing

provides insight into comprehension, educators feel that testing decreases teaching time and makes it almost impossible to perform activities that are related to the subject being taught that are not included in the textbook.<sup>76</sup> Furthermore, students begin to have a negative stance towards learning when it appears that higher test scores, not learning, is the end goal.<sup>77</sup> In an effort to decrease testing, I sought to determine the effectiveness of identical pre and posttests. If only one type of testing approach is fostered, students are not encouraged to process and think in other ways; therefore, a natural instinct will be to use the same approach that is used in the classroom on the standardized state-wide exam, which might require a different approach. The goal of this study was to demonstrate whether or not identical pre and posttest accurately assess student learning and the effect testing had on teachers and their implementation of information gained during professional development. Because learning is more important than assessing student learning, a relatively stress-free environment for teachers is essential so that students can enjoyably learn about STEM at an impressionable time period, resulting in the necessary foundation needed to have a successfully, rewarding scientific career.

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## CHAPTER 2. RECOMBINANT PRODUCTION AND *IN VIVO* CHARACTERIZATION OF A THIOPURINE S-METHYLTRANSFERASE FROM *LEPTOSPIRA BORGPIETERSENII*

### Summary

Leptospira are pathogenic spirochetes that can cause the pervasive zoonotic bacterial disease, Leptospirosis. *Leptospira* colonize organs such as the kidney and liver and cause adverse effects in its hosts. The two genomes of two pathogenic species, *L. interrogans* and *L. borgpetersenii*, have been sequenced and although these species share numerous genes, there are some genes that remain unique to each species. LBJ0800 is not present in any sequenced spirochete strains except for *L. borgpetersenii*. In this study, I describe cloning, recombinant protein expression, and in vivo characterization of a thiopurine S-methyltransferase (LBJ0800) unique to a limited number of *L. borgpetersenii* strains that cause fulminant infections in golden Syrian hamsters, and are not detected in other spirochete genomes. LBJ0800 is not present in any sequenced spirochete strains except for *L. borgpetersenii*. ELISA experiments and immunohistochemistry studies indicate that although LBJ0800 is a unique gene/protein, it is not expressed during infection at detectable levels.

### Introduction

Leptospirosis is a worldwide zoonosis that affects both humans and animals.<sup>1</sup> There are several pathogenic species of *Leptospira*, with *L. interrogans* and *L. borgpetersenii* being the most common in human and animal infections.<sup>2</sup> Recognized as an emerging infectious disease, *Leptospira* causes infections ranging from chronic to acute potentially life-



threatening clinical diseases.<sup>3,4</sup> These bacteria colonize in the kidneys of infected animals and are excreted through their urine, contaminating soil and various water sources.<sup>5</sup> Although humans and animals are susceptible to this disease via direct contact with an infected animal or indirect contact via environmental sources, rodents are the main reservoirs responsible for transmission; humans are only accidental hosts, acquiring the bacteria through mucosal membranes, scrapes and cuts.<sup>6,7</sup>

Characterizing leptospiral proteins is an essential step in identifying potential virulence factors. Identifying proteins involved in the pathogenesis of *Leptospira* may result in possible new and innovative approaches to prevent this infectious disease. Insight into the pathogenesis of leptospirosis has been gained by the expression and characterization of outer membrane proteins (OMPs) such as LipL46, LipL41, LipL36, LipL32, and LipL21.<sup>6,8,9,10</sup> Immunohistochemistry and the use of ELISAs are common methods utilized to detect OMPs expressed during infection, in part because these proteins are often expressed at higher levels than other proteins in the bacterial cell and are at the interface between the bacteria and the host.<sup>11,12</sup> In this report, LBJ0800, a thiopurine methyltransferase is identified and analyzed.

## Materials and Methods

### Materials and general methods

Enzymes and reagents used for molecular biology procedures, DNA Ladders and deoxynucleotide triphosphates were purchased from Invitrogen, BD Biosciences, and New England Biolabs. Oligonucleotides were synthesized by The DNA Facility of the Iowa State University Office of Biotechnology (Ames, IA). Protein molecular weight standards were obtained from GE Healthcare Life Sciences. The QIAprep Spin Miniprep kit was obtained

from Qiagen and the Champion pET Directional TOPO Expression Kit was obtained from Invitrogen. All chemicals were obtained from Sigma Aldrich unless otherwise stated. The DNA procedures, including plasmid DNA isolation, agarose gel electrophoresis, and transformation of *E. coli*, were performed according to standard techniques (Sambrook et al. 1989) and manufacturer's instruction. Protein concentrations were determined with the Bio-Rad protein assay method (Bradford 1976).

### **Bacterial strains and growth conditions**

Genomic DNA of *Leptospira borgpetersenii*, obtained from *L. borgpetersenii* strain JB197 (Bulach et al 2006), was used for all cloning experiments. OneShot Top10 chemically competent cells *Escherichia coli* cells (Invitrogen, Carlsbad, CA) and PCR-Blunt pET101 vector (Invitrogen, Carlsbad, CA) was used for directional cloning of PCR products. *Escherichia coli* BL21 Star (DE3) competent cells (Invitrogen, Carlsbad, CA) were used in combination with the T7 expression system (pET101 vector; Invitrogen, Carlsbad, CA) for expression of the thiopurine *S*-methyltransferase. *Escherichia coli* cells were grown on Luria Bertani (BD Biosciences, San Jose, CA) medium at 37 °C in an incubator shaker at 225 rpm. Carbenicillin was added at 50 mg/ml to make the selective media.

### **PCR amplification, cloning, expression, and purification of enzyme**

The LBJ0800 gene was amplified from JB197 genomic DNA by PCR synthesis using two oligonucleotide primers. The forward primer, 5'-CACCATGGACACTAATTTTTGGC-3,' and the reverse primer, 5'-ATCGGTCGATTGTTTCAACAATAACC-3,' were designed from the putative thiopurine *S*-methyltransferase of *L. borgpetersenii*. The cycling

parameters were 95 °C for 5 min, followed by 35 cycles of 94 °C for 15 sec, 60 °C for 30 sec, 68 °C for 2 minutes, with a final elongation step of 72 °C and holding step for 10 °C. The amplified DNA was cloned in pET101 vector using topoisomerase to yield constructs for the expression of putative thiopurine *S*-methyltransferase containing a C-terminally fused His<sub>6</sub> tag. The LBJ0800/pET101 construct was transformed into *E. coli* expression strain BL21 Star (DE3) (Invitrogen, Carlsbad, CA). Transformants were grown in LB medium broth containing 50 µg/ml at 37 °C for approximately 18 hours in a shaking incubator. Recombinant expression was induced with 0.4mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 16 hours at 16 °C (Houston et al. 2011) after an OD<sub>600</sub> reached 1.0. Cells were harvested by centrifugation at 4000 g and the QIAexpress Ni-NTA Fast Start Kit and protocol (Qiagen) were utilized for the purification of the recombinant 6xHis-tagged protein under native conditions. The purified protein was dialyzed into PBS using a Slide-A-Lyzer Dialysis Cassette, 10 MWCO (Thermo Scientific, Rockford, IL) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot using wet transfer techniques (Bio-Rad Laboratories, Hercules, CA). The Limulus Amebocyte Lysate (LAL) Endotoxin Test (Lonza, Walkersville, MD) and EndoTrap blue 5/1 (Biovendor) were used for the detection, quantification, and removal of bacterial endotoxins.

### **LBJ0800 Specific Antisera Production**

All animal experiments described were reviewed and approved by the National Animal Disease Center's Institutional Animal Care and Use Committee and were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Washington, DC).

Two female New Zealand White rabbits approximately 8-weeks old were immunized for production of polyclonal antiserum to recombinant LBJ0800. Purified recombinant protein LBJ0800 was suspended in sterile saline at a concentration of 1mg/ml and used according to manufacturer's directions to prepare eventual adjuvant emulsions (below). The doses used to immunize the rabbits are summarized in the table below (Table 2.1.). Adjuvant was rehydrated with sterile saline according to manufacturer's directions and emulsion of protein and adjuvant made by mixing with two 3 mL all-plastic syringes connected by an adjuvant bridge (Vita Needle Luer Lock Adaptor, Vita Needle Company, Needham, MA) for several minutes.

Injection protocols were modified slightly from manufacturer's recommendations. One rabbit, named Marple, was immunized using MPL+TDM adjuvant (Sigma M6661) containing monophosphoryl Lipid A (isolated from *Salmonella minnesota*) and synthetic trehalose dicorynomycolate in squalene oil, Tween 80 and water. Marple received booster injection at two, four and seven weeks using the MPL+TDM adjuvant preparation according to the following regimen, MPL+TDM adjuvant was administered 50  $\mu$ l in each of six sites intradermally (over the back), 200  $\mu$ l intramuscularly (quadriceps of one hind leg), 125  $\mu$ l in each of three sites subcutaneously (over the neck and each shoulder). The second rabbit, named Maxine, was initially immunized using the TiterMax® Gold adjuvant (Sigma T2684), and received booster injections at two weeks using soluble protein, at four weeks using TiterMax® Gold and ultimately at seven weeks with the MPL+TDM adjuvant preparation. TiterMax® Gold adjuvant was administered according to the following regimen, 100  $\mu$ l intramuscularly (quadriceps of one hind leg), 125  $\mu$ l in each of five sites subcutaneously

(over the neck, over each shoulder, and over the quadriceps of each hind leg). The two-week booster consisted of soluble recombinant protein 50  $\mu$ l in each of two sites given subcutaneously (over each shoulder). The four-week booster followed the initial TiterMax® Gold regimen, and a final booster given at seven weeks employed the MPL+TDM adjuvant preparation and injection protocol.

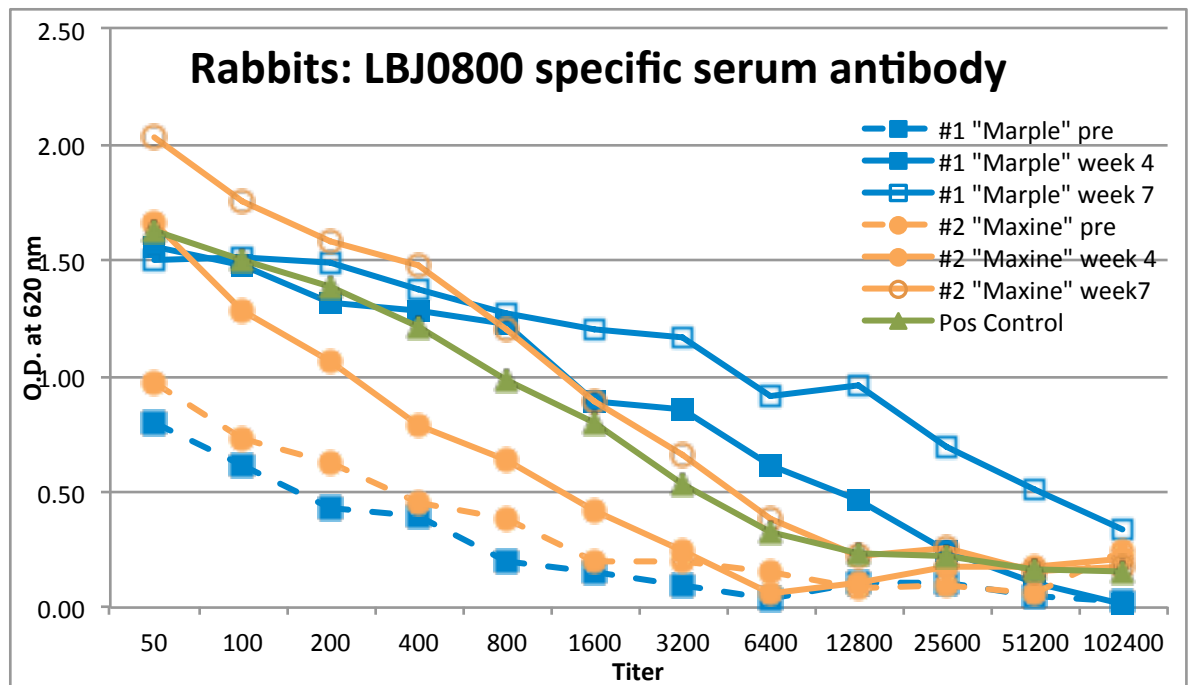
Serum antibody titers were checked before immunization and at 2 weeks following each booster immunization. Peripheral blood was collected from the lateral ear vein under mild sedation using acepromazine administered subcutaneously. Serum was separated by centrifugation and stored at -20 °C until assayed by ELISA (Figure 2.1.). Upon demonstration of satisfactory titers, terminal exsanguination was performed under ketamine/xylazine anesthesia.

Serum samples collected prior to immunization and every two weeks following immunization were tested for LBJ0800-specific antibodies. Immulon 2 flat bottom ELISA plates (Dynatech Laboratories, Chantilly VA) were coated overnight with 1  $\mu$ g/mL LBJ0800. Plates were washed with phosphate buffered saline (PBS, pH 7.4) containing 0.5% Tween 20 (PBS-T) and blocked for two hours with PBST+ 3% gelatin (Type B, Sigma). Plates were washed and individual serum samples were serially diluted in PBST and incubated overnight at 4 °C. High titer rabbit sera from NVSL to serovar Hardjo 197 was used as a positive control. On the third day, plates were washed again with PBST and horseradish peroxidase-conjugated goat anti-rabbit IgG (H&L) (Kirkregard & Perry Laboratories (KPL), Gaithersburg MD) (1:10,000 dilution) was added. After incubating for two hours, plates were washed and 100  $\mu$ l of SureBlue Reserve TMB Microwell Peroxidase Substrate (KPL) was added to each well. After a 15-minute incubation, stop solution (TMB BlueSTOP, KPL) was

added and changes in optical density (OD) were measured spectrophotometrically at 600 nm. The titer was determined as reciprocal of highest dilution resulting in OD greater than or equal to 0.5.

<b>Rabbit</b>	<b>1<sup>st</sup> immunization</b>	<b>2-week booster</b>	<b>4-week booster</b>	<b>7-week booster</b>
<b>1 “Marple”</b>	Adjuvant: MPL+TDM	Adjuvant: MPL+TDM	Adjuvant: MPL+TDM	Adjuvant: MPL+TDM
	0.5 mg LBJ0800	0.5 mg LBJ0800	0.5 mg LBJ0800	0.5 mg LBJ0800
<b>2 (Red Spot) “Maxine”</b>	Adjuvant: TiterMax Gold	Adjuvant: None	Adjuvant: TiterMax Gold	Adjuvant: MPL+TDM
	0.5 mg LBJ0800	0.1 mg LBJ0800	0.5 mg LBJ0800	0.5 mg LBJ0800

**Table 2.1.** Adjuvants and doses of protein administered



**Figure 2.1.** Serum antibody responses as measured by ELISA, pre-immunization, two weeks following the week 4 and week 7 immunizations with LBJ0800 protein

## ELISA

Immulon, flat-bottom, 2HB, clear polystyrene 96-well microtitre plates were coated overnight at 4 °C with 50 µl per well of 1µg/ml purified LBJ0800. Plates were blocked for 2 hours at room temperature with PBS containing 0.05% Tween 20 (PBS-T). Wells were incubated overnight at 4°C with 100 µl per well of a 1:50 dilution of pre and post challenge *L. borgpetersenii* serovar Hardjo strains JB197 and 203 antisera and washed four times with PBST. Wells were incubated with 100 µl per well of a 1:10,000 dilution of Sheep anti-Bovine IgG-heavy chain Antibody, HRP Conjugated (BETHYL Laboratories, Inc., Montgomery, TX) for 2 hours at room temperature, followed by five washes with PBST. ELISA plates were developed by adding 100 µl per well of Sure Blue Reserve TMB Microwell Peroxidase Substrate (1-Component) (KPL, Inc., Gaithersburg, MA) for 15

minutes in the dark at room temperature. The reaction was stopped by adding a 100  $\mu$ l volume of TMB BlueSTOP Solution (KPL, Inc., Gaithersburg, MA) and the absorbance at 655 nm was measure immediately.

### **Immunohistochemistry**

The processes used to obtain and prepare tissues from infected and uninfected hamsters for immunohistochemistry have been modified from methods described elsewhere (Matsunaga et al., 2006; Eshghi et al., 2012). Golden Syrian hamsters were inoculated either with *L. interrogans* serovar Pomona strain RM211 or *L. borgpetersenii* serovar Hardjo strains JB197 and 203. After euthanization of moribund and healthy uninfected hamsters, liver and kidney tissues were removed and fixed in 10% buffered zinc formalin and paraffin-embedded. Serial 4  $\mu$ m sections of kidney and liver tissue were cut. Paraffin was removed from tissue sections with xylene and ethanol, using standard procedures. To visualize cell and tissue organization and structure, sections were stained with haematoxylin and eosin (H/E). Tissue sections were prepared by initially blocking non-specific antigen sites using 10% normal goat serum/PBS at room temperature for 60 min, prior to incubation at 4 °C with primary antibody, Anti-LBJ0800 used at a 1:100 dilution. Normal goat serum/PBS block was used as a negative control and Anti-LipL-32, at a 1:200 dilution, was used as a positive control for leptospire on all tissue sections from both infected and uninfected hamsters. Sections were washed with PBS to remove unbound antibody and then incubated at room temperature for 60 min in the dark with a 1:800 dilution of Alexa Fluor 594 F(ab')<sub>2</sub> Fragment of Goat Anti-Rabbit IgG (H+L) secondary antibody (Invitrogen). Sections were then immediately incubated for 10 min with a 1:3000 dilution of 4',6-diamidino-2-phenylindole



(DAPI). After rinsing with PBS, slides were mounted with ProLong Gold Antifade reagent (Invitrogen). All images were captured on a Spot RT color CCD camera mounted on a Nikon Eclipse E800 microscope. All fluorescent images were captured under identical exposure conditions.

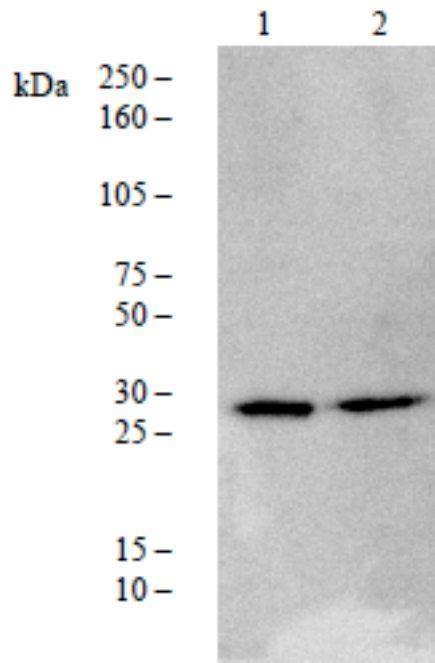
Cardiac blood smears and homogenized tissue from infected hamsters' liver and kidney were airdried, fixed in acetone, and airdried again. Spots of *Leptospira borgpetersenii* serovar Hardjo strains JB197 and 203 organisms of actively growing cultures were airdried and processed similarly as the paraffin section post paraffin removal. Freshly cut liver and kidney from infected hamsters were placed in optimal cutting temperature (OCT) embedding compound and stored at -80 °C. Cryosections were cut 7 µm thick, allowed to air dry, and fixed in acetone. Visualization and fluorescent immunohistochemistry for blood smears, spots of organisms from cultures, and frozen tissue were identical to the formalin fixed paraffin embedded tissue sections. (Data not shown)

## Results

### Cloning and Expression of LBJ0800

An open reading frame predicted to encode the thiopurine *S*-methyltransferase (645 bp) was PCR amplified and directionally cloned into pET101 with a polyhistidine tag at the C terminus generating plasmid, LBJ0800/pET101. To confirm the presence of a recombinant thiopurine *S*-methyltransferase, the expression construct was introduced into *E. coli* BL21 Star (DE3) cells, which were then grown in LB broth for IPTG-initiated induction of the protein. Cells were harvested by centrifugation, resuspended in Lysis Buffer and purified by

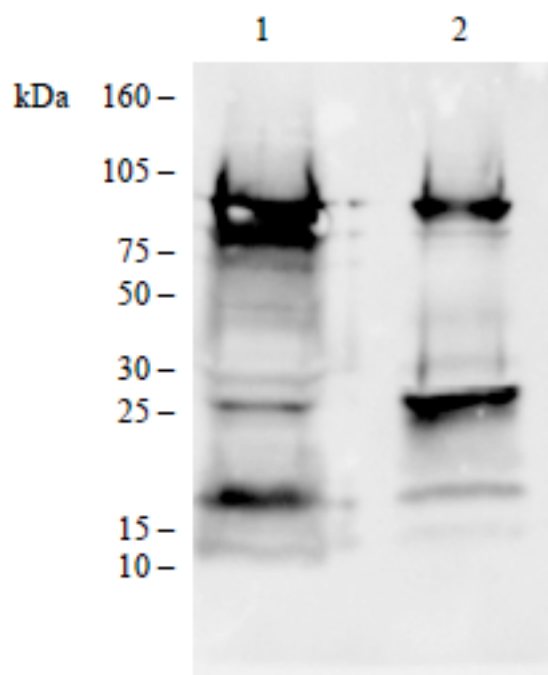
a Ni-NTA metal-affinity column. Western blot analysis confirmed the purified, recombinant protein with a molecular mass of approximately 28 kDa (Figure 2.2.).



**Figure 2.2.** Western blot of the purified polyhistidine tagged thiopurine *S*-methyltransferase expressed in *E. coli*, purified using a Ni-NTA metal-affinity column, and detected by Anti-V5 Antibody. *Lanes 1 and 2:* 1<sup>st</sup> and 2<sup>nd</sup> elutions of LBJ0800, respectively.

### Antisera Production to LBJ0800

Two rabbits were immunized with the recombinant protein, LBJ0800 and either adjuvant, MPL+TDM (Marple) or TiterMax Gold (Maxine). Serum antibody titers were checked before immunization and at 2 weeks following each booster immunization. Animals were euthanized, upon demonstration of satisfactory titers. LBJ0800 was recognized by the post-bleed antiserum from “Marple,” which is observed in Figure 2.3.



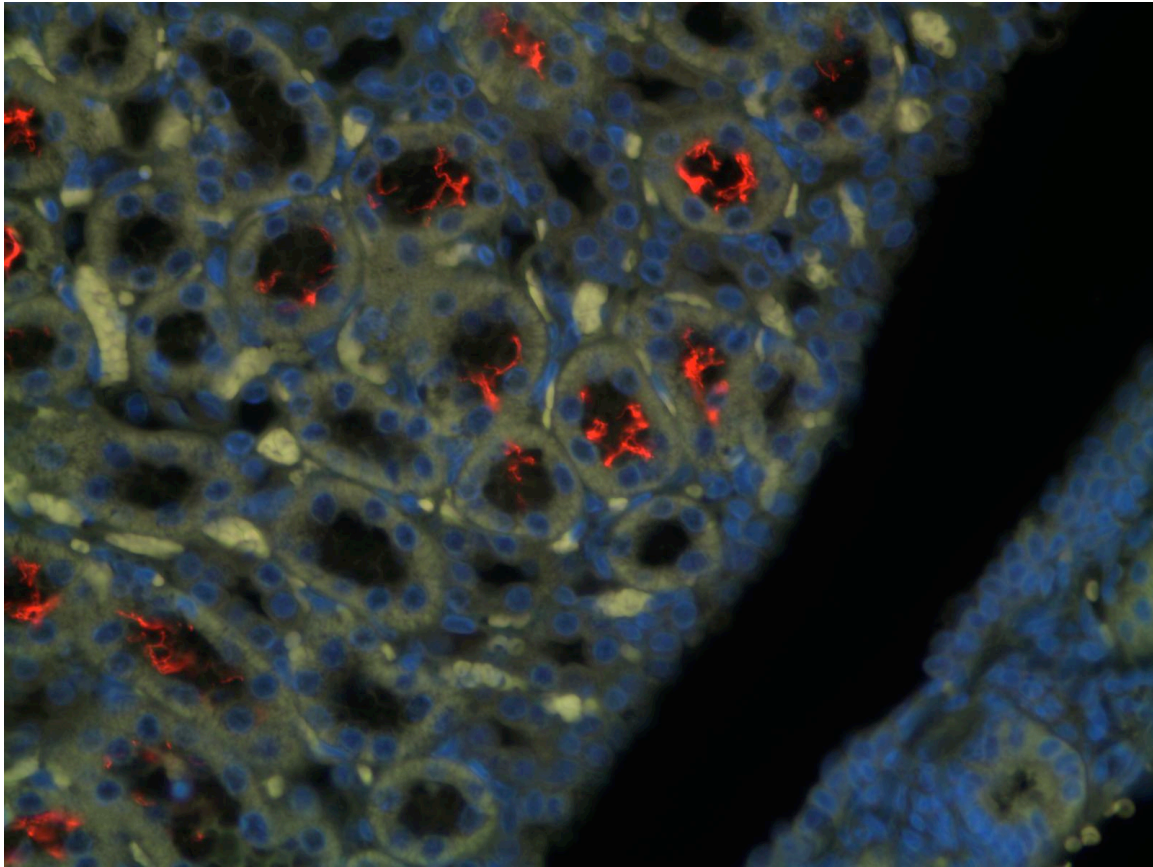
**Figure 2.3.** Immunoblot displaying reactivity of LBJ0800 post-bleed antisera from “Marple” with LBJ0800. *Lane 1:* Supernatant; *Lane 2:* purified thiopurine *S*-methyltransferase at ~ 28 kDa.

### ELISA

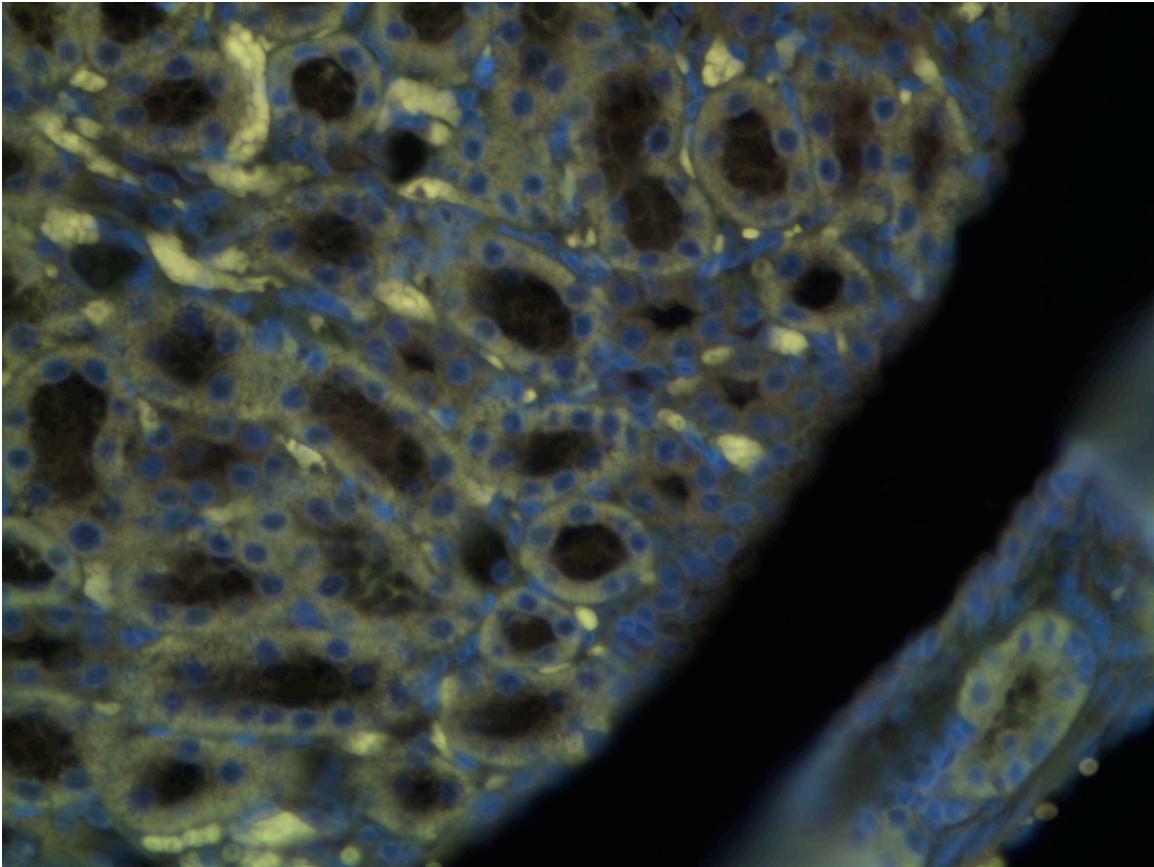
Pre and post challenge sera of cows infected with *L. borgpetersenii*, serovar Hardjo strains 197 and 203 were utilized to determine if LBJ0800 was expressed during leptospirosis infection. Fetal bovine serum (FBS) and PBS were used as negative controls. FBS and PBS displayed insignificant reactivity levels to LBJ0800. Comparable levels of reactivity to LBJ0800 was observed when pre and post challenge JB197 and 203 sera were applied to the wells, indicative of undetectable expression levels of LBJ0800 during infection (data unshown).

### **Immunohistochemistry with LBJ0800 antisera**

To determine if the thiopurine *S*-methyltransferase was expressed in vivo, uninfected and infected tissues from hamsters challenged with *Leptospira borgpetersenii*, serovar Hardjo strains 203 and 197 were studied. Antigen expression by leptospire in blood vessels, the kidney, and liver was examined. In Figure 2.5., organisms within the convoluted tubules of kidney sections were detected with antisera to LipL32. Organisms were not detected in the exact kidney sections for pre and post bleed antisera to LBJ0800 (Fig. 2.6. and 2.7.). Leptospire in between hepatic cells are obvious with antisera to LipL32 (Figure 2.8.). Unfortunately, there is no antigen detection within the liver sections for pre and post bleed antisera from immunized rabbit to LBJ0800 (Figures 2.9. and 2.10.).

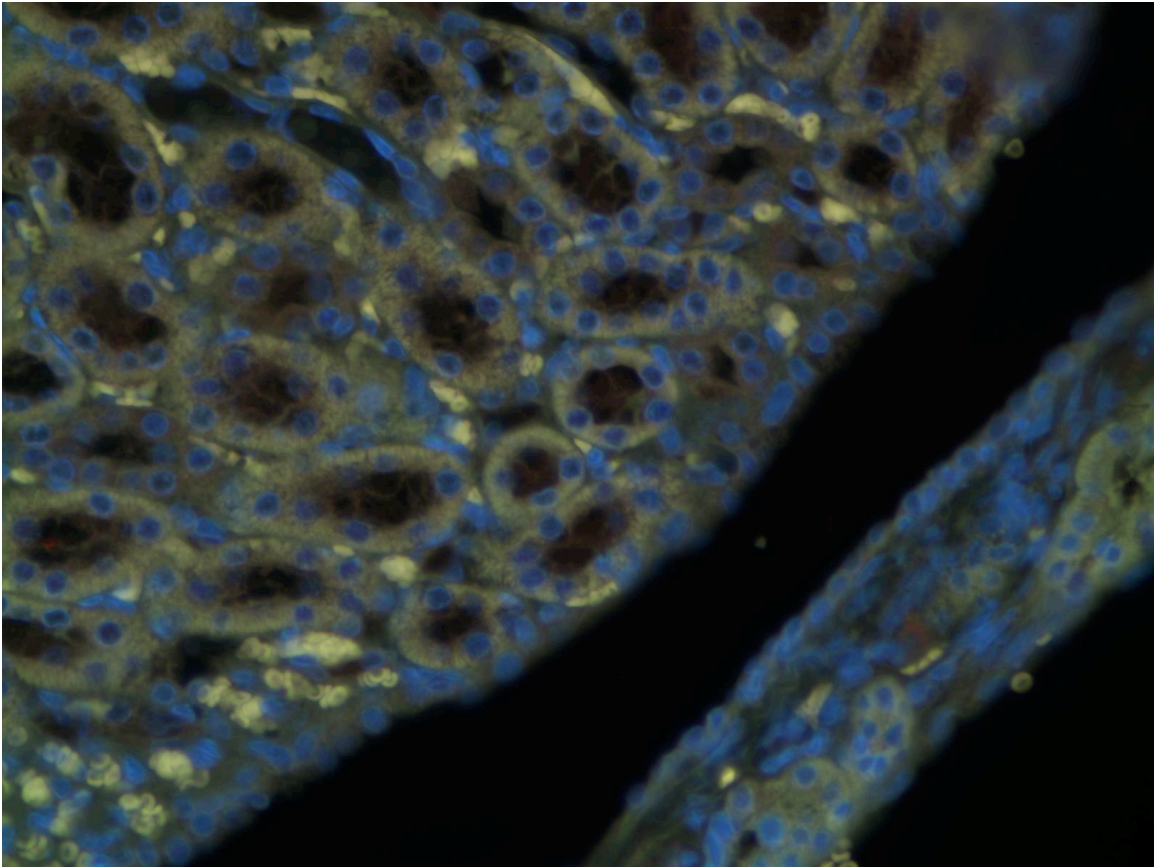


**Figure 2.4.** Strain 203 Infected Hamster's Kidney with LipL32 Antibody.  
Blue: Chromatin (DAPI stain); Yellow: Red blood cells (Autofluorescence); Red: Spirochetes (Alexa Fluor 594 F (ab')<sub>2</sub> Fragment of Goat Anti-Rabbit IgG (H&L) secondary antibody)

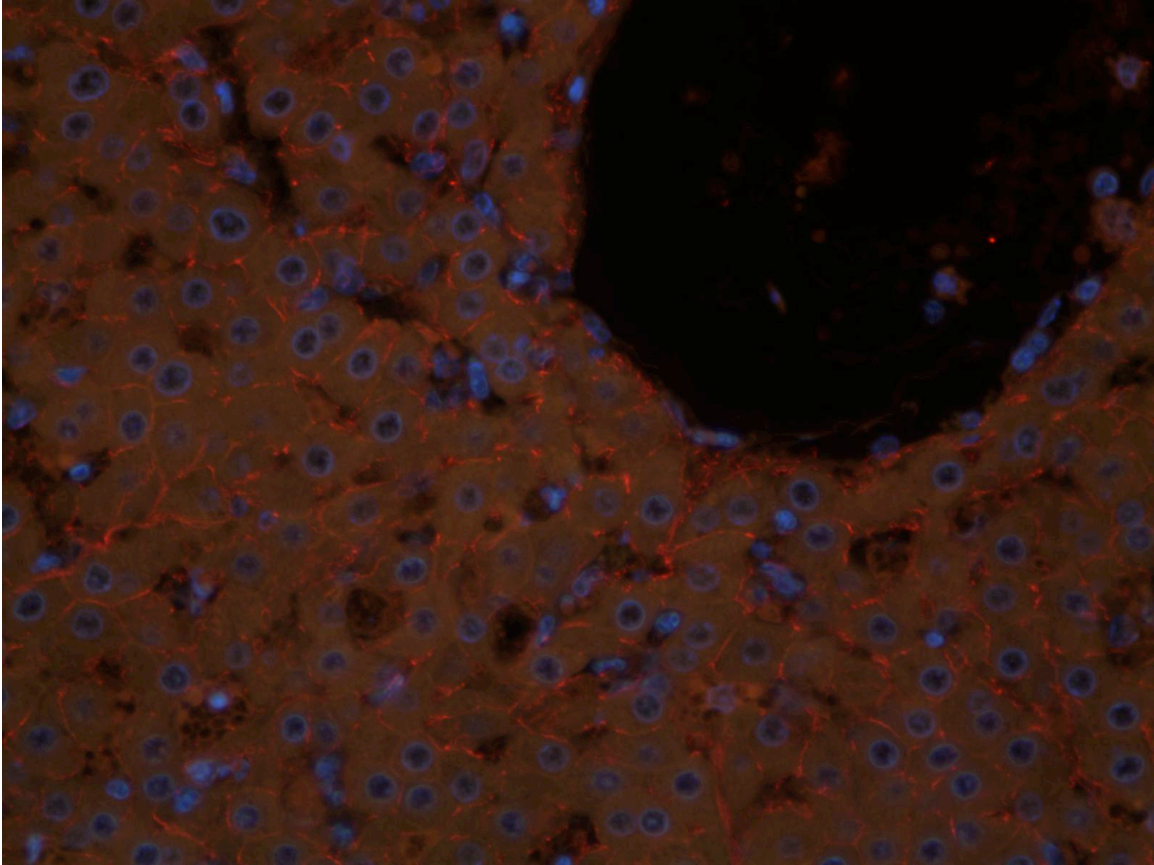


**Figure 2.5.** Strain 203 Infected Hamster's Kidney with LBJ0800 Pre-bleed Antibody.  
Blue: Chromatin (DAPI stain); Yellow: Red blood cells (Autofluorescence)



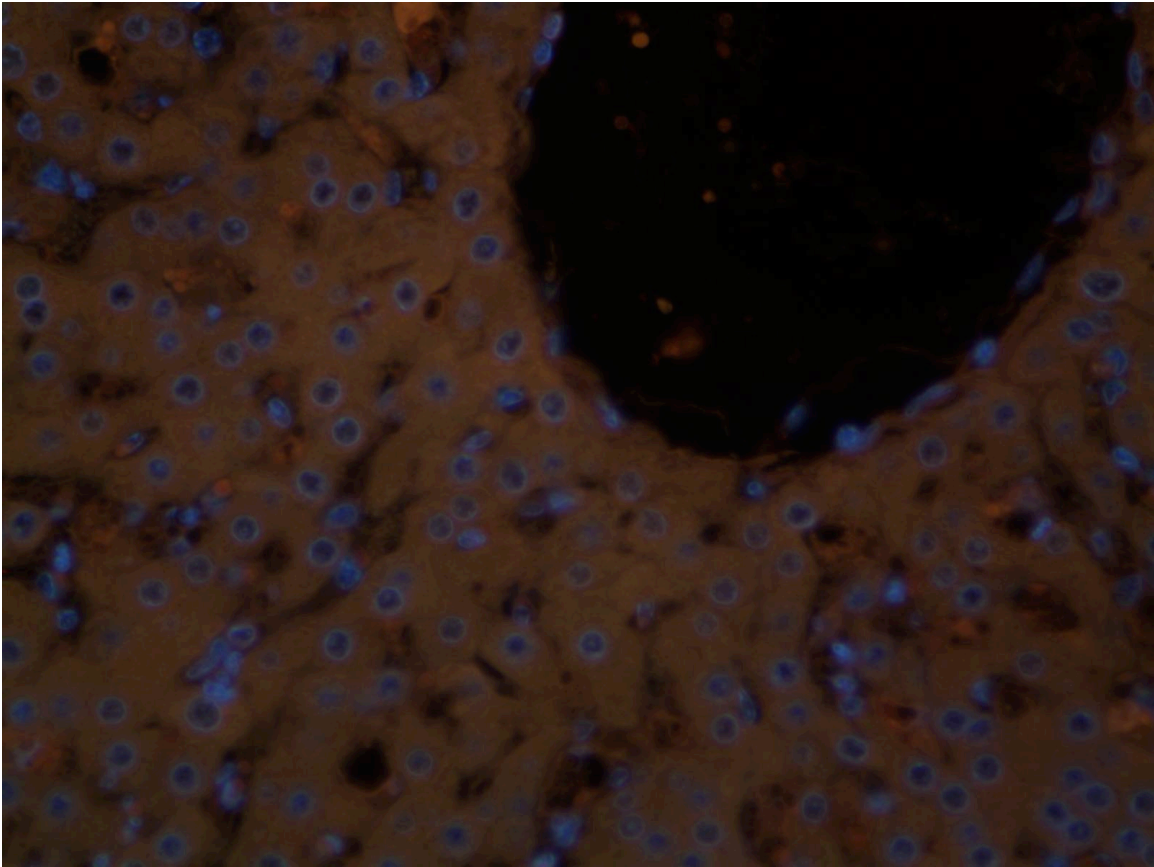


**Figure 2.6.** Strain 203 Infected Hamster's Kidney with LBJ0800 Post-bleed Antibody.  
Blue: Chromatin (DAPI stain); Yellow: Red blood cells (Autofluorescence)

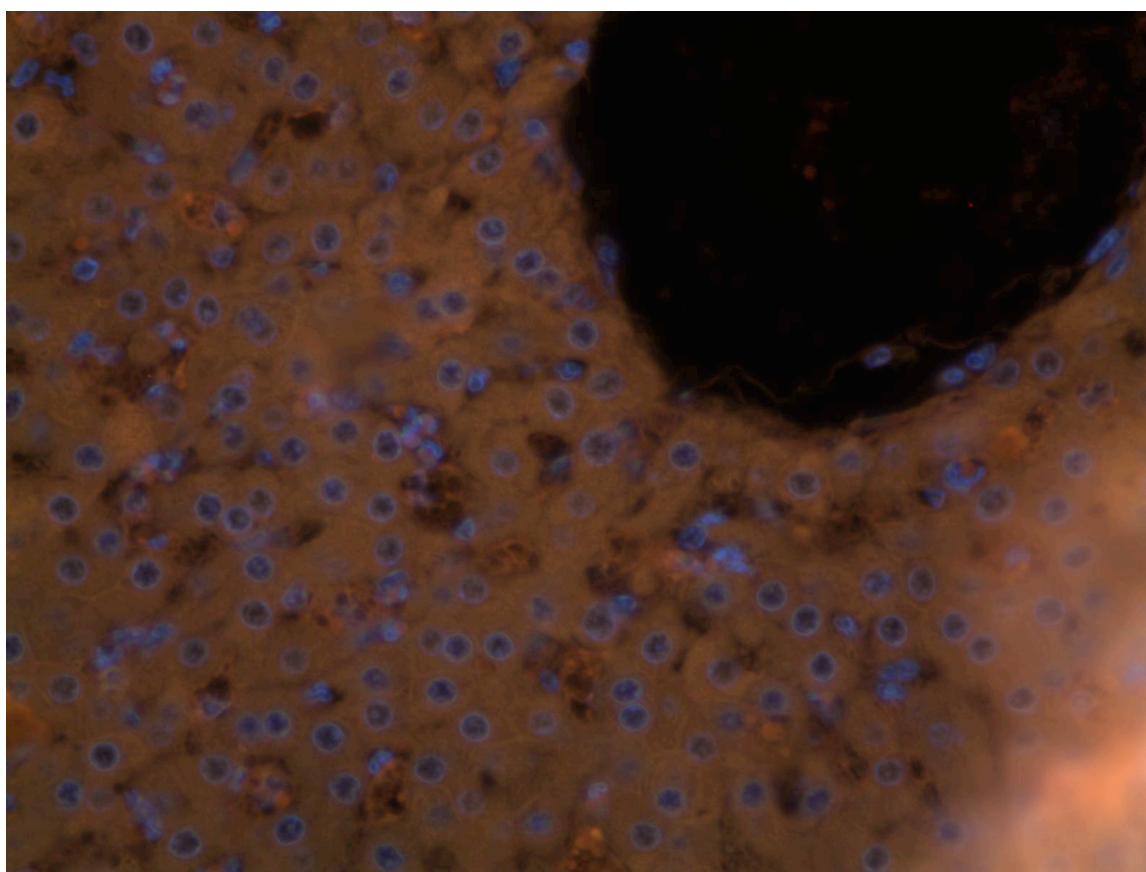


**Figure 2.7.** JB197 Infected Hamster's Liver with LipL32 Antibody. Blue: Chromatin (DAPI stain); Red: Spirochetes (Alexa Fluor 594 F (ab')<sub>2</sub> Fragment of Goat Anti-Rabbit IgG (H&L) secondary antibody)





**Figure 2.8.** JB197 Infected Hamster's Liver with LBJ0800 Pre-bleed Antibody. Blue: Chromatin (DAPI stain); Red: Nonspecific residual binding (Alexa Fluor 594 F (ab')<sub>2</sub> Fragment of Goat Anti-Rabbit IgG (H&L) secondary antibody)



**Figure 2.9.** JB197 Infected Hamster's Liver with LBJ0800 Post-bleed Antibody. Blue: Chromatin (DAPI stain); Red: Nonspecific residual binding (Alexa Fluor 594 F (ab')<sub>2</sub> Fragment of Goat Anti-Rabbit IgG (H&L) secondary antibody)

### Conclusion

In this study, the purification and in vivo expression of LBJ0800, a thiopurine *S*-methyltransferase, is described. A purified, polyhistidine tagged thiopurine methyltransferase was successfully expressed and detected by Anti-V5 Antibody (Invitrogen, Grand Island, NY) on an immunoblot at the expected mass of approximately 28 kDa. Sera against the recombinant protein was produced to satisfactory titers and utilized on tissues (kidney and liver) inoculated with *Leptospira interrogans* serovar Pomona strain RM211 or *Leptospira borgpetersenii* serovar Hardjo strains 203 and JB197. After staining, no antigen

was detected during growth *in vivo*. An ELISA on sera from animals infected with strains 203 and JB197 was also performed to determine if LBJ0800 induced the development of antibodies during infection, an indirect method to determine *in vivo* expression of the enzyme. PBS and FBS, the latter consisting of no antibodies, was utilized as negative controls. Comparable levels of reactivity to LBJ0800 was observed with both pre and post challenge JB197 and 203 sera resulting in no detection of LBJ0800 being expressed during infection. Because *L. borgpetersenii* grows slowly and poorly *in vitro*, it is possible that LBJ0800 is expressed at levels below detection using the methods used in this study.<sup>13</sup>

Genome sequence evidence indicates that LBJ0800 is not present in any of the sequenced spirochetes, *Brachyspira*, *Borrelia*, *Treponema*, except *Leptospira borgpetersenii* strain JB197 and other closely related “type B” strains of serovar Hardjo, making it a novel gene in spirochetes as well as *Leptospira* pathogenesis. Two strains of *L. borgpetersenii* serovar Hardjo, JB197 and 203 have the ability to cause infection with varied virulence in hamsters.<sup>14</sup> JB197 establishes an acute infection in hamsters, similar to that recognized in humans, soon after exposure to *Leptospira* and quickly disseminates throughout the body affecting the brain, pancreas, liver, small intestine and kidneys, resulting in clinical signs of infection such as pulmonary hemorrhaging and leukocyte aggregation.<sup>14,15</sup> When hamsters are exposed to the related “type A” non-virulent strain 203, no clinical signs of infection are observed, creating an asymptomatic, chronic infection.<sup>14,16</sup> The presence of LBJ0800 in “type B” strains and not “type A” gives reason to speculate that it is involved in the pathogenesis of *Leptospira* and thus merits studying. Future work is focused on enzymatic characterization.

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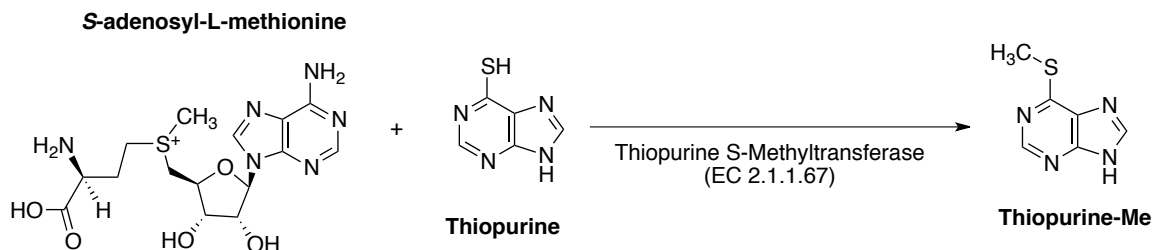
### CHAPTER 3. PURIFICATION AND IDENTIFICATION OF A NOVEL THIOPURINE *S*-METHYLTRANSFERASE FROM *LEPTOSPIRA BORGPIETERSENII* USING MASS SPECTROMETRY ANALYSIS

#### Summary

Human thiopurine *S*-methyltransferases (hTPMT) modulate the cytotoxic effects of aromatic and heterocyclic sulfhydryl compounds, such as 6-mercaptopurine and 6-thioguanine, by using them as methyl acceptor substrates, resulting in partial inactivation. In addition to using these thiopurines as substrates, previous studies show that bacterial thiopurine *S*-methyltransferases (bTPMT) can methylate inorganic and organic selenium as well as tellurite. To date, only bTPMTs originating from pseudomonads have been reported. In this study, an approximately 28 kDa, novel thiopurine *S*-methyltransferase from *Leptospira borgpetersenii* a spirochetal bacterium is characterized.

#### Introduction

Human thiopurine *S*-methyltransferase (hTPMT) is the cytosolic enzyme responsible for modulating cytotoxic effects of thiopurines by catalyzing the reaction that utilizes them as methyl acceptors and *S*-adenosyl-L-methionine (SAM) as a methyl donor (Scheme 3.1).<sup>1,2</sup> Azathioprine, 6-thioguanine, and 6-mercaptopurine are thiopurines used for the treatment of inflammatory bowel disease (IBD), organ transplantations, and acute lymphoblastic leukemia.<sup>3</sup> Within cells, these prodrugs are metabolized to thioguanine nucleotides (TGNs) and employ their cytotoxic effects by either incorporating themselves into DNA or RNA, triggering programmed cell death or inhibiting purine synthesis through the metabolite, methylthioinosine monophosphate.<sup>4,5</sup> By methylating these purine antimetabolites, TPMT is able to effectively inactivate a percentage of the administered dose.<sup>5</sup>



**Scheme 3.1.** Basic enzymatic reaction catalyzed by thiopurine *S*-methyltransferase

Bacterial thiopurine *S*-methyltransferases (bTPMTs) can methylate inorganic and organic selenium and tellurite as well as thiopurines.<sup>6,7,8</sup> Up to the present time, only pseudomonad bTPMTs have been reported.<sup>9,10</sup> Herein, a bTPMT from *Leptospira borgpetersenii* is described. *Leptospira* consists of many saprophytic, intermediate pathogenic, and pathogenic species, with *L. interrogans* (transmitted via contaminated water) and *L. borgpetersenii* (acquired via host to host transmission) causing the majority of leptospirosis cases.<sup>11,12</sup> Transmitted by rodents and other reservoir hosts, leptospirosis is an emerging infectious zoonosis that generates infections ranging from acute to mild clinical manifestations.<sup>13</sup> Causing headaches, organ failure, and possible death in humans and chronic infections with renal and hepatic failure in animals as well as abortion and infertility in cattle, leptospirosis is a worldwide public health concern.<sup>14</sup> Because this gene is not found in any other sequenced spirochetes, purification and identification of this novel gene by mass spectrometry analysis will contribute to spirochetal research and known bTPMTs.



## Materials and Methods

### Materials and General Methods

Enzymes and reagents used for molecular biology procedures, DNA Ladders and deoxynucleotide triphosphates were purchased from Invitrogen, BD Biosciences, and New England Biolabs. Oligonucleotides were synthesized by The DNA Facility of the Iowa State University Office of Biotechnology (Ames, IA). Protein molecular weight standards were obtained from GE Healthcare Life Sciences. The QIAprep Spin Miniprep kit was obtained from Qiagen and the Champion pET Directional TOPO Expression Kit was obtained from Invitrogen. All chemicals were obtained from Sigma Aldrich unless otherwise stated. The DNA procedures, including plasmid DNA isolation, agarose gel electrophoresis, and transformation of *E. coli*, were performed according to standard techniques (Sambrook et al. 1989) and manufacturer's instruction. Protein concentrations were determined with the Bio-Rad protein assay method.<sup>15</sup>

### Bacterial Strains and Growth Conditions

Genomic DNA of *Leptospira borgpetersenii*, obtained from *L. borgpetersenii* strain JB197 (Bulach et al 2006), was used for all cloning experiments. OneShot Top10 chemically competent cells *Escherichia coli* cells (Invitrogen, Carlsbad, CA) and PCR-Blunt pET101 vector (Invitrogen, Carlsbad, CA) was used for directional cloning of PCR products. *Escherichia coli* BL21 Star (DE3) competent cells (Invitrogen, Carlsbad, CA) were used in combination with the T7 expression system (pET101 vector; Invitrogen, Carlsbad, CA) for expression of the thiopurine S-methyltransferase. *Escherichia coli* cells were grown on Luria



Bertani (BD Biosciences, San Jose, CA) medium at 37 °C in an incubator shaker at 225 rpm. Carbenicillin was added at 50 mg/ml to make the selective media.

### **PCR Amplification, Cloning, Expression, and Purification of Enzyme**

The LBJ0800 gene was amplified from JB197 genomic DNA by PCR synthesis using two oligonucleotide primers. The forward primer, 5'-CACCATGGACACTAATTTTTGGC-3,' and the reverse primer, 5'-ATCGGTCGATTGTTTCAACAATAACC-3,' were designed from the putative thiopurine *S*-methyltransferase of *L. borgpetersenii*. The cycling parameters were 95 °C for 5 min, followed by 35 cycles of 94 °C for 15 sec, 60 °C for 30 sec, 68 °C for 2 minutes, with a final elongation step of 72 °C and holding step for 10 °C. The amplified DNA was cloned in pET101 vector using topoisomerase to yield constructs for the expression of putative thiopurine *S*-methyltransferase containing a C-terminally fused His<sub>6</sub> tag. The LBJ0800/pET101 construct was transformed into *E. coli* expression strain BL21 Star (DE3) (Invitrogen, Carlsbad, CA). Transformants were grown in LB medium broth containing 50 µg/ml at 37 °C for approximately 18 hours in a shaking incubator. Recombinant expression was induced with 0.4mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 16 hours at 16 °C (Houston et al. 2011) after an OD<sub>600</sub> reached 1.0. Cells were harvested by centrifugation at 4000 g and the QIAexpress Ni-NTA Fast Start Kit and protocol (Qiagen) were utilized for the purification of the recombinant 6xHis-tagged protein under native conditions. The purified protein was dialyzed into PBS using a Slide-A-Lyzer Dialysis Cassette, 10 MWCO (Thermo Scientific, Rockford, IL) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot using wet transfer techniques (Bio-Rad Laboratories, Hercules, CA).

## Mass Spectrometry

Samples were analyzed on and Agilent 1200/6130 high performance liquid chromatographic mass spectrometry (HPLC-MS) system. The mobile phases were (A) 94.9% v/v water (EM Science, Billerica, MA), 5% v/v acetonitrile (EM Science), 0.1% v/v formic acid (Sigma-Aldrich, St. Louis, MO). Ten microliters of sample extract were injected onto a 2.1 mm diameter, 100 mm long column packed with 3.5  $\mu\text{m}$  Zorbax-SB C18 particles (Agilent). The gradient conditions were 100% A from 0-2 minutes, linear ramp to 80% B at 6 minutes, hold at 80% B for 3 minutes; the flow rate was 0.35 mL/min, and the column was re-equilibrated with 100% A for 3.5 minutes between each analysis. The 6130 mass spectrometer was equipped with a multimode electrospray/atmospheric pressure chemical ionization (ESI/APCI) source and was operated positive ion ESI mode with 2000 volts on the heated capillary and charging electrodes, 5 L/min of nitrogen drying gas @ 300 °C, 150 °C vaporizer temperature, and 60 psi of nebulizer gas pressure. Mass spectra were recorded by scanning the quadrupole from  $m/z$  100-500 every 1.25 seconds throughout the separation. Agilent Chemstation software was used for all data processing operations.

The molecular weight of thiopurine *S*-methyltransferase was obtained using a Waters CapLC/LCT HPLC-MS system (Waters Corporation, Beverly, MA). The mobile phases were (A) 94.9% v/v water (EM Science, Billerica, MA), 5% v/v acetonitrile (EM Science), 0.1% v/v formic acid (Sigma-Aldrich, St. Louis, MO). Five microliters of sample extract were injected onto a 0.5 mm diameter, 100 mm long column packed with 5  $\mu\text{m}$  Biobasic C8 particles (Phenomenex Corporation, Torrance, CA). The gradient conditions were hold at 10% B for 0.5 minutes, then a linear ramp to 90% B at 14 minutes and hold at 90% B for 3

minutes. The flow rate was 15  $\mu\text{L}/\text{min}$ , and the column was re-equilibrated with 10% B for 4 minutes between injection cycles. The ESI needle was held at +3.6 kV, the nitrogen desolvation gas flow rate was 285 L/hr at 125  $^{\circ}\text{C}$ , and the cone gas was set to 20 L/hr. Time-of-flight mass spectra were recorded from  $m/z$  600-2300 at a rate of 1 Hz. The MaxEnt1 module of MassLynx 4.1 (Waters) was used to interpret the charge state distribution obtained by ESI and calculate the uncharged molecular weight.

### **Mass Spectrometry Based Enzyme Assay**

The mass spectrometry based enzyme assay was first described by Mizanur et al, 2007.<sup>16</sup> Substrates, concentrations, and temperature, were adjusted to fit this study. The enzymatic reaction was initiated by the addition of 2mM 6-Guanine, 6-Thioguanine, or 6-Mercaptopurine (20  $\mu\text{l}$ ) (each in separate reactions) and 5 mM of *S*-Adenosyl-L-Methionine (SAM) (20  $\mu\text{l}$ ). Purified LBJ0800 enzyme solution (15  $\mu\text{l}$ ) was added and reactions were carried out at 37  $^{\circ}\text{C}$  for 35 minutes. 15 $\mu\text{l}$  of the reaction was quenched by the addition of 30  $\mu\text{l}$  of 70% methanol/water. Aliquots of the reaction mixtures were diluted with 65  $\mu\text{l}$  of acetonitrile/water/triethylamine (35/65/0.2). These samples were subjected (10  $\mu\text{l}$ ) to analysis via ESI-MS to detect the formation of 6-Methylguanine, 6-Methylthioguanine, and 6-Methylmercaptapurine in comparison to a control reaction containing heat-killed enzyme.

### **Multiple Sequence Alignment**

A multiple sequence alignment of the amino acids of TPMT was performed using Biology Workbench 3.2 (<http://seqtool.sdsc.edu/CGI/BW.cgi>).

## Protein 3D Structure Prediction

Molecular figures were generated using PyMol.<sup>17,18,19</sup>

## Results

### Amino Acid Sequence Alignment

To compare the leptospiral TPMT with other known TPMTs mammalian and bacterial sources, a BLAST search was initiated with the amino acid sequence of the enzyme and then aligned using Biology Workbench 3.2 (Figure 3.2.) The enzyme showed significant alignments with two conserved domains: (1) *S*-adenosyl-L-homocysteine binding and (2) thiopurine binding. Arg152 (Arg123 in lbjTPMT) has been shown to directly interact with substrate binding, decreasing  $V_{max}$  and increasing  $K_m$  for 6-mercaptopurine, the methyl acceptor (Figure 3.2).<sup>20</sup> To study the gene more in detail, LBJ0800 was cloned and expressed in an *E. coli* strain.

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gTPMT      1 MDGTRTSLDIEEYS DTEVQKNQVLTLEEWQDKWVNGKTAFHQEQGHQLLKKHLDTFLK GK
hTPMT      1 MDGTRTSLDIEEYS DTEVQKNQVLTLEEWQDKWVNGKTAFHQEQGHQLLKKHLDTFLK GK
chTPMT     1 MDGTRTSLDIEEYS DTEVQKNQVLTLEEWQDKWVNGKTAFHQEQGHQLLKKHLDTFLK GK
cTPMT      1 MDDTSTLTDVKEY PDTEVQKNRVLTLEEWREKQVVDGKIGFHQEQGHQLLKKHLDTFLK GE
dTPMT      1 MDKTRTFLDVKEY PDTEVQKNRVLTLEEWQEKWVSRRI GFHQEQGHQLLKKHLDTFLK GE
mTPMT      1 -----MSLDMKEHPDAEVQKNQVLTLEDWKEKQVTRHISF HQEQGHQLLKKHLDTFLK GQ
lbjTPMT    1 -----MDTNFWHQKWEKNEIAFHSEVNPLLVENIDKLSLAE
psTPMT     1 -----GSHQSEVNKDLQQYWSLNVVV

gTPMT      61 SGLRVFFPLCGKAVEMKVFADRGHSVVGVEISELGIQEFFTEQNL SYSEEPITEIPGTKV
hTPMT      61 SGLRVFFPLCGKAVEMKVFADRGHSVVGVEISELGIQEFFTEQNL SYSEEPITEIPGTKV
chTPMT     61 SGLRVFFPLCGKAVEMKVFADRGHSVVGVEISELGIREFFT EQNL SYSEEPITEIPGTKV
cTPMT      61 NVLRVFFPLCGKAVEMKVFADRGHCVVGVEISELGIREFFT EQNL SYSEEPIMEIPGAKV
dTPMT      61 NGLRVFFPLCGKAVEMKVFADRGHSVVGVEISELGIREFFAEQNL SYTEEPIVEIPGGKI
mTPMT      56 SGLRVFFPLCGKAIEMKVFADRGHTVVGVEISEIGIREFFAEQNL SYTEEP LAE IAGAKV
lbjTPMT    38 -NGRIFLPLCGKTLDIAMLSRGRFVVGAELSKIAIEQLFQEL ---GVEPKISKAA-EL
psTPMT     23 -GARVLVPLCGKSQDMSVLSGQGYHVVGAELESEA AVERYFTER ---GEQPHITSQG-DF

gTPMT      121 FKSSSGNISLYCCSIFDLPRTNIGKFDMIWIRGALVA INPGDRKCYADTMLSLLGKKFQY
hTPMT      121 FKSSSGNISLYCCSIFDLPRTNIGKFDMIWIRGALVA INPGDRKCYADTMLSLLGKKFQY
chTPMT     121 FKSSSGNISLYCCSIFDLPRTNIGKFDMIWIRGALVA INPGDRKCYADTMLSLLGKKFQY
cTPMT      121 FKSSSGNISLYCCNLFDLPRVNI GKFDRIWIRGALVAVNPGDRKCYTDIMLSLTRKGFY
dTPMT      121 FKSSSGNISLYCCSLFDLPRANIGKFDRIWIRGALVA INPGDRERYADIMLSLTRKGFY
mTPMT      116 FKSSSGNISLYCCSIFDLPRANIGKFDRIWIRGALVA INPGDHDRIYADIILSLLRKEFQY
lbjTPMT    92 ERYSAHHIDIFVGDIFHLTAASLGFVDAIYIRASLVALPEDTSKHYVKHLM EIT-NTAPQ
psTPMT     77 KVYAAPGIEIWC GDFFALTARDIGHCAAFYIRAMIALPADMRERYVQHLEALMPQACSG

gTPMT      181 LLCVLSYDPTKHPGPPFVYVPHAEIERLFGKICNIRCLEKVDAFEER--H KSWGIDCLFEK
hTPMT      181 LLCVLSYDPTKHPGPPFVYVPHAEIERLFGKICNIRCLEKVDAFEER--H KSWGIDCLFEK
chTPMT     181 LLCVLSYDPTKHPGPPFVYVPHAEIERLFGKICNIRCLEKVDAFEER--H KSWGIDCLFEK
cTPMT      181 LLAVLSYDPTKHPGPPFVYVDAEIKNLFGSTCNIHCLEKVDVFEER--H KSWGIDYIVEK
dTPMT      181 LLAVLCYDPTKHAGPPFVYVPEAEIKKLFSGICNIHCLEKVDVFEEQ--H KSWGIDYIEK
mTPMT      176 LVAVLSYDPTKHAGPPFVYVPSAELKRLFGTKCSMQCLEEVDAL EER--HKAWGLDYLF EK
lbjTPMT    151 LLICYEYDQSKMAGPPFSIRS GEVQKHRYRNHYHFNLI VSKNVVG---GLK GKCEATENV
psTPMT     137 LLITLEYDQALLEGPPFSVPQTW LHRVMSGNWEVTKVGGQDTLHSSARGLKAGLERMDEH

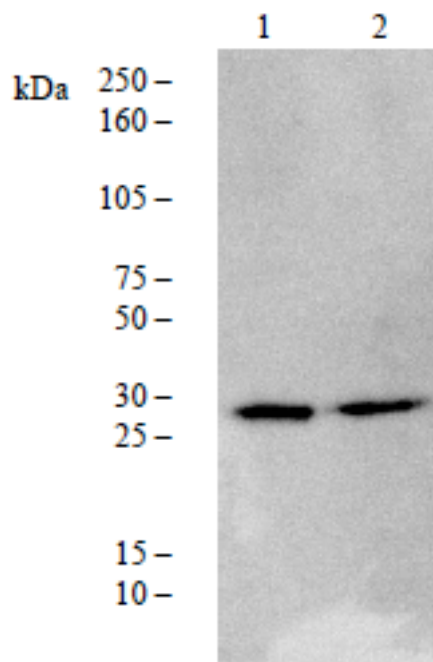
gTPMT      239 LYLLTEK--
hTPMT      239 LYLLTEK--
chTPMT     239 LYLLTEK--
cTPMT      239 LYLLTEK--
dTPMT      239 LYLFTEK--
mTPMT      234 LYLLTEK--
lbjTPMT    207 WLLLKQSTD
psTPMT     197 VYVLERV--

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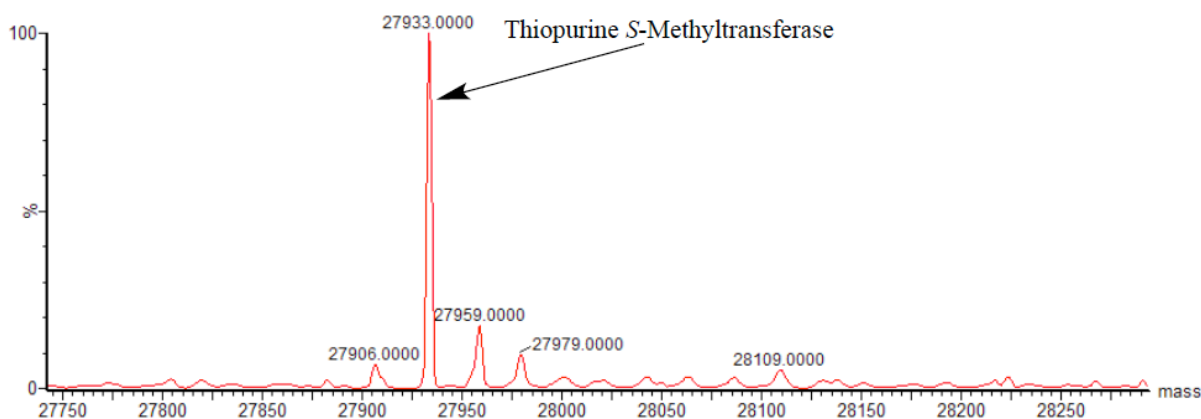
**Figure 3.1.** Alignment of TPMT and related sequences with LBJ0800 (lbjTPMT). Mammalian TPMTs: gorilla (gTPMT, AAX37643), human (hTPMT, AAB27277), chimpanzee (chTPMT, AAX37639), cat (cTPMT, Q6E1C1), and dog (dTPMT, AAL18006), mouse (mTPMT, AAC25919). Bacterial TPMTs: *Pseudomonas syringae* (psTPMT, PDB lpjz) and *Leptospira borgpetersenii* (lbjTPMT). Conserved residues are highlighted in blue. Arg123 in lbjTPMT, a critical residue in substrate binding, is highlighted in blue and denoted with a black rectangle. Lysine 55 in lbjTPMT (Lys40 in psTPMT) is a residue in the active site of the 3D predicted structure by I-TASSER and is highlighted in blue and surrounded by a black rectangle.

### Cloning, Expression, and Purification of LBJ0800

The open reading frame predicted to encode the putative thiopurine methyl *S*-methyltransferase (645 bp) was PCR amplified and directionally cloned into pET101 with a polyhistidine tag at the C terminus to generate plasmid, LBJ0800/pET101. To confirm the presence of a functional recombinant thiopurine *S*-methyltransferase, the expression construct was introduced into *E. coli* BL21 Star (DE3) cells, which were then grown in LB broth for IPTG-initiated induction of the protein. Cells were harvested by centrifugation, resuspended in Lysis Buffer and purified by a Ni-NTA metal-affinity column. Western blot and mass spectrometry analysis confirmed the purified recombinant protein with a molecular mass of approximately 28 kDa (Figure 3.3 and Figure 3.4.).



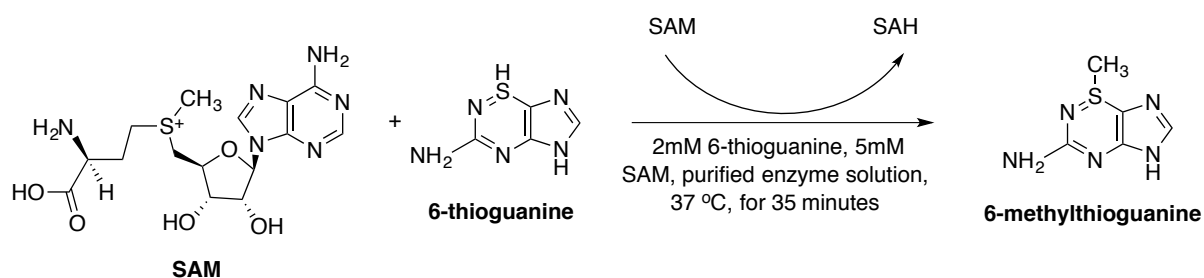
**Figure 3.2.** Western blot of the purified polyhistidine tagged thiopurine *S*-methyltransferase expressed in *E. coli*, purified using a Ni-NTA metal-affinity column, and detected by Anti-V5 Antibody. *Lanes 1 and 2:* 1<sup>st</sup> and 2<sup>nd</sup> elutions of LBJ0800, respectively.



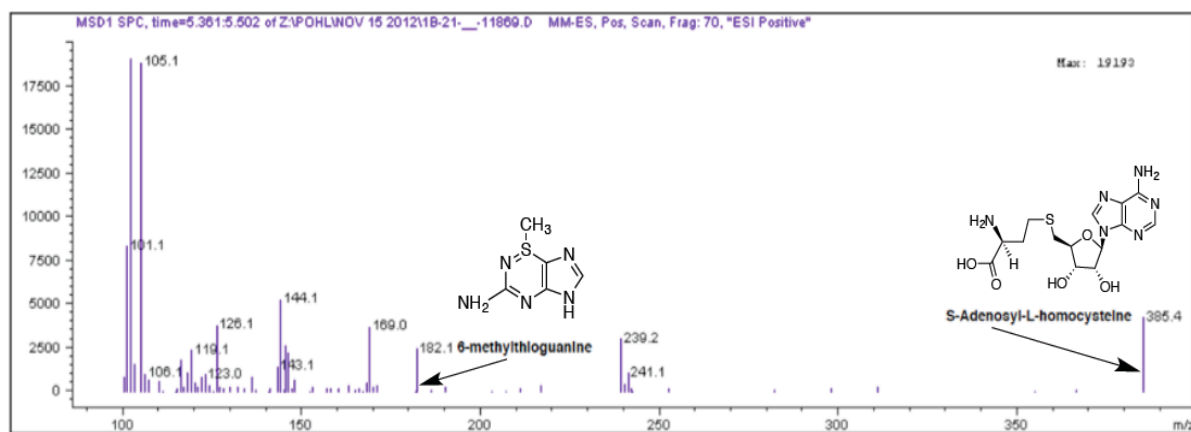
**Figure 3.3.** Mass spectrum of LBJ0800, thiopurine *S*-methyltransferase (lbjTPMT). The mass spectrum confirms the molecular mass of approximately 28 kDa of the recombinant protein, lbjTPMT.

### Enzymatic Activity LBJ0800

Thiopurines such as 6-thioguanine, 6-mercaptopurine, and azathioprine are well known substrates for TPMTs.<sup>3</sup> TPMT utilizes *S*-adenosyl-L-methionine as a methyl donor and thiopurines as the methyl acceptors. In this study, 6-mercaptopurine, 6-thioguanine, and its analogue, guanine served as methyl acceptors in separate reactions to determine LBJ0800's enzymatic capability as a TPMT. After a thirty-five min incubation, only the methylated form of 6-thioguanine, 6-methylthioguanine, was observed (Scheme 3.1 and Figure 3.5).



**Scheme 3.2.** lbjTPMT enzymatic reaction

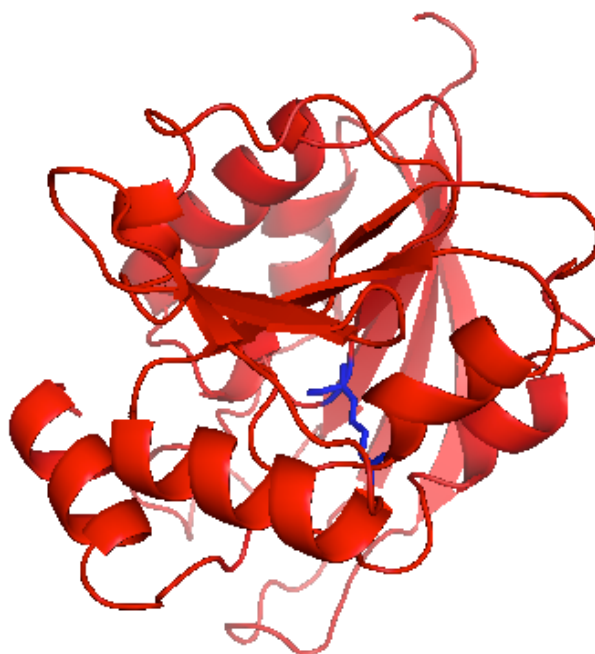


**Figure 3.4.** Mass spectrum demonstrating 6-methylthioguanine (182.1 m/z) and the byproduct, S-adenosyl-L-homocysteine

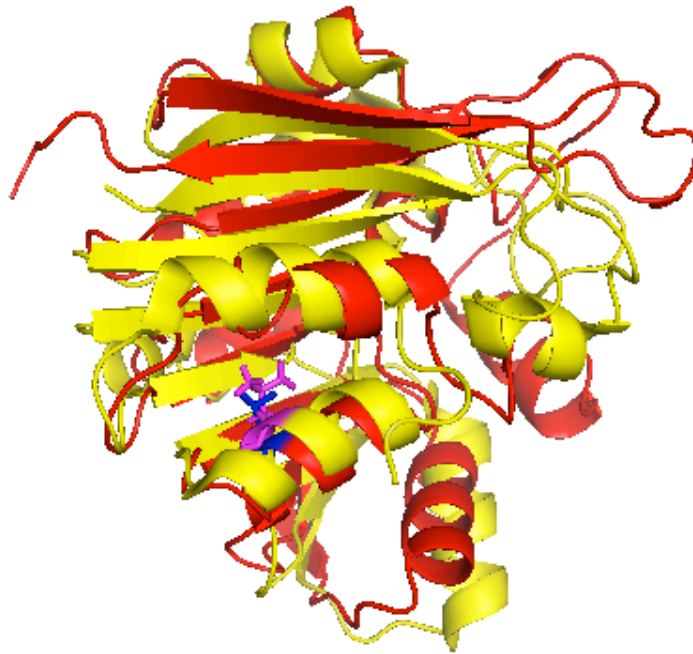
### Protein 3D Structure Prediction

After enzymatic activity was confirmed, a three dimensional structure of lbjTPMT was predicted to gain insight into lbjTPMT's putative structure and aligned with psTPMT for comparison (Figure 3.6.). The tertiary structure prediction was performed by I-TASSER server. Out of three generated similar models of the sequence, the best one was chosen to predict the structure employing the criteria of good alignment with template, a C-score of 0.99, a TM score of  $0.85 \pm 0.08$ , and a RMSD value of  $3.5 \pm 2.4 \text{ \AA}$ . *Pseudomonas aeruginosa* thiopurine S-methyltransferase (psTPMT) is the only other reported bTPMT and thus its structure is aligned with lbjTPMT's for comparison (Figure 3.7.).





**Figure 3.5.** 3D predicted structure of lbjTPMT. Eight  $\alpha$ -helices, seven  $\beta$  sheets and sixteen coils were predicted using I-TASSER. Arg123 (in blue), a critical polar residue in substrate binding, is predicted to be a buried residue within the hydrophobic interface of one of the coiled regions.



**Figure 3.6.** Alignment of 3D predicted structure of lbjTPMT (red) and psTPMT (yellow). Located in the active site within the hydrophilic interface of the  $\alpha$ -helix region, Lys55, a predicted highly exposed residue in lbjTPMT (in blue), and Lys40 (in magenta) are only conserved in bTPMTs.

### Conclusion

In this study, a novel leptospiral bTPMT was purified and its activity identified by mass spectrometry. A purified, polyhistidine tagged thiopurine methyltransferase was successfully expressed and detected by Anti-V5 Antibody (Invitrogen, Grand Island, NY) on an immunoblot at the expected mass of approximately 28 kDa. Because there is plenty genomic DNA information and little functional data, confirmation of the biochemical function was needed. Therefore, the identification and function of lbjTPMT, which is to methylate purines, was performed using mass spectrometry analysis. Methylating 6-

thioguanine and not its analogue, guanine, or 6-mercaptopurine demonstrates substrate specificity. Perhaps Lys55 in lbjTPMT (Lys40 in psTPMT) contributes to the specificity observed. Although Arg123 in lbjTPMT (Arg) is conserved in all TPMTs and is the most critical residue in substrate binding (Peng, 2008), Lys55 (Lys40 in psTPMT) is only conserved in bTPMTs and is located in the active site.

Purines, specifically 6-TGNs, incorporate themselves into leukocyte DNA as false bases, inhibiting their morphology and replication process thus creating cytotoxicity.<sup>21</sup> *Leptospira borgpetersenii* possess the gene, lbjTPMT, to methylate these molecules to modulate their cytotoxic effects. Because leukocyte aggregation during infection has only been observed in strain JB197 and lbjTPMT methylates the only purine associated with leukocytes, lbjTPMT might play a role in this phenomenon.<sup>22</sup> In addition to lbjTPMT exclusively methylating thioguanines and possibly contributing to leptospirosis, genome sequence evidence indicates that it is not present in any of the sequenced spirochetes, *Brachyspira*, *Borrelia*, *Treponema*, except *Leptospira borgpetersenii* strain JB197 and other closely related “type B” strains of serovar Hardjo, making it a novel gene in spirochetes as well as *Leptospira* pathogenesis. Future work includes kinetically characterizing this enzyme and designing other methods to determine its expression during infection to gain insight into its role in *Leptospira* pathogenesis.

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## CHAPTER 4. ASSESSMET OF IDENTICAL PRE AND POSTTESTS AS A LEARNING COMPREHENSION TOOL AND THE IMPACT TESTING HAS ON SCIENCE TEACHER EDUCATION

A paper submitted to *The Journal of Science Teacher Education*

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### Abstract

Identical multiple choice questions for pre and posttesting have become a favorite among educators. While this method is convenient, by giving identical posttest questions as those on the pretest, which one is being assessed: memorization or comprehension? Presumably, if comprehension, then there will not be a statistically significant difference in the scores of the comprehension tests taken by students who had identical post and pretest questions versus those who had different post and pretest questions. The goal of this study is to demonstrate whether or not identical pre and posttests accurately assess student learning by exploiting the manner in which questions are presented to discuss the effect testing has on teachers and their implementation of information gained during professional development.

*Keywords:* Student learning · Comprehension · Middle School Classroom · Assessment · Professional Development<sup>1</sup>

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<sup>2</sup> Primary researcher, an author, and author for correspondence

<sup>3</sup> Primary data analyst and an author

<sup>4</sup> An author and major professor

## Introduction

Selecting appropriate techniques to assess student learning remains a concern for school administrators. No Child Left Behind Act of 2001 mandates that schools receiving funding from the federal government must create learning objectives, methods to evaluate achievement of those objectives, and finally, administer an annual standardized exam as a means to gauge student learning (Sclafani, 2003). A national survey of middle and high school teachers stated that 60.5% (n=97) of participants thought that standardized testing would not improve student learning in science (Aydeniz & Southerland, 2012). Despite this point of view, teachers administer tests throughout the school year to prepare students for the statewide-standardized exam. While administrators are able to assess student learning from this standardized exam, repeating testing enhances memory for the material being tested (Richland et al., 2009). Although a survey of 63 undergraduate students concluded that tests are merely assessments and are not linked to how much one has learned (Richland et al., 2009) because “one question cannot gauge whether a student knows or understands the specific topic the question is covering” (Aydeniz & Southerland, 2012), a similar study has not been completed in the middle school classroom by middle school students to determine if the same theory applies to less experienced learners. Furthermore, determining whether identical or different questions on pre and posttests enhance memorization has not been studied either. Whether application questions vs. multiple choice questions better assess student learning still remains debatable (Garavalia et al., 2003). In this study, we aim to determine if there is any effectiveness to the identical pre and posttest strategy and determine the impact, if any, it has on comprehension by utilizing various approaches to ask the same question. We then employ the results to discuss how testing, specifically multiple choice

testing, influences science teachers' teaching and the impact it has on their implementation of information learned during professional development.

## **Methods**

### **2.1 Description of Assessments**

Pretest: Fifteen multiple choice questions on the subject of weather and climate created by the school district

District's Posttest: Fifteen multiple choice questions on the subject of weather and climate created by the school district (identical to the pretest)

GK-12 Posttest: Fifteen multiple choice questions covering the same weather and climate topics as the pre and posttests but with different questions created by the author

Comprehension Exam: A fifteen-point application (fill in the blank and open response question) test covering the same weather and climate topics created by the author

### **2.2 Limitations of Study**

Designing an assessment tool (i.e. standardized exams) that meets all students' needs can be challenging because there are various types of learning and testing styles. Simply being taught a certain subject does not result in comprehension (Vermunt, 1996). All students obtain, process, and recollect information presented to them in different ways (James and Gardner, 1995). Similarly, over time, students develop test-taking preferences. Some prefer multiple choice tests while others would rather take essay exams because they "reflect students' knowledge in the subject matter tested" (Zeidner, 1987). Learning and testing preferences for this study were not evaluated; therefore, test results could be skewed. For



example, a student who is better at taking an application test could have been randomly selected to take the district's posttest and not the GK-12 posttest. As a result, true knowledge gained is difficult to measure. However, the scores and results are still valid because it is a true representation of the dilemma that occurs daily in the educational school system.

### **Participants**

The study was administered in a public, middle school science classroom during the weather and climate unit. A maximum of eighty-one sixth grade students from three blocks (A, B, and C) with identical instructors participated in the aforementioned assessments. Due to unforeseen circumstances, there were 4 students that did not take the school district's pretest and their results on subsequent assessments were omitted for any paired analysis, resulting in a maximum of seventy-seven students involved in pre and posttesting. The comprehension exam was given a week after both posttests were administered and seventy-three students were present on that particular day. Students that did not take either posttest or the comprehension exam did not have his or her scores included in any comparative analysis involving both of these testing groups, resulting in seventy-three students' scores involved in all 4 comparisons. The number of students that participated in each assessment group is indicated in Table 4.1.

<b>Block</b>	<b>Pretest</b>	<b>District's Posttest</b>	<b>GK-12 Posttest</b>	<b>Comprehension Exam</b>
<b>A</b>	27	14	13	24
<b>B</b>	27	14	13	26
<b>C</b>	23	12	11	23
<b>Total</b>	77	40	37	73

**Table 4.1.** Number of students by block that participated in each assessment

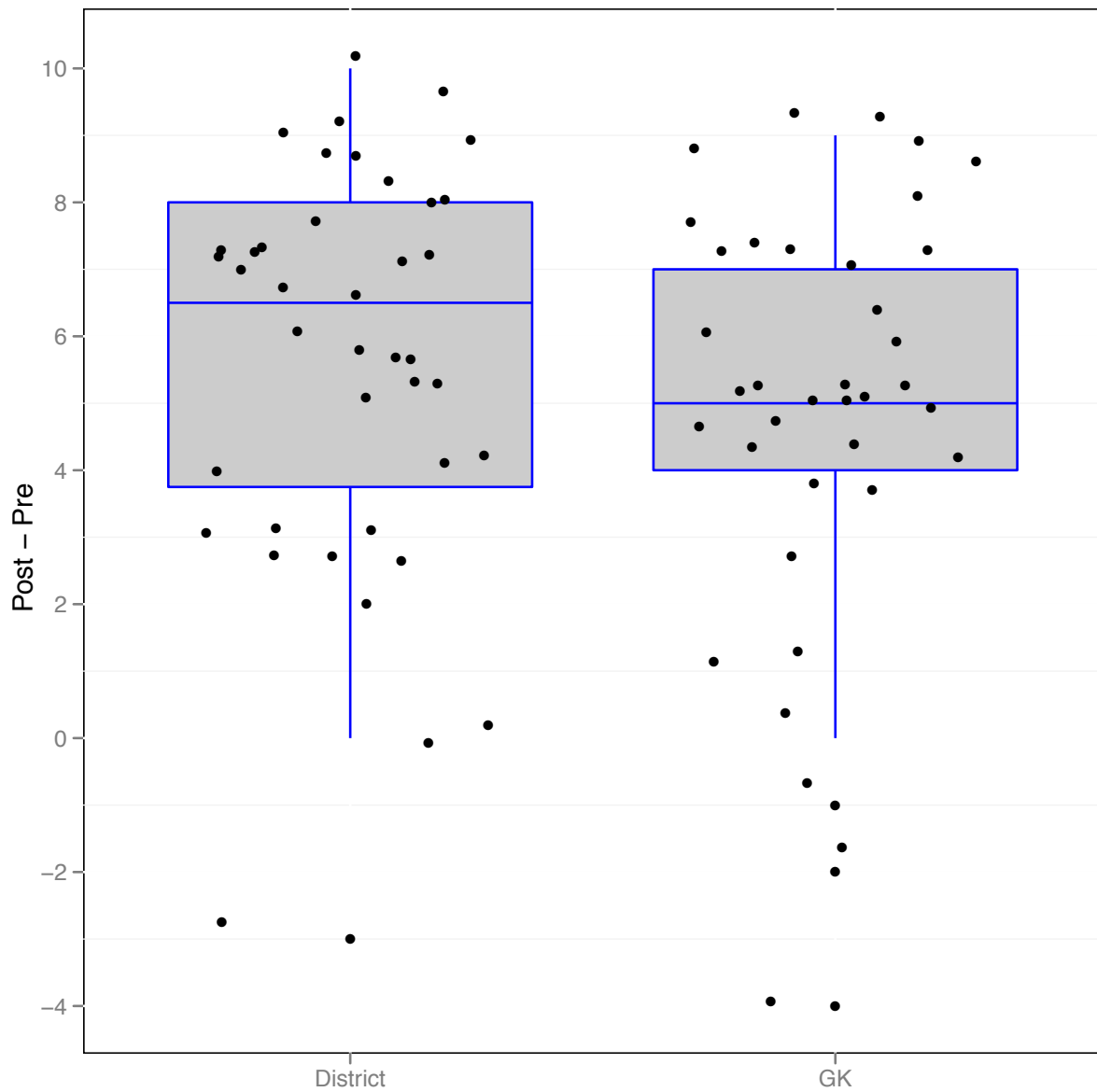
Before the weather and climate unit began, all seventy-seven students completed the pretest. At the conclusion of the unit, students were given either the school district's posttest or the GK-12 posttest. For posttesting purposes, within each block, students were randomly divided into two groups with the assignment of completing either the school district's posttest or the GK-12 posttest. To avoid the development of any bias from the students and/or instructors, this random assignment occurred immediately before the posttests' administration. As stated previously, the comprehension exam was given a week after both posttests were administered and seventy-three students were present on that particular day.

## Results

Four comparisons were made, each of which addressed a key objective of the study. The first two comparisons assessed if there had been an improvement made by the students over the semester. This improvement would be marked by an increase in the scores on the post examinations. The third comparison determined the degree of improvement among the students who took the district's posttest and the students that took the GK-12 posttest. The last of the comparisons gauged whether or not taking a posttest that differed from the pretest made a difference on the performance on the comprehension exam.

#### 4.1 Post and Pretest Differences

In the analysis of the exam scores of the students, we considered a paired analysis between the district's posttest and the pretest, as well as a paired analysis of the GK-12 posttest and the pretest. The data, as displayed in Figure 4.1., indicates that a vast majority of the students demonstrated improvement characterized by their respective differences in test scores being greater than zero. In both cases, the null hypothesis of a difference of zero among the exams was rejected with a P-VALUE  $< 0.0001$ . This implies that there is significant evidence, as indicated by assessment performance, that students have improved in performance over the semester.



**Figure 4.1.** Paired differences of both assessment groups between post and pretests. There is a significant difference (increase) among the performance from the beginning to the end of the unit between both groups (District and GK-12). Graphically, the majority of the score “points” are above zero. In both cases, the null hypothesis of a difference of zero among the exams was rejected with a P-VALUE of  $<0.0001$ .

Exams	Mean Difference	Test Statistics	P-Value
District Post – Pre	5.700	12.275	< 0.0001
GK-12 Post – Pre	4.919	9.558	< 0.0001

**Table 4.2.** Paired t-test results for post & pretest differences

#### 4.2 District's Posttest vs. GK-12 Posttest

To test if the differences observed between the students whom took the GK-12 posttest and the district's posttest were significantly different, we considered an independent two sample T-Test on the paired differences. Information on the two sets of paired differences is displayed in Table 4.3. Based on the data observed, there is not enough evidence to conclude that there is a difference between pre & posttest differences among the district and GK-12 testing groups, (P-VALUE = 0.2635). Both groups have a similar distribution, which can also be viewed in Figure 4.1.

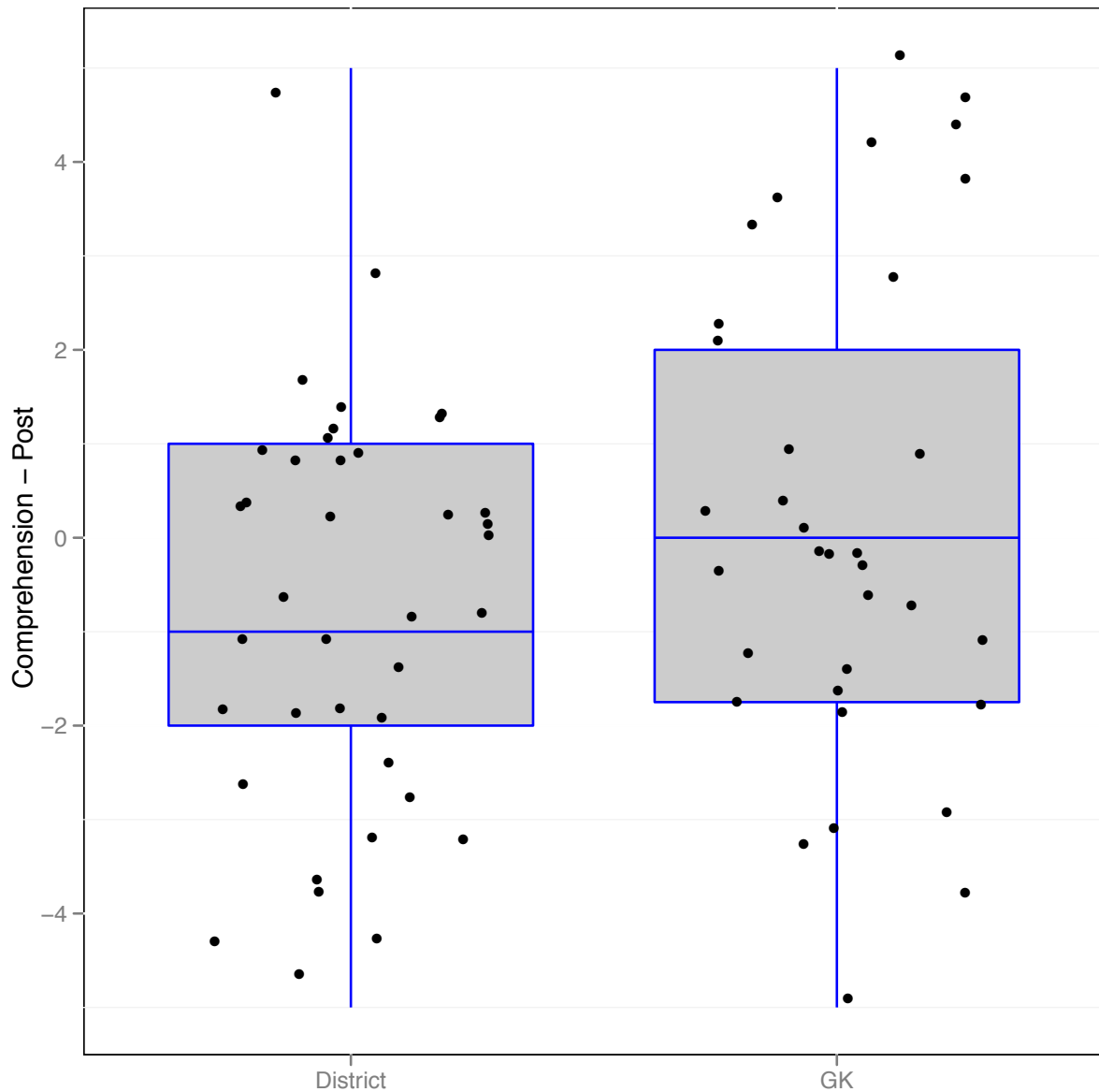
	Sample Size	Mean	Standard Deviation
District Post	40	5.700	2.940
GK-12 Post	37	4.919	3.130

**Table 4.3.** Summary statistics of the pre & post differences by posttest group

#### 4.3 Posttest Group and Comprehension Exam

The comprehension exam was designed to gauge the overall subject understanding of the students. Students who took the district's posttest were compared to students who took the GK-12 posttest. The comparison was made with an independent two sample T-Test comparing the paired differences of Comprehension - Posttest for both testing groups. There was not enough evidence to suggest that there is a significant difference in performance on

the comprehension exam among students who took the district's posttest and students who took the GK-12 posttest, P-VALUE = 0.07572. The distribution of the comprehension exam scores overlaps substantially in Figure 4.2.



**Figure 4.2.** Paired differences of both assessment groups between the comprehension exam and the posttests. There was not enough evidence to suggest that there is a significant difference in performance on the comprehension exam among students who took the district's posttest and students who took the GK-12 posttest, P-VALUE of 0.07572. The

distribution of the comprehension exam scores overlaps substantially.

### **Conclusion**

While learning is the primary goal for educational systems, educators feel that testing decreases teaching time and makes it almost impossible to perform hands-on activities that are related to the subject being taught (Smith, 1991). “Teachers frequently report that the pressure to raise test scores encourages them to emphasize instructional and assessment strategies that mirror the content and format of the state test, and to devote large amounts of classroom time to test preparation activities” (Abrams et al., 2003). Although standardized testing is mandated, at what point is student learning more important than assessing student learning? The findings in this study demonstrate that while students displayed an increase of knowledge between the pre and both posttests as characterized by the results in Section 4.1, there was not a significant difference when comparing the district’s posttest group to the GK-12 posttest group as indicated in Section 4.2. Another focus of the study was to determine if the different posttesting strategy had an effect on overall comprehension. The results of this study (Section 4.3) reveal that there was not a significant indicator suggesting that having different questions on the posttest had any impact on comprehension.

### **Discussion**

Pretesting is a method utilized to evaluate what, if anything, a student knows about particular topics before they are taught (Sheran & Sarbaum, 2012). While assessing student learning is essential, too many tests could become overwhelming and foster a negative stance towards learning for students (Bangert-Drowns et al., 1991). Being that there was not a

significant difference in the test scores between the pretest vs. district's posttest and the pretest vs. GK- 12 posttest, it is clear that the manner in which the questions are asked is irrelevant in this case. However, there was a significant difference between the pretest score and the scores of both posttests, implying that students did learn, which is expected. If the curriculum is not modified to tailor the students' needs as reflected by the pretest, how effective is the method of pretesting? Is giving a pre and posttest for the purpose of having documented proof that a student has learned *something* worth the decrease in instruction time and the elimination of countless activities that enhance learning during the school day? If possible, it would be advantageous for administrators to eliminate pretesting and only administer a unit posttest, if needed, to prepare students for the statewide-standardized exam. As a result, teachers would have extra time to put to practice what was learned during their professional development courses and create fun, thought-provoking activities that reinforce the topics taught, providing students with applicable and intriguing experiences.

### **Implications for Science Teachers**

Professional development opportunities are vital to public education (National Commission on Teaching and America's Future 2003). These opportunities can be formal, i.e. organized learning settings such as online or classroom educational courses and school-organized staff development workshops or informal, i.e. reading educational scholarly literature, casual conversations with administrators and parents, and collaborations with fellow teachers (Feiman-Nemser, 2001; Desimone, 2009). Richter and colleagues found that teachers were more likely to participate in formal training throughout the middle of their career (17-30 years of teaching) during the "phase of experimentation and activism," whereas



teachers in the beginning (1-6 years of teaching) and end (30 plus years of teaching) prefer informal training (Richter et al., 2011). Regardless of the uptake method of information, the ultimate goal is that teachers partaking in professional development activities are learning innovative means to present educational information to students in a meaningful, interactive manner. While the objective of many professional development methods is to fulfill the aforementioned, if a teacher is physically involved in a professional development activity, but mentally dealing with the pressure from administrators, the government, and parents concerning higher exam scores and the *seemingly, straight-forward paved road* to achieve them or have his livelihood possibly become affected, how effective is the training experience (Smith 1991)? Students, co-workers, administrators, and personal situations are all factors that can affect how a teacher perceives and implements the information presented (Boardman & Woodruff, 2004).

Because science can be more informational than practical, memorization is usually the method utilized to learn the material (Wee et al., 2007) and multiple choice testing is the tool frequently used for its evaluation (Douglas et al., 2012). Most educators administer multiple choice tests as a mode to assess student learning because it is quicker to grade, resulting in an efficient use of time. One issue with using multiple choice tests is that an educator's teaching style will become geared toward the test, reducing the usage of the various teaching styles learned during professional development (Smith, 1991). The well-known cliché, "If you don't use it, you will lose it," is simply stated in two words, "Limited use," by Boardman and Woodruff (2004). In their study, they found that educators felt as if the information gained during professional development activities must correlate to techniques to improve standardized exam scores and that if they did not, they were less likely

to implement them because the necessity of students to do well decreased their desire to try new teaching methods (Boardman & Woodruff, 2004). When teachers are only exercising one teaching technique, it appears as if they do not have a complete warehouse of teaching styles to complete the inventory of students' needs, with the primary need being to learn. Science teachers' teaching capacities become limited when they continually teach a certain way for one ultimate goal, higher standardized exam scores. Is reducing their styles of teaching the appropriate sacrifice in hopes of students performing better on standardized exams? In our study, we concluded that whether a student is asked a multiple choice or application question is irrelevant. Both group of students, those who had identical pre and posttest multiple choice questions and those who had different pre and posttest (multiple choice versus application questions) displayed an increase in knowledge on the comprehension exam. Since the primary goal of education is to learn, science teachers should get a break. It is imperative that science teachers stop feeling the pressure of needing to focus primarily on increasing the standardized exam scores because this only leads to the teaching, creation, and administering of more assessment tools as well as a concern for the teachers' physical, mental, and emotional well-being. Healthy, reasonably stress-free teachers will allow for the building upon and utilization of their educational courses and professional development training to create fun, thriving, and exciting learning environments for students to take a leading role in their education, i.e. increasing their standardized exam scores by being involved in the various activities and teaching styles provided by their teachers.

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## CHAPTER 5. CONCLUSION

### What We Have Learned

To gain insight into the biology of *Leptospira*, a putative thiopurine *S*-methyltransferase (LBJ0800) was investigated. The open reading frame predicted to encode the putative thiopurine *S*-methyltransferase (645 base pairs) was PCR-amplified and directionally cloned into pET101 with a polyhistidine tag at the *C*-terminus to provide plasmid, LBJ0800/pET101. To confirm the presence of a functional recombinant thiopurine *S*-methyltransferase, the expression construct was introduced into *E. coli* BL21 Star (DE3) cells, which were then grown in LB broth for IPTG-initiated induction of the protein. Cells were harvested by centrifugation, resuspended in Lysis Buffer and purified by a Ni-NTA metal-affinity column. Western blot and mass spectrometry analysis confirmed the identity of the purified recombinant protein with a molecular mass of approximately 28 kDa. In the second chapter, *in vivo* characterization of the putative thiopurine *S*-methyltransferase was discussed. It was determined by ELISA experiments and immunohistochemistry studies that although LBJ0800 is a unique protein, it was not expressed during infection at detectable levels. This observation could possibly be a result of *Leptospira* growing slowly and poorly *in vitro*, host cells needing to be present to trigger expression, or conditions utilized could be too stringent. In chapter 3, research efforts focused on characterizing the biochemical function of the gene product of the putative thiopurine *S*-methyltransferase. Demonstrating substrate specificity, LBJ0800 methylated 6-thioguanine, validating its putative function as a thiopurine *S*-methyltransferase, now referred to as lbjTPMT. Arg 152 (Arg123 in lbjTPMT) has been shown to directly interact with the enzyme substrate upon binding and is conserved

in all sequenced thiopurine *S*-methyltransferases; therefore, now that the enzymatic function has been confirmed, future work could focus on the role for this arginine residue in the *Leptospira* version of the protein. In Chapter 4, appropriate techniques to accurately assess student learning and the impact testing has on science teacher education were studied by determining the effectiveness of identical pre and post testing. We concluded that there was a significant difference between the pretest score and scores of posttests, implying that students did learn, which is expected. However, having different questions on the posttest did not have any impact on comprehension. Given that too many tests can negatively impacts teachers' physical, mental, and emotional well-being as well as foster a negative stance towards learning for students, if the curriculum is not modified to tailor the students' needs as reflected by the pretest, it was determined that the pretest was unnecessary. Time could be better utilized creating fun, thriving, exciting learning environments for students to take a leading role in their education.

### **Future Work**

Future work includes the optimization of *in vitro* enzymatic expression conditions and designing other methods to determine lbjTPMT's role in *Leptospira* during infection. Since lbjTPMT's biochemical function has now been confirmed, the first step is to determine its optimal enzymatically-active expression conditions to kinetically understanding this enzyme. Temperature, pH, incubation times, and substrate and purified enzyme concentrations are all factors that effect enzyme activity. Next, known inhibitors of this class of enzymes such as 3,4-dimethoxy-5-hydroxybenzoic acid (DMHBA), 3-aminosalicylic acid, 4-aminosalicylic acid, and 5-aminosalicylic acid can be utilized to monitor the effect each, as

well as a combination of the four, has on the overall enzymatic activity.<sup>1</sup> Because ljbTPMT has many residues that are conserved in other TPMTs, specifically, one essential to substrate binding, point mutations could be prepared to determine whether these residues are as important for catalysis of methylating aromatic and heterocyclic sulfhydryl compounds in *L. borgpetersenii* as they are in other TPMTs. After determining the effect of point mutations, if any, the next study would be to insert LBJ0800 into the non-infectious saprophyte, *L. biflexa*. Observing the phenotype produced will give direct insight into its role in general, as well as indirectly give insight into its role in the pathogenesis of *L. borgpetersenii* (i.e. if the insertion of this gene causes *L. biflexa* to become an intermediate pathogenic or pathogenic species). Challenging hamsters with this modified species of *L. biflexa* and comparing the manifestations of infection and immunohistochemistry with those of hamsters challenged with *L. borgpetersenii* will contribute to the aforementioned studies, resulting in several ways to gain understanding of ljbTPMT's role in the *Leptospira* life cycle.

Since it has been concluded that identical pre and post testing is not an accurate assessment of learning comprehension in the middle school classroom, an initial future study is to alter the curriculum to meet the students' needs as reflected on the pretest. By implementing the necessary changes in the curriculum (i.e. increasing instruction taught in areas in which students performed poorly and decreasing instruction in areas where adequate understanding is demonstrated), teachers are actively solving a critical issue that is oftentimes overlooked in the classroom, which is the fact that if students are not taught the fundamentals in the beginning, it is very difficult to build a solid foundation upon which more learning can take place. A follow-up study would be to obtain the scores of those students who took a pre test and the curriculum was improved to satisfy the students'

learning needs and compare it to the scores of students who did not take a pretest to determine if pre and posttests are needed before the state-wide standardized exam. A final study would include a student survey querying them about their stance on pre and post testing and their preference of preparation before the state-wide standardized exam. Although students should not create all learning procedures in the classroom, their feedback is important because students possess different learning styles, resulting in various forms of comprehension. Hands-on activities, applicable experiences, repetition via worksheets, and memory games are all acceptable learning tools by which students comprehend new material. When testing is decreased, more time is available for these various forms of learning tools to be implemented, resulting in increased comprehension, which produces higher state-wide standardized exam scores.

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The Lord is my rock and my fortress and my deliverer;

My God, my strength in whom I will trust;

My shield and the horn of my salvation, my stronghold. I will call upon the Lord, who is worthy to be praised.” Psalm 18:1-3a

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