Xylitol and capsular gene expression in *Streptococcus pneumoniae*

Paula Kurola,¹ Terhi Tapiainen,² Tarja Kaijalainen,¹ Matti Uhari² and Annika Saukkoriipi¹

Correspondence
Paula Kurola
paula.kurola@thl.fi

¹National Institute for Health and Welfare, Oulu, Finland

²University of Oulu Department of Pediatrics, Oulu, Finland

Xylitol is a sugar alcohol that inhibits the growth and adherence of *Streptococcus pneumoniae*. In clinical trials, xylitol has been shown to decrease the occurrence of acute otitis media in day-care children but did not decrease nasopharyngeal carriage of the pneumococci. It has also been shown that xylitol affects the ultrastructure of the pneumococcal capsule. Here, it was hypothesized that xylitol might affect the expression of pneumococcal capsular genes. Capsule gene expression levels were studied in 24 clinical pneumococcal isolates and one ATCC strain (49619) by using a real-time RT-PCR method targeting the mRNA of the second gene of the pneumococcal capsular locus, the *cpsB* gene. The isolates were exposed to 5 % glucose, 5 % xylitol and control medium (brain heart infusion medium containing 10 % fetal bovine serum) for 2 h. *cpsB* gene expression levels were measured by using a relative quantification method with calibrator normalization where the 16S rRNA gene of pneumococcus was used as a reference. Exposure to xylitol lowered *cpsB* gene expression levels significantly compared with those in the control ($P=0.035$) and glucose ($P=0.011$) media. This finding supports previous results where exposure to xylitol changed the ultrastructure of the pneumococcal capsule and could explain further the high clinical efficacy of xylitol in preventing otitis media.

Received 9 April 2009

Accepted 9 July 2009

INTRODUCTION

Xylitol is a five-carbon sugar alcohol that has been used as a sweetening substitute for sucrose due to its preventative effect on dental caries (Mäkinen *et al.*, 1995). Xylitol reduces the growth of α -haemolytic streptococci, including *Streptococcus pneumoniae* (Tapiainen *et al.*, 2001; Söderling *et al.*, 2008), and prevents the adherence of pneumococci and *Haemophilus influenzae* to nasopharyngeal cells (Kontiokari *et al.*, 1998). In clinical trials, xylitol given in the form of chewing gum or syrup decreased the occurrence of acute otitis media in day-care children by 30–40 % (Uhari *et al.*, 1996, 1998). Nasopharyngeal carriage of pneumococci remained unaffected and xylitol was shown to be ineffective in preventing pneumococcal mucosal colonization in rats (Uhari *et al.*, 1996; Kontiokari *et al.*, 1999).

In a previous study (Tapiainen *et al.*, 2004), we found that, after exposure to xylitol, the cell wall of pneumococci became more diffuse and the polysaccharide capsule became ragged, yet the bacteria remained viable. We hypothesized that xylitol could affect the expression of pneumococcal capsular genes. The *cpsB* gene has been shown to be essential for encapsulation and for regulation

of the production of capsular polysaccharide (CPS; Morona *et al.*, 2004). Here, we investigated the effect of xylitol on expression of the *cpsB* gene.

METHODS

Bacterial isolates and growth conditions. Twenty-four clinical *S. pneumoniae* isolates and one ATCC 49619 (serotype 19F) strain were studied. Four of the clinical isolates (serotypes 6B, 9V, 14 and 23F) were obtained from routine middle ear effusion samples from children and, together with the ATCC strain, have been used in a previous study demonstrating changes in the ultrastructure of *S. pneumoniae* after xylitol exposure (Tapiainen *et al.*, 2004). The rest of the clinical isolates were obtained from middle ear effusion samples from children attending an otitis media prevention trial that assessed surgical procedures in the prevention of acute otitis media.

The isolates were plated on blood agar plates and incubated at 36–37 °C in a 5 % CO₂ atmosphere. After overnight incubation, bacterial colonies were collected with a sterile loop in 3 ml brain heart infusion (BHI) medium containing 0.2 % glucose supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS; Cambrex). Bacteria were grown at 36–37 °C in a 5 % CO₂ atmosphere to an OD₆₅₀ of 0.3–0.4 (approx. 5 h). The test media were prepared by adding 5 % D-glucose (w/v; Merck) or 5 % xylitol (w/v; Sigma) to the control medium (BHI supplemented with 10 % heat-inactivated FBS) and sterilized by filtration (0.22 µm pore-sized filter; Corning). The xylitol concentrations used in this study were chosen on the basis of a previous experimental study and xylitol concentrations achieved in

Abbreviation: CPS, capsular polysaccharide.

the saliva of children who consumed the same amount of xylitol products as used in previous clinical trials (Uhari *et al.*, 1996, 1998; Tapiainen *et al.*, 2002).

Three hundred microlitres of the bacterial suspension was transferred into 3 ml of the test media containing 5 % glucose or 5 % xylitol or into the control medium, and incubation at 36–37 °C in 5 % CO₂ was continued for 2 h. After 2 h of growth, 200 µl of the suspension was transferred into 400 µl of an RNA-stabilizing solution (Bacteria Protect Reagent; Qiagen), incubated for 5 min at room temperature and centrifuged for 10 min at 5000 g. The supernatant was discarded and the pellet was stored at –70 °C.

RNA isolation and RT-PCR. RNA was extracted from the RNA-stabilized cell pellets with an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. RNA was eluted in 30 µl RNase-free water and 5 µl of the eluate was subjected to reverse transcription. Removal of genomic DNA and first-strand cDNA synthesis was performed with a QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. Quantitative real-time PCRs were carried out using a LightCycler Instrument (Roche Diagnostics). The PCRs of the gene of interest (*cpsB*) and the reference gene (16S rRNA) were performed in separate PCR runs but using the same cDNA sample, which was diluted 1:100 using sterile RNase- and DNase-free water. All PCRs were performed in triplicate.

For the target gene, *cpsB*, six primers were designed with Primer3 software (<http://frodo.wi.mit.edu/>) using published sequences of the *S. pneumoniae cpsB* gene (GenBank accession nos CR931632–CR931722). The oligonucleotides included two forward primers and four reverse primers and they were all used in the same reaction mixture. Primer pair wzh2_F (forward) and wzh2_R (reverse) recognized serotypes 25F and 38. The rest of the serotypes were amplified with a primer set including one forward primer (wzh_F) and three reverse primers (wzh_R, wzh_R2 and wzh_R3; Table 1). Amplicon sizes were 117–169 bp and all of the serotypes of pneumococcus were expected to amplify based on an analysis with the *S. pneumoniae* CPS BLAST Server available online (http://www.sanger.ac.uk/Projects/S_pneumoniae/CPS/). The 20 µl reaction mixture contained 1 × LightCycler Fast Start DNA Master SYBR Green I mixture (Roche Diagnostics), 3 mM MgCl₂, 0.5 µM each primer and 2 µl cDNA. The protocol consisted of a pre-incubation step at 95 °C for 10 min and 45 cycles of denaturation at 95 °C for 10 s, annealing at 63 °C for 5 s and extension at 72 °C for 8 s. After cycle 15, the annealing temperature was decreased by 1 °C in every cycle until the target temperature of 58 °C was reached. Fluorescence was measured after each extension step. A melting-curve analysis was performed by heating at a rate of 20 °C s^{–1} to 95 °C, cooling at 20 °C s^{–1} to 60 °C and holding for 30 s, and finally heating slowly at 0.1 °C s^{–1} to 95 °C with continuous fluorescence measurement. Samples with a crossing point value (the cycle number at which the

fluorescence of the sample exceeds that of the background) below 40.00 and a melting peak above 80 °C were considered positive.

The real-time PCR targeting the 16S rRNA gene of *S. pneumoniae* was developed based on the publication by Rogers *et al.* (2007). The sequences of the primers amplifying a 53 bp fragment of the 16S rRNA gene are presented in Table 1 (16S-F and 16S-R). The 20 µl reaction mixture contained 1 × LightCycler Fast Start DNA Master SYBR Green I, 3 mM MgCl₂, 0.5 µM each primer and 2 µl cDNA. The protocol consisted of a pre-incubation step at 95 °C for 10 min and 40 cycles of denaturation at 95 °C for 10 s, annealing at 65 °C for 3 s and extension at 72 °C for 8 s. Fluorescence was measured after each extension step. A melting-curve analysis was performed by heating at 20 °C s^{–1} to 95 °C, cooling at 20 °C s^{–1} to 60 °C and holding for 30 s, and finally heating slowly at 0.1 °C s^{–1} to 95 °C with continuous fluorescence measurement. Samples with a crossing point value below 35.00 were considered positive.

Calculation of the relative amount of the target gene was based on the crossing point of the sample and the efficiency of the PCR. Differing from the commonly used 2^{–ΔΔCT} method (Livak & Schmittgen, 2001), we used relative standard curves for efficiency correction. To determine the efficiencies of the two PCRs, external standard curves were prepared separately for the target gene and the reference gene by amplifying serial dilutions of pneumococcal DNA (strain ATCC 6305) containing 50–500 000 pneumococcal genome equivalents. Calibrator-normalized gene expression levels were determined by using the relative quantification tool in the LightCycler software version 4.05 (Roche Diagnostics). The software calculates the relative ratio of the target gene to the reference gene for each sample and normalizes for different detection sensitivities of target and reference genes by dividing the target:reference ratio of each sample by the target:reference ratio of a calibrator, which is included in each PCR run. This normalizes all samples within a run and also provides a constant calibrator point between the PCR runs. As a calibrator, we used cDNA transcribed from RNA from *S. pneumoniae* strain ATCC 6305 in its exponential growth phase.

Statistical analysis. Statistical analyses were carried out using SPSS version 16.0 (SPSS Inc.). Statistical significance was assessed by using analysis of variance for repeated measures after logarithmic transformation of the data. In the case of statistically significant results, different test media were compared in pairs. *P* values were adjusted using the Bonferroni method.

RESULTS AND DISCUSSION

Capsular gene expression levels were measured by using RT-PCR and a relative quantification method. Our RT-PCR method targeted the mRNA of the *cpsB* gene, which is

Table 1. Primers used in the real-time PCRs

Primer	Sequence (5'→3')	T _m (°C)
wzh_F	TAGATGACGGTCCCAAGTCA	54.4
wzh_R	GAGTTTCAAACATGCCCTTG	54.5
wzh_R2	ATTCCCCGAACCTGAAGAAAG	56.0
wzh_R3	TCTTTTGCAATTTCACGAACC	56.0
wzh2_F	GTTGGCTTGTCATGTCCTT	56.6
wzh2_R	ATTCGGTGAAACGACTCCTG	56.4
16S-F	TGAGGTAACCGTAAGGAGCCA	58.3
16S-R	TCACCCCAATCATCTATCCCA	56.6

conserved among pneumococcal serotypes. *cpsB* encodes a phosphotyrosine protein phosphatase that is required to dephosphorylate CpsD (Morona *et al.*, 2002), an autophosphorylating protein tyrosine kinase that has been shown to negatively regulate CPS production (Morona *et al.*, 2000). CpsB therefore plays an important part in the regulation of CPS production (Morona *et al.*, 2002). We used the 16S rRNA gene as a reference gene in our PCR method. Other studies applying RT-PCR to investigate pneumococcal capsule gene expression have also used the 16S rRNA gene as a reference (LeMessurier *et al.*, 2006; Hathaway *et al.*, 2007; McEllistrem *et al.*, 2007) and studies with other bacteria have actually evaluated 16S rRNA as the optimal reference for their RT-PCR application (Eleaume & Jabbouri, 2004; Tasara & Stephan, 2007). We also tested another reference gene, *gyrB*, which was used in a previous publication (Oggioni *et al.*, 2006). Thus, all samples were also analysed with a *gyrB* PCR and the gene expression levels between growth media differed significantly (data not shown). However, the ratio of target crossing point to reference crossing point of the calibrator was found to be more stable between PCR runs when the 16S rRNA gene was the reference and it was therefore used in this study.

In our previous study, we found that xylitol affects the appearance of the pneumococcal capsule after 2 h of exposure to xylitol, but that 30 min of exposure caused no change in the morphology of pneumococci (Tapiainen *et al.*, 2004). Here, we first tested both time points, as the changes in gene expression levels could have been observed as early as 30 min after exposure to xylitol. However, when the first five isolates were analysed, no differences in the capsule gene expression levels were seen among the growth media after 30 min of exposure. Therefore, the single time point of 2 h was selected.

After 2 h of exposure to the test medium, gene expression levels differed significantly between the test media ($P=0.005$) (Fig. 1). The means of the gene expression ratios were 3.63 [95 % confidence interval (CI) 1.86–7.08], 3.54 (95 % CI 1.82–7.08) and 0.95 (95 % CI 0.30–3.09) in

the control, glucose and xylitol media, respectively. *cpsB* gene expression levels were significantly lower in xylitol than in the control medium, with a mean difference of 0.577 in the log-transformed gene expression ratios (95 % CI for the difference 0.33–1.12, $P=0.035$). Exposure to xylitol also lowered gene expression levels when compared with glucose exposure, the mean difference in the log-transformed gene expression ratios being 0.570 (95 % CI for the difference 0.11–1.03, $P=0.011$). Capsular gene expression levels in the glucose medium did not differ significantly from those in the control medium (mean difference of 0.007, 95 % CI -0.28 to 0.26 , $P=1.0$).

The results showed that xylitol alters the capsule gene expression of pneumococci and support our previous findings of the effect of xylitol on the ultrastructure of pneumococci, especially the observed smaller size and ragged appearance of the polysaccharide capsule (Tapiainen *et al.*, 2004). The observed alterations in capsular gene expression could also further explain the efficacy of xylitol in preventing otitis media observed in previous studies where xylitol decreased the occurrence of otitis media but did not reduce pneumococcal carriage either in children during a clinical trial or in an animal model (Uhari *et al.*, 1998; Kontiokari *et al.*, 1999). CPS is the principal virulence factor of *S. pneumoniae*, and encapsulated pneumococci are usually more virulent than their unencapsulated counterparts (Kim & Weiser, 1998). Thus, the reductive effect of xylitol on capsular gene expression could alter the virulence of pneumococci and make them less capable of causing infections such as otitis media.

A xylitol concentration of 5 % was used in this study. In previous studies, growth inhibition was detected after exposure to 1 % xylitol and morphological changes were visualized by scanning electron microscopy after exposure to 0.5–5 % xylitol (Kontiokari *et al.*, 1995; Tapiainen *et al.*, 2004). A xylitol concentration of 5 % was reached in the saliva of children only for 10–15 min after chewing xylitol gum or consuming a xylitol mixture in doses used in earlier

Fig. 1. Calibrator-normalized capsular gene expression levels in different test media. The bar indicates the mean of the log-transformed data. Exposure to xylitol lowered the *cpsB* gene expression levels significantly compared with those in the control ($P=0.035$) and glucose ($P=0.011$) media. Capsular gene expression levels in the glucose medium did not differ significantly from those in the control medium ($P=1.0$).

clinical trials (Tapiainen *et al.*, 2002). If xylitol acts only as an anti-adhesive agent (Konttiokari *et al.*, 1998), the expected efficacy would be less than observed in the clinical trials, in which regular consumption of xylitol after each meal five times per day was successful in preventing acute otitis media by 40 % (Uhari *et al.*, 1996, 1998). Thus, our finding of xylitol's effect on capsular gene expression of pneumococci further explains the results of our previous clinical trials. The effect on capsular gene expression, however, is not likely to be very long term if alternative carbon sources are available for pneumococci between xylitol doses, as xylitol administered in only three daily doses, i.e. not after each meal, failed to prevent acute otitis media (Hautalahti *et al.*, 2007).

Twenty-five isolates of 12 different serotypes were analysed in this study, and as there were only a few isolates with the same serotype, the differences in gene expression levels between different serotypes were not analysed further. However, two isolates had extremely high capsular gene expression levels: 172 and 157 in the control medium, 45.9 and 302 in glucose, and 157 and 119 in the xylitol medium. These two isolates were of serotype 38.

In conclusion, we found that xylitol significantly decreases capsular gene expression levels in *S. pneumoniae* isolates. This finding supports our previous results where exposure to xylitol changed the ultrastructure of pneumococci (Tapiainen *et al.*, 2004) and could further explain the high clinical efficacy of xylitol in preventing otitis media (Uhari *et al.*, 1996, 1998).

ACKNOWLEDGEMENTS

This study was supported by the Päivikki and Sakari Sohlberg Foundation. We thank E. Korhonen, L. Kuisma and A. Ojala for their excellent work in the laboratory.

REFERENCES

- Eleaume, H. & Jabbouri, S. (2004). Comparison of two standardisation methods in real-time quantitative RT-PCR to follow *Staphylococcus aureus* genes expression during in vitro growth. *J Microbiol Methods* **59**, 363–370.
- Hathaway, L. J., Böttig, P. & Mühlemann, K. (2007). In vitro expression of the first capsule gene of *Streptococcus pneumoniae*, *cpsA*, is associated with serotype-specific colonization prevalence and invasiveness. *Microbiology* **153**, 2465–2471.
- Hautalahti, O., Renko, M., Tapiainen, T., Konttiokari, T., Pokka, T. & Uhari, M. (2007). Failure of xylitol given three times a day for preventing acute otitis media. *Pediatr Infect Dis J* **26**, 423–427.
- Kim, J. O. & Weiser, J. N. (1998). Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of *Streptococcus pneumoniae*. *J Infect Dis* **177**, 368–377.
- Konttiokari, T., Uhari, M. & Koskela, M. (1995). Effect of xylitol on growth of nasopharyngeal bacteria in vitro. *Antimicrob Agents Chemother* **39**, 1820–1823.
- Konttiokari, T., Uhari, M. & Koskela, M. (1998). Antiadhesive effects of xylitol on otopathogenic bacteria. *J Antimicrob Chemother* **41**, 563–565.
- Konttiokari, T., Svanberg, M., Mattila, P., Leinonen, M. & Uhari, M. (1999). Quantitative analysis of the effect of xylitol on pneumococcal nasal colonisation in rats. *FEMS Microbiol Lett* **178**, 313–317.
- LeMessurier, K. S., Ogunniyi, A. D. & Paton, J. C. (2006). Differential expression of key pneumococcal virulence genes in vivo. *Microbiology* **152**, 305–311.
- Livak, K. J. & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402–408.
- Mäkinen, K. K., Bennett, C. A., Hujoel, P. P., Isokangas, P. J., Isotupa, K. P., Pape, H. R., Jr & Mäkinen, P. L. (1995). Xylitol chewing gums and caries rates: a 40-month cohort study. *J Dent Res* **74**, 1904–1913.
- McEllistrem, M. C., Ransford, J. V. & Khan, S. A. (2007). Characterization of in vitro biofilm-associated pneumococcal phase variants of a clinically relevant serotype 3 clone. *J Clin Microbiol* **45**, 97–101.
- Morona, J. K., Paton, J. C., Miller, D. C. & Morona, R. (2000). Tyrosine phosphorylation of CpsD negatively regulates capsular polysaccharide biosynthesis in *Streptococcus pneumoniae*. *Mol Microbiol* **35**, 1431–1442.
- Morona, J. K., Morona, R., Miller, D. C. & Paton, J. C. (2002). *Streptococcus pneumoniae* capsule biosynthesis protein CpsB is a novel manganese-dependent phosphotyrosine-protein phosphatase. *J Bacteriol* **184**, 577–583.
- Morona, J. K., Miller, D. C., Morona, R. & Paton, J. C. (2004). The effect that mutations in the conserved capsular polysaccharide biosynthesis genes *cpsA*, *cpsB*, and *cpsD* have on virulence of *Streptococcus pneumoniae*. *J Infect Dis* **189**, 1905–1913.
- Oggioni, M. R., Trappetti, C., Kadioglu, A., Cassone, M., Iannelli, F., Ricci, S., Andrew, P. W. & Pozzi, G. (2006). Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis. *Mol Microbiol* **61**, 1196–1210.
- Rogers, P. D., Liu, T. T., Barker, K. S., Hilliard, G. M., English, B. K., Thornton, J., Swiatlo, E. & McDaniel, L. S. (2007). Gene expression profiling of the response of *Streptococcus pneumoniae* to penicillin. *J Antimicrob Chemother* **59**, 616–626.
- Söderling, E. M., Ekman, T. C. & Taipale, T. J. (2008). Growth inhibition of *Streptococcus mutans* with low xylitol concentrations. *Curr Microbiol* **56**, 382–385.
- Tapiainen, T., Konttiokari, T., Sammalakivi, L., Ikäheimo, I., Koskela, M. & Uhari, M. (2001). Effect of xylitol on growth of *Streptococcus pneumoniae* in the presence of fructose and sorbitol. *Antimicrob Agents Chemother* **45**, 166–169.
- Tapiainen, T., Renko, M., Konttiokari, T. & Uhari, M. (2002). Xylitol concentrations in the saliva of children after chewing xylitol gum or consuming a xylitol mixture. *Eur J Clin Microbiol Infect Dis* **21**, 53–55.
- Tapiainen, T., Sormunen, R., Kajjalainen, T., Konttiokari, T., Ikäheimo, I. & Uhari, M. (2004). Ultrastructure of *Streptococcus pneumoniae* after exposure to xylitol. *J Antimicrob Chemother* **54**, 225–228.
- Tasara, T. & Stephan, R. (2007). Evaluation of housekeeping genes in *Listeria monocytogenes* as potential internal control references for normalizing mRNA expression levels in stress adaptation models using real-time PCR. *FEMS Microbiol Lett* **269**, 265–272.
- Uhari, M., Konttiokari, T., Koskela, M. & Niemelä, M. (1996). Xylitol chewing gum in prevention of acute otitis media: double blind randomised trial. *BMJ* **313**, 1180–1184.
- Uhari, M., Konttiokari, T. & Niemelä, M. (1998). A novel use of xylitol sugar in preventing acute otitis media. *Pediatrics* **102**, 879–884.