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# Investigation of glycoside hydrolases and calcium in Cytophaga hutchinsonii cellulose utilization

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# Investigation of glycoside hydrolases and calcium in *Cytophaga hutchinsonii* cellulose utilization

By

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Dr. Matthew Kaproth: Committee Member

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**Abstract**

*Cytophaga hutchinsonii* is a soil bacterium that can utilize cellulose as a carbon source. The cellulose utilization system of *C. hutchinsonii* has been predicted to be novel and its endoglucanases could potentially be used in the production of cellulosic based biofuels. These enzymes could be used to process cellulose to glucose or other soluble sugars, which can be further converted to ethanol by yeast fermentation. Current biofuels utilize starches, primarily from corn, which has direct competition with farming space for food crops. This study focuses on observing the growth of *C. hutchinsonii* knockout mutant strains on cellulose substrate to better understand how the endoglucanase genes influence cellulose utilization. Attempts were made to create mutants lacking GH8 endoglucanase genes, but was unsuccessful. The previously generated GH9 and GH5 mutants were grown on cellulose with varying amounts of calcium, which is known to be a metallocofactor in enzyme activity. Normal cell growth was observed in the absence of calcium when cellulose was the sole carbon source. Higher concentrations of calcium inhibited cell growth on cellulose or caused cell death. However, we suggest that calcium concentrations <5mM be tested to determine an optimal concentration of calcium for growth on cellulose.

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## 1. Introduction

The production of biofuels from lignocellulose biomass and the conversion of crystalline cellulose to soluble fermentable sugars is an essential step. This process requires multiple enzymes and is of interest in the search for more productive methods of utilizing cellulose (1). Current strategies to reduce the enzyme harvesting costs have not been productive. A search for new cellulolytic systems or enzymes is greatly needed to make biofuels more prominent with lower costs of production.

*Cytophaga hutchinsonii* is a commonly found aerobic Gram-negative soil bacterium that belongs to the phylum Bacteroidetes. This bacterium has been shown to efficiently digest crystalline cellulose by a novel mechanism that is currently not fully understood (2, 3). Genomic and functional analyses point towards the idea that *C. hutchinsonii* proteins involved in the digestive process of cellulose may be unique compared to other bacteria that use cellulose as a carbohydrate source (4, 5). Researchers have been working on understanding the mechanics of how *C. hutchinsonii* utilizes cellulose, and previous studies have developed genetic manipulation methods necessary for studying this organism (6, 7, 24, 25, 26). Previous work has also utilized proteomic analysis to determine which of *C. hutchinsonii*'s enzymes and proteins were expressed when this bacterium was grown on cellulose substrates (8). Other conducted research has investigated the various classes of enzymes that are involved in the cellulolytic activities of *C. hutchinsonii.* These enzymes belong to the glycoside hydrolase (GH) families GH5, GH8, and GH9 (5).

We used genetic and biochemical methods to investigate the functions of the glycoside hydrolase endoglucanases in *Cytophaga hutchinsonii,* which have not been extensively studied. The increased knowledge of cellulolytic systems in bacteria may allow for the decreased dependency from classical fossil fuels, and starch-based biofuels that require the use of food crops. Research efforts on this topic will increase understanding of the intricacies of cellulose turnover in many ecosystems.

## 2. Literature Review

### Cellulose

Cellulose is the most abundant biopolymer on Earth and is a major potential source to produce liquid biofuels (9). This carbohydrate is often found in nature in its linear β-1,4-linked glucose polymers that forms into an organized crystalline structure (10). As a major component of plant tissue, cellulose is often wrapped in lignin to give rise to lignocellulose. Lignocellulose is a rigid structure that gives structural integrity to plants to handle the elements, such as potential wind. Cellulose can be degraded by enzymes to produce glucose, a fermentable sugar that can be used to create cellulosic ethanol. The issues to utilizing lignocellulose as a biofuel are the cost of cellulases and pretreatment to remove lignin. Fundamental research into cellulases made by microbes may lead to a sufficient cost reduction to make cellulosic ethanol a viable source of biofuels (11).

### Cellulase systems

Many microorganisms are unable to utilize cellulose due to its stable glycosidic bonds, but several fungi and bacteria have evolved various strategies to utilize cellulose as a carbon energy source (12). Cellulolytic bacteria species that are more understood than *C. hutchinsonii* produce an array of enzymes that work together to digest cellulose. These enzymes have been identified as endoglucanases (EGs), cellobiohydrolases (CBHs), and β-glucosidases (BGs). EGs initiate the digestion cellulose by cleaving the amorphous glycosidic chains of cellulose by hydrolysis. CBHs will then attack the ends

of the broken chains and break the crystalline orientation, leading to the release of cellobiose. BGs are tasked with the final step of hydrolyzing cellobiose to glucose (13). The recently discovered lytic polysaccharide monooxygenases (LPMOs) are also involved in the degradation of cellulose by some organisms (14).

Currently, there are two well-studied strategies that bacteria utilize to digest cellulose: The noncomplexed cellulase system and complexed cellulase system (Fig. 1). In the noncomplexed system, secretion of extracellular enzymes begins the process of breaking apart the crystalline cellulose. EGs degrade the amorphous region that will allow for exoglucanases (CBHs) to flank the ends of the polymer chains to release cellobiose. BGs in the area, notably outside of the cell, will breakdown the cellobiose into

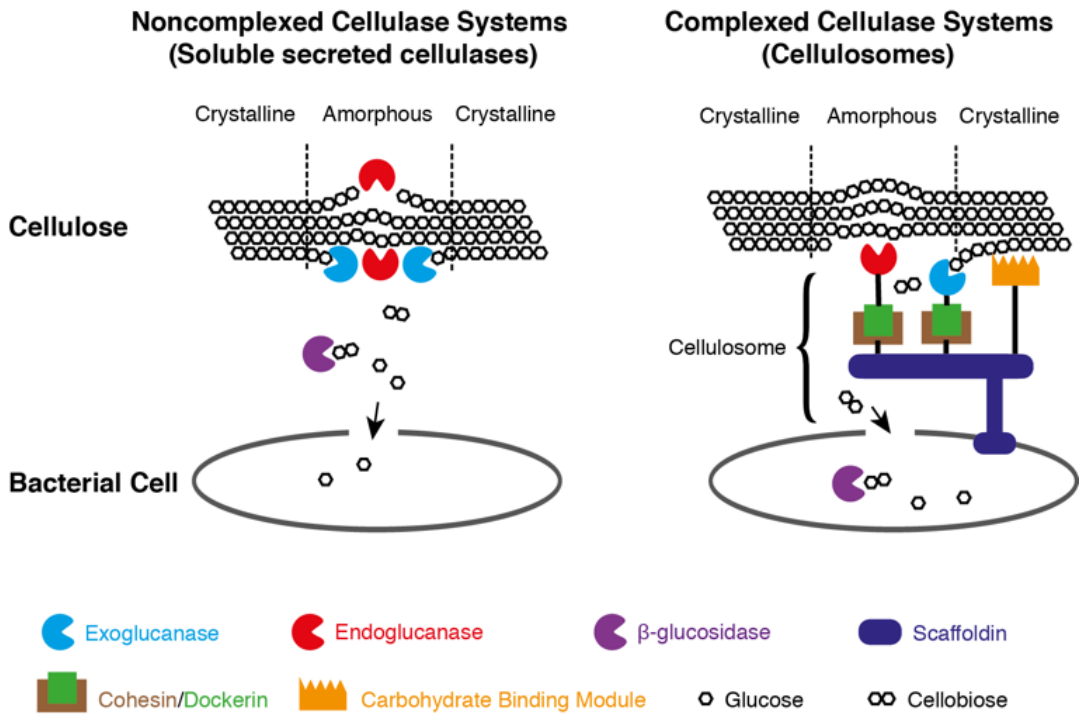


**Figure 1. Two cellulolytic mechanisms employed by known cellulose digesting bacteria.** (4) Left: Noncomplexed cellulase system (Free-cellulase system) Right: Complexed cellulase system. Both mechanisms are not utilized by *C. hutchinsonii*.

glucose molecules. The processed glucose will then enter the cytoplasm and be used for metabolic processes (15). The second strategy, the complexed cellulase system, involves a scaffold protein organized structure called a cellulosome, which is typically attached to the bacterial cell surface. The cellulosome contains a carbohydrate binding molecule that allows the cell to anchor itself with the cellulose substrate. Additionally, on the cellulosome, EGs and CBHs are positioned in a manner that allows for organized cooperation in the breakdown of cellulose (16).

**Bacteroidetes**

The phylum *Bacteroidetes* consists of Gram-negative, either aerobic or anaerobic, rod-shaped bacteria that are prevalent in soil, water, and animal microbiomes (17, 18). Within this phylum, a secretion system that is important to many members is the Type IX secretion system (T9SS) (19). The T9SS system allows cell motility and acquisition of essential nutrients.

Many members of *Bacteroidetes* utilize polysaccharides involving unique cell-associated enzymes and transporters that cut the polysaccharide into smaller products called oligomers, which are then actively transported into the periplasm by the starch utilization system proteins SusC and SusD (7). The oligomers are further digested by enzymes in the periplasm to produce monomers, which will then be transported in the cytoplasm. This strategy is efficient in the digestion of polysaccharides and more notably prevents the organisms from feeding surrounding neighbors by not allowing any degraded substrate to sit in the open environment (20). Cellulose utilization studies

within the phylum are currently only known to occur in *C. hutchinsonii,* and its mechanism of cellulose digestion is likely to be novel.

### *Cytophaga hutchinsonii* cellulose utilization

The ability of *C. hutchinsonii* to digest cellulose is efficient and was recognized almost a century ago, but the mechanism is still not completely understood (2–4). Currently, there is limited knowledge of the enzymes or proteins that are involved in the uptake of cellulose *C. hutchinsonii.* Individual cells of *C. hutchinsonii* must attach directly to cellulose for efficient digestion to occur. The mechanism of cell adhesion to cellulose is not well understood, but current research suggests it to be the responsibility of novel cellulose-binding proteins (4). In addition, it was previously observed that there were minimal traces of soluble reducing sugars in the medium during the process of cellulose digestion, and that the cells are efficient in the uptake of these sugars (21, 22). This process of soluble reduced sugar uptake is of interest because it was tested that *C. hutchinsonii* does not seem to use SusC-like and SusD-like protein channels to uptake the sugars. Successful gene deletions of the *sus*C and *sus*D loci in *C. hutchinsonii* showed that mutants did not have any issues growing on cellulose substrates (7).

Genome analyses currently indicates that *C. hutchinsonii* proteins involved in binding and digestion of cellulose are unusual and it may employ a novel cellulose utilization system (4, 5). *C. hutchinsonii* does not produce cellulosomes found in the complex cellulase system. There are also no exoglucanase homologs and is likely to digest cellulose using only endoglucanases. This was suggested from the observation of

*C. hutchinsonii* needing to be in direct contact with the substrate as it breaks down cellulose. It does not seem to release large quantities of enzymes into the environment based on proteomic analyses (7). The evidence suggests that a novel mechanism is being used in cellulose digestion.

By understanding how this efficient cellulose degrader functions, it may be possible to utilize *C. hutchinsonii* in the production of biofuels from wasted plant material that is often discarded.

**Glycosidic hydrolases of *C. hutchinsonii***

Besides the absence of free cellulases and cellulosomes, another unique aspect of the *C. hutchinsonii* cellulolytic system includes a novel repertoire of predicted cellulolytic enzymes and novel carbohydrate-binding modules (CBMs). It has been revealed from genomic testing that 9 predicted cellulolytic EGs and 8 β-glycosidases that may be involved in cellulose digestion (21, 23–25). These enzymes belong to the glycoside hydrolase families GH5, GH8 and GH9.

In a 2016 study by Zhu et al. it was observed that *cel5B* and *cel9*C have a major role in cellulose utilization (21). The *cel9*C knockout showed a deficiency when grown on cellulose substrate. A double deletion of *cel5*B and *cel9*C resulted in a mutant that was incapable of utilizing cellulose, but still managed to grow when glucose was introduced. These two endoglucanases reside in the periplasm and are diagramed in Figure. 2.

Previous research by Taillefer et al. found that the GH8 EGs may be potential active cellulases as all six EGs from this family were seen to be highly expressed when

*C. hutchinsonii* cells were growing on cellulose substrate (8). The gene loci for these six GH8 EGs on the genome of *C. hutchinsonii* are tagged as CHU_1075, CHU_1240, CHU_2852, CHU_3440, CHU_3441, and CHU_3727 (8). Investigation of the GH8 EGs may help increase understanding of how *C. hutchinsonii* uniquely utilizes its array of enzymes to breakdown and process cellulose.

**Essential calcium cofactor for glycoside hydrolase family 9**

In a study conducted by Zhang et al. (26), they observed that calcium ions were a necessary metallocofactor in the function of Cel9C. The protein Cel9C was observed to be important in *C. hutchinsonii*'s cellulose utilization(21). In Zhang's study, they isolated the enzyme and then proceeded to study its properties and relationship with calcium. With the addition of 5 mmol of calcium, the Cel9C enzyme exhibited stability and became more reactive. There have not been any studies conducted on how an increase in calcium in the environment may affect *C. hutchinsonii* as a whole organism. Additional calcium may allow *C. hutchinsonii* to be more efficient at breaking down cellulose. It is also possible that calcium may become toxic to the cells at a higher concentration.

**Type IX secretion system and gliding motility of *Cytophaga hutchinsonii***

The T9SS is a major component of the *C. hutchinsonii* gliding motility machinery as it gives the cell the ability to glide around surfaces, instead of using flagella or pili to move around. This unique technique of moving across surfaces may be directly linked with cellulose digestion and allow the cell to penetrate deeper in the cellulose crystalline matrix, allowing more cellulose to be digested from a single source (5). In a recent study,

it was observed that the T9SS in *C. hutchinsonii* is directly linked to the accumulation of calcium, which is an essential metal ion associated with cellulose utilization. The current model of *C. hutchinsonii* cellulose utilization is diagrammed in Figure 2.

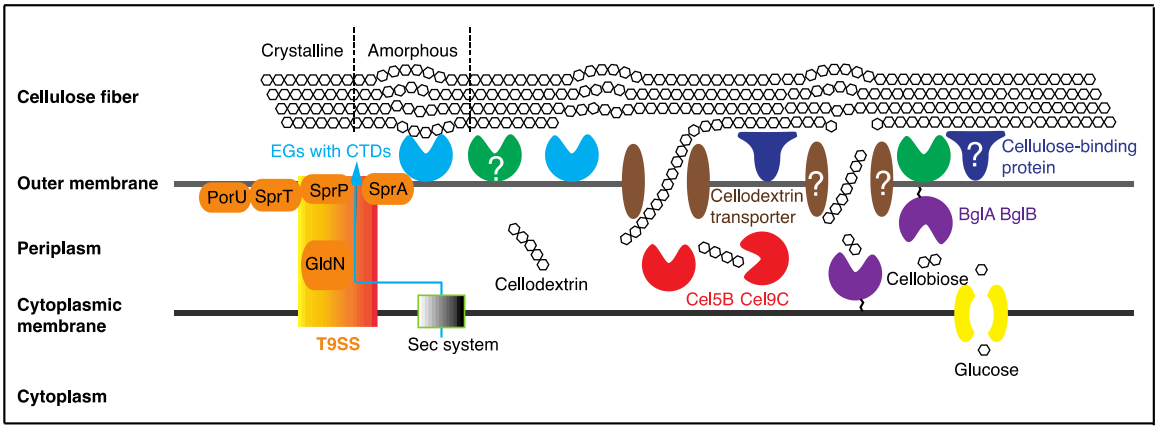


**Figure 2. Current model of *C. hutchinsonii* cellulose utilization and the Type 9 secretion system within the periplasm** (4). Within the periplasmic space, Cel9C and Cel5B work in tandem to breakdown cellulose fragments into cellobiose, which is further broken down into glucose and then transported into the cytoplasm.

## 3. Methodology

### Culture methods

Peptone yeast extract (PYE-10, 10 g/liter peptone, 0.5 g/liter yeast extract, 4 g/liter glucose, pH 7.3) was utilized as media for the growth of all *C. hutchinsonii* strains. For situations that required precise control of a medium's contents, modified Stanier medium (10 mM $KNO_3$, 4.4 mM $K_2HPO_4$, 0.8 mM $MgSO_4$, 0.07 mM $FeCl_3$, 0 mM / 5 mM / 10 mM $CaCl_2$, pH 7.3) (2) was used. Strains of *E. coli* were grown in LB medium (27).

All strains were stored with 15% glycerol in a -80°C deep freezer. After each new strain was isolated, a single colony was inoculated into 3 ml of the appropriate liquid media and incubated overnight. One milliliter of this overnight culture was then added to 250 µl of a 75% glycerol solution in a cryotube. The cryotube was then appropriately labeled and stored at -80°C. A second tube was prepared from the same culture and stored in a separate freezer as a back-up.

### Target genes for mutagenesis

The deletion of several genes was attempted in this study. Target genes and their purported functions are listed in Table 1. The deletion of these genes in the various GH8 mutants may have allowed us to observe the effects of these enzymes on the utilization of cellulose via growth analysis.

**Targeted gene deletion systems**

To take advantage of the available genome information and investigate gene functions in vivo, an unmarked nonpolar gene deletion system *in C. hutchinsonii* was previously developed and is shown in Figure 3 (4). The system uses *sacB* as the counter selectable marker, encoding a periplasmic levansucrase that confers sucrose sensitivity to *C. hutchinsonii*. This method constructs gene deletions in the wild-type strain or any other markerless backgrounds. The *sacB* gene with a *Bacteroidetes* promoter was cloned onto the suicide plasmid pLYL03 (28–30) that does not replicate in *C. hutchinsonii* to generate pYT313 (29, 31). The plasmid pYT313 contains the *ermF* gene that confers erythromycin resistance to *C. hutchinsonii.*

To construct chromosomal deletions, we clone upstream and downstream regions of the target gene into pYT313 and introduce the deletion constructs into *C. hutchinsonii* by conjugation or electroporation. Homologous recombination results in integration of the plasmids into the genome, selected by erythromycin resistance. A second recombination event, selected by sucrose resistance, removes the plasmids along with the genes of interest by the action of *sacB*, resulting in the final markerless deletion on the chromosome (Fig. 3). Using this approach, we can isolate gene deletion mutants. Importantly, this approach can be used iteratively to construct strains with multiple deletions. This method is thus well suited to analyze *C. hutchinsonii* cellulose utilization, which involves the action of multiple proteins with overlapping functions (21). This strategy can also be used to produce mutants with single amino acid changes or with added peptide tags in vivo, allowing us to study the functions of certain amino acids, to

localize *C. hutchinsonii* cellulases, or to purify natively expressed wild type or mutant cellulases to study enzymatic activities. Modified versions of pYT313 with different multiple cloning sites, to facilitate deletion of diverse regions of the genome were also developed (19). All strains and plasmids used in this research are listed on Table 1.

A gene deletion strategy as described in Figure 3 was used to attempt the generation of mutant strains of *C. hutchinsonii*. Primers to amplify the upstream and downstream regions of the target genes were created using the MacVector software (Version 18.2.5) (Table 2). These regions were then amplified by polymerase chain reaction (PCR) using Phusion DNA polymerase (Thermo Fisher Scientific), digested with restriction enzymes (Table 2), and ligated onto the suicide vector pYT313, generating deletion constructs (Table 1).

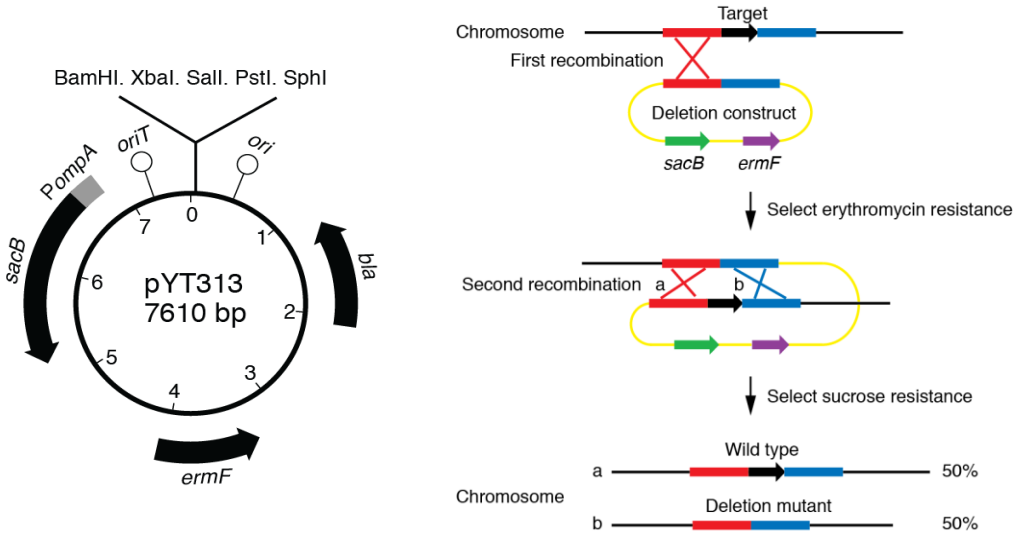


**Figure 3. Targeted gene deletion systems in *C. hutchinsonii*. Left)** Map of pYT313. Upstream and downstream regions from the gene of interest were cloned into the multiple cloning site. **Right)** Regions shown in red and blue are upstream and downstream regions of the target gene. Green, purple, and yellow denote regions of the plasmid. *sac*B is a counter selectable marker used by the gene deletion plasmid pYT313. *sac*B confers sucrose sensitivity on *C. hutchinsonii* mutants. *ermF* confers erythromycin resistance on *C. hutchinsonii* (4).

**Table 1. Strains and plasmids used in this study.**

| Strains and Plasmids | Description | Resource |
|---|---|---|
| *E. coli* | | |
| DH5αMCR | Strain used for gene cloning | (21) |
| S17-1 λ pir | Strain used for gene transfer | (29) |
| | | |
| *Cytophaga hutchinsonii* | | |
| ATCC 33406 | Wild-type | ATCC |
| CHU_428 | Streptomycin resistant WT | (7) |
| CHU_511 | *Δcel5B* mutant | (21) |
| CHU_538 | *Δcel9C* mutant | (21) |
| CHU_542 | *Δcel5BΔcel9C* mutant | (21) |
| | | |
| Plasmids | | |
| pYT313 | Suicide vector/cloning construct | (31) |
| pYT162 | Shuttle vector and control | (29) |
| pCV02 | Used to delete CHU_3727 (Cel8E) | This study |
| pCV05 | Used to delete CHU_1240 (associated with GH8) | This study |
| pCV06 | Used to delete CHU_3440-3441(Cel8C/Cel8D) | This study |
| pCV10 | Used to delete CHU_2852 (Cel8B) | This study |

## Primer design

Genome sequencing and analysis of *C. hutchinsonii* have been completed by previous works (4, 5, 8, 21, 30, 32). Using the software MacVector (Version 18.2.5), primers (Table 2) were designed to delete the loci of the GH8 genes. Restriction enzymes sites were added to the primers. Primers were synthesized by Integrated DNA

Technologies, Inc. Primers were diluted to 10μM in double distilled water and mixed thoroughly before use. Primer solutions were stored at freezing temperatures.

**Table 2. Primers used in this study.**

| Primer | Sequence and Description |
|---|---|
| YZ0096 | 5' – GCTAGGGATCCGCTTAAAATGATCGATGTGC – 3'; Forward primer to amplify upstream region of CHU_1240. BamHI site added. Product size 3095 b.p. with primer YZ0097 |
| YZ0097 | 5' – GCTAGTCTAGAATCAAATGTGTACGTTACCG– 3'; Reverse primer to amplify upstream region of CHU_1240. XbaI site added. Product size 3095 b.p. with primer YZ0096 |
| YZ0098 | 5' – GCTAGTCTAGAGAACTGTCAGACGGAACATA – 3'; Forward primer to amplify downstream region of CHU_1240. XbaI site added. Product size 2975 b.p. with primer YZ0099 |
| YZ0099 | 5' – GCTAGGTCGACTATCAAAGGTGGTAATGACG – 3'; Reverse primer to amplify downstream region of CHU_1240. SalI site added. Product size 2975 b.p. with primer YZ0098 |
| YZ0100 | 5' – GCTAGGGATCCCAATACTTCAACAGGCAC – 3'; Forward primer to amplify upstream region of CHU_2852. BamHI site added. Product size 2971 b.p. with primer YZ0101 |
| YZ0101 | 5' – GCTAGGTCGACAAATGAGCTTCGGTTAGT – 3'; Reverse primer to amplify upstream region of CHU_2852. SalI site added. Product size 2971 b.p. with primer YZ0100 |
| YZ0102 | 5' – GCTAGGTCGACCTTACGGTATGGGAATCCTA – 3'; Forward primer to amplify downstream region of CHU_2852. SalI site added. Product size 2970 b.p. with primer YZ0103 |
| YZ0103 | 5' – GCTAGGGATCCTTAATTGTCCAGACAGAACGA – 3'; Reverse primer to amplify downstream region of CHU_2852. SphI site added. Product size 2970 b.p. with primer YZ0102 |
| YZ0104 | 5' – GCTAGGGATCC GCTTTAGCATCAAATGGATCTG – 3'; Forward primer to amplify upstream region of CHU_3440-3441. BamHI site added. Product size 2959 b.p. with primer YZ0105 |
| YZ0105 | 5' – GCTAGGTCGACTACATGAATCGTATCACCTGG – 3'; Reverse primer to amplify upstream region of CHU_3440-3441. SalI site added. Product size 2959 b.p. with primer YZ0104 |
| YZ0106 | 5' – GCTAGGTCGACAACATACAGAATTTCCCCGAT – 3'; Forward primer to amplify downstream region of CHU_3440-344. SalI site added. Product size 2689 b.p. with primer YZ0107 |
| YZ0107 | 5' – GCTAGGCATGCAAATTCACACAATATCCACCG – 3'; Reverse primer to amplify downstream region of CHU_3440-3441. SphI site added. Product size 2689 b.p. with primer YZ0106 |
| YZ0108 | 5' – GCTAGGGATCCATGTCTATTCCAATCACCGT – 3'; Forward primer to amplify upstream region of CHU_3727. BamHI site added. Product size 2760 b.p. with primer YZ0109 |
| YZ0109 | 5' – GCTAGGTCGACTCCTGCAGGTGTATTAATCT – 3'; Reverse primer to amplify upstream region of CHU_3727 SalI site added. Product size 2760 b.p. with primer YZ0108 |
| YZ0110 | 5' – GCTAGGTCGACATTGAAATGCCGGTAGCTG – 3'; Forward primer to amplify downstream region of CHU_3727. SalI site added. Product size 2947 b.p. with primer YZ0111 |
| YZ0111 | 5' – GCTAGGCATGCTACCTGTTGATTTTGCCATTG – 3'; Reverse primer to amplify downstream region of CHU_3727. SphI site added. Product size 2947 b.p. with primer YZ0110 |

**Sequencing of plasmids**

To ensure that the plasmids were accurate, the regions of interest were sequenced and analyzed. Regions of interest were amplified using Phusion PCR as previously described. The PCR products were then loaded into an agarose gel with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific). After electrophoresis, a gel extraction was performed using the Gel/PCR DNA Fragments Extraction Kit from IBI Scientific. The products was then stored at -20°C until they could be sequenced. Concentration of each sample were measured using the NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer at a wavelength of 260nm.

Gel-extraction products were then prepared and sent to GENEWIZ to be sequenced. A DNA alignment was performed on the sequences in MacVector to confirm the correct regions were present on the suicide vector.

**Conjugation**

*E. coli* DH5αMCR cells with the finished plasmid deletion construct is unable to donate the plasmid to the wild-type *C. hutchinsonii*, because this *E. coli* strain lacks conjugation pili. The donor strain S17-1λ pir received the plasmid from DH5αMCR, via plasmid prep and transformation, before conjugation was done. Methods on the conjugation process used in this research for *C. hutchinsonii* have been previously established by Zhu and McBride (29). The *cel*8A mutant has already been obtained through this process.

The conjugation procedure that was used in this study follows: The ATCC strain of *C. hutchinsonii* was streaked onto PYE10 (0.4% glucose, 7g/L agar) from a -80◦C freezer. Cells were grown for 48 hours before selecting a single colony to streak again onto PYE10 to increase purity and viability of cells. This new plate was grown for another 48 hours. A few colonies were selected, and lawn streaked onto PYE10 (0.4% glucose, 15 g/L agar), and grown for 48 hours. The S17-1λ *E. coli* with the plasmid construct was streaked onto LB with ampicillin (100 μg/mL) from a -80◦C freezer and grown for 16-20 hours. Once isolated colonies have grown on the LB plate, a colony was selected and inoculated into 5 mL of LB with ampicillin and grown for 16-20 hours. After 48 hours of growth, *C. hutchinsonii* cells were scraped off the plate and suspended in 5mL of Stanier liquid media inside a 15 mL conical sealed tube.

The 5 mL of *E. coli* was transferred to a separate 15 mL conical tube. The tubes were centrifuged at 3000 x g for 6 minutes. The supernatant was removed, and *E. coli* was washed with Stanier and centrifuged under the same conditions to remove the lingering ampicillin. After the supernatant was removed from both conical tubes, cells were resuspended in one tube with 200μL of Stanier and mixed with the cells in the other conical tube. Mixed until *C. hutchinsonii* and *E. coli* cells were homogenous. The cell mixture was spot plated onto a Stanier plate with glucose (0.1% glucose). The conjugation period was 48 hours. After the conjugation period had passed, the cell spot was resuspended into 2 mL of liquid Stanier. After mixing thoroughly, 100 μL of the sample was plated onto PYE10 with erythromycin (30 μg/mL). This process was replicated 20 times for each individual conjugation system. Any *C. hutchinsonii* cells that

have incorporated the plasmid construct will form colonies on the plates after 14 days. Colonies would have to be confirmed by PCR as this process generates 50% mutant and 50% wild-type strains due to the outcomes of homologous recombination.

**Electroporation**

Another method that was used to transfer the plasmid constructs to *C. hutchinsonii* was by electroporation. Successful gene transfer and electroporation parameters have been established and methods follow as demonstrated by Xu (30). Different voltages and plasmid concentrations have been used and tested in our lab. *C. hutchinsonii* cells were made competent by washing with cold 10% glycerol three times and stored at -80$^{o}$C in 10% glycerol. Optimization on the protocol has yet to be published, as seen with poor mutagenesis efficiency. Control efficiency with the shuttle vector pYT162 has showed promise.

Electroporation settings on the pulser (BioRad Gene Pulser) was listed as: 12 kV/cm, 200 Ω, 25 μF. One millimeter electroporation cuvettes were used (BioRad, 0.1cm Electroporation cuvette). After shock, it was observed in our lab that a 24-hour pre-erythromycin expression period during cell recovery resulted in more colonies expressing erythromycin resistance with the shuttle vector pYT162. All electroporation samples were plated onto PYE10 with erythromycin (30 μg/mL) and grown for 48 hours before colonies were observed.

**Filter paper digestion by *C. hutchinsonii* cells**

Wild-type and mutant *C. hutchinsonii* strains were grown at 25°C for 2 days on PYE10 with 15 g/liter agar, at which time will be scraped off the plates, suspended in 1 ml of Stanier medium with no calcium, and pelleted by centrifugation at 4,200 x g for 3 min to remove residual carbohydrates. Cells were suspended in Stanier medium with no calcium to a concentration of 1.0 at OD600. Then 3 µL of cells were spotted onto a 30-mm-diameter Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, United Kingdom) overlaid on Stanier agar with different additions of calcium (0 mmol, 5 mmol). The Stainer agar will supply cells with essential metals necessary for growth and development. Cells were incubated at 25°C for fifteen days, and growth and cellulose digestion was documented using a camera.

**Growth curve analysis**

Cells were prepared the same as the filter paper assay, after washing the cells in Stanier with no calcium and achieving an OD600 = 1.0, 100µL of the cell mixture was inoculated into 50 ml of Stanier (0 mmol, 5 mmol, 10 mmol calcium) in 250-ml flasks. Cellulose was added to each flask to a concentration of 0.1% cellulose (0.1g/100mL). Each flask was then incubated in a shaker set at 25°C and 200 rpm. Growth was presented as log function (micrograms of cell protein per milliliter). Growth assays were done in triplicates, and the error bars included showed standard deviations (21, 29). Due to the nature of *C. hutchinsonii* being bound to the cellulose substrate and cellulose being non-soluble in water, growth will be indicative by protein abundance. A Bradford assay

protocol was used to determine the growth of the wild-type control and knockout mutants. From the Bradford assay kit, a standard curve was also generated to analyze total protein acquired. The Bradford assay protocol followed as previously done in (21).

# 4. Results

## Construction of deletion plasmids

Four deletion constructs for CHU_1240, CHU_2852, CHU_3440-3441, and CHU_3727 were made in this study shown in Table 1. Digestion of plasmids (Fig. 4) and plasmid sequencing results indicated that the deletion constructs had the correct amplified regions used to flank the gene of interest to generate knockout mutants. Verifying the presence of the erythromycin selection marker was also done to each deletion construct by PCR.

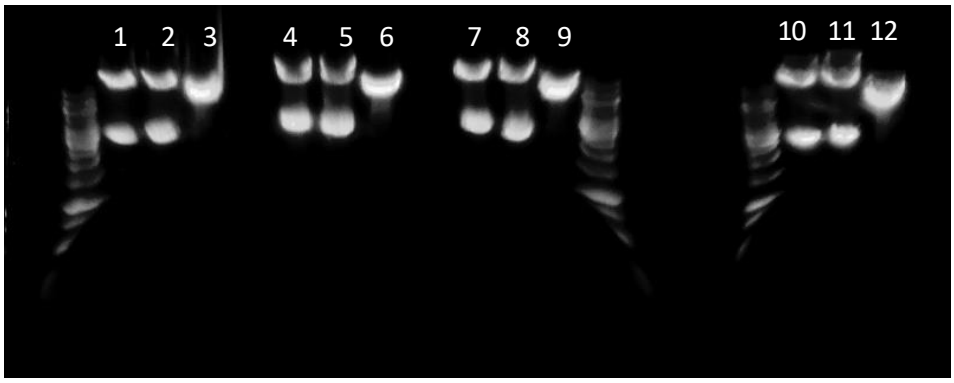


**Figure 4. Gel image of a digestion utilizing restriction enzymes to confirm plasmid constructs.** Lanes 1-3 is a digestion of the upstream (BamHI, SalI), downstream (SalI, SphI), and entire fragment (BamHI, SphI) of pCV02. Lanes 4-6 is a digestion of the upstream (BamHI, XbaI), downstream (XbaI, SalI), and entire fragment (BamHI, SalI) of pCV05. Lanes 7-9 is a digestion of the upstream (BamHI, SalI), downstream (SalI, SphI), and entire fragment (BamHI, SphI) of pCV06. Lanes 10-12 is a digestion of the upstream (BamHI, SalI), downstream (SalI, SphI), and entire fragment (BamHI, SphI) of pCV10.

## Optimization of electroporation conditions using pYT162

After shock with the settings at 12 kV/cm, 200 Ω, 25 μF, a recovery period of 24 hours was optimal for cells to express erythromycin resistance from the control plasmid pYT162. It was observed that cells harvested from log phase were more likely to receive

pYT162 and express erythromycin resistance. Additions of 10 mM $MgCl_2$ or 10 mM $CaCl_2$ to the glycerol wash did not have any positive or negative effects on the ability of the cells to take up pYT162. Total number of colonies expressing erythromycin resistance observed at 8 hours of recovery was 10≥ colonies. With a recovery period of 24 hours, total number of colonies expressing erythromycin resistance was 100≤ colonies.

**Construction of deletion mutants**

We were unable to generate any GH8 mutants in this research. Both conjugation and electroporation were utilized in attempts to transfer the deletion constructs to *C. hutchinsonii* without success. We are confident that the control plasmid pYT162 was successfully transferred inside the cells, which showed erythromycin resistance when grown on PYE10 with erythromycin.

**Filter paper growth analysis**

For 0 mmol of calcium in the Stanier agar, growth was observed with all tested strains except for CHU_542, which served as a negative control (Fig. 5). CHU_538 showed slower growth on cellulose when compared to that of CHU_428 and CHU_511. This finding correlates to what was observed by Zhu et al. 2016 (21). There was no growth observed on the cellulose filter paper for all the strains when 5 mmol of calcium was added to the Stanier agar (Fig. 6).

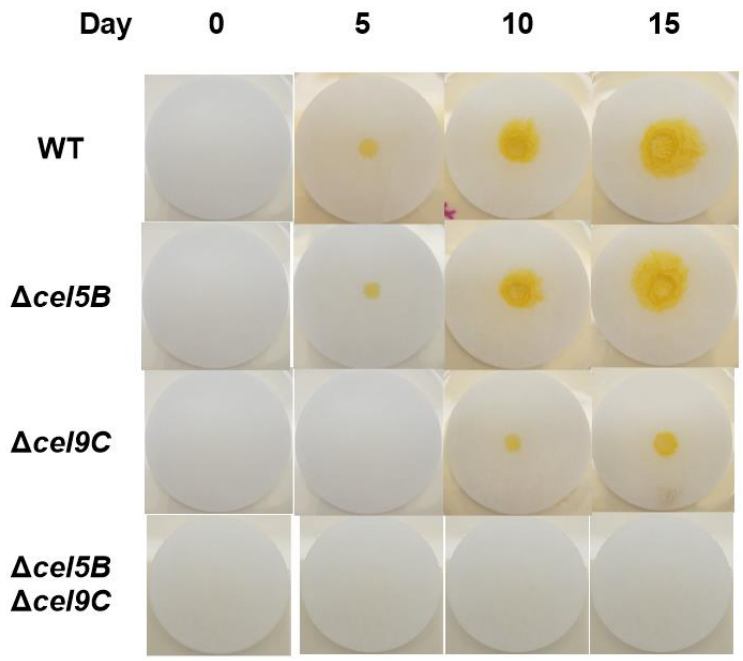


**Figure 5. Filter paper growth assay with 0mmol of calcium.** Growth of WT, Δ*cel5B*, Δ*cel9C*, and Δ*cel5*BΔ*cel9*C over 15 days. Stanier agar containing 0 mmol of calcium overlaid with a 30 mm Whatman filter paper.

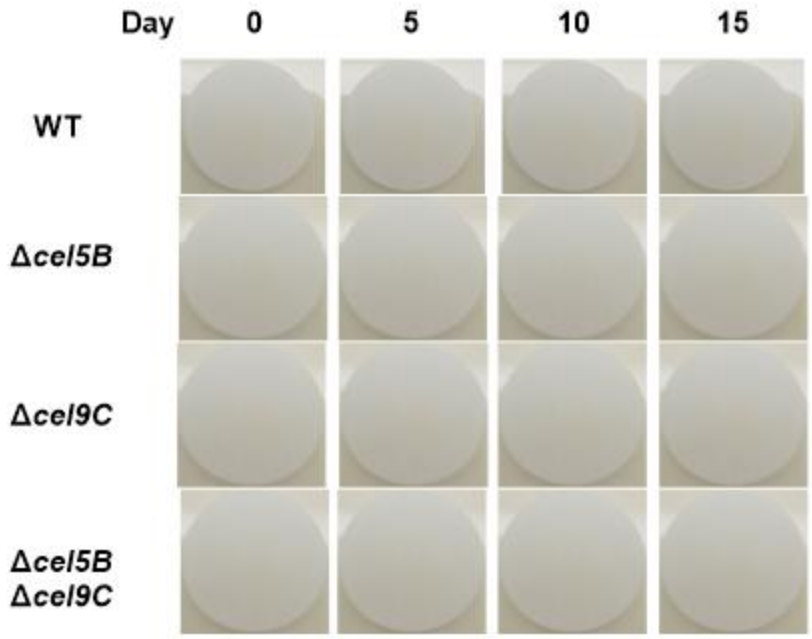


**Figure 6. Filter paper growth assay with 5 mmol of calcium.** Growth of WT, Δ*cel5B*, Δ*cel9C*, and Δ*cel5*BΔ*cel9*C over 15 days. Stanier agar containing 5 mmol of calcium overlaid with a 30 mm Whatman filter paper.

## Growth curve analysis

Modified Stanier media with different concentrations of calcium was used to observe cell growth with 0.1% cellulose added. Growth was observed in the absence of calcium, except for CHU_542 which did not grow, as expected. There was no growth with any strain in the presence of 5 mmol or 10 mmol calcium (Fig. 7). Cells were likely killed or inhibited. A Bradford assay and standard curve was utilized to measure total cell protein content (µg/mL).

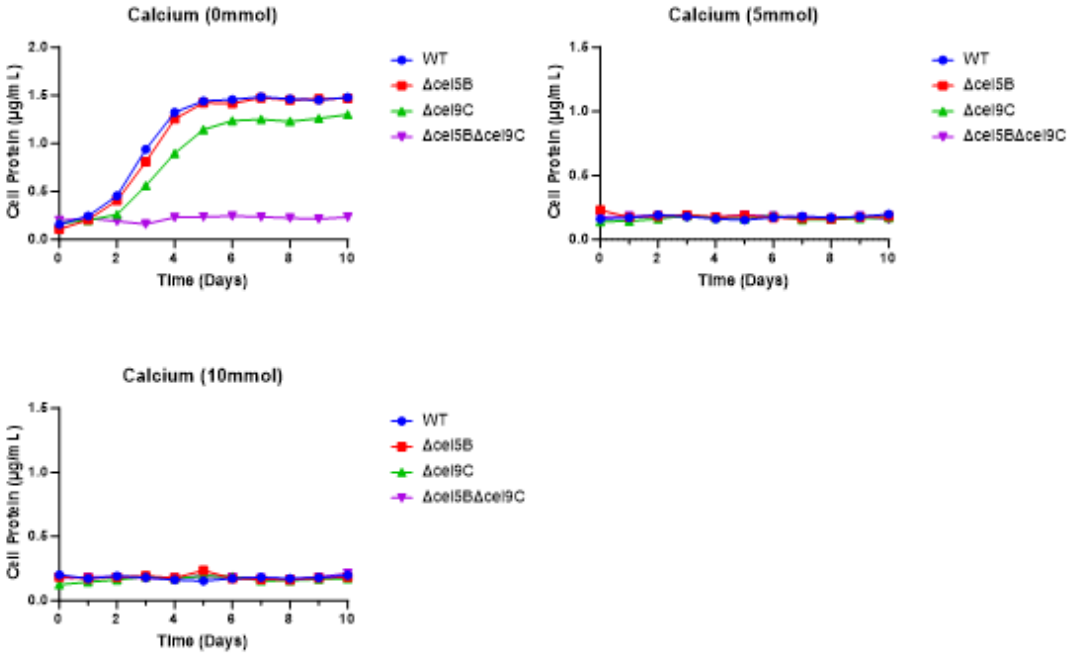


**Figure 7. Effects of calcium on cellulose utilization by *C. hutchinsonii*.** Growth curves of the WT, Δ*cel5B*, Δ*cel9C*, and Δ*cel5*BΔ*cel9*C in 0 mmol, 5 mmol, and 10 mmol of calcium. All strains were performed in triplicate. Error bars displayed as standard deviation. Error bars not displayed due to lack of deviation among triplicate samples.

## 5. Discussion

This study emphasized the current difficulties with the current genetic tools used to manipulate *C. hutchinsonii*. Conjugation and electroporation were both used as methods of transferring the deletion construct to the cells. The primary issue may not have been whether or not the plasmid was received by *C. hutchinsonii,* but rather the plasmid not being integrated into the genome. It is currently unknown why *C. hutchinsonii* expresses a low recombination rate.

Both electroporation and conjugation have been successful in creating deletion mutants in *C. hutchinsonii* in other studies, however with low efficiency. This study tried to explore methods to increase the likelihood that genome integration of the suicide vector carrying the deletion construct. Cell densities were increased during the conjugation and electroporation protocol, but no mutants were achieved during the time frame of this study. Cells were also harvested during different phases of growth in attempts to increase DNA transfer efficiency. Xu et al. (30) observed in their study that cells harvested in the early lag phase were more likely to express antibiotic resistance from their shuttle vector. However, we found the cells harvested from the log phase tended to receive the tested plamid pYT162, a shuttle vector with an erythromycin resistance marker, with a higher chance.

During our electroporation protocol, it was observed that cells that were given pYT162 grew relatively well on PYE-10 agar with erythromycin after a 24-hour recovery period after shock. Additional time for recovery saw that less colonies being produced in

the presence of erythromycin, which can be attributed to cells quickly ridding themselves of excess genetic material if they are not needed.

GH5 and GH9 mutants have been successfully made by Zhu et al. 2016. These endoglucanase mutants were not created directly from the ATCC background. A streptomycin resistant strain of *C. hutchinsonii* was used as the deletion background to create the GH5 and GH9 mutants (21). Conjugation was also the preferred gene transfer method used in this study. In our study, the deletion background was the ATCC strain. It may be worth attempting to use the CHU_428 as the deletion background for creating the GH8 mutants by conjugation in a revisited future study.

In other studies, with the members of phylum Bacteroidetes, there has been success in genetic manipulation tools. The model study organism *Flavobacterium johnsoniae*, also uses a conjugation system. It has since been modified to achieve high efficiency in mutagenesis. The triparental conjugation technique has been developed to transfer the deletion constructs to the target *F. johnsoniae* strain. An *E. coli* helper strain is used in addition to a donor *E. coli* strain (DH5α-mcr) and recipient *F. johnsoniae* strain. This helper strain contains the plasmid pRK2013 which encodes the genes required to construct a conjugation pilus(33). This plasmid allows the donor strain to transfer the target deletion construct to the recipient strain. Success in generating mutants in *F. johnsoniae* is known to be high. The triparental conjugation method used in *F. johnsoniae* may be tested in *C. hutchinsonii.*

In another study conducted by Sloboda and Zhu at Minnesota State University, Mankato in 2022 showed that foreign plasmids can be sliced up in the cytoplasm by their study organism's, *Flavobacterium psychrophilum*, restriction enzymes (35). This would result in a plasmid that would likely not be integrated into the genome. To protect the integrity of the plasmid carrying the deletion construct, another plasmid was developed to methylate those restriction enzyme sites on the deletion construct. This process is called pre-methylation, and it protects the plasmid from being destroyed by host restriction enzymes because of the methylation. The same issue may also exist in *C. hutchinsonii* and cause low DNA transfer efficiency.

Difficulties with the genetic manipulation of *C. hutchinsonii* was predicted by research done by Xu et al., in which they state that the absence of *rec*B and *rec*C in the genome of *C. hutchinsonii* may be one of the reasons for its low recombination efficiency. To increase recombination frequency, it is partly dependent on the length of homologous sequences. With larger homologous sequences having a higher efficiency (30). Our own homologous sequences were about 3 kb in size, which was well within that range of predicted higher efficiency for plasmid integration and recombination to occur.

The inability to generate any GH8 mutants in *C. hutchinsonii* was likely a result of coincidence in the time frame experimentation was carried out. With additional trials of electroporation and conjugation, it is likely a mutant strain would have been developed. Future studies on *C. hutchinsonii* should be directed towards the optimization of the genetic tools and methods. Many other members within this phylum have had their

gene deletion protocols optimized to yield high mutant strain generation. Currently, there is no clear explanation on the low success rate of generating mutants in *C. hutchinsonii.*

Another purpose of this study was to observe the growth of *C. hutchinsonii* and selected mutants when exposed to different concentrations of calcium. Stanier media is originally prepared with about 0.1 mmol of calcium in 1L. This concentration was manipulated to give final concentrations of 0 mmol, 5 mmol, and 10 mmol of calcium. In the study that was conducted by Zhang et. al., they observed that 5 mmol of calcium yielded maximum activity of the Cel9C enzyme. Their experiment was not done within the organism, as they induced and extracted Cel9C from *E. coli*.

Based on our results, 5 mmol of calcium was likely lethal to *C. hutchinsonii*. The filter paper assay and growth curve both demonstrated a lack of ability to grow. It is not surprising, as a 5-fold increase in the amount of calcium in the system can cause imbalances with the cells. Although 5 mmol of calcium was shown to be good for enzyme stability, the same cannot be said for cell integrity. The same experiments were done for 10 mmol of calcium, and results were the same showing no proliferation of cells.

It should also be noted that calcium was introduced to the media as calcium chloride. So, with the additional calcium ions in solution, more chlorine anions were also present in the growth media. The large amounts of ions and anions in solution could both have attributed towards likely cell incompetence. The pH of the growth media was buffered and was controlled to be at around 7.3. Even with stabilizing the pH of the

growth environment, the ions and anions are still present and may pose imbalances in the cell. Metabolism and cell membrane function were predicted to be negatively influenced.

The absence of calcium was observed not to affect the growth of *C. hutchinsonii* when cellulose is the sole carbohydrate source. Our findings suggest that *C. hutchinsonii* is fully capable of growing well without calcium, which was considered to be an essential metallocofactor in many GH9 proteins. Zhang et al., 2015 ran an experiment that looked at the reactivity of Cel9C, an essential protein in cellulose utilization later determined by Zhu et al., 2017, in the absence of calcium and found that function was minimal. There are a few explanations for this phenomenon.

The GH5, GH8, or other GH9 endoglucanases may have a major role in the utilization of cellulose when the Cel9C activity is down regulated by the lack of available calcium in the environment. It is possible that these enzymes or other cellulolytic enzymes are produced and use different metal ions as their respective cofactors. In a review paper published in 2021 by Smethrust et al., they discuss the possibility of biological systems being adaptive to the changing environments in Earth's history (34). Available metal ions necessary for functioning biological systems changed many times, so using similarly charged ions was necessary.

In our Stanier media that was modified to exclude calcium ions, other metals ion with similar charges to that of calcium persisted in the growth solution. Magnesium was present, and it is possible that Cel9C was utilizing magnesium as a secondary metallocofactor to carry out normal functions in degrading cellulose. Experiments that

would include the exclusion of calcium and magnesium are likely not possible. The absence of multiple essential metal ions would lead to cell death or inhibition of replication.

There is also a concern that trace amounts of calcium may have persisted in the conditions where calcium was unwanted. Cell cultures of *C. hutchinsonii* were grown on PYE-10, and yeast extract is rich in minerals with calcium being included. Although cells were washed with Stanier free of calcium before being inoculated into various growth systems, lingering calcium could have still been present. To further optimize the procedures done in this study, the use of EDTA would allow for removing those trace amounts of calcium in solution.

Cellulose is the most abundant biopolymer on Earth and the tools to utilize the available carbon source are lacking. Additional fundamental research of cellulases produced by the many microorganisms on our biosphere could lead to discovering stable and efficient enzymes. Current incentives to continue to use amylase to process starch instead of using cellulases to process cellulose is the higher costs of producing cellulases. In this research we have continued to add knowledge about the cellulose utilization mechanism in *C. hutchinsonii*.

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