Role of hyaluronidase in *Streptococcus intermedius* biofilm

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Streptococcus intermedius is found in biofilms on teeth and as a commensal member of the gastrointestinal and urinary floras, but may also be associated with deep-seated purulent infections and infective endocarditis. *S. intermedius* produces hyaluronidase, an enzyme that breaks down hyaluronan (HA), a major component of the extracellular matrix of connective tissue. We investigated the involvement of hyaluronidase in *S. intermedius* biofilm formation and dispersal as well as adhesion to human cells. The hyaluronidase activity and expression of the *hyl* gene were higher in growth media supplemented with HA. Inactivation of the *S. intermedius* hyaluronidase resulted in a mutant that formed up to 31 % more biofilm in media supplemented with HA. Hyaluronidase added to the medium caused dispersal of *S. intermedius* biofilm. Adhesion to epithelial cells was similar in the wild-type and the hyaluronidase mutant. We concluded that hyaluronidase may be important for *S. intermedius* detachment from biofilms but not for adhesion to epithelial cells. The ability of *S. intermedius* to detach from the surface and to spread may be crucial in the pathogenicity of this micro-organism.

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INTRODUCTION

Streptococcus intermedius is a commensal member of the human oral, gastrointestinal and urinary floras, but may also be associated with deep-seated purulent infections, particularly in the brain and liver (Claridge *et al.*, 2001; Whiley *et al.*, 1992). In the oral cavity, *S. intermedius* is mainly found in biofilms on teeth, but may be associated with periodontal disease and implantitis (Tanner *et al.*, 1997). In subcutaneous abscesses induced by dental plaque in mice, *S. intermedius* was found in approximately 50 % of the cases (Okayama *et al.*, 2005). Like other oral streptococci, *S. intermedius* may be implicated as a causative agent of infective endocarditis (Gossling, 1988; Hamada & Slade, 1980; Piscitelli *et al.*, 1992; Whiley *et al.*, 1992).

Hyaluronan (HA) is a major component of the extracellular matrix of connective tissue and is expressed by many cell types, including keratinocytes (Agren *et al.*, 1997). In epithelial cells, HA may mediate the adherence of bacteria exhibiting HA-binding surface proteins (Aoki *et al.*, 2004). HA is a high-molecular-mass polysaccharide, made up of alternating *N*-acetylglucosamine and glucuronic acid residues linked by glycosidic bonds (Girish & Kemparaju, 2007). HA has been suggested to support cell proliferation and migration during challenges like wounding and inflammation (Tammi *et al.*, 2002). In the oral cavity HA is present in saliva (Pogrel *et al.*, 1996), in the

epithelium of the oral mucosa (Tammi *et al.*, 1990), and in gingival exudates from sites of chronic gingivitis (Last & Embery, 1987). HA is also found in sites associated with *S. intermedius* purulent infections, including brain (Ruoslahti, 1996), heart (Camenisch *et al.*, 2000) and liver tissues (Nyberg *et al.*, 1988).

HA may be cleaved by bacterial hyaluronidase at the β -1-4 linkage, the products being unsaturated disaccharides (Hynes & Walton, 2000). The main hyaluronidase function is claimed to be provision of bacterial nutrients by the enzymic depolymerization of HA (Starr & Engleberg, 2006). The disaccharides produced may be transported and metabolized intracellularly. Another important hyaluronidase function is HA degradation in connective tissues, which may allow the spread of pathogens or toxins from an initial site of infection (Feldman *et al.*, 2007; Starr & Engleberg, 2006). Interestingly, the severity of gingivitis in young male adults correlated with their salivary hyaluronidase activity (Rovelstad *et al.*, 1958). Among oral streptococci, only *S. intermedius* and *Streptococcus constellatus* produce hyaluronidase, with *S. intermedius* strains showing a higher frequency of enzymic activity (Takao, 2003). The *S. intermedius* hyaluronidase also breaks down chondroitin sulphate, although degradation rates are less than 8 % of the rate with HA (Shain *et al.*, 1996). The hyaluronidase in *S. intermedius* is closely related to that in *Streptococcus pneumoniae*, including conserved regions associated with carbohydrate binding, enzyme activity and cell wall anchoring domains (Takao, 2003). Most of the hyaluronidase activity is found in the culture

Abbreviation: HA, hyaluronan.

supernatant (Homer *et al.*, 1997). Production of bacterial hyaluronidase in the area of the gingival crevice may interrupt the integrity of the periodontal tissue by breaking down HA, leading to periodontal destruction and spread of toxins. The presence of the anchoring domains may also suggest a role for the enzyme on the bacterial cell surface.

