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Growth of *Bacillus subtilis* in Phosphate Limited Media Reduces Susceptibility to Antibacterial Activity of Chitosan

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Chitosan is a commercially available derivative of chitin that has been extensively studied for its antimicrobial properties. Recent studies point to the disruption of cell membranes as a possible mode of action that is facilitated by a strong positive charge from protonation of free amino groups in acidic to neutral conditions. This study investigated the impact of cell surface charge on susceptibility to chitosan by using phosphate limited media to induce a conversion of cell wall moieties. Based on previous studies, phosphate limitation would cause the conventional teichoic acid moieties on *Bacillus subtilis* to convert to the less negatively charged teichuronic acid moieties. Exposure to chitosan after using phosphate limited media resulted in increased resistance to cell death as determined by a permeability staining assay. While this experiment pointed to electrostatic interaction between negatively charged teichoic acids and positively charged chitosan as a possible reason for the strong antimicrobial activity of chitosan, quantitative analyses were not performed to assess the magnitude of the effect on charge.

Chitosan is a positively charged polysaccharide consisting of β-1,4-linked glucosamine and N-acetylglucosamine produced by acidic deacetylation of chitin (3, 5). It is used in a wide range of industrial settings as a flocculating agent, a hydrating agent, and a broad antimicrobial agent (7). Studies by Pan *et al*. (5) elicited the ability of chitosan to disrupt membrane integrity and increase permeability in lactic acid bacteria; however, the mechanism behind this observation was not investigated. Another study by Raafat *et al.* showed that resistance to chitosan increased with the knock-out of teichoic acids (6). Teichoic acids are negatively charged surface moieties that comprise a major component of Gram-positive cell walls (9, 11).

Based on the observations of Pan *et al.* (5) and Raafat *et al.* (6), we hypothesized that the mode of action of chitosan is dependent on the net charge of the cellular surface whereby smaller net negative charge imparts increased resistance to chitosan. To test our hypothesis, we chose a model based on the conversion of teichoic acids to teichuronic acids in *Bacillus subtilis* under inorganic phosphate limitation conditions. Teichuronic acid is a phosphate-poor polymer made during phosphate starvation. It is composed of glucuronic acid to satisfy the requirement for an anionic polymer (1, 2, 8, 10). Since phosphate contributes the majority of negative charge on the cell wall in the form of teichoic acid (8) and it is replaced by the less negative glucuronic acid in the form of teichuronic acid, it can be inferred that the effective charge of the cell wall is decreased after the transition to teichuronic acids from teichoic acids.

Without a suitable assay to determine surface charge, this study relied on reproducing the observations of Grant (2) to assume teichoic acid to teichuronic acid transition had occurred. By using differential staining of bacteria before and after the transition, we were able to compare susceptibility of bacteria to chitosan. The results from our study support our hypothesis as cells grown under phosphate limitation were less permeabilized as visualized with fluorescence microscopy.

MATERIALS AND METHODS

Bacteria and culture conditions. *Bacillus subtilis* WB746 was obtained from the MICB 421 culture collection at the University of British Columbia Department of Microbiology and Immunology.

The base media used was adapted from Grant's phosphate-limited media (2). The composition (per liter) was: $MgCl_2$•$6H_2O$ - 8.3 mg; $MnCl_2$•$4H_2O$ - 19.8 mg; NH_4Cl - 535 mg; Na_2SO_4 - 106 mg; NH_4NO_3 - 96.5 mg; $CaCl_2$•$4H_2O$ - 148 mg; L-glutamic acid - 1.35 g; Tris-HCl - 15.7 g; D-glucose - 5 g (autoclaved separately); $FeCl_3$ - 0.59 mg (filter sterilized). The base media was supplemented with (per liter) 0.02 ml of a trace element solution containing (per ml): $CuSO_4$•$5H_2O$ - 5mg; $MnCl_2$•$4H_2O$ - 2 mg; $ZnSO_4$•$7H_2O$ - 65 mg; $FeSO_4$•$7H_2O$ -5 mg; HCl - 100 mg. The pH was adjusted to 7.0 using NaOH. A 0.4 M solution of KH_2PO_4 was prepared separately and added to cultures to achieve starting concentrations of 0.125 mM PO_4 and 2.5 mM PO_4.

Preparation of chitosan. Low molecular weight chitosan (Sigma, #448869) was dissolved in 1% (w/v) acetic acid (0.1 g chitosan dissolved in 10 ml acetic acid). To get a final chitosan

concentration of 2 g/L in the culture, 1 ml of the dissolved chitosan was added to 4 ml of culture for a final volume of 5 ml.

