Effect of Chitosan on the Growth and Murein Hydrolase Activity of *Streptococcus mutans* with Mutations in the *cid* and *lrg* Two Component Autolytic Regulatory System.

Martina Feldmann, Robby Puttock, Bei Wang and Keely Wu
Department of Microbiology & Immunology, University of British Columbia

Chitosan is a naturally occurring polysaccharide with antimicrobial properties. This study investigated whether chitosan could induce the LytSR two-component autolytic regulatory system, specifically the *cid* and *lrg* operons, found in *Streptococcus mutans*. A turbidity assay and a murein hydrolase assay were used to observe autolytic activity in chitosan-treated and non-treated cultures of wildtype *S. mutans, cidAB* and *lrgAB* mutants, and *E. coli*. The turbidity assay showed that growth of all bacterial strains was inhibited and cell lyses occurred when cells were incubated in the presence of chitosan. Cultures treated with and without chitosan both showed decreased murein hydrolase production in the murein hydrolase assay.

Chitosan is a widely used antimicrobial compound formed by the alkaline N-deacetylation of chitin, the main component of fungal cell walls and crustacean exoskeletons (7,13,14). It can inhibit the growth of a wide range of microorganisms such as bacteria, fungi, and yeast (10,14). Due to it's properties as a polymeric cationic macromolecule with positively charged amino groups, chitosan is able to penetrate the cell wall of Gram positive bacteria because it is composed chiefly of peptidoglycan and lacks an outer membrane. Therefore, it is less capable of interacting with both the cytoplasmic and outer membrane of Gram negative bacteria (3,13). However, low molecular weight chitosan has the ability to pass through the cell wall (18) as studies have confirmed the presence of chitosan oligomers, inside *E. coli* cells treated with chitosan (11).

Murein hydrolases are a ubiquitous family of broad and diverse enzymes that specifically cleave structural components of the bacterial cell wall (11). These enzymes have been previously shown to participate in a number of important biological processes during cell growth and division, including daughter cell separation, cell wall growth, and peptidoglycan recycling and turnover (11). However, over-expression of murein hydrolase genes can lead to the destruction of the cell wall and subsequent cell lyses. Because of their potentially lethal capacity to hydrolyze the cell wall, tight control is exercised over the expression and activity of murein hydrolases (5).

CidAB and *lrgAB* are a pair of discistronic operons that regulate murein hydrolase activity and in turn, cell lysis (10). A two-component system encoded upstream of *lrgAB*, known as LytSR, is required for *lrgAB* expression but not for *cidAB* expression. The LytSR-regulated dicistronic operon encodes two membrane-associated proteins, designated LrgA and LrgB that are involved in the control of murein hydrolase activity through anti-holin proteins (1,2,5). *lrgAB* has an inhibitory effect on murein hydrolase activity while *cidAB* has a stimulatory effect on murein hydrolase activity. The *cidAB* operon is located downstream of *cidR* which is proposed to be a transcriptional regulator for *cidAB*. LytSR affects murein hydrolase activity which in turn affects autolysis (1,19). In *Streptococcus mutans*, these operons have a critical role in autolysis. As such, these genes are subject to regulatory control due to the fact that they control lysis and cell death (15,19). The *lrg* and *cid* operons encode membrane proteins responsible for the regulation of murein hydrolase activity. These gene products share structural similarities with the holin protein families in bacteriophages (17). Holin proteins in bacteriophages control the timing and onset of cell autolysis. Holin genes frequently contain a "dual start motif" that produces two different protein products known as holins and anti-holins (5). Studies by Jolliffe *et al.* (1,9) revealed that the energized state of the bacterial membrane was a crucial determinant in the regulation of autolysis and murein hydrolase activity. Specifically, agents that dissipate the proton gradient of cellular membranes were shown to induce autolysis (1,9).

This study tested the effect of chitosan on murein hyrdolase activity and growth in *cid* and *lrg* mutants of *S. mutans*. Chitosan is known to strongly upregulate the *lrg* operon during treatment with chitosan, which leads to production of antiholin proteins (13). Antiholins act in opposition and inhibit holins that are produced by the *cid* operon; therefore, transportation of hydrolases from the intracellular to extracellular matrix is repressed. However, the persistent premature expression and upregulation of *lrgAB* establishes an abnormal

accumulation of *lrgAB* gene products within the membrane, dissipating the membrane potential gradually. When the threshold is reached, the antiholin proteins from *lrgA* flip orientation to function as holins. Hydrolase genes that are normally expressed under these conditons now have more channels through which they can uncontrollably leave the cell, resulting in early cell death.

MATERIALS AND METHODS

Bacterial strains. Four bacterial strains were used for experimental purposes. Three strains of *S. mutans* UA159 (wildtype), SAB118 (D*cidA* : : WKm^r *cidAB*$^-$) and SAB115 (D*lrgAB* : : WKm^r *lrg*AB) were provided by Dr. Robert A. Burne of the Department of Oral Biology, College of Dentistry, University of Florida. *E. coli* B23 was obtained from the MICB 421 culture collection in the Microbiology and Immunology Department of the University of British Columbia.

Growth conditions. All strains were grown overnight in Todd Hewitt broth (Difco #56886JK, Fluka-Sigma #T1438) in a micro-anaerobic environment at 37ºC. All overnight cultures were inoculated with Todd Hewitt media treated with 1% acetic acid or 0.5 g/litre of chitosan (Sigma, #448869) dissolved in 1% acetic acid. Todd Hewitt broth with 1% acetic acid was used as a control to ensure observed effects were due to the chitosan and not the acetic acid. The media pH was slightly acidic at 6.9 to ensure chitosan activity.

Turbidity assay. Bacterial growth was measured as turbidity at OD_{600} using a Beckman Spectronic 20D spectrophotometer. The treated Todd Hewitt media sample with 0.5 g/litre of low molecular weight chitosan dissolved in 1% acetic acid was used as a blank. Each strain (*S. mutans cidAB*, *lrgAB*, UA159 (wildtype) and *E. coli* B23) was inoculated in Todd Hewitt media treated with 0.5 g/litre chitosan dissolved in 1% acetic acid and untreated Todd Hewitt media with 1% acetic acid. The untreated Todd Hewitt media sample with 1% acetic was used as a blank. The treated and untreated samples were measured at 600 nanometers at 10 minute intervals. For the chitosan treated samples, a 24 hour measurement was taken. The 24 hour samples were incubated at 37ºC in a micro-anaerobic environment.

Preparation of cells for the hydrolase assay. 15 ml cultures were grown in Todd Hewitt media overnight for 18 hours then diluted to 0.7 OD_{600}. The samples were treated with and without chitosan to a final concentration of 0.5 g/litre and incubated at 37ºC with shaking for 96 min.

Preparation of autolysins. Preparation methods for extracellular and intracellular autolysins were followed as described in Mani *et al.* (11). To collect extracellular autolysins, 15 ml cultures were centrifuged (6,000 x g, 5 min at 21ºC) to remove cells. The culture supernatant was used for the determination of extracellular hydrolase activity. The pelleted cells were retained for use in isolating intracellular hydrolases. Soluble autolytic activity present in the cytoplasm was isolated from the pellets after mechanical disruption of the cells with a FastPrep-24 bead-basher (M.P. Biomedicals, Irvine, California). The cellular debris was removed by centrifugation (6,000 x g, 5 min at 21ºC) and the supernatant was used to assay intracellular autolysin activity. Extracellular and intracellular autolysins were combined for determination of total autolysin activity and dialyzed for 24 hours in distilled water. The samples were then freeze-dried and concentrated 10X for use in the hydrolase assay.

Determination of total autolysin activity and hydrolase assay for autolytic activity. To determine autolytic activity, freeze-dried *Micrococcus lysodeikticus* cells were resuspended (1 mg/ml) in 0.1 M Tris buffer adjusted to a pH of 7.3. Enzyme extracts and assay cells were added in equal proportions and incubated at 37ºC with shaking. Turbidity was measured at 15 minute intervals at OD_{600} using a Beckman Spectronic 20D spectrophotometer.

Plate count. Bacterial strains were incubated at 37ºC in a micro-anaerobic environment in treated (0.5 g/litre chitosan dissolved in 1% acetic acid) and untreated (1% acetic acid) Todd Hewitt media. The untreated strains, *S. mutans* (*cidAB*, *lrgAB* and AU159 (wildtype)) and *E. coli* B23 were incubated for 50 minutes and the treated strains were incubated for 70 min. From each of the 8 samples, 100 μl of post-incubation culture was spread onto a 1.5% Todd Hewitt agar plate for a total of 16 plates with replicate plating. 100 μl of each of the treated 24 hour samples was spread onto 1.5% Todd Hewitt agar plates in replicate. All plates were incubated at 37ºC in a micro-anaerobic environment established by wrapping parafilm around each plate for approximately 24 hours.

Cell staining. Samples taken from the 24 hour incubated overnight chitosan treated and untreated plates were stained with 1% fuchsin dissolved in water and viewed under 1000x magnification to observe cell arrangement.

RESULTS

Growth of *S. mutans* strains and *E. coli* B23 with and without chitosan. Figure 1 depicts the exponential growth curve of the *S. mutans* strains and *E. coli* B23. Under micro-anaerobic conditions, *E. coli*, *lrgAB* and wildtype strains displayed identical exponential growth trends. The *cidAB* strain displayed a similar trend initially but began to enter the late exponential phase earlier than the other strains. Based on Figure 1, each strain had a steady exponential increase with similar growth rates. The slowest growth rate was observed in the *cidAB* mutant, which was approximately 20% slower than the *S. mutans* wildtype. As seen in Figure 2, chitosan had a dramatic effect on bacterial growth when compared to the control growth curve (Fig. 1). Chitosan had a negative effect on bacterial growth and the turbidity measurements decreased steadily over a 70 minute time period. In the presence of chitosan, the turbidity values of all of the strains decreased immediately. The cell lysis rates were the same for all of the *Streptococcus* strains, with *E. coli* having a slightly slower rate.
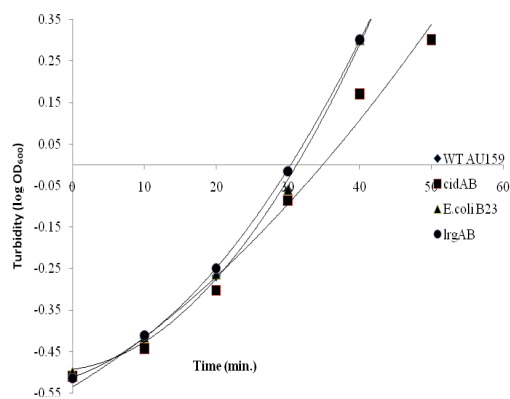

FIG.1. Control growth curve of overnight cultures of *S. mutans* (*lrgAB*, *cidAB* and wildtype) and *E. coli* B23 grown in Todd Hewitt media and incubated at 37ºC in a micro-anaerobic environment.
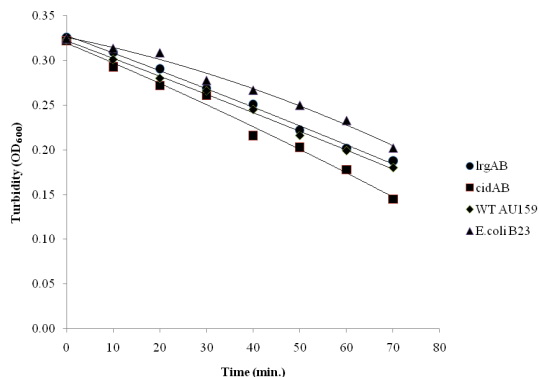
FIG. 2. Effect of chitosan on overnight cultures of *S. mutans* (*lrgAB*, *cidAB* and wildtype) and *E. coli* B23 incubated at 37ºC in a micro-anaerobic environment grown in Todd Hewitt media with 0.5 g/litre chitosan.

All of the samples that were not treated with chitosan resulted in a confluent lawn on all plates. However, on the plated samples treated with chitosan there was an astounding decrease in the number of colonies with an average of 11 colonies on the *S. mutans cidAB* sample, 9 colonies on the *S. mutans* wildtype sample and *S. mutans lrgAB* sample, and 3.5 colonies on the *E. coli* B23 sample.

Cell staining of *S. mutans* strains and *E. coli* B23 incubated for 30 minutes in the presence and absence of chitosan. All stained samples had a non-distinct cell arrangement when viewed microscopically. There were singles, pairs and clusters of cells visible. There was no difference between the chitosan treated and untreated samples nor between any of the bacterial strains. This procedure was undertaken to determine if chitosan had an effect on the cellular arrangement of cells which could indicate a change in the binary fission process.

Hydrolase accumulation in cells treated with chitosan. Figure 3 shows the decrease in turbidity for samples of *M. lysodeikticus* incubated with hydrolases isolated from *S. mutans* and *E. coli*. The turbidity for all eight samples of bacteria decreased in varying amounts. The control was composed of inactivated freeze-dried

Micrococcus suspended in Tris buffer and combined with concentrated and dialyzed Todd Hewitt media. No significant differences were observed for this sample over the incubation period. Of the four samples treated with chitosan, we observed the largest decrease (most hydrolase production) in the *lrgAB* knockouts and the smallest decrease was observed in the *cidAB* knockout. All of the untreated samples produced a relatively similar amount of hydrolase; an exception to this was the *lrgAB* knockout, which produced a significantly higher amount of hydrolase. Interestingly, the untreated sample of the *lrgAB* strain and the chitosan treated sample of the *lrgAB* mutants produced similar amounts of hydrolase; Chitosan upregulates the *lrgAB* gene, which is nonfunctional in this mutant. Also, the chitosan treated and untreated *E. coli* B23 samples showed similar levels of hydrolase production. The addition of chitosan clearly affected the two component regulatory system and in turn the hydrolase activity.

DISCUSSION

The *S. mutans cidAB* operon has been previously shown to enhance murein hydrolase activity and cell death (5,16). The *lrgAB* operon encodes a negative effector of these processes and is positively regulated by the LytSR two-component regulatory system and lies downstream from the *lytS* and *lytR* genes (1,12). Using BLAST, the *cidAB* and *lrgAB* operons are seen to be highly conserved in Gram positive bacteria. The LrgA protein of *S. mutans* has a 92% similarity to the Lrg protein family in *E. coli*. The proteins that are homologous to holin and anti-holin are well conserved within bacteria.

Previous experiments have shown that chitosan has an effect on the LytSR two-component regulatory system, and that the *cidAB* and *lrgAB* in *Streptococcus mutans*, affects murein hydrolase activity and autolysis (5,16,17) through induction of *lrgAB* by chitosan. The *lrgAB* operon encodes an anti-holin and the *cidAB* encodes the holin counterpart (1,5). *cidA* and *lrgA* gene products regulate murein hydrolase activity in a similar fashion as holins and anti-holins, respectively (1,5,12).

TABLE 1. Growth results of samples incubated with and without chitosan for 50 and 70 min and 100 µl plated on Todd Hewitt 1.5% agar incubated for 24h at 37ºC in a micro-anaerobic environment.

Sample	Treatment	Number of Colonies
S. mutans UA159 (wildtype)	1% Acetic acid	Confluent lawn
S. mutans UA159 (wildtype)	Chitosan + 1% acetic acid	9
S. mutans SAB115 (*lrgAB*)	1% Acetic acid	Confluent lawn
S. mutans SAB115 (*lrgAB*)	Chitosan + 1% acetic acid	9
S. mutans SAB118 (*cidAB*)	1% Acetic acid	Confluent lawn
S. mutans SAB118 (*cidAB*)	Chitosan + 1% acetic acid	11
E. coli B23	1% Acetic acid	Confluent lawn
E. coli B23	Chitosan + 1% acetic acid	3.5

The holin is a shorter form of the protein while the anti-holin is slightly longer with only a few extra positively charged amino acids such as lysine and arginine at the N-terminus. These anti-holins have extra positive charges that have a drastic effect on how the N-terminus of LrgA is oriented in the membrane.(5) The extra positive charges orient the N-terminus of the hydophobic anti-holin into the cytoplasmic side of the inner membrane (6,8). The CidA holin, which lacks the positive charges, is localized in the periplasmic side of the membrane. The presence of the N-terminal inside topology of the LrgA anti-holin in the presence of the chitosan-energized membrane confers an inhibitory effect to the CidA holin (15). There is an accumulation of holin protein within the membrane which gradually dissipates the proton gradient (1).

The growth and division of cells in the exponential phase occurs through a strictly controlled metabolism of the murein sacculus by murein hyrdolase to enlarge the sacculus through the insertion of new subunits. The hydrolases are also responsible for separating the septum during cell division. Compounds such as chitosan, dissipate the proton gradient of cellular membranes below a threshold force of more than 85 mV which is required to maintain cellular intergrity (1). Once a certain threshold is reached, the N-terminus of the anti-holin flips to the periplasmic side of the membrane. This conformational change establishes the anti-holin function as a holin, therefore creating a complete and rapid de-energization of the membrane potential (10). The reduced ability to maintain a homeostatic energy balance due to dissipation of membrane potential triggers this cascade in order to regain balance or induce cell death in order to recycle cellular components (15, 19).

Comparing Figure 2 to Figure 1, chitosan largely inhibited the growth of bacteria in the turbidity measurements. Based on Figure 1, each strain grew rapidly with similar growth rates with the *cidAB* mutant having the slowest growth rate. The *cidAB* mutant lacks the *cidAB* gene and therefore is unable to produce the holin and murein hydrolase needed for exponential growth, therefore establishing a slower growth rate. The *E .coli* and wildtype have the same growth rate (Fig. 1). The *lrgAB* mutant had a slower growth rate compared to the wildtype- approximately 9% lower, thus not establishing a significant difference in biological system.

The four strains that were incubated with 0.5 g/litre chitosan displayed the opposite effect on the turbidity compared to those not treated with chitosan. Each strain had a steady decrease in rate with *E. coli* having the lowest lysis rate compared to the *S. mutans* strains (Fig. 1). The *lrgAB* knockout mutant had a steady decrease due to its inability to inhibit the expression of the *cidAB* operon which increases murein hydrolase activity. The
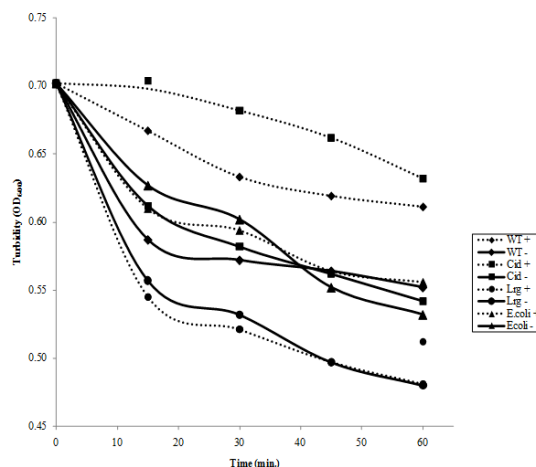
FIG. 3. Hydrolase assay for supernatant of 8 samples (*E. coli* B23, *S. mutans cidAB*, *S. mutans lrgAB* and S*. mutans* wildtype, incubated in the presence of chitosan) incubated 60 min, taking turbidity readings every 15 min at OD$_{600}$. The changes in turbidity of a culture of *M. lysodeikticus* were measured after addition of hydrolases isolated from the *S. mutans* and *E. coli* strains.

cidAB operon is expressed normally in the early exponential phase but no *lrgAB* operon is present to inhibit the hydrolase activity during the late exponential phase. Since there is no hydrolase inhibition, uncontrollable cell lysis occurs. The *cidAB* knockout mutant also has a steady decrease similar to the *lrgAB* mutant.

According to our hydrolase assay, a significant difference was observed in the levels of hydrolase production when cells were incubated with 0.5 g/litre chitosan as opposed to the untreated control. We measured both the intracellular and extracellular components of the four bacterial strains treated or untreated with chitosan in order to assay for total hydrolase activity. The plate counts before and after using the fast prep- 24 beadbasher showed cells were lysed effectively from a confluent lawn of bacteria to a rough estimate of 400 colonies after seven cycles of beadbashing (Fig. 3). All strains were expected to have similar amounts of cells since the turbidity of the cultures was measured and equalized before the incubation start time.

Chitosan effectively induced an inhibitory effect on the production of hydrolases in the Gram positive *S. mutans* strains and had almost no effect on the Gram negative *E. coli* (Fig. 3). The LrgA protein of *S. mutans* has a 92% similarity to the Lrg protein family in *E. coli* and would be expected to also be affected by treatment with chitosan. However, contrary to our expectations, the hydrolase results for *E. coli* treated with chitosan were very close to the levels of hydrolase produced in the untreated *E. coli* samples. This data suggested that

chitosan did not have an obvious effect on *E. coli*, as opposed to *S. mutans*. This piece of information seems to agree with past research in which chitosan was shown to have less effect on Gram negative bacteria (7). Nevertheless, cells can have multiple hydrolases. It is possible that the same type of hydrolase in *E. coli* were inhibited by chitosan while other types of hydrolases were not. Since the hydrolytic assay we did is quite crude, only one type of hydrolase might be enough to sustain cell lysis.

For the wildtype samples, the chitosan treated strain produced less hydrolase as compared to the untreated ones, which is what was expected. The effect of chitosan is to increase the production of anti-holins (13) by upregulating the *lrg* operon, and therefore hydrolases cannot be transported efficiently to the extracellular matrix. In the untreated samples, there is no strong upregulation of the *lrg* operon, so the bacterial cells can export normal levels of hydrolase across the membrane. In the treated samples, chitosan strongly upregulates the *lrg* operon, leading to increased anti-holin production and subsequent inhibition of holins which leads to decreased hydrolase levels.

Both the chitosan treated and untreated samples of the *lrg* knockout strain showed similar amounts of hydrolase production (Fig. 3). This is in accordance with our initial expectations. Chitosan upregulates the *lrg* operon, which works to reduces murein hydrolase production by inhibiting the holins produced by the *cid* operon (13). In the *lrg* strain under both treated and untreated conditions, the *lrg* operon could not be stimulated to inhibit hydrolase production; therefore, these two samples, with or without treatment of chitosan, were expected to produce the same amount of hydrolases.

Interestingly, the results for the *cid* knockout strains showed that cells treated with chitosan produced a lower level of hydrolases than the untreated samples. Theoretically, this strain cannot produce holins due to the absence of the *cid* operon and therefore should not be able to export hydrolase; therefore, cells are expected to produce low levels of hydrolase. In regards to the chitosan treated strain of the *cid* knockout, even though there is an upregulation for the *lrg* operon, the anti-holin produced has no target to stimulate. In the chitosan treated samples, the upregulation of the *lrg* operon leads to the production of more anti-holins. As previously explained, after a certain threshold is reached the N-terminus of the anti-holin will flip to the periplasmic side of the membrane and begin to function as a holin (10). In the untreated sample, the same would not be expected due to the lack of chitosan to upregulate the *lrg* operon. Therefore, more hydrolases would be expected in the treated samples compared to untreated ones. Nevertheless, our data indicated the

contrary and the untreated strains produced higher levels of hydrolase than that of the treated sample. One possible reason for this could be due to the complex interaction between proteins and genes in the LytSR system; it is possible that other genes may be involved in the regulation of hydrolase production in *S. mutans* other than the *cid* operon.

An important trend that we observed was that all of the untreated bacterial samples produced roughly equal amounts of hydrolases (Fig.3). When cells are not treated with chitosan, there is no upregulation of the genes and all strains are expected to exhibit similar levels of hydrolases required for normal cell growth and function. An exception to this was observed for the *lrg* strain for the previously explained reason.

In conclusion, we received contradicting results from our experiment. The turbidity assay results proved that chitosan plays an important role in bacterial cell lysis, but the levels of hydrolases produced by all four tested strains of bacteria were similar and there was no significant difference in lysis rates between all the strains when treated with chitosan. On the other hand, the hydrolase assay indicated chitosan functions on the two component system of *cid* and *lrg* operons and regulates the transportation of hydrolases from intracellular to extracullar. Chitosan works directly on *lrg* operon, so only the bacteria with *lrg* genes were affected by chitosan. And it is also proved that the *lrg* operon produces anti-holins that inhibit the transportation of hydrolases and therefore repress cell lysis. The *Cid* operon is involved in the process of cell lysis as well, since the *cid* knockout strains treated with chitosan showed different results from those of the wildtype. We can conclude that the *cid* operon functions in the opposite way to the *lrg* operon, which means it produces holins that can increase the transport of hydrolases and boost cell lysis. However, it is hard to prove the mechanism of how the *cid* operon works from our data. Some specific data points cannot be well explained using our hypothesis. Furthermore, it is shown that chitosan had no distinct effect on Gram negative bacteria compared to Gram positive strains, which may due to the difference of the cell membrane structure between them.

FUTURE EXPERIMENTS

Contradictory findings were obtained in our experiment. Our turbidity assay data showed that all samples treated with chitosan were lysed at the same rate, and therefore, similar amounts of hydrolases were expected to be produced from all strains. In contrast, the hydrolase assay saw significant differences in the amount of hydrolase produced from the four treated samples. As opposed to assaying for total hydrolase, future research can consider experimenting with

extracelluar and intracellular hydrolases independently to determine the contribution of each part to the total hydrolase activity. Future considerations include the investigation on the entry time of chitosan into the Gram positive and Gram negative bacteria. Also, since there are several different hydrolases present in bacteria, future experiments would have to be limited to only one specific hydrolase. Hydrolase knockout bacterial strains with only one intact hydrolase should be used to eliminate any variable hydrolase activity and focus on one specifically. Through the specific focus on one hydrolase, the effect of chitosan can be more effectively analyzed. Since LytSR also has more components, *cidA*, *cidB*, *lrg*A, *lrgB*, knockout strains of each component should be utilized for a more extensive analysis of the LytSR and the effect chitosan has on it.

REFERENCES

1. **Ahn,SJ., K.C. Rice, J. Oleas, K. W. Bayles, and R. A Burne.** 2010. The *Streptococcus mutans cid* and *lrg* systems modulates virulence traits in response to multiple environmental signals. Microbiology. **156**:3136-47
2. **Bayles, K. W**. 2003. Are the molecular strategies that control apoptosis conserved in bacteria?. Trends in Microbiol. **11**:306-311
3. **Dyahningtyas, T. E.**. 2010. Potency of chitosan as a bioactive edible coating for preservation of meat of common shrimps (*Crangon crangon*). Department of Chemistry. University of Hamburg, Germany.
4. **Garg, P., Y. J. Park, D. Sharma, and T. Wang**. 2010. Antimicrobial effect of chitosan on the growth of lactic acid bacteria strains known to spoil beer. J. Exp. Microbiol. Immunol. **14**:7-12
5. **Groicher, Kajetan H., B. A. Firek, D. F. Fujimoto, and K. W. Bayles.** 2000. The *Staphylococcus aureus lrgAB* Operon Modulates Murein Hydrolase Activity and Penicillin Tolerance. J. Bacteriol. **182**: 1794-1801
6. **Helander, M., E. L. Nurmiaho-Lassila, R. Ahvenainen, J. Rhoades, and S. Roller**. 2001. Chitosan disrupts the barrier properties of the outer membrane of Gram negative bacteria. Int. J. Food Microbiol. **71**: 235-244
7. **Hong, K. No, N. Y. Park, S. H. Lee, and S. P. Meyers.** 2002.Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. Int. J. Food Micrbobiol. **74**: 65-72
8. **Jolliffe, L. K., R. J. Doyle and U. N. Streips**.1981. The energized membrane and cellular autolysis in *Bacillus subtilis*. Cell. **25**:753-763
9. **Kong, M., X.G. Chen, K. Xing, and H.J. Park**. 2010. Antimicrobial properties of chitosan and mode of action: A state if the art review. Int. J. Food Microbiol. **144**:51-63.
10. **Liu, X. F.,Y. L.Guan, D. Z.Yang, Z. Li, and K. D. Yao.** 2001. Antibacterial action of chitosan and carboxymethylated chitosan,. J. Appl. Polymer Sci. **79**:1324-1335.
11. **Mani, N., P. Tobin, and R. K. Jayaswal.** 1993. Isolation and characterization of autolysis-defective mutants of *Staphylococcus aureus* created by Tn917-lacZ mutagenesis. J. Bacteriol. **175**:1493-1499
12. **Patton, T. G.** 2005. The regulation of *lrg* and *cid* operons and their effect on viability and lysis in *Staphylococcus aureus*. University of Idaho, United States.
13. **Raafat, D., K. von Bargen, A. Haas, and H.G. Sahl**. 2008. Insights into the mode of action of chitosan as an antimicrobial compound. Appl. Environ. Microbiol. **74**:3764-3773
14. **Rabea, E. I., M. E.-T. Badawy, C. V. Stevens, G. Smagghe, and W. Steurbaut.** 2003.Chitosan as antimicrobial agent: Applications and mode of action. Biomacromolecules **4**:457–1465
15. **Rice, K. C. and K. W. Bayles.** 2008. Molecular control of bacterial death and lysis. Microbiol. Mol. Biol. Rev. **72**:85-109
16. **Rice, K. C., B. A. Firek, J. B. Nelson, S. J. Yang, T. G. Patton, and K. W. Bayles.** 2003. The *Staphylococcus aureus cidAB* operon: Evaluation of its role in regulation of murein hydrolase activity and penicillin tolerance. J. Bacteriol. **185**:2635-2643
17. **Rice, K. C., T. Patton, S. J. Yang, A. Dumoulin, M. Bischoff, and K. W. Bayles.** 2004. Transcription of the *Staphlococcus aureus cid* and *lrg* murein hydrolase regulators is affected sigma factor B. J.Bacteriol.**186**:3029-3037
18. **Richardson, S.C.W., H.V.J. Kolbe, and R. Duncan.** 1999. Potential of low molecular mass chitosan as a DNA delivery system: biocompatibility, body distribution and ability to complex and protect DNA. Int. J. Pharm. **178**:231-243
19. **Sharma-Kuink, B. K., E. E. Mann, J. S. Ahn, L. J. Kuechenmeister, P. M. Dunman, and K. W. Bayles**. 2009. The *Staphylococcus aureus* LytSR two -component regulatory system affects biofilm formation. J Bacteriol. **191**:4767-4775