Deleting *tonB4* and *tonB6* in *B. thetaiotaomicron*

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IINTRODUCTION

The Bacteroidetes phylum has been studied extensively because of its ability to digest a wide array of complex carbohydrates. This trait enables Bacteroidetes species to thrive in competitive environments, such as the human gut microbiome (McKee et al., 2021). Complex carbohydrates, also known as polysaccharides, are composed of a plethora of monosaccharide subunits, joined by different types of glycosidic linkages. Therefore, several highly-specific systems of enzymes are needed to completely digest polysaccharides and use them for primary metabolism (Grondin et al., 2017). Humans are only able to intrinsically digest a handful of dietary carbohydrates—simple starches, lactose, and sucrose, in particular. This fact has sparked interest in studying the Bacteroidetes species that inhabit the human gut microbiome and consume otherwise indigestible complex carbohydrates. These species provide many symbiotic benefits; for instance, they produce short-chain fatty acids through hydrolysis and fermentation of otherwise indigestible carbohydrates, which can then be absorbed and utilized by the epithelial cells of the gut.

A feature of the Bacteroidetes genome is the presence of polysaccharide utilization loci (PULs), a term used to describe localized, coregulated genes whose products orchestrate the detection, transportation, and digestion of specific complex carbohydrates (Grondin et al., 2017). The first such coregulated system was discovered in *Bacteroides thetaiotaomicron* (*B. theta*). *B. theta* is a prevalent bacterium in the human gut microbiome and has been characterized widely as a model organism for carbohydrate digestion. Several studies have identified eight coregulated genes which produce the proteins required for saccharification of starch oligosaccharides and polysaccharides. These eight genes were coined the starch utilization system (Sus), becoming the prototypical model for complex carbohydrate utilization.

All PULs encode proteins which are homologous to SusC and SusD (Grondin et al., 2017). SusC encodes an outer membrane-spanning TonB-dependent transporter (TBDT), the main channel through which oligosaccharides enter the periplasm. SusD encodes a cell surface glycan-binding protein (SGBP) which aids in recognition and initial binding of starch substrates, although substrate binding is often assisted by other SGBPs. Several CAZymes are responsible for breaking down the polysaccharides. Saccharification begins with the action of a cell surface-anchored endoglycanase—the resulting oligosaccharides move through TBDT into the periplasm, where several linkage-specific glycan hydrolases can act. The resulting monosaccharides can then be transported into the cytosol for metabolism.

The focus of this project is TonB, a protein involved in the Ton Complex. While the Ton Complex genes are not a part of the Sus loci, its products work in tandem with TBDTs and play an integral role in the translocation of starch (Ratliff et al., 2022). The Ton Complex is a molecular motor in the inner membrane of *B. theta* that uses a proton gradient to permit the active transport of starch oligosaccharides through TBDTs. The proton motive force is harnessed by ExbB and ExbD to create energy that is transferred to TonB. When energized, TonB pulls on the Ton Box, unfolding the plug domain of TBDTs. This conformational interaction creates the space needed for starch oligosaccharides to move through the channel of TBDT. This model is largely based on characterization of TonB in *E.* coli—further work is currently being done to characterize TonB in *B. theta*. In *E. coli,* TonB is anchored to the inner membrane and the Exb system by its N-terminal transmembrane helix (Figure 1). TonB has a proline-rich periplasmic domain that spans the entire periplasm, enabling its C-terminal domain to reach the outer membrane.

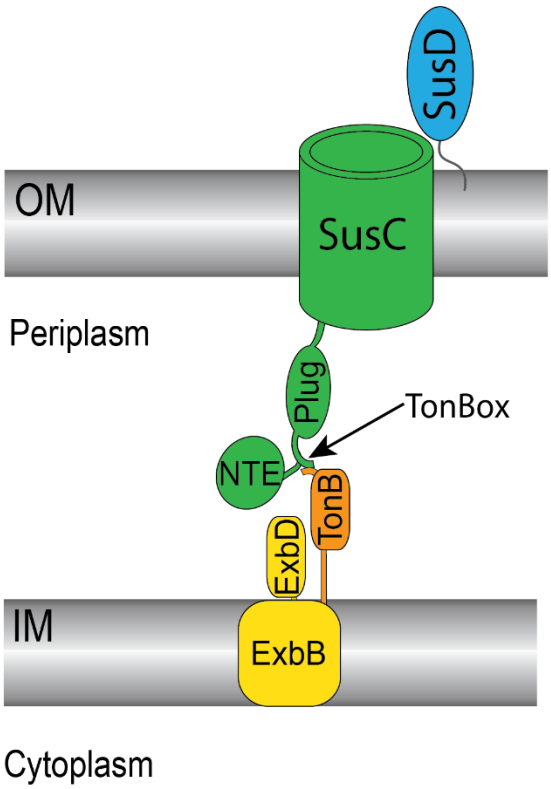

Figure 1. Predicted arrangement of SusC, SusD, and the Ton Complex. TBDT SusC is shown in green with its N-terminal extension domain and TonBox. TonB is in orange and is shown interaction with the plug domain at the TonBox.

Understanding the *tonB* genes and the overall starch utilization system of *B. theta* has greater implications for human health. First, by studying the intake system of dietary fibers, we are able to design more effective fiber supplements or functional foods that increase the relative abundance of beneficial bacteria, such as *B. theta* (Cequeira et al., 2020). By examining *tonb*, we are developing a fuller model of Sus, the intake system for starch. Second, an analysis of *tonb* in *B. fragilis* revealed that *tonB3*, one of its *tonB* homologs, is essential for pathogenesis in *B. fragilis* infections (Parker et al., 2022). Thus, TonB proteins of pathogenic bacteria may serve as potential targets in novel antimicrobial treatments, which has been made relevant by the rise of antibiotic resistance. By developing models of *tonB* in *B. theta* and other symbiotic microbes,

future studies will be able to design antimicrobial treatments that are selective for pathogenic microbes and have a minimal impact on beneficial bacteria.

This project is a deletion of two homologs of *tonB* in *B. theta.* Eleven *tonB* homologs have been identified by searching the *B. theta* genome for its C-terminal Pfam domain (Pollet et al., unpublished). The function of each homolog is unclear. Previous characterization of *tonB* homologs have been conducted in *R. anatipestifer* and *B. fragilis*. Characterization in the former concluded that each *tonB* product performs a different role (Liao et al., 2015); however, characterization in the latter concluded that a single *tonB* is responsible for the transport of several different polysaccharides (Parker et al., 2022). In-frame full gene deletions of each of the eleven *tonB* genes suggests that TonB4 is the main TonB protein involved in transport: deletion of TonB4 increased the lag phase of *B. theta* growth on potato amylopectin and other starch substrates. In the Δ *tonB4* strain, a large increase in the abundance of TonB6 was found in the cell membrane—the cells likely express *tonB6* to utilize starch after the prolonged lag phase. This suggests that TonB6 may act as a backup to TonB4, working with TBDTs in its absence.

Previous attempts to design a Δ*tonB4*/Δ*tonB*6 *B. theta* strain using a standard counter-selectable allelic exchange procedure have been unsuccessful. Introducing the second deletion through homologous recombination on existing Δ*tonB4* and Δ*tonB6* strains did not succeed (Pollet et al., unpublished). FudR was used to select for cells which have experienced the second crossover event and lost the suicide vector; none of the cells which had both deletions grew on FudR. This observation is evidence for a model in which either a functional *tonB4* or *tonB6* gene is necessary for *B. theta* cells to survive. In this project, we try a new system in which a plasmid with an inducible copy of either *tonB4* or *tonB6* is transformed into a deletion strain of the same gene. This allows *B. theta* to express a functional copy of either *tonB4* or *tonB6* while the other is

deleted through the same counter-selectable allelic exchange procedure. Given that this strain is able to grow, if expression of the inducible gene is turned off, and the cells continue to survive, there may be a third *tonB* homolog that is expressed. Thus, the research question of the project is: is it essential for either *tonB4* or *tonB6* to be expressed?

The first step of creating this double-deletion strain is to amplify and isolate the *tonB4* and *tonB6* genes from *B. theta* genomic DNA. Each gene is combined with the *pNBU2_erm_P1T_DP-GH023* plasmid (Lim et al., 2017). This plasmid integrates the gene behind an anhydrotetracycline(aTC)-inducible P1T_DP promotor, allowing for controlled expression of the gene insert. We use transform the plasmid into electrocompetent S17-1|*pir E. coli* cells through electroporation; the plasmid contains an *E. coli* RP4 mobilization region to maintain copies of the plasmid and an ampicillin resistance gene (*bla*) for selection in *E. coli*. Then, we conjugate the aTC-*tonB4* and aTC-*tonB6* plasmids into *B. theta* through homologous recombination. The plasmid contains an *E. coli* RP4 mobilization region to enable conjugation and an erythromycin resistance gene (*ermB*) for selection in *B. theta*. The NBU2 mobilizable transposon integrates the plasmid at one of two $tRNA^{ser}$ attachment sites in the *B. theta* genome. The last step is to introduce the second *tonB* deletion through homologous recombination, using the pExchange-*tdk* counter-selectable vector.

We were able to successfully construct four Δ*tonB*6/aTC- *tonB6 B. theta* strains. Three of the strains have inserted the plasmid at $tRNA^{ser}$ attachment site 2 (att2), and one has inserted the plasmid at attachment site 1 (att1). The project ended after attempting to delete *tonB4* on a Δ*tonB*6/aTC-*tonB*6 strain, while inducing expression of *tonB*6 using anhydrotetracycline. There are stocks of ten potential Δ*tonB*4/Δ*tonB*6/aTC-*tonB*6 strains, but screening needs to be done to confirm the deletion of *tonB4.* Generating this strain will help establish that *B. theta* is dependent

on the presence of either TonB4 or TonB6. If the deletion was successful, the next step of the project is to grow this Δ *tonB*6/Δ*tonB*4 strain on various substrates to explore the role of other TonB proteins. In the future, this strain can be used for further characterization of the TonB-TBDT interaction.

METHODS AND MATERIALS

Amplifying *tonB4* and *tonB6* gene flanks using B. theta genomic DNA

The *tonB4* gene was amplified the following forward and reverse primers: GAAATAAAGACATATAAAAGAAAAGACACCATGGAAGTTAAAAAATCACCCAAGGCAGAC (F), CTGTTCCATCACTGGAAGATAGGCAATTAGTTACTGCAATCTGAACATTACAGGAACTG (R). The *tonB6* gene was amplified the following forward and reverse primers: GAGACGAAATAAAGACATATAAAAGAAAAGACACCATGGAAGCCAAAAAATCAAAAAAG (F), CTGTTCCATCACTGGAAGATAGGCAATTAGCTACTGCAGTCTGAATACTACAGGTAC (R). Reactions included 10µL of 2x Phusion (ThermoFisher Scientific) Master Mix, 1µL of each of the forward and reverse primers at 10µM, and 1µL of B. theta genomic DNA as the template. Thermocycling conditions were as follows: 98°C for 30s, 98°C for 10s, 55°C for 15s, 72°C for 30s, 72°C for 120s. Steps 2-4 (denaturation, annealing, elongation) were repeated 35 times. Because Phusion has an elongation rate of 1kB/15s, a 30-second elongation cycle was used to allow for amplification of products up to 2kB. Gel electrophoresis was used to confirm the presence of 686bp products.

Amplifying pNBU2 plasmid backbone

The pBNU2 plasmid backbone was amplified with linear forward (CTAATTGCCTATCTTCCAGTGATGGAACAG) and reverse (GTGTCTTTTCTTTTATATGTCTTTATTTCGTCTCTATCAC) primers. Reactions included 10µL of 2x Phusion (ThermoFisher Scientific) Master Mix, 1µL of each of the forward and reverse primers at 10µM, and 1µL of the SGBP-B* pNBU2_erm_P1T_DP-GH023 plasmid from Foley et al. as the template. Thermocycling conditions were as follows: 98°C for 30s, 98°C for 10s, 55°C for 15s, 72°C for 75s, 72°C for 120s. Steps 2-4 (denaturation, annealing, elongation) were repeated 35 times. Because Phusion has an elongation rate of 1kB/15s, a 75-second elongation cycle was used to allow for amplification of products up to 5kB. Gel electrophoresis was used to confirm the presence of 4971bp products.

Cleaning PCR of amplified gene flanks and backbone

A PCR cleanup was conducted on the amplified *tonB4* and *tonB6* flanks and pNBU2 backbone. Procedures are adapted from a Qiagen Kit. For each sample, 80µL of Buffer PB (5 M Gu-HCl, 30% isopropanol) was used to bind 16µL of PCR products (5:1 buffer to PCR sample). 750µL of Buffer PE (100mL: 80mL 100% ethanol, 1mL 1M Tris HCl pH 7.5) were used to wash the column, and 30µL of water was used to elute. Products were quantified using a NanoDrop One (ThermoFisher Scientific).

Combining *tonB4* and *tonB6* with aTC-pNBU2 plasmid backbone

An NEBuilder® HiFi DNA Assembly Kit was used to combine the amplified *tonB4* and *tonB6* flanks and pNBU2 backbone. The *tonB4* reaction included 18µL of NEBuilder HiFi DNA Assembly Master Mix, 100ng (7.04µL of 16.1ng/µL) plasmid, 27.6ng (1.71µL of 14.2ng/µL),

and 1.25µL of deionized water. The *tonB6* reaction included 18µL of NEBuilder HiFi DNA Assembly Master Mix, 100ng (14.9µL of 6.7ng/µL) plasmid, 27.6ng (2.17µL of 12.8ng/µL), and 0.93µL of deionized water. Reactions were incubated in a thermocycler at 50°C for 15 minutes.

Transforming aTC-*tonB4* and aTC-*tonB6* plasmids into competent S17-1 l *pir*

A transformation into competent S17-1 | *pir E. coli* cells was done for both plasmids using the same procedure. 10µL of ligation reaction was dialyzed by leaving it on nitrocellulose membrane floating on distilled H_2O for 15 minutes. 3µL of ligation reaction was added to 100µL of *E. coli*. Cells were electroporated at 2.5kV and incubated at 37°C for 1 hour. Cells were plated on LB-Amp300 plates to select for transformed cells and incubated overnight at 37°C. A liquid culture was made with 10mL LB and 15µL of 200mg/mL Amp to be used for a glycerol stock.

Screening *E. coli* DNA for insertion of gene into plasmid.

An adaptation of QIAprep's Miniprep Kit was used to purify the plasmid DNA from S17-1 l *pir*/aTC-*tonB* cells—the same procedure was used for both the *tonB4* and *tonB6* strain. 250µL P1 Buffer (50mM Tris HCl pH 8.0 at ~25°C, 10 mM EDTA, 100 ug/mL RNaseA), 250µL P2 Buffer (200 mM NaOH, 1% SDS w/v), 350µL N3 Buffer (4.2M guanidine hydrochloride, 0.9 M potassium acetate pH 4.8), and 500µL of Buffer PE were used to resuspend, lyse, neutralize, and wash the cells. Cells were eluted with 40µL deionized water. Products were quantified using a NanoDrop One (ThermoFisher Scientific). The purified plasmids were amplified using forward and reverse primers for both the pNBU2 backbone and *tonB4* or *tonB6* respectively. The same primers and settings as before were used. Gel electrophoresis was used to verify that the *tonB* genes were inserted into the transformed plasmid.

Conjugating pNBU2 into *B. theta*

Overnight cultures of S17-1 l *pir*/aTC-*tonB4* were grown in LB and ampicillin at 37°C. Overnight cultures of *B. theta* Δ*tonB4* were grown in TYG at 37°C in the anaerobic chamber. The same two cultures were grown for *tonB6*. Cultures were sub-diluted to obtain similarly sized pellets. Pellets of each culture were washed with LB without Ampicillin, combined into 1mL TYG, plated on BHI + 10% horse blood agar plates, and incubated aerobically at 37°C for 24 hours.

Selecting for conjugated *B. theta* using Gent/Erm

The combined cells were removed and resuspended in 1mL TYG. 100µL of the resuspended conjugation and 100µL of a 10-fold dilution of the conjugation were plated on BHI-blood agar plates with gentamicin (Gent, 200µg/mL) and erythromycin (Erm, 25µg/mL) and incubated anaerobically at 37°C for 3 days. At this point, only Δ*tonB6*/aTC-*tonB6* strains grew. 6 individual resistant colonies of this strain were re-streaked on BHI-blood agar plates with Gent and Erm and incubated anaerobically at 37°C for 2 days. Single colonies of each single-recombinant were made into TYG stocks.

Isolating genomic DNA from Δ*tonB6*/aTC-*tonB6* strains

An adaptation of Qiagen's DNeasy Blood and Tissue Kit was used to isolate genomic DNA from each of the 6 Δ*tonB6*/aTC-*tonB6* strains .500µL of overnight culture for each strain were pelleted and resuspended in 50µL sterile PBS. 180µL of ATL was added and vortexed. 200µL of AL and 200µL of ethanol was added and vortexed. The mixture was centrifuged at 13xg for 1 minute. 500µL Buffer AW1 and 500µL Buffer AW2 were used for the first and second washes. 100µL of water was used to elute.

Screening Δ*tonB6*/aTC-*tonB6* for integration of plasmid strains at only one site

The NBU2 mobilizable transposon targets one of two $tRNA^{ser}$ attachment sites in B. theta. PCR reactions with primers that flank each site are run for each of the 6 Δ*tonB6*/aTC-*tonB6* strains. Attachment site 1 (att1) was amplified with the following forward and reverse primers: CCTTTGCACCGCTTTCAACG (F), TCAACTAAACATGAGATACTAGC (R). Attachment site 2 (att2) was amplified with the following forward and reverse primers: TATCCTATTCTTTAGAGCGCAC (F), GGTGTACCTGGCATTGAAGG (R). Reactions included 10µL of 2x Phusion (ThermoFisher Scientific) Master Mix, 1µL of each of the forward and reverse primers at 10µM, and 1µL of BtΔ*tonB6*/pNBU2-aTC + *tonB6* DNA. Thermocycling conditions were as follows: 98°C for 30s, 98°C for 10s, 55°C (att1) and 58°C (att2) for 30s, 72°C for 45s, 72°C for 120s. Steps 2-4 (denaturation, annealing, elongation) were repeated 35 times. Because Phusion has an elongation rate of 1kB/15s, a 30-second elongation cycle was used to allow for amplification of products up to 3kB. Gel electrophoresis was used to confirm the presence of products in only one of two reactions for each strain. Expected product sizes are approximately 1000bp (att1) and 500bp (att2).

Knocking out *tonB4* using *ΔtonB4* pExchange plasmid

A stock of *ΔtonB4* pExchange plasmid from Pollet et al. was transformed into S17-1 | *pir E. coli* cells using the same electroporation technique as before. This plasmid was conjugated into Δ*tonB*6/aTC-*tonB*6 using the same method as before. Six single-recombinant strains were grown overnight and made into glycerol stocks after Gent/Erm selection. 1mL of each overnight was pooled together, and 100µL of this solution was transferred to FudR plates to screen for the crossover. 500µL of PBS with 200ug/mL anhydrotetracycline was soaked into the plates to promote expression of *tonB6*. After 4 days of growth, 10 single recombinant strains were grown overnight and stocked.

RESULTS AND DISCUSSION

The goal of this project was to create a Δ*tonB4*/Δ*tonB6* strain of *B. theta* that contains an aTC-inducible version of either *tonB4* or *tonB6*. First, we worked on inserting the inducible gene into an existing deletion strain. Throughout the project, steps to create a Δ*tonB4*/aTC-*tonB4* strain and a Δ*tonB6*/aTC-*tonB6* strain were completed in parallel.

The first step of creating these strains was to amplify *tonB4* and *tonB6* from isolated *B. theta* genomic DNA, so that they can each be inserted into *pNBU2_erm_P1T_DP-GH023,* the plasmid that contains an aTC-inducible promoter. Amplification was successful, as verified by the bands of expected sizes around 700bp (Figure 2A). After conducting a PCR cleanup, DNA concentration was quantified at 14.2ng/µL for *tonB4* and 12.8ng/µL for *tonB6*.

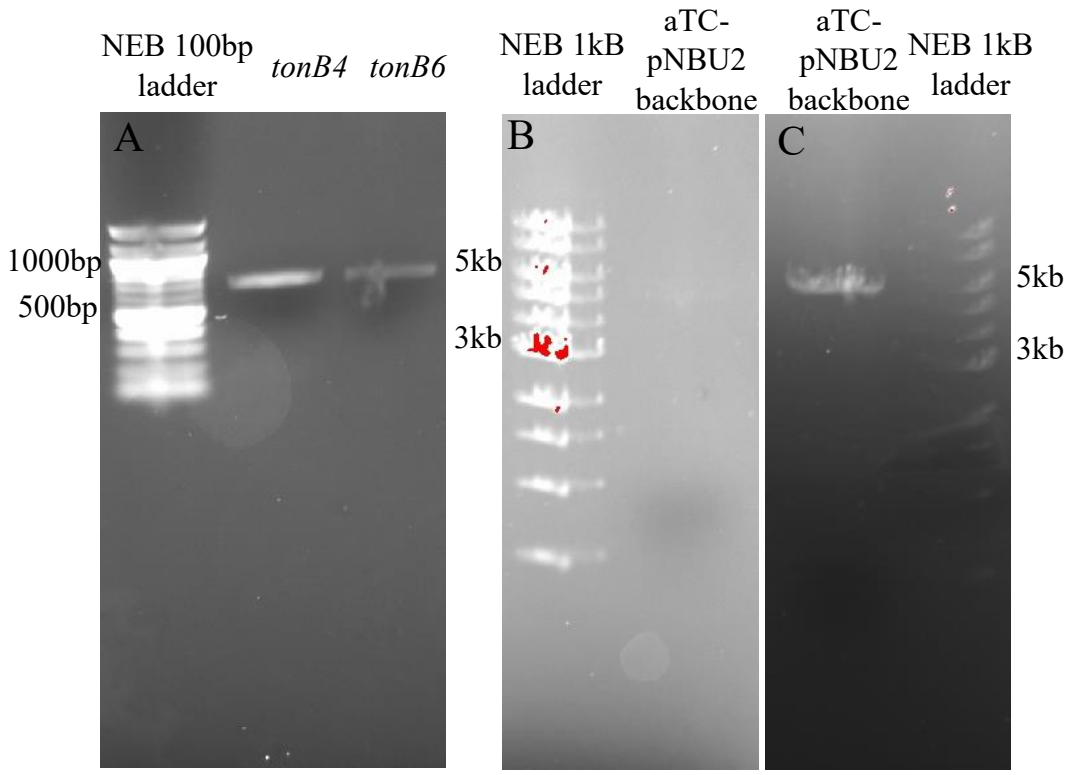

Figure 2. Amplification of *tonB4* and *tonB6* from B. theta genomic DNA (A) and amplification of aTC-pNBU2 plasmid backbone (B, C). All parts of the final transformation plasmid are amplified individually. *tonB4* and *tonB6* are 686bp and 691bp respectively. The plasmid backbone is 4971bp. DNA from the first backbone amplification (B) was used to create a plasmid with *tonB6*, and DNA from the second backbone amplification (C) was used to create a plasmid with *tonB4*.

The pNBU2-backbone was also amplified from a stock. In using gel electrophoresis to verify successful amplification, only a faint band was visible at the expected size of approximately 5000bp (Figure 2B). Quantification confirmed a low concentration of DNA at 6.7 ng/µL, which was not enough for the construction of both an aTC-*tonB4* and an aTC-*tonB6* plasmid. Another batch of pNBU2-backbone was amplified; this time, the band was much more visible, and the concentration of the new batch was quantified at 16.1ng/µL. It's unclear why the first amplification yielded such a low concentration of DNA, but it's likely that it was a result of a pipetting error during PCR setup.

The next step is to assemble the aTC-*tonB4* and aTC-*tonB6* plasmids using Gibson assembly. Since *B. theta* does not readily take up plasmids from the environment, the plasmids are designed to first be transformed into *E. coli* (Lim et al., 2017). The aTC-*tonB4* and aTC-*tonB6* plasmids are each transformed into S17-1|*pir E. coli* cells through electroporation. Since the plasmid contains an ampicillin resistance gene (*bla*), the cells were grown on LB-Amp plates to select for successfully transformed cells. DNA from each of these *E. coli* strains was isolated and amplified using *tonB4* and *tonB6* primers to ensure that the plasmids contained the *tonB4* and *tonB6* gene inserts. In the gel, bands at around 700bp confirmed successful assembly of the

plasmid and transformation into *E.coli* (Figure 3). Quantification corroborated this gel results with concentration of *tonB4* at 52.2ng/µL and concentration of *tonB6* at 52.3ng/µL.

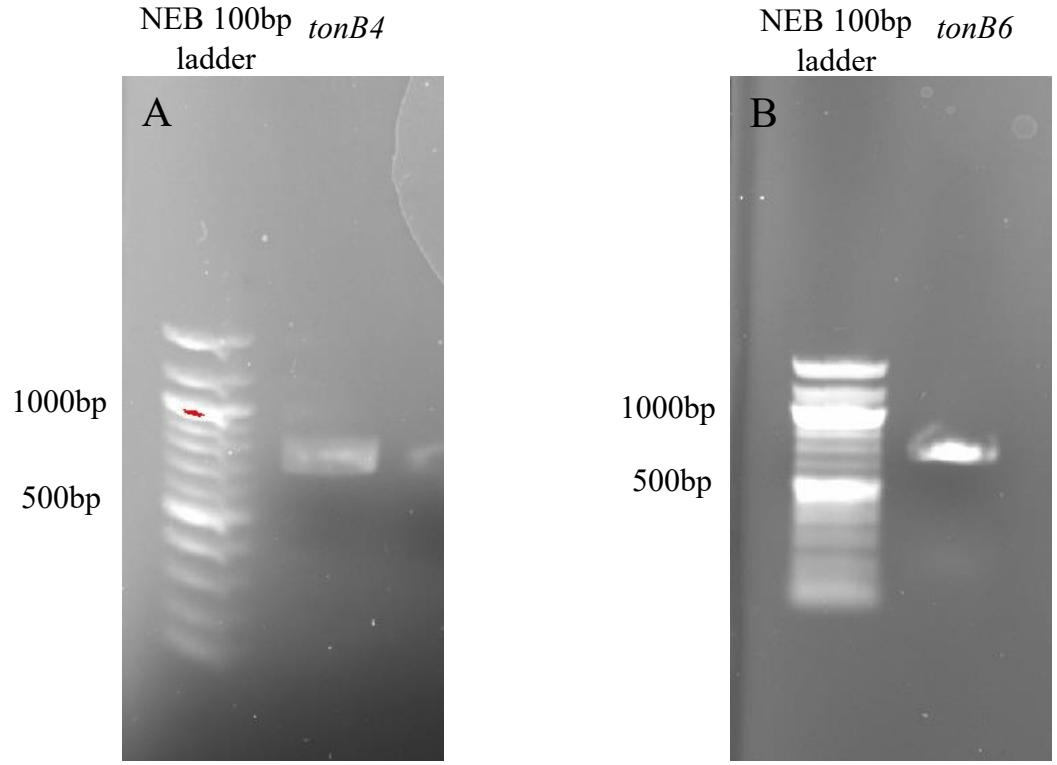

Figure 3. Amplification of DNA from transformed S17 *E. coli* with aTC-*tonB4* (A) and aTC-*tonB6* (B). Electrocompetent S17 *E. coli* cells were transformed with the new plasmids containing an Amp resistance gene and aTC-*tonB4* or aTC-*tonB6*. Successfully transformed cells were selected for by growing on Amp plates. Primers that flank *tonB4* and *tonB6* were used to amplify this DNA to check for insertion of the *tonB* genes into the plasmid.

To conjugate the plasmid into *B. theta*, *E. coli* aTC-*tonB4* was grown with *B. theta* Δ*tonB4*, and *E. coli* aTC-*tonB6* was grown with *B. theta* Δ*tonB6*. To select for successfully conjugated *B. theta* cells, the bacteria were grown on Gent/Erm plates anaerobically. *E. coli* does not grow anaerobically, but the pNBU2-backbone contains an erythromycin resistance gene (*ermB*) allowing B. theta to grow on these plates. The conjugation of E. coli aTC-*tonB6* with B.

theta *ΔtonB6* resulted in growth on Gent/Erm, suggesting the successful generation of Δ*tonB6*/aTC-*tonB6* strains; however, the conjugation of E. coli aTC-*tonB4* with B. theta Δ*ton*B4 did not. It's unclear why Δ*ton*B4/aTC-*ton*B4 strains were unable to grow, but since the Δ*tonB6*/aTC-*tonB6* strains were sufficient to move on, we did not make further attempts to make any Δ*tonB4*/aTC-*tonB4* strains. Six individual colonies of the Δ*tonB6*/aTC-*tonB6* strain, which represent single-recombinant strains, were re-streaked. Stocks were made from single colonies of each single-recombinant strain.

Screening of each of these single-recombinant Δ*tonB6*/aTC-*tonB6* strains is necessary to confirm a successful integration of aTC-*tonB6* into the genome. Even though selection was done by growing cells in Gent/Erm, *B. theta* is able to survive from free plasmids that haven't integrated into the genome, so screening is essential for moving on. The NBU2 mobilizable transposon of the pNBU2-backbone targets one of two $tRNA^{ser}$ attachment sites (att1 and att2) in *B. theta*. DNA from each of the six strains, as well as a control, is isolated and amplified using primers which flank each site. These reactions were run in a gel. If the att1 band matches the att1 band of the control, then the aTC-*tonB6* gene has integrated into the att1, and if the att2 band matches the att2 band of the control, then the gene has integrated into site 2. Initially, gel electrophoresis revealed that only site 1 amplifications were successful (Figure 4).

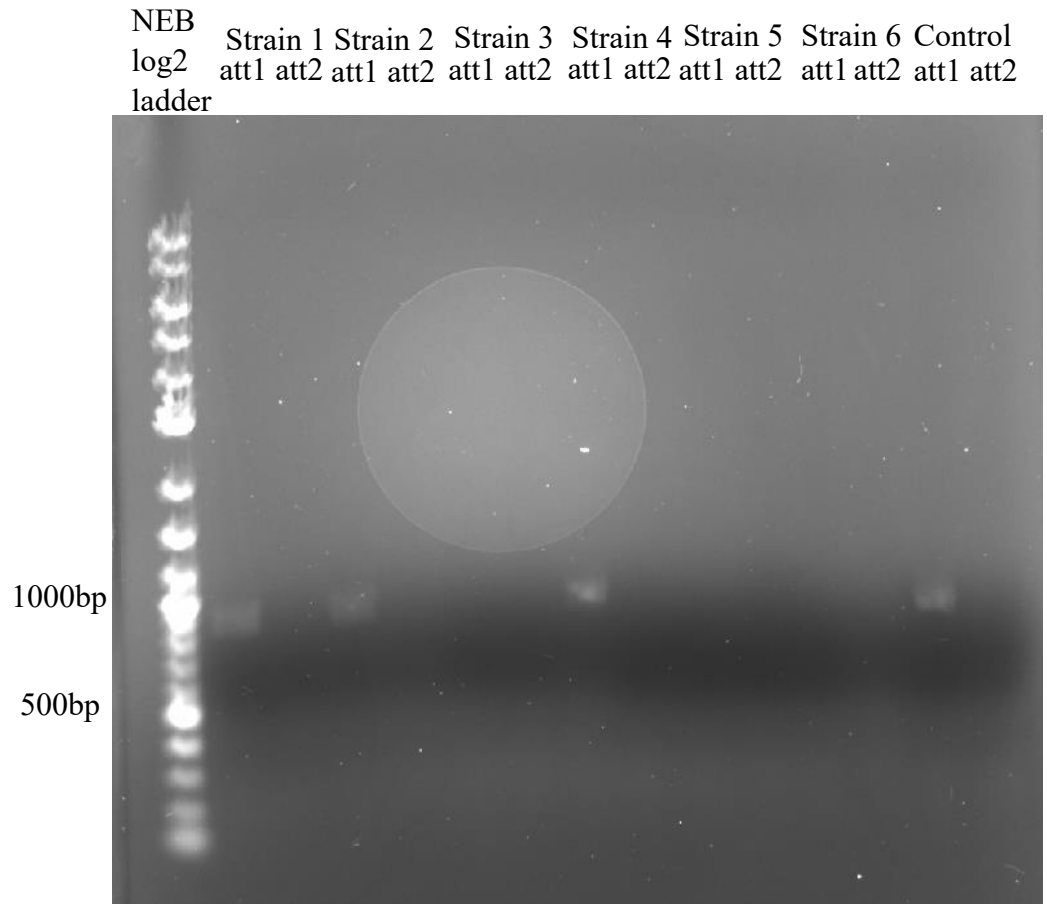

Figure 4. Amplification of DNA from B. theta control and each Δ*tonB6*/aTC-*tonB6*strain using NBU att1 and NBU att2 primers. The NBU2 mobilizable transposon targets one of two tRNA^{ser} attachment sites (att1 and att2) in *B. theta*. PCR reactions with primers that flank each site are run for all of the 6 *B. theta* ΔtonB6/aTC-*tonB6* strains. A successful conjugation strain has a band that matches the control for one of the two attachment sites.

Amplification of att2, even from the control, did not succeed–this indicated that failure of amplification was a result of a procedural error. After remaking the 10μM solution of both att2 primers, amplification was successful using the DNA from a *B. theta* control as the template (Figure 5). Higher annealing temperatures also appeared to promote amplification of att2 (Figure 5).

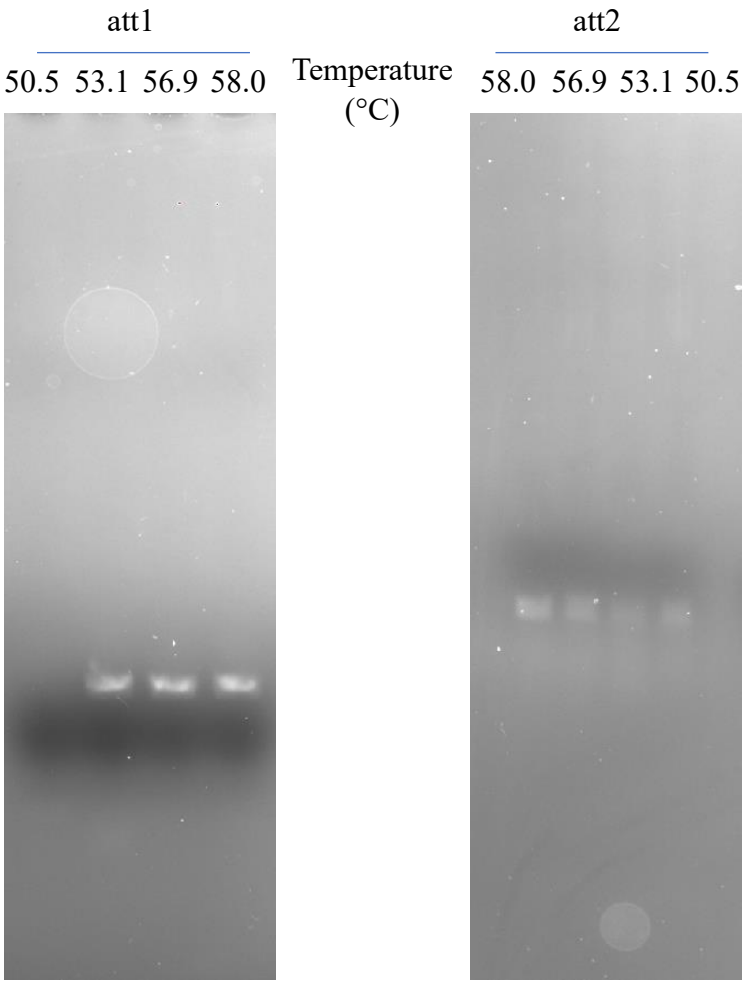

Figure 5. Amplification of DNA from *B. theta* control at different temperatures using attachment site 1 (A) and attachment site 2 (B) primers. The NBU2 mobilizable transposon targets one of two $tRNA^{ser}$ attachment sites (att1 and att2) in B. theta. PCR reactions with primers that flank each site are run for normal *B. theta* genomic DNA to find ideal annealing temperature for future PCRs.

Amplifications using a multi-temperature thermocycler yielded the best gels. After amplifying att2 using the new primer solutions and at an annealing temperature of 58°C, gel electrophoresis confirmed that four of the six Δ*tonB6*/aTC-*tonB6 B. theta* strains were successful (Figure 6). Three of the strains (1, 2, and 4) have integrated the plasmid at att1, and one (5) has

integrated the plasmid at att2. Strains 3 and 6 appear to have unsuccessfully integrated the plasmid. The faint band of att1 in strain 3 was a result of a pipetting error during gel loading.

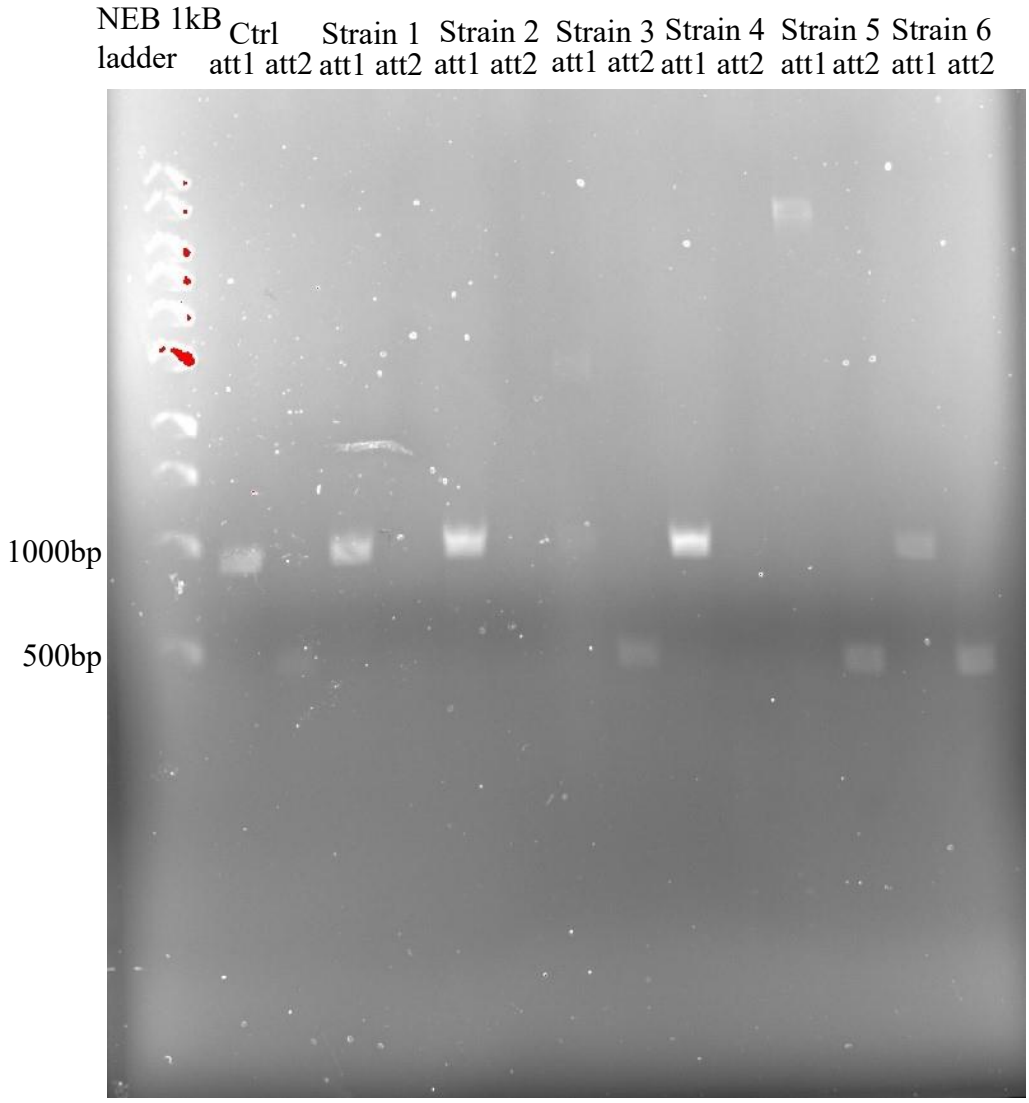

Figure 6. Amplification of DNA from B. theta control and each Δ*tonB6*/aTC-*tonB6* strain using NBU att1 and NBU att2 primers. The NBU2 mobilizable transposon targets one of two $tRNA^{ser}$ attachment sites (att1 and att2) in B. theta. PCR reactions with primers that flank each

site are run for all of the 6 Δ*tonB6*/aTC-*tonB6* strains. A successful conjugation strain has a band that matches the control for one of the two attachment sites.

The project ended after attempting to delete *tonB4* on a Δ*tonB*6/aTC-*tonB*6 strain, while inducing expression of *tonB*6 using anhydrotetracycline. There are stocks of ten potential Δ*tonB*4/Δ*tonB*6/aTC-*tonB*6 strains, but without screening, it's impossible to determine whether the deletion of *tonB4* was successful. If screening reveals that a strain had both *tonB4* and *tonB6* knocked out, then we will grow that strain on a medium without aTC. If these cells survive when expression of *tonB6* is turned off, the next step of the project is to grow this Δ*tonB6*/Δ*tonB4* strain on various substrates to explore the role of other TonB proteins.

First, we will grow the strain on glucose or maltose, as *B. theta* does not use the Sus system to transport any of these carbohydrates—the *tonB* deletions should have no effect. Next, we will grow the strain on starch substrates to look for an extended lag phase of growth. Its lag phase will be compared to other Δ*tonB* deletion strains. If *B. theta* is able to grow on starch, then another *tonB* homolog is likely compensating for the absence of TonB4 and TonB6. Last, we can use membrane proteomics to quantify the abundance of TonB homologs in the cell membrane: an increase in the abundance of another homolog suggests further redundancy in function.

In the future, this strain can be used for further characterization of the TonB-TBDT interaction. Understanding this interaction is important for building a fuller model of the starch utilization system in *B. theta*. Since Sus is the prototypical model of polysaccharide utilization loci in *Bacteroides*, building this model will pave the way for studies that investigate the role of proteins involved in the PULs of other bacteria. PULs in bacteria that inhabit the human gut microbiome are particularly relevant to human health. Studying these PULs enables us to engineer compounds which target these bacteria. One such compound is a dietary supplement

that helps increase the abundance of bacteria that help us make use of otherwise indigestible dietary fibers. Another is an antibiotic which targets the TonB proteins of pathogenic bacteria while being minimally harmful to the TonBs of beneficial bacteria (Parker et al., 2022); the presence of redundant TonB homologs in *B. theta* may allow it to survive while pathogenic bacteria are eliminated. This project is a part of a broader mission to understand the microorganisms that play vital roles in the human body.

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