Growth assay. Bacteria from a single colony of TSA media were grown for 24 hours in base media supplemented with KH_2PO_4 to a final concentration of 2.5 mM shaking at 37°C. The culture was then diluted (about 1 in 15) into fresh medium at 37°C to an initial turbidity of 0.15 to 0.18 (OD_{500} in a spectrophotometer, 1 cm light path). Small-scale cultures (30 - 50 ml) were supplemented with KH_2PO_4 to final concentrations of 0.125 mM and 2.5 mM and grown at 37°C in a reciprocating shaking water bath set at 200 rpm. Growth was measured through optical density at a wavelength of 500 nm in 15 to 30 minute intervals with 1/5 dilutions as necessary.

Fluorescent staining assay. Differential fluorescent staining was adapted from the procedure used by Pan *et al*. (5) to differentiate between chitosan affected and unaffected cells based on membrane integrity. Exponentially growing cells were obtained by inoculating fresh media with overnight cultures and supplemented with KH_2PO_4 to final concentrations of 0.125 mM and 2.5 mM. Cultures were incubated at 37°C and 200 rpm with 4 ml samples drawn at 3 and 7 hours. Experimental samples were treated with 1 ml of activated chitosan at a final concentration of 2 g/L while separate control samples were treated with 1 ml of 1% acetic acid or 1 ml of toluene. Cultures were incubated at room temperature for 10 minutes. Samples were centrifuged in an Eppendorf Model 5424 microfuge at 7000 x g for 3 minutes. Pellets were resuspended in 1.5 ml of 0.85% (w/v) NaCl, vortexed for one minute, and microfuged again at the same settings. After this wash step, the pellet was resuspended in 1 ml of 0.85% NaCl and stained with 3 µl of 20 mM propidium iodide (Cat. No. P4170, Sigma-Aldrich) and 3 µl of 0.01% w/v acridine orange (Cat. No. A4921, Sigma-Alrich). Stained samples were vortexed and incubated in the dark at room temperature for 15 minutes. Two washes were conducted by microfuging at the same settings followed by resuspending in 1 ml of 0.85% NaCl. A wet mount was prepared and observed under a Zeiss Axiostar Plus fluorescence microscope with a 490 nm excitation filter and a 510 nm barrier filter. Pictures were taken through the eyepiece with a Canon EOS Digital Rebel XSi camera at ISO 1600, aperture f=3.5, and 1/6s shutter speed.

RESULTS

Growth of *B. subtilis* in phosphate limited media. Characteristics of the growth of *B. subtilis* in base media supplemented with different concentrations of phosphate are shown in Figure 1. Both phosphate limited (0.125 mM PO_4) and control cultures (2.5 mM PO_4) grew slowly but similarly during the initial 3 hours, followed by logarithmic growth at different rates for approximately 4 hours. Growth in phosphate limited media diverged significantly at around the 4.5 hour time point and reached a maximum optical density of approximately 1.0 OD_{500} units lower than the culture with excess phosphate.

Based on the findings by Grant (2), phosphate limited media caused *B. subtilis* to have an abrupt end in exponential growth with the exhaustion of inorganic phosphate in the medium. This was visualized by a break in the growth curve at approximately 5.5 hours. In the experiment by Grant (2), the abrupt end in exponential growth was followed by a short lag phase then by a slower growth phase, described as a biphasic

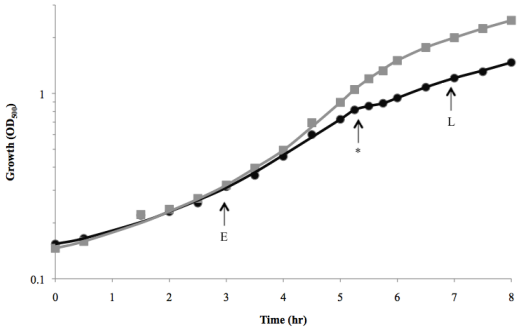

FIG. 1. Effect of phosphate limitation on *B. subtilis* growth rate. Growth in phosphate limited media of 0.125 mM [PO_4] (●) and media with excess phosphate at 2.5 mM [PO_4] (■) was monitored. E and L represent early and late sampling time points for fluorescent staining. The * represents the point when inorganic phosphate was depleted in the media.

pattern of growth. This same pattern can be seen in Figure 1, implying that inorganic phosphate was exhausted in the phosphate limited media past the break point.

Fluorescent staining and visualization of cells treated with chitosan. The effect of chitosan was visualized through differential staining with propidium iodide (PI) and acridine orange (AO) to determine the extent of membrane permeabilization. PI, a polar hydrophilic dye, identified dead cells by binding to DNA, staining them red, but was excluded from cells with intact cellular membranes. AO, on the other hand, was a nonspecific dye that also bound to DNA but was able to cross the membrane of both living and dead cells, staining living cells green. Controls and experimental samples can be seen in Figure 2.

Positive control cultures were subjected to toluene, puncturing the cellular membrane of all cells and causing them to appear red under fluorescence microscopy. Negative control cultures were subjected to 1% acetic acid in the buffered media, causing minimal cell death and appearing as mostly green under the fluorescent microscope.

Experimental samples treated with chitosan showed results that differed from both positive and negative controls. Chitosan treated cells sampled at 3 hours showed higher prevalence of clumping and a higher proportion of red-stained cells than green-stained cells. Chitosan treated cells grown in phosphate limited media sampled at 7 hours also showed aggregation but had a higher proportion of green-stained cells than red-stained cells. Chitosan treated cells grown in media with excess phosphate sampled at 7 hours showed aggregation that was similar to the chitosan treated cells,

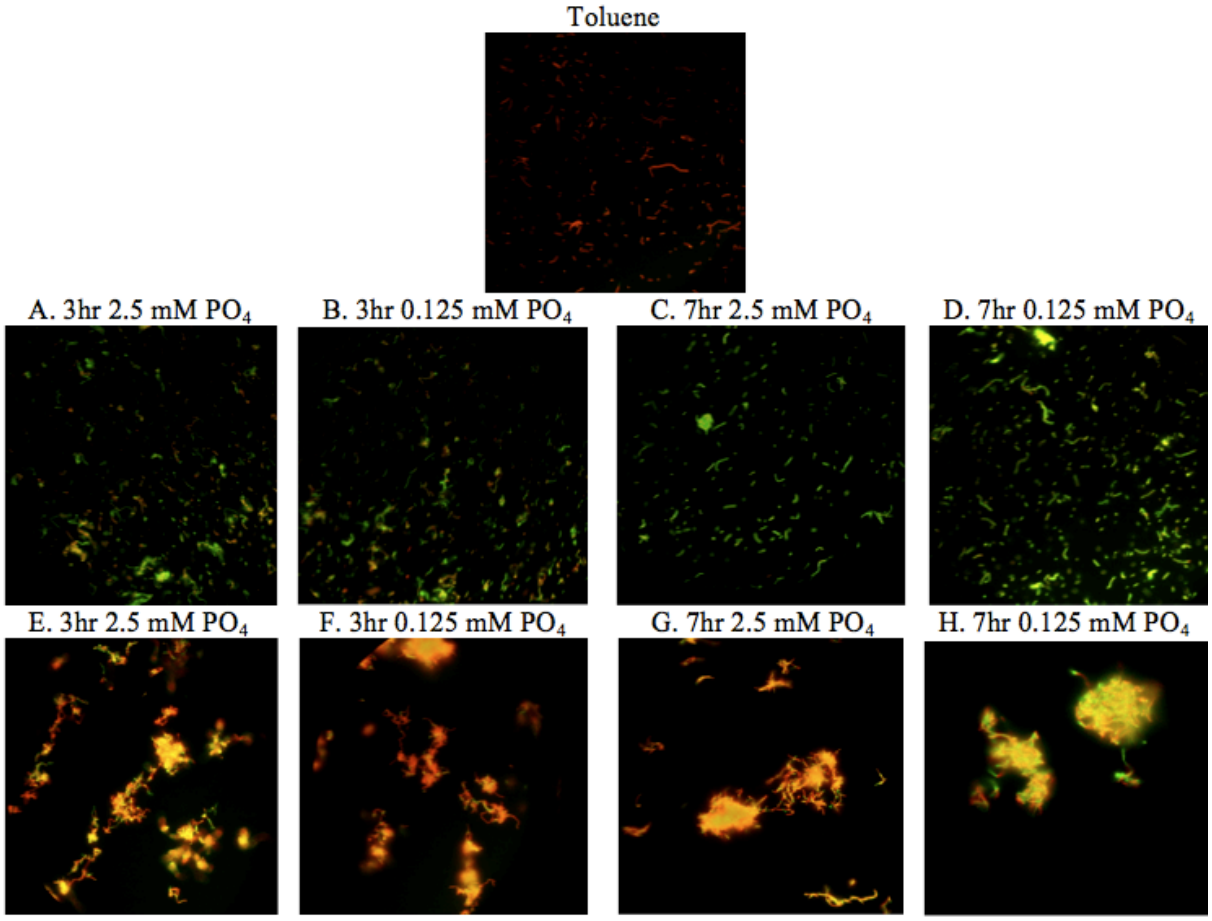

FIG. 2. Effect of phosphate limitation on the relative viability of cells treated with chitosan. Fluorescent visualization of *B. subtilis* WB746 using propidium iodide (20 mM) and acridine orange (0.01% w/v). Negative controls (A-D) were subject to acetic acid (0.2% v/v) for 10 min. Treatment samples (E-H) were subjected to 2.0 g/L chitosan for 10 min. Positive control: Toluene (100 µl/ml). Perforated cells stain red and intact cells stain green. Cells were viewed through a 400X objective lens. Photos represent an average trend over 20 fields.

but had more red-stained cells, similar to the 3 hour sampling point. However, the difference in color between the two treatments of the phosphate limited cultures suggests that something in the cell wall has changed to cause resistance to the effects of chitosan.

The general trend in chitosan treated samples showed an increase in aggregation compared to the acetic acid and toluene controls. As chitosan is a polymer of positive charges, it acts as a flocculation agent (4), this result was consistent with expectations. There was no noticeable difference in magnitude of aggregation among chitosan treated cultures so no conclusion can be made as to the effect of reduced teichoic acid levels on aggregation action by chitosan.

A difference in chitosan resistance was detected in the phosphate limited culture at the L sampling point. Based on the results reported by Grant (2), teichoic acid had finished converting into teichuronic acid by this point. These cells, with less negative cell moieties, showed a higher proportion of living cells compared to the cells with more negative cell moieties. However, the proportion of living cells was still lower compared to negative controls treated with 1% acetic acid. Quantitative analysis of living to dead cell ratios could not be accomplished as three-dimensional aggregation caused by chitosan made many cells indistinguishable. However, visual inspection over numerous fields showed obvious differences between the samples.

DISCUSSION

The phosphate and uronic acid content of bacterial cell walls after the break point was consistent with replacement of teichoic acid by teichuronic acid (2). This observation was the basis of an expected decrease in negative charge for phosphate-deprived cells sampled at the late (L) time point in Figure 1. As a result of this transition at the break point, both cultures at the early (E) sampling point as well as the culture with excess phosphate at the L sampling point were expected to have relatively higher proportions of teichoic acid and thus, greater negative charge compared to the phosphate limited culture at the L sampling point

Based on Figure 1, there was a clear decrease in bacterial growth in phosphate limited media compared to media with excess phosphate. This observation was expected and was required to infer that L sampling points in phosphate limited media had transitioned to the less negative teichuronic acid cell moieties. Previous experiments clearly demonstrated that phosphate in teichoic acid was rapidly released at the break point using acid-extraction of teichoic acids and other quantitative measurements. A similar bacterial strain and culture conditions were used in this experiment.

The images from the fluorescence microscopy in Figure 2 showed that the phosphate limited cells in the L sampling point were indeed more resistant to antibacterial effects of chitosan. This observation was consistent with observations made by Raafat *et al.* of the chitosan effect on *Staphylococcus aureus* cells with knocked-out tagO genes resulting in a lack of teichoic acids (6). Since teichuronic acids are still negatively charged cell moieties, complete resistance to chitosan was not expected nor observed.

Compared to the images of lactobacillus strains in the experiment by Pan *et al.* (5), similar levels of cell death were observed when comparing chitosan treated and untreated cultures with excess phosphate. However, the flocculation effect of chitosan was significantly more pronounced in this experiment while the effect seemed to be nonexistent in observations made by Pan *et al.* (5). This difference can be attributed to the difference in final chitosan concentration: 2 g/L in this experiment compared to 0.5 g/L in experiments performed by Pan *et al.* (5) Preliminary growth curves with chitosan pointed at insufficient chitosan concentrations to significantly impact growth past 30 minutes if 0.5 g/L was used. The four-fold increase in concentration allowed a more significant observable difference in place of quantitative analyses. The aggregations in this experiment were most likely caused by connection of bacteria via chitosan polymers due to opposing charges. The observation that clumping still occurred but with less death suggests that the charge threshold for cell death is lower than that for clumping.

The results from this experiment showed that phosphate limitation reduced the susceptibility of *B. subtilis* to chitosan after depletion of inorganic phosphate in the media. More definitive tests would be required to deduce that it was specifically a difference in surface charge that yielded this result, but this is the theoretical basis of the observation.

FUTURE DIRECTIONS

In order to fully assess the potential role of cell surface charge on chitosan resistance, a quantitative approach to determining surface charge between the E and L sampling points needs to be established. It is our recommendation that isoelectric focusing assays be pursued in obtaining this key information as difficulties with electrostatic-interaction chromatography assays yielded inconclusive results (data not published). It is expected that isoelectric focusing will show that *B. subtilis* growing for an extended period of time in phosphate limited media will have a lower cell wall charge and migrate accordingly. A quantitative method for determining cell death after chitosan exposure would allow for a correlative relationship to be established between surface charge and susceptibility to chitosan. The issue encountered in this experiment involved extensive aggregation of chitosan treated bacteria, preventing accurate microscopic counts or plate counts. This is an inherent problem with chitosan as it is a flocculation agent used in industry for this exact quality. If these aggregations can be resolved, it is expected that the percentage of cells permeabilized by chitosan is much lower in the phosphate limited cultures compared to the phosphate rich cultures.

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