

**PRODUCTION AND SECRETION OF HETEROLOGOUS
IMMUNOMODULATORS IN *BACTEROIDES THETA* IOTAOMICRON**

JEFFREY TINGLEY
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Department of Biological Sciences
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PRODUCTION AND SECRETION OF HETEROLOGOUS IMMUNOMODULATORS
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JEFFREY TINGLEY

Date of Defence: August 28th 2019

Dr. D. Wade Abbott Thesis Co-Supervisor Agriculture and Agri-Food Canada Lethbridge, Alberta	Research Scientist	Ph.D.
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Dr. L. Brent Selinger Thesis Co-Supervisor	Professor	Ph.D.
---	-----------	-------

Dr. G. Douglas Inglis Thesis Examination Committee Member Agriculture and Agri-Food Canada Lethbridge, Alberta	Research Scientist	Ph.D.
---	--------------------	-------

Dr. Dmytro Yevtushenko Thesis Examination Committee Member	Associate Professor	Ph.D.
---	---------------------	-------

Dr. Tony Russell Chair, Thesis Examination Committee	Associate Professor	Ph.D.
---	---------------------	-------

Abstract

Chronic and acute inflammation in the colon significantly impact human and animal health, and productivity in agriculture. Unfortunately, current treatments are not very effective for long-term management and treatment is costly. This research project has helped address these limitations by engineering a colonic bacterium, *Bacteroides thetaiotaomicron*, to produce tumor necrosis factor receptor 2 (TNFR2), α -1-antitrypsin (A1AT) and elafin. These anti-inflammatory proteins were stitched to *BtAnchor* and *BvSecrete* signal peptides that facilitate their trafficking to the outer surface and external environment of the cell, respectively. Protein targets were initially produced and localized within the Gram-negative, model organism, *Escherichia coli*, and elafin demonstrated statistically significant inhibition of elastase. Protein targets: A1AT, TNFR2, and elafin were successfully produced in a dual-glycan responsive *B. theta* strain previously developed in the Abbott lab. The strains developed as part of this project represent novel tools for reducing of inflammation in agricultural livestock and human medicine.

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List of Abbreviations

A1AT	α -1-antitrypsin
AGP	antimicrobial growth promoter
APC	antigen presenting cell
BHI	brain heart infusion
BT_#	<i>B. theta</i> VPI 5482 gene #
BtAnchor	Bt_3698 signal peptide
BvMPK_#	<i>Bacteroides vulgatus</i> mpk gene #
BvSecrete	BvMPK_0228 signal peptide
CT	cytotoxic T-cell
DAMP	damage-associated molecular pattern
DC	dendritic cell
DGM	distal gut microbiota
FUdR	5-Fluoro-2'-deoxyuridine
GFP	green fluorescence protein
GIT	gastrointestinal tract
IBD	inflammatory bowel disease
IC ₅₀	half maximal Inhibitor concentration
Ig	immunoglobulin
IL-#	interleukin
ILF	isolated lymphoid follicle
IPTG	thiogalactopyranoside
LAB	lactic acid bacteria
LB	lysogeny broth
Lol	localization of lipoproteins pathway
LTi	lymphoid tissue inducer cell
MeOH	ethanol
MHC	major histocompatibility complex
MM	<i>Bacteroides</i> minimal media
mmHg	millimeter of mercury
NET	neutrophil elastase trap
NETosis	neutrophil extracellular trap cell death
Ni-NTA	Ni ²⁺ -nitrilotriacetic acid
NK	natural killer
NOD	nucleotide-binding domain
N-SP	N-terminal signal peptide
PAMP	pathogen-associated molecular pattern
pBT_#	<i>B. theta</i> VPI 5482 gene # promoter
PSA	<i>B. fragilis</i> Polysaccharide A

PUL75	<i>B. theta</i> polysaccharide utilization locus 75
PVDF	polyvinylidene fluoride
SCFA	short-chain fatty acid
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide
SEC	general secretion pathway
SP#	signal peptidase #
T#SS	type # secretion system
TBS	tris-buffered saline
TBS-T	tris-buffered saline + Tween-20
TCR	t-cell receptor
TDK	thymidine kinase
TGFβ-1	transforming growth factor-β1
T _H	helper T-cell
TLR	toll-like receptors
TNFR	tumor necrosis factor receptor
TNF-α	tumor necrosis factor α
T _{Reg}	regulatory T-cell
TYG	tryptone yeast extract glucose

Chapter 1 Literature Review and Introduction

1.1 The gastrointestinal tract (GIT)

The GIT is a complex organ system that extends from the mouth to the anus composed different digestive compartments, including the stomach, small intestines, and the colon; and is composed of tissues layers from the mucosa to the serosa [1]. Each of these compartments a specific function. Together this organ system is responsible for the ingestion of nutrients, mechanical and limited chemical breakdown of food, absorption of nutrients and water, excretion of waste, and provides optimal conditions for symbiotic microbial growth.

1.1.1 The colonic mucosa

The colon's mucosa is structured around a long tube (lumen) and has crypts that can extend down into the submucosa. The mucosa contains a variety of epithelial cell types, including enterocytes, endocrine cells, and goblets cells which act as a barriers, participate directly in immune functions, and secrete peptides, hormones and mucins into lumen [2-4]. Goblet cells are primarily involved with the production of mucus in the colon[5]. The major component of colonic mucus in the colon is the heavily glycosylated protein Muc2. Mucins are largely composed of *O*-linked, and in smaller quantities *N*-linked, oligosaccharide side chains. *O*-linked glycans are attached to mucins through free hydroxyl groups of serine and threonine and are composed of N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, L-fucose, and negatively charged components, such as sialic acid and terminal sulfate groups [6]. The interaction of mucins with water and metal ions gives rise to the high viscosity of mucus. Multiple factors can affect mucus viscosity including: mucin production and

concentration, mucin–mucin di-sulfide interactions, pH, bacterial mucin foraging, metal ion concentrations, and sulfation and negative charged monosaccharides [7]. Host and bacterial enzymes can proteolytically cleave mucins, expanding the mucus volume up to four times which gives rise to the appearance of a mucus bi-layer [7, 8].

The colon is home to the densest population of microorganisms known to interact with human tissues ($\sim 10^{11}$ microbial cells/mL) [9]. This community is referred to as the distal gut microbiota (DGM). Bacteria within the DGM are found in much higher concentrations in the lumen, which contains the less viscous “outer mucus layer” in comparison to colonization levels of the dense “inner mucus layer” [10]. Horizontal (proximal to distal) and vertical (epithelial crypts to lumen - radial) gradients exist within the colon, which create unique environments for DGM microorganisms. From the proximal colon to the distal colon, mucus viscosity increases [11], while concentrations of antimicrobial peptides [12] and oxygen [13] decrease. Oxygen concentrations can drastically differ in the vertical gradient as well, as concentrations within the crypts and intestinal tissue can reach 40 mmHg [10] while luminal concentrations are as low as 1 mmHg [10]. This oxygen gradient is well-documented and has been viewed using green fluorescence protein (GFP), as binding of GFP with oxygen is necessary for proper folding [14]. Facultative anaerobes can use oxygen in the mucus layers for aerobic respiration; however, in low oxygen concentrations, fermentation or anaerobic respiration with alternative electron acceptors are used for ATP synthesis [15]. As such, oxygen that diffuses from epithelial cells is consumed by facultative anaerobes, causing lower oxygen concentrations in the lumen [16]. This results in diverse populations of bacteria, partitioned on the basis of their oxygen requirements, which include: facultative anaerobes, aero-

tolerant anaerobes, and obligate anaerobes [17]. Other metabolic phenotypes exist. For example: *Bacteroides fragilis*, a colonizer of colonic crypts [18], is considered a “nanaerobe” as it has been shown to consume small amounts of oxygen through the use of cytochrome bd complex [17, 19].

Mucus viscosity between the inner and outer layer drastically affects microbial colonization. The inner mucus layer, once thought to be sterile, is now postulated to be a reservoir for diverse microbial species for repopulation of the colon following dysbiosis [18, 20]. Microheterogeneity in mucus viscosity creates differential chemical environments for selection of bacterial morphologies [11], mucus foraging [21] and mucus-binding capabilities [22]. Mucin *O*-glycans are a prominent nutrient source for members of the microbiota [22]. For example, *Bacteroides thetaiotaomicron* (*B. theta*), a prominent saccharolytic commensal bacterium in the colon, is well-studied in its ability to utilize *O*-linked oligosaccharides [23] and can produce a variety of mucolytic carbohydrate-active enzymes and metallopeptidases for the deconstruction of mucin [23, 24].

1.2 Mucosal immunology

Due to its exposure to and constant interaction with the external environment, the colon has developed a vast repertoire of defense mechanisms. These cell lineages are designed to take up, recognize and respond to antigens, and provide a physico-chemical barrier against the external environment. These include cells of the leukocyte lineage: dendritic cells, neutrophils, macrophages, T- and B-cells; and colonic epithelial cells (colonocytes) [2]. These cells appear in two locations: inductive sites and effector sites. Inductive sites compose the gut associated lymphoid tissue, and are sites of antigen sampling and corresponding T- and B- cell proliferation [25], and include colonic patches

and isolated lymphoid follicles (ILF) [26]. In contrast, effector sites are defined as areas where mature cells perform their actions in the lamina propria. All of these cells are generally classified as cells of the innate and adaptive immune systems.

1.2.1 Innate immunity

The innate immune system provides protection through chemical and physical barriers, incites stimulation/participation of adaptive immune cells, and induces recognition and removal of invading organisms/antigens [27]. Cells of the innate immune system delineate from common myeloid progenitors, leading to macrophages, dendritic cells (DCs), granulocytes, mast cells; and lymphoid: which include natural killer cells (NK) and innate T-cells [28, 29]. DCs and macrophages phagocytize bacteria and antigens and are the primary antigen-presenting cells (APC) in the immune system. DCs are primarily involved in antigen sampling from the lumen and delivering antigens to inductive sites, while macrophages generally do not travel to inductive sites [30]. Antigens are then presented to lymphocytes within inductive sites or surrounding immune cells on major histocompatibility complexes (MHC). The granulocytes include eosinophils, basophils, and neutrophils [31]. Eosinophils, basophils, and mast cells are well studied for their role in allergic reactions; however, they are suspected to have a larger role in immune function including releasing pro-inflammatory cytokines and histamine [1, 32]. Eosinophils have been found to undergo eosinophil extracellular trap cell death, a non-apoptotic cell death pathway with the release of DNA and proteases in a process similar to that of neutrophils [33]. Neutrophils are highly destructive cells with the ability to release a variety of antimicrobial peptides, phagocytize bacteria, and create neutrophil elastase traps (NETs) through controlled cell death (NETosis). NETs involve a

massive release of chromatin, DNA and proteases from neutrophils. This can result in cell death, and this process seems to be a last resort as proteases cause damage to host tissue [34]. NETs can be directly induced by activation of Toll-like-receptors (TLRs) and the inflammasome (Figure 1.1) [34]. TLRs and nucleotide-binding oligomerization (NOD)-like receptors allow traditional APCs and a variety of cell lineages to respond to ‘danger’ signals including: pathogen-associated molecular patterns (PAMPs) and damage-associated molecular pattern (DAMPs), as microorganisms and stress/danger antigens, respectively [35]. PAMPs and DAMPs activate an innate immune system signaling complex, the inflammasome, which activates caspase. Caspase-1 activates pro-inflammatory cytokines such as tumor necrosis factor α (TNF- α), and can induce cell death [36]; these cytokines have intimate relationships with the adaptive immune system. NK cells are cytotoxic and recognize non-self-signals; however, they do not have the ability to adapt to new antigens. NK immunoglobulin-like receptors recognize infected cells that have downregulated self-antigens which targets cells for death using proteases and perforin to puncture cell membranes [37].

Innate T-cells are similar to T-cells of the adaptive immune system in that they have T-cell receptors (TCRs), however, they are less specific and are generally considered to straddle the line between the innate and adaptive immunity. Examples of innate T-cells include NK T-cells, intraepithelial T-cells, and lymphoid tissue inducer cells (LTi). NK T-cells have invariant TCRs which target the MHC type protein CD1 and recognize lipid antigens [38]. IETs are long-living T-cells which have both pro- and anti-inflammatory properties including targeting infected epithelial cells for cell death, and promoting tolerance to food

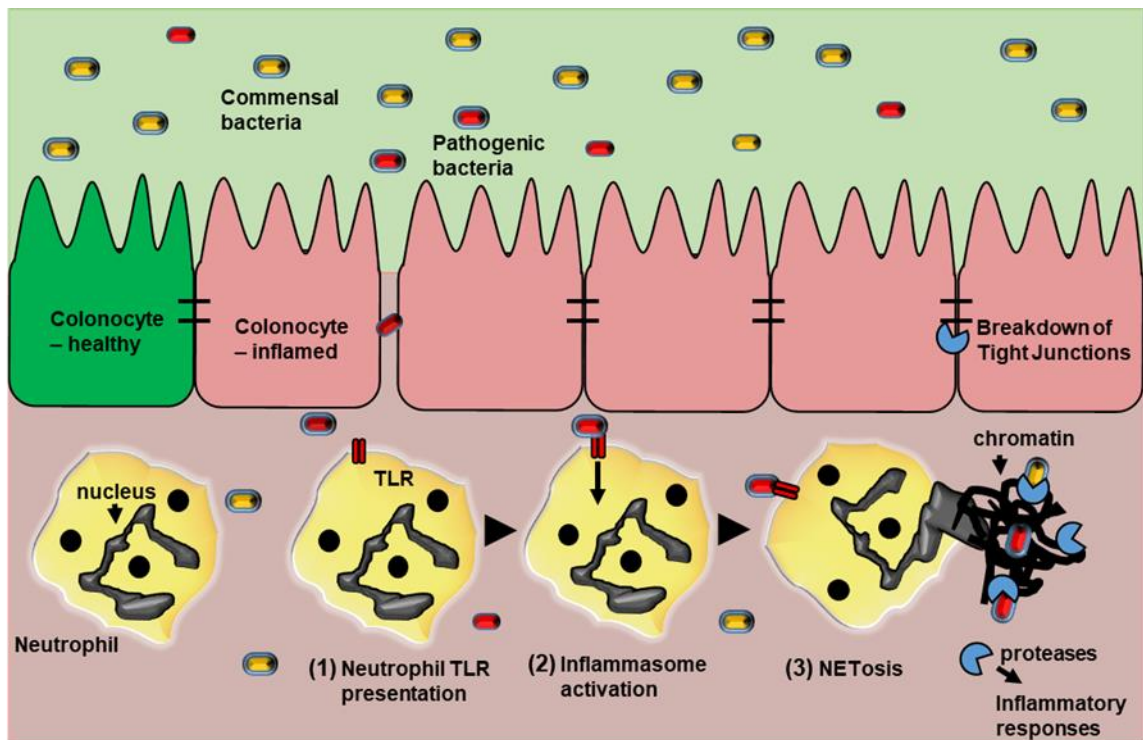


Figure 1.1: Induction of NETosis in neutrophils through TLR activation. (1) Neutrophils contain surface receptors called TLRs which interact with antigens, such as bacterial LPS. (2) Interaction of TLRs with the appropriate antigen stimulate the production of the inflammasome and NETosis. (3) NETosis involves the formation of NETs composed of DNA, chromatin, and proteases, including serine proteases, such as neutrophil elastase. These proteases induce pro-inflammatory pathways, breakdown epithelial tight junctions, and breakdown of bacterial outer membrane proteins.

antigens by the release interleukin-10 (IL-10) and transforming growth factor β -1 (TGF β -1) [3]. LTi cells are important in the development of lymphoid tissue including ILF and cryptopatches in other organisms [39]. The complement system is a major contributor to promoting inflammation and pathogen clearance and targeting pathogens for removal [40]. Complement proteins are produced by hepatocytes in the liver; however, they are also produced by various innate immune cells [40].

Although not generally considered immune cells, colonocytes provide a barrier between the external environment and the underlying tissue and are the first line of defense between foreign antigens and bacteria. Colonocytes can recognize PAMPs and DAMPs and participate in the construction of inflammasomes. As well, antimicrobial peptides β -defensins are produced in small amounts from colonocytes [41]. Specialized colonocytes throughout the GI tract can provide chemical and molecular defenses including mucus production, secretion of anti-microbial peptides, and cell-signaling molecules [2]. The most prevalent specialized colonocytes are goblet cells, primarily known for producing mucin proteins. In addition, goblet cells also produce trefoil factor 3 which appears to aid in epithelial layer repair and regeneration [42]. Other specialized colonocytes are much fewer in number including enteroendocrine cell types [43], tuft cells [44], cup cells [44], and M-cells [45] present at inductive sites.

1.2.2 Adaptive immunity

The remainder of the lymphocyte progenitor line, including T-cells and B-cells, comprise the adaptive immune system. This system is characterized by having the ability to adapt and respond to new antigens to provide for more efficient protection when a second exposure to a past stimulus occurs, termed immunological memory. T- and B-cells

recognize their antigens through T-cell receptors (TCRs) and B-cell receptors (*i.e.* antibodies) which are highly variable due to genetic recombination [46]. TCRs are made up of variable α and β gene segments or, in smaller amounts, γ and δ gene segments [47]. Naïve T-cells mature into T-cells which have TCRs and other co-receptors, including glycoproteins CD4+ and CD8+, and co-stimulatory ligands. These surface glycoproteins determine which MHC class to interact with, as CD4+ generally interact with MHC class 2 and CD8+ with MHC class 1 [48]. The TCR-MHC interaction is generally accompanied by both co-stimulatory and co-inhibitory factors [49]. These interactions determine whether T-cells mature into either effector cells or anergic, non-responsive cells [49]. Co-inhibitory receptors appear on T-cells after 1-3 days, inducing an exhaustive state, preventing chronic inflammation [50]. There is a variety of CD4+ and CD8+ coated cells including Helper T cells (T_H), cytotoxic T-cells (CTs), and certain regulatory T-cells (T_{regs}). T_H (CD4+CD3+) interact with MHC class 2 complex and generally produce cytokines and chemokines that aid in the proliferation of B-cells, aid the phagocytic capacity of monocytes, and develop T-cell memory [51]. There is a variety of T_H cells including T_{H1} , T_{H2} , T_{H9} , T_{H17} , T_{Reg} , and T_{FH} (follicular helper) each with their own armory of cytokines which can cross-regulate one another, and stimulate pro- and anti-inflammatory responses from immune competent cells [50]. CD8+ CT cells are restricted to binding to MHC class 1 receptors and are destructive cell types. CTs recognize self-antigens on MHC class 1, which are distributed across almost all cells, and stimulate apoptosis of the target cell [51]. T_{Reg} cells express the CD4+ co-receptor and the CD25+ receptor, and develop in the thymus instead of the mucosal tissues. T_{Reg} cells are immunosuppressive cells which can inhibit APC activity and produce inhibitory cytokines, such as TGF β -1 and IL-10 [52, 53].

Memory T-cells (both CD4+ and CD8+) contain surface glycoproteins from a previous immune response. When their antigens reappear they become 're-activated' and migrate to the site of infection [54].

B-cells are primarily known for their role in adaptive immunity, and production of antibodies. antibodies are made up of a heavy chain and a light chain which get their receptor diversity through joining variant gene segments together [55]. The heavy chains also contain a constant (C) region, which determines its immunoglobulin (Ig) subtype: IgA, IgM, IgG, IgE, and IgD. Switching from IgM to IgA subtypes is possible due to recombination, occurring after activation of mature B cells. Activation can occur through T-cell dependent and independent manners; interaction with gram-negative lipopolysaccharides and capsular polysaccharides instigates T-cell independent activation of the mature B cells [56]. Mature B cells express IgM however, IgA is the most predominant antibody in the colon [25], and results from B-cell differentiation and recombination [57]. IgA generally form dimers which can interact with antigens specifically and non-specifically [58].

1.2.3 Signaling molecules

Immune competent cells produce a vast array of signaling molecules to interact with other cells to attenuate cascades, suppress or stimulate inflammation, or signal cellular repair. Cytokines are among the most well-known signaling molecules, and can be secreted by almost every cell in the body [59]. Cytokines can be functionally pleiotropic, they can form synergies and antagonize. Cytokine categories include ILs, interferons (IFNs), TNF ligands, growth factors, and chemokines [60]. IL superfamilies are grouped by structural similarities and the receptors they share, or their roles in immunity [60]. IFN class 1 are

responsible for inhibiting virally infected cells from replication; whereas, class 2 stimulates type 1 immune responses, inducing the secretion of more IFNs and TNF subfamilies [61]. The TNF family is composed of TNF- α , lymphotoxin, and B-cell activating factors. TNF- α is a potent pyrogenic immunomodulator that is secreted by various immune cells including T_H cells, macrophages, DCs, and NK cells, and recognized through tumor necrosis factor receptors. It increases the amount of MHC class 1 and 2 expression, leading to an increase in T- and B-cell activation, and promotes destruction of tissues [62]. Cytokine growth factors include TGF β -1, a highly pleiotropic cytokine that is generally an immunosuppressive cytokine, and is produced by T_{Reg} cells among others [63]. Chemokines, the largest sub-category of cytokines, are a specialized sub-type of cytokines designed to manipulate the localization of cells and regulate immune cell migration [64]. Chemokines are important in general immune function and also the development of secondary lymphoid organs, such as ILFs, which requires LT_i cells to be brought to areas of future development [39].

1.2.4 Microbe-immune system interaction

The immune system has been tailored to respond to and co-exist with the DGM. The DGM contribute largely to a state of colonic immune homeostasis through direct interaction with the host immune system. DCs at effector and inductive sites can sample commensal bacteria, which can lead to the activation of antigen specific B-cells to produce IgA [65]. IgA can then traverse through the mucus layers and regulate the population of commensal through antigen specific and non-specific methods [25]. Commensal bacteria, also reduce the amount of habitable space for pathogens reducing the risk of infection, providing colonization resistance [66]. The disturbance of the normal colon microbiota,

dysbiosis, can lead to infection [66]. Microbe-host interactions commonly occur through carbohydrate interactions (Figure 1.2). Foreign invaders typically present PAMPs on their cell surface that are recognized by various membrane bound TLRs and cytosolic receptors like the NOD-like receptors [67, 68]. Although, this is not always the case as *Escherichia coli* lipopolysaccharide can directly activate caspase through a TLR-independent mechanism [69]. Capsular polysaccharides from *B. fragilis* and other *Bacteroides* spp. directly interact with immune cells through their Zwitterionic charge [70]. *B. fragilis* polysaccharide A (PSA) (Figure 1.2) and B are taken up by APCs and can promote both abscesses and immunosuppression; however, it is not clear how and when these polysaccharides contribute to these outcomes [70]. Short-chain fatty acids (SCFA) including butyrate, propionate, and acetate (Figure 1.2) produced by fermentation of dietary fibre which reaches the colon by the microbiota can lead to the activation of T_{Reg} cells, modulate host immunity, inhibit pathogens, and provide a source of nutrients for colonocytes [71, 72].

1.3 Immune dysfunction

Due to the complexity of the immune system and the interactions with the surrounding environment there is a variety of ways immune dysfunction can occur. Host immune systems must differentiate between inert molecules, such as food and ‘danger’ antigens such as PAMPs/DAMPs. When these responses are deregulated, autoimmune diseases from allergies to chronic intestinal inflammation occur. The exact mechanism of food intolerance is not well understood, but there are some known factors. For instance, the absence of T_{Reg} cells in organisms can lead to allergic reactions towards food [73]. Environmental stressors including temperature and flight/fight responses can alter proper

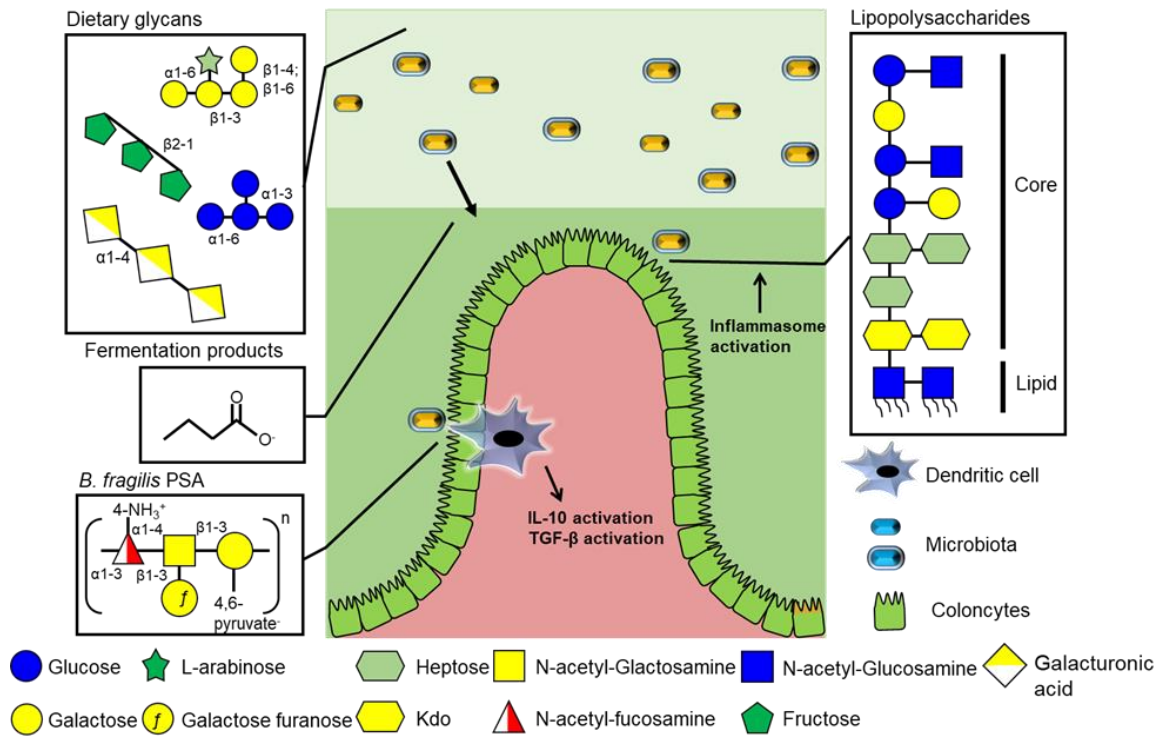


Figure 1.2: Carbohydrates and fermentation products in the colon. Carbohydrates play important roles in bacterial and human metabolism. Dietary polysaccharides such as homogalacturonan, fructooligosaccharides, and arabinogalactan; and exopolysaccharides, such as dextran, are utilized by the distal gut microbiota. Fermentation of the carbohydrates produces short-chain fatty acids including butyrate, which are utilized by the host colonocytes. Lipopolysaccharides and capsular polysaccharides, such as PSA from *B. fragilis*, interact directly with host colonocytes and immune cells to stimulate or suppress the immune system.

immune function by directly interfering with cell signaling or indirectly reducing the amount of energy available for initiating and maintaining immune response [74]. The immune system requires a vast amount of energy [75]; therefore, reducing energy availability will prevent proper functioning. Likewise, immune stimulation will increase energy utilization and reduce the amount of energy readily available for other bodily processes, such as growth [75].

1.3.1 Cost of immune dysfunction

Intestinal immune dysfunction in humans, both acute and chronic, is costly. Chronic enteritis, including inflammatory bowel diseases (IBD), Crohn's disease, and ulcerative colitis, annually cost thousands of dollars per patient, either directly through medical expenses or indirectly through loss of productivity on the economy [76, 77]. Symptoms can range from abdominal pain and diarrhea to colorectal cancer under extreme cases of inflammation. IBD can occur through host-pathogen interactions, genetic predisposition, alteration of the normal microenvironment, and other environmental stressors [78], although the underlying mechanisms are not completely known and may involve a combination of factors [79]. The microbiota may be a key diagnostic for IBD as patients have been observed to have a less diverse and more unstable microbiota [80, 81]. Conversely, acute inflammatory diseases are an interesting paradigm. Acute inflammation is a foundation of the immune system, activating the immune response in response to a negative stimulus to remove the stimuli and repair damage. However, diarrhoeal disease is responsible for roughly 1.3 million deaths annually [82]. Poor standards of living and sanitation in developing countries is correlated with an increase in enterotoxigenic *E. coli*, *Shigella* spp., and *Campylobacter* spp. [83]. In agriculture, the energetic cost of

inflammation, and the subsequent loss of growth, is a significant concern, as a lack of physical growth in livestock directly affects downstream product revenues [84].

1.3.2 Current Solutions

There are a number of promising drugs to treat IBD or IBD symptoms in humans, including chemical therapies, immunotherapies, and corticosteroids. Chemical therapies including mesalamine and azathioprine, have demonstrated toxic side-effects for the host, patients also experience relapse after treatment [85], while immunotherapies such as infliximab can be in the thousands per year per patient. While long term corticosteroid use runs the risk of bone loss [86]. In addition, the delivery of these compounds to inflamed tissues is difficult, which requires higher dosages to be administered. Colon-targeted therapies taken orally have to pass through the upper GIT, including the highly acidic stomach [87]. In acute inflammation, diagnostics of disease is expensive or technically challenging, and although there are promising results using antibiotics, these treatments are saved for severe cases as identification of etiology (bacterial or viral) is difficult in developing countries [83].

Antimicrobial growth promoters (AGPs) used in animal production have been used historically to improve food safety and increase growth rates and animal weights. As of 2018, administration of antibiotics require veterinary prescription and claims of growth promotion caused by medically important antimicrobials have been removed [88]. This is in large part due to public concern and the awareness of antibiotic-resistance genes being transferred from bacteria found in animals to bacteria found in humans [89]. Recently antibiotic resistance to colistin, a “last-resort” antibiotic in human health, has been found in livestock [90]. Complications associated with infections caused by antibiotic-resistant

bacteria is predicted to be a leading cause of death by 2050 [91]. Therefore, there is an urgent need to find alternatives to AGPs. The growth promoter mechanism of AGPs, however, is still unclear as it may be a combination of microbiota modulation and modulating host immune responses [92]. As modulators of the gut microbiota, probiotics and prebiotics represent potential alternatives to AGPs.

1.4 Microbe intervention

1.4.1 Prebiotics

Prebiotics are non-digestible carbohydrates, which promote the proliferation of beneficial microorganisms [93]. Diet has long been known to affect both core community members and transient microbes of the gut [94]. Dietary fibers and resistant starch that pass into the colon is utilized by gut microbes and fermented into alcohols, gases, and organic acids including SCFAs such as butyrate (Figure 1.2) [15]. Butyrate administered at 80 mM to mice suffering from *Citrobacter rodentium*-induced inflammation showed increased weight gain and host cytokines involved in suppressing the immune response [72]. The use of prebiotics to stimulate SCFA production by bacteria, specifically butyrate and lactate, or SCFA-producing community members is an active area of research [95]. Popular prebiotic substrates in this regard include arabinogalactan [96], fructooligosaccharides [95], and lactic acid polysaccharides such as dextran [97] (Figure 1.2). [95]. Recently, the butyrate-producing *Faecalibacterium prausnitzii* was shown to utilize homogalacturonan oligosaccharides (Figure 1.2) as well as fructooligosaccharides, [95, 98]. Human milk oligosaccharide and similarly structured oligosaccharides represent a promising avenue in prebiotics as they promote the growth of certain probiotic *Bifidobacterium* spp. [99].

1.4.2 Probiotics

Probiotics are live microorganisms that provide a benefit when supplied in sufficient quantities [100]. In order to be considered a probiotic, the species must be known; fecal microbiota transplants, although useful in some situations for treatment of infections from *Clostridium difficile* [101], therefore, are not considered a probiotic treatment [100]. This issue was attempted to be resolved using a fecal substitute, RePOOP, which is comprised of a known bacterial composition to prevent unforeseeable infections seen in fecal microbiota transplants [102]. Probiotics have been shown to benefit numerous areas of human health including anxiety and depression [103], obesity [104], and inflammation [105]. Probiotics have also shown to benefit animal health, including chickens [106] and cattle [107]. Probiotic studies are numerous and health benefits surrounding probiotics are emphasized; however, heterogeneity between study design and individual effects between study participants make it difficult to make conclusive statements about the benefit of certain probiotic strains. Host-specific factors, including epithelial barrier integrity may also diminish the benefits of probiotics [108]. With sufficient research, probiotics may be used to effectively aid in human and animal health and production.

1.4.3 Synbiotics

A major issue facing the adoption of probiotics is colonization and engraftment of the bacterium within the colon. In large part, ‘synbiotics’, or the combinatorial use of prebiotics and probiotics [109], attempts to resolve this issue. Selective growth of a probiotic with a prebiotic provides a potential mechanism to overcome colonization resistance by supplying a foreign probiotic with a dietary prebiotic [110]. This can be based

upon stochastic processes like colonization order [20] or nutrient depletion/preferences which prevents community reprogramming [111]. Recently, bioengineering of *B. theta* and *Bacteroides stercoris* to utilize a unique nutrient, porphyran, allowed for successful colonization of intestinal crypts [112].

The benefits of synbiotics compared to individual pro- and prebiotics have not fully been elucidated [109]. In preliminary studies, researchers studying ulcerative colitis have demonstrated that there is a significant quality of life improvement with patients using synbiotics over prebiotics and probiotics [113]. Biomin® IMBO is composed of *Enterococcus faecium*, a prebiotic substrate from chicory, and substances from algal populations, and has been shown to efficiently promote growth in broiler chickens [114], but similar to human models, all components are not always tested individually. Further investigation of the synergism that exist between prebiotic-probiotic regimes will require carefully designed factorial experiments with consistent treatments and biological systems.

1.5 Bioengineering

Bioengineering of microorganisms has allowed for diagnostics and therapies in diverse environments including the colon [115] and soil [116]. Due to the ever-growing convenience and opportunities for bioengineering there has been a growing focus on regulatory practices, such as biocontainment and standardization of parts. Engineered biological parts that have been studied for their function and have been engineered to a specific design can be placed in the Registry of Standardized Parts (http://parts.igem.org/Main_Page). Organisms must be scrutinized for their potential to escape confined ecosystems, i.e. ‘biocontainment’ and engineered organisms or genetic elements must be prevented from being transferred to other biological systems. To mitigate

risks in using genetically-modified organisms, engineered bacteria that can be removed from the environment after would be ideal. As well, genetic stability and the transfer of genetic components from the engineered species to native ecosystem members must be prevented [117]. Engineering organisms for colonic health has been researched in a number of colonic community members including *E. coli*, *Lactococcus* spp., and *Bacteroides* spp. [118].

1.5.1 *E. coli*

E. coli has been a model laboratory prokaryote due to its quick growth period, ability to grow with or without oxygen, and ability to utilize multiple nutrient sources [119]. Due to the popularity and genetic tractability the lac operon [120] and techniques such as plasmid transformation [121], extensive genetic tools have been developed for *E. coli*. For these reasons, *E. coli* is thought to be a promising chassis for bioengineering therapies for colonic health, and as a result, many studies have been performed using this microorganism. Recently, *E. coli* Nissle 1917 was designed to detect a quorum sensing molecule from *Pseudomonas aeruginosa*, and in response produce both a *P. aeruginosa* toxin and a biofilm-degrading enzyme [122]. In this study, *E. coli* strains were shown to be stable for up to three weeks in mice; however *E. coli* Nissle 1917 has been shown to be stable for up to 6 months in humans [123]. To address issues with biocontainment, *E. coli* C321.ΔA was designed to utilize non-standard amino acid, bipA, for essential genes [124]. The escape frequency (frequency of an organism escaping their designed environmental conditions) of this strain in the absence of bipA was low at 10^{-11} escapees per colony forming unit, however this strategy requires continuous production and delivery of bipA which is costly.

The use of *E. coli* as a therapeutic chassis does have short-comings. Although a prevalent member of the microbiota, the low abundance of *E. coli* in healthy individuals does raise questions for its efficacy as a therapeutic chassis [118]. Furthermore, its inability to colonize the inner mucus layer [21] prevents the use of *E. coli* as long-term therapeutic and for diverse niches in the colon, such as near the epithelial cell layer.

1.5.2 Lactic acid bacteria (LAB)

Lactic acid bacteria such as *Lactococcus* spp. are well studied probiotics, and similar to *E. coli*, have been well studied because of their growth proficiency and useful biochemical pathways for industry [125]. Inducible and constitutive promoters, chromosomal and plasmid modification, and protein secretion systems have been extensively developed for these bacteria [125]. Steidler *et al.* 2000, engineered a strain of *Lactococcus lactis* producing IL-10 which reduced colitis severity in mice [126]. In 2006, a thymine dependency was engineered into this strain, and it was put into phase 1 clinical trials [127]. Although this trial was successful with 8 of 10 patients showing reduced severity of colitis, many relapsed after treatment was withdrawn and phase 2 trials failed to show significant reduction in colitis symptoms [118]. Currently *L. lactis* strains producing trefoil factor 1 [128] and anti TNF- α nanobodies [129] are in clinical trials sponsored by ActoGeniX N.V. As with *E. coli*, the largest concern to date with LAB probiotics and engineered strains is the inability to colonize the gut [130]; however, short-term delivery of products is still a promising avenue.

1.5.3 *Bacteroides* spp.

The *Bacteroides* genus is a prevalent and abundant colonizer of the microbiota. Species are generally considered to be part of the ‘core’ DGM and some strains have shown to be stable for years within the colon [131]. Although well-studied in humans and mice, it is also found in the digestive systems of other mammalian species, including cattle and poultry [132]. Within the colon, *B. theta* and *B. fragilis* have demonstrated the ability to penetrate and colonize the inner mucus layer and even the epithelial crypts in germ free mice [20]. *Bacteroides* spp. are well known as carbohydrate ‘generalists’ as they possess the ability to utilize a multitude of complex carbohydrates. The genetic elements within Bacteroidetes dedicated to carbohydrate utilization are organized into polysaccharide utilization loci (PULs) of which *Bacteroides* spp. can contain hundreds [133]. PULs are composed of at least one pair of a SusC/D-like (TonB-dependent transporter and surface glycan binding protein, respectively), a PUL regulatory gene, and a cohort of carbohydrate active enzymes [133]. Although the PUL definition was initially coined in 2006 [134], the first PUL to be characterized in *B. theta* was the starch utilization system [135].

Due to the abundance of *Bacteroides* spp. within the colon and its ability to colonize intestinal mucus and crypts, *Bacteroides* spp. have become an attractive target for bioengineering to treat colonic diseases. Previously, *Bacteroides ovatus* has been used to produce IL-2, TGF β -1, keratinocyte growth factor-2 (KGF2) under the control of a xylan-inducible promoter [136-138]. All three studies produced biologically active proteins and were secreted to the media. Optimization of *Bacteroides* spp. protein production including the identification of ribosomal binding site sequences and promoter sequences have been shown drastically increased production of a reporter protein [139, 140]. Plasmids for

homologous recombination into the chromosome [141] along with constitutive [20] and carbohydrate-inducible promoters [142] have been discovered and characterized, which provide options for tailored strategies to bioengineer new genetics parts into *Bacteroides* spp. Whitaker *et al.* used tools to design *Bacteroides* spp. with adjustable levels of GFP and mCherry reporter proteins [20]. In addition, genetic parts were adapted to create a strain of *B. theta*, *B. theta* ‘Double Trouble’, which contains a carbohydrate-inducible feedback loop utilizing dextran and arabinogalactan to increase promoter activation [143]. As evidenced by these recent studies, *Bacteroides* spp. represent promising chassis for colonic biological therapeutics and diagnostics, with the added advantages of providing long-term solutions for patients.

1.6 Bacterial secretion systems

Bacterial secretion systems are essential for interaction with the external environment. Protein secretion systems are divided into nine types (T1SS-T9SS) with different subtypes (T6SSⁱ⁻ⁱⁱⁱ) [144]. Proteins can be targeted to the external environment or the outer membrane via a one- or two-step process. Two-step secretion systems, generally T2SS and T5SS, require the general secretory (SEC) or twin-arginine pathway to first transport proteins from the cytoplasm to the periplasm [145]. Although, the protein secretion mechanisms in Bacteroidetes is not well known, bioinformatics analyses indicated that T1SS, T6SSⁱⁱⁱ, and T9SS are present in a number of *Bacteroides* spp., and T5dSS and T4SS components have also been discovered in some members [144, 146, 147]. Homologs to T1SS, and the recently discovered T5dSS have been discovered *B. theta* [146]; however, T6SS or T9SS secretion systems have not been identified in *B. theta* to date [148, 149]. KEGG analysis indicates that the *B. theta* reference genome, *B. theta* VPI-

5482, does have a majority of the SEC pathway components (SecD/F, SecG, SecY, SecA, YajC, and YidC) (Figure 1.3) yet annotations are missing for the chaperone protein SecB, and SecE transmembrane component [150-153]. Protein export can be achieved in a chaperone-independent manner [145], but SecE has been shown to be essential for signal peptide transport [154]. *B. theta* VPI-5482 also contains annotated, homologous sequences to components of the localization of lipoproteins pathway (Lol) – Lgt, (BT_3118) and LspA (BT_0808). Lipoproteins can be transported into the periplasm using the SEC pathway and attached to the outer membrane using the Lol pathway [155].

1.7 Candidate immunomodulatory protein targets

1.7.1 Tumor necrosis factor receptor 2

TNF- α is a pleiotropic cytokine involved in many pro-inflammatory pathways. TNF- α secretion and binding can cause increased blood flow in microenvironments, activation of macrophages and their production of pro-inflammatory cytokines, and survival of T-effector cells [156]. In many IBD patients, TNF- α is overexpressed from a variety of both immune and non-immune cell types [157] while in broiler chickens, heat stress drastically increases TNF- α production [158]. Therefore, in human and poultry models, the increased levels of TNF- α prolong inflammatory conditions and may potentially cause collateral damage to surrounding tissues. Two main receptors for TNF- α , TNFR1 and TNFR2, are present throughout the intestines with TNFR1 being ubiquitous and TNFR2 displayed only on immune cells. TNFR2 has a greater binding affinity for membrane-bound TNF- α , which has been found to play a larger role in chronic inflammation than soluble TNF- α [60, 156]. Receptor shedding naturally occurs, releasing

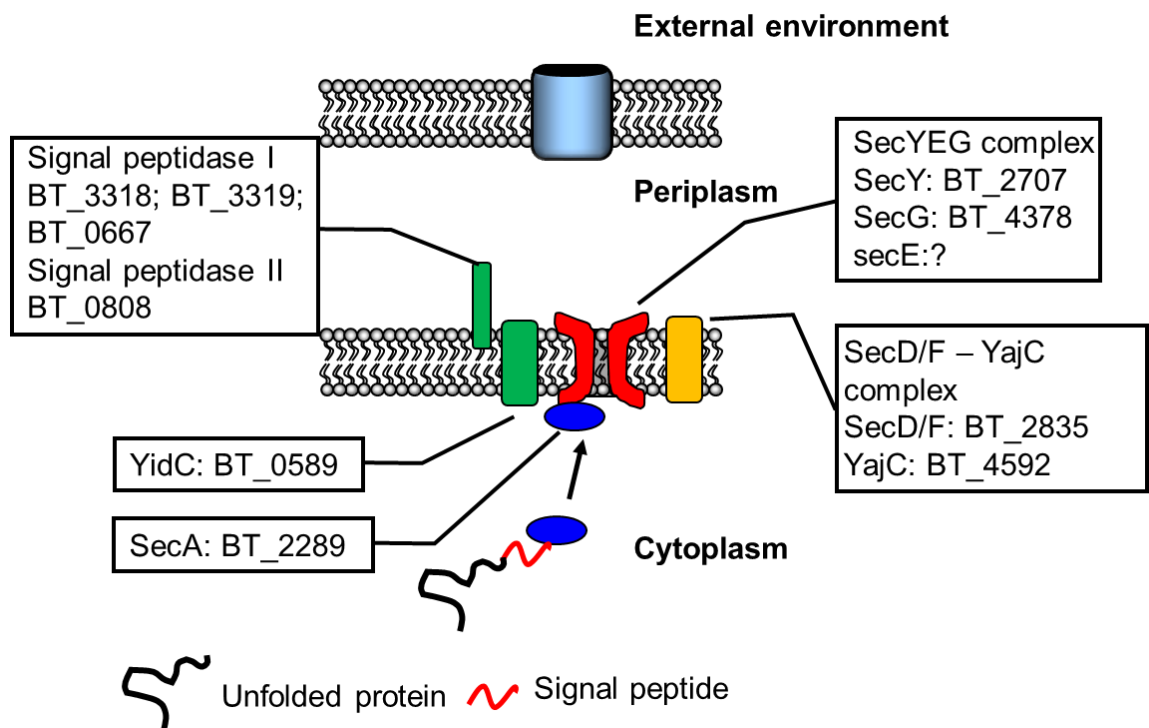


Figure 1.3: SEC pathway components homologs in *B. theta* VPI-5482. *B. theta* appear to secrete proteins into the periplasm through a chaperone (SecB)-independent mechanism. A major transmembrane component, SecE, is also not annotated in *B. theta* VPI-5482. Currently, a secretion mechanism for localization of proteins from the SEC targeted proteins from the periplasm to the external environment is unknown. Protein is targeted to the SECYEG cytoplasmic membrane complex while the process of membrane transport is powered through the ATP motor (SecA) and translocation is enhanced by SecD/F-YajC, complex and YidC. Signal peptides targeted to the external environment are cleaved by signal peptidase I.

soluble TNFR2 competing for binding with membrane-bound TNFR2 [159]. Thus, exogenous, soluble, TNF- α -binding targets including TNFR2 could be introduced in order to bind to excess TNF- α , and diminish pro-inflammatory signaling [160].

1.7.2 Serine protease inhibitors

Serine proteases are generally produced by neutrophils and are used as antimicrobials for intracellular and extracellular bacterial degradation, as well as for cytokine/chemokine modification to further promote neutrophil migration [161]. As they can breakdown immunoglobulins, epithelial tight junctions, and have pro-inflammatory effects (Figure 1.1) [162], serine proteases are generally tightly regulated via control of their production and secretion, and through the production of serine protease inhibitors. Examples of serine protease inhibitors include: the α -1-antitrypsin (A1AT), and the alarm inhibitor elafin [161]. The balance of proteases and their inhibitors is skewed towards proteases in cases of chronic enteritis, which causes deleterious outcomes for the host [163]. This balance however can be manipulated as exogenous elafin secreted by *L. lactis* has been shown to mitigate inflammation [164], and purified A1AT demonstrates anti-inflammatory properties through a similar mechanism [165].

1.8 Hypothesis

Functional human immunomodulatory proteins, TNFR2, A1AT, and elafin, fused to *Bacteroides* spp. N-terminal signal peptides, *BtAnchor* and *BvSecrete*, can be produced and trafficked to the outer membrane surface and external environment, respectively, in *E. coli* and *B. theta*.

1.9 Project outline and objectives

This project will bioengineer strains of *B. theta* that produce and secrete immunomodulators. The pINT plasmid, previously developed in the Abbott lab and adapted from pEXCHANGE-tdk [166], will be used to integrate targets into the *B. theta* Δ tdk Δ PUL75 strain and the *B. theta*::P02683091::P30900267 Δ tdk Δ PUL75 strain ‘Double Trouble’. *B. theta* Δ tdk Δ PUL75 has deletions of PUL75, responsible for utilization of homogalacturonan (a major component of pectin), and the thymidine kinase gene. The tdk mutation aids in screening for homologous recombination, and the deletion of PUL75 allows for a genomic locus sufficient for genome insertion without significant enlargement of the *B. theta* genome. The Double Trouble strain contains a feedback loop controlled by dextran and arabinogalactan, which increases activity levels of reporter proteins [143]. Targets will be induced by the dextran inducible promoter BT_3090 (pBT_3090), and targeted for secretion into the media by stitching on the *B. vulgatus* carbohydrate esterase family 8 secretion signal peptide (*Bv*Secrete) or targeted to the outer membrane using the *B. theta* starch utilization system SusG outer membrane signal peptide (*Bt*Anchor).

1.10 Objectives

1. Create N-terminal signal peptide tagged immunomodulators: A1AT, TNFR2, elafin
2. Validate the stability, localization, and function of protein targets produced within *E. coli*.
3. Generate and validate *B. theta*- Δ tdk- Δ PUL75 strains under the control of carbohydrate-inducible promoters for the heterologous production and secretion of:

A) *BvSecrete_A1AT*, B) *BvSecrete_Elafin*, C) *BvSecrete_TNFR2*, D)
BtAnchor_A1AT, E) *BtAnchor_Elafin*, and F) *BtAnchor_TNFR2*

Chapter 2 Materials and Methods

2.1 Target and plasmid construction

Gene fragments encoding TNFR2 extracellular domain (Human), A1AT (Murine), and elafin (Murine) were commercially synthesized (Bio Basic) and sub-cloned into the NheI and XhoI sites of a pET-28a expression plasmid. DNA fragments encoding an N-terminal signal peptide (N-SP) *BvSecrete* from *BvMPK_0228* (pectinesterase) or *BtAnchor* from *BT_3698* (amylase) were fused to the TNFR2, A1AT, and elafin DNA sequence using a 5' forward primer containing the N-SP DNA sequence and a terminal NcoI restriction site sequence. The immunomodulator-specific 3' reverse primer removed the native stop codon and added a terminal XhoI restriction site sequence. The removal of the native stop codon allowed for addition of a C-terminal hexa-histidine tag encoded by plasmids used in this study.

Targets destined for *B. theta* expression were either cloned behind pBT_3090 sequence of pINT, which contains a compatible multiple cloning site using an NcoI restriction site, or PCR “stitched” to the sequence. Stitching of targets to pBT_3090 was done using overlapping PCR by first adding a C-terminal portion of the pBT_3090 sequence to the N-terminus of target genes. The pBT_3090 sequence and the modified target protein coding sequence was amplified together to create a pBT_3090-target protein fusion. The forward primer for pBT_3090 contains the BglIII restriction site sequence.

Three target variants (untagged, *BvSecrete*, and *BtAnchor*) of each immunomodulatory target were cloned into the pET-28a expression plasmid. Targets were digested with NcoI and XhoI and subsequently cloned into similarly digested pET-28a plasmid. Targets were cloned into the pINT_DX plasmid behind the pBT_3090 promoter

sequence. Targets stitched to the pBT_3090 DNA sequence were cloned into the pINT_DX plasmid using BglII and XhoI cut sites. pINT_DX plasmids contain 5' and 3' flanks surrounding target proteins which are homologous to the flanking regions of the PUL75 locus, which allows for chromosomal integration of targets into the Δ PUL75 loci in the *B. theta* genome. All plasmids were validated using PCR and sequence verification. Eighteen pINT constructs were developed for this project (Table 2.1)

2.2 *E. coli* protein production and localization

2.2.1 Culturing of *E. coli* and heterologous gene over expression

pET-28a plasmids were transformed into chemically competent *E. coli* BL21 Star (DE3) cells for protein overexpression. Transformed bacterial cells were grown in LB (1.0% w/v Bacto-tryptone, 0.5% w/v yeast extract, and 171 mM NaCl) medium supplemented with 50 μ g/mL kanamycin as a selective agent. Once the OD_{600 nm} of the culture reached 0.7 protein expression was induced by the addition of IPTG to a final concentration of 250 mM. The cell culture was incubated at 16 °C for 16 h.

2.2.2 Cell Lysis

1 mL of OD_{600 nm} 1.0 cell culture was harvested by centrifugation at 3,000 x g for 5 min and re-suspended in 50 μ L (20X concentrated) BugBuster Primary Amine-Free Solution (EMBMillipore #70923). Cell suspensions were incubated for 10 min at room temperature to lyse cells, and lysates were centrifuged at 17,000 x g for 5 min. Insoluble and soluble fractions were separated and stored at -20 °C until needed for analysis by SDS-PAGE and Western blot.

Table 2.1: Plasmids used in this study

Plasmid	Notes	Source
pET-28a	<i>E. coli</i> expression vector	Novagen, Cat.69864-3
pET-28a_A1AT	Contains A1AT under IPTG	This study
pET-28a_BvSecrete-A1AT	Contains BvSecreteA1AT under IPTG	This study
pET-28a_BtAnchor-A1AT	Contains BtAnchorA1AT under IPTG	This study
pET-28a_Elafin	Contains Elafin under IPTG	This study
pET-28a_BvSecrete-Elafin	Contains BvSecreteElafin under IPTG	This study
pET-28a_BtAnchor-Elafin	Contains BtAnchorElafin under IPTG	This study
pET-28a_TNFR2	Contains TNFR2 under IPTG	This study
pET-28a_BvSecrete-TNFR2	Contains BvSecreteTNFR2 under IPTG	This study
pET-28a_BtAnchor-TNFR2	Contains BtAnchorTNFR2 under IPTG	This study
pINT_3090		Jones <i>et al.</i> 2019
pINT_DX		Jones <i>et al.</i> 2019
pINT_3090_A1AT	Contains A1AT under DX	This study
pINT_3090_BvSecrete-A1AT	Contains BvSecreteA1AT under DX	This study
pINT_3090_BtAnchor-A1AT	Contains BtAnchorA1AT under DX	This study
pINT_3090_Elafin	Contains Elafin under DX	This study
pINT_3090_BvSecrete-Elafin	Contains BvSecreteElafin under DX	This study
pINT_3090_BtAnchor-Elafin	Contains BtAnchorElafin under DX	This study
pINT_3090_TNFR2	Contains TNFR2 under DX	This study
pINT_3090_BvSecrete-TNFR2	Contains BvSecreteTNFR2 under DX	This study
pINT_3090_BtAnchor-TNFR2	Contains BtAnchorTNFR2 under DX	This study
pINT_DX_A1AT	Contains A1AT under DX	This study
pINT_DX_BvSecrete-A1AT	Contains BvSecreteA1AT under DX	This study
pINT_DX_BtAnchor-A1AT	Contains BtAnchorA1AT under DX	This study
pINT_DX_Elafin	Contains Elafin under DX	This study
pINT_DX_BvSecrete-Elafin	Contains BvSecreteElafin under DX	This study
pINT_DX_BtAnchor-Elafin	Contains BtAnchorElafin under DX	This study
pINT_DX_TNFR2	Contains TNFR2 under DX	This study
pINT_DX_BvSecrete-TNFR2	Contains BvSecreteTNFR2 under DX	This study
pINT_DX_BtAnchor-TNFR2	Contains BtAnchorTNFR2 under DX	This study

2.2.3 Protein purification

Bacterial culture cells were harvested by centrifugation at 3,000 x *g* for 20 min at 4 °C. The cell pellet from 1L of bacterial culture was resuspended in 25 mL of lysis buffer (20 mM Tris pH 8.0, 500 mM NaCl), and cells were homogenized by two passes of sonication (1 min on, 1 min off for 2 min total; 30% amplitude; Fisher Scientific). Cellular debris was removed by centrifugation at 17,000 x *g* for 1 hour at 4 °C. The soluble supernatant was loaded onto Ni²⁺-nitrilotriacetic acid (Ni-NTA) resin and protein was eluted using a 10-500 mM imidazole (in 20 mM Tris pH 8.0 and 500 mM NaCl) gradient. Proteins were dialyzed into 20 mM Tris pH 8.0, 400 mM NaCl, and subsequently concentrated 10X using Amicon™ Bioseparations Stirred Cells and Amicon™ centrifuge units, MWCO 10kDa (EMD Millipore). Proteins were quantified with a Nanodrop 2000c (Thermo Scientific) using extinction coefficients calculated from Protpram [167]. Protein purification was monitored throughout by SDS-PAGE and Western blot.

2.2.4 Media fraction collection (*Bv*Secrete localization)

To determine localization of the *Bv*Secrete tagged targets, media samples were collected along with cell pellets. After harvesting of bacterial cells as above, spent culture media was run through a 0.45 µm filter to further remove bacterial contamination and loaded onto Ni-NTA resin as above. Proteins were purified and dialyzed as describe above for cell lysates, and purification was monitored throughout by SDS-PAGE and Western blot.

2.2.5 Whole cell dot blot (*BtAnchor* localization)

To determine localization of the *BtAnchor* tagged targets to the outer membrane of *E. coli* dot blot assays were performed as previously described [168]. Briefly, two 1 mL samples of induced OD_{600 nm} 1.0 cultures were harvested by centrifugation at 3,000 x g for 5 min, washed in Tris-buffered saline (10 mM Tris-HCl pH 7.5, 150 mM NaCl) (TBS) three times, and then re-suspended in 100 µL TBS with or without 100 µM proteinase K. Cells were incubated at 37 °C for 16 h under agitation. Cells were washed three times with TBS, dotted on an methanol-wetted polyvinylidene difluoride (PVDF) membrane, and allowed to dry. Once dry, PVDF membranes were wetted in methanol and blocked in 5% (w/v) casein in TBS containing 0.5% (v/v) Tween-20 (TBS-T) at 4 °C with agitation for 24 h. Membranes were then incubated with 1:2500 rabbit anti hexa-histidine polyclonal IgG-HRP conjugated primary antibody (Bethyl in TBS-T with 5% (w/v) casein for 24 h at 4°C with gentle agitation. Blots were washed three times in TBS-T buffer and visualized colourimetrically using an Opti-4CN™ Substrate Kit (Bethyl Laboratories #A190 114P).

2.3 Homologous recombination and generation of *B. theta* strains

pINT_DX plasmids were transformed into *E. coli* S17-1λpir cells and positive transformants were selected with 100 µg/mL ampicillin. 5 mL of *E. coli* S17-1λpir cells were grown in LB supplemented with ampicillin until OD_{600 nm} 0.6-0.8. Strains of *B. theta* were grown in 5 mL TYG [1% Bacto™ Tryptone (BD; 211705), 0.5% Yeast Extract Bacteriological grade (VWR; J850), 4.1 mM L-cysteine, 0.2% glucose, 0.1 M KPO₄ pH 7.2, 2.2 µM vitamin K₃, 40 µL/mL TYG Salts (2 mM MgSO₄·7H₂O, 119 mM NaHCO₃, and 34.2 mM NaCl), 28.8 µM CaCl₂, 1.4 µM FeSO₄, 4.4 µM resazurin, 1.0 µL/mL (v/v) histidine/hematin (1.9 mM hematin, and 200 mM L-histidine, 1000X stock solution] at 37

°C in anaerobic atmosphere (85% N₂, 10% CO₂, 5% H₂) until OD_{600nm} 0.6-0.8. *E. coli* S17-1λpir donor culture and *B. theta* recipient cultures were pelleted by centrifugation at 3,000 x g and re-suspended together in 1 mL of TYG before being spread on BHI agar [3.7% Bacto™ Brain Heart Infusion (BD; 237500), 8.3 mM L-cysteine, 10 mL/L hemin (0.77 mM hemin in 0.01 M NaOH), and 0.2% NaHCO₃ agar] with no supplemented antibiotic. Cultures were grown at 37 °C aerobically for 16 h, and biomass was scraped off plates and suspended in 5 mL TYG, and 200 µL of the resulting cell suspension was spread on BHI agar supplemented with 200 µg/mL gentamycin and 25 µg/mL erythromycin for selection of successfully mated donor and recipient strains. Cells were re-streaked on BHI agar containing gentamycin and erythromycin for further confirmation in selection. 8-10 isolates were arbitrarily selected and grown overnight in TYG at 37 °C in anaerobic atmosphere overnight and pooled. Once pooled, 100 µL of culture was grown on BHI agar supplemented with 200 µg/mL 5-Fluoro-2'-deoxyuridine (FUdR) selection agent to select for the removal of the pINT_DX backbone, required to remove resistance to gentamycin and erythromycin. Again, 8-10 isolates were re-streaked on BHI-FUdR agar to obtain pure isolates. Validation of strains was done by colony PCR and Sanger sequencing using primers both internal and external to the ΔPUL75 locus to confirm proper sequence and integration into the locus (Table 2.2).

2.4 *B. theta* protein production and localization

2.4.1 Culturing of *B. theta*

B. theta strains were grown in TYG at 37 °C in an anaerobic atmosphere until OD_{600nm} 0.8. Cells were harvested by centrifugation at 3,000 x g for 10 min. Cell pellets were washed twice with 2X minimalized medium (MM) [pH 7.2: 200 mL/L (v/v) 10X

Table 2.2: Strains used in this study

Strains	Notes	Source
<i>E. coli</i> BL21 Star (DE3)	Protein production strain	ThermoFisher, Cat.601003
<i>E. coli</i> S17-1 lambda pir	recA pro hsdR RP4-2-Tc::Mu-Km::Tn7 λ- pir	PMID_6340113
<i>B. thetaiotaomicron</i> VPI-5482 Δtdk, ΔPUL75	Deletion of <i>Bt</i> 4108- <i>Bt</i> 4124. FUDr resistant	Jones <i>et al.</i> 2019
<i>B. thetaiotaomicron</i> VPI-5482 Δtdk, ΔPUL75::3090_A1AT	Contains A1AT under DX	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δtdk, ΔPUL75::3090_BvSecrete-A1AT	Contains <i>Bv</i> SecreteA1AT under DX	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δtdk, ΔPUL75::3090_BtAnchor-A1AT	Contains <i>Bt</i> AnchorA1AT under DX	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δtdk, ΔPUL75::3090_Elafin	Contains Elafin under DX	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δtdk, ΔPUL75::3090_BvSecrete-Elafin	Contains <i>Bv</i> SecreteElafin under DX	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δtdk, ΔPUL75::3090_BtAnchor-Elafin	Contains <i>Bt</i> AnchorElafin under DX	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δtdk, ΔPUL75::3090_TNFR2	Contains TNFR2 under DX	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δtdk, ΔPUL75::3090_BvSecrete-TNFR2	Contains <i>Bv</i> SecreteTNFR2 under DX	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δtdk, ΔPUL75::3090_BtAnchor-TNFR2	Contains <i>Bt</i> AnchorTNFR2 under DX	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δtdk, ΔPUL75::DX_A1AT	Contains A1AT under DX	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δtdk, ΔPUL75::DX_BvSecrete-A1AT	Contains <i>Bv</i> SecreteA1AT under DX	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δtdk, ΔPUL75::DX_BtAnchor-A1AT	Contains <i>Bt</i> AnchorA1AT under DX	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δtdk, ΔPUL75::DX_Elafin	Contains Elafin under DX	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δtdk, ΔPUL75::DX_BvSecrete-Elafin	Contains <i>Bv</i> SecreteElafin under DX	This study

<i>B. thetaiotaomicron</i> VPI-5482 Δ tdk, Δ PUL75::DX_ <i>BtAnchor-Elafin</i>	Contains <i>BtAnchorElafin</i> under DX	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δ tdk, Δ PUL75::DX_TNFR2	Contains TNFR2 under DX	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δ tdk, Δ PUL75::DX_ <i>BvSecrete-TNFR2</i>	Contains <i>BvSecreteTNFR2</i> under DX	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δ tdk, Δ PUL75::DX_ <i>BtAnchor-TNFR2</i>	Contains <i>BtAnchorTNFR2</i> under DX	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δ tdk, Δ PUL75, p <i>Bt3091</i> ::pAG, p <i>Bt0267</i> ::pDX	Dextran regulator under arabinogalactan promoter, and arabinogalactan regulator under dextran promoter.	Jones et al. 2019
<i>B. thetaiotaomicron</i> VPI-5482 Δ tdk, Δ PUL75::DX_A1AT, p <i>Bt3091</i> ::pAG, p <i>Bt0267</i> ::pDX	Contains A1AT under DX. Double Trouble system.	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δ tdk, Δ PUL75::DX_ <i>BvSecrete-A1AT</i> , p <i>Bt3091</i> ::pAG, p <i>Bt0267</i> ::pDX	Contains <i>BvSecreteA1AT</i> under DX. Double Trouble system.	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δ tdk, Δ PUL75::DX_ <i>BtAnchor-A1AT</i> , p <i>Bt3091</i> ::pAG, p <i>Bt0267</i> ::pDX	Contains <i>BtAnchorA1AT</i> under DX. Double Trouble system.	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δ tdk, Δ PUL75::DX_Elafin, p <i>Bt3091</i> ::pAG, p <i>Bt0267</i> ::pDX	Contains Elafin under DX. Double Trouble system.	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δ tdk, Δ PUL75::DX_ <i>BvSecrete-Elafin</i> , p <i>Bt3091</i> ::pAG, p <i>Bt0267</i> ::pDX	Contains <i>BvSecreteElafin</i> under DX. Double Trouble system.	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δ tdk, Δ PUL75::DX_ <i>BtAnchor-Elafin</i> , p <i>Bt3091</i> ::pAG, p <i>Bt0267</i> ::pDX	Contains <i>BtAnchorElafin</i> under DX. Double Trouble system.	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δ tdk, Δ PUL75::DX_TNFR2, p <i>Bt3091</i> ::pAG, p <i>Bt0267</i> ::pDX	Contains TNFR2 under DX. Double Trouble system.	This study
<i>B. thetaiotaomicron</i> VPI-5482 Δ tdk, Δ PUL75::DX_ <i>BvSecrete-TNFR2</i> , p <i>Bt3091</i> ::pAG, p <i>Bt0267</i> ::pDX	Contains <i>BvSecreteTNFR2</i> under DX. Double Trouble system.	This study

***B. thtaiotaomicron* VPI-5482 Δ tdk,** Contains *BtAnchor*TNFR2 under DX. This study
 Δ PUL75::DX_*BtAnchor*-TNFR2,
p*Bt*3091::pAG, p*Bt*0267::pDX Double Trouble system.

Bacteroides salts solution pH 7.2 (999 mM KH₂PO₄, 30 mM NaCl, 17 mM (NH₄)₂SO₄), 20 mL/L (v/v) Balch's vitamins pH 7.0 (36.5 μM p-aminobenzoic acid, 4.5 μM folic acid, 8.2 μM biotin, 40.6 μM nicotinic acid, 10.5 μM calcium pantothenate, 13.3 μM riboflavin, 14.8 μM thiamine HCl, 48.6 μM vitamin B6, 73.8 nM vitamin B12, and 24.2 mM thiocetic acid), 20 mL/L (v/v) amino acid solution (5 mg/mL amino acids: alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine), 20 mL/L (v/v) purine/pyrimidine solution pH 7.0 (1 mg/mL adenine, guanine, thymine, cytosine, and uracil), 20 mL/L (v/v) trace mineral solution pH 7.0 (1.7 M ethylenediaminetetraacetic acid, 12.2 M MgSO₄·7H₂O, 3 M MnSO₄·H₂O, 17.1 M NaCl, 359.7 mM FeSO₄·7H₂O, 901.1 mM CaCl₂, 347.7 mM ZnSO₄·7H₂O, 40.1 mM CuSO₄·5H₂O, 161.7 mM H₃BO₃, 41.3 mM Na₂MoO₄·2H₂O, and 84.1 mM NiCl₂·6H₂O), 4.4 μM vitamin K₃, 2.9 μM FeSO₄·7H₂O, 14.4 μM CaCl₂, 2 mM MgCl₂·6H₂O, 7.4 pM vitamin B12, 16.5 mM L-cysteine, and 2 μL/mL (v/v) histidine/hematin], and re-suspended in MM solutions containing 0.5% select carbohydrate. *B. theta*ΔtdkΔPUL75 strains were grown in MM containing either glucose (control) or dextran from *Leuconostoc mesenteroides* (150,000 MW; Sigma) to induce target gene expression; *B. theta*ΔPUL75Δtdk::pbt3091::pAG, pbt0267::pDX strains were grown in MM containing glucose (control), dextran, arabinogalactan from larch wood (Megazyme), or a mixture of dextran and arabinogalactan to induce target gene expression.

2.4.2 Cell lysis

Overnight cultures (5 mL) were grown in an anaerobic atmosphere for 16 h at 37 °C. Cells were harvested and lysed, and samples of soluble and insoluble protein fractions were taken as described above (2.2.3).

2.4.3 Media fraction collection (*Bv*Secrete localization)

To determine localization of the *Bv*Secrete-tagged targets, media samples were collected along with cell pellets. Proteins were purified from spent media as described above (2.2.4).

2.4.4 Whole cell dot blot (*Bt*Anchor localization)

To determine the presence of the *Bt*Anchor-tagged targets on the outer membrane of *B. theta*, dot blots were performed as described above (2.2.5) with one minor change; cells were harvested by centrifugation at 3,000 x g in 10 min intervals.

2.5 Protein analysis (SDS-PAGE and Western blots)

Samples were mixed 1:1 with 2X SDS loading buffer (2 M glycerol, 1.28 M β -mercaptoethanol, 125 mM Tris pH 6.8, 140 mM SDS, 60 μ M bromophenol blue) and denatured at 95°C for 5 min. Samples were analyzed by SDS-PAGE, in gels containing 15% acrylamide. For Western blot analysis, protein was transferred from the SDS-PAGE gel to a PVDF membrane by wet blotting (30 V, 16 h, 4 °C). Membranes were incubated with 5% (w/v) casein in TBS-T at 4 °C with agitation for 24 h. Membranes were then incubated with 1:2500 rabbit anti hexa-histidine polyclonal IgG-HRP conjugated primary antibody (Bethyl) in TBS-T with 5% (w/v) casein for 24 h at 4°C with gentle agitation.

Blots were washed three times in TBS-T buffer and visualized colourimetrically using an Opti-4CN™ Substrate Kit (Bethyl Laboratories #A190 114P).

2.6 Elastase activity assay

Activity of secreted protease inhibitors was determined using the Enzchek® Elastase Assay kit (Molecular Probes). In this assay, bovine elastin substrate labeled with green fluorescence BODIPY dye (DQ elastin) at 25 µg/mL is cleaved by elastase from pig pancreas (0.2 U/mL), resulting in the release of fluorophore-conjugated peptides and a correlated increase in detected fluorescence. Fluorescence was quantified using a Synergy HT Multi-detection plate reader ($\lambda_{\text{ex}} = 485 \text{ nm}$, $\lambda_{\text{em}} = 528 \text{ nm}$). Protease inhibitors A1AT and elafin with the *Bv*Secrete and *Bt*Anchor signal peptides, and the pET-28a vector control purified in **2.2.3** were incubated with elastase prior to the addition of elastin substrate. Standard concentration curves were generated using 0.2 mM, 0.1 mM, 0.05 mM, 0.01 mM, and 0.005 mM protease inhibitors and vector controls. Positive and negative assay controls were done using standard kit solutions. Data were collected for three technical replicates and analyzed using GraphPad Prism 8.00 (GraphPad software). The linear portion of the fluorescence curves were used to calculate Inhibitor Concentration₅₀ (IC₅₀), and the relative activity of the elastase assay with 5 µM was plotted. An unpaired t-test was conducted between the means of the sample treatments.

2.7 TNFR2-TNF binding assay

Binding of *Bv*Secrete-TNFR2 and *Bt*Anchor-TNFR2 to TNF- α was validated using native-PAGE electrophoresis. *Bv*Secrete-TNFR2 and *Bt*Anchor-TNFR2 at 100µM or 10µM of purified protein was incubated with 50µM TNF- α (abcam) for 2 hours at room

temperature. The resulting samples were mixed 1:4 with 5X native sample buffer (62.5 mM Tris pH 6.8, 40% glycerol, 0.01% Bromophenol Blue), and run on 15% native-PAGE gels at room temperature for 35 min at 200 V. Gels were visualized using coomassie blue staining.

Chapter 3 Results

3.1 Designing protein constructs for localization in *E. coli* and *B. theta*

To target the secretion of TNFR2, elafin, and A1AT, two N-SPs were selected for validation in this thesis: *BtAnchor*, from the *B. theta* SusG amylase for localization and anchoring to the outer-membrane of the bacterium, and *BvSecrete* from *Bacteroides vulgatus* carbohydrate esterase family 8 (CE8) for secretion into the environment [168]. *BvSecrete* is predicted by the online webserver SignalP [169] to contain a SPI sequence and be secreted into the medium (99% confidence) (Figure 3.1A), while *BtAnchor* is predicted to be contain a SPII sequence (99% confidence) (Figure 3.1B), which would be lipidated at the N-terminal cysteine and surface-exposed (Figure 3.1C) [170]. Previously, V6 and DSB nanobodies targeting *C. jejuni* were successfully targeted in *E. coli* using these signal peptides [168]. Therefore, it is anticipated that *BvSecrete* and *BtAnchor* should provide a compatible system for secretion in both *E. coli* and *B. theta*. In addition, untagged constructs of each enzyme were generated to be used as a control to gauge the level of heterologous production within the cell. All of these constructs were developed in the pET-plasmid system and the pINT-plasmid system for expression in *E. coli* and *B. theta*, respectively.

3.2 Protein production in *E. coli*

Protein localization in *E. coli* was monitored by Western blot following induction of protein expression, cell lysis, and fractionation of soluble and insoluble cellular components (Figure 3.2). In the untagged constructs, bands corresponding to the predicted

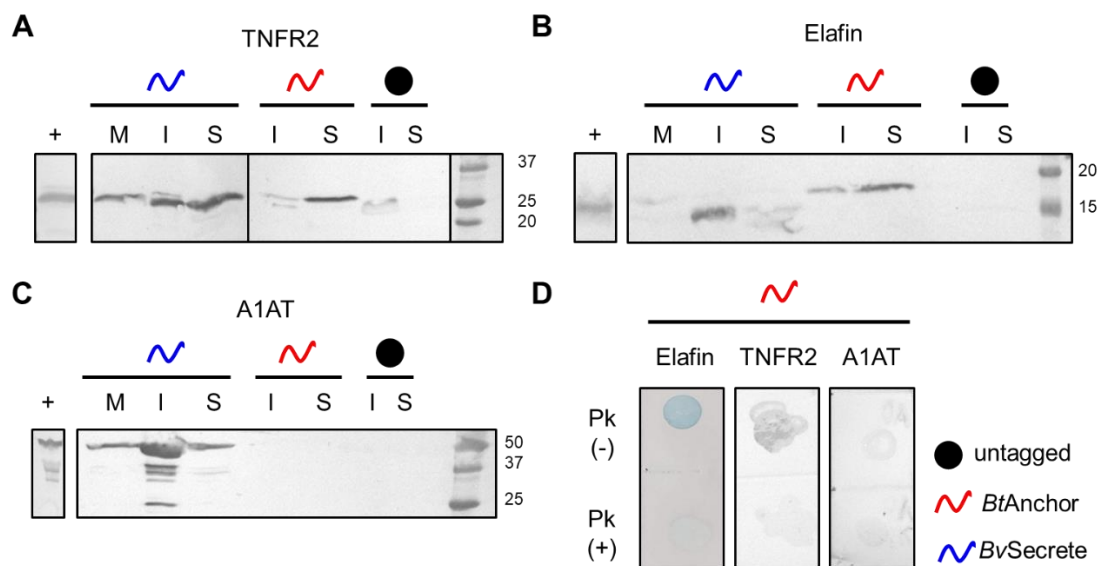


Figure 3.2: *E. coli* production and localization of immunomodulatory proteins. Protein production of untagged and signal peptide-tagged (A) TNFR2, (B) elafin, and (C) A1AT were monitored by Western blot using His₆-specific antibodies. M: 10X concentrated media fraction; I: insoluble fraction; S: soluble fraction. (D) Dot blot of *BtAnchor*-tagged proteins with and without proteinase K (Pk) treatment of cells to remove surface bound proteins visualized using His₆-specific antibodies.

size of elafin, and A1AT are absent from both insoluble and soluble fractions, likely indicative that both proteins were either not produced in detectable quantities or the His₆-tag epitope was not available for binding by the antibody [171]. A significant band at the position expected for TNFR2 was observed in the insoluble fraction (Figure 3.2A), which suggests the protein is not folding properly or it is unstable. Addition of the N-SPs appeared to improve protein production levels and folding. Bands indicating the presence of *BtAnchor*-TNFR2 and *BtAnchor*-elafin were detected in the soluble and insoluble fraction (Figure 3.2A and B); and bands equivalent to all *BvSecrete* targets were observed in the soluble and insoluble fractions (Figure 3.2A, B, and C). However, a band corresponding to *BtAnchor*-A1AT was not observed. Soluble proteins were purified by Ni-NTA affinity chromatography and used as positive protein controls in Western blots.

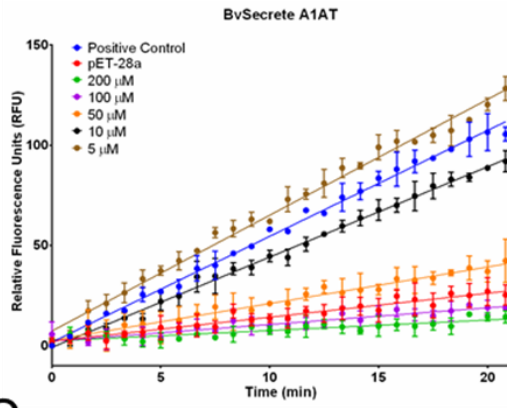
To determine if the proteins were being trafficked to different cellular locations in *E. coli*, cells were fractionated. To evaluate if *BvSecrete* peptides targeted the protein for extracellular secretion, media fractions of induced cultures were collected and concentrated. *BvSecrete*-A1AT, *BvSecrete*-TNFR2, and *BvSecrete*-elafin were all successfully localized to the media fraction (Figure 3.2A, B, and C); although, *BvSecrete*-elafin was present in low quantities. Next, to evaluate if *BtAnchor* could successfully target protein cargo to the outer membrane surface, dot blots \pm proteinase K were conducted. Based upon the disappearance of signal in the presence of proteinase K, *BtAnchor*-TNFR2 and *BtAnchor*-Elafin appeared to be successfully targeted to the outside of the cell (Figure 3.2D). However, no detectable levels of *BtAnchor*-A1AT appeared on the outer membrane of *E. coli*, which may have resulted from the low levels of overall protein production, that

the structure of this protein is not compatible for secretion in *E. coli*, or that the epitope is not accessible when the protein is attached to the cell surface (Figure 3.2D).

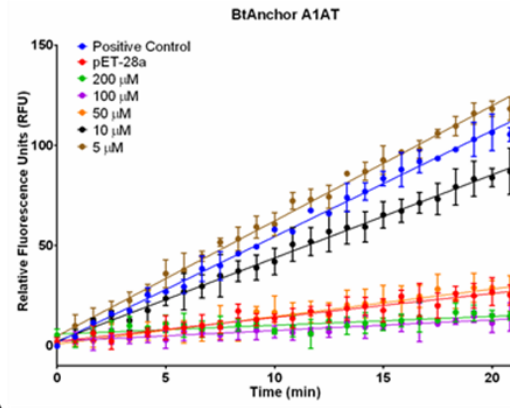
3.3 Activity assay for protease inhibitors A1AT and elafin

To determine if recombinant protein were functional, *BvSecrete-A1AT*, *BvSecrete-elafin*, *BtAnchor-A1AT* and *BtAnchor-elafin* were purified from *E. coli* and assayed with the Enzchek® Elastase Assay kit (Molecular Probes). In this assay, elastase cleaves a fluorophore-conjugated substrate resulting in an increase of fluorescence, which can be quantified over time (Figure 3.3A-D). Elastase inhibitors are then added in increasing concentrations, and if functional, will decrease the relative fluorescence. Non-specific proteins purified from an empty pET-28a vector *E. coli* lysate (undiluted) was included as a negative control. *BvSecrete-A1AT*, *BtAnchor-A1AT*, and *BtAnchor-elafin* were found to significantly inhibit porcine elastase above the negative control at 200 μM ($p < 0.001$), although inhibition at lower concentrations (50 μM and less) was not as strong for *BtAnchor-A1AT*, and *BtAnchor-elafin* when compared to the pET-28a vector control (Figure 3.3A, B, and D). This observation suggests that *E. coli* lysate may contain a protein or compound that weakly inhibited the reporter enzyme. In comparison, *BvSecrete-elafin* inhibited porcine elastase at all of the dilutions tested ($\geq 5 \mu\text{M}$), suggesting that this protein is a specific inhibitor (Figure 3.3C). IC_{50} values from the linear portion of the reaction curve were calculated to determine the inhibition activity of target proteins. (Figure 3E);

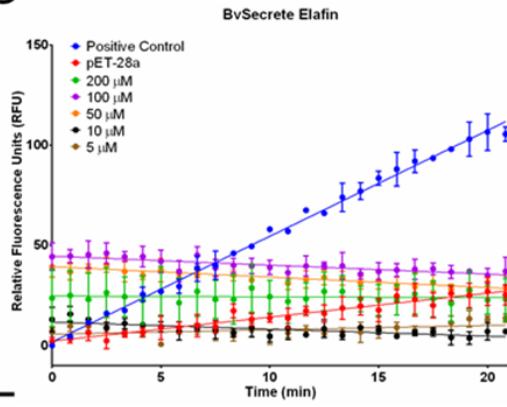
A



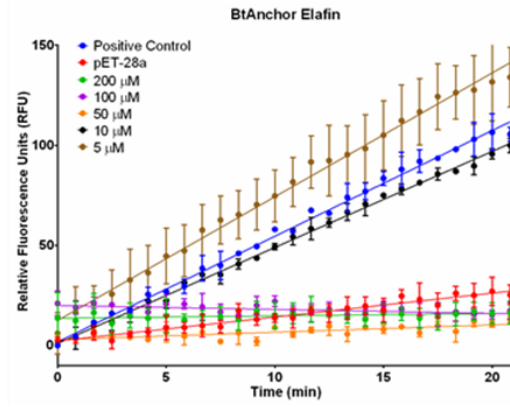
B



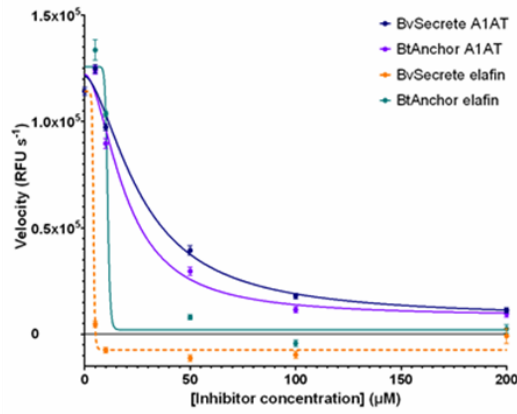
C



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E



F

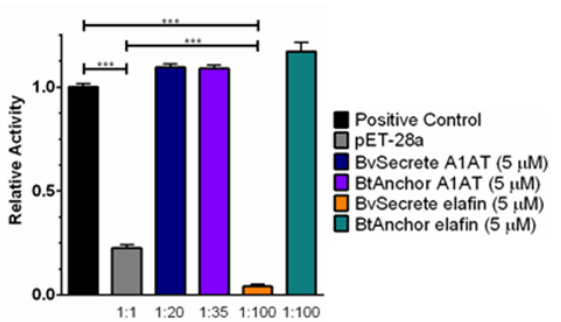


Figure 3.3: Fluorescence-based serine protease assay of neutrophil elastase activity.

The rate of cleavage of BODIPY-conjugated substrate (Enzchek® elastase assay) was monitored over time ($\lambda_{\text{ex}} = 485 \text{ nm}$, $\lambda_{\text{em}} = 528 \text{ nm}$), with the linear portion of the reaction shown. The enzymatic assay was performed without (positive and negative controls) or with increasing dilutions (200-5 μM) of *Bv*Secrete-A1AT (**A**), *Bt*Anchor-A1AT (**B**), *Bv*Secrete-elafin (**C**), or *Bt*Anchor-elafin (**D**). pET-28a vector control was included as a recombinant protein expression control to screen for *E. coli* inhibition. For all panels, error bars represent the standard error calculated from duplicate (positive control) or triplicate values (all others). The initial rates of substrate cleavage by porcine neutrophil elastase were obtained in the presence of 5 μM *Bv*Secrete-A1AT (navy), *Bt*Anchor-A1AT (purple), *Bv*Secrete-elafin (orange), and *Bt*Anchor-elafin (teal), and concentrated pET-28a contaminant (grey). (**E**) Data from the four inhibitor constructs were fit to a four-parameter dose-response curve in GraphPad Prism 8 (GraphPad Software). IC_{50} values were calculated from the fits, with the exception of *Bv*Secrete-elafin (not enough data points). For all panels, error bars represent the standard error calculated from duplicate (positive control) or triplicate values (all others). (**F**) Data were normalized to the activity of elastase in the absence of inhibitor (black). A significance difference ($p < 0.001$) is marked above the bars, and dilution factors for each target is marked below the graph.

however, the affinity of *Bv*Secrete-elafin to porcine elastase was too high to get a confident IC_{50} value (Figure 3.3E). Inhibition of porcine elastase at 5 μ M from all inhibitors was plotted on a bar graph with the undiluted, pET-28a vector control. (Figure 3.3F).

3.4 TNFR2-TNF binding assay

*Bv*Secrete-TNFR2 and *Bt*Anchor-TNFR2 were investigated for their ability to bind to TNF- α (Figure 3.4A). *Bv*Secrete-TNFR2 or *Bt*Anchor-TNFR2 ran as a smear when run on native-PAGE electrophoresis, suggesting that they were not adopting a single, compact structure. Incubation with soluble TNF- α did not result in a visible band shift for TNF- α (100 μ M or 10 μ M; Figure 3.4B). When 100 μ M *Bt*Anchor-TNFR2 and *Bv*Secrete-TNFR2 were incubated with TNF- α band disappearance was observed, suggesting a potential interaction between TNF- α and the TNFR2 samples.

3.5 Construction of engineered *B. theta* strains for carbohydrate-inducible immunomodulator expression

To determine if the *Bt*Anchor and *Bv*Secrete peptides improved the stability and / or yields of protein production in *B. theta* and correctly trafficked protein cargo, the constructs were engineered for insertion into *Bacteroides* spp. compatible vectors. Multiple cloning- and strain-based strategies have been developed for the introduction of genetic material into the *B. theta* chromosome and for the induction of target genes within *B. theta* [143]. The pINT_DX plasmid is designed for integration of genetic material into the *B. theta* PUL75 locus by double cross over events using 5' and 3' sequences homologous to regions flanking PUL75 homologous flanking regions, with a dextran-inducible pBT_3090 promoter (Figure 3.5A). Gentamycin and erythromycin resistance genes allow for

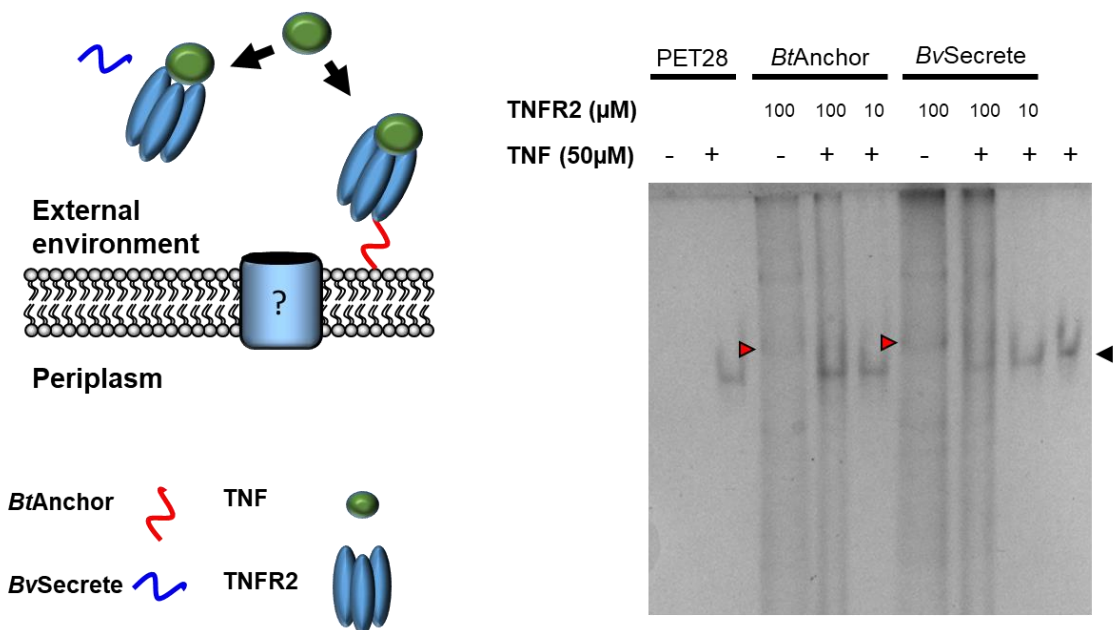


Figure 3.4: *BvSecrete*-TNFR2 and *BtAnchor*-TNFR2 TNF- α binding assay. (A) Depiction of localized *BvSecrete* and *BtAnchor* interaction with TNF- α . **(B)** Native-PAGE gel of TNF- α binding to the pET-28a vector control, and N-SP tagged TNFR2 at 100 μM and 10 μM . Black arrow represents TNF- α (17 kDa). Red arrows indicate band disappearance in the TNFR2-TNF mixture.

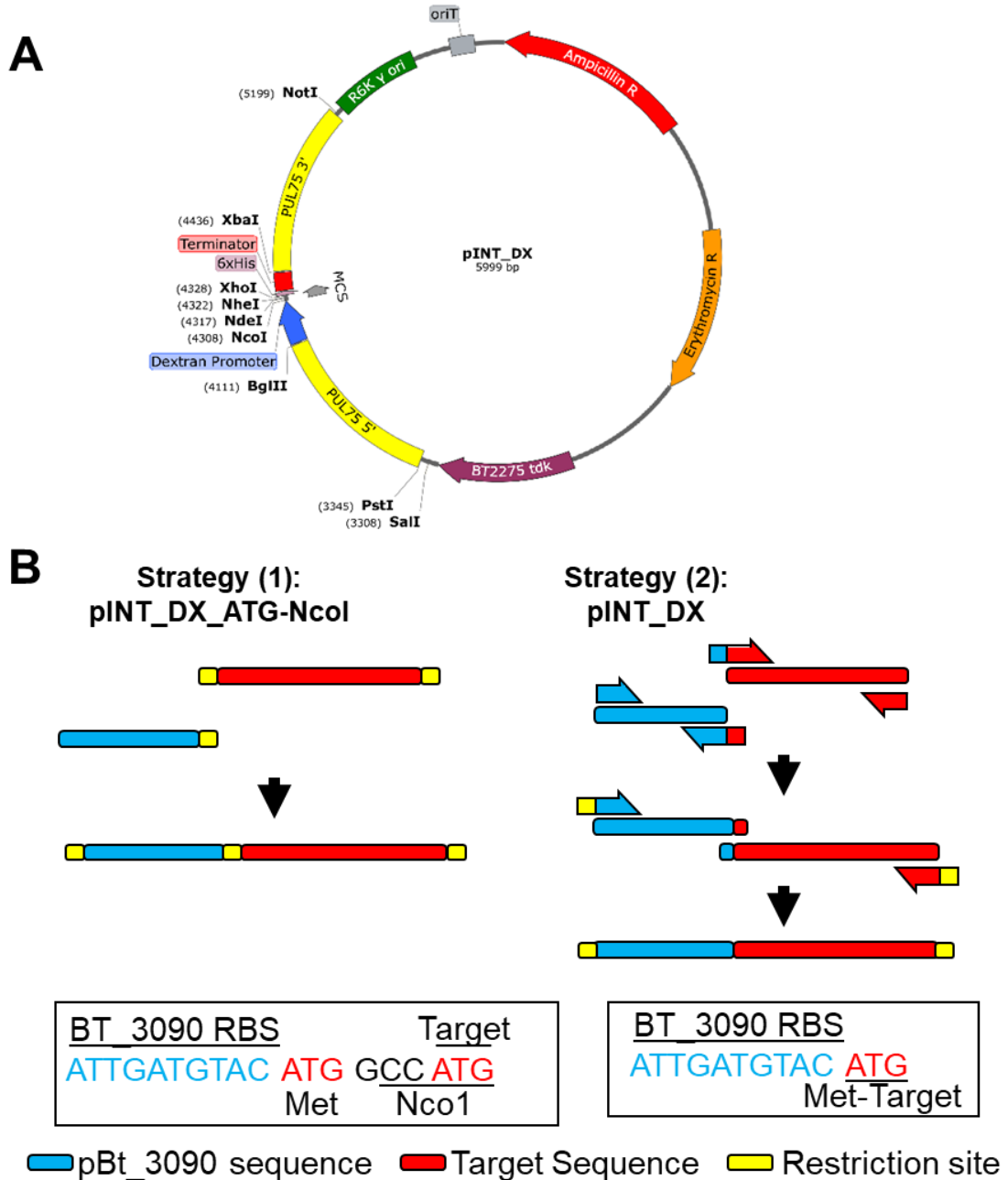


Figure 3.5: Strategies for plasmid design for homologous recombinant in *B. theta*. (A) Plasmid map of the pINT plasmid used in this study for integration of genetic material into the PUL75 locus. (B) Cloning strategies of pINT plasmids. Strategy (1) involves cloning the target DNA sequence behind pBT_3090 using a NcoI restriction site, whereas strategy (2) fuses the target DNA sequence to the pBT_3090 sequence.

antibiotic selection, but are excised in the second crossover event, and therefore, are not present in the final strain. The *tdk* protein is responsible for the phosphorylation of FUdR, resulting in F-dUMP, which inhibits thymidylate synthase (ThyA) and prevents DNA replication. Inclusion of FUdR in culture medium will inhibit growth of strains with an intact *tdk* gene, allowing for selection of only those strains that have had successful integration of genetic material.

Two strategies were conducted to clone the target immunomodulators into pINT_DX plasmids (Figure 3.5B). In the first strategy, target DNA sequences were cloned behind the pBT_3090 promoter using an *Nco*I cut site with the addition of an 'ATG GCC' sequence, allowing for direct subcloning from pET-28a with the genes remaining in frame. In the second strategy, amplicons were generated with the pBT_3090 sequences directly fused to the target proteins, enabling cloning of *Bv*Secrete and *Bt*Anchor protein constructs.

The pINT integration plasmids were conjugated into two different *B. theta* recipient strains in this study: *B. theta* Δ *tdk* Δ PUL75 and *B. theta* Δ *tdk* Δ PUL75 Double Trouble. Gene induction in *B. theta* Δ *tdk* Δ PUL75 is compatible with direct activation of the pBT_3090 dextran-inducible promoter (Figure 3.6A). In this system, dextran from *L. mesenteroides*, a homopolysaccharide consisting of α 1-6-glucose chains with α 1-3 glucose side chains, is transported into the periplasm through BT_3090 TonB-dependent transporter (SusC-like). Derivative dextran oligosaccharide products are predicted to activate BT3091 SusR-like [142]. The activation of SusR activates the pBT_3090 promoter, which induces the genes in the dextran PUL (PUL48), including BT_3090, and in theory, the target proteins controlled by pBT_3090. In comparison, induction of target proteins in the Double Trouble

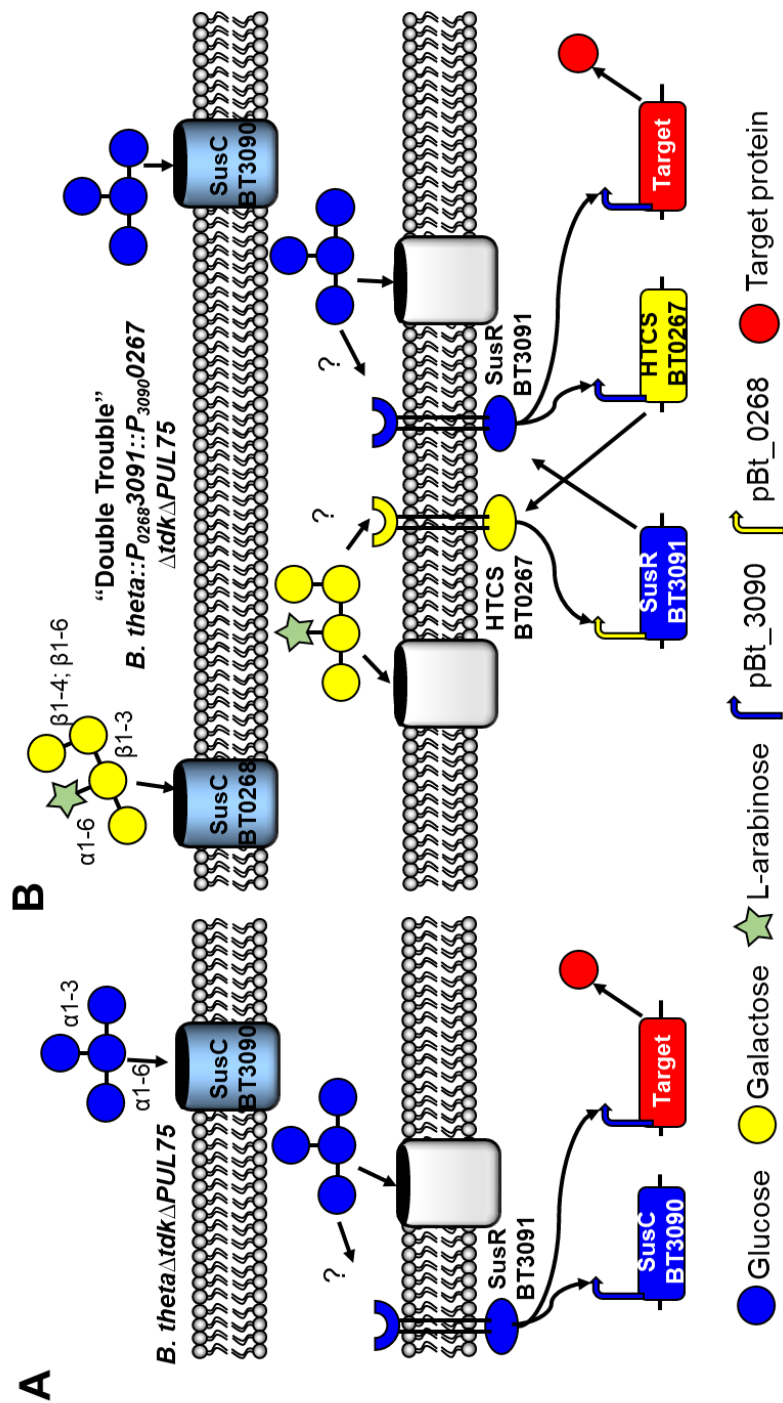


Figure 3.6: Regulatory *B. theta* strains used in this study for target gene induction. (A) Regulation of target proteins in *B. theta* Δ tdk Δ PUL75 VPI-5482 and (B) *B. theta*::P02683091::P30900267 Δ tdk Δ PUL75 Double Trouble in dependent on single dextran- and dual dextran/arabinogalactan-inducible promoter systems.

been engineered to be activated by arabinogalactan. These mechanisms create a dual-glycan expression system, which increases the fidelity and potency of transgene expression as induction has been observed to be increased thousand-fold over single-promoter systems [143].

3.6 Mammalian immunomodulators can be produced in *B. theta*

To evaluate the proficiency of protein production in *B. theta*, a total of 27 strains were constructed and sequence-verified (Table 2.2). Analysis by Western blots revealed that the single glycan responsive *B.theta* Δ tdk Δ PUL75 strains did not generate any detectable protein in the different fractions. A non-specific band corresponding to ~25 kDa present on Western blots was observed for all fractions and strains that likely represents a non-specific antibody interaction with a native *B. theta* protein. In contrast, three different constructs successfully produced protein in the Double Trouble strains. These included: *BtAnchor-elafin*, *BtAnchor-TNFR2*, and *BvSecrete-A1AT* (Figure 3.6A and B). Consistent with what was previously reported for the Double Trouble system, protein production was only visible in the presence of both dextran or arabinogalactan, but not when the carbohydrates provided as sole carbon sources [143]. Notably, protein was only observed when the lysate fractions were concentrated 50-fold, which was two-fold more than what was required to measure protein production in *E. coli*. No protein was observed in the medium (100X) of *BvSecrete-A1AT* culture (Figure 3.6 B). likewise, *BtAnchor-TNFR2* nor *BtAnchor-elafin* appeared to be localized to the outer membrane (Figure 3.6C).

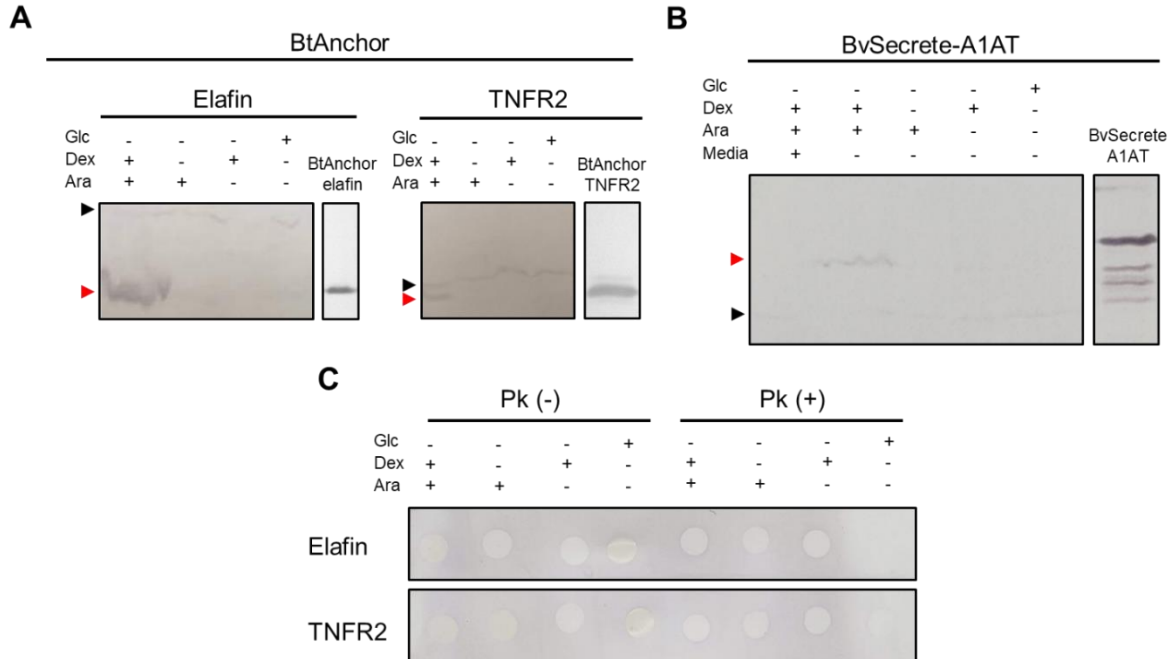


Figure 3.7: Protein production and localization of target immunomodulators in *B. theta* Double Trouble strains. (A) Production of *BtAnchor*-Elafin and *BtAnchor*-TNFR2. Protein signal of the immunomodulatory targets is only present in the “mixture” with both dextran and arabinogalactan as a carbon source. (B) Protein production of *BvSecrete* A1AT from the Double Trouble strain. Similar to other results, target protein was visible only in the dextran/arabinogalactan mixture. No observable target protein was localized to the medium (100X). In A and B the black arrow indicates a ~25kDa band which appears in all Double trouble” samples regardless of carbohydrate source. (C) Dot blot of *BtAnchor*-elafin Double Trouble and *BtAnchor*-TNFR2 Double trouble. No observable protein was localized to the outer surface of the cell wall. Legend as follows: G- glucose, A- arabinogalactan, D - Dextran, M – dextran and arabinogalactan mixture, + - positive control purified from Ni-NTA affinity chromatography, Pk – proteinase K.

Chapter 4 Discussion

Engineering bacteria to produce beneficial proteins is a promising avenue for the treatment of human and animal diseases. The use of colonic bacteria, such as *E. coli* and *B. theta*, to produce immunomodulatory proteins as therapeutics for intestinal inflammation expands on this concept. *E. coli* is a well-studied Gram-negative enteric bacterium that can be used for the production and purification of heterologous therapeutic proteins in mammals, including proteins of host origin [172]. *B. theta* is a stable colonizer of the colon in humans, chickens, mice, and pigs [139, 173]. Due to its abundance within the colon, and the recent surge of available genetic parts for protein production within this microorganism [139, 143], *B. theta* is a promising platform for therapy in the colon. In this thesis, the secretion and activity of immunomodulatory proteins A1AT, elafin, and TNFR2 produced in *E. coli* and *B. theta* was investigated.

Previously, Murine A1AT, Murine elafin, and Human TNFR2 have not been produced in *E. coli*; however, recombinant forms of murine elafin had been purified from yeast [174] and human cell lines [175]. Other studies had successfully used *E. coli* to produce human A1AT (63% identity to murine) with variable results [176], and human TNFR1, a homolog of TNFR2 [177], from the insoluble fraction. To build upon these findings and determine how the differences in sequence relatedness and biological source of these proteins impact their production efficiencies in a bacterial expression system required further study. In the current study, recombinant murine A1AT and elafin were not produced in *E. coli* at detectable levels, while TNFR2 was produced in low quantities in the insoluble fraction (Figure 3.2A-C). Protein production can be affected by a number of mechanisms, including disulfide bond formation and post-translational glycosylation

[178]. Both elafin [174] and TNFR2 [179] require the thiol side chain of cysteines (SH) to form covalent bonds with one another, which stabilizes protein structure; however, in the reducing environment of the cytoplasm, these bonds are prevented from forming [180]. To circumvent this, unfolded proteins can be secreted into the oxidizing environment of the periplasm, which promotes proper disulphide bond formation and protein folding [180]. In addition, protein glycosylation can impact protein folding, solubility, stability, and function. TNFR2 contains multiple sites of glycosylation, which have been shown to effect both its stability and binding [179]. Unfortunately, an unglycosylated control was not included to enable a direct comparison with the solubility results in this study. Both A1AT orthologues have N-glycosylation sites, although a lack of glycosylation has not been shown to affect human A1AT function or stability when produced within *E. coli* [176].

The addition of the N-SPs, *Bv*Secrete and *Bt*Anchor, increased yields of all three target immunomodulators in the soluble fraction (Figure 3.2A-C), underpinning that *Bacteroides* spp. derived N-SPs can aid in the production and purification of proteins in *E. coli*. In this regard, addition of N-SPs may represent an approach for increasing the yield of soluble protein for other proteins produced in *E. coli*. Also, all three immunomodulatory targets were secreted to the media when fused to *Bv*Secrete (Figure 3.2A-C), while *Bt*Anchor-elafin and *Bt*Anchor-TNFR2 were successfully localized to the outer membrane (Figure 3.2D). N-SPs target the nascent proteins for secretion into the periplasm where folding conditions, including a reducing environment and bacterial chaperones, promote proper disulfide bond formation [180]. Previously, leader sequences from the Gram-negative *Erwinia carotovora* (PelB) [181] and Gram-positive *Bacillus* spp. (G1) [182] have been included in heterologous protein expression plasmids designed for use in *E. coli* for

localization to the periplasm and/or medium. Recently, N-SPs from *Bacteroides* spp. were shown to be effective when expressing recombinant proteins in *E. coli* [183]. Recombinant production in *E. coli* does not always result in functional protein, therefore, it was necessary to develop and conduct biochemical assays to determine if A1AT and elafin inhibited neutrophil elastase, and if TNFR2 could bind to TNF- α .

The Enzchek® assay monitors the level of fluorescence that is produced when neutrophil elastase cleaves its substrate. In the presence of an inhibitor, a loss of activity is represented by a loss of fluorescent signal. Surprisingly, I observed that the pET-28a control sample was able to inhibit neutrophil elastase when used as a control (Figure 3.3B); however, this interaction appeared to be non-specific and likely resulted from contaminating *E. coli* proteins that eluted from the Ni-NTA affinity resin. *E. coli* is known to possess native bacterial serine protease inhibitors, including ecotin, a 16 kDa serine protease inhibitor that has inhibitory activity against trypsin and neutrophil elastase [184]. Therefore, protein samples were diluted upwards of 100X to dilute the effect of contaminating proteins and to generate dose response curves (Figure 3.3A). IC₅₀ values were estimated for *Bv*Secrete-A1AT (21 μ M), *Bt*Anchor-A1AT (15 μ M), and *Bt*Anchor-elafin (17 μ M). *Bv*Secrete-elafin appeared to have the strongest inhibitory effect on protease activity (200 μ M-5 μ M), which prevented the calculation of accurate IC₅₀ values. In the literature, the affinity of elafin for neutrophil elastase is estimated to be sub-nanomolar [185]. This result suggests that *Bv*Secrete-elafin produced in *E. coli* may be a promising candidate for future design of immunomodulatory bacterial strains or recombinant elastase inhibitors. The other proteins examined in this study did not demonstrate inhibitory activity above the background. Further efforts to improve their

yields and stability may help overcome these challenges and improve their potency. For example, A1AT irreversibly binds to elastase via a drastic conformational change in A1AT [165]. In this regard, its failure to inhibit neutrophil elastase may have resulted from the addition of a N-terminal peptide preventing the required conformational change.

To determine if recombinant TNFR2 could interact with TNF α , binding assays were conducted in solution and visualized by native gel electrophoresis. Under these conditions, the band corresponding to TNF α was not observed to shift when incubated with *Bv*Secrete-TNFR2 or *Bt*Anchor-TNFR2 (Figure 3.4B). However, some bands in the 100 μ M *Bv*Secrete-TNFR2 and *Bt*Anchor-TNFR2 disappeared when incubated with TNF- α . When visualized by native gel electrophoresis, the TNFR2 sample resolved as a complex pattern, which may be a result of protein stability or contamination with *E. coli* proteins. The disappearance of individual bands, however, indicates there may be an interaction with proteins in this sample with TNF- α . If interaction with TNFR2 and TNF- α is occurring it may be at a low concentration and be difficult to visualize by this method. Alternatively, TNFR2 stability and binding have been shown to be affected by glycosylation, modifications which would not occur in *E. coli* [179]. Therefore, proper binding of TNFR2 to TNF- α may be affected. As TNFR2 binds more strongly to trimerized, membrane-bound TNF α than soluble TNF α [186], more complex bioassays, such as cell lines expressing membrane-bound TNF α , may be required to provide physiological conditions suitable for binding. Alternatively, more sensitive techniques such as isothermal titration calorimetry (ITC) may be used. ITC is frequently used for the characterization of protein-protein interactions, and has previously been shown to elucidate the interaction of TNFR components with TNF α [187].

While protein expression and localization were observed in *E. coli*, the effect of the N-SPs on heterologous protein production in *B. theta* remained to be determined. The N-SPs fused to the proteins were native to *B. theta*, which may provide further benefits for the folding process. Also, *B. theta* colonizes the mammalian gut, which would provide long-term and stable delivery of these proteins to inflamed tissues in the colon. Therefore, three N-SP variants (*BtAnchor*, *BvSecrete*, and untagged) for each immunodulator were cloned into the pINT plasmids using two strategies, resulting in a total of eighteen *B. theta* chromosomal integration plasmids. The pINT plasmid was selected over other *Bacteroides* expression systems, such as pNBU2 (one-step chromosome integration) and pEP (episomal plasmid) for two main reasons: the pINT system enables the antibiotic resistance genes to be eliminated from the genome of *B. theta*, and chromosomal integration would help maintain the stability of exogenous genes throughout the lifecycle of the cell and through replication without the use of antibiotic selection. Genes of interest were incorporated into the PUL75 locus, which was deleted from strains used within this study to provide a recommissioned section of the genome for the expression of foreign genetic material without significantly affecting genome size. This approach was previously used to demonstrate function a heterologous reporter gene, NanoLuc [140]. Immunomodulatory targets were initially cloned behind the pBT_3090 promoter using restricted sites engineered into the amplicon and pINT plasmid (Figure 3.5B). This method has the advantage of being relatively quick; however, it results in extraneous sequence at the 5' end of the gene. Using this approach, there was not any detected protein in these strains. Concurrent studies suggested that the addition of the 5' 'ATG G' site significantly reduced gene expression in the reporter gene NanoLuc in the pINT construct (unpublished).

Although this result may be gene target specific, it prompted a second engineering strategy which was to fuse pBT_3090 directly to each target by overlapping PCR (Figure 3.5B). Fused products were integrated into the *B.theta*ΔtdkΔPUL75 and *B.theta*ΔtdkΔPUL75-Double Trouble recipient strains. The second strain was selected because it has previously been shown to increase protein levels in *B. theta* [143]. In this study, NanoLuc activity in the *B.theta*ΔtdkΔPUL75 strain was ~300-fold when strains were grown in dextran over glucose and ~1,100-fold when strains were grown in a mixture of dextran and arabinogalactan in the *B.theta*ΔtdkΔPUL75-Double Trouble strain. Additionally, this strain also provided greater control in regulating gene expression when induction relies upon the presence of two chemically distinct substrates from different biological sources [143], a novel feature for *Bacteroides* spp. expression platforms.

Of the twenty seven strains generated in this study, protein production was only detectable when *BtAnchor*-TNFR2, *BtAnchor*-elafin and *BvSecrete*-A1AT was expressed in the *B.theta*ΔtdkΔPUL75-Double Trouble strain in the presence of dextran and arabinogalactan (Figure 3.6A and B). Bands corresponding to purified protein controls were visible when cell lysates were 50X concentrated, which was approximately double the amount required for visualization in *E. coli* cell lysate fractions. This result is not surprising as *E. coli* BL21 has been optimized for hyperexpression and stability of heterologous proteins, including a viral T7 RNA polymerase/promoter system, and the deletion of proteases [188], whereas, *B. theta* has not. Although, there have been recent attempts to optimize protein production in *Bacteroides* spp. including optimized ribosomal binding sites and report proteins [20, 139]. Importantly, this marks the first-time mammalian proteins have been produced in the *B. theta*. In addition to the expected band,

an intrinsic protein band (~25 kDa) was observed in all strains and in all carbohydrate sources. This suggested that a constitutively-produced *B. theta* protein cross-reacts with the anti-His₆ antibody used in this study (Figure 3.6A and B).

While protein production was visible in the lysate of *B. theta*, there was not any indication the proteins were being trafficked to the outer membrane or into the media (Figure 3.6A-C). Although secretion of IL-2, TGFβ1, and KGF2 have been demonstrated in *B. ovatus* using the *B. fragilis* metalloprotease fragilysin N-terminal SP (SPII) [136-138], there has not been a report of secreting a heterologous protein from *B. theta*. For the *B. ovatus* proteins, secretion was very inefficient, with an estimated to be 300-800 pg/mL. The above strains also demonstrated efficacy *in vivo* indicating that protein concentrations around 300-800 pg/ml may be effective for inflammation mitigation. These concentrations fall below the detection limit of the α-His antibody used in my study [189]. Therefore, strains developed in this study may be producing undetectable, yet therapeutic concentrations of target immunomodulators. Alternatively, this may also reflect physiological differences in how proteins are trafficked in *B. ovatus* and *B. theta*. Recently, it was discovered that many lipoproteins from *B. theta* are released into the medium in outer-membrane vesicles which may lead to a novel target N-SPs designed for heterologous protein secretion [170]. In addition, annotation of *B. theta* secretion systems are currently incomplete and important components of the secretion system have not been identified and may be absent. For example, SecE, an essential membrane component of the SEC pathway in *E. coli*, has not been annotated in *B. theta* VPI-5482. Interestingly, SecE homologous sequences were found in *Bacteroides* spp., but contain only one of the three transmembrane stretches found in *E. coli*; although, this was found to be sufficient for

protein secretion [190]. Upon further analysis using TBLASTN [191], a sequence corresponding to a potential SecE component in *B. theta* VPI-5482 was found and compared to other *Bacteroides* species as well as outgroup sequence from *E. coli* (Figure 4.1). More research will be required to determine the differences in protein secretion in *Bacteroides* spp., insights which could be used to engineer systems for improved protein stability and trafficking yields.

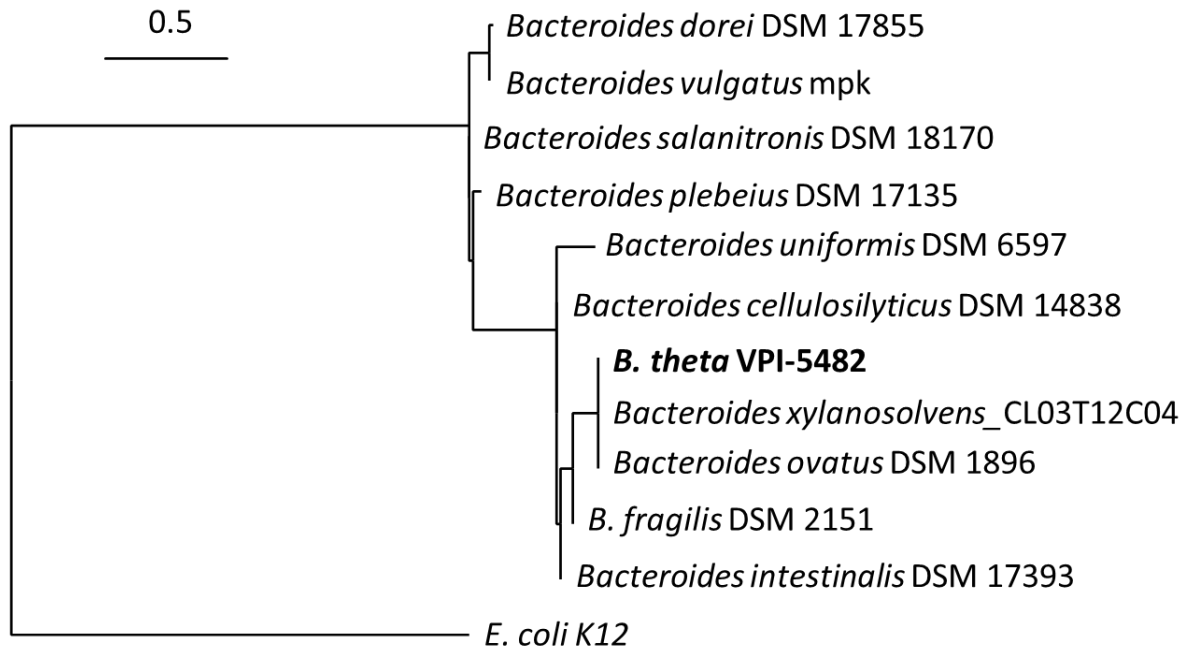


Figure 4.1: Phylogenetic tree of the SecE component of *Bacteroides* spp. Sequences of the SecE component from *Bacteroides* spp. were collected and the amino acid sequences were run against the DNA genome sequence of *B. theta* VPI-5482 using TBLASTN [191]. Sequences were aligned using MUSCLE [192] and the tree was generated using FastTree2 [193].

Chapter 5 Conclusions and Future Directions

In this project, nine plasmids were designed for the episomal production and secretion of mammalian immunomodulatory proteins from *E. coli*. Protein production and solubility of all three immunomodulatory proteins was increased with the addition of novel N-terminal *Bacteroides* spp. N-SPs. All three targets were secreted into the medium with *BvSecrete*, while elafin and TNFR2 were localized to the outer membrane of *E. coli* using *BtAnchor*. These N-SPs represent novel tools for the production and purification of proteins from *E. coli* systems. Importantly, *BvSecrete*-elafin demonstrated activity against neutrophil elastase, indicating this construct may be useful for mitigating inflammation. To determine if the immunomodulators could be produced in *B. theta*, twenty-seven strains were created. *BtAnchor*-TNFR2, *BtAnchor*-elafin, and *BvSecrete*-A1AT were successfully produced in *B. theta*, although no protein localization was observed. This project has demonstrated that the production of mammalian proteins within the colonic microorganism *B. theta* is feasible, and further work may lead to the successful generation of an immunomodulatory strain of *B. theta*.

5.1 Genetically modified organisms (GMOs)

Although this project represents a promising technique for immunotherapy, there are clear concerns which need to be addressed. The immune system is essential for organism health and some parts of it are in constant interaction with the environment. Acute inflammation is required for normal homeostasis of the colon, and there is a fine line between acute, self-limiting inflammation and chronic inflammation [194]. Interference

with immune regulation for therapeutic applications represents a valid concern, and engineered systems will have to be carefully designed and deployed in order to correctly stimulate beneficial immune responses. Additionally, developing biocontainment and biosecurity measures will be required to control the use of GMOs and mitigate public health concerns [124]. Public opinion on GMOs is mixed, with a 2018 Canadian survey indicating 37% of Canadians believe GMOs are safe, 34% believe they are unsafe, with the remainder unsure [195]. and although 44% of Canadians believe that we do not understand the potential health effects, over 50% believe that GMO testing and research is sufficient while 30% was unsure [195]. Therefore, that more education on the benefits and threats of GMOs is required; however, skepticism and scrutiny over they implementation should be on a case-by-case basis.

5.2 Future Directions

This thesis provided several conclusions that will assist in future efforts to produce mammalian immunodulator proteins in a bacterial system:

1. *Bacteroides* spp. N-SPs increased the production, stability, and trafficking of proteins in *E. coli*
2. Production of proteins within the *B.theta* Δ tdk Δ PUL75 Double Trouble platform represents a key stepping stone for future bioengineering strategies.

Moving forward, efforts to improve the production of immunomodulators in enteric bacteria could be invested in several ways.

1. Improve the Double Trouble system. New and more potent promoters from *B. theta* or other *Bacteroides* spp. could be discovered. Tuning expression of these promoters could be studied using other dual-glycan approaches. The response of

promoters within the host and during different stages of bacterial physiology could be studied.

2. Niche engineering and strain engraftment. Future work must include methods to engraft strains into the healthy microbiota. This be done through engineering the ability to compete with a unique nutrient [112].
3. Previously, BT_3090 induction has been shown to be increased within a biofilm community, which suggests that protein targets should be studied in more sophisticated systems, such as chemostats or animal models where bacteria exist predominantly in biofilms [196].
4. Screen other *Bacteroides* spp. N-SPs to determine their effects on protein production and/or localization. With genetic tools and engineering strains now in hand, production of other N-SPs and their effect on solubility and localization could be studied and ranked. Recently, it was discovered that a sub-set of lipoproteins in *B. theta* are trafficked to outer membrane vesicles as well as to the outer membrane surface [170]. The use of SPs that could localize protein targets to outer membrane vesicles may greatly increase the opportunity of proteins to interact with the colonic epithelium. Therefore, the exploration of novel genetic parts from *Bacteroides* spp. may further enhance protein production and secretion of heterologous proteins.
5. Investigate the secretion potential of other bacterial systems. *Lactococcus* spp. are Gram-positive microorganisms commonly used for bioengineering applications for the intestine. Protein production and secretion systems continue to be optimized in these microorganisms [197], they may represent a useful platform for

immunomodulator secretion into the GIT, albeit for short-term therapy as *Lactococcus* spp. have a transient nature in the colon [130].

6. Bioassay of *E. coli* BvSecrete-elafin and *B. theta* Double Trouble BvSecrete-elafin. The transient nature of *E. coli* strains within the colon does represent a potentially effective strategy for inflammation mitigation. As well, *B. theta* Double Trouble BvSecrete-elafin concentration may be therapeutic, even if was not detectable in this study. Therefore, Bioassays would provide increased verification of the therapeutic potential of these strains.
7. Attempt to codon optimize constructs for the expression host. Codon optimization can affect translation speed and errors, and can increase protein production in *E. coli* by up to 70% [198]. For example, elafin has been optimized for *B. theta* using Codon Optimization OnLine (COOL) (Figure 5.1) [199].

With many potential avenues to explore, including the use of novel *Bacteroides* N-SPs or improved promoter systems and regulatory systems, selection of alternative bacterial species, or optimization of coding sequences for bacterial expression systems, bioengineering of microorganisms to treating inflammation in the GIT remains a promising venture.

> Native elafin

GCA	GCT	GTT	ACT	GGT	GTC	CCA	GTC	AAG	GGT	CAG	GAT	ACC	GTG
AAG	GGT	CGT	GTT	CCT	TTC	AAC	GGT	CAG	GAT	CCA	GTG	AAA	GGC
CAG	GTG	AGC	GTA	AAA	GGT	CAG	GAC	AAA	GTT	AAA	GCG	CAG	GAG
CCG	GTT	AAA	GGC	CCG	GTT	TCC	ACG	AAA	CCG	GGT	TCT	TGC	CCG
ATC	ATC	CTG	ATC	CGT	TGC	GCG	ATG	CTG	AAC	CCG	CCG	AAT	CGC
TGT	CTG	AAA	GAC	ACC	GAC	TGT	CCG	GGC	ATT	AAA	AAA	TGC	TGC
GAA	GGC	TCT	TGC	GGC	ATG	GCC	TGT	TTT	GTA	CCG	CAA	TAA	

> Optimized for *B. theta* elafin

GCA	GCC	GTA	ACC	GGA	GTA	CCT	GTC	AAA	GGT	CAG	GAT	ACG	GTG
AAA	GGA	CGT	GTT	CCT	TTC	AAT	GGA	CAA	GAT	CCG	GTG	AAG	GGA
CAG	GTA	AGT	GTG	AAA	GGG	CAG	GAC	AAA	GTA	AAA	GCA	CAG	GAA
CCG	GTT	AAA	GGA	CCG	GTA	TCT	ACC	AAA	CCG	GGA	TCT	TGT	CCT
ATT	ATT	CTG	ATC	CGC	TGT	GCC	ATG	CTG	AAT	CCG	CCG	AAC	CGT
TGT	CTG	AAA	GAT	ACA	GAT	TGT	CCG	GGT	ATC	AAG	AAA	TGT	TGC
GAA	GGA	AGT	TGC	GGA	ATG	GCT	TGT	TTT	GTT	CCG	CAA	TAA	

Figure 5.1: Codon optimized sequence of elafin for *B. theta*. Optimization was done using COOL. Codon changes are highlighted in yellow; 50:98 codons were changed (51%).

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