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Mildly Inhibitory Effects of Sodium Polyanethol Sulfonate and pH on the Growth of Wild-Type, *lrgAB*, and *cidAB* Mutants of *Streptococcus mutans*

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The *lrgAB* and *cidAB* operon encodes for anti-holin-like proteins and holin-like proteins respectively, which are mechanisms of control for endolysin activity in *S. mutans*. The upregulation of *lrgAB* genes by chitosan is purported to cause increased cell lysis in *Streptococcus mutans* by promoting the action of extracellular murein hydrolases. These enzymes can be inhibited by polyanethol sulfonate (SPS). This study tested whether SPS at the pH levels needed to dissolve chitosan is inhibitory to *S. mutans*. By testing the effects of pH and SPS separate from chitosan in *lrgAB*, *cidAB*, and wild-type *S. mutans*, we can test whether the reagents are suitable to use in assessing the effects of chitosan on the genes. A turbidity assay was used to monitor the growth of wild-type, *cidAB*, and *lrgAB* mutants in SPS and non-SPS treated Todd-Hewitt broth over a 75 minute period at pH 5, 6, and 7.2. Results showed that 0.5% SPS was capable of slowing the growth of all strains at pH 6 and 7.2, with the greatest effect on *lrg*AB mutants and the least obvious effect on *cid*AB mutants. Cell growth for all strains was generally arrested at pH 5 regardless of the presence of SPS. Because SPS showed inhibitory effects on growth by itself, its use in chitosan studies as an inhibitor of endolysin is questionable.

Cell lysis is understood as the breakdown of cells by viral and enzymatic mechanisms resulting in a decrease in cell integrity. Understanding the various processes by which cell lysis occurs offers insight for research on novel antimicrobial agents. A way of inducing cell lysis is through upregulation of autolytic pathways involving murein hydrolases, also known as endolysins or autolysins (10). Murein hydrolases are a family of enzymes that cleave bacterial cell wall components and are involved in various cell processes in cell wall turnover during cell expansion and division. However, due to their capability of hydrolyzing cell walls, murein hydrolase expression is tightly regulated to prevent overexpression leading to cell wall destruction and, subsequently, cell death (16).

Chitosan is a compound that can be synthesized by N-deacetylation of chitin, a major component of extracellular skeletons of arthropods, which has various anti-microbial properties (10, 23). Previous research has shown that one mechanism of chitosan's antimicrobial properties operates through manipulation of the *lytSR* regulatory system by upregulation of the *lrgAB* operon of *Streptococcus mutans* (10, 22). In *S. mutans*, *lrgAB* and *cidAB* genes play a critical role in controlling autolysis by regulating membrane embedded proteins that stimulate murein hydrolase activity (15, 24). *LrgAB* genes encode the proteins, lrgA and lrgB, which inhibit the effects of murein hydrolase, which are anti-holin-like proteins (1, 4, 14). In comparison, transcription of *cidAB* genes results in the synthesis of counteracting holin-like proteins responsible for promoting murein hydrolase activity (1, 4, 14). Holin-like-proteins are proteins that normally promote endolytic activity by exporting intracellular murein hydrolases to the extracellular membrane compartment, while anti-holin-like proteins are proteins that revert this step by inhibiting the formation of the murein hydrolase transport channels (14).

In *S. mutans*, chitosan induced expression of *lrgAB* leads to proton dissipation (10, 22, 23). According to a model using holins and anti-holins from bacteriophage, proton dissipation induces the flipping of anti-holins, causing them to act as holins and subsequently export endolysin (15). Flipping occurs when the N-terminus of the anti-holin switches to the periplasmic side as a result of the rapid loss of the electrochemical gradient (23). The upregulation of *lrgAB*, coupled with the proton dissipation effects of chitosan, could potentially explain the mechanism through which chitosan causes cell lysis (10, 22, 23).

To test whether chitosan-induced cell lysis is actually triggered by endolysin activity, sodium

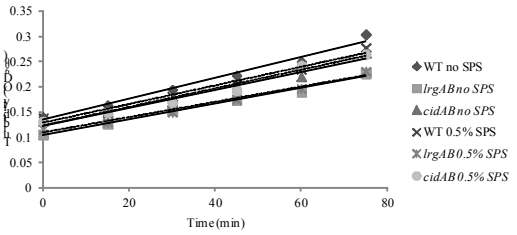

FIG. 1. Growth increases of *S.mutans* without SPS and with 0.5% SPS at pH 7.2 (n=3)

polyanethol sulfonate (SPS) can be used to inhibit the effects of chitosan-induced endolysin activity. SPS is an anionic detergent containing a benzene sulfonate per molecule. Previous research conducted by Wecke *et al.* revealed that SPS has an inhibitory effect on murein hydrolases in *Staphylococci* (29). In another study, SPS successfully inhibited the antibacterial endolysin lysostaphin, which is produced by *Staphylococcus simulans,* and is known to induce cell wall lysis in other species of Gram-positive bacteria (29). Other than being able to inhibit the bactericidal activity of penicillin (28), SPS is also commonly used to inactivate immune components that will otherwise cause bacterial death and decrease bacterial yield in patient blood samples (19, 29). Furthermore, in a study involving *Peptostreptococcus*, SPS was shown to be inhibitory for growth of anaerobic cocci (13). The inhibition of autolysin activity by SPS is purported to function through binding to techoic acid residues on cell walls (29). Although it has not been confirmed, the binding of SPS to cell wall is suggested to create steric hindrance, thus inhibiting the interaction between autolysin and its substrate (29). Since chitosan has to be dissolved in an acidic solution to activate its antimicrobial activity (8, 11), the effects of low pH on the growth of *S. mutans* and the activity of SPS were chosen as the focus of this study.

MATERIALS AND METHODS

Bacterial Strains. Three strains of *S. mutans* UA159 (wild-type), SAB118 (D*cid* : : WKmr *cidAB*¯) and SAB115 (D*lrg* : : WKmr *lrgAB*¯) were 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, pH 6.9) in a micro-anaerobic environment at 37ºC. After overnight incubation, the natural pH increased to 7.2 and were adjusted to either pH 5 or 6 or left unadjusted at pH 7.2. Polyanetholesulfonic acid sodium salt (Sigma, #P2008-5G) was added directly into the test tubes to reach final concentration of 0.25%, 0.5%, or 1.0%. Overnight cultures that were adjusted to pH 5, pH 6, and pH 7.2 without the addition of SPS were used as controls.

Turbidity Assay. Bacterial growth was measured by turbidity reading at 600 nm. Turbidity readings were taken at 15-minute intervals for a total of 75 minutes. Cultures were incubated at 37ºC in a shaking water bath throughout the assay. Todd-Hewitt broth with 0%, 0.25%, 0.5%, or 1.0% SPS was used as blanks.

TABLE 1. Percentage changes in cell density of *S.mutans* after 75 minutes at pH 7.2.

Type of Strains	Treatment Condition		Effect of addition of 0.5% SPS
	without 0.5% SPS	with 0.5% SPS	
wild-type	116%	101%	-15%
cidAB	103%	101%	-2%
lrgAB	114%	99%	-15%

RESULTS

SPS affects growth of *S. mutans* at pH 7.2. Figure 1 depicts exponential growth of *lrgAB, cidAB*, and wild-type strains of *S.mutans* over a period of 75 minutes. In the absence of SPS, all three strains exhibited similar growth patterns relative to each other although the wild-type strain showed the highest turbidity reading throughout the assay, followed by *cidAB* and *lrgAB* mutants. Similar slopes between the three strains under non-SPS condition in Figure 1 represent the relatively consistent rate of growth for all of them under the same condition. Looking at the calculated percentage changes in cell density presented in Table 1, 2, and 3, all strains show expected results of higher growth rate under physiological pH of 7.2 than when they are in placed in lower pH condition of 6 and 5. At the end of incubation, *lrgAB*, *cidAB*, and wild-type strains in the non-SPS treated group grew by 114%, 103%, and 116%, respectively. On the other hand, the SPS-treated strains grew by 99%, 101%, and 101%, respectively. Thus, the addition of 0.5% SPS into the culture resulted in a lesser increase of cell growth in *lrgAB* mutant and wild-type strain by approximately 15% while a lesser increase by 2% was seen in *cidAB* mutant. This implied that the proteins encoded by *cidAB* played a certain role in supporting the growth of cells in the presence of 0.5% SPS under physiological pH of 7.2.

SPS slowed growth of *S. mutans* at pH 6. Figure 2 shows decreased growth rates for all three strains upon incubation at pH 6 in comparison to the exponential growth rate observed in Figure 1. Rather than showing the same pattern of doubled cell concentration after 75 minutes seen at pH 7.2, the bacteria had a modest increase of around one third of its

TABLE 2. Percentage changes in cell density of *S.mutans* after 75 minutes at pH 6.

Type of Strains	Treatment Condition		Effect of addition of 0.5% SPS
	without 0.5% SPS	with 0.5% SPS	
wild-type	36%	17%	-19%
cidAB	26%	22%	+4%
lrgAB	39%	21%	-18%

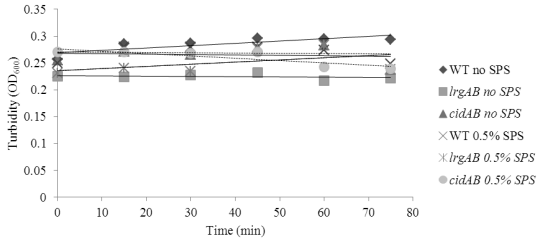

FIG. 2. Growth increases of *S. mutans* without and with 0.5% SPS at pH 6 (n=3)

starting density for all three strains at pH 6. Contrary to the result at pH 7.2, *cidAB* mutant show the highest cell density throughout the assay under both treatment conditions, followed by *lrgAB* and wild-type strains sequentially. In the absence of SPS, the percentage increase of cell density was found to be the highest in *lrgAB* at 39% while wild-type and *cidAB* showed a lower increase of 36% and 26% respectively. Conversely, the percentage increase of cell density when the samples were incubated with 0.5% SPS indicated a lesser growth increase in all strains in comparison to the non-SPS samples. *CidAB* mutants indicated a 22% increase, *lrgAB* mutants revealed a 21% increase and wild-type had a 17% increase in cell density. The extent of drop in cell growth upon addition of 0.5% SPS at pH 6 culture were relatively similar to the values obtained from pH 7.2 samples. *LrgAB* and wild-type strains still suffered greater reduction in cell growth at 19% and 18% respectively than the *cidAB* mutants at 4%.

The growth of *S. mutans* displayed variability among three strains at pH 5 with and without SPS treatment. Figure 3 shows that pH 5 condition limited the growth of three strains to a large extent. In the absence of SPS, the growth of wild-type strain was completely halted while *cidAB* mutant had a 11% decrease in cell density (Figure. 3). *LrgAB* mutant is the only one still exhibiting an increase of 3% in cell concentration. Unlike the relatively consistent reduction pattern in cell growth found in cultures treated at pH 6 and pH 7.2, addition of SPS to *S.mutans a*t pH 5 revealed more interesting results. While *cidAB* and *lrgAB* mutants showed reduced cell growth after treatment with SPS by 7% and 1% respectively, wild-type strain exhibited an unusual increase of 15% in cell density. This result suggests that presence of SPS inhibited the destructive effect of high acid condition at pH 5 on the wild-type strain only. Overall, addition of SPS led to an increase of growth rate in wild-type and *cidAB* by 15% and 4%, respectively, while causing a decrease in growth rate of *lrgAB* by 4%.

Based on the results from the three pH conditions, it was observed that SPS exerted the least negative effect on cell growth of *cidAB* mutants. *LrgAB* mutants, however, were consistently shown to suffer the greatest reduction in cell growth upon addition of SPS. From the general pattern of reduced cell growth across the three strains in three different pH conditions, it can be concluded that 0.5% SPS caused a mild inhibitory effect on the cell growth. The extent of this inhibition fluctuated and was independent of the concentration of the SPS used (data not shown).

DISCUSSION

The data indicated that addition of SPS to all three strains of *S. mutans* at all but one pH condition decreased their rates of growth compared to non-SPS treated samples, with the exception being the addition of SPS in wild-type and *cidAB* mutant at pH 7.2, (Tables 1, 2, 3). This suggests that SPS has an inhibitory effect on cell growth in *S. mutans*. The exact mechanism by which SPS functions is mostly unknown (29), though previous studies have postulated that SPS binds to teichoic acids on the cell wall in order to inhibit the activity of murein hydrolase (29). In the study by Wecke *et al.,* they reported no negative effect of SPS on cell growth, in contrast to our results (29). An explanation for our results is that altering teichoic acid by SPS binding to it could affect its normal cell functions. Teichoic acids are important structures that maintain the structural integrity of cell walls by attracting cations such as magnesium and calcium, as well as forming a matrix with peptidoglycan that regulates autolysin, nutrient, and protein transport (17). Binding of SPS to techoic acids in *S. mutans* could potentially hinder these crucial homeostatic functions of teichoic acid. Furthermore, it has been shown that *S. aureus* mutants lacking techoic acids have altered autolysis rates, where techoic acids are proposed to be important for morphogenesis and cell division (3, 9,

TABLE 3. Percentage changes in cell density of *S.mutans* after 75 minutes at pH 5.

Type of Strains	Treatment Condition		Effect of addition of 0.5% SPS
	without 0.5% SPS	with 0.5% SPS	
wild-type	0%	15%	+15%
cidAB	-11%	-7%	+4%
lrgAB	3%	-1%	-4%

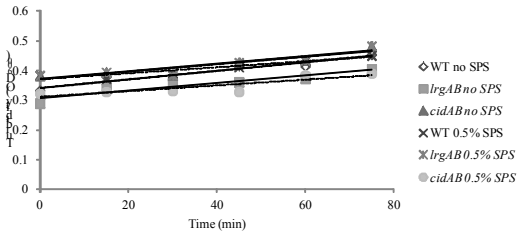

FIG. 3. Growth increases of S. *mutans* without SPS and with 0.5% SPS at pH 5 (n=3)

18). The binding of SPS to teichoic acids is also quite unusual, as both teichoic acids and SPS exhibit negative charges, the binding of which is unknown. Thus, the net charge of the cell wall is altered and made overly negative, which can potentially affect cell growth negatively (29).

SPS has also been shown to have inhibitory effects in various strains of *Peptostreptococcus*, a facultative anaerobe, in broth with SPS concentration ranging from 0.025 to 0.05% as well as exhibiting significant zones of inhibition with disks containing 5% SPS (13). Because *S. mutans* is also an anaerobe, SPS may have similar, but evidently milder inhibitory effects. In addition, researchers have conducted experiments to test the effects of the anionic surfactant dodecyl benzene sulfonate on the growth of *Escherichia coli*, in which partial lysis of cells were reported (20). The significance is that SPS is an anionic surfactant with an alkyl benzene sulfonate structure similar to dodecyl benzene sulfonate. The *E. coli* growth curves showed a consistently lower cell density in all cells grown with the surfactant (20). Such structural similarity prompts the hypothesis that both chemicals may act in similar ways and affect cell growth.

The most interesting observation is the difference between *lrgAB* mutant and *cidAB* mutant growth when subject to SPS (Tables 1, 2, 3). Specifically, *lrgAB* mutants were most affected by SPS, while *cidAB* mutants were least affected. We hypothesize that this phenomenon is tightly linked with the physiology and replication mechanisms of the cell. A previous study has proven that because *cidAB* gene products are used to form a holin-like murein hydrolase channel and *lrgAB* gene products act to inhibit the formation of the channels (5, 6, 27, 31, 32), *lrgAB* mutants to have the highest amount of murein hydrolase in the inner wall zone, while *cidAB* mutants have the least amount of murein hydrolase (10). Murein hydrolase is an integral part of cell wall replication because it catalyzes the hydrolysis of the link between N-acetylmuramic acid residues and L-amino acid residues in peptidoglycan, regulates cell wall growth, separates cell walls of daughter cells during cell division, and monitors peptidoglycan turnover (30). Hence, *cidAB* mutants were affected the least by SPS because the enzymes were already relatively low in concentration in the inner wall zone in *cidAB* mutants in the first place. Because of this, *cidAB* mutants have a relatively more stable cell wall and a lower growth rate due to reduced cell wall development. In contrast, *lrgAB* mutants exhibited a higher cell growth rate because its *cidAB*-encoded murein hydrolase channels were not inhibited, enabling higher levels of murein hydrolase activity in the inner wall zone. As a result, endolysins were able to hydrolyze peptidoglycan to allow insertion of new peptidoglycan units, which subsequently results in unhindered cell growth (5, 6). *LrgAB* mutants therefore had the most significant decrease in cell growth when SPS was added because of the inhibitory effects on murein hydrolase activity. It must also be stressed that the *cidAB* and *lrgAB* mechanism of murein hydrolase export and action is potentially only one of the many processes by which the cell utilizes enzymes necessary for cell wall hydrolysis (30). Evidence supports this hypothesis as *S. mutans* growth persisted despite inhibition of murein hydrolase action and export.

Table 1 shows that the *lrgAB* mutant is not different phenotypically from the wild-type strain in terms of the rate of cell growth during the time of the experiment. Previous studies have found that expression of *lrgAB* genes is repressed in the early stage of exponential growth, which starts around two hours after a fresh culture is inoculated with the bacteria, and will increase greatly towards the later stage of exponential growth at six hours, with expression peaking at twelve hours (1). Since the experiment started 1.5 to 3 hours after the fresh culture was inoculated, followed by a 75 minute long turbidity assay, it is likely that the cells were still in the earlier stage of exponential growth. The naturally lower concentration of *lrgAB* gene products in the early stage of exponential phase may explain why no obvious differences in terms of cell growth for the

lrgAB mutant and the wildtype strain were observed during this period. This is so even though prior studies have showed that *lrgAB* mutants have a higher level of murein hydrolase activity in comparison to the wild-type strain, since lrgA and lrgB are not present to inhibit the formation of murein hydrolase channels (5, 6). However, defects in the *lrgAB* gene may have a more pronounced effect on cell growth and survival when the cell enters later exponential or stationary phase, when the level of *lrgAB* gene products are naturally higher, rather than in the earlier stages where expression of *lrgAB* gene is lower.

On the other hand, *cidAB* mutants seemed to be phenotypically different than the wild-type strain during the period tested. As shown in both Table 1 and Figure 1, growth rate of *cidAB* mutants consistently lagged behind the *lrgAB* mutants and the wild-type strain by about 10%. This lowered rate of growth of the *cidAB* mutants appeared to be pH independent, as the rate of growth for *cidAB* mutants is about 10% lower than the wild-type and the *lrgAB* mutants across all the pH levels tested, as shown in Tables 1, 2 and 3. Experssion of the *cidAB* genes are responsible for the formation of murein hydrolase transporters, which are essential for the transport of murein hydrolases in the inner wall zone (22). Without murein hydrolases in the inner wall zone, *cidAB* mutants have reduced ability replace or insert new cell wall components, results in lower cell wall turnover which negatively impacts cell growth and division (30). Consistent with the need for fast cell growth during the exponential phase, studies have found that the expression of the *cidAB* gene is highest in the early stage of the exponential phase, and gradually decreases toward the later stages of cell growth, such as the lag phase (1). This explains the observation that *cidAB* mutants exhibited lower rate of growth in comparison to the wild-type strain in the early phase of cell growth.

Previous studies have stated that acid tolerance is a virulence factor of *S. mutans* (2). However, *S. mutans* grown in aerobic conditions have lower acid tolerance (26), and the *lrg* and *cid* systems seemed to be necessary for tolerating acid stress under aerobic growth (1). However, our results showed that the relationship of the growth rates between the *cidAB, lrgAB,* and wild-type strains seems to be consistent in the range of pH levels tested. Growth rate of *lrgAB* and the wild-type strains were very similar, and the growth rate of *cidAB* mutants is about 10% lower than the wild type strains across all pH levels tested, as shown in Tables 2 and 3, as well as Figures 2 and 3. The rates of growth for all three strains were lower at lower pH levels. For example, at pH 6, the rate of growth for all three strains has decreased about two thirds in comparison to that at pH 7.2. as shown in Table 2 and Figure 2. At pH 5, the rates of growth for all three strains were almost completely arrested, as shown in Table 3 and Figure 3. Since the wild-type strain did not exhibit higher growth rate at lower pH levels in comparison to the *cidAB* and *lrgAB* mutants, our results did not agree with the previous finding that the presence of *lrg* and *cid* system was necessary for cell growth under acidic conditions (1). Our results also suggest that despite acid tolerance being a virulence factor of *S. mutans*, the bacteria did not grow as readily at lower pH levels in comparison to a more neutral pH of 7.2. These findings were significant when we attempted to combine chitosan treated *S. mutans*. As demonstrated by previous literature, chitosan needs to be dissolved in an acidic solution so its antimicrobial properties may be activated (11). However, since the rate of growth is lower at lower pH levels, it is hard to conclude if the lower rate of growth is due to lower pH levels or the antimicrobial properties of chitosan.

The findings showed that *cidAB* mutant consistently lagged behind the *lrgAB* mutant and the wild-type strain during early exponential stage growth. This effect was observed when the cells were grown in aerobic condition at pH 5, 6, and 7.2. Our findings showed that, contrary to previous literature on similar organisms such as *Peptostreptococcus* and *S. aureus*, SPS itself had an inhibitory effect on cell growth in *S. mutans* across all strains and pH levels, with the exception of *cidAB* and wild-type in pH 7.2. Hence, the use of SPS as a reagent to reverse chitosan induced endolysin activity is questionable.

FUTURE DIRECTIONS

Due to formation of an insoluble white gel-like precipitate between chitosan and SPS, the ability of SPS to reverse chitosan-induced lysis was not assessed. The non-uniform distribution of precipitates produced inconsistent readings and made the turbidity assay a non-feasible approach. Moreover, the acidic pH (~4.5) needed to dissolve chitosan was proven to be detrimental to the growth of *S. mutans*, which made it impossible to interpret whether the result was due to the effect of chitosan or the acidic environment.

An alternate approach to using SPS would involve pre-treating the cells with SPS for approximately 10 minutes prior to adding chitosan in order to prevent cell wall disintegration (29). Therefore, future consideration could include performing the experiment by sequential addition of chitosan after SPS instead of adding them simultaneously. Growth and morphological

observations could also be performed by using light microscopy to assess the effect of SPS on cell wall integrity. The fact that SPS itself has inhibitory effect on cell growth has to be taken into account and appropriate controls have to be set up in order to distinguish between the effect of chitosan and SPS on cell death.

Another modification would involve using a water-soluble form of chitosan. Using this form of chitosan, acidification of culture could be avoided, although one would have to test whether this particular form of sugar polymer were still able to induce the the expression of *lrgAB* genes. To enable enumeration of the cells in culture, a technique involving light microscopy or plate counting could be utilized instead of turbidity assays. However, this would require a method for shearing *S. mutans* and separating the individual colony from the chain form.

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