Metabolic Engineering of Seaweed Polysaccharides in Therapeutic Human Gut Microbes

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I. Abstract

Probiotic bacteria often struggle to effectively colonize the human gut when administered as a medical intervention. Studies have shown that a predictive factor for whether a probiotic bacterial species will be able to effectively colonize a gut is whether or not it can occupy a carbon-utilization niche that is not already taken by a preexisting species. The carbohydrates porphyran and agarose found in red seaweed, such as nori, represents such a unique carbon source that is largely absent from the western diet. Research has shown that the colonization of porphyran-digesting gut microbes can be finely controlled by feeding porphyran to the host animal. This ability to tune and control colonization of the gut microbiome by engineering a synthetic metabolic niche could be broadly useful for many medical applications utilizing therapeutic gut bacteria to increase the efficacy and affordability of these treatments.

The aim of this project is to elucidate how the porphyran or agarose utilization metabolic pathway can be transferred to a phylogenetically distant gut microbe that is used therapeutically. A goal of this project is to engineer Escherichia coli to digest and utilize the carbohydrates found in red seaweed and engineer its ability to colonize the gut. E. coli is an easily cultured and genetically tractable model organism. Our approach is to engineer E. coli to metabolize porphyran or agarose by expressing and secreting novel porphyranase and agarase enzymes from seaweed-degrading bacteria, such as Bacteroides plebeius. We will express the enzymes and modulate expression of native metabolism genes in order to optimize E. coli for porphyran or agarose degradation. This optimization will be informed by genome-scale metabolic modeling and flux balance analysis.

We report initial results characterizing the enzymatic activity of the cloned porphyranases and agarases in E. coli. Enzyme activity was assayed using polyacrylamide carbohydrate gel electrophoresis (PACE) to identify oligomeric carbohydrate breakdown products and by quantification of reducing sugars using 3,5-dinitrosalicylic acid (DNS) colorimetric assays. We also report on the use of the hemolysin type I secretion system and the flagellar type III secretion system to secrete the porphyranase and agarase enzymes from E. coli.

II. Introduction:

The gut microbiome has been linked to an increasing amount of human illnesses and ailments including asthma, autism, autoimmune disease, some cancers, depression, anxiety, diabetes, inflammatory bowel syndrome (IBS), obesity, ect.[1] Currently the gut microbiome can be utilized for treatment through fecal microbiota transplants and genetically engineered drug producing bacteria. Treatment involving manipulation of the gut microbiome often runs into many challenges including the problem of wash out of the desired strain.[2] This means that the beneficial bacteria employed do not permanently colonize the gut due to competition with the microorganisms already established in the gut flora that efficiently utilize various substrates and metabolic niches. Recent advancements in research and accumulation of data on host-microbiome interactions through sequence based analysis have highlighted the importance of the human gut microbiome in health and disease. Host physiology, metabolic functions, immune system function, digestion and nutrition are some of the many integral systems which are dependent on the gut microbiome.[3] Due to the success of fecal microbiota transplant treatments, genetically engineered microbes for therapeutic purposes are a hot topic of research from pathogen elimination to drug delivery systems.[4]

One major factor in the success of a newly introduced strain of bacteria to the gut microbiome is carbon availability.[5] A possible route to combat this problem would be to introduce a strain which uses a carbon source unique to the current gut environment, therefore eliminating competition with the commensal gut bacteria. A benefit of this approach would also be that treatment using this strain can be fine tuned without impacting the current community of bacteria. This means that the treatment strain can be introduced and removed more easily.

In 2005, a novel gut microbe species was isolated from Japanese individuals for its ability to utilize a unique carbohydrate as a carbon source.[6] Porphyran is a complex, sulfated marine polysaccharide which can be isolated from culinary nori, otherwise known as red algae of the genus *Porphyra.* This species is well known for its use in many Japanese dishes including sushi. Species which are able to utilize porphyran have been shown to better engraft to the gut and outcompete gut bacteria which have already colonized. [7,8] Both porphyran and agarose are carbohydrates found in red seaweed which have structures made up of repeating galactose monomers.

Through gut metagenome analysis, it was shown that novel porphyranases and agarases are not present in the gut microbiota of North American individuals as they are in Japanese individuals. This is a crucial factor in the success of future engineered strain treatment success as it assures a unique metabolic niche in North American individuals.[9] The ability to access a unique carbon source allows for little if any competition for resources from already established microbes in the gut of an individual. More reliable and tunable colonization of medically administered bacteria in the human gut is necessary for any future treatment applications.

The goal of this project is to genetically engineer a strain of *Escherichia coli* that is able to break down and utilize porphyran or agarose as a carbon source. The future applications of this project involve using this strain to improve colonization or engraftment of future gut microbiome treatments by engineered *E. coli.* These experiments aim to produce a more user friendly strain which efficiently colonizes the human gut to carry out its functional purpose.

The techniques in synthetic biology and genome scale metabolic modeling will be used to accomplish the goal of engineering a synthetic metabolic niche in a laboratory strain of *E. coli* K12 MG1655, BL21 and the probiotic Nissle 1917 strain, to secrete, digest and utilize the carbohydrates found in red seaweed known as porphyran and agarose. This is important because it creates a unique carbon utilization niche allowing for better engraftment or colonization and persistence of engineered strains for possible treatments for human gut microbiome related ailments or diseases.

This will be accomplished by transforming genes from a novel polysaccharide utilization locus (PUL) found in a human gut bacterium *Bacteroides plebeius* into *E. coli* and modulating gene expression of the newly transferred genes and native metabolism genes. The ultimate goal of this project is to engineer an *E.coli* strain that can degrade and metabolize porphyran and the agar derivatives of porphyran. Computational metabolic analysis using flux balance analysis will be applied to estimate the growth rate, nutrient uptake and product secretion rates for the bacterium *B. plebeius* and the engineered *E. coli* strains in communities of common gut bacteria using MATLAB software. Wet lab experiments will be designed to construct and assay the strains informed by modeling predictions.

III. Literature Review

Discovery of porphyran utilization gene cluster in gut microbes:

In 2005, Kitahara et al. isolated gut microbes from Human faecal samples of Japanese individuals. They were able to isolate multiple Gram negative rods through culture dependent and independent methods. The 16S rRNA genes were sequenced and the species were categorized based on both phylogenetic and phenotypic qualities. Two novel strains stood out due to slightly varying sequencing results. Among those isolated was *Bacteroides plebeius,* an obligate anaerobe that can use a complex sulfated marine polysaccharide known as porphyran which is found in red seaweed.[6]

The genes necessary to break down and use porphyran were discovered to be in a specific cluster of genomic DNA, the polysaccharide utilization loci (PUL). Hehemann *et al*. were originally researching the diversity of marine glycosyl hydrolases of common marine organisms, seeking to characterize carbohydrate active enzymes that target unique marine carbohydrates. They isolated a marine bacterial species closely related to Bacteroides, *Zobellia galactanivorans* from red seaweed and analyzed its genome. There were five genes distantly related to agarases

and carrageenases that were in the glycosyl hydrolase family, yet they lacked critical recognition residues specific to agars and carrageenans.

They cloned and expressed these genes in *E. coli* to test for substrate specificity and only two of the genes produced protein for further experimentation. After testing the enzymatic activity of the proteins against a panel of marine polysaccharide, they only found activity on the porphyran found in red algae (Figure 1). Next they looked for sequence homology of these genes across all published genomes and discovered that *B. plebeius* most likely obtained its ability to utilize porphyran as a carbon source through horizontal gene transfer from marine Bacteroides related to *Z. galactanivorans*.(Figure 2)[10]

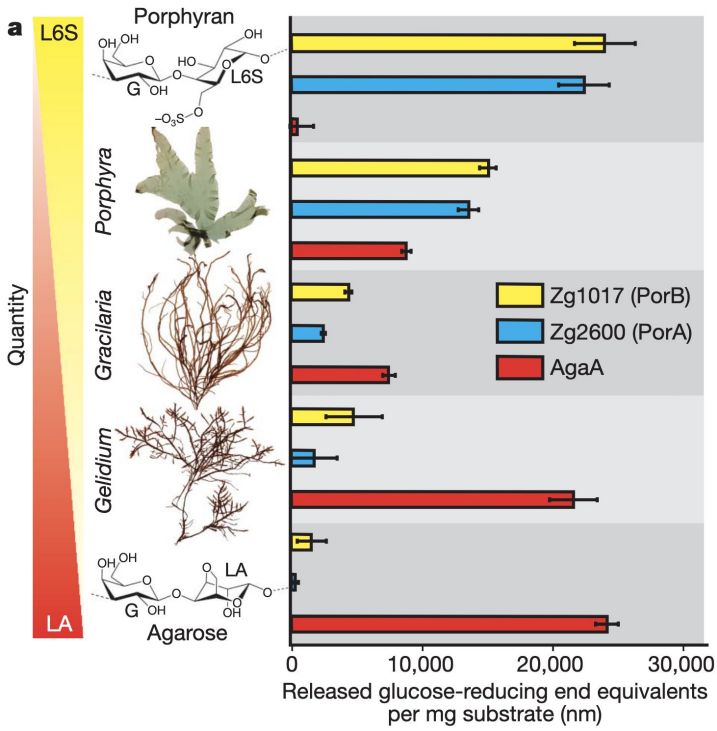

Figure 1. **Enzymatic activity based on 1689 (PorA) and 1693 (PorB) digests on crude algal extracts.** PorA and PorB protein from novel genes were screened on crude algal extracts and compared to AgaA; the species on which the enzymes were active are displayed. The bars show the amount of produced glucose-reducing end equivalents (n = 3, error bars represent mean ± s.d.). The activities on crude extracts are compared with that on pure porphyran (top) and agarose (bottom). Hehemann et al. (2010) [10]

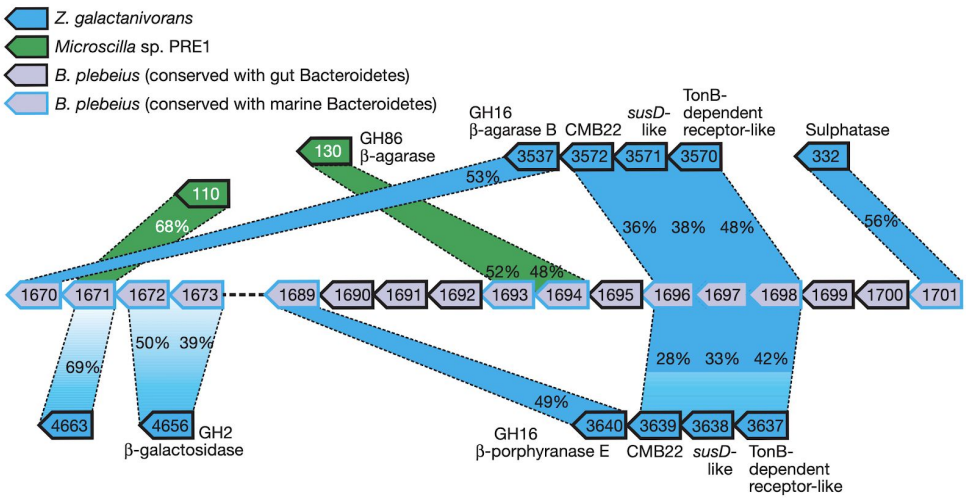

Figure 2. **Sequence similarity of porphyran utilization gene cluster between novel gut microbe and marine bacteria.** Shown is the sequence identity between *B. plebeius*, *Microscilla* sp. PRE1 and *Z. galactanivorans* proteins. Six of these genes (*Bp1670*, *Bp1671*, *Bp1689*, *Bp1693*, *Bp1694* and *Bp1696*) are conserved with marine bacteria, but are absent in the genomes of other gut *Bacteroides*. Hehemann et al. (2010)

A paper by Boraston *et al.* characterized two β- porphyranases encoded by the polysaccharide utilization locus from *B. plebeius*, and uncovered molecular details of porphyran hydrolysis. They wanted to understand the mechanism behind the degradation of seaweed galactans by human gut microbes. In order to do this, they tested the growth and enzymatic activity of 291 human gut Bacteriodetes on agarose, κ-carrageenan, and porphyran. This led them to discover that very few specific strains were capable of breaking down a specific complex carbon source. Using quantitative PCR, they saw that all 35 of the observed genes from *B. plebeius*'s PUL were activated in the presence of porphyran.[11]

<u>Porphyran utilization is a metabolic niche in the human gut microbiome:</u>

Through gut metagenome analysis, it was shown that porphyranases and agarases are present in the gut microbiota of North American individuals at a much lower rate than they are in Japanese individuals. They used the following criteria when analysing available genomic datasets of both Japanese and North American individuals: *E*-value $<10^{-15}$, score >50, identity >50% and also applied the Fischer's exact test to confirm the probability of porphyranase sequence occurrence in gut microbiota.[10]

Synthetically creating a unique carbon utilization niche gives a newly introduced engineered bacteria improved persistence in colonization of the host gut.[9] They also show improved strain engraftment due to transfer of a 21 gene fragment from *Bacteroides plebeius* PUL to a naïve Bacteroides strain, *Bacteroides thetaiotaomicron,* that could not previously use porphyran as a carbon source. This was also shown to improve strain engraftment or colonization in mouse model experiments. Controlled colonization has been shown by the Sonnenburg group

at Stanford University, they observed gut colonization of *Bacteroides ovatus,* a strain isolated for its ability to utilize porphyran as a carbon source.

This was accomplished by feeding each experimental mouse group a restricted lab diet for one week they equilibrated their gut flora. Then, human microbiota samples were introduced to some mice, others maintained the conventional mouse microbiota, and there were also es-germ free mice. Each was fed a diet containing porphyran to observe the abundance of exogenous porphyran utilizing strain. Tuning of colonization is shown by introduction of porphyran in the diet to mice with both a PUL+ and PUL- strain (Figure 3).[7]

The Alm lab at MIT used orthogonal niche engineering along with dietary intervention to show improved colonization of the species *B. plebeius* in mice with a porphyran supplemented diet. They were interested in testing the ability to control the growth of target organisms in order for future therapeutic applications. To accomplish this, they introduced *B.plebeius* as the constant for seaweed-using (+) bacteria and a strain incapable of using porphyran as a carbon source (-) to mice and supplemented their diet with either constant or sporadic doses of porphyran. They then observed the abundance of the bacteria over the short and long term to test how well the controllability through this mechanism would hold. Over the long term, control of (+) strain bacteria by introduction of porphyran was no longer effective meaning that if this mechanism were to be applied to medical intervention in the future, a fine tuned treatment regime would be necessary.[8]

<u>Genome scale metabolic in silico modeling for informed predictions</u>:

A recently established and quickly expanding tool in synthetic biology which serves great value is culture independent analysis through *in silico* modeling of microbial communities. We can estimate the success of an engineered strain of bacteria within a microbial community, such as the gut microbiome, using genome scale metabolic modelling and flux balance analysis in silico.[12]

The energy load needed for the engineered *E. coli* strain expressing genes from the *B. plebeius* porphyran utilization locus can be identified and the *E. coli* further manipulated to better handle the task of degrading porphyran. This theoretical computer model can be designed to predict complications with real world application of engineered strains such as competition for resources with common species of commensal gut flora or the impacts of new byproduct formation on the community.[13]

Metabolic pathways can be incorporated into these models and used to determine interactions between species and growth media such as rate of nutrient consumption.[14] We can better understand the mechanism of the porphyran degradation pathway to manipulate wet lab and growth experiments accordingly.

<u>Potential applications of non-Bacteroides species for gut microbiome engineering:</u>

Current bacterial species able to utilize this unique carbon niche have disadvantages for their use in human gut microbiome treatments. Most all to date are intestinal or marine Bacteroidetes which are complex organisms to work with. They are not well studied to date, making all culture techniques, experiments, and genetic manipulation more difficult. Culturing of obligate anaerobes and human gut microbes, such as *B. plebeius*[16] are inherently more difficult than that of *E. coli.*[17] Both *B. plebeius* and *E. coli* have an optimal growth temperature of 37°C which is ideal for the human gut environment.

E. coli is widely studied and much more genetically tractable. It is also a facultative anaerobic making it easier to work with right on a lab bench rather than in an anaerobic chamber. *E. coli* also grows at a much faster rate than that of *B. plebeius.* The nutrient requirements for *E. coli* are more simple,[18] which in turn causes culture media to be less specific and also relatively easier to make. *E. coli* has been shown to use a variety of different carbon and even nitrogen sources for growth,[19] this fact supports our hypothesis that it will be able to grow using the breakdown products from porphyran if given the genes to create and secrete the proper enzymes to degrade it.

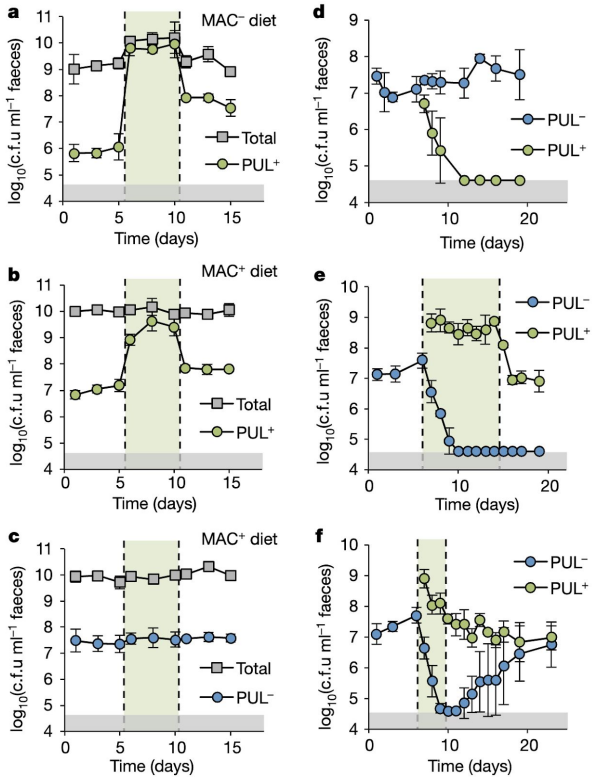

Figure 3. **Density of (PUL+)strain and a strain lacking the ability to utilize seaweed polysaccharides (PUL-), and total culturable anaerobes (Total) in feces of conventional mice.** Periods of administration of porphyran in drinking water indicated by green shading (**a–c**: 1% w/v (maximum possible), **d–f**: 0.1% w/v (to induce 1-log above PUL+)). **a**, Mice colonized with PUL+ and fed a MAC-deficient diet or **b**, MAC-rich diet demonstrated toggling of strain abundance upon administration of porphyran. **c**, Mice colonized with PUL- and fed a MAC-rich diet showed no change in strain abundance with administration of porphyran. **d–f** Mice eating a MAC-rich diet were colonized with PUL− for 6 days, and challenged with PUL+ on day 6. **d**, PUL+ is excluded by PUL− in the absence of porphyran. **e**, PUL+ displaces PUL− with access to porphyran for eight days. **f**, PUL+ and PUL− stably co-exist after a three day pulse of porphyran. Error bars indicate standard deviation, n=4 for all experiments. Gray shaded boxes represent limit of detection Time (days). Sonnenburg et al. (2018)[7]

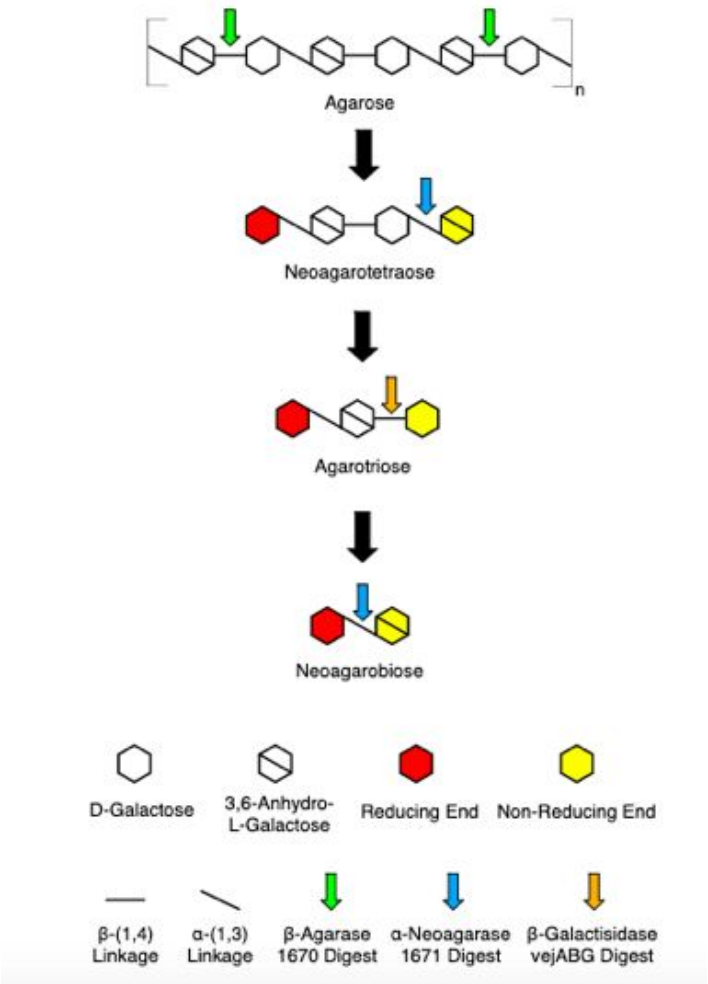

Figure 4. Degradation pathway of agarose by agarases 1670 (β-agarase), 1671 (α-neoagarase), and vejABG (β-galactisidase). The colored arrows indicate the location where each of the enzymes cleaves and the black arrows show the breakdown product resulting from the enzymatic digest.

IV. Methodology:

Safety training was completed in preparation for the experiments performed and was maintained through the project duration including: laboratory safety training, hazardous waste management training, autoclave use and procedures, biological safety, and fire safety training. Experimental techniques that were required for this project include: anaerobic and aerobic culturing methods, sterile technique, media preparation, plasmid construction and purification, polymerase chain reaction (PCR), primer design using the Snapgene software, Golden Gate assembly, heat shock transformation, both agarose and SDS page gel electrophoresis, gel extraction, sample sequencing, protein purification.

The freeze-dried ampoule of *B. plebeius* DSM 17135[15] was inoculated into liquid culture in/on strain specific media in anaerobic conditions at 37°C for 72 hours. The media was made following recipes provided by DSMZ for Tryptone Yeast Glucose (TYG) media, Chopped Meat media, Sheep's Blood agar (SBA), ect.[15]. The cells were boiled in order to lyse them and collect the genomic DNA using the Qiagen Gram positive DNA isolation kit. Cells were collected from a liquid culture, which was pelleted by centrifugation for 10 minutes at 3,000 rpm in a tabletop microcentrifuge. PCR using primers designed to amplify porphyran utilization genes including gene 1693 encoding for porphyranase A and gene 1689 encoding for porphyranse B. Gene 1670 (agarase A), gene 1671 (agarase B) and *vejABG* (agarase) were ordered and synthesized from Twist due to culturing issues with the *B. plebeius* strain .

PCR and colony PCR (cPCR) products were analyzed using agarose DNA gel electrophoresis and imaging. Golden Gate/Type IIs DNA assembly was used to clone desired genes (e.g. porphyranases and agarases) into plasmids with alternating antibiotic markers, type I secretion tags, type three secretion system genes, Histidine tags, and flexible linker sequences. Ribosome binding site tuning using the Ribosome Binding Site calculator[22] were incorporated at three translation rates (1000, 10,000, and 100,000) Snapgene software was used to create and visualize expression constructs for these genes of interest.

Heat shock transformation was used to transform assembled plasmids into *E. coli* K12 DH5A, DH10B, MG1655, BL21 and probiotic *E. coli* Nissle 1917. The samples were sent out for Sanger sequencing to further analyze and confirm results. Glycerol stocks were made of the successful strains. These strains were first induced using IPTG for expression of the proteins without coexpression of the secretion system *hlyBD*. The cells were then lysed using the BPer lysis kit to obtain whole cell protein content and test for enzymatic activity using the 3,5-dinitrosalicylic acid (DNS) assays to quantify abundance of reducing ends produced in enzymatic digestion of the polysaccharides porphyran and agarose (Figure 6,7).

The lysate was then used in a nickel column purification of the His-tagged enzymes. The extracted protein was also used to run SDS-PAGE gels to determine if there is a band at the correct size for the proteins of interest (Figure 9). After enzymatic activity was confirmed from cytoplasmic expression, secretion was attempted by induction of expression of both the enzymes (IPTG) and secretion system (aTc) to confirm secretion and activity of the enzymes.

The crude porphyran extraction protocol[7] from raw culinary nori (red seaweed) was repeated and optimized for use in enzymatic activity assays until purified porphyran became commercially available . This was done by autoclaving a 5% w/v solution of dried nori with water, doing an ethanol precipitation and using dialysis tubing to separate the desired large marine polysaccharide product. To determine porphyran and agarose degradation activity, polyacrylamide carbohydrate gel electrophoresis (PACE) and Thin Layer Chromatography (TLC)[20] (Figure 8)against carbohydrate standards were used. Characterization of the enzymatic activity of his-tagged secreted proteins, including porphyranases and agarases, purified from media using the Thermo scientific spin purification protocol. Sodium dodecyl

sulfate–polyacrylamide gel electrophoresis (SDS-PAGE gels) was used to analyse the products (Figure 9).

A project aimed towards creating a genome scale metabolic model using flux balance analysis in MATLAB software was started in order to make modeling predictions which would guide the wet lab experiments. A literature search for enzyme kinetics of the porphyran degradation pathway to incorporate into the metabolic model was being conducted. Computational modeling in a constraints based model were to be applied to estimate the growth rate, nutrient uptake and product secretion rates for the bacterium *B. plebeius* and the engineered *E. coli* strains in communities of common gut bacteria. Functions were included in which you could manipulate variables of nutrient availability and consortia to observe changes in growth rate and determine the significance of the presence of specific molecules and enzymes in growth media/ environment.

V. Results

Genetic Construction:

Enzyme expression constructs were created by hierarchical assembly. 1670, 1671, *vejABG*, 1689, and the *hylBD* secretion genes were synthesized by Twist biosciences, while 1693 was successfully PCR amplified out of *B. plebeius* genomic DNA. These genes were then cloned into the pNH6 plasmid backbone that served as the starting point for future assemblies. Different versions of these genes were cloned in the pNH6 backbone by iPCR and type IIs assembly that had 6x His tags and secretion signals at the N- and C-termini. The pNH6 versions of these genes were then assembled by IIs assembly into part plasmid backbones with RBS variants of different strengths using annealed oligonucleotides to create the various RBSs. These part plasmids were then cloned into transcriptional unit backbones with a promoter (P_{Tac}) and then cloned into the expression plasmid pAN871 either with or without the *hlyBD* secretion system.

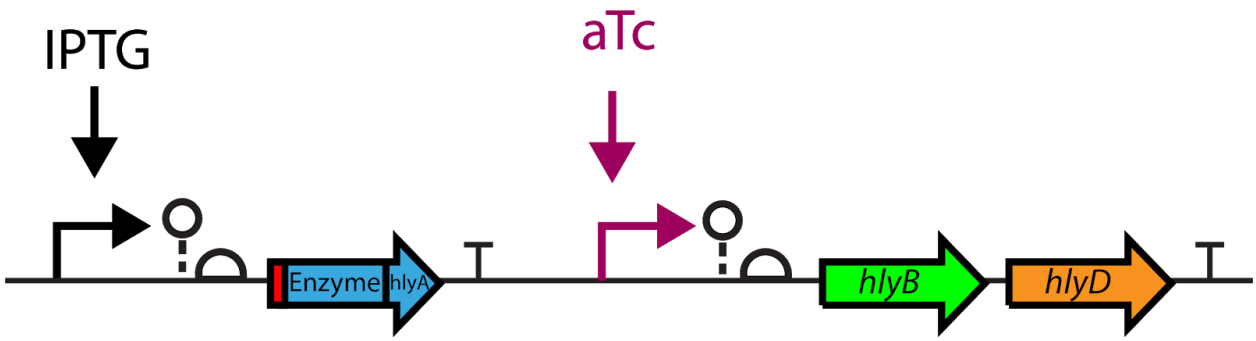

Figure 5. Genetic construct using Histidine tag fusion to each of four enzymes (porphyranases and agarases) with type I secretion system.

Gene	Ribozymes	RBS 1000	RBS 10000	RBS 1000 w/ His	RBS 10000 w/ His
1693	RiboJ10	CACTGAGCCCAAGTTAACGACTATACACC	AACAAATTTTAATCGGAGGACATATTG	TACCCTTACCACCACTGGGCGTTCT	TATCCAGGAAGGAGCGTCATTGT
1689	RiboJ53	AAGGGACCGCTCCAAACCTAGGAACTTA	ATCGAAGAAGGAGGTATTACT	CTCACATCAAACCAGGAAGATTAATA	CGGCATCAGCACAACAAATTAACACGTAATTCATACTCCCATATCAAACAATCCATAGGTTTTTT
1670	RiboJ51	TAATAGGAACGGCCCGAAC	TACAACGGAGCGGGGAAGGTACTCA	AGTTATCGAAAGATCCGAGGAATAACTTAACT	TCGCCACGCCCATTCAACGCCAGGGAGGGATTTG
1671	riboJ	AGAAATTCAACGGAATAACTCGAT	AGATTGACTCCGGGGGTTACAGTAC	TTGTTTCGCTAGCACCTGGGGAGGCATTGT	TTTGGTAACGTACGCGGGAAGGTAAT

Figure 6. Sequence of ribosome binding site variants used for each gene of the enzymes. Oligonucleotides were ordered from twist and annealed, then incorporated in the genetic construct.

Genetically Engineered Strain Microscopy:

Each strain was then cultured in *Luria Broth* (LB) for 12 hours and back diluted to an estimated optical density of 0.01, incubated for 2.5 hours and induced with IPTG for 3 hours. The BL21 strain cells for 1670, 1689 and 1693 enzymes showed no noticeable signs of toxicity. The microscopy images of the 1671 agarase gene showed possible toxicity. After running the sequence of the gene through a signal peptide detecting program, a possible gram negative signal sequence was identified. The truncated version of the 1671 gene was incorporated into the new cloning and showed improved cell health(Figure 7)[21].

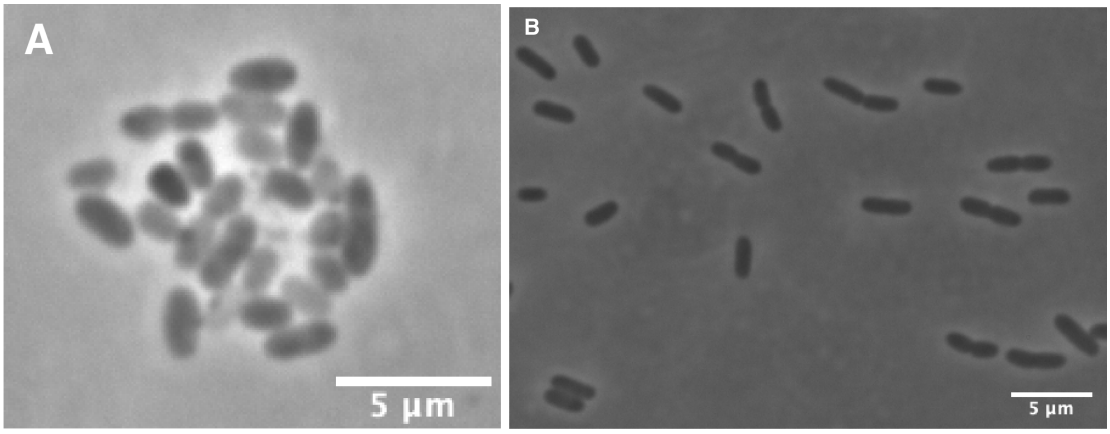

Figure 7. Microscopy showing difference in cell health between regular and truncated version of 1671 agarase gene. (A) *E.coli* K12 DH10B with full length version of agarase (1671) gene. (B) *E. coli* K12 DH110B with truncated version of 1671.

Enzymatic activity assays:

Enzymes collected from cell lysate of BL21 strains were used to digest 0.1% solutions of porphyran and agarose overnight. After conducting a DNS assay (Figure 8) optical densities were recorded to quantify the amount of reducing ends. Increased reducing ends were observed by agarase 1670, the truncated version of 1671 (Figure 9), and porphyranases 1689 and 1693. The vejABG agarase enzyme was not functional, possibly due to a mutation in the DNA sequence or improper folding of the protein product. The exact optical density measurements for each were recorded however were not accessible at this time.

TLC was used to visualize the size of the enzymatic digestion products (Figure 10). Results were inconclusive, the control standard D-galactose was expected to travel past the larger sized breakdown products because it the monomer of these structures, however this was not observed in any of three TLC experiments. This could be due to an incorrect development solvent for the specific qualities of agarose and porphyran digested products. An SDS-PAGE gel of the protein product collected from the cell lysate of BL21 strains for each enzyme was run and also yielded inconclusive results. This could have been due to improper purification of the enzymes of interest.

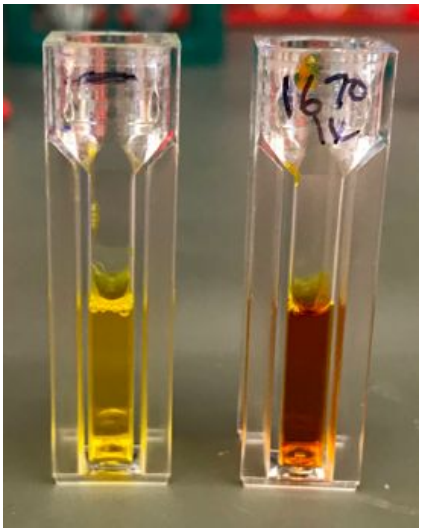

Figure 8. Example of DNS assay showing positive result for enzymatic activity by digest of 0.1% agarose solution from 1670 strain lysate. Shown is a negative control (left) compared to an overnight digest of agarase 1670 on agarose. The darker color indicates the presence of reducing ends which are a result of enzymatic cleavage.

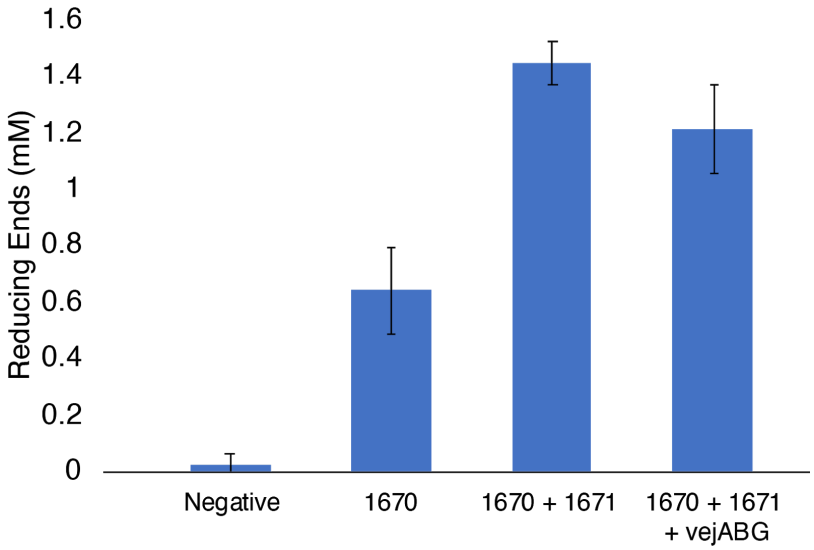

Figure 9. Results of DNS assay for agarase enzyme digests. This shows enzymatic activity of both agarase 1670 and 1671 in combination. There was not increased reducing ends created with the addition of vejABG agarase which was not expected. We can conclude that this enzyme is not functional possibly due to a genetic mutation or a misfolding of the protein product.

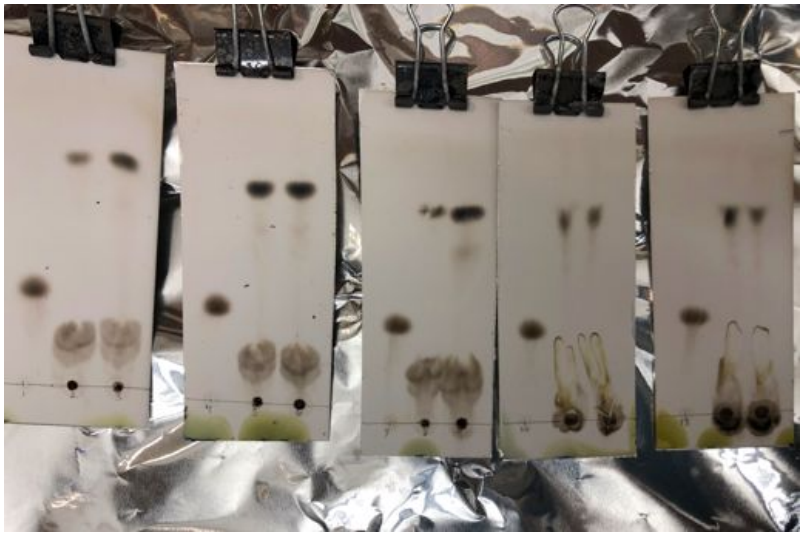

Figure 10. Thin Layer Chromatography (TLC) of agarase and porphyranase overnight digestions on agarose and porphyran. Silica gel plates were spotted with 20 µL of each sample 2 µL at a time and the far left lane of each plate was 2 µL of concentrated standard D-galactose. Plates were run for about an hour in 3:1:1 water: acetic acid: somthing running buffer and then developed with 10% sulfuric acid.

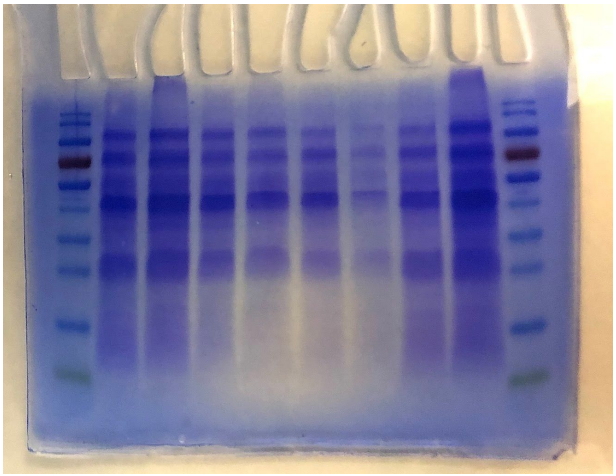

Figure 11. Agarose and porphyran enzymatic digest products. SDS-PAGE gel with coomassie stain. The information on which sample is in each lane was not accessible at this time. Without knowing which enzyme was being purified and indistinguishable significant bands due to improper separation, this experiment was unsuccessful.

VI. Conclusions

Through these experiments it was shown that these strains of genetically engineered *E. coli* can produce functional agarase (1670, truncated 1671) and porphyranase (1689, 1693) enzymes. Transfer of a unique seaweed carbohydrate metabolism from a human gut microbe into *E. coli* for improved colonization of a future probiotic still seems viable. Based on observations of the brightfield microscopy and enzymatic assays from cell lysate of the expression constructs transferred into Nissle 1917, the constructs will need to be tailored more specifically to result in the same level of functionality from the BL21 engineered strains.

The future course of experiments for this project is clear to achieve the goals of the project. They include the products of enzymatic digestion by the porphyranases and agarases to be analyzed against carbohydrate standards using the PACE method. Agarase vejABG will be re-evaluated to troubleshoot the lack of functionality observed for this enzyme. Once it is made functional, all of the enzymes for agarose digestion will be purified and added into minimal media with agarose as the sole carbon source to test if *E. coli* can grow on the breakdown products.

Complete cloning for transfer of the porphyran or agarose metabolism into Nissle 1917 and MG1655 will be conducted. Experiments with transfer of enzymes needed for agarose digestion will be conducted for the gram positive short peptide quorum sensor *Bacillus subtilis*. This organism has been used for probiotic purposes in previous literature and is well known for producing and secreting relatively large amounts of protein as compared to *E. coli*. *B. subtilis* could also possess transport proteins that could import oligosaccharide breakdown products of agarose instead of being limited to monomers. It is also a genetically tractable species similar to the reasons for having *E. coli* as the target species originally.

VII. References

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