In nature micro-organisms grow preferentially in highly specialized biofilm communities (Costerton *et al.*, 1995). Biofilm formation is a dynamic process where extracellular polymers provide the matrix of densely aggregated surface-adherent micro-organisms (Costerton *et al.*, 1999). A wide range of factors may be important for biofilm integrity and stability, including bacterial surface proteins, bacterial enzymes, and amount and types of nutrients in the environment (Branda *et al.*, 2005; Sutherland, 2001). HA present in saliva and connective tissue might serve as a source of nutrients or adhesion substrate and thereby affect biofilm formation. It is also possible that HA may form part of the complex extracellular polymer matrix of biofilms. Hyaluronidase might also be involved in biofilm formation through different mechanisms. While surface bound, hyaluronidase might function as an adhesin. Surface-bound and cell-free enzymes might influence nutrient availability, or promote detachment of cells from the biofilm. Several studies have shown the presence of hyaluronidase at sites of infection (Hashioka *et al.*, 1994; Takao *et al.*, 1997; Unsworth, 1989). However, little is known about the specific role of HA and bacterial hyaluronidase in biofilm formation and dispersal. The ability to express hyaluronidase and to form biofilms may be critical for the pathogenesis of *S. intermedius*. The aim of this study was therefore to investigate whether biofilm formation or dispersal, as well as adhesion to epithelial cells, may be affected by hyaluronidase.

METHODS

Bacterial strains and culture media. The *S. intermedius* strains used in this study were the type strain NCTC 11324 and its isogenic hyaluronidase mutant SI009 (Hyl⁻, described below). The strains were stored at -20 °C in brain heart infusion broth (Difco) supplemented with 15 % (v/v) glycerol.

Growth conditions. Growth rate in liquid culture was monitored in trypticase soy broth (TSB, Difco) supplemented or not with HA (1 µg ml⁻¹, Sigma) or in minimal medium (MM) (Homer *et al.*, 2001) supplemented with glucose (1.8 mg ml⁻¹) or HA (up to 5 mg ml⁻¹) as carbohydrate source. Bacterial cultures were incubated at 37 °C in a 5 % CO₂ aerobic atmosphere. OD₆₀₀ was measured at different time intervals until stationary phase.

Construction of the hyaluronidase-defective mutant. The *S. intermedius* hyaluronidase gene was inactivated by insertion duplication, using the streptococcal integration vector pSF151 (Tao *et al.*, 1992). The hyaluronidase preliminary sequence information from *S. intermedius* UNS 35 was kindly provided by Dr Karen A. Homer, KCL, London. The forward primer FP062 (5'-CAGAATCCAAG-ATGGCTCTTACATTGACC-3') and the reverse primer FP063 (5'-ACTCTAGAGCGCGGCTTAAGTCCATTAATTC-3') were used to amplify a 207 bp region of the hyaluronidase gene. The amplicon and

pSF151 were digested with *EcoRI* and *XbaI*, ligated, and used to transform *S. intermedius* NCTC 11324. Transformation was conducted as previously described (Petersen *et al.*, 2001). The transformants were selected by plating the cells on Todd-Hewitt broth agar plates containing 5 % horse serum and kanamycin (500 µg ml⁻¹). An isogenic mutant was randomly selected for further characterization and named SI009. The correct inactivation site was confirmed both by Southern hybridization with probes for pSF151 or *hyl*, and by DNA sequencing using the forward primer FP039 (5'-AGCGGATAACAATTTCCACAGGA-3') corresponding to the distal region of pSF151 and the reverse primer FP063.

RNA isolation and real-time PCR. Total RNA from *S. intermedius* grown in TSB or in TSB supplemented with HA was extracted at various time points with the High Pure RNA isolation kit (Roche) according to the manufacturer's recommendation, except that the cells were incubated at 37 °C for 30 min in 100 µl lysis buffer containing 20 mg lysozyme ml⁻¹ and 100 U mutanolysin ml⁻¹. DNase I was used during the RNA extraction to remove remaining DNA. RNA concentration was adjusted to 100 ng ml⁻¹, and samples were stored at -70 °C until use.

