

## Gram-Positive *S. mitis* Become Sensitive to Colistin and Nalidixic Acid when Grown in Xylitol

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### Abstract

The generation time of diplococcal *viridans group streptococci*, *S. mitis* is unusually prolonged when grown in rich broth containing xylitol (2% or higher concentrations). These xylitol-exposed streptococci predominantly grow in chains of varying lengths with heterogeneity of color: pink, purple and pink and purple combined. The pink portion of these bacterial chains appears to be thinner than the purple portion indicating a reduction in cell wall thickness. In support of optical microscopy, scanning electron microscopy of the same bacterial population demonstrates a considerable reduction of sizes but still in chains. In these chains, there is a variation in size indicating that all members of the diplococcal *S. mitis* population are not equally affected. Apparently, the partially affected viable population of *S. mitis*, becomes sensitive to colistin and nalidixic acid when grown in xylitol.

**Keywords:** Xylitol; Cell wall thickness; *S. mitis*; Colistin; Nalidixic acid; Electron microscopy

### Introduction

In 1928 Dr. Fred Griffith recorded in his pathology report that the two kinds of colonies, rough and smooth colonies of *S. pneumoniae* are seen in microbiological solid growth media (e.g. blood agar plate). Until now these colonies have not been appropriately analyzed. However, such morphological variations (opaque and transparent) of bacterial colonies are also true for several diplococcal Gram-positive pathogens including *S. mutans* (dental) and *S. mitis*. Significantly, these diplococcal Gram-positive pathogens have low G-C content (< 40%) but they differ in their pathogenesis. In support of our statement, it has been known that the virulence associated proteins CBP's, CbpA, PspA and PepA are found only in *S. pneumoniae* except *S. mitis* is phylogenetically related to *S. pneumoniae* [1,2]. Above all, this pathogen is becoming increasingly resistant to modern medicines, antibiotics and surface antigen based polyvalent vaccines and therefore, the disease "bacterial pneumonia" has created a new concern. In order to save children from pneumonia, alternative therapies which affect bacterial genetic ability used to communicate among the members of their own community are urgently needed. Signal based communication is also controlling their gene transfer ability.

A low-calorie sugar alcohol, xylitol, has long been promoted as a preventive agent for dental caries primarily caused by *Streptococcus mutans* [3-5]. Based on our previous work, we believe that, in addition to dental caries, an appropriately developed preventive use of xylitol may also reduce the incidence of community acquired pneumonia, blood stream infections, meningitis, sinusitis and acute otitis media in children [2-9]. The causative bacterium of these diseases is primarily the Gram-positive diplococcal *S. pneumoniae* which does not differ from the *viridans group streptococci*, specifically *S. mitis*, in morphology and metabolism [2-9]. Importantly, these diplococcal pathogens also carry loci for the partial utilization of xylitol [2,10]. Studies on *S. pneumoniae* have shown that an exposure to xylitol causes alteration of surface structure(s) and decreases the bacterial adherence to both biotic and abiotic surfaces [7,11]. This observation is further supported by more recent work showing that the xylitol damages the surface ultra-structure of Pneumococci when compared to the effect of fructose or sucrose [7,12]. We have also observed that the *S. mitis* shows neither

lysis nor any bactericidal effect while they are grown to saturation in the presence of xylitol. Our analyses by the Raman spectroscopy have previously confirmed formation of a xylitol-derivative that stably affects the cell division of the parents (an irreversible genetic effect) [2]. We report here that xylitol-treated *S. mitis* becomes sensitive to the combination of antibiotics, colistin (polymyxin B or E) and nalidixic acid, resembling the behavior of Gram-negative *E. coli* K-12. The major difference of these Gram-positive and Gram-negative bacteria is their cell wall thickness [13].

### Material and Methods

A genetically pure culture of *S. mitis* in brain heart infusion (BHI) was grown overnight in a CO<sub>2</sub> jar. After approximately 24 hours, the culture was diluted to 10<sup>-4</sup> and 10<sup>-5</sup> in BHI and allowed to grow for 2 to 3 hours. Then, 0.1 ml from each dilution was spread onto trypticase soy agar (TSA), and TSA containing colistin and nalidixic Acid (CNA) plates, and incubated for 24 to 72 hours under three different conditions: a) 37°C without CO<sub>2</sub>, b) 37°C with CO<sub>2</sub> and c) 25°C (room temperature). The titer was the same under all three conditions. We have used 2 different strains obtained from ATCC collection: ATCC 6249 (closely related to *S. pneumoniae*) and *S. mutans*: ATCC 25175 (dental pathogen). Interestingly, *S. mutans* shows a growth problem at a pH higher than 7 (unpublished data).

To establish the growth curve for *S. mitis*, all overnight cultures in BHI medium were diluted 1:50 by adding 0.1 ml of each culture to two separate tubes containing 4.0 ml of fresh BHI and allowed to grow at 37°C in a water bath shaker. Samples were withdrawn at five different

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time intervals and each sample diluted to  $10^{-4}$  in phosphate buffered saline. After dilution, 0.1ml of each culture was spread onto the TSA plates and incubated at 37°C. The bacterial titer was determined on a solid agar media by counting the colony forming units (CFU). The growth curve for the *S. mitis* in 2% xylitol was established by preparing an overnight culture in BHI and incubating in a candle jar for 24 hours. This culture was diluted 100-fold. Then, 1 ml was transferred to two separate tubes. The first tube was a control to which nothing was added. To the second tube, 0.1 ml of a 20% xylitol (stock solution) was added. Both tubes were incubated in a water bath at 37°C with shaking. Samples were withdrawn at different time intervals. At each interval, 0.1 ml was diluted to  $10^{-3}$  or  $10^{-4}$  in phosphate buffered saline and spread onto the TSA and CNA plates. The plates were incubated at 37°C and the colonies were counted after 24 hours. The logarithmic growth of the bacteria was monitored by counting the colonies to establish the number of colony forming units in different rich agar (solid) media. There was no hemolysis or lysis of intact bacteria in blood agar media. The MIC procedures to examine the alteration of the antibiotic sensitivity patterns induced by the presence of xylitol in the *S. mitis* growth media were also modified [14].

### Scanning electron microscopy (SEM)

*S. mitis* was grown in BHI containing 2% xylitol for overnight at 37°C, and fixed with 2.5% glutaraldehyde. The sample was washed serially with 25% absolute ethanol. After shadowing with gold for 5 seconds, the sample was visualized under JEOL JSM-7600F at 15 kV [15].

## Results

### Altered growth pattern of *S. mitis*

To further confirm our observations, we followed the growth pattern of the *S. mitis* in rich broth (TSB) with and without xylitol. Our data (Figure 1) demonstrated that they grew much slower in the presence of xylitol. Their generation time was prolonged, causing a 50-fold reduction of the final titer compared with the control grown similarly but without xylitol. Furthermore, unlike the growth pattern of the Gram-negative *E. coli*, the diplococcal *viridans* group streptococci (*S. mitis*) did not show any lag period but immediately initiated growth when their overnight cultures were diluted in the fresh nutrient broth. In the presence of xylitol (2%), this growth rate declined but continued at a much slower rate to the titer of  $10^7$ /ml while the titer of the control culture (0% xylitol) nearly reached a titer of  $10^9$ /ml during the same period of growth. A marked decline in the growth rate occurred after one hour in 2% xylitol. On the basis of our growth curve as presented

in Figure 1, we cannot compute the generation time but after 5 hours of growth, the xylitol-exposed *S. mitis* were unable to reach the same titer as the control ( $10^7$ /ml vs.  $10^9$ /ml).

The maximum effect was observed at the 2% xylitol. At concentrations of xylitol above 2%, the rate of growth did not decrease further but it reached a plateau. Our data confirms that xylitol can influence the *S. mitis* metabolism even in the rich broth containing dextrose. This finding is in agreement with the thesis work of Dr. Tapiainen [7]. To minimize the population of dead or old cells, we have diluted our overnight culture 10,000-fold and examined the morphological homogeneity of the diplococcal population immediately after dilution in fresh broth [2]. The overnight culture was also grown in an anaerobic jar with gentle shaking but the final titer did not differ even if they were grown aerobically.

### Xylitol-induced alteration of antibiotic sensitivity

We grew the *S. mitis* in rich broth with varying concentrations of xylitol (0% to 6%) from early log-phase to saturation ( $10^4$ /ml to  $10^9$ /ml) (Figure 1). The xylitol-exposed bacteria were then spread onto three different growth media: blood agar, TSA, and TSA containing antibiotics colistin and nalidixic acid (CNA). After 24 hours of incubation at 37°C with and without CO<sub>2</sub>, an approximately equal number of colonies grew on all three media; but, colonies on CNA were much smaller in size compared to the colonies grown on blood agar and TSA media that contained no antibiotics. When we transferred these small colonies (Lederberg's technique) to another fresh CNA plate containing 2% xylitol, the transferred colonies showed a serious inhibitory effect and became visible only as micro-colonies after 48 hours of incubation at 37°C. This data leads to a conclusion that these micro-colonies are due to the complete inhibition of cell multiplication (or cell division), or both, and the partial utilization of xylitol has made them sensitive to the combined action of antibiotics, colistin and the nalidixic acid (1 µg/ml each). The colistin and nalidixic acid could reach their targets located in the membrane of *S. mitis* after their growth for 5-7 generations in 2% of xylitol. However, such inhibitory effect was much less when these antibiotics were added separately. To confirm this observation, an agar dilution test was performed after the required modification of the protocol of Baker et al. as reported below [14].

### Measurement of antibiotic sensitivity by MIC

In the agar dilution test, the bacteria were diluted in Muller-Hinton broth to reach an approximately titer of  $10^6$  CFU and spread on the surface of the TSA medium [2]. In our experiment these TSA plates contained varying concentrations of antibiotics (0% to 100 µg/ml), colistin and nalidixic acid separately or together. However, the stock solutions of these two antibiotics were prepared in sterile distilled water and filtered through Millipore filter discs, with a pore size of 2 µm. After autoclaving, the solid agar TSA medium was cooled to 47°C by leaving them in a water bath for 45 minutes and then different concentrations of antibiotics were added (Table 1). We have also recorded that the commercially available CNA media containing these antibiotics lose their potency after autoclaving but still Gram negative *E. coli* K-12, *S. typhimurium* and numerous air-borne bacteria do not show any growth during their storage at room temperature or even after 48 hours of incubation at 37°C with or without CO<sub>2</sub>. In support of this observation, the Gram-positive *S. mitis* have conditionally become sensitive to antibiotics as evidenced by the formation of micro-colonies in the presence of colistin (polymyxin B or E) and nalidixic acid when used in combination (Table 1).

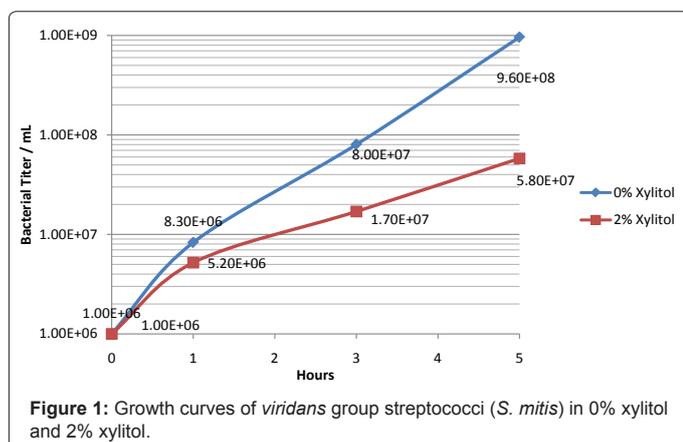


Figure 1: Growth curves of *viridans* group streptococci (*S. mitis*) in 0% xylitol and 2% xylitol.

### Comparison of xylitol-grown *S. mitis* by optical microscopy and SEM

At all concentrations of xylitol, the bacteria are similarly grown to saturation (5-hours) with and without shaking. When these xylitol-

exposed populations are visualized by the century old Gram-staining technique, the majority of the bacteria grow in chains and display a combination of color both purple and pink and a very few purple diplococci individuals (Figure 2B). Obviously, the pink segment of the

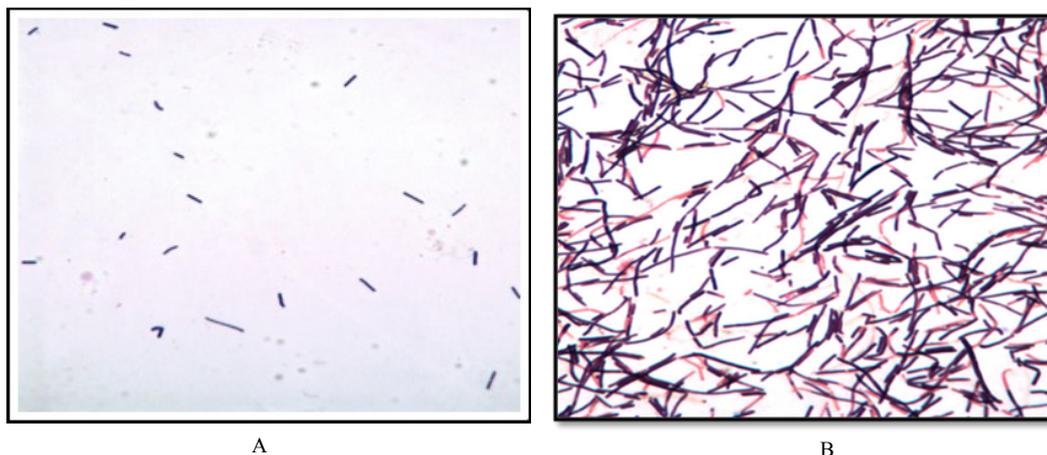
Bacteria grown with and without 2% xylitol	*TSA	TSA with Colistin and Nalidixic acid				
		1µg/ml	2µg/ml	5µg/ml	10µg/ml	15µg/ml
<i>S. mitis</i> without xylitol	+	+	+	+	+	+/-
<i>S. mitis</i> in 2% xylitol for 2.5 Hr **	+	+	-	-	-	-
<i>S. mutans</i> without xylitol	+	+	-	-	-	-
<i>S. mutans</i> in 2% xylitol	+	-	-	-	-	-
<i>E. coli</i> K-12 without xylitol	+	-	-	-	-	-
<i>E. coli</i> K-12 in 2% xylitol	+	-	-	-	-	-

"+" means growth, "-" means no growth, "+/-" means very little growth

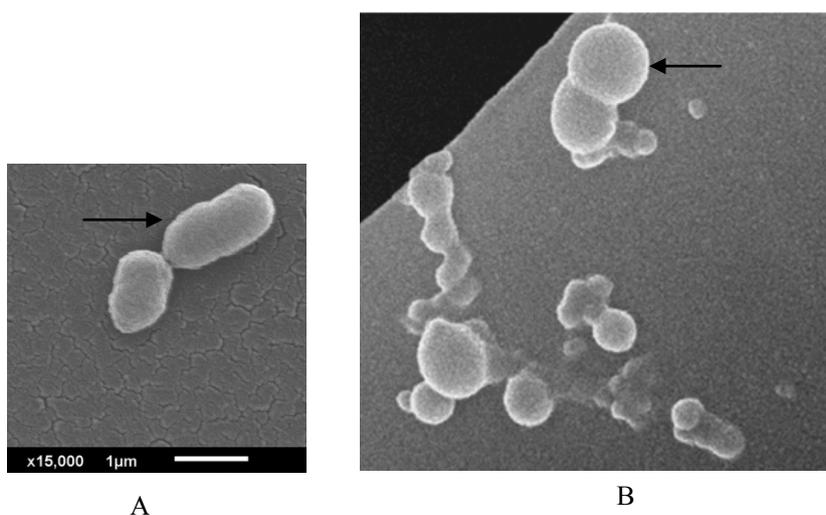
\*Plating was repeated on Mueller-Hinton Agar as well as TSA

\*\* *S. mitis* when grown beyond 3 hours in the presence of xylitol, starts showing a significant decrease in their titer as measured by their colony forming units (cfu). Therefore, their antibiotic sensitivity pattern was compared after 2.5 hours of growth at 37°C.

**Table 1:** Antibiotic sensitivity of *S. mitis* in 2% xylitol as measured by agar dilution test.



**Figures 2A and B:** Gram-stain of *S. mitis* control (A) and (B) exposed to 2% xylitol. Figure 2B shows *S. mitis* in chains with a combination of colors. They represent two different growth phases and therefore their titer differs.



**Figures 3A and 3B:** Scanning electron micrograph of *S. mitis* in 2% xylitol. There is heterogeneity of sizes after exposing to xylitol. Partially damaged bacteria are larger, still smaller than the normal size. Arrow shows the normal size *S. mitis* (~1.8 µm).

same purple chain appears very thin and fragile. Failure of crystal violet to stably interact with the xylitol-grown Gram-positive bacteria leads to a conclusion that the cell wall thickness has been affected. SEM analysis of the same population exposed to 2% xylitol confirms their presence in a long chain but in heterogeneity of sizes (Figure 3B). Figure 3A shows bacterial control without xylitol. In Figure 3B there are a few larger bacteria in the same chain. These are probably the live ones which have been partially affected by xylitol but are still capable of forming colonies [12]. These colonies become sensitive to nalidixic acid and colistin when used in combination (Table 1). In the same micrograph there is a presence of normal size diplococcal parent (about 1.8  $\mu\text{m}$ ). The optical density values at 580  $\mu\text{m}$  showed no difference between the control and the 2% xylitol-exposed population. What is more, the optical density values do not show any difference indicating no lysis.

## Discussion

Unlike penicillin or lysozyme, xylitol does not lyse the bacterial cell wall, but bacterial multiplication is gradually affected. Evidently, the cell division mechanism of diplococcal bacteria belonging to both Gram-positive and Gram-negative needs further exploration. It is well-established that the duplication and subsequent error-free segregation of chromosomes into daughter cells requires an effective association with cell wall/membrane complex (cytokinesis) [16]. Our data indicates that the stable presence of xylitol derivative, formed by partial metabolism of xylitol, in *S. mitis* interferes in their normal biosynthesis and therefore their generation time is prolonged (Figure 1). The optical densities of the bacterial cultures with and without xylitol do not differ very much but the xylitol-exposed diplococci grow in long chains regardless of their chain lengths. Obviously, Griffith's rough and smooth colonies can be explained by revisiting his old data when we accept that this pathogen grows in chains (latent or stationary phase) and they will produce a single colony (rough) instead of separate colonies (Palchaudhuri et al, Ms. in preparation). We have also observed by the Gram staining technique that the majority of the xylitol-exposed diplococci are stably present in chains with heterogeneity of color within the same chain (Figure 2B). SEM analysis of the same population exposed to 2% xylitol confirms their presence in a long chain and shows heterogeneity of sizes (Figure 3B). The pink is indicative of death or reduction of cell wall thickness resembling the staining pattern of Gram negative *E. coli*. Consequently, those that survive the exposure to xylitol become sensitive to the combination of the antibiotics colistin and nalidixic acid. We speculate that the reduction of cell wall thickness

caused by the accumulation of xylitol derivative may interfere in their normal cell division.

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