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Role of *Streptococcus mutans* Eukaryotic-Type Serine/Threonine Protein Kinase in Interspecies Interactions with *Streptococcus sanguinis*

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Abstract

Objective—Interspecies interactions of oral streptococci involve the production and excretion of antimicrobial compounds to compete successfully during colonization. Bacteriocin production by *Streptococcus mutans* and hydrogen peroxide (H₂O₂) production by *Streptococcus sanguinis* have been demonstrated as crucial for the clinical relevant antagonism between both species. A potential target of H₂O₂ is the cell-envelop of *S. mutans*. In the present study, the role of cell-envelop associated eukaryotic serine/threonine protein kinase (STPK) in *S. mutans* during interspecies competition has been investigated.

Design—Allelic replacements via homologous recombination of the STPK encoding gene with a kanamycin resistant determinant has been constructed. The mutant has been screened for the susceptibility towards cell-envelope stress. A previously developed spotting assay was used to simulate interspecies competition.

Results—The STPK⁻ mutant showed an increased susceptibility toward envelop-stress caused by H₂O₂ and was significantly more inhibited during interspecies competition assays.

Conclusions—*S. mutans* is able to sense antimicrobial compounds excreted by competing species and can potentially adjust the cell-envelop toward an increased resistance.

Introduction

Multispecies bacterial communities such as found in the oral biofilm are competing among the available space and nutrients. The competition involves several mechanism including the excretion of inhibiting substances against competitors (1). This was shown for the clinical relevant competition between the opportunistic pathogen *S. mutans* and the commensal *S. sanguinis*. *S. mutans* strategy relies on the cell-density regulated production of antimicrobial peptides, mutacin I and IV (2,3). *S. sanguinis*, although known to produce the bacteriocin sanguicin (4), mainly competes with other oral streptococci by excreting H₂O₂ (1,5). H₂O₂ is bacteriostatic at low concentrations and able to inhibit *S. mutans* growth by interfering with

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the glycolytic pathway (6). Oxidative stress triggered by higher H₂O₂ concentrations may interfere with protein biosynthesis and can cause methionine oxidation to methionine sulfoxide of cytoplasmatic and cell envelope proteins. Methionine sulfoxide might lead to conformational changes of proteins, which eventually could not be repaired by the cell (6,7).

Cell-envelope stress is common to bacteria and specific proteins are involved in sensing changes to the envelope structure (8). A recent report showed that a eukaryotic-type serine/threonine protein kinase (STPK) in *Enterococcus faecalis*, PrkC, is involved in the antimicrobial resistance toward cell-envelope active compounds like cephalosporins and bile salt (9). STPKs are widely encoded in the genomes of bacteria. They have distinct architectural domains comprised of the kinase catalytic domain and additional functional domains, including phosphatases, glycoproteases and others (10). One class of functional domains belongs to a larger family of penicillin binding proteins, called PASTA (for penicillin binding protein and serine/threonine kinase associated domain) (11). They are found as a part of several STPKs mainly in Gram-positives, including *Bacillus subtilis*, *Streptococcus pneumoniae* and *E. faecalis* (9,12,13). *S. mutans* encodes a STPK, PknB, with similar architecture compared to PrkC and a PknB⁻ mutant showed an altered biofilm phenotype, lower transformation efficiency and reduced acid tolerance (14). In this report we provide evidence that the STPK of *S. mutans* is important in reacting to H₂O₂ produced by *S. sanguinis* during interspecies competition.

Materials and Methods

Bacterial strains and media

S. mutans UA140 (15) and UA159 (16), *S. sanguinis* SK36 (17) and the isogenic UA140 PknB⁻ mutant were routinely grown at 37°C in Todd Hewitt broth (TH; Difco, Sparks, MD) or on TH agar plates in 5% CO₂. *S. mutans* serotype *e* and *f* were kindly provided by Dr. J. Merritt, University of Oklahoma Health Sciences Center. When indicated, THY medium (Todd Hewitt medium supplemented with 0.2% yeast extract (EMD; Darmstadt, Germany) was used. *Escherichia coli* DH5α cells were grown aerobically at 37°C in Luria Bertani medium (Difco). When required kanamycin was supplemented at the following concentration: 800 µg ml⁻¹ for *S. mutans*, and 25 µg ml⁻¹ for *E. coli*. Hydrogenperoxide (H₂O₂) was purchased from BDH (Distributor VWR; West Chester, PA). Plates were stored overnight for drying on air before using in the spotting assay described below. Growth curves of *S. mutans* UA140 and PknB⁻ mutant were measured using a Bioscreen C analyzer, version 2.4 (Oy Growth Curves AB Ltd., Finland), which simultaneously measured the development of turbidity in multiple cultures.

Construction of PknB⁻ mutant

Standard recombinant DNA manipulations were performed essentially as described before (18). The deletion of *pknB* in *S. mutans* was created by double cross-over homologous recombination. To generate the constructs for the deletion, two fragments corresponding to the up and downstream regions of *pknB* in *S. mutans* were generated by polymerase chain reaction (PCR) using GoTag DNA polymerase (Promega; Madison, WI) and specific oligonucleotides listed in Table 1. Oligonucleotides pknB1 and pknB2 for *S. mutans* incorporated restriction enzyme (RE) sites for *Xba*I and *Bam*HI during amplification of the up stream fragments. Oligonucleotides pknB3 and pknB4 incorporated RE sites for *Hind*III and *Xho*I during amplification of the down stream fragments, respectively. After RE digestion of the PCR products, fragments were ligated into compatible RE sites on pBSK-kan (gift of Dr. J. Merritt, University of Oklahoma Health Sciences Center) up and down stream of a kan-cassette (gene *aphA*) conferring kanamycin resistance in streptococci. The correct insertion of the up and down stream fragments was confirmed by PCR and RE digestion. The resulting plasmid pKNB-Sm was used as template to PCR amplify the up and down stream fragments including the kan-

cassette using oligonucleotides *pknB1* and *pknB4*. The PCR products were subsequently transformed into *S. mutans* using a transformation protocol described earlier (19). Correct kan-resistant clones were identified by colony PCR for the absence of the *pknB* encoding region. To ensure that no expression of *pknB* occurred in the *PknB⁻* mutant, RNA was isolated and cDNA synthesized essentially as described before (18). Semi-quantitative Real-Time PCR was performed with oligonucleotides *pknB-RT-F* and *pknB-RT-R*, hybridizing in the coding region of *pknB*, using a G-STORM GS1 thermocycler (Gene Technologies Limited; Essex, Great Britain). Equal volumes of the PCR reaction after 25 cycles were analyzed on a 1% agarose gel.

Susceptibility to H₂O₂

Overnight cultures of the wild type or the *PknB⁻* mutant were grown in THY until mid-logarithmic phase ($A_{600\text{ nm}} \sim 0.5$). Two ml were mixed with 4 ml hand warm soft-agar THY (0.7% agar) and poured onto a THY plate. Sterile filter disks (BD; Sparks, MD) infused with 10 μ l of 30% H₂O₂ were aseptically placed on top of the solidified soft-agar in the center of the plate. Growth inhibition was measured as the diameter of the zone of inhibition after overnight incubation as commonly done for agar diffusion assays. The diameter of wild type and *pknB* mutant from five independent plates each was measured and the wild type diameter set to 1. The increase in inhibition diameter of the *pknB* mutant was calculated relative to the wild type.

Competition assay on solid medium

To compare competitive growth between wild type and *PknB⁻* mutant, a protocol described previously was used with modifications (18). Briefly, 8 μ l of an overnight culture of *S. sanguinis* in THY medium adjusted to an absorption at 600 nm of 0.8 was inoculated onto a THY agar plate as the pioneer colonizer. After incubation overnight (16 h) at 37°C in 5% CO₂, 8 μ l of *S. mutans* wild type or *PknB⁻* mutant adjusted to an absorption at 600 nm of 0.8 was inoculated next to the pioneer colonizer such that the colonies almost touched each other. The plate was incubated overnight. Growth inhibition was assessed by measuring the proximal zone of inhibition at the intersection with the pioneer colony (see Fig. 2A).

Statistical analysis

Statistical analysis of data was performed with the QuickCalcs online calculators (<http://www.graphpad.com/quickcalcs/index.cfm>) using the *t* test software to compare the means of two groups. The data were considered significantly different if the two-tailed *P* value was < 0.05.

Results

Inactivation of *pknB* in *S. mutans*

The role of *S. mutans* STPK homolog in acid tolerance, biofilm formation and competence has been investigated before, but a potential role in biofilm ecology has not been addressed (14). A knockout mutant encompassing the protein kinase domain and the three PASTA domains (Fig. 1A) has been constructed and non-expression of the gene in the *PknB⁻* mutant confirmed (Fig. 1B). The *pknB* gene is the last gene in an operon including the phosphoprotein phosphatase gene *pppL*. A polar effect on downstream genes is therefore not expected. Growth of the mutant seems to be only slightly impaired compared to the wild type when grown in TH medium, while no difference was observed with THY medium (Fig. 1C). All consecutive experiments were therefore performed using THY as growth medium.

Decreased fitness of the PknB⁻ mutant during *S. mutans* – *S. sanguinis* interactions

The excretion of mutacins by *S. mutans* and H₂O₂ by *S. sanguinis* are considered important virulence factors (1). Mutants unable to produce either H₂O₂ or mutacins are less competitive as determined by sequential inoculation experiments to simulate early and later colonizers (1,18). To learn if the later colonizer is able to react to the presence of antimicrobials produced by the pioneer colonizer, a spotting assay described earlier (1) was used to determine the susceptibility of *S. mutans* wild type and PknB⁻ mutant to H₂O₂ produced by *S. sanguinis*. The antimicrobial compound is excreted by the early colonizer during growth and diffuses through the agar surrounding the spotted colony, creating a concentration gradient. As shown in Fig. 2A, *S. mutans* wild type was less susceptible to the H₂O₂ produced by *S. sanguinis* compared to the PknB⁻ mutant. Statistical analysis using the *t*-test to compare two means showed a significant difference between wild type and mutant (Fig. 2B). This result indicates a protective function of the STPK in *S. mutans* in the interspecies interaction with H₂O₂ producing oral streptococci.

To confirm that the inhibition is caused by H₂O₂ excreted by *S. sanguinis*, and to exclude any strain specific inhibition effect, a *S. sanguinis* pyruvate oxidase mutant (Pox⁻) (18) was tested in its ability to inhibit *S. mutans* strains UA140, UA159 and serotype *e* and *f* strains. As shown in Fig. 2C, the *S. sanguinis* wild type was able to inhibit all tested *S. mutans* strains, while the H₂O₂ deficient mutant failed to inhibit the growth of all tested competitor strains.

Inactivation of *pknB* in *S. mutans* increased sensitivity to H₂O₂

The PknB⁻ mutant did not show any significant growth defects under normal condition, consistent with reports for *Staphylococcus aureus*, *E. faecalis* and *S. mutans* strain UA159 (9,14,20). The described phenotype of impaired growth at pH5 in *S. mutans* UA159 was confirmed for *S. mutans* UA140 (data not shown).

Next, the direct susceptibility to H₂O₂ of the *S. mutans* PknB⁻ mutant was tested with an agar diffusion assay. The PknB⁻ increased in the susceptibility compared to the wild type (Fig. 3). This increase was statistically significant.

Discussion

STPKs carrying the functional PASTA domain have been suggested as important status sensors for the cell-envelope of Gram-positive bacteria. Mutations of the STPKs in *E. faecalis*, *S. mutans* and *S. pneumoniae* all increased the susceptibility of these organisms toward cell-envelop stress caused by low pH, increased osmolality or certain cell-envelop targeting antibiotics (9,13,14). We now provide evidence that the STPK of *S. mutans* is required to withstand ecological stress in the interspecies competition of oral streptococci. Commensal *S. sanguinis*, considered as beneficial early colonizer of the oral biofilm engages in interspecies competition with other streptococci, including *S. mutans* by excreting inhibiting amounts of H₂O₂. Likewise *S. mutans* UA140 excretes the bacteriocins mutacin I and IV (1). Both antimicrobials are able to interfere with the bacterial cell-envelope causing considerable stress, growth inhibition or death of the susceptible species (21,22). Our results show that functional expression of *pknB* in *S. mutans* is required to cope with lower concentrations of H₂O₂. The inhibition zone was greater for the PknB⁻ mutant indicating a concentration dependent effect. The biological significance of H₂O₂ in the oral environment is not entire clear, since enzymes able to detoxify H₂O₂ are present in saliva, e.g. salivary lactoperoxidase (sLPO). We have demonstrated earlier that sLPO is able to influence the competition between oral streptococci (23). We suggest that the effect of H₂O₂ is most likely to occur in the microenvironment of the oral biofilm, only affecting near neighbors. Decreased H₂O₂ tolerance was also reported for a STPK mutant of *S. pneumoniae* (24).

Clearly, oral streptococci face several stress situations and the interspecies competition is only one example. In addition, there is host-induced stress due to food intake that increases saliva flow, changes osmolality and lowers the pH. Therefore a variety of stresses might be sensed by STPK of oral streptococci. Surprisingly all sequenced genomes of oral streptococci seem to encode only one PknB homolog. The response of the cell to diverse cell-envelop stresses must be regulated downstream of the STPKs. PknB itself lacks any obvious DNA binding domains. A protein domain search using the putative *pknB* homologs of *Streptococcus agalactiae*, *Streptococcus thermophilus*, and *S. sanguinis* did not reveal any DNA-binding domains indicating a common down-stream regulatory mechanism. This suggests that an interaction with another cytoplasmatic protein is most likely to occur. Saskova *et al.* demonstrated that the STPK in *S. pneumoniae* is involved in global control of gene expression, including functional genes for cell-wall metabolism, DNA repair, and oxidative stress response (24). Further studies showed that STPK of *S. pneumoniae* is able to interact with the α -subunit of the RNA polymerase, RpoA, and suggesting RpoA as downstream regulator of gene expression in the cell-envelop stress response (24,25). If a similar regulation exists in *S. mutans* awaits conformation.

The offensive strategies of *S. sanguinis* and *S. mutans* have been characterized in detail, but less information is available for defensive or protective measurements of oral streptococci in interspecies competition. So far only *S. gordonii*'s strategy to enzymatically inactivate the cell-density dependent signal controlling the expression of *S. mutans* bacteriocins was described (26). A recent study by Perry *et al* identified the HK/RR11 two-component system as important to adapt to oxidative stress, demonstrating that *S. mutans* might have alternative ways to cope with certain stresses (27). HK/RR11 regulates several stress responsive genes in *S. mutans* including DNA-damage-inducible protein P, an alkaline-shock protein homolog and osmoprotectant amino acid ABC transporter (27). The response of *S. mutans* toward cell-envelop stress might well be coordinated between the STPK and the two-component signal transduction system HK/RR11.

In conclusion, we have shown the important role of the STPK in *S. mutans* during interspecies competition. *S. mutans* and most likely other oral streptococci as well sense antimicrobials secreted during competitive growth and adapt with yet to be identified mechanism to this ecological stress.

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Fig. 1. Protein architecture of PknB and mutant characterization. A) Domain architecture of *S. mutans* PknB, TM = trans membrane domain. B) Semi quantitative real-time PCR of *pknB* expression in wild type (WT) and *PknB*⁻ mutant ($\Delta pknB$). C) Growth curves in TH and THY medium. Solid squares = wild type; void squares = *PknB*⁻ mutant.

Fig. 2.

Interspecies growth inhibition. A) Picture demonstrating the effect of *S. sanguinis* H₂O₂ on *S. mutans* wild type (WT) and PknB⁻ mutant ($\Delta pknB$). B) Measurements of the proximal zones of inhibition. 1 = wild type, 2 = PknB⁻ mutant. Presented are averages and standard deviations of 5 experiments. * $p < 0.05$. C) Inhibition of different *S. mutans* strains by *S. sanguinis* wild type and pyruvate oxidase mutant.

Fig. 3. Effect of H₂O₂ on the growth of *S. mutans* wild types and PknB⁻ mutant. Presented is the relative increase of the inhibition zone with the wild type set to 1. 1 = wild type, 2 = PknB⁻ mutant. Shown are averages and standard deviations of 5 experiments. * $p < 0.05$.

Tables
Oligonucleotides used in this Study

Oligonucleotide	Sequence
pknB1	GCTTATCTAGATCGGATTATGAATTGGGTCA
pknB2	GCTTAGGATCCGGAGAATCCGATAACGACCA
pknB3	GCTTAAAGCTTTCAAGTGCGACGACAACTTC
pknB4	GCTAGCTCGAGAACTTGAGAGATTACTCGCCAGA
pknB-RT-F	ACCAATTCAATGCTGGGTTC
pknB-RT-R	GGAATATGCCCTGTCAGCAT