Complementary DNA templates were created from 50 ng RNA using the Transcriptor First Strand cDNA Synthesis kit (Roche), following the manufacturer's protocol. The primer pair FP112 (TGCTGAA-AAAGTGCAACAGG) and FP113 (ATCAAGCCAAGCATTCCATC) was used to investigate *hyl* expression by real-time PCR. To normalize the data, the primer pair FP116 (TGAAGAAGGTTTTCGGATCG) and FP117 (CGCTCGGGACCTACGTATTA) was used to amplify a sequence in the housekeeping 16S rRNA gene. Real-time PCR was carried out in the ABI Prism 7700 detection system (Applied Biosystems) using qPCR Mastermix for SYBR Green I (Eurogentec). The gradient thermocycling programme was set for 40 cycles at 95 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s, with an initial cycle at 95 °C for 10 min. During each cycle, the accumulation of PCR products was detected by monitoring the increase in fluorescence from double-stranded-DNA-binding SYBR green. Dissociation curves were run immediately after the last PCR cycle by plotting the fluorescence intensities against temperatures as the set point temperature (55 °C) was increased by 1 °C for 30 s (41 cycles). To exclude DNA contamination of RNA samples, replicate control assays were performed in which reverse transcriptase was omitted. Data were collected and analysed using the software and graphics program Mx4000 version 4.00 (Stratagene). Standard curves were obtained for both *hyl* and 16S rRNA genes. The relative differences in expression were analysed with the Relative Expression Software Tool (version 1.9.12) (Pfaffl *et al.*, 2002). Each assay was performed in triplicate in three independent experiments.

Hyaluronidase activity. Measurement of hyaluronidase activity was based on the colorimetric method described by Reissig *et al.* (1955), slightly modified by Asteriou *et al.* (2001). It determines the concentration of reducing β-N-acetyl-D-glucosamine ends generated from HA hydrolysis. N-Acetyl-D-glucosamine (Sigma A 8625) was used as a standard. Briefly, bacteria from the second overnight culture were transferred to 1.5 ml TSB. The supernatants from the wild-type and Hyl⁻ mutant were collected and frozen at different time points. Premixed HA solution (200 µl of 1 mg ml⁻¹ solution) containing 5 mM ammonium acetate at pH 5 was incubated with 100 µl culture supernatant at 37 °C for 1 h. The reaction was stopped by adding 50 µl borate solution, followed by vortexing and incubation in a boiling water bath for 3 min. After cooling, 1.5 ml of a 10-fold diluted p-dimethylaminobenzaldehyde (DMAB) solution was added to each tube. In this method, the N-acetyl-D-glucosamine generated from degradation of HA is converted to a furan derivative, which reacts with the DMAB to form a red complex. The colour was determined spectrophotometrically at 590 nm after 20 min incubation at 37 °C.

Each assay was performed in triplicate in three independent experiments.

Biofilm assay. Biofilm formation was assessed using the microtitre plate assay as previously described (Petersen *et al.*, 2004). *S. intermedius* wild-type and Hyl⁻ were grown in two successive overnight cultures in TSB and diluted 1:200 in TSB or TSB supplemented with HA (1 µg ml⁻¹). Then 500 µl aliquots were inoculated into wells of polystyrene flat-bottom 24-well microtitre plates (Nunc). The plates were incubated at 37 °C in a 5% CO₂ aerobic atmosphere for 18 h. The biofilms were collected from some wells after 18 h. In other wells, fresh medium with and without HA was added and biofilm formation was allowed to continue for another 18 h. To assay biofilm detachment, biofilms formed for 18 h were treated with hyaluronidase (bovine testes, Sigma, 450 U mg⁻¹, final concentration 225 U ml⁻¹) for 3 h or 18 h at 37 °C in a 5% CO₂ aerobic atmosphere. The biofilms were washed twice with distilled water and suspended in 500 µl fresh TSB by scraping the bottom and lateral walls of the wells with a disposable cell scraper adapted to fit into the wells. In separate wells, total growth was measured by collecting the biofilm and planktonic cells together in the 500 µl medium used during incubation. To determine the amount of planktonic cells, the biofilm values were subtracted from corresponding total growth (biofilm and planktonic) values, as determined by OD₆₀₀. The OD₆₀₀ values of the suspended cells were measured. Each assay was performed in triplicate in three independent experiments.

The hyaluronidase activity of the wild-type was measured in a similar assay, except that 3 ml 1:200 culture was used in each well of 6-well microtitre plates. After 18 h, 3 ml fresh medium was added and incubated for a further 18 h. The biofilm and planktonic cells were collected from the wells by scraping and centrifugation. The supernatant was used to assess the hyaluronidase activity. The *S. intermedius* Hyl⁻ mutant was included as negative control and bovine hyaluronidase at different concentrations as positive control.

To evaluate the hyaluronidase activity in biofilm cells, *S. intermedius* was grown for 18 h in TSB medium supplemented or not with HA, as described above. Biofilm cells were collected and resuspended in TSB supplemented with HA and adjusted to an OD₆₀₀ of 1. The suspension was centrifuged, resuspended to a calculated OD₆₀₀ of 10 and incubated at 37 °C in a 5% CO₂ aerobic atmosphere for 1 h. The suspