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Chitosan Inhibits pBR322-Amp^R transformation in *Escherichia coli* DH5α

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The effect of chitosan, a polycationic biopolymer, during transformation in Gram-negative *Escherichia coli* DH5α cells was investigated in this study. Chitosan was hypothesized to enhance transformation efficiency by transient membrane permeabilization in *E. coli* DH5α cells through electrostatic interactions. Transformation efficiency of chitosan-mediated transformation was compared to the conventional heat shock method and the optimal conditions to use chitosan were determined by performing a pH assay, minimum-inhibitory concentration assay, and DNA optimization assay. In comparison to the heat shock method, chitosan-supplemented transformation did not enhance bacterial transformation in *E. coli* DH5α cells. Furthermore, chitosan-supplementation in heat shock transformation resulted in a decrease in transformation efficiency. The effect of chitosan on cell growth was further investigated and cell aggregates were observed in chitosan-treated *E. coli* DH5α cells in addition to reduced cell growth. Results from this study showed that chitosan inhibits transformation in Gram-negative *E. coli* DH5α cells in comparison to the heat shock method of bacterial transformation. The mechanism of inhibition may be due to the formation of a cell-chitosan complex that physically excluded DNA, resulting in decreased transformation efficiency.

Transformation, the introduction of foreign genetic material in bacterial cells, is a phenomenon that is poorly understood. Heat shock is the conventional method to perform transformation wherein the plasmid DNA is mixed with competent bacterial cells and the plasmid DNA-cell mixture is subsequently heated to 42 ºC, which is believed to impart physical holes in the membrane, allowing the plasmid DNA to enter the cell (1). In this process, the loss in cell viability is inevitable and remains to be one of the limiting factors in transformation efficiency (2).

Chitin is a ubiquitous polymer of N-acetylglucosamine that can be found in the exoskeletons of insects, crustacean shells, and walls of certain fungi (3). Chitosan, a derivative of chitin, can be obtained by the N-deacetylation of chitin and has a wide range of applications in the biotechnological industry (4). Specifically, the antimicrobial properties of chitosan have been extensively studied due to its broad spectrum of effects. In addition to antifungal activity and yeast inhibition, chitosan exhibits bactericidal effects in various Gram-negative and Gram-positive bacteria (3-5).

In Gram-negative bacteria, chitosan has been reported to exert its antimicrobial activity by disrupting membrane integrity. Chitosan, a relatively insoluble macromolecule in aqueous solutions, can be made soluble by protonation of the NH_2 group on carbon-2 below its pKa (pH: 6.3). The poly-cationic form of chitosan is thought to be interacting with anionic components of the outer membrane such as lipopolysaccharides and various protein. The electrostatic interaction between chitosan and the components in the outer membrane disrupts membrane integrity by increasing membrane permeability. (3)

Interestingly, Kean *et al* demonstrated that chitosan could also be used for gene delivery in epithelial breast cancer cells (6). In their study, chitosan and its tri-methylated variants were complexed to plasmid DNA to transfect MCF-7 breast cancer cells. Transfection with chitosan resulted in a greater efficiency than polyethylenimine, a common transfection agent used for mammalian cell transfection (6). Consistent with membrane permeabilization effect observed in bacterial cells, the mechanism of transfection in mammalian cells is also dependent on the electrostatic interactions between chitosan and the cell membrane (6). Chitosan has thus been proposed as a non-viral gene delivery vector in eukaryotic mammalian cells. The use of chitosan in bacterial transformation has not been reported in literature. Chitosan-mediated transformation may provide a novel method in gene delivery in bacterial cells if exogenous gene delivery is also enhanced. This study attempted to assess the use of chitosan as an alternative to the conventional heat shock as a method to transform bacterial cells.

It is hypothesized that chitosan enhance transformation efficiency by transient membrane permeabilization in *E. coli* DH5α cells through electrostatic interactions. In this study, chitosan inhibited transformation in Gram-negative *E. coli* DH5α cells in comparison to the conventional heat shock method of bacterial transformation. The mechanism of inhibition may be due to chitosan forming a complexes with the cells to physically exclude exogenous DNA, resulting in a decreased transformation efficiency.

MATERIALS AND METHODS

Bacterial Strain and Culture Condition. *E. coli* DH5α was used as the host cell and pBR322-Amp^R plasmid was used as the vector in order to assess the effect of chitosan on plasmid transformation. Ampicillin drug selection was used to distinguish transformants from non-transformed cells. Both cells and vectors were obtained from the MICB 421 culture collection at the University of British Columbia Department of Microbiology and Immunology. Lysogeny broth – Lennox (LBL) was used instead

of regular Lysogeny broth with 5 g less sodium chloride as indicated in Hancock manual (7).

Preparation of Chitosan. 1 g of chitosan (Sigma, #448869) was added to 50 ml of water. Once the powder was well distributed, 50 ml of 2% (v/v) acetic acid was added to make 1% (v/v) acetic acid in order to fully dissolve the chitosan. The final chitosan concentration was 10,000 ppm. The solution was solubilized by stirring for at least 30 minutes, and then was diluted to the desired concentration for subsequent experiments with 1% acetic acid.

Preparation of Competent cells. The method of preparing competent cells was adapted from the Hancock Lab methods (7). *E. coli* DH5α cells were first grown overnight in LBL, and the overnight culture was used to inoculate each of four 100 ml aliquots of fresh LBL broth. The cells were incubated at 37 ºC with shaking and allowed to grow up to an OD_{600} of 0.2-0.4 before they were put on ice for 10 minutes. To harvest the cells the cultures were centrifuged at 10,000 rpm for 5 minutes with Bio-Rad benchtop centrifugation, then the supernatant was removed and the cells were resuspended in ice cold 0.1 M $CaCl_2$ and left on ice for 20-40 minutes. Next, the centrifugation step was repeated and the cells were resuspended in 8 ml of 15% glycerol, 0.1 M $CaCl_2$ freezing solution. These competent cells were stored at -80 ºC.

pH Assay. pH assays were carried out to find the lowest pH that DH5α cells could tolerate, since chitosan was made in an acidic solution. Three aliquots of 1% acetic acid solution were prepared and titrated to pH 4.5, 5.5 and 6.0 respectively with 5 M NaOH. The pH adjusted acid was then added to DH5α cells at 1:1 ratio. Cells were exposed to each pH condition for 1 minute, 3 minutes, 5 minutes, 10 minute and 15 minutes. A positive control was also prepared with $CaCl_2$ buffer, which is the buffer used to prepare competent cells. After exposure to acetic acid, 1 ml of LBL broth was added to each cell aliquot and cells were incubated at 37 ºC for 1 hour. Cells from each condition was then plated onto LBL agar plates and incubated overnight at 37 ºC. Upon observing an absence of cell inhibition in the above assay, another pH assay was carried out with a lower pH in a 96-well plate. 50 µl of 1% acetic acid at pH 2.5 (uptitrated) was added to 5 µl of cells and 45 µl of LBL broth. The solution was then incubated overnight at 37 ºC.

Minimum Inhibitory Concentration (MIC) Assay. The MIC assay was performed in order to find the lowest concentration of chitosan the cells could tolerate. The method was adapted from a previous MIC assay done by Hancock *et al* (8). A final chitosan concentration of 3.13 - 100 ppm, achieved by a serial dilution with a dilution factor of 2, at pH 4.5 (adjusted with NaOH from the chitosan stock) was used for the MIC assay. The mixture of cells and chitosan was incubated at 5 minute and 15 minute intervals before being spread on LBL agar plates, which were incubated at 37 ºC overnight. These time points were chosen, as the same incubation times were used for the chitosan supplemented transformations during subsequent experiments. A modified MIC assay was performed in a 96-well plate using up to 200 ppm chitosan at both pH 6 and 2.5. Each well contained 5 µl cells, 50 µl chitosan and 145 µl LBL broth. The plates were incubated overnight at 37 ºC, and cell growth was judged based on turbidity. A control with the addition of 1% acetic acid without chitosan was used for each MIC assay.

DNA Optimization Assay. The amount of pBR322-Amp^R to introduce into the competent *E. coli* DH5α cells was determined by assessing transformation efficiency. Transformation of *E. coli* DH5α cells was performed using a bacterial transformation protocol from Hancock's online database (7). A range of pBR322-Amp^R (10 pg, 100 pg, 1 ng, 10 g, and 100 ng) was combined with 50 µl of prepared competent *E. coli* DH5α cells. Cell-plasmid mixtures were incubated at 4 ºC for 30 minutes and placed in a 42 ºC water bath for 1 minute. Samples were then incubated at 4 ºC for 1 minute and 1 ml LBL broth was added to each for a 1 hour incubation at 37 ºC with shaking. 100 µl of the transformed cells were plated on LBL plates supplemented with ampicillin (0.05 µg/ml) and incubated at 37 ºC overnight before performing colony count to assess transformation efficiency.

Transformation with Chitosan Compared to Heat Shock and Chitosan Complemented Heat Shock. Transformation of DH5α cells with chitosan was carried out and compared with the heat shock method (positive control) to assess whether chitosan enhances transformation. 10,000 ppm chitosan was prepared as described previously and then diluted to 100 ppm. 50 µl of the diluted chitosan solution and 2 ml of pBR322-amp^r plasmid DNA (10 ng) were then added to 50 µl of competent DH5α cells and incubated at room temperature for 15 minutes. 1 ml of LBL broth was then added to the mixture and incubated at 37 ºC for 1 hour. 100 µl of the chitosan treated culture was then spread onto LBL agar plates with ampicillin (0.05ug/ml) and incubated overnight at 37 ºC. Colony forming units were counted the next morning. Transformation by the regular heat shock method was carried out in parallel with chitosan transformation as a positive control. A negative control with no DNA added was also carried out to ensure absence of contamination. Duplicates were done for each condition.

24-hour Growth Assay. 24-hour growth assay method was adapted from a 24-hour growth assay experiment with chitosan using Gram-positive lactic acid bacteria by Pan *et al* (3). An overnight culture of *E. coli* DH5α was grown until an OD_{600} of 0.2 was reached to obtain cells in the exponential growth phase. Three conditions including LBL broth (7 ml), 7 ml + 70 µl of chitosan, and 7 ml + 70ul of 1% acetic acid were prepared and incubated as blank, chitosan-treated, and 1% acetic acid-treated, respectively. After 24 hours of incubation at 37 ºC, the turbidity of the samples was measured using the Pharmacia Biotech® Ultrospec® 3000 Spectrophotometer at an absorbance of 600 nm.

Chitosan-mediated Precipitation Assay. Using the optimal conditions as previously determined, 10 ng of pBR322-Amp^R was combined with 50 µl *E. coli* DH5α competent cells and incubated at 4 ºC for 20 minutes. A range of chitosan concentrations (100, 50, 25, 12.5, 6.25, and 3.1 ppm) was added to the cell-plasmid DNA mixtures and incubated for 15 minutes. 1 ml of LBL broth was then added and the samples were incubated at 37 ºC before plating 100 µl onto LBL plates supplemented with ampicillin (0.05 µg/ml). The bacterial plates were incubated at 37 ºC overnight before performing cell counts and evaluating the degree of precipitation by visually assessing the presence and size of the precipitate.

Fluorescence Staining Assay For Staining Permeabilized Cells. A fluorescence staining technique was adapted from a previous experiment (3) where groups of gram positive lactic acid bacteria were treated with chitosan in order to assess the antimicrobial activity of chitosan via cell membrane disruption. Upon cell permeabilization, the membrane permeant acridine orange dye can enter the cell and dye the cell red-orange, whereas propidium iodide is membrane impermeant and will only dye intact cell membranes. For the control group, *E. coli* DH5α in exponential growth phase was obtained by inoculating an overnight culture in LBL and was incubated at 37 ºC until an OD_{600} of 0.2 was reached. The positive control group was treated with toluene to a final concentration of 100 µl/ml, whereas the negative control group was treated with acetic acid to a final concentration of 66.7 µl/ml. The two treatment groups were incubated at 37 ºC for 24 hours. After overnight incubation, washing and staining steps were performed. The cells were pelleted by centrifugation at 7,000 g for 10 minutes, then

resuspended by three minutes of vortexing and washed with 1 ml of 0.85% NaCl. The cells were then centrifuged again at 7,000 g for 5 minutes. This washing step was repeated two more times. After the final wash, cells were resuspended in 0.85% NaCl and stained with 3 µl of a 1:1 mixture of 20 mM propidium iodide (PI, Cat. No. P4170, Sigma-Aldrich®) and 0.01% w/v acridine orange (AO, Cat. No. A4921, Sigma-Aldrich®). Stained samples were immediately vortexed for a few seconds, and then incubated in the dark for 15 minutes. The dyes were removed by centrifuging the cells at 7,000 g for 5 minutes and resuspending in 1 ml of 0.85% NaCl. A wet mount for each sample was prepared and observed under a Zeiss Axiostar Plus fluorescence microscope set with a 490 nm excitation filter and a 510 nm barrier filter. Photos were taken with an iPhone 5S via the microscope eyepiece. For the experimental group, the *E. coli* DH5α cells were treated with chitosan, then heat shocked before proceeding with the washing and staining steps.

RESULTS

Competent *E. coli* DH5a cells tolerated acidic pH 4.5-6, withstood chitosan concentrations up to 200 ppm, and yielded consistent transformants with 10 ng of pBR322-Amp^R. A pH assay was performed in order to determine if competent *E. coli* cells would be able to tolerate an acidic environment. Chitosan is dissolved in 1% acetic acid for protonation and solubility (6). A higher degree of protonation may result in a stronger electrostatic interaction with negatively-charged components of the outer membrane. In the first pH assay, E. coli cells were exposed to a pH of 4.5, 5.5, 6.0 by the addition of pH-titrated 1% acetic acid and incubated for 1, 3, 5, 10 and 15 minutes. After overnight incubation on LBL plates, lawns of cells were observed at each pH conditions for all incubation periods. A second pH assay was performed to determine if *E. coli* cells were able to tolerate an even lower pH. In a 96-well plate, microcultures of *E. coli* cells were exposed to an extended range of pH (2.5, 4.5, 5.5, and 6.0) and cell growth was assessed by turbidity. Cell growth, marked by the turbidity, was observed for all pH conditions relative to the negative control.

A MIC assay was carried out in order to determine the minimum concentration of chitosan the *E. coli* cells could tolerate, to use in the transformation experiments. Initially, cells were plated on LBL agar plates after incubation at 5 minute and 15 minute intervals with 3.13-100 ppm of chitosan at pH 4.5. However, results showed a lawn of cells under all the concentrations of chitosan used (Data not shown). As such, modifications were made and further MIC assays were prepared with up to 200 ppm final chitosan concentration, along with overnight incubation in 96 well plates. The pH of the chitosan was decreased to 2.5 to increase protonation of the chitosan, since the previous pH assays showed cell viability at this low pH level. Despite the higher chitosan concentration and overnight incubation, cell growth was still observed in the form of turbidity in the 96 well plates (Data not shown), and it was concluded that the *E. coli* cells were viable at up to 200 ppm of chitosan.

Since transformation efficiency (TE) varies with the batch of competent cells and isolated plasmid DNA, the amount of DNA that would yield the highest TE with consistent colony numbers had to be determined. A range of pBR322-Amp^R (10 pg, 100 pg, 1 ng, 10 g, and 100 ng) was combined with 50 µl of prepared competent *E. coli*

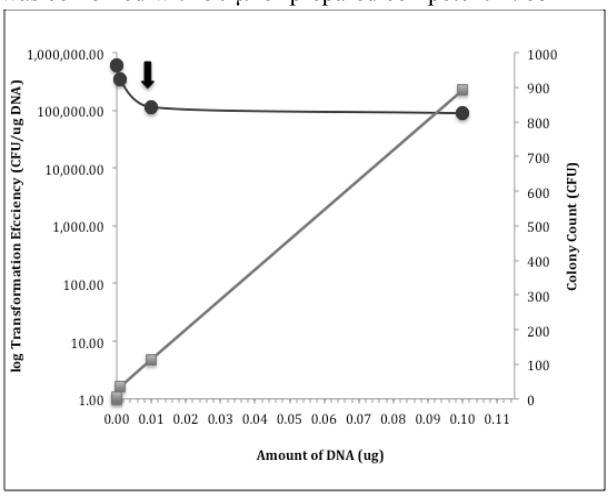

FIG 1 Comparison of Transformation Efficiency with varying amounts of pBR322-Amp^R in competent *E. coli* DH5*α* competent cells. A range of pBR322-Amp^R (10 pg, 100 pg, 1 ng, 10 g, and 100 ng) was combined with 50 µl of prepared competent *E. coli* DH5α cells. 10 ng (as indicated) of plasmid DNA was the amount at which moderate colony count with sufficient transformation efficiency were obtained.

DH5α cells. By comparing transformation efficiency using varying amounts of pBR322-Amp^R, it was found that 100 pg resulted in the highest TE (Fig. 1).

Upon several attempts to repeat the experiment, however, the transformation efficiency for 100 pg and 1 ng was difficult to reproduce due to inconsistent colony numbers. In some replicates, an absence of colonies was observed and statistical analysis could not be performed. In contrast, 10 ng of pBR322-Amp^R demonstrated consistent colony numbers and transformation efficiency, and was used in subsequent assays (Fig. 1).

Chitosan-mediated transformation showed significantly low transformation efficiency compared to conventional heat-shock method. As shown in FIG 2, no colony forming units (CFUs) were observed on plates with chitosan supplemented cells. In contrast, the heat shocked sample had 266 CFUs on average. A white precipitate was observed in the chitosan treated cells after the 1 hour incubation.

Chitosan complemented heat shocked sample showed significantly low transformation efficiency compared to heat shocked sample. As shown in Fig 3, transformation efficiency of pBR322-Amp^R into DH5α cells is significantly inhibited by chitosan. Heat shock treated DH5α cells showed 266 colonies and chitosan decreased the CFU count to 17.

Chitosan reduced *E. coli* DH5*α* growth but cells remained viable. The role of chitosan in inhibiting DNA uptake during bacterial transformation was examined since transformation seem to be negatively affected by the addition of chitosan. A 24-hour growth assay was

performed using 100 ppm of chitosan and the turbidity was quantified by measuring OD_{600} that was not done in the previous MIC assay. Turbidity measurements were not

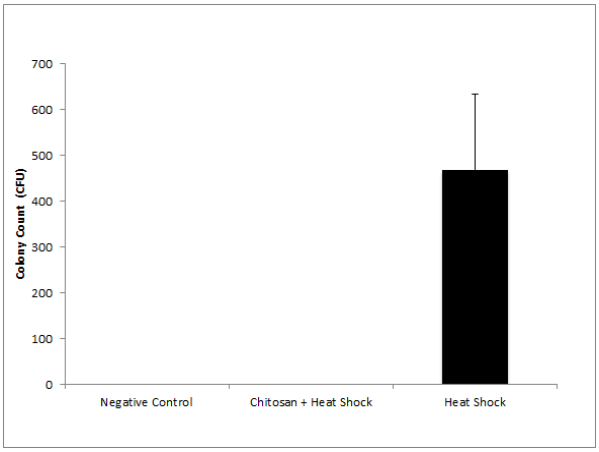

FIG 2 Number of Colony Forming Units observed in chitosan treated cells and heat shocked cells. DH5α cells were treated with 100 ppm of chitosan for 15 minutes at room temperature, and heat shocked cells were incubated at 42 ºC for 1 minute. 468 colonies were observed in the heat shock transformation and no colonies were observed in the chitosan-treated transformation.

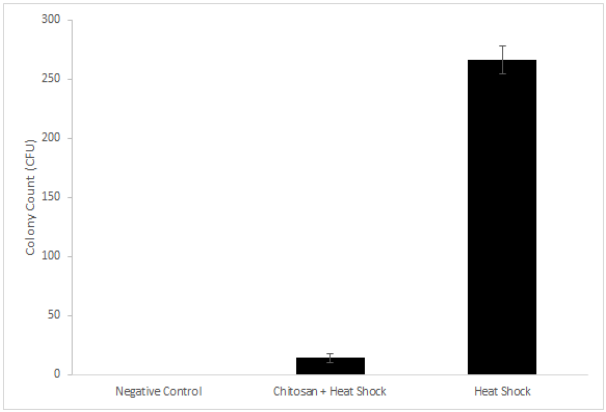

FIG 3 Number of Colony Forming Units observed in chitosan supplemented heat shock and conventional heat shock. DH5α cells were treated with 100 ppm of chitosan for 15 minutes at room temperature and heat shocked for 1 minute at 42 ºC. 100 µl of LBL broth was spread on the LBL-Amp agar plate. Heat shock method resulted in 266 colonies. Chitosan complemented heat shock resulted in 17 colonies.

taken then since the goal was to assess of growth that was visually determined by absence/presence of turbidity.

In the 24-hour growth assay, the culture and reagent volumes were significantly scaled up (39-fold) in comparison to the MIC assay in order to better assess any macroscopic differences between the samples. During the course of the experiment, a precipitate was observed in the chitosan-treated cells that was absent during the MIC assay (Fig. 4B). Turbidity measurements were still taken for the untreated, 1% acetic acid-treated, and chitosan-treated samples, where all of the samples were thoroughly re-suspended to obtain OD_{600} reading. The results show that chitosan extensively reduced *E. coli* DH5α growth by an extensive reduction in culture turbidity (Fig. 4A).

Chitosan caused cell precipitation in *E. coli* DH5α cells. To further investigate the relationship between chitosan and the precipitate observed in the 24-hour growth experiment, a precipitation assay was performed in which *E.coli* DH5α cells were transformed with 10 ng of pBR322-amp^r using varying concentrations of chitosan (100, 50, 25, 12.5, 6.25, and 3.1 ppm) and incubated for 15 minutes. During the incubation period, precipitates were observed at higher chitosan concentrations (Fig. 4C). Furthermore, the results also showed that the degree of precipitation may be positively correlated with the concentration of chitosan; a higher degree of precipitation was observed in the samples containing higher chitosan concentrations (Fig. 4C). The cell-plasmid DNA mixtures were plated on selective plates and cell count analysis showed an absence of colony formation, except for a single colony on 100 ppm chitosan-treated cells (Table 1). The identity of the precipitate was determined to be a chitosan-cell complex due to same physiological effect observed during the 24-hour growth assay.

Chitosan caused *E. coli* DH5α aggregation and heat shock may enhance cell perforation. Positive control treated with toluene became perforated as we anticipated, whereas negative control treated with acetic acid did not disturb the cell membrane. 27 out of 56 were perforated cells dyed with red colour (Fig. 5) in untreated cells. 8 out of 22 cells were observed in the heat shock treated cells as an intact cells, but 14 red dyed perforated cells were observed in the same field under the 510 nm excitation filter (Fig. 5). Interestingly, large cell clumps were observed in the chitosan supplemented heat shock treated cells (Fig. 5). Since all perforated and intact cells were aggregated into large masses, the aggregates fluoresced under both the 490 nm and 510 nm excitation filters (Photo under 510 nm excitation filter is not shown).

DISCUSSION

In this experiment, the role of positively charged chitosan in the transformation of pBR322-Amp^R into *E. coli* DH5α cells was investigated. Chitosan is known to be well protonated at lower pH levels, and is typically dissolved in 1% acetic acid. In order to find an optimum condition where chitosan is protonated enough to disrupt the cell membrane and aid in plasmid delivery, but still retain cell viability, pH assays and MIC assays were performed.

The first pH assay was set up to mimic transformation conditions instead of using the heat shock method in 96 well plates. Inhibition of cell growth at a specific pH in the range at a specific exposure time was expected. However, cell growth was observed in all conditions. This result suggested that DH5α cells may have been able to tolerate an even lower pH. Therefore, another pH assay was carried out wherein cells were subjected to pH 2.5, which is the natural pH of 1% acetic acid, for 15 minutes and overnight; however, cell growth was

observed again. This observation led to the conclusion that DH5α cells were able to tolerate fairly acidic conditions as low as pH of 2.5 for at least 24 hours. Therefore, all further experiments involving chitosan

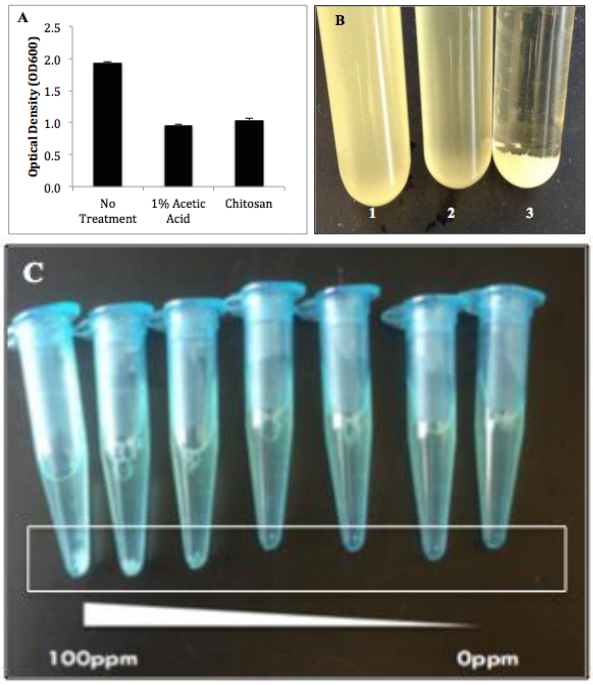

FIG 4 Chitosan reduced *E. coli* DH5α growth and caused precipitation (A) *E. coli* DH5α cells were incubated for 24 hours in LBL broth, LBL and 1% acetic acid, or LBL and 100 ppm chitosan (final concentration). Cell turbidity was measured at OD_{600} and extensive decrease in OD_{600} was observed in 1% acetic acid and chitosan-treated cells. (B) Precipitation formation during the 24-Growth Assay in (1) untreated, (2) 1% acetic acid and (3) chitosan-treated cells. Extensive precipitation was observed in chitosan-treated cells. (C) Chitosan-mediated Precipitation Assay. *E. coli* DH5α cells were transformed with 10 ng of pBR322-amp^r and incubated with varying chitosan concentration (100, 50, 25, 12.5, 6.25, 3.1 ppm) for 15 minutes. Precipitates (enclosed area) were clearly observed at chitosan concentration of 100, 50, and 25 ppm.

were carried out without adjusting the pH of the 1% acetic acid (pH 2.5).

Since it was found in previous literature that the MIC for chitosan on *E. coli* DH5α is 20 ppm (8), several assays were carried out in order to confirm the MIC of chitosan. The first assay yielded inconclusive results where no inhibition of growth was observed, and it was thought that the 5 and 15 minute incubation times were insufficient for chitosan to exert any physiological effect. As such, the following assay was carried out with an overnight incubation at pH 6. However, the result was also inconclusive because the cells grew under all chitosan concentrations up to 200 ppm. It was then hypothesized that pH 6 was too high and resulted in a decrease in protonation of the amine group on the chitosan, which inherently rendered it unable to interact with the negatively charged components of the bacterial cell membrane to cause permeabilization. Therefore, the next assay attempt was carried out at pH 2.5 to increase protonation, since previous pH assays showed cell viability at this low pH level. This assay still showed cell growth under all the chitosan concentration levels,

TABLE 1 The effect of varying chitosan concentration on *E. coli* DH5α colony count. *E.coli* DH5α cells were transformed with 10 ng of pBR322-Amp^R using varying concentrations of chitosan (100, 50, 25, 12.5, 6.25, and 3.1 ppm) and incubated for 15 minutes. Colony count was only observed with chitosan concentration at 100 ppm.

[Chitosan] (ppm)	Colony Count
3.1	0
6.25	0
12.5	0
25	0
50	0
100	1

so it was concluded that the cells could tolerate up to 200 ppm of chitosan at pH 2.5 and that subsequent transformation experiments could be done within this range.

The absence of transformation in chitosan treated samples suggested that chitosan inhibits transformation, instead of enhancing transformation in DH5α cells. The appearance of precipitates and clear supernatant in the chitosan-treated samples suggested that chitosan may have attracted the cells, which have negatively charged membranes (3), and formed chitosan-cell complexes. This observation was further supported by the microscope image shown (Fig. 5C) where cell aggregates were observed. In addition, a comparison of the microscope figures of the heat shock-treated sample (Fig. 5B) and the untreated sample indicates that the heat shock method may enhance cell membrane perforation, since more red dyed cells were present. A chitosan-mediated precipitation assay was then carried out to further investigate the relationship between chitosan and aggregate formation. Results indicated that chitosan was positively correlated with precipitation in which a higher degree of precipitation was observed at higher chitosan concentrations. This physiological effect of chitosan on *E. coli* DH5α cells may be the critical factor that is responsible for inhibiting bacterial transformation. Chitosan may inhibit DNA uptake by binding to negatively-charged outer membrane through electrostatic interactions, and forming polymers with other chitosan-cell complexes. It is also likely that chitosan can form a complex with the plasmid DNA, which can inhibit bacterial DNA uptake through other mechanisms. Interestingly, in other studies where 250 ppm chitosan at pH 5.6 was used, no precipitation of Gram-negative bacteria was observed (4). Therefore, it was speculated that the low pH used in this study may have promoted cell aggregation through excess protonation.

Due to the absence of transformation in the first experiment, another transformation efficiency assay was performed to test whether chitosan coupled with

the heat shock method had any effect on transformation. As shown in chitosan-supplemented heat shock inhibited transformation (Fig. 4), and cell precipitation was observed once again. The consistent formation of

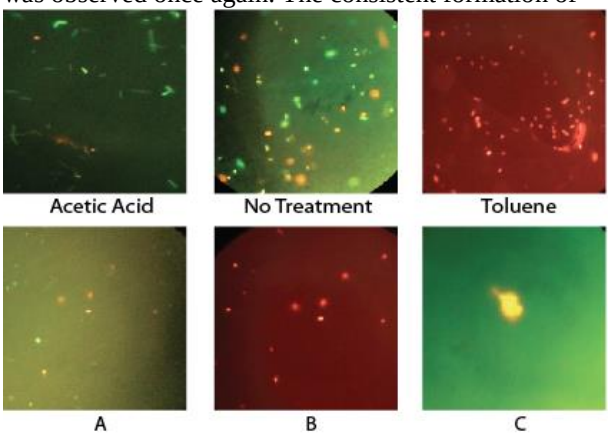

FIG 5 Fluorescence staining and visualization. (a) Heat shock treated cells were stained using propidium iodide (20 mM) and acridine orange (0.01% w/v). Intact cells were stained green with propidium iodide under 490 nm excitation filter. (b) The same heat shock treated and stained cells were observed under 510 nm excitation filter. Perforated cells were stained red with acridine orange. (c) chitosan complemented heat shock treated cells were observed under 490 nm excitation filter. The positive control group was treated with toluene (100 µl/ml), resulting in perforated cell membranes showing bright red colour, and the negative control group was treated with acetic acid (66.7µl/ml). The experimental negative control treated with acetic acid and toluene did not undergo heat shock. The cells were viewed under a 1000x objective lens. Photos represent an average trend over 20 fields

precipitation in chitosan treated samples led to the speculation that chitosan inhibited transformation by aggregating DH5α cells or pDNA, or both. The fact that chitosan is used as a fining agent to precipitate eukaryotic yeast cells during the process of beer-making supports the findings of this experiment (J. Smith, personal communication).

Another observation was that this precipitation only occurred in the transformation experiments and the 24-hour growth assay; no precipitation was observed in the MIC assays. It was speculated that the absence of aggregates in the MIC assays was due to the relatively small scale of the experimental set-up; the final volume of the chitosan/cell mixtures was only 200 µl, compared to the 1 ml during the transformation experiments.

Once the inhibitory effect of chitosan on bacterial transformation was established, a 24-hour growth assay was performed in order to further investigate and verify the effect of chitosan in *E. coli* DH5α cell growth. The data from this assay demonstrated that chitosan reduced *E. coli* DH5α cell growth that was not previously observed in the MIC assay in which OD_{600} was not taken. This was in line with our expectations due to previously reported chitosan antimicrobial activity in literature (3-5). These results further verified that *E. coli* DH5α cells were still viable under 100 ppm chitosan, which supports the MIC assay data. In addition, the presence of cell aggregates was evident in the chitosan-treated cell sample during the 24-hour growth assay. Overall, these findings indicated that chitosan inhibit bacterial transformation in Gram-negative *E. coli* DH5a that may be due to chitosan precipitating cells and/or forming polymers with other chitosan/cell complexes.

FUTURE DIRECTIONS

A further test can be performed to decrease the precipitation observed during transformation by carrying out the transformation at a higher pH. Doing this will decrease the amount of protonation on chitosan, rendering it less positive, which may lead to less interactions with the negative cell membrane and therefore, lower the precipitation. Also, future experiments can be carried out to investigate the detailed mechanism of how chitosan interferes with the transformation process by performing transformation in different pH adjusted with acetic acid. Possible tests include investigating the interaction between chitosan and DNA using DNA-tagged fluorescence assays, or the effects of acetic acid in the transformation process. This experiment can also be repeated using tri-methylated chitosan, which is more stable and is soluble in water.

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