2015

Characterization of Exopolysaccharide (EPS) Produced by Bacillus subtilis Mutants

Alexander Argianas
Loyola University Chicago

Recommended Citation
http://ecommons.luc.edu/luc_theses/3124

This Thesis is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Master’s Theses by an authorized administrator of Loyola eCommons. For more information, please contact ecommons@luc.edu.

This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License.
Copyright © 2015 Alexander Argianas
LOYOLA UNIVERSITY CHICAGO

CHARACTERIZATION OF EXOPOLYSACCHARIDE (EPS)
PRODUCED BY BACILLUS SUBTILIS
MUTANTS

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

PROGRAM IN INFECTIOUS DISEASE AND IMMUNOLOGY

BY
ALEXANDER CHARLES ARGIANAS
CHICAGO, IL
DECEMBER 2015
ACKNOWLEDGEMENTS

I would first like to thank Dr. Katherine L. Knight for giving me the opportunity to learn and grow as a scientist in her lab. Dr. Knight is an impressive mentor and I highly value the time she has taken to guide me towards developing a more thoughtful and lucid scientific mind. I will always remember her and the impact she has had on my development.

I want to thank the professors in the Department of Microbiology and Immunology who have given their time, on multiple occasions, to ask me about my project and discuss with genuine interest the problems I’m trying to address in my research. It means a lot to me that I was able to learn in an environment where professors throughout the department are dedicated to my own development as a scientist.

I want to thank Dr. Paul O’Keefe for championing my involvement in the InDIRI program. I was given a chance to learn and I greatly appreciate and will continue to build upon the foundations I have laid as a basic scientist. I’ve benefited from our discussions on my research and his thoughtful input.

I want to thank my fellow lab members for always challenging me to communicate my ideas clearly, succinctly and offering suggestions on improvement. I am appreciative to lab members who were mentors in and outside of the lab and am appreciative of their time and efforts.
I want to thank my family and loved ones for their continual support throughout the processes and for their words of encouragement. In particular, I want to thank my mother and father for supporting me to continually strive to be a better version of myself and for instilling in me the importance of hard work and humility. I appreciate the struggles my parents faced as the first generation to go to college and the sacrifices they have made so that I would have a better life. I am eternally thankful that they have given me the freedom to pursue what I am passionate about in life and have encouraged me to follow my dreams to their fullest extent.

My experiences in Loyola’s M.S. program in Infectious Disease and Immunology have been the most meaningful and rewarding education I have had. I hope to build on what I have learned and continue to contribute to the scientific body of knowledge in the future.
# TABLE OF CONTENTS

ACKNOWLEDGMENTS iii

LIST OF TABLES vii

LIST OF FIGURES viii

LIST OF ABBREVIATIONS x

ABSTRACT xiii

CHAPTER ONE: REVIEW OF LITERATURE 1
   Gut Host Microbe Interaction 3
   Probiotics 6
   The Extracellular Matrix and Formation of Biofilms 8
   Bacterial Polysaccharides and Their Immunomodulatory Activity 10
   Glycosyltransferases 14
   The epsA-O Operon and Characteristics of EPS 15
   *Citrobacter rodentium* as a Model of Enteric Disease 16
   Methods for the Analysis and Study of Complex Carbohydrates 18

CHAPTER TWO: MATERIALS AND METHODS 22
   Pellicle and Biofilm Formation Assays 22
   Purification of EPS 23
   Comparison of EPS Size Isolated from *Bacillus subtilis* mutants 24
   Detection of Similar Epitopes of EPS Isolated from Various *Bacillus subtilis* Mutants 25
   EPS Binding to Peritoneal Cells 25
   Polysaccharide Composition Enzyme Assay 26
   Mice 28
   Bacterial Strains and Spore Preparation 28
   Isolation and Identification of Bacteria Contained in Natto 30
   *Citrobacter rodentium* Disease Model 30
   Serum Inflammatory Chemokine ELISA 32
   Allergic Sensitization to Ovalbumin 33
   Generation of Bone marrow Derived Mast Cells and Peritoneal Derived Mast Cells 34
   Mast Cell Degranulation Assay 36
   Statistical Analysis 38
### CHAPTER THREE: RESULTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characterization of Biofilm Formation by <em>Bacillus subtilis</em> mutants</td>
<td>39</td>
</tr>
<tr>
<td>DNA Sequence Analysis of Genes in the <em>epsA-O</em> Operon and</td>
<td></td>
</tr>
<tr>
<td>Comparison to Homologous Genes</td>
<td>43</td>
</tr>
<tr>
<td>Characterization of EPS Produced by <em>Bacillus subtilis</em> mutants</td>
<td>44</td>
</tr>
<tr>
<td>Recognition of EPS Isolated from <em>Bacillus subtilis</em> by anti-EPS serum</td>
<td>49</td>
</tr>
<tr>
<td>Recognition of EPS by Peritoneal Cells</td>
<td>50</td>
</tr>
<tr>
<td>Genes in the <em>epsA-O</em> Operon are Required for Protection from the</td>
<td></td>
</tr>
<tr>
<td>Enteric Pathogen, <em>Citrobacter rodentium</em></td>
<td>52</td>
</tr>
<tr>
<td>Protection from <em>Citrobacter rodentium</em>-Associated Disease by <em>Bacillus</em></td>
<td>60</td>
</tr>
<tr>
<td><em>subtilis var. Natto</em></td>
<td></td>
</tr>
<tr>
<td>Characterization of Immunomodulatory Effects of Protective EPS</td>
<td>66</td>
</tr>
<tr>
<td>Isolated from <em>Bacillus subtilis</em> mutants</td>
<td></td>
</tr>
</tbody>
</table>

### CHAPTER FOUR: DISCUSSION

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Unique and Novel Approach to Studying Polysaccharide Biosynthesis</td>
<td>72</td>
</tr>
<tr>
<td>Summary of Findings</td>
<td>72</td>
</tr>
<tr>
<td>Confirmation of Protective Strains and Future Models</td>
<td>77</td>
</tr>
<tr>
<td>Potential Probiotics and Caution of Their Use</td>
<td>79</td>
</tr>
<tr>
<td>Further Study of the Enzymes Encoded in the <em>epsA-O</em> Operon</td>
<td>80</td>
</tr>
<tr>
<td>Optimization of Purified EPS and Polysaccharide Biosynthesis</td>
<td>81</td>
</tr>
<tr>
<td>Analysis of the Properties of EPS Produced by <em>Bacillus subtilis</em> mutants</td>
<td>82</td>
</tr>
<tr>
<td>Concluding Remarks</td>
<td>83</td>
</tr>
</tbody>
</table>

### REFERENCES

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>References</td>
<td>84</td>
</tr>
</tbody>
</table>

### VITA

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>VITA</td>
<td>97</td>
</tr>
<tr>
<td>Table</td>
<td>Page</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>1. Determination of monosaccharide composition of EPS from <em>B. subtilis</em> strains</td>
<td>47</td>
</tr>
<tr>
<td>2. Percent composition of glucose and mannose from <em>B. subtilis</em></td>
<td>48</td>
</tr>
<tr>
<td>3. MALDI-TOF MS analysis of bacteria isolated from Natto</td>
<td>61</td>
</tr>
<tr>
<td>4. Characterization of <em>B. subtilis</em> mutants</td>
<td>71</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Illustration of the order of the 15 genes in the <em>epsA</em>-<em>O</em> operon</td>
<td>16</td>
</tr>
<tr>
<td>2.</td>
<td>Illustration of the enzymatic reactions in a monosaccharide composition assay</td>
<td>27</td>
</tr>
<tr>
<td>3.</td>
<td>Model of <em>C. rodentium</em> infection</td>
<td>31</td>
</tr>
<tr>
<td>4.</td>
<td>Model of allergic sensitization to ovalbumin</td>
<td>33</td>
</tr>
<tr>
<td>5.</td>
<td>Equation for the calculation of percent of mast cell degranulation</td>
<td>37</td>
</tr>
<tr>
<td>6.</td>
<td>Illustration of mast cell degranulation assay</td>
<td>37</td>
</tr>
<tr>
<td>7.</td>
<td>Effect of deletion to genes in the <em>epsA</em>-<em>O</em> operon on biofilm and pellicle formation in <em>B. subtilis</em></td>
<td>41</td>
</tr>
<tr>
<td>8.</td>
<td>Effect of deletion in genes in the <em>epsA</em>-<em>O</em> operon on biofilm and pellicle formation of <em>B. subtilis</em></td>
<td>42</td>
</tr>
<tr>
<td>9.</td>
<td>Effect of deletions of genes in the <em>epsA</em>-<em>O</em> operon on biofilm formation in <em>B. subtilis</em></td>
<td>43</td>
</tr>
<tr>
<td>10.</td>
<td>Assessment of the size of EPS isolated from different <em>B. subtilis</em> strains</td>
<td>45</td>
</tr>
<tr>
<td>11.</td>
<td>Primary repeating structure and linkage of EPS-J</td>
<td>48</td>
</tr>
<tr>
<td>12.</td>
<td>Modified western SDS-PAGE performed on EPS from <em>B. subtilis</em> strains</td>
<td>50</td>
</tr>
<tr>
<td>13.</td>
<td>Binding of EPS from <em>B. subtilis</em> strains to peritoneal cells</td>
<td>52</td>
</tr>
</tbody>
</table>
14. Levels of serum KC, an inflammatory chemokine, in mice treated with *B. subtilis* spores and infected with *C. rodentium*

15. Colonization of *B. subtilis* mutants and *C. rodentium*

16. Effect of *B. subtilis* mutants on *C. rodentium*-associated disease 10 days post-infection

17. Effect of *B. subtilis* mutants on *C. rodentium*-associated disease 10 days post-infection

18. Representative photos of H&E stained longitudinal colonic sections from mice treated with *B. subtilis* and infected with *C. rodentium*

19. Colonization of *B. subtilis* var. *Natto* and *C. rodentium*

20. Effect of *B. subtilis* var. natto on *C. rodentium*-associated disease 10 days post-infection

21. Effect of *B. subtilis* var. natto on *C. rodentium*-associate disease 10 days post-infection

22. Representative photos of H&E stained longitudinal colonic sections from mice treated with *B. subtilis* and infected with *C. rodentium*

23. Serum levels of allergen specific IgG1 in sensitized mice

24. Purity of cultured PDMC and BMDMC isolated from the peritoneum

25. Morphology of mast cells treated with EPS

26. Percent degranulation in a mast cell degranulation assay

27. Percent degranulation in a mast cell degranulation assay
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δgene</td>
<td>Functional Disruption of a gene</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt</td>
</tr>
<tr>
<td>Abs</td>
<td>Antibodies</td>
</tr>
<tr>
<td>ACK</td>
<td>Ammonium-Chloride-Potassium</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenine Diphosphate</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>ASF</td>
<td>Altered Schaedler Flora</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenine Triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BM</td>
<td>Bone Marrow</td>
</tr>
<tr>
<td>BMDMC</td>
<td>Bone Marrow Derived Mast Cell</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CV</td>
<td>Crystal Violet</td>
</tr>
<tr>
<td>ddH&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>De-Ionized Water</td>
</tr>
<tr>
<td>DNP</td>
<td>2,4-Dinitrophenol</td>
</tr>
<tr>
<td>d.p.i.</td>
<td>Days post infection</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
</tbody>
</table>
EPS  Exopolysaccharide
EtOH  Ethanol
FACS  Fluorescence Activated Cell Sorting
FCS   Fetal Calf Serum
FITC  Fluorescein
FPLC  Fast Protein Liquid Chromatography
g     Gravity
G6P   Glucose-6-Phosphate
G6P-DH Glucose-6-Phosphate Dehydrogenase
GIT   Gastrointestinal Tract
H&E   Hematoxylin and Eosin
HK    Hexokinase
HMW   High Molecular Weight
IBD   Inflammatory Bowel
i.p.  Intraperitoneal
kD    Kilodaltons
LB    Luria-Bertani
LMW   Low Molecular Weight
LPS   Lipopolysaccharide
mA    Mili-Amps
mg    milligram
mL    Milliliter
mM    Milimolar
MΦ  Macrophage
NADP+  Nicotinamide-Adenine Dinucleotide Phosphate
NADPH  Nicotinamide adenine dinucleotide phosphate
OD  Optical Density
O/N  Overnight
OVA  Ovalbumin
PBS  Phosphate Buffered Saline
PDMC  Peritoneal Derived Mast Cell
PGI  Phosphoglucone Isomerase
PMI  Phosphomannose Isomerase
PSA  Phenol Sulfuric Acid
RPMI  Roswell Park Memorial Institute
RT  Room Temperature
TEA  Triethanolamine
TLR  Toll-Like-Receptor
TMB  3,3′,5,5′-Tetramethylbenzidine
TMCH  Transmissible Murine Colonic Hyperplasia
mg  Migrogram
µL  Microliter
SDS-PAGE  SDS-Polyacrylamide Gel Electrophoresis
V  Volts
WT  Wild Type
ABSTRACT

*Bacillus subtilis* is a probiotic bacterium that can protect against the murine-specific attaching and effacing (A/E) pathogen *Citrobacter rodentium*. *C. rodentium* displays many features observed with the human pathogen enteropathogenic *E. coli* (EPEC). Protection by *B. subtilis* is mediated by exopolysaccharide (EPS), which is encoded by the epsA-O operon that contains 15 genes. Disruption of one of these genes, *epsH*, which appears to encode a putative glycosyltransferase, leads to the loss of protective capacity by *B. subtilis* and by EPS. EPS is a major component of the *B. subtilis* biofilm and the ΔepsH mutant is unable to form a biofilm. The goal of this project was to characterize EPS produced by *B. subtilis* with mutations to the 15 genes of the epsA-O operon and assess how single deletions to the genes affect phenotype (biofilm formation), and the capacity to protect mice from disease caused by *C. rodentium*. We found that mutations in 13 of the 15 genes eliminated the probiotic activity of *B. subtilis*, even though some of the mutants developed biofilms. Our goal is to compare the structures of EPS from protective and non-protective mutants, and identify a small molecule that can be used as a therapeutic to prevent disease caused by enteropathogens.
CHAPTER ONE

REVIEW OF LITERATURE

The immunomodulatory benefits from interactions of the immune system with commensal bacteria in the intestine have long been appreciated (Shanahan 2002). The gastrointestinal tract (GIT) is colonized by upwards of $10^{13}$ to $10^{14}$ bacteria, with the majority of bacteria residing in the colon. Bacteria in the GIT provide the host with nutrients, extract calories from host food, and modify the host immune system to help maintain a state of homeostasis (Guarner and Malagelada 2003; Hardy et al. 2013; Hooper, Littman, Macpherson 2012). Bacteria provide these benefits through production of a variety of molecules, including polysaccharides, such as polysaccharide A (PSA) of Bacteroides fragilis, which interacts with the host and induces mucosal tolerance (Mazmanian et al. 2005). Bacterial carbohydrates, such as PSA from B. fragilis, can be complex and contain several different sugars linked by glycosidic bonds. In addition, the carbohydrates can differ in glycosidic linkages ($\alpha$ or $\beta$), linear or branched structure, and degree of polymerization (monomers, oligomers, and polymers), making these carbohydrates highly diverse.

Perhaps nowhere else is the diversity of carbohydrates greater than in the substances secreted by bacteria to form a biofilm. Bacterial biofilms, some of which adhere to host tissue, are a key structure that provides interaction between host and bacteria. Biofilms are comprised of mono- or multi-species bacterial communities encased in an extracellular matrix (ECM)
Great diversity are found in the different carbohydrate polymers in the ECM of bacteria and these structures mediate a high degree of interaction between host and bacterial communities (Anderson et al. 2003; Fujimura et al. 2010; Hall-Stoodley, Costerton, Stoodley 2004; Jones and Knight 2012; Macfarlane and Dillon 2007). Bacterial polysaccharides have also been shown to enhance virulence by allowing pathogens to evade the host immune system. Two of the best-studied bacterial polysaccharides are polysaccharide A (PSA), produced by *Bacteroides fragilis*, and exopolysaccharide (EPS), produced by *Bacillus subtilis*, both of which have been shown to prevent colitis (Jones and Knight 2012; Jones et al. 2014; Onderdonk et al. 1977; Round and Mazmanian 2010; Tzianabos et al. 1993).

The Gram-positive spore forming soil bacterium *B. subtilis*, which produces EPS, exists either as a unicellular planktonic cell or in a nonmotile aggregate biofilm (Lemon et al. 2008). Mature biofilms consist of non-motile cells encapsulated in an ECM, which is primarily composed of EPS and poly-N-acetyl-glucosamine (Jones et al. 2014; Roux et al. 2015). The undomesticated strain of *B. subtilis* (NCBI 3610) forms colonies with wrinkled architecture on LB agar plates and a pellicle between the air liquid interface when grown in liquid medium (Branda et al. 2001; Kearns et al. 2005). Formation of a biofilm or pellicle is dependent on the production of EPS, which occurs in the cytoplasm of the cell and is secreted by an unknown mechanism. Following secretion, EPS is localized in the protein scaffold of the extracellular matrix (Driks 2011; Guttenplan, Blair, Kearns 2010). Synthesis of EPS is regulated by the genes encoded in the 15-gene *epsA-O* operon and the *tapA-sipW-tasA* operon. Once EPS is produced by *B. subtilis* it is
most likely stabilized by repeating amyloid protein, TasA, on the extracellular surface of the bacteria. The interaction between EPS and the protein scaffold of TasA and TapA is unknown (Driks 2011; Guttenplan, Blair, Kearns 2010; Romero et al. 2011).

A single oral dose of *B. subtilis* spores can protect mice from enteric disease caused by *C. rodentium*, and the protective activity is provided by EPS, which is composed of mannose (~88%) and glucose (12%) (Jones et al. 2014). *B. subtilis* spores with deletions to *epsH*, a glycosyltransferase of the *eps* operon, and EPS isolated from a *epsH* mutant (Jones and Knight 2012; Kearns et al. 2005; McLoon et al. 2011), are unable to protect from this disease and do not form a biofilm (Jones and Knight 2012). The goal of this study was to characterize EPS produced by *B. subtilis* mutants of the 15 gene *epsA-O* operon and to determine which genes are required for biofilm formation and which are required for protection from disease caused by *C. rodentium*.

**Gut Host Microbe Interaction**

The microbiome or microorganisms that naturally colonize the host cover the skin and gastrointestinal tract (GIT) and are believed to have become resident microbes during years of co-evolution (Chung et al. 2012; Hardy et al. 2013; Ley, Peterson, Gordon 2006; Macfarlane and Dillon 2007; Madan et al. 2012; Nowrouzian et al. 2003). In exchange for an environment favorable for their growth, host microbes provide nutrients, extract calories from food, shape the immune system, impact development of allergies, aging, mood, and alter susceptibility to disease (Bravo et al. 2011; Hardy et al. 2013; Horton et al. 2015; Thorburn et al. 2015; Wu et al. 2010).
The human microbiome has considerable species diversity and includes the genera *Bacteroidetes*, *Actinobacteria*, and *Firmicutes*. As humans age, there are changes to the microbial populations in the GIT. The microbiota composition fluctuates over the lifetime of its host due to changes in environment, diet, and infection (Gareau, Sherman, Walker 2010; Li, Bihan, Methé 2013; Martínez et al. 2015; Saraswati and Sitaraman 2014).

The diet of the host provides a way for bacteria to be introduced to the GIT and influences the health of the host. Foods rich in metabolites such as butyrate, a short chain fatty acid, serve as a source of energy for host colonic epithelial cells, and regulate mucosal gene expression, differentiation and apoptosis (Kelly et al. 2015). These foods, or prebiotics, also play a role in priming the environment to be favorable for certain species of bacteria. On the luminal surface of epithelial cells in the GIT, continuous layers of mucus are secreted from goblet cells. This coating of mucus is between 100-200µm thick and protects against adhesion and invasion by harmful bacteria. Often, mucus secreted by the host has complex carbohydrate structures and can only be degraded by several hydrolytic enzymes usually sourced from multiple bacteria (Macfarlane, Bahrami, Macfarlane 2011; Resta-Lenert and Barrett 2003). Mucins, or heavy molecular weight proteins that coat surface epithelial cells, are extensively glycosylated, constitutively secreted by goblet cells, and their polymerization provides a structural foundation for mucus (Hardy et al. 2013). Colonization with certain bacteria, such as *Lactobacillus rhamnosus* and *Lactobacillus plantarum* 299v, can up-regulate mucin production, thereby preventing attachment by enteropathogens, such as *E. coli* O157:H7 (Curtis et al. 2014; Mack et al. 1999). In addition, species previously thought to
exert negative effects on the host, such as *Helicobacter pylori*, have begun to be appreciated for their potential benefits. Recent data on the relationship between *H. pylori*, thought to be responsible for peptic ulcer disease, noncardia gastric cancer, and esophageal cancer, may also afford protection against esophageal adenocarcinoma, allergic asthma and inflammation, rhinitis, and atopy (Blaser and Atherton 2004; Blaser 2008; Macfarlane, Bahrami, Macfarlane 2011; Qiu et al. 2011; Reibman et al. 2008).

Due to the huge diversity of the microbiota, it is difficult to identify which bacteria are key players in immunomodulation. To better understand the species and mechanisms on host immunomodulation and immune system development, germ-free animals, or animals previously unexposed to microorganisms, are used. A benefit to these animals is that they limit the diversity of the microbiota and provide some degree of control for bacteria that are introduced to a host (Macpherson and Harris 2004; Tlaskalová-Hogenová et al. 2011). Once individual species of interest are identified they can be isolated and used for individualized treatments which can serve as an attractive alternative to conventional therapies and pharmaceuticals (Macfarlane, Bahrami, Macfarlane 2011; Thomas, Ockhuizen, Suzuki 2014). The use of beneficial bacteria involved in alleviation of disease has already led to improved outcomes for diseases of the GIT. Improved outcomes include repression of pathogenic microbial growth, prevention of irritable bowel disease (IBD), and prevention of complications from ileal pouch-anal anastomosis (IPAA) (Fujimura et al. 2010; Gionchetti et al. 2003; Hooper, Littman, Macpherson 2012; Macpherson and Harris 2004; O'shea et al. 2009; Stecher and Hardt 2008).
Probiotics

Probiotics, from the Greek ‘pro bios’ (for life), are microbial organisms that contribute to human health and are implicated for their potential protective effects against inflammatory bowel disease (IBS), antibiotic associated and infectious diarrhea, metabolic syndrome, allergic disorders, mood disorders, and cancer (Bravo et al. 2011; Gareau, Sherman, Walker 2010; Hume 2011; Wang et al. 2015). These microbes can influence the activity of a number of cells of the innate immune system, including but not limited to: natural killer T cells, dendritic cells, macrophages, epithelial cells, as well as cells of the adaptive arm of the immune system such as Th1 cells, Th2 cells, Th17 cells, Treg cells, and B cells (Hardy et al. 2013; Hooper, Littman, Macpherson 2012). Bacteria shown to affect these immunomodulatory activities consist of strains from the genera *Lactobacillus*, *Bifidobacterium*, *Bacillus*, and *Pediococcus* (Guandalini 2011; Hardy et al. 2013; Hume 2011; Wang et al. 2015). Of these genera, only one, *Bacillus*, displays capability to form spores. Spore-producing bacteria can be used as probiotics; spores are easy to store and are able to survive the gastric acid of the stomach, successfully colonizing the GIT of the host (Hong, Le, Cutting 2005).

Probiotics have favored niches in the GIT, and these niches are altered when changes to the diet are made. In the GIT, ‘functional foods/prebiotics,’ non-digestible food ingredients, such as indigestible dietary fiber/carbohydrates, primes the environment for probiotics. These non-digestible food ingredients may act cooperatively to selectively promote the growth and activity of one or more beneficial probiotic species and stimulate activity of pre-existing commensal bacteria (Perez-Vilar and Hill 1999; Roberfroid et al. 2010; Thorburn et al. 2015).
Before the probiotic strains responsible for protection and immune-modulation were identified, the protective benefits of fermented and cultured foods had been appreciated in fermentation processes throughout history. Fermented and cultured foods were present in the Middle East, Ancient Greece and Asia because of their ability to alleviate illness and aid human health (Hume 2011); however, their mechanisms of protection were not well understood. In 1905, Stamen Grigorov, a Bulgarian microbiologist, was the first to identify the potential health benefits of *Lactobacillus bulgaricus* (*Lactobacillus delbrueckii* ssp. *bulgaricus*) (Metchnikoff 1908). In 1908, Ilya Metchnikoff expanded this finding, developing the hypothesis that human health and longevity were associated with the ingestion of lactic acid producing bacteria. Thus the first steps leading to a better understanding of how certain foods protect against disease was elucidated.

Metchnikoff reasoned that Bulgarian peasants lived longer and consumed large quantities of sour milk containing cultures of what are now known as *Lactobacillus bulgaricus*. In 1954, Werner Kollath proposed the term probiotic to designate “active substances essential for healthy development of life” (Guarner and Malagelada 2003; Hume 2011). The term was further modified by Lilly and Stillwell in 1965 to describe the “substances secreted by one microorganism that stimulate the growth of another” and again in 1974 by Parker et. al. to include organisms and substances that contribute to intestinal microbial balance (Hume 2011; Soccol et al. 2010). Currently, probiotics are identified as live microorganisms which when consumed in adequate amounts, confer a health effect on the host (FAO/WHO 2002; Sanders 2008).
The term ‘probiotic’ has evolved, but problems with their use persist. Bacteria found in commercial products continue to be widely mislabeled, and mechanisms of protection are often poorly characterized (Hong, Le, Cutting 2005; Huys et al. 2013; Michail et al. 2006; Ngo Thi Hoa et al. 2000; Sanders et al. 2010). Additionally, some bacteria labeled as ‘probiotics’ may actually do more harm than no treatment at all (Besselink et al. 2008). Future individualized therapies will benefit from a better understanding of how probiotics confer protection and identification of which molecules are responsible for protection. Additionally, individual molecules are generally easier to directly administer over live active cultures (Lemon et al. 2008; Soccol et al. 2010) and reduce the risk of potential negative interactions between host and live organisms.

**The Extracellular Matrix and Formation of Biofilms**

Biofilms are a key structure in the interaction between the host and bacteria. Biofilms are ubiquitous in nature and are comprised of tightly associated mono- or multi-species bacterial communities that are encased in an extracellular matrix (ECM) (Flemming and Wingender 2010). The ECM is a primary component of the biofilm and develops over a series of different stages in response to external signals. In early stages, planktonic cells congregate at the site of initiation of the biofilm and differentiate into non-motile, matrix-producing cells that attach to a surface. These non-motile matrix-producing bacteria form bundles, grow, and aggregate. When the environment is not favorable for the bacterial community, cells lyse or, if they are spore forming bacteria, produce spores and the biofilm begins to break down (Flemming and Wingender 2010; Vlamakis et al. 2013).
The formation of a biofilm is regulated at different stages via diverse mechanisms. A well-studied regulatory mechanism responsible for production of a biofilm is quorum sensing (QS); the quorum sensing regulatory pathway is known to influence production of an ECM. Quorum sensing allows cell-cell communication, expression of required genes in a cell-density dependent manner, and the production, release, and detection of chemical signaling molecules. There are two QS processes for bacteria, autoinducer-1 (AI-1), common in intra-species communication and autoinducer-2 (AI-2), associated with inter-species interaction (Vu et al. 2009). An example of a QS peptide is ComX, which functions as a pheromone and triggers production of surfactin, which is involved in mediating a subpopulation of cells to produce an ECM (Marvasi, Visscher, Casillas Martinez 2010). However, these are species-specific mechanisms and are not necessarily general. Disease-causing bacteria often use the molecules of the ECM to facilitate adherence to host tissues, evade detection by the host immune system, and prevent phagocytosis (Nwodo, Green, Okoh 2012).

Molecules of the ECM are not all detrimental to the host, and exopolymeric substances secreted by commensal bacteria can actually protect against disease (Jones et al. 2014; Mazmanian and Kasper 2006). The best-studied exopolymeric substance is polysaccharide A (PSA), one of eight distinct polysaccharides synthesized by the commensal B. fragilis. B. fragilis is a Gram-negative obligate anaerobe, and it has a capsular polysaccharide complex that co-opts Treg lineage differentiation and induces mucosal tolerance (Huang, Lee, Mazmanian 2011; Mazmanian et al. 2005). As mentioned previously, EPS from the commensal B. subtilis is also well studied and
protective against the enteropathogen *C. rodentium* (D'Arienzo et al. 2006; Hooper et al. 2001; McLoon et al. 2011).

**Bacterial Polysaccharides and their Immunomodulatory Activities**

Over 99% of microorganisms on Earth live within biopolymers (Rehm 2010; Vu et al. 2009). The first discovery of a bacterial polymer dates back to the mid-nineteenth century with the discovery of dextran, a microbial polysaccharide in wine, by Louis Pasteur. Bacterial polysaccharides are diverse structural products composed of glycosidically-linked sugars, and classified as homopolysaccharides or heteropolysaccharides. Homopolysaccharides are composed of one monosaccharide in repeating units (cellulose, curdlan, dextran, levan, pullulan,) while heteropolysaccharides are composed of two or more monosaccharides and are usually present as multiple copies of oligosaccharides with three to eight residues (alginate, gellan or xanthan) (Freitas, Alves, Reis 2011; Galván et al. 2013; Kumar, Mody, Jha 2007; Öner 2013; Ruffing and Chen 2006).

Bacterial polysaccharides have varying chemical structure and monomer composition, as well as physiochemical, and rheological properties. A bacterial polysaccharide from one species of bacteria may vary greatly depending on the strain it is produced from, making generalizations about the characteristics of polysaccharides difficult (Öner 2013). The diverse and unique properties of bacterial polysaccharides makes them attractive molecules in food, pharmaceutical, cosmetic, oil drilling, and paper manufacturing industries (Rehm 2010; Roca et al. 2015; Schmid, Sieber, Rehm 2015). The production costs associated with microbial EPS are also high and inhibit use
on a wider scale (Freitas, Alves, Reis 2011; Öner 2013; Rehm 2010; Rehm 2015; Roca et al. 2015). Most carbohydrate food ingredients used today were introduced between 1940 and 1965. Some of the best-studied and commercially exploited bacterial polysaccharides include alginate, gellan, glucan, guar, hyaluronan, levan, succinoglycan, and levan (Freitas, Alves, Reis 2011; Galván et al. 2013; Öner 2013; Ruffing and Chen 2006). With annual production of bacterial polysaccharides at an estimated 2,000 tons for dextran and 100,000 tons of xanthan alone, these bacterial products, their synthesis, and their properties are of great value to industry (Rehm 2010). Although bacterial carbohydrates are of high importance, their synthesis, physical properties, and structures remain poorly characterized.

Elucidation of the structure and composition of polysaccharides are important because minor changes in structure of the polysaccharide can alter recognition between host receptor and polysaccharide and the development of disease. Bacteria such as Neisseria meningitides, Streptococcus pneumonia, group B streptococci, and Staphylococcus aureus all produce forms of capsular polysaccharides that have varying levels of virulence. N. meningitides, a gram-negative bacteria, produces multiple polysaccharides that are serologically distinct. Of the 13 serogroups produced by N. meningitides, six serogroups (A, B, C, W-135, X, and Y) cause disease (Stephens, Greenwood, Brandtzaeg 2007; Tzeng and Stephens 2000; Van Kranenburg et al. 1999; Zughaier 2011). Understanding how changes to bacterial polysaccharide can reduce virulence is of importance for developing targeted therapeutics. Bacterial polysaccharides coat the extracellular surfaces of bacteria and contribute to the development of a robust extracellular matrix and formation of a biofilm (Guttenplan, Blair, Kearns 2010; McLoon...
et al. 2011) and are also often necessary for energy storage, adhesion, and immune evasion (Kumar, Mody, Jha 2007; Rehm 2010; Schmid, Sieber, Rehm 2015; Sutherland 2001). Extracellular polysaccharides, such as capsular polysaccharides, are known to enhance virulence (Cuthbertson et al. 2009; Nanra et al. 2013; O'Riordan and Lee 2004; Stephens, Greenwood, Brandtzaeg 2007) and are implicated in a number of difficult-to-treat infectious diseases (Djeribi et al. 2012; Hall-Stoodley, Costerton, Stoodley 2004; Parsek and Singh 2003; Zegans et al. 2002).

As mentioned above, the best-studied exopolysaccharides with beneficial immunomodulatory activities are PSA, produced by B. fragilis, and EPS produced by B. subtilis. EPS produced by B. subtilis, is thought to confer protection through a TLR-4 dependent pathway. Mice injected with EPS develop increased levels of M2 macrophages (ϕ), which are capable of suppressing T-cell proliferation in vitro. It is important to note that EPS is not the only bacterial polysaccharide B. subtilis produces. A number of neutral polysaccharides add to the structure of the exopolymeric matrix including poly-N-acetyl glucosamine (PNAG), and type I (β-2,6-linked D-fructose units) and type II (fructose and glucose polymer linked by α-glycoside bonds) polymers. Of these bacterial polysaccharides produced, Type I and II polymers are synthesized outside the cell by the extracellular enzyme levansucrase and are therefore extracellular polysaccharides (Dogsa et al. 2013; Marvasi, Visscher, Casillas Martinez 2010; Schmid, Sieber, Rehm 2015). Although there are other polysaccharides produced by B. subtilis, EPS and PNAG are the primary components of the ECM (Jones et al. 2014; Roux et al. 2015).

The production of bacterial polysaccharides generally involves multiple enzymatic steps with precursor biosynthesis occurring inside the cell and
polymerization(secretion) localized in the cell envelope. Presently, there are four mechanisms known for the production of carbohydrate polymers in bacteria: the Wzx/Wzy-dependent pathway, the ATP-binding cassette (ABC) transporter-dependent pathway, the synthesize-dependent pathway, and the extracellular synthesis pathway used by a single sucrase protein.

The biosynthesis pathways of polysaccharides are not fully understood and different biosynthesis pathways encode multiple types of glycosyltransferases (GTS), polymerizing and branching enzymes, and sugar modifying enzymes. The majority of cell-surface bacterial polysaccharides are produced from the Wzx/Wzy dependent assembly pathway (Islam and Lam 2014). Bacterial polysaccharides from the Wzx/Wzy pathway include bacterial surface heteropolysaccharides, such as emulsan produced by *Acinetobacter lwoffii*, succinoglycan produced by *Sinorhizobium meliloti*, and the type I and 4 capsular polysaccharides produced by *Escherichia coli*. The synthesis of these molecules by the Wzx/Wzy dependent pathway begins with individual repeating lipid-linked units that are anchored at the inner membrane. The polysaccharide chain grows at its reducing end and additions are assembled sequentially by a number of GTS at the cytoplasmic face of the inner membrane (IM). Polysaccharides then translocate across the cytoplasmic membrane by a Wzx protein, a flippase. Polymerization of the polysaccharide is also accomplished by a block transfer mechanism that utilizes the polymerase protein, Wzy. The Wzy protein functions at the periplasmic space and facilitates the export of the polymer to the extracellular environment where it’s attached to the bacterial surface as a capsular polysaccharide (CPS) or released as EPS.
Most CPS are synthesized by the ABC transporter dependent pathway. Polysaccharides are synthesized by GTSs at the cytoplasmic face of the inner membrane and depending on the number of GTSs involved, the polysaccharides are either homo or heteropolymeric. Transport of synthesized CPCs occurs through tripartite efflux pump complexes that span the inner membrane and periplasmatic proteins (Schmid, Sieber, Rehm 2015; Whitney and Howell 2013; Willis and Whitfield 2013).

Arguably a less complicated mechanism is the synthase dependent pathway, which results in secretion of completed polymer strands across the membranes and cell wall and is independent of a flippase. Polysaccharide polymerization and translocation is completed by a single synthase protein and products are generally homopolymeric. Some enzymes in the synthase-dependent pathway are bi-functional and perform both polymerization and secretion (Schmid, Sieber, Rehm 2015).

While a general understanding of the mechanisms for the synthesis of bacterial polysaccharides is known, further characterization is necessary to better understand the enzymes involved in polysaccharide synthesis.

**Glycosyltransferases**

The large variety and complexity of oligosaccharide and polysaccharide structure is in part due to the enzymes such as GTSs, glycosidases, glycan phosphorylases, and polysaccharide lysases, which synthesize their production. GTSs are a class of enzymes that catalyze glycosidic bond formation and utilize sugar donors with a nucleoside.
phosphate or a lipid phosphate-leaving group (Lairson et al. 2008). There are two known classes of GTSs that result in retention (retaining GT) or net inversion (inverting GT) at the anomeric carbon relative to the phospho-sugar donor substrate (Gavin, Jon, Toone 2009; Lairson et al. 2008). As is true for polysaccharide biosynthesis, the catalytic mechanisms of GTS are also poorly understood. Additionally, GTSs vary in their substrate specificity and sequence and are classified into two superfamilies on the basis of structural fold, GT-A and GT-B. Both GT-A and GT-B GTSs contain Rossman folds (Gavin, Jon, Toone 2009; Sánchez-Rodríguez et al. 2014), or structural motifs found in proteins that bind cofactors. Incidentally, SpsA, which is produced by B. subtilis, was the first GTS shown to adopt a GT-A fold (Lairson et al. 2008).

The epsA-O Operon and Characteristics of EPS

Genes responsible for the production of EPSs are generally clustered (Dertli et al. 2013; Van Kranenburg et al. 1999) and identifiable by the multiple GTSs, polymerases, and secreting enzymes they encode (Guttenplan, Blair, Kearns 2010; Schmid, Sieber, Rehm 2015). EPS is produced by 15 different genes encoded in the eps operon of B. subtilis. The 15 genes encoded in the operon have sequences that suggest a variety of enzymatic activities including 6 glycotransferases, 2 pyruvyltransferases, as well as a kinase, flippase, acetyltransferase, and aminotransferase (Roux et al. 2015). At least one of these genes, epsE, encodes for a bifunctional enzyme that promotes matrix synthesis and inhibits motility (Guttenplan, Blair, Kearns 2010).

EPS secreted from B. subtilis with a deletion to epsH, a glycosyltransferase, does not inhibit all C. rodentium associated disease markers, or modulate the immune system
compared to WT EPS (Jones and Knight 2012; Jones et al. 2014). It is believed that
*epsH* is an important enzyme that contributes to the formation of a protective EPS. The
use of strains of *B. subtilis* with deletions to the genes of the *eps* operon is a unique and
novel approach to identify immunomodulatory forms of a bacterial polysaccharide.

Figure 1. Illustration of the order of the 15 genes of the *epsA-O* operon. Labels below
the gene indicate the suggested type of enzyme the gene encodes. Red text is to highlight
the large number of putative glycosyltransferases found in the operon.

*Citrobacter rodentium* as a Model of Enteric Disease

*Citrobacter rodentium* is a Gram negative (G-) attaching and effacing (A/E)
mouse specific enteropathogen, previously known as *Citrobacter freundii* (Luperchio et
al. 2000; Schauer et al. 1995). *C. rodentium* induces actin filled membranous protrusions,
pedestals, at the site of attachment. Following pedestal formation, an A/E lesion forms
and the pathogen breaches the epithelial barrier infecting the host. *C. rodentium* is used in
infection models in mice to study diseases such as enteropathogenic *E. coli* (EPEC),
enterohaemorrhagic *E. coli* (EHEC), Crohn’s, ulcerative colitis (Chandrakesan et al. 2014;
Higgins et al. 1999). These are diseases that have few prophylactic treatments available.
*C. rodentium* is genetically similar to the human attaching and effacing (A/E) pathogens which attach and adhere to the intestinal epithelium, destroy microvilli, displace commensal microbiota, and deplete goblet cells. *C. rodentium* shares 67% of genes with EPEC and EHEC (Petty et al. 2010) including the locus of enterocyte effacement pathogenicity island (Garmendia, Frankel, Crepin 2005), which encodes effector proteins and a type III secretion system. It also expresses many of the same effectors of EPEC and EHEC, such as a type IV pilus, which is known to increase colonization (Mundy et al. 2003).

Following infection with *C. rodentium*, mice develop transmissible murine crypt hyperplasia (TMCH), which is accompanied by intestinal inflammation during colitis. TMCH is caused by thickening of the colonic mucosa which is a result of excessive induction of epithelial regeneration and repair mechanisms (Barker, De Wetering, Clevers 2008; Barthold and Jonas 1977; Luperchio and Schauer 2001; Mundy et al. 2005; Schauer and Falkow 1993a; Schauer and Falkow 1993b). Mice also develop colitis and this contributes to the overgrowth of *C. rodentium* and an overall reduction of total bacterial diversity in the GIT (Mundy et al. 2005). TMCH is triggered by the loss of epithelial barrier integrity and the transit of bacteria into the sterile lamina propria. Mice with TMCH generally experience cycles of biphasic disease (progressive and regressive phases) that last 2-3 weeks (Chandrakesan et al. 2010; Luperchio and Schauer 2001). During the progressive phase, fibroblast growth factor 7 and IL-11 are produced by pericryptal myofibroblasts. Other uncharacterized signals trigger cell proliferation via activation of WNT-β-catenin, signal transducer and activator of transcription3 (stat3), and phosphatidylinositol 3-kinase-AKT-β-catenin signaling pathways (Brown et al. 2011;
Gibson et al. 2010; Papapietro et al. 2013). The regressive phase involves pathogen clearance and resolution of colitis and inflammation and a return to normal intestinal homeostasis (Chandrakesan et al. 2010).

Probiotics including *Lactobacillus rhamnosus*, *Lactobacillus helveticus*, and *Bifidobacterium breve* UCC2003 have been shown to prevent *C. rodentium* colonization (Hardy et al. 2013). The gut commensal *Bacteroides thetaiotaomicron* is thought to exacerbate infection and increase gut permeability (Curtis et al. 2014) and contribute to worsening of disease. *B. subtilis* has been shown to prevent symptoms of disease (D’Arienzo et al. 2006; Mazza 1994) and it is now believed that EPS produced by *B. subtilis* is responsible for protection against *C. rodentium* associated disease (Jones and Knight 2012; Jones et al. 2014). Interestingly, protection by EPS is independent of colonization levels of *C. rodentium* suggesting that EPS modulates the immune system of the host (Jones and Knight 2012).

**Methods for the Analysis and Study of Complex Carbohydrates**

Bacterial polysaccharides can be extremely large and vary in molecular weight (a few thousand to several million Daltons), and their study requires a unique set of methods (Cui 2005; Kumar, Mody, Jha 2007). Carbohydrates are heterogeneous and differ in their primary structure, charge, linkages (α- and β-) and degree of polymerization, and have macromolecular characteristics including linear and branching structure. These variations give polysaccharides a number of properties and makes their study difficult.

Successful analysis of polysaccharides relies on the removal of other substances that interfere with analysis and results are influenced by how the sample was prepared.
Low molecular weight carbohydrates can be extracted with hot 80% ethanol while water-soluble polysaccharides can be precipitated with >70% ethanol and a low molarity salt solution (Benhura and Chidewe 2011; Cui 2005; Davis and Clapp 1961; Eliasson 2006; Healey et al. 2014; Nielsen 2010; Smidsrod and Haug 1967; Weising et al. 2005).

Much of what is known about bacterial carbohydrates comes from their use in industry (Vu et al. 2009; Walt et al. 2012). Xanthan gum, produced by the bacterium Xanthomonas campestris, is a unit-repeating polymer and is most likely the best-studied extracellular polysaccharide. This high molecular weight (3x10^5-8x10^6 Da) bacterial polysaccharide is used in industry and agribusiness and valued for its solubility in either hot or cold solutions, high viscosity at low concentrations, and cellulose-like backbone. Although it was cleared for use by the FDA in 1969 and its synthesis has been extensively studied, the polymerization and export of xanthan is poorly characterized as is the biosynthesis of other bacterial polysaccharides (Cui 2005; Galván et al. 2013; Hassler and Doherty 1990; Kumar, Mody, Jha 2007; Roca et al. 2015).

Basic analysis has historically been limited to the presence or absence of carbohydrate in a sample. Assays such as the phenol-sulfuric acid assay (Dubois et al. 1956) and the anthrone assay are two tests used to determine the presence of carbohydrate content; however, phenol-sulfuric acid assays are more commonly used (Nielsen 2010). In the presence of a strong acid and heat, carbohydrates undergo a series of reactions that lead to the formation of furan derivatives such as furfuraldehyde and hydroxymethyl furaldehyde. A dehydration reaction is followed by newly formed furan derivatives which condense with themselves and phenolic compounds if present, and produce dark colored complexes which can then be measured at 490nm (Nielsen 2010).
Although useful in detecting carbohydrate in a sample, any measurement of the total sugar is an estimate based on the reactivity of the sugar used as a standard (historically glucose) and many different sugars in solution react differently with the assay reagents (Cui 2005; Nielsen 2010). Additionally, samples can be assayed for the presence of a carbohydrate via a modified phenol sulfuric acid assay which does not use a phenol solution and therefore reduces reagents and chemical interaction (Albalasmeh, Berhe, Ghezzehei 2013). There are also enzymatic methods of analysis but these methods require a basic knowledge of the polysaccharide composition (Gawehn 1988).

Presently, advances in the field of carbohydrate analysis have created a number of new methods that enable the determination of the structure of complex polysaccharides. These include fast atom bombardment mass spectrometry (FAB-MS), matrix-assistant laser desorption ionization mass spectrometry (MALDI-MS), electrospray ionization mass spectrometry (ESI-MS), nuclear magnetic resonance (NMR) spectroscopy, Correlation Spectroscopy (COSY), Total Correlated Spectroscopy (TOCSY), and Distortionless Enhancement by Polarization Transfer (DEPT). All of these techniques allow for the solving of structure and composition with some being more precise than others. A problem that persists is that these methods require specialized equipment and reference databases that are not always available (Bubb 2003; Cui 2005; Walt et al. 2012). NMR is presently one of the most powerful techniques for carbohydrate analysis. Use of NMR in conjunction with other analytical methods allows for a characterization of a complex polysaccharide including detailed structural information of carbohydrates, identification of monosaccharide composition, elucidation of α- or β- anomeric
configurations, establishment of linkage patterns, and identification of large polymer sugar units in oligosaccharides and/or polysaccharides (Bubb 2003; Cui 2005).
CHAPTER TWO
MATERIALS AND METHODS

Pellicle and Biofilm Formation Assays

To assess pellicle formation of *B. subtilis* mutants, overnight (O/N) bacterial starter cultures were diluted 1:100 in a total volume of 2mL per well in a 24-well tissue culture dish and incubated without agitation for 24 hr at 37°C. Following incubation, pellicle formations at the air-liquid interface were assessed with bright field microscopy and photos taken.

To assess biofilm formation, O/N bacterial starter cultures were diluted 1:100 and 25μL was plated onto plates containing Luria-Bertani (LB) media (Difco) with 1.5% agar (Beckman Dickinson). The plates were incubated without agitation for 24 hr at 37°C. Biofilm formation was assessed with bright field microscopy and photos taken. Strength of biofilm formation was quantified by microtiter dish biofilm formation assay as described by O'Toole (O'Toole 2011). Briefly, 100μL of a 1:100 dilution of starter culture was plated and incubated for 16 hr. Plates were washed twice with water and 125μL of a 1% crystal violet (CV) solution was added to wells and incubated at room temperature (RT) for 20 min. Plates were washed with deionized water (dH₂O) four times and 150μL of a 30% acetic acid solution was added to wells of the microtiter plate to solubilize remaining CV. The 150μL of solubilized CV was then transferred into a new flat-bottomed microtiter dish and the absorbance of the liquid measured at 550nm.
Purification of EPS

To isolate and purify EPS produced by *B. subtilis* mutants, 10mL of LB broth was inoculated from a frozen glycerol stock and incubated for 16 hr at 250 RPM at 37°C. A 1:1000 dilution of bacterial starter cultures was made in 1L of LB broth and incubated for 16 hr at 37°C. Cultures were pelleted at 4500 x g for 20 min at RT and the supernatant collected. Supernatants were precipitated with 50% ethanol (EtOH) and incubated O/N at -20°C. Precipitates were pelleted at 15000 x g for 20 min at 4°C, re-suspended in 100mL of 0.1M Tris, and treated with DNase (67µg/mL) and RNase (330µg/mL) for 4 hr at 37°C. DNase and RNase digested samples were digested with Proteinase K (40 µg/mL) for 4 hr at 56°C and precipitated with 100mL 50% EtOH and incubated O/N at -20°C. Precipitates were pelleted at 15000 x g for 30 min at 4°C.

EPS pellets were re-suspended in 0.1M Tris with a working concentration of sodium azide and loaded on a size exclusion column with a Sepharcryl 1000 (allyl dextran and N,N’-methylene bisacrylamide) matrix. Fractions containing EPS were identified by a modified phenol sulfuric acid assay as described in Albalasmeh et. al. (Albalasmeh, Berhe, Ghezzehei 2013). Briefly, 30µL of fraction sample volume was added to a 96-well microtiter dish and 90µL of concentrated H₂SO₄ (18M) was added to wells. Microtiter dishes were then placed on the surface of a water bath set at 68°C for at least 30 min and the absorbance measured at 315 nm on a UV-Vis Spectrophotometer (BMG Labtech, Omega SPECTROstar).

Phenol sulfuric acid assay positive fractions were pooled and precipitated with 50% EtOH and incubated O/N at -20°C. Precipitates were collected at 15000 x g, re-suspended in tissue culture grade water and dialyzed O/N at 4°C in 1L of tissue culture
grade water. Dialysis cassettes (Life Technologies, Slide-A-Lyzer) with a molecular weight cut off of 10K were used to remove small impurities, salts, and remaining sodium azide in EPS samples. Dialyzed samples were precipitated with 50% EtOH O/N at -20°C. Precipitates were collected at 12500 x g for 20 min at 4°C and dried in a vacuum centrifuge.

Purified EPS from B. subtilis strains used in this study were re-suspended in 0.1M Tris or 1X phosphate buffered saline (PBS).

<table>
<thead>
<tr>
<th>10X PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
</tr>
<tr>
<td>pH to 6.45</td>
</tr>
</tbody>
</table>

**Comparison of EPS Size Isolated from Bacillus subtilis Mutants**

To assess the size of pre-column purified EPS from various B. subtilis mutants, 100μL of 50μg/mL EPS was loaded into a fast performance liquid chromatography (FPLC) apparatus (GE, AKTA protein purification system) with a Superose 6 (agarose matrix) 10/300 GL Size Exclusion Tricorn column (GE). Solutions of 20mg/mL Blue Dextran (>2,000KD), 6mg/mL apoferritin (450kD), 6mg/mL IgG (150kD), and a 10M fructose were also loaded on the column to serve as reference points of size.
Detection of Similar Epitopes of EPS Isolated from Various *Bacillus subtilis* Mutants

To assess if EPS isolated from *B. subtilis* mutants had similar epitopes to WT EPS, modified western SDS-PAGE was performed. Samples of pre-column EPS were prepared as described previously and samples were boiled for 10 min at 95°C. A 5% stacking gel and 10% resolving gel were prepared and a total volume of 30µL was loaded onto a western SDS-PAGE apparatus consisting of 5µL of 6X sample buffer, and 25µL of a 1mg/mL sample of EPS. EPS samples were run at 100 Volts (V) for 1.5 hr and transferred to nitrocellulose membrane (LifeTechnologies) O/N at 100mA at 4°C. Free binding sites on nitrocellulose membranes were blocked with 5% skim milk in 1X TBST for 2 hr and incubated with a 1:10000 dilution of 1mg/mL primary rabbit anti-EPS serum Ab in 1X TBST with 5% bovine serum albumin (BSA). Nitrocellulose membranes were treated with a 1:10000 dilution of a 1mg/mL secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (LifeTechnologies) in 1X TBST with 5% BSA for 30 min Immunoblots were developed using Immun-Star HRP (BioRad).

EPS Binding to Peritoneal Cells

To assess the level of binding of EPS from *B. subtilis* mutants to macrophages (MΦ) isolated from the peritoneal cavity, cells from the peritoneal cavity were analyzed by flow cytometry using a BD FACSCanto II flow cytometer. Approximately 2 million cells were collected from C57Bl/6 mice by peritoneal lavage and red blood cells lysed by a 5 min incubation with ammonium-chloride-potassium (ACK) Lysing Buffer (LifeTechnologies). Cells were washed once in 1mL of FACS buffer, 1X PBS, 10% Fetal
Calf Serum (FCS), and pelleted by centrifugation at 300 x g for 5 min. Pellets were re-suspended in a volume of 100µL FACS buffer. One µL of 1mg/mL EPS was added to the cells and incubated for 30 min at 4°C. Samples were washed with 2mL FACS buffer and the pellet re-suspended in 100µL. One µL of 1mg/mL rabbit anti-EPS serum Abs was added to samples and incubated for 30 min at 4°C. Cells were washed with 1mL of FACS buffer and the pellet re-suspended in 100µL. Cells were stained for the presence of macrophage markers with 1µL of a 1mg/mL rat anti-mouse F4/80 (BioLegend, Catalog #123117) MΦ marker conjugated to allophycocyanin (APC) and 1µL of a 1mg/mL goat anti-rabbit IgG Ab conjugated to fluorescein isothiocyanate (FITC) (Lifetechnologies, Catalog#65-6111), and incubated for 30 min at 4°C. Tubes were washed with FACS buffer and the pellet re-suspended in a final volume of 300µL of FACS buffer. Samples were analyzed by flow cytometry using the BD LSRFortessa or BD FACSCanto II flow cytometers; data were analyzed by the Flow Jo flow cytometry program.

**Polysaccharide Composition Enzyme Assay**

To identify the monosaccharide composition of EPS produced by *B. subtilis* mutants, EPSs were assayed for percentage of D-mannose, D-Fructose, and D-Glucose as described with slight modifications by Gawehn et. al. (Gawehn 1988). Briefly, 30µL of 10mg/mL EPS was treated with 4M trifluoroacetic acid (TFA) for 18 hr at 95°C (Biermann 1988; Fengel and Wegener 1979; Galanos, Lüderitz, Himmelspach 1969). Samples were dried and re-suspended in 10µL of 0.1M Tris. Enzyme reactions were carried out in a 200µL total reaction volume. In a 96-well micotiter dish, 188µL of 45.6mM triethanolamine buffer (TEA), 2µL of 3µg/µL EPS, 2µL of 0.55mM adenosine
tri-phosphate (ATP), 2µL of 0.36mM nicotinamide adenosine-di-phosphate (NADP), and 2µL of 4.6U/mL hexokinase was added to each well and measured for base absorbance at 340 nm.

<table>
<thead>
<tr>
<th>TEA Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triethanolamine</td>
</tr>
<tr>
<td>MgCl₂</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
</tr>
</tbody>
</table>

Enzyme preparations of 2µL 0.9U/mL of Glucose-6-Phosphate Dehydrogenase (G6PDH), 2µL of 2.3U/mL Phosphoglucose Isomerase (PGI), and 2µL of 4U/mL Phosphomannose Isomerase (PMI) was added in succession and incubated at RT for 20 min, 10 min, and 30 min respectively and absorbance measured at 340 nm at each step.

Figure 2. Illustration of the enzymatic reactions in a monosaccharide composition assay. HK – Hexokinase, G6P-DH – Glucose-6-Phosphate Dehydrogenase, PGI – Phosphoglucose Isomerase, PMI – Phosphomannose Isomerase
Mice

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Usage Committee at Loyola University Medical Center, Maywood, IL. C57BL/6 founders were from Charles River Laboratory (Wilmington, MA), and mice utilized for experiments (4 to 6 weeks of age) were bred at Loyola University, Chicago, IL. Sterile standard chow and tap water were given to mice *ad libitum*.

**Bacterial Strains and Spore Preparation**

The 32 *B. subtilis* strains used in this study were provide by Dr. Daniel Kearns (Indiana University, Bloomington, IN) and are as follows: wild-type NCBI 3610, DK1943 (Δ*epsA*), DK1806 (Δ*epsB*), DK1807 (Δ*epsC*), DS4248 (Δ*epsD*), DS2152 (Δ*epsE*), DS4164 (Δ*epsF*), DS7499 (Δ*epsG*), DS6776 (Δ*epsH*), DK1758 (Δ*epsI*), DS4166 (Δ*epsJ*), DK2055 (Δ*epsK*), DS7432 (Δ*epsL*), DS4901 (Δ*epsM*), DS4900 (Δ*epsN*), DK1759 (Δ*epsO*), DK1960 (Δ*epsA* Δ*sinR* Δ*tasA*), DK1821 (Δ*epsB* Δ*sinR* Δ*tasA*), DK1822 (Δ*epsC* Δ*sinR* Δ*tasA*), DK7724 (Δ*epsD* Δ*sinR* Δ*tasA*), DS2369 (Δ*epsE* Δ*sinR* Δ*tasA*), DS7722 (Δ*epsF* Δ*sinR* Δ*tasA*), DS7760 (Δ*epsG* Δ*sinR* Δ*tasA*), DS7148 (Δ*epsH* Δ*sinR* Δ*tasA*), DK1781 (Δ*epsI* Δ*sinR* Δ*tasA*), DS7723 (Δ*epsJ* Δ*sinR* Δ*tasA*), DK2057 (Δ*epsK* Δ*sinR* Δ*tasA*), DS7761 (Δ*epsL* Δ*sinR* Δ*tasA*), DS7726 (Δ*epsM* Δ*sinR* Δ*tasA*), DS7725 (Δ*epsN* Δ*sinR* Δ*tasA*), DK1782 (Δ*epsO* Δ*sinR* Δ*tasA*), and were generated as described by Kearns et. al. (Kearns et al. 2005). Briefly, strains of *B. subtilis* mutants were generated using insertion deletion with long flanking homology PCR and
transformed into competent cells of *B. subtilis* strain PY79 and transferred to *B. subtilis* strain 3610.

*B. subtilis* spores were generated via exhaustion (Monteiro et al. 2005). Briefly, O/N bacterial starter cultures were diluted 1:100 in Difco sporulation medium (DSM);

<table>
<thead>
<tr>
<th>Difco Sporulation Medium (DSM) – per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto nutrient broth (Difco)</td>
</tr>
<tr>
<td>10% (w/v) KCl</td>
</tr>
<tr>
<td>1.2% (w/v) MgSO₄ • 7H₂O</td>
</tr>
<tr>
<td>NaOH, pH 7.6</td>
</tr>
<tr>
<td>Ca(NO₃)₂</td>
</tr>
<tr>
<td>MnCl₂</td>
</tr>
<tr>
<td>FeSO₄</td>
</tr>
</tbody>
</table>

and incubated for 48 hr 250 RPM at 37°C. Bacteria were pelleted at 5000 x g for 15 min at 4°C and washed three times in ice-cold sterile water. Pellets were re-suspended in sterile water and heat shocked at 68°C for 30 min to kill any remaining vegetative cells. Samples were washed three times with ice-cold water and stored at 4°C. Spore preparations were assessed for spore concentrations by culturing serial dilutions. On the day of administration, 10⁹ CFU *B. subtilis* spores were washed with ice-cold sterile water, re-suspended in 200μL 1X PBS, and given to mice via oral gavage (o.g.).

For infection studies, *C. rodentium* ATCC 51459 was cultured for 16 hr in LB media, washed once in 1X PBS and an infectious dose of 5X10⁸ CFU was re-suspended in 200μL of sterile PBS for administration to mice by o.g.
Isolation and Identification of Bacteria Contained in Natto

Three brands of Natto (soybeans fermented with *B. subtilis*), Hiruzen (Natto 1), Marukin (Natto 2), and Mizkan (Natto 3), were obtained from a local grocery store, diluted in LB media and particulates removed. Samples were heat shocked for 45 min at 70°C and plated on LB media with 1.5% agar. Single colonies were selected and analyzed using matrix assisted laser desorption ionization – time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik Maldi Biotyper). Sample time of flights were measured against clinical and research databases and scored for match to most likely bacterial species (Bruker Research Database).

Citrobacter rodentium Disease Model

Specific-pathogen-free C57Bl/6 mice were orally administered *B. subtilis* spores (10⁹ in 200µL in 1X PBS) 24 hr prior to infection with 5X10⁸ CFU of *C. rodentium* by o.g. Age- and gender- matched mice were utilized for each experiment. To assess disease, mice were euthanized 10-11 days post infection and tissues collected. Mouse feces were collected and scored for degree of diarrhea. Fecal samples were scored as described (Jones and Knight 2012) using a scale from 1 to 4 with a score of 1 = no diarrhea; 2 = slightly soft stool - mild diarrhea; 3 = very soft stool - moderate diarrhea; 4 = unformed stool - severe.

To measure colonization with *B. subtilis*, feces were homogenized in 500µL of sterile 20% glycerol in 1X PBS, and serial dilutions were cultured on selective LB with 1.5% bacto agar to determine the total number of *B. subtilis* CFU. Vegetative cells were killed by heating at 68°C for 30 min, and serial dilutions were plated onto LB medium
plates to quantify the spores. Plates were incubated overnight at 37°C; the resulting colonies are represented as the number of CFU per gram of feces.

To measure colonization by *C. rodentium*, feces taken 10-11 days post infection were homogenized in 500µL of sterile 20% glycerol in 1X PBS, and serial dilutions were cultured on selective MacConkey agar plates. Plates were incubated overnight at 37°C, and only small colonies that displayed the characteristic pink center surrounded by a white rim were counted. The level of colonization was calculated and expressed as the number of CFU per gram of feces.

To assess for *C. rodentium* induced colonic alterations, distal colons from euthanized mice were collected and fixed overnight in 10% formalin-buffered phosphate, embedded in paraffin and hematoxylin and eosin (H&E) stained. Longitudinal tissue sections were examined for epithelial hyperplasia and goblet cell loss with bright field microscopy and photos taken.

![Figure 3. Model of *C. rodentium* infection.](image-url)
Serum Inflammatory Chemokine ELISA

To assess the level of serum inflammatory keratinocyte chemoattractant (KC) cytokine, enzyme-linked immunosorbent assays (ELISA) were performed. Mictrotiter plates (96-well) were coated with 100µL of a 2.0µg/mL mouse KC capture antibody (R&D Systems, Catalog #DY453, Part #840325) and incubated overnight at 4°C. Plates were washed with ELISA washing buffer (0.05% Tween20 in 1X PBS) three times and free binding sites blocked with 300µL of a 1X reagent diluent (R&D Systems, Catalog #DY995) and incubated for 1.5 hr at RT. Plates were washed three times and 100µL of serum samples and standards were added and incubated for 2 hr at RT. Plates were washed three times and 100µL of a 200ng/mL biotin labeled rat anti-mouse KC detection Ab in carbonate buffer (R&D Systems, Catalog #DY453, Part#840326) was added and incubated for 2 hr at RT. Plates were washed three times and 100µL of a 200ng/mL Streptavidin-HRP reagent (R&D Systems, Catalog #DY453, Part #893975) was added and incubated at RT for 30 min. Plates were washed three times and 100µL of a working concentration of 3,3’,5,5’-tetramethylbenzidine (TMB) (LifeTechnologies, Catalog #00-2023) was added and the absorbance measured at 405 nm. Concentrations of KC were calculated against a standard solution of recombinant mouse KC (R&D Systems Catalog #DY453, Part #840327).

<table>
<thead>
<tr>
<th>Carbonate Buffer</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂CO₃</td>
<td>15 µM</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>34.5 µM</td>
</tr>
<tr>
<td>pH</td>
<td>9.5</td>
</tr>
</tbody>
</table>
**Allergic Sensitization to Ovalbumin**

To assess sensitization to ovalbumin, C57Bl/6 mice were orally gavaged with $10^9$ CFU of *B. subtilis* spores at day -3, and -1. At day 0, mice were injected intraperitoneally (i.p.) with 100µL ovalbumin (100µg/mL) in 1X PBS (OVA) (SIGMA Fraction V) and 100µL of 20mg/mL alum in 1X PBS (Thermo Scientific Catalog #77161, Lot #OD182171). Blood from sensitized mice was collected at day 14 and sera was assessed by ELISA for levels of IgG1 and OVA-specific IgG1.

![Model of allergic sensitization to ovalbumin.](image)

To measure levels of total IgG1 from sera of sensitized mice, 96-well microtiter plates were coated with 100µL of a 2µg/mL rat anti-mouse IgG1 primary antibody in carbonate buffer and incubated O/N at 4°C. Plates were washed three times with ELISA washing buffer, blocked with 100µL of a 3% BSA in 1X PBS solution, and then incubated for two hours at RT. Plates were washed three times and 100µL of a 500ng/mL standard diluted in duplicate two-fold six times was added. Serum (100µL of 1:20000)
was added and plates were incubated for 1 hr. at RT. Plates were washed three times and 100µL of a 2µg/mL biotinylated rat anti-mouse IgG1 secondary antibody (BioLegend, Catalog #406604) added and incubated for 1 hr at RT. Plates were washed three times and 100µL of a 1:3000 Streptavidin-HRP solution added and incubated in the dark at RT for 30 min. Plates were washed three times and 100µL of a working concentration of 3,3’,5,5’-tetramethylbenzidine (TMB) added and absorbance measured at 405 nm.

To measure levels of OVA-specific IgG1 from sera of sensitized mice, 96-well microtiter plates were coated with 100µL of 10µg/mL OVA in carbonate buffer pH 9.2 and incubated O/N at 4°C. Plates were washed three times, blocked with 100µL of 3% BSA in 1X PBS and incubated for 2 hr at RT. Plates were washed three times and 100µL of serum (1:20,000) was added and plates were incubated for 1 hr. at RT. Plates were washed three times and 100µL of a 2µg/mL biotinylated rat anti-mouse IgG1 secondary antibody (Biolegend, Catalog #406604) added and incubated for 1 hr at RT. Plates were washed three times and 100µL of a 1:3000 Streptavidin-HRP solution added and incubated in the dark at RT for 30 min. Plates were washed three times and 100µL of a working concentration of TMB added and absorbance measured at 405 nm.

**Generation of Bone Marrow Derived Mast Cell (BMDMC) and Peritoneal Derived Mast Cell (PDMC).**

Peritoneal cells from C57Bl/6 mice were collected by peritoneal lavage in Roswell Park Memorial Institute media (RPMI) (LifeTechnologies, Catalog #31800) with 1X RPMI additive and pelleted at 300 x g at 4°C for 5 min. For generation of BMDMCs,
femurs and tibias from mice were harvested and bone marrow (BM) collected. Pellets were washed once in RPMI and spun at 300 x g at 4°C for 5 min. Pellets were re-suspended in 5mL cell culture medium (RPMI with additive and 10% FCS) and transferred into a small cell culture flask. Cells were incubated at 37°C and 5% CO₂.

<table>
<thead>
<tr>
<th>10X RPMI Additive – per liter</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>L-glutamine (200mM)</td>
<td>120 mL</td>
</tr>
<tr>
<td>MEM Vitamin Solution (100X)</td>
<td>100 mL</td>
</tr>
<tr>
<td>Penicillin Streptomycin (10,000U/mL)</td>
<td>50 mL</td>
</tr>
<tr>
<td>Fungizone (250µg/mL)</td>
<td>20 mL</td>
</tr>
<tr>
<td>Non-Ess AA (100X)</td>
<td>100 mL</td>
</tr>
<tr>
<td>ESS AA (50X)</td>
<td>100 mL</td>
</tr>
<tr>
<td>Heps (1M)</td>
<td>100 mL</td>
</tr>
<tr>
<td>Na Pyruvate (100mM)</td>
<td>100 mL</td>
</tr>
<tr>
<td>7.5% NaHOC₃</td>
<td>260 mL</td>
</tr>
<tr>
<td>Gentamicin (50mg/mL)</td>
<td>30 mL</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>35 µL</td>
</tr>
<tr>
<td>Tissue culture grade H₂O</td>
<td>70 mL</td>
</tr>
</tbody>
</table>

On day three, non-adherent cells were discarded and 5mL of medium with 30ng/mL SCF and 10ng/mL IL-3 was added and placed back into the incubator (37°C, 5% CO₂). At day 6, 5mL of fresh medium was added with 30ng/mL SCF and 10ng/mL IL-3 preparations to flasks. Cell cultures were assessed for mast cell purity by flow cytometry and stained with FcεRIα and CD117 cell markers. At day 9 or 10, approximately 300,000 non-adherent cells were assessed for purity by staining with hamster anti-mouse FcεRI (BioLegend, Catalog #134314) in 100µL with 1µL of a 1mg/mL, rat anti-mouse CD117 (BioLegend, Catalog #105807), and rat ant-mouse FcBlock (CD16/32) (BioLegend Catalog #101319). Samples were analyzed by flow
cytometry using the BD LSRFortessa or BD FACSCanto II machines; data were analyzed by the Flow Jo flow cytometry program.

To assess the morphology of BMDMC and PDMC, cells were washed in FACS buffer and spun at 300 x g for 5 min. To visualize purified BMDMC or PDMCs, approximately 200,000 purified cells were washed and suspended in 100μL of 1X Tyrode’s buffer. Cells were spun on a glass slide with a cytospin centrifuge and fixed, stained with Diff-Quick and morphology visualized on bright field microscopy.

<table>
<thead>
<tr>
<th>1X Tyrode’s Buffer – per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepes</td>
</tr>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td>CaCl₂</td>
</tr>
<tr>
<td>MgCl₂</td>
</tr>
<tr>
<td>Glucose</td>
</tr>
<tr>
<td>BSA</td>
</tr>
</tbody>
</table>

**Mast Cell Degranulation Assay**

To assess mast cell degranulation 5mL of approximately 200,000 cells per mL of BMDMC or PDMC in RPMI were treated with 5μL of 10mg/mL EPS. Cells were primed with 2μg/mL of 1mg/mL IgE anti-DNP at 37°C O/N. BMDMC or PDMC were derived as described previously. Cells were washed in 1X Tyrode’s buffer and spun at 1000 RPM at (RT) for 5 min. Pellets were re-suspended in 100μL of 1X Tyrode’s buffer and 10μL added in duplicate to a 96-well V-bottom plate. Serially diluted (20,000ng/mL, 2,000ng/mL, 200ng/mL, 20ng/mL and 2ng/mL) 2,4-dinitrophenol (DNP) were added to each sample (10μL) and incubated at 37°C for 60 min. Plates were centrifuged at
1000rpm for 5 min and 10µL of supernatant from V-bottom plate was transferred to a flat bottom plate and the remaining supernatant aspirated. Pellets from V-bottom plates were treated with 20µL of 0.5% Triton-X in 1X Tyrode’s buffer and transferred to a 96-well flat bottom plates. A solution of p-Nitrophenyl-N-acetyl-beta-D-glucosaminidine (p-NAG) substrate (50µL of 4mM) was added to each well in a 96-well plate containing supernatant and cell lysate and incubated for 60 min at 37°C. Glycine (0.2M), pH 10.7 (150µL) was added to each well and the absorbance measured at 405 nm. To calculate the percent of mast cell degranulation, the optical densities (O.D.) of samples were multiplied twice and average cell supernatant was divided by average cell supernatant and average cell lysate and multiplied by 100.

\[
= 100 \times \left( \frac{2 \times \text{Average cell supernatant O.D.}}{(2 \times \text{Avg. cell supernatant O.D.}) + (2 \times \text{Avg. cell lysate O.D.})} \right)
\]

Figure 5. Equation for the calculation of percent of mast cell degranulation.

Figure 6. Illustration of mast cell degranulation assay; treatment, priming and challenge.
Statistical Analysis

The number of times experiments were repeated are as indicated in results. Experiments were analyzed using Student’s $t$-test and error bars denote standard errors of the mean (SEM). Differences were considered statistically significant if $P$ was $< 0.05$. (* $= <0.05$; ** $= 0.01$; *** $= 0.001$; **** $= 0.0001$). NS = not significant.
CHAPTER THREE

RESULTS

Characterization of Biofilm Formation by Bacillus subtilis Mutants

B. subtilis from a WT undomesticated strain forms a robust biofilm and pellicle while an ΔepsH mutant does not. The epsA-O operon encodes for enzymes that aid in the formation of biofilms and pellicles (Branda et al. 2006). To assess if deletions of individual genes in the epsA-O operon affected the ability of B. subtilis to form biofilms or pellicles, pellicle and biofilm formation assays for each of the 15 gene strains were performed. Deletions of 11 of these genes resulted in the loss of both pellicle and biofilm formation. Four strains, ΔepsA, ΔepsB, ΔepsJ, and ΔepsO were still capable of forming a full biofilm or pellicle in the absence of the strains’ corresponding deleted gene (Figures 7A and 8A). The biofilms produced by strains of ΔepsC, ΔepsI, ΔepsK, and ΔepsN B. subtilis were capable of forming what look like weak biofilms but unable to form pellicles. A complete lack of pellicle and biofilm formation was exhibited in ΔepsD, ΔepsE, ΔepsF, ΔepsG, ΔepsH, ΔepsL, and ΔepsM strains (Figures 7A, 8A, and 9). Of the strains quantitated for the strength of biofilm produced, only ΔepsJ formed a statistically significant biofilm that was comparable to WT B. subtilis (Figure 9).

Deletion of sinR, a global repressor gene of the epsA-O operon, and tasA, an extracellular protein, results in increased EPS production as well as increased release of EPS into culture medium (Branda et al. 2006; Chu et al. 2006). Deletion of genes in the epsA-O, in addition to sinR and tasA deletions, provides a means to study EPS produced
by these mutant strains. These triple mutants, ΔepsA-O, ΔsinR, and ΔtasA, are derivatives of *B. subtilis* DS991 (ΔtasA and ΔsinR). EPS from *B. subtilis* DS991 was previously demonstrated to protect mice from *C. rodentium*-associated disease. Strains, with three deletions to *sinR, tasA* and *epsA-O* gene demonstrated a reduced capacity to form a biofilm (Figures 7B and 8B).
Figure 7. Effect of deletion to genes in the *epsA-O* operon on biofilm and pellicle formation in *B. subtilis*. Photos of biofilm columns represent phase contrast images of colonies grown overnight on LB plates with 1.5% agar, showing effects of mutations in the indicated genes on the formation of a biofilm. Photos from pellicle columns depict top-down images of microtiter wells (24-well plate) in which cells were grown in LB media for 24 hr at 37°C. All strains used are as indicated in Table 4. A. Biofilm and pellicle formation of strains of *B. subtilis* with a single deletion to a gene of the *epsA-O* operon. B. Biofilm and pellicle formation of strains of *B. subtilis* with a deletion to a gene of the *epsA-O* operon, *sinR*, and *tasA*. 
Figure 8. Effect of deletion in genes of the epsA-O operon on biofilm and pellicle formation in *B. subtilis*. A. Biofilm and pellicle formation of strains of *B. subtilis* with a single deletion to a gene of the epsA-O operon. B. Biofilm and pellicle formation of strains of *B. subtilis* with a deletion to a gene of the epsA-O operon, sinR, and tasA. All strains are as indicated in Table 4. Data provided by our collaborator, Dr. Daniel Kearns, Indiana University, Bloomington, IN.
Figure 9. Effect of deletions of genes of the epsA-O operon on biofilm formation in *B. subtilis*. Bars represent the average absorbance (550 nm) of crystal violet as a measurement of biofilm in wells in a 96-well microtiter dish following aspiration, crystal violet staining, and addition of ETOH. Bacterial cultures were grown in LB media and incubated at 37°C for 24 hr. Strains used are as indicated in Table 4. Biofilms of *B. subtilis* mutants were significant compared to ΔepsH when P<0.05(*) (Student’s t-test). Error bars represent the SEM.

DNA Sequence Analysis of Genes of the epsA-O Operon and Comparison to Homologous Genes

EPS produced by *B. subtilis* is thought to be synthesized by the enzymes encoded in the epsA-O operon. To identify and compare genes of the epsA-O operon to homologous genes, sequences of the epsA-O operon in *B. subtilis* strain NCBI 3610 were obtained from the NCBI website. Amino acid sequences from all epsA-O operon genes (epsA, epsB, epsC, epsD, epsE, epsF, epsG, epsH, epsI, epsJ, epsK, epsL, epsM, epsN, epsO) were aligned with *Escherichia coli* and *Staphylococcus aureus* sequences using the
basic local alignment search tool (BLAST) from the NCBI website. Of the 15 genes of the \textit{epsA-O} operon, 6 genes, \textit{epsD}, \textit{epsE}, \textit{epsF}, \textit{epsH}, \textit{epsJ}, and \textit{epsL}, have sequences that suggest they code for a glycosyltransferase. Two genes, \textit{epsE} and \textit{epsL} seem to have bi-functional activity. Additionally, the remaining genes of the operon may code for modulator of a tyrosine kinase, a tyrosine kinase, a UDP-sugar epimerase, a membrane protein, 2 pyruvyl transferases, flippase, an acetyltransferase, and an aminotransferase (\textbf{Table 4}) (Data provided by our collaborator, Dr. Daniel Kearns, Indiana University, Bloomington, IN). The large number of glycosyltransferases suggest that carbohydrate modification may play an important role in EPS production.

\textbf{Characterization of EPS Produced by \textit{Bacillus subtilis} Mutants}

To assess the size of EPS synthesized by \textit{B. subtilis} strains, samples of EPS were run on a fast protein liquid chromatography apparatus with standards of multiple sizes to provide points of reference. Nine different samples of over-producing and over-secreting strains of \textit{B. subtilis} with single deletions of genes of the \textit{epsA-O} operon were used including all strains with deletions of genes that encode for a putative glycosyltransferase; \textit{ΔepsD}, \textit{ΔepsE}, \textit{ΔepsF}, \textit{ΔepsH}, \textit{ΔepsJ}, and \textit{ΔepsL}. Samples were run on a Superose 6 matrix, a composite of cross-linked agarose gel (GE Lifesciences), and fractions were assayed for the presence of carbohydrates by the phenol sulfuric acid assay. All samples eluted from the column at similar volumes, suggesting the size/shape of these polysaccharides are generally similar to each other. The samples eluted between the IgG (150kDa) and fructose standards, suggesting that EPS in all of the mutants is less than 150 kDa (\textbf{Figure 10}).
Differences in the absorbance were found among the different forms of EPS, suggesting that some forms of EPS, such as ΔepsM and ΔepsN, secrete more EPS than other strains, such as ΔepsG or ΔepsH (Figure 10). We conclude that all B. subtilis mutant strains tested make EPS that is similar in size to WT EPS.

Figure 10. Assessment of the size of EPS isolated from different B. subtilis strains. Graph depicts the absorbance of fractions eluted from a size exclusion column with a Superose 6 matrix. Samples of EPS from strains of B. subtilis are as indicated in Table 4. Blue arrows represent standards of Blue Dextran (>2000kD), apoferritin (480kD), IgG (150kD), and fructose which were used as reference points.

EPS from WT B. subtilis is composed of different monosaccharide constituents, mannose (88%) and glucose (12%) (Jones et al. 2014). To assess if there were differences in the carbohydrate composition of EPS isolated from different B. subtilis strains WT EPS, and EPS from ΔepsH and ΔepsF strains were analyzed using an enzymatic test. WT
EPS protects from disease caused by *C. rodentium* and EPS from ΔepsH does not. If there are differences in the composition of EPS from protective and non-protective *B. subtilis* strains, then it might help identify the type of monosaccharide important for protection.

The enzymatic test measures the change in absorbance that is a result of NADPH production from enzyme and monosaccharide interactions. Monosaccharides were hydrolyzed and phosphorylated by Hexokinase (HK). Glucose-6-phosphate dehydrogenase (G6PDH), which identifies glucose-6-phosphate as its substrate, and phosphomannose isomerase (PMI), which identifies mannose-6-phosphate as its substrate, were used to measure the levels of glucose and mannose in EPS from WT *B. subtilis* as well as EPS from ΔepsH and ΔepsF. There were no discernable differences in glucose or mannose content between the three EPS samples used (Table 1, A-C). These data confirm our current knowledge of WT EPS and suggest that the composition of EPS from two *B. subtilis* mutants is similar; however, this assay may not have a sufficiently high level of sensitivity to identify differences.
EPS from WT *B. subtilis* characterized by gas chromatography and mass spectroscopy suggests that WT EPS has multiple glycosyl linkage residues. Major glycosyl linkage residues include a 2,6 linked Mannopyranosyl residue (2,6-Man) (31.8% of total EPS), terminal mannopyranosyl residue (t-Man) (29.9% of total EPS), and a 3 linked mannopyranosyl residue (2-Man) (15% of total EPS) (Jones et al. 2014). To assess the composition of polysaccharide samples, with a more sensitive assay, and to identify

### Table 1. Determination of Monosaccharide Composition of EPS from *B. subtilis* Strains

Table values represent the absorbance of samples of EPS from strains of *B. subtilis*. Strains of *B. subtilis* are as indicated in Table 4. Columns indicate the enzyme used in reaction as well as time of reaction prior to measurement. Sucrose and Mannose were used as positive and negative controls respectively. **A.** Absorbance of WT EPS **B.** Absorbance of WT EPS and EPS from ΔepsH **C.** Absorbance of WT EPS and EPS from ΔepsF. Samples were diluted as indicated (1:2, 1:4, and 1:8).
linkages in EPS from *B. subtilis* strains, gas chromatography, mass spectrometry, and NMR spectroscopy were performed on samples of EPS by our collaborator, Dr. Neil Price, USDA. EPS from WT *B. subtilis*, as well as EPS from ΔepsE, ΔepsJ, and ΔepsM strains were used. If there are large differences in the composition and linkages of EPS from non-protective mutant strains of *B. subtilis*, such as ΔepsM, when compared to WT *B. subtilis* or protective strains, such as ΔepsJ and ΔepsE, then the differences in monosaccharide composition and linkages may identify important residues for protection.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mannose (%)</th>
<th>Glucose (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT <em>B. subtilis</em></td>
<td>73.87</td>
<td>26.13</td>
</tr>
<tr>
<td>ΔepsE</td>
<td>76.27</td>
<td>23.73</td>
</tr>
<tr>
<td>ΔepsJ</td>
<td>88.31</td>
<td>11.69</td>
</tr>
<tr>
<td>ΔepsM</td>
<td>82.14</td>
<td>17.86</td>
</tr>
</tbody>
</table>

**Table 2. Percent composition of glucose and mannose from *B. subtilis* strains.** Compositional analysis by gas chromatography and mass chromatography. EPS from strains are as indicated in Table 4. Data provided in collaboration with Dr. Neil P.J. Price, NCAUR-ARS-USDA, Peoria, IL.

![Primary repeating structure and linkage of EPS-J](image)

**Figure 11. Primary repeating structure and linkage of EPS-J.** Structure and linkage of EPS from *B. subtilis* ΔepsJ. Structure was determined from data gathered by permethylation linkage and NMR spectroscopy. Data provided in collaboration with Dr. Neil P.J. Price, NCAUR-ARS-USDA, Peoria, IL

Samples of WT EPS had mannose and glucose levels consistent with previous studies (Jones et al. 2014). Samples of EPS from *B. subtilis* mutants had levels of mannose and glucose that were similar to WT EPS (Table 2). Additionally, preliminary
NMR experiments and analysis on EPS from ΔepsJ suggests similar composition and linkage that has been reported from WT EPS (Data not shown). These data confirm our current knowledge of the composition of WT EPS and suggest that the composition of EPS from B. subtilis mutants may be similar in monosaccharide composition, and in the case of EPS from ΔepsJ, similar linkage (Figure 11).

**Recognition of EPS Isolated from Bacillus subtilis Mutants by Anti-EPS Serum**

WT EPS is recognized by polyclonal rabbit anti-EPS serum. To assess if EPS from B. subtilis mutants produce EPS similar to WT EPS, a modified western blot was performed. If EPS from B. subtilis strains is similar to WT EPS, then polyclonal rabbit anti-EPS serum should also recognize EPS from other B. subtilis strains. All mutant forms of EPS were recognized by polyclonal rabbit anti-EPS serum. This suggests that EPS from B. subtilis mutants is relatively similar to WT EPS. Additionally, EPS samples did not migrate into the stacking gel or resolving gel and are primarily at the interface between buffer and stacking gel (5%) or interface between stacking gel and resolving gel (8%). This suggests that EPS samples were too large or did not have a sufficient negative charge to migrate into the gel (Figure 12).
Recognition of EPS by Peritoneal Cells

WT EPS binds to peritoneal cells and exerts an immunomodulatory effect (Jones et al. 2014). EPS from a ΔepsH mutant binds to peritoneal cells less well than WT EPS. Results from the characterization of EPS from B. subtilis strains suggest that EPS is made by these strains and that it is similar in composition to WT EPS. To determine if EPS from other B. subtilis strains binds to peritoneal cells, we performed flow cytometry and assessed the binding of EPS to cells that had been incubated with EPS and rabbit anti-EPS serum. Data suggest that EPS from B. subtilis strains binds the surface of peritoneal cells at varying levels. EPS from ΔepsD, ΔepsE, ΔepsF, ΔepsG, ΔepsH, and ΔepsL bind peritoneal cells similar to WT EPS (Figure 13). More EPS binding from ΔepsJ, ΔepsM, and ΔepsN is found that with WT EPS. These data suggest that EPS binding to peritoneal
cells is not dependent on the ability of the \textit{B. subtilis} strain to form a biofilm as EPS from \textit{ΔepsJ}, a strain that does form a biofilm, and EPS from \textit{ΔepsM} and \textit{ΔepsN}, strains that do not form biofilms, bind peritoneal cells \textbf{(Figure 7A, Figure 8A, Figure 13)}.

EPS from \textit{ΔepsH} was not previously believed to bind to peritoneal cells. Additionally, EPS from \textit{B. subtilis} strains used in this experiment was not column purified. Taken together, these data suggest that pre-column purified EPS from a number of \textit{B. subtilis} mutants can bind to peritoneal cells. Alternatively, there may be something in the pre-column sample that also binds peritoneal cells and is recognized by the rabbit anti-EPS serum.
Figure 13. Binding of EPS from *B. subtilis* strains to peritoneal cells. Flow cytometric analysis of EPS binding to the surface of peritoneal cells from naïve C57Bl/6 mice. Granulocytes were selected on forward and side scatter. The x-axis represents fluorescent intensity of rabbit anti-EPS serum. The y-axis represents normalized cell count. EPS from strains are as indicated in Table 4. FITC-conjugated goat anti-rabbit Ig was used to detect rabbit anti-EPS Ab.

**Genes in the epsA-O Operon are Required for Protection from the Enteric Pathogen, *Citrobacter rodentium***

Mice treated with WT *B. subtilis* spores by oral gavage or WT EPS by intraperitoneal injection and then infected with *C. rodentium* do not display characteristic
symptoms of disease. Mice treated with spores or EPS from a ΔepsH mutant and then infected with *C. rodentium* are not protected from *C. rodentium*-associated disease (Jones and Knight 2012). To determine which genes encoded in the epsA-O operon are needed to confer protection against *C. rodentium*, *B. subtilis* spores with a single gene from the epsA-O operon deleted were given to mice by oral gavage and mice were infected with *C. rodentium*. If certain genes in the epsA-O operon are required to produce a protective form of EPS, then mice treated with *B. subtilis* with deletions of the genes of the epsA-O operon will not be protected from *C. rodentium*-associated disease.

All mice treated with *B. subtilis* had detectable levels of *B. subtilis* in their stool five days post treatment. Additionally, all mice treated with *C. rodentium* had detectable levels of the pathogen on day 10 of the experiment (Figure 15, A-B). These data suggest that any symptoms of disease exhibited by mice were not due to lack of *B. subtilis* persistence nor to a lack of colonization by the infectious pathogen, *C. rodentium*. Furthermore, colonization with *B. subtilis* mutants did not seem to correlate with protection, as spores of ΔepsJ and ΔepsK mutants were found at relatively high spore concentrations in mice; however, disease markers suggest that ΔepsJ is protective while ΔepsK is not (Figure 15A and Figures 16-17, A-D). Conversely, colonization with *C. rodentium* did not correlate with disease severity, as mice treated with ΔepsA and ΔepsB spores were colonized with a two fold difference of *C. rodentium* (Figure 15B), but showed disease markers that were relatively the same (Figures 16-17, A-D, Figure 18).

Mice treated with WT *B. subtilis* spores do not develop loose and unformed stools, while mice treated with ΔepsH spores do. Of the 15 mutant strains of *B. subtilis* tested, 13 did not protect mice from developing diarrhea. Mice treated with *B. subtilis*
spores of ΔepsE and ΔepsJ did not develop diarrhea (Figure 17C). In addition to diarrhea, mice with C. rodentium-associated disease have enlarged spleens relative to body weight. To assess if B. subtilis strains could protect from splenic inflammation, the spleens of mice were harvested at day 10 or 11 post infection with C. rodentium. Of the mice treated with B. subtilis mutants, 6 of 15 treatment groups did not have enlarged spleens. Mice treated with B. subtilis mutants ΔepsE and ΔepsJ had spleen to body weight ratios similar to PBS control (Figure 17D).

The large intestines from mice infected with C. rodentium exhibit changes to the colonic architecture. These changes include elongation of crypts of the large intestines, crypt hyperplasia, (Mundy et al. 2005) and loss of goblet cells (Jones and Knight 2012). Mice treated with WT B. subtilis spores do not have elongated crypts or lower levels of goblet cells. Uninfected mice have a crypt height of approximately 200µm. Of the 15 B. subtilis mutant strains tested, two, ΔepsE and ΔepsJ did not have signs of elongated crypts or loss of goblet cells. Mice treated with ΔepsL spores had the largest crypt height; and therefore, the most inflamed colons of all groups. Mice treated with spores of ΔepsE and ΔepsJ also had no change to goblet cells over untreated mice (Figure 16, A-B).

From these data, we conclude that only two B. subtilis mutants, ΔepsE and ΔepsJ, conferred complete protection, suggesting that most genes in the epsA-O operon are necessary to produce a protective form of EPS.

Interestingly, mice treated with EPS from WT B. subtilis and infected with C. rodentium have lower levels of the inflammatory chemokine KC. To assess for protection against C. rodentium by B. subtilis spores, an ELISA to assay the level of KC in sera was
performed. Data suggest that there is no correlation between protection and KC cytokine in mice treated with *B. subtilis* spores (Figure 14 and Figures 16-17, A-D).

**Figure 14.** Levels of serum KC, an inflammatory chemokine, in mice treated with *B. subtilis* spores and infected with *C. rodentium*. Assessment of *B. subtilis* spores on *C. rodentium*-associated disease 10 days post-infection (dpi) of WT mice. *B. subtilis* strains are as indicated in Table 4. Results are averages from at least 3 mice. Error bars represent the SEM.
Figure 15. Colonization of *B. subtilis* mutants and *C. rodentium*. Results are averages from two independent experiments; a total of 6 mice were assessed. Error bars represent the SEM. **A.** Persistence of spores of *B. subtilis epsA-O* mutants in the colon of mice for a period of five days post oral gavage. **B.** Colonization of *C. rodentium* in feces of mice at day 10 or 11.
Figure 16. Effect of *B. subtilis* mutants on *C. rodentium*-associated disease 10 days post-infection. Infection-induced alterations in colon which were quantified by measuring crypt heights and goblet cells per crypt height. Results are averages from two independent experiments; a total of 6 mice were assessed for each group. Error bars represent the SEM. A. Distal sections of fixed colons were measured for crypt height. Columns at or below red bar suggest no *C. rodentium*-associated disease. B. Number of goblet cells per crypt height. Columns at or above red bar suggest no *C. rodentium*-associated disease. Treatments with *B. subtilis* spores and infection with *C. rodentium* were compared to naïve mice. Treatments were significant compared to naïve untreated mice when *P* < 0.05. Data were analyzed by Student’s *t*-test, * = <0.05; ** = 0.01; *** = 0.001; **** = 0.0001; NS = not significant.
Figure 17. Effect of *B. subtilis* mutants on *C. rodentium*-associated disease 10 days post-infection. Infection-induced symptoms of *C. rodentium*-associated disease. C. Diarrhea score D. Spleen to weight ratio. Results are averages from two independent experiments; a total of 6 mice were assessed for each group. Error bars represent SEM. Treatments with *B. subtilis* spores and infection with *C. rodentium* were compared to naïve mice. Columns at or below red bar suggest no *C. rodentium*-associated disease. Treatments were significant compared to naïve untreated mice when P< 0.05. Data were analyzed by Student’s t-test, * = <0.05; ** = 0.01; *** = 0.001; **** = 0.0001; NS = not significant.
Figure 18. Representative photos of H&E stained longitudinal colonic sections from mice treated with *B. subtilis* and infected with *C. rodentium*. Samples include untreated mice (Naïve-PBS), *B. subtilis* and infected with *C. rodentium* (3610 + Cr), *B. subtilis* mutants ΔepsE and ΔepsJ and infected with *C. rodentium*, and mice infected only with *C. rodentium*. Black bars on the lower right hand corner indicate a measurement of 200µm. Data shown are representative of results from three independent experiments.
Protection from C. rodentium-Associated Disease by Bacillus subtilis var. natto

Natto is a traditional fermented soybean dish eaten in a number of Asian countries. Traditional Natto is fermented in the presence of B. subtilis var. natto and these strains may confer protection against C. rodentium-associated disease.

Bacterial isolates from all three brands of Natto had similar colony morphology with some being hazier than others and did not have the characteristic colony wrinkling present in B. subtilis NCBI 3610 (Data not shown). By MALDI-TOF MS analysis, all bacterial isolates were identified as B. subtilis (Table 2).

All mice treated with B. subtilis var. natto spores had detectable levels of B. subtilis following treatment that were comparable to WT B. subtilis spores. Additionally, all mice infected with C. rodentium were colonized with the pathogen at day 10 or 11. These data suggest that mice were colonized by B. subtilis and C. rodentium (Figure 19B).

To assess protection from C. rodentium-associated disease, we measured the crypt heights of mice treated with Natto spores and infected with C. rodentium. Mice receiving spores from any of the 3 Natto isolates had on average, shorter crypt heights, more goblet cells per crypt, and significantly better stool scores than mice infected with C. rodentium alone. These data suggest that mice treated with B. subtilis from Natto were protected from diarrhea and infection-induced alterations to the colon (Figure 20-21, A-C). Additionally, the spleen to weight ratios of mice treated with Natto 1, Natto 2, and Natto 3 were similar to PBS controls and WT B. subtilis suggesting that mice treated with
spores from Natto had significantly less systemic inflammation than mice treated with *C. rodentium* alone (Figure 21D).

Although mice treated with Natto 3 spores had significantly reduced crypt heights, their stool scores, reduced spleen to body weight ratio, and goblet cells per crypt (Figure 19:1-2, A-D, Figure 21) suggest that mice treated with spores from Natto 3 were protected. Collectively, these data suggest that a commercially available product can protect against enteropathogenic disease; and more importantly, that some brands confer more protection than others.

<table>
<thead>
<tr>
<th>Analyte ID</th>
<th>Organism Best Match</th>
<th>Score Value</th>
<th>Organism Second Best match</th>
<th>Score Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natto 1: Two colonies selected</td>
<td><em>Bacillus subtilis</em></td>
<td>2.155; 2.035</td>
<td><em>Bacillus subtilis</em></td>
<td>2.155, 1.974</td>
</tr>
<tr>
<td>Natto 2: Two colonies selected</td>
<td><em>Bacillus subtilis</em></td>
<td>2.138, 2.118</td>
<td><em>Bacillus subtilis</em></td>
<td>2.078, 2.102</td>
</tr>
<tr>
<td>Natto 3</td>
<td><em>Bacillus subtilis</em></td>
<td>2.117</td>
<td><em>Bacillus subtilis</em></td>
<td>2.09</td>
</tr>
</tbody>
</table>

Table 3. MALDI-TOF MS analysis of bacteria isolated from Natto. All isolates with different colony morphologies were selected for analysis. Analyte ID indicates product, best match and second best match indicate the primary and secondary species. A Score value >2 is significant to the species level (Bruker MALDI-TOF MS Research Database).
Figure 19. Colonization of *B. subtilis var. natto* and *C. rodentium*. Results are averages from two independent experiments; a total of 6 mice were assessed. Error bars represent the SEM. **A.** Persistence of *B. subtilis var. natto* spores in the colon of mice for a period of five days post oral gavage. **B.** Colonization of *C. rodentium* in feces of mice 10 or 11 dpi.
Figure 20. Effect of *B. subtilis* var. *natto* on *C. rodentium*-associated disease 10 days post-infection. Infection-induced alterations in colon were assessed by measuring crypt heights and goblet cells per crypt height. Results are averages from two independent experiments; a total of 6 mice were assessed for each group. Error bars represent the SEM. **A.** Distal sections of fixed colons were measured for crypt height. Bars at or below red bar suggest no *C. rodentium*-associated disease. **B.** Number of goblet cells per crypt height. Columns at or above red bar suggest no *C. rodentium*-associated disease. Treatments with *B. subtilis* spores and infection with *C. rodentium* were compared to naïve mice. Treatments were significant compared to naïve untreated mice when P<0.05. Data were analyzed by Student’s *t*-test, * = <0.05; ** = 0.01; *** = 0.001; **** = 0.0001; NS = not significant.
Figure 21. Effect of *B. subtilis* var. *natto* on *C. rodentium*-associated disease 10 days post-infection. Infection-induced symptoms of *C. rodentium*-associated disease. C. Diarrhea score and D. Spleen to weight ratio. Bars above the red line indicate no protection. Columns at or below red bar suggest no *C. rodentium*-associated disease. Results are averages from two independent experiments; a total of 6 mice were assessed for each group. Error bars represent SEM. Treatments with *B. subtilis* spores and infection with *C. rodentium* were compared to naïve mice. Treatments were significant compared to naïve untreated mice when \( P<0.05 \). Data were analyzed by Student’s *t*-test, * = \(<0.05\); ** = 0.01; *** = 0.001; **** = 0.0001; NS = not significant.
Figure 22. Representative photos of H&E stained longitudinal colonic sections from mice treated with *B. subtilis* and infected with *C. rodentium*. Photos are representative of colon sections from untreated mice (Naïve-PBS), treated with *B. subtilis* spores and infected with *C. rodentium* (3610 + Cr), treated with *B. subtilis var. natto* spores and infected with *C. rodentium* (Natto 1 + Cr, Natto 2 + Cr, or Natto 3 + Cr), and mice infected only with *C. rodentium*. Black bars on the lower right hand corner indicate a measurement of 200µm. Data shown are representative of results from three independent experiments.
Characterization of Immunomodulatory Effects of Protective EPS isolated from 

*B. subtilis* mutants

Mice treated with WT *B. subtilis* spores or WT EPS and sensitized to OVA do not develop OVA specific Abs (Swartzendruber and Knight, unpublished data). If strains of *B. subtilis* are protective in a *C. rodentium* model of disease, then these same strains may also display immunomodulation in other models. We tested if *B. subtilis* mutant strains that are either protective or not protective in the *C. rodentium* disease model behave similarly in the OVA sensitization model. WT and mutant *B. subtilis* spores from strains ΔepsH, ΔepsF, and ΔepsJ were orally administered to mice 3 days prior to intraperitoneal injection of OVA and after 14 days, we used ELISA to determine the level of serum IgG1 anti-OVA. We found that mice treated with spores from ΔepsJ strain had low levels of IgG1 anti-OVA Ab, similar to that from mice treated with WT *B. subtilis* spores. In contrast, mice treated with spores from ΔepsH and ΔepsF, strains that do not confer protection against *C. rodentium*, did not decrease the level of IgG1 anti-OVA, compared to mice that received no *B. subtilis*. Mice treated with ΔepsH had low OVA-specific IgG1 and mice treated with ΔepsF had higher levels of OVA-specific IgG1. These data suggest that a strain of *B. subtilis* protective against *C. rodentium*, ΔepsJ, is immunomodulatory in a sensitization model. Interestingly, a strain of *B. subtilis* that did not confer protection against *C. rodentium*, ΔepsH, seems to have some immunomodulatory effect as there was a reduced level of OVA-specific Abs in serum from these mice (Figure 23). Taken together, this preliminary experiment demonstrates that strains of *B. subtilis* not protective in a *C. rodentium* model may still exert an
immunomodulatory effect on the host, and that $\Delta$epsJ, a strain protective in a *C. rodentium* model, is also immunomodulatory in other models of study.

![Figure 23. Serum levels of allergen specific IgG1 in sensitized mice.](image)

Bacterial polysaccharides can modulate allergic responses (Meier, Bein, Jaques 1957) and mast cell cultures treated with WT EPS do not degranulate in an *in vitro* assay. To investigate if strains of *B. subtilis* with immunomodulatory effects modify degranulation in mast cells, BMDMCs or PDMCs (Figure 24) were treated with purified EPS from $\Delta$epsF and $\Delta$epsJ. Interestingly, compared to naïve BMDMCs, BMDMCs treated with EPS-J have a different morphology and do not degranulate (Figure 25, A-B). The perimeter around EPS-J treated mast cells have what look to be small circular morphologies while naïve mast cells have a characteristic granule appearance. This may
suggest that mast cells treated with immunomodulatory EPS causes gradual movement of mast cell granules to the perimeter of the cell.

Data from degranulation assays also suggest that there is a difference between mast cells cultured from the bone marrow and mast cells cultured from the peritoneal cavity. These preliminary experiments suggest that PDMCs seem to degranulate while BMDMCs do not seem to degranulate. Additionally, both PDMCs and BMDMCs have high absorbance following challenge and degranulation over naïve mast cells. This may suggest that the granules themselves are different in composition as there is less absorbance regardless if mast cells degranulated or not (Figures 26-27). Collectively, these data suggest that EPS-J has immunomodulatory activity and that it somehow modulates the morphology of mast cells and potentially their contents.

**Figure 24. Purity of cultured PDMC and BMDMC isolated from the peritoneum.** Cells were stained with mast cell markers (CD117 and FcεRI). FACS plots represent mast cell purity from peritoneal (PDMC) and bone marrow (BMDMC) isolated from the peritoneal cavity of naïve C57Bl/6 mice.
Figure 25. Representative photos of mast cells before and after treatment with EPS-J. Photos represent difference in morphology of naïve BMDMC (A.) and BMDMC treated with EPS from ΔepsJ. BMDMCs in culture were cytospun on glass slides and stained using Diff-Quick reagents. Images represent magnification at 100x. Red arrows indicate circular objects found along the perimeter of mast cells.

Figure 26. Percent degranulation in a mast cell degranulation assay. Percent degranulation (A.) and absorbance of lysate from degranulated PDMCs (B.). PDMCs were cultured for 10 days, half were treated with purified EPS from B. subtilis ΔepsJ. Cells were then treated with IgE anti-DNP and challenged with DNP.
Figure 27. Percent degranulation in a mast cell degranulation assay. Percent degranulation (C.) and absorbance of lysate from degranulated BMDMCs (D.). BMDMCs were cultured for 10 days, half were treated with purified EPS from *B. subtilis ΔepsJ*. Cells were then treated with IgE anti-DNP and challenged with DNP.
Table 4. Characterization of *B. subtilis* mutants. Strain column indicates strain of *B. subtilis* used. Mutation column indicates which genes are deleted in the strain. Proposed function indicates the function of the deleted gene determined through BLAST. Phenotype is determined by the morphology of *B. subtilis*. Biofilm is indicated by the growth of bacteria and degree of opacity; ++ (strong biofilm), + (weak biofilm), and ---- (no biofilm). Pellicle formation is indicated by formation of a biofilm at the air liquid interface; ++ (strong pellicle), + (weak pellicle), and ---- (no pellicle). ETOH: Sup precipitate are indicated as + (precipitate) or ---- (no precipitate) when a visible precipitate formed after addition of EtOH (50% final) to supernatant from an overnight culture of *B. subtilis*. Protection from disease is indicated as + (protection) or ---- (no protection) when mice are protected by three of four symptoms of disease (diarrhea, goblet cell loss, crypt hyperplasia, and spleen to weight ratio). WT = Wild Type; ND = Not Determined; WM = Wrinkled Morphology; WFM = Wrinkled and Flat Morphology.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation</th>
<th>Proposed Function</th>
<th>Phenotype</th>
<th>Biofilm</th>
<th>Pellicle</th>
<th>Precipitate</th>
<th>Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCBI 3610</td>
<td>WT</td>
<td>Full operon present</td>
<td>WM</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DS991</td>
<td>Δ asrR Δ atsA</td>
<td></td>
<td>WM</td>
<td>++</td>
<td>++</td>
<td>---</td>
<td>+</td>
</tr>
<tr>
<td>DK1943</td>
<td>Δ epsA</td>
<td>Modulator of EpsB Tyrosine Kinase</td>
<td>WM</td>
<td>++</td>
<td>++</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>DK1806</td>
<td>Δ epsB</td>
<td>Tyrosine Kinase</td>
<td>WM</td>
<td>++</td>
<td>++</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>DK1807</td>
<td>Δ epsC</td>
<td>UDP-Sugar Epimerase</td>
<td>WM</td>
<td>+</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>DS4248</td>
<td>Δ epsD</td>
<td>Glycosyltransferase</td>
<td>---</td>
<td>-</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>DS2152</td>
<td>Δ epsE</td>
<td>Glycosyltransferase, Flagellar clutch</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>++</td>
<td>---</td>
</tr>
<tr>
<td>DS4164</td>
<td>Δ epsF</td>
<td>Glycosyltransferase</td>
<td>---</td>
<td>-</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>DS7499</td>
<td>Δ epsG</td>
<td>Membrane Protein of Unknown Function</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>DS6776</td>
<td>Δ epsH</td>
<td>Glycosyltransferase</td>
<td>---</td>
<td>-</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>DK1758</td>
<td>Δ epsI</td>
<td>Pyruvyl Transferase</td>
<td>WM</td>
<td>+</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>DS4166</td>
<td>Δ epsJ</td>
<td>Glycosyltransferase</td>
<td>WM</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>---</td>
</tr>
<tr>
<td>DK2055</td>
<td>Δ epsK</td>
<td>Floppase</td>
<td>WM</td>
<td>+</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>DS7432</td>
<td>Δ epsL</td>
<td>Glycosyltransferase, Phosphotransferase</td>
<td>---</td>
<td>-</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>DS4901</td>
<td>Δ epsM</td>
<td>Acetyltransferase</td>
<td>WM</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>DS4900</td>
<td>Δ epsN</td>
<td>Aminotransferase</td>
<td>WM</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>DK1759</td>
<td>Δ epsO</td>
<td>Pyruvyl Transferase</td>
<td>WM</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>---</td>
</tr>
<tr>
<td>Natto 1</td>
<td>ND</td>
<td></td>
<td>WM</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Natto 2</td>
<td>ND</td>
<td></td>
<td>WM</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Natto 3</td>
<td>ND</td>
<td></td>
<td>WM</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>DK1760</td>
<td>Δ epsA Δ asrR Δ atsA</td>
<td>Modulator of EpsB Tyrosine Kinase</td>
<td>FM</td>
<td>-</td>
<td>++</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DK1822</td>
<td>Δ epsB Δ asrR Δ atsA</td>
<td>Tyrosine Kinase</td>
<td>FM</td>
<td>-</td>
<td>---</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DK1732</td>
<td>Δ epsC Δ asrR Δ atsA</td>
<td>UDP-Sugar Epimerase</td>
<td>FM</td>
<td>-</td>
<td>---</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DK7724</td>
<td>Δ epsD Δ asrR Δ atsA</td>
<td>Glycosyltransferase</td>
<td>FM</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DS2169</td>
<td>Δ epsE Δ asrR Δ atsA</td>
<td>Glycosyltransferase, Flagellar clutch</td>
<td>FM</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DS7722</td>
<td>Δ epsF Δ asrR Δ atsA</td>
<td>Glycosyltransferase</td>
<td>FM</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DS7760</td>
<td>Δ epsG Δ asrR Δ atsA</td>
<td>Membrane Protein of Unknown Function</td>
<td>FM</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DS7148</td>
<td>Δ epsH Δ asrR Δ atsA</td>
<td>Glycosyltransferase</td>
<td>FM</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DK1781</td>
<td>Δ epsI Δ asrR Δ atsA</td>
<td>Pyruvyl Transferase</td>
<td>FM</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DS7725</td>
<td>Δ epsJ Δ asrR Δ atsA</td>
<td>Glycosyltransferase</td>
<td>FM</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DS2057</td>
<td>Δ epsK Δ asrR Δ atsA</td>
<td>Floppase?</td>
<td>FM</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DS7761</td>
<td>Δ epsL Δ asrR Δ atsA</td>
<td>Glycosyltransferase / Phosphotransferase</td>
<td>FM</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DS7726</td>
<td>Δ epsM Δ asrR Δ atsA</td>
<td>Acetyltransferase</td>
<td>FM</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DS7725</td>
<td>Δ epsN Δ asrR Δ atsA</td>
<td>Aminotransferase</td>
<td>FM</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DK1782</td>
<td>Δ epsO Δ asrR Δ atsA</td>
<td>Pyruvyl Transferase</td>
<td>FM</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
A Unique and Novel Approach to Studying Polysaccharide Biosynthesis

Carbohydrate polymers, specifically bacterial polysaccharides, have a wide variety of uses in public, private, and academic settings. Presently, their compositions, biosynthesis, and mechanisms of immunomodulation are poorly understood. This study used a unique and novel approach to better understand the synthesis of a complex polysaccharide and its immunomodulatory activity. Using numerous approaches, investigators have tried to characterize the enzymes that are involved in the biosynthesis of bacterial polysaccharides (Freitas, Alves, Reis 2011; Öner 2013; Rehm 2010; Roux et al. 2015; Schmid, Sieber, Rehm 2015; Wang et al. 2000). To our knowledge, our study, which focuses on changes to a complex polysaccharide and not the enzymes or genes involved in its biosynthesis, is one of the first to investigate what changes occur to a polysaccharide during its biosynthesis.

Summary of Findings

EPS from WT *B. subtilis* contributes to the formation of a robust biofilm and pellicle, and protects against all disease markers of the murine enteropathogen *C. rodentium*. A single deletion of the *epsH* gene of the *epsA-O* operon inhibits the ability of *B. subtilis* to form a strong biofilm and protect mice from *C. rodentium*-associated disease. Mutants of *B. subtilis*
with deletions of the genes encoded in the \textit{epsA-O} operon were assessed for their ability to form a biofilm or pellicle, and their ability to protect against \textit{C. rodentium}-associated disease. EPS purified from \textit{B. subtilis} strains were characterized for their physical and chemical properties.

The results from the biofilm and pellicle formation assays confirm that EPS is a key component that contributes to the formation of a biofilm. Experiments that assess the biofilm and pellicle formation of \textit{B. subtilis} strains provide limited information on the characteristics of EPS produced by these mutants. There is no correlation with the formation of a biofilm following deletion of the genes in the \textit{epsA-O} operon and protection from \textit{C. rodentium}. Spores of \textit{ΔepsE}, a non-biofilm forming strain, or spores of \textit{ΔepsJ}, a biofilm forming strain, were both protective against \textit{C. rodentium}-associated disease. Therefore, the ability to form a biofilm is independent of protection.

Our results indicate that there is no single gene in the \textit{epsA-O} operon responsible for protection from the enteropathogen \textit{C. rodentium}. Additionally, lack of protection from \textit{C. rodentium} is not due to the lack of a polysaccharide being produced. To our knowledge, all \textit{B. subtilis} mutants produce some form of polysaccharide. From preliminary experiments, it also seems that the monosaccharide composition from \textit{ΔepsE}, \textit{ΔepsF}, \textit{ΔepsH}, \textit{ΔepsJ}, and \textit{ΔepsM} does not correlate with protection. Interestingly, EPS from \textit{B. subtilis} with a deletion to \textit{epsF}, a glycosyltransferase, elutes from a size exclusion column similar to EPS from WT \textit{B. subtilis}. This is contrasted with EPS from a \textit{ΔepsH} mutant that elutes after WT EPS suggesting it is smaller in size. The use of EPS from an \textit{epsF} mutant may serve as a better negative control than EPS from an \textit{epsH} mutant as it elutes out more closely with WT EPS preparations.
A closer analysis of the composition of EPS suggested that there was a high degree of similarity in the mannose and glucose concentrations in mutant forms of EPS. Conformational studies were completed using NMR spectroscopy and suggest that, from the samples tested, (ΔepsE, ΔepsJ, and ΔepsM) the majority of EPS is made of mannose and a minority is made of glucose. There does not seem to be a correlation with mannose or glucose concentration and protection or immunomodulatory activity. Therefore, protection by EPS is not due to the type or amount of monosaccharide found in EPS. The glycosidic bonds present in protective forms of EPS, such as the α1,6-Man or α1,2-Man that are present in EPS from ΔepsJ, may be key linkages for immune modulation. Studies on the bonding profiles in EPS from ΔepsE, which is protective, and ΔepsF, which is not protective, are already underway.

From preliminary NMR analyses of EPS from ΔepsJ, the data suggest that a long polymer is present. Loss of any the enzymes encoded by the epsA-O operon may result in altered EPS structure. These modifications could eliminate or mask residues important for interaction with the immune system. These deletions could also eliminate or drastically reduce EPS production or limit the stability of the polysaccharide such that it degrades faster than normal. Until better understandings of either enzyme activity, or differences between individual forms of EPS are elucidated, the protective residues will remain a mystery.

Peritoneal cells collected from B57Bl/6 mice intraperitoneally injected with WT EPS are believed to become anti-inflammatory alternatively-activated M2-Mϕ. Mice treated with WT EPS developed higher levels of M2-Mϕ than mice treated with EPS from ΔepsH. When peritoneal macrophages from mice treated with WT EPS are co-
cultured with naive CD4+ T cells, \textit{in vitro} T-cell proliferation is suppressed (Paynich and Knight, unpublished data). Preliminary experiments using EPS from \(\Delta\text{eps}J\), which is protective against \textit{C. rodentium}, and \(\Delta\text{eps}F\), which is not protective against \textit{C. rodentium}, suggest that \(\Delta\text{eps}J\) does skew cells of the peritoneal cavity to become M2-Mϕ and these cells can suppress T cell proliferation while cells treated with \(\Delta\text{eps}F\) cannot (Data not shown). This experiment should also be repeated with EPS from \(\Delta\text{eps}E\). If EPS from \(\Delta\text{eps}E\) is protective against \textit{C. rodentium}-associated disease, then it should also exert immunomodulatory activities similar to WT EPS and EPS from \(\Delta\text{eps}J\).

It is believed that glycosyltransferases, of which the \(\text{eps}A-O\) operon has six putative genes (\(\Delta\text{eps}D, \Delta\text{eps}E, \Delta\text{eps}F, \Delta\text{eps}H, \Delta\text{eps}J, \Delta\text{eps}L\)), are involved in the synthesis of EPS. If glycosyltransferases are involved in the synthesis of EPS, then deletion of these genes should result in loss of immunomodulatory activity. Surprisingly, deletions of two putative glycosyltransferases, \(\text{eps}E\) and \(\text{eps}J\), did not affect protection. Future studies may benefit from investigating the remaining 4 glycosyltransferases to identify their activities and substrates. In terms of protection against \textit{C. rodentium}-associated disease, it does seem that the remaining 13 genes of the \(\text{eps}A-O\) operon are required for protection. Of these 13 remaining gene products, 4 encode for glycosyltransferases.

The lack of protection conferred by 13 of 15 \textit{B. subtilis} mutants suggests that multiple genes of the \(\text{eps}A-O\) operon are necessary to produce a protective form of EPS. Enzymes produced by genes encoded in the \(\text{eps}A-O\) operon most likely act sequentially to generate functional EPS. It is known that some enzymes, such as EpsB are dependent on the activity of EpsA. Additionally, there may be additional bi-functional enzymes,
such as EpsE, and the loss of multiple functions contributes to changes of EPS produced (Guttenplan, Blair, Kearns 2010). Furthermore, gene products may directly interact with each other, may produce necessary substrates for downstream enzymes, or modify EPS in such a way that it is not recognized by other enzymes responsible for the next stage of EPS synthesis. Elucidation of the order in which proteins act on the formation of EPS would clarify the biosynthetic pathway of EPS, identify functionally redundant enzymes, and contribute to our understanding of the biofilm matrix of B. subtilis.

While some mutants do not confer protection in a C. rodentium-disease model, such as ΔepsH spores, they may have immunomodulatory activity. In a model assessing sensitization and development of IgG1, WT EPS or B. subtilis spores inhibited the development of IgG1. As a negative control, mice were treated with spores from two mutant groups that do not protect against C. rodentium-associated disease, ΔepsH and ΔepsF. Treatment of mice with B. subtilis ΔepsH spores prevents these mice from developing IgG1 antibodies and is similar to the suppression of IgG1 antibodies observed with treatment of WT B. subtilis and ΔepsJ. Conversely, mice treated with ΔepsF, which is not protective against C. rodentium, is also unable to suppress IgG1. Collectively, these data suggest that there is a very specific component or structure of EPS that is interacting with the host and perhaps, the component or structure of EPS needed for immunomodulatory activity changes with the models and approaches employed to study EPS. There may be multiple residues with immunomodulatory activity. Unique structures of EPS that can suppress C. rodentium-associated disease may be different than those that suppress the development of IgG1 antibodies.
When EPS from strains of *B. subtilis* were tested for reactivity against rabbit anti-EPS antibody, which was generated by immunization with WT EPS, all mutant forms of EPS bound this Ab. This finding suggests that the polyclonal antibodies present in rabbit anti-EPS serum recognize a similar form of residue on the multiple EPSs from *B. subtilis* strains. This idea is corroborated with the NMR analysis of purified EPS from ΔepsE, ΔepsJ, and ΔepsM mutants showing that the structures are very similar to each other and to WT EPS. Additionally, there is a high degree of similarity in the sugar linkages of at least one strain, ΔepsJ to WT EPS. Further analysis should focus on the structural differences of protective forms of EPS compared to non-protective forms of EPS.

**Confirmation of Protective Strains and Future Models**

The initial studies of protection mediated by EPS were in a *C. rodentium* model of disease. Since these initial experiments, EPS has been shown to confer protection in an anaphylaxis model of disease (Swartzendruber and Knight, unpublished data) and a model of experimental autoimmune encephalitis (Osborne and Knight, unpublished data). Other models of disease may prove useful in exploring the potential uses of EPS as a therapeutic. Spores of *B. subtilis* from ΔepsE, ΔepsH, and ΔepsJ have some form of immunomodulatory activity, and they may be beneficial for future studies. Additionally, as previously mentioned, spores or purified EPS from ΔepsF may serve as a better negative control than ΔepsH, which has demonstrated immunomodulatory activity in a model of antigen sensitization.

In our model of infection with *C. rodentium*, treatment of mice with *B. subtilis*
spores from WT, ΔepsE, and ΔepsJ mutants protected against disease. This model mimics EPEC in humans and would be easy to modify in order to test an EHEC model of disease using *C. rodentium* that expresses shiga toxin. Treatment of mice with these spores and then infection with *C. rodentium* that expresses the stx phage, which carries the genes encoding Shiga toxin, would provide further evidence for the protective ability of EPS. Additionally, protection via EPS in this model would be of particular interest because it would demonstrate that immunomodulation mitigates the damaging and debilitating effects of Shiga toxin associated with EHEC. EHEC is a prevalent disease in the United States (Crim et al. 2014) and identification of a new therapeutic would be of value.

Finally, experiments have demonstrated that treatment of BMDMCs with WT EPS prevents mast cells from degranulating when primed with IgE and challenged with corresponding antigen (Swartzendruber and Knight, unpublished data). Preliminary experiments suggest that mast cells treated with EPS from ΔepsJ also do not degranulate mast cells. Additionally, mast cells treated with EPS from ΔepsJ look characteristically different than untreated mast cells. BMDMCs treated with EPS from ΔepsJ appear “glassy” with what appears to be granules outside the cell on/near the periphery. It is possible that EPS causes slow release of granules, which would explain why no degranulation occurs when sensitized cells are challenged with allergen at a later time point. Future experiments that follow the morphological changes of mast cells over time may provide support for this hypothesis and provide a timeline for EPS action.
Potential Probiotics and Caution of Their Use

A number of probiotic strains of bacteria are either from heritage collections isolated from the environment or mass-produced by industry. Depending on the handling of these strains, there is the possibility of some level of variation if they are handled in a way that favors certain characteristics/traits. All three strains of *B. subtilis* var. *natto* were protective against *C. rodentium*-associated disease; however, there was variation in the degree of protection as well as the phenotypic characteristics of these strains when grown on petri dishes. Polysaccharides can vary greatly depending on the strain and substrates used (Öner 2013). EPS contributes to the taste and texture of Natto and most likely, *B. subtilis* strains are different between the different brands of Natto.

Selection of desired characteristics of bacteria that affect taste, texture, and smell of fermented foods may be beneficial for product development; however, they may irreparably harm the immunomodulatory effects of *B. subtilis*. Furthermore, studies using bacteria from industry are hampered by the secrecy these companies maintain over production of these ‘probiotic’ strains.

In previous work, mice that were treated with purified EPS and infected with *C. rodentium* did not have an increase in serum KC inflammatory chemokine. In contrast, mice treated with *B. subtilis* spores and then infected with *C. rodentium* had elevated levels of KC. It was expected that treatment with spores would also inhibit serum KC inflammatory chemokine but this was not the case. In fact, mice treated with WT *B. subtilis* had levels of KC inflammatory chemokine similar to infection with *C. rodentium* alone. These data highlight the complicated nature of using a live organism and highlight the importance of delivering purified EPS. While probiotics, such as *B. subtilis*, may
protect from pathogens, treatment with a live organism may have unintended consequences to the host.

Probiotics are a benefit to society, however, the lack of understanding regarding the mechanisms involved in protection is a problem associated with their use. We live in an age where the dissemination of information is quick and easy, regardless of the accuracy, and as probiotics are relatively un-regulated, they may harm those seeking health and wellness.

**Further Study of the Enzymes Encoded in the epsA-O Operon**

This study was not focused on the enzymatic activities of the proteins encoded by the epsA-O operon; however, a better understanding of the enzymes encoded by the epsA-O operon will enhance our understanding of bacterial polysaccharide biosynthesis. Alternatively, now that it is known that 13 of 15 genes of the epsA-O operon are necessary for protection, future studies may benefit from strains of *B. subtilis* with multiple gene deletions to better pinpoint and characterize EPS biosynthesis.

Genes of the epsA-O operon may be coupled in their production for EPS as is the case in other polysaccharides such as Xanthan. Additionally, other studies have demonstrated improved yield by overexpressing genes involved in bacterial polysaccharide production (Galván et al. 2013). Application of these methods to EPS synthesis may prove useful in the production of EPS.

Furthermore, GTs are generally promiscuous in the substrates they utilize. If GTs in the epsA-O operon are promiscuous in substrate usage, this would also modify the quality of EPS produced. Alternatively, alterations to the growth medium or
substrates available to *B. subtilis* may produce EPS that is characteristically different from *B. subtilis* grown in LB.

**Optimization of Purified EPS and Polysaccharide Biosynthesis**

Throughout this study, spores were used in place of purified EPS. Spores germinate into vegetative cells and treatment with live organisms increases the number of potential interactions occurring in the host as demonstrated in experiments that assessed the level of serum KC inflammatory chemokine. Synthesis of polysaccharides *in vitro* are described in the literature and provide a way to optimize and study the activities of single enzymes involved in polysaccharide biosynthesis (Braat et al. 2006; Carignatto et al. 2011; Faijes and Planas 2007; Kobayashi 2007; Steidler et al. 2000; Woodward et al. 2010; Zhang et al. 2003). The additional products that *B. subtilis* produces may hamper or counteract the protective effects exerted by EPS in such a way that EPS’s efficacy is not as high as when it is delivered as a purified substance. Delivery of purified EPS would therefore be an ideal approach for future studies. It would also be advantageous to encapsulate EPS so it can bypass the gastric acid, and serve as an alternative therapy for immunocompromised patients when administration of live organisms is contraindicated.

Currently, production of EPS yields 10-15mg/L. Concentrations using genetically modified bacterial polysaccharide producing strains can be as high as 188g/L (Ruffing and Chen 2006). The are numerous reports on the optimization and increased yield of bacterial polysaccharides through various methods. Methods to increase EPS yield include: increasing the pool of polysaccharide precursors (mannose and glucose for EPS), over-expression of gene(s) involved in synthesis, insertion of multiple plasmids encoding
enzymes needed for polysaccharide production, higher ethanol (v/v) during precipitation, precipitation with the low molarity salts, and use of a better carbon source to list a few (Schmid, Sieber, Rehm 2015; Thorne et al. 2000; Zhang et al. 2003). A potential caveat to these optimization techniques is that EPS may lose its immunomodulatory activity and thus any product should be tested for protective effects.

**Analysis of the Properties of EPS Produced by *Bacillus subtilis* Mutants**

The lack of simple methods for the analysis of polysaccharides has limited research. Of the methods currently available for the analysis of polysaccharides, combined use of various NMR techniques and mass spectrometry offer the best way to study a number of properties including polysaccharide structure and composition (Bubb 2003; Roux et al. 2015).

NMR approaches used in this thesis have already proven useful for understanding the structure of mutant forms of EPS. The forms of EPS identified enzymatically, and by NMR, suggest there are relatively low and varying levels of glucose and mannose in the samples of EPS tested. NMR analysis has also proven useful in demonstrating that a single long polysaccharide may be present in samples tested. The structure of the immunomodulatory carbohydrate, PSA from *B. fragilis*, was resolved through NMR spectroscopy and provided information to show how surface-exposed charges could interact with MHCII molecules (Baumann et al. 1992; Cobb et al. 2004; Kalka-Moll et al. 2002). Although EPS is not currently known to have a charge, elucidation of the structure of EPS from various *B. subtilis* mutants will provide a better understanding of residues
present and may help elucidate the roles that enzymes of the *epsA-O* operon play in EPS biosynthesis.

Analyses by NMR and mass spectrometry will enable closer inspection of the composition and types of residues found in samples of EPS and allow for comparison between protective and non-protective forms of EPS. Elucidation of the components of EPS produced by Δ*epsJ*, which behaves in a similar manner to WT EPS, shows a high degree of similarity to WT EPS. EPS from Δ*epsE*, which is believed to be protective, and Δ*epsF*, which is not believed to be protective, are ideal for further study.

While not typically used, pulse chase experiments, using radiolabeled carbon, have been used successfully in the past to study bacterial polysaccharide synthesis (Robyt, Yoon, Mukerjea 2008). Employing pulse chase methods, the first experiments used radiolabeled $^{14}$C-sucrose successfully followed the transfer of sucrose molecules to the reducing end of a growing dextran polymer.

**Concluding Remarks**

In summary, this work has sought to characterize a complex polysaccharide, EPS, using strains of *B. subtilis* with deletions of the genes encoded in the *epsA-O* operon. Through the use of a *C. rodentium* disease model, 13 genes of the *epsA-O* operon of *B. subtilis* were identified as being necessary for prevention of inflammatory disease induced by the enteric pathogen. Consistent with the idea that *B. subtilis* can provide protection from disease, we isolated and identified *B. subtilis* in Natto products that also protected mice from disease, thus providing a clinical approach towards treatment.
Critically, Natto is a product that has been available for decades and is safe for human consumption.

Collectively, these data highlight the complicated nature of bacterial polysaccharide biosynthesis, demonstrate that protection is available from commercially available sources, and provide a new avenue to study the enzymes needed for protection. We can begin compositional studies to examine the similarities and differences of protective EPS. Further study should help elucidate and advance the field of carbohydrate polymer research, provide a rationale for further study of EPS from epsA-O operon mutants, and may aid in identification of other bacterial EPSs with immunomodulatory activity.

While this work has provided a better phenotypic understanding of EPS, the immunomodulatory activity, and structural analysis of EPS are needed for a true understanding of how these polysaccharides interact with the host.
REFERENCES


VITA

The author, Alex Argianas, was born in Elmhurst, IL to Charles and Connie. He received his Bachelor of Science in Biology and Spanish and a Certificate in Theology from Benedictine University (Lisle, IL) in August of 2012. Alex was introduced to research at Benedictine by studying the impact of nitrogen and phosphorus concentrations on plant species and subsequent female butterfly oviposition preference with Dr. Cheryl Heinz. He also studied the prevalence of Sudden Cardiac Death in University Athletes in collaboration with Young Hearts 4 Life.

Following graduation, Alex worked in the clinical infectious disease laboratory at Lurie Children’s Hospital until matriculating at Loyola University in 2013. Shortly after starting his graduate studies, he joined the lab of Dr. Katherine L. Knight where he studied the production of a complex polysaccharide produced from the commensal bacteria \( B. \ subtilis \). His focus was on the identification of genes of the EPS operon necessary to confer immunological protection from enteropathogenic disease.

While at Loyola, Alex served as treasurer of the graduate student council, volunteered weekly at the employment center of DuPage Pads tutoring the homeless and completed the requirements for the Leadership in Science honors program. Alex was also nominated and awarded the Spirit of Martin Luther King, Jr. award. After completion of his MS degree, Alex will be working in the lab of Dr. Francis Alonzo and teaching at Benedictine University. Alex plans to continue his education in the biomedical sciences
and aspires to be a clinical scientist that studies chronic diseases and provides medical care to underserved and marginalized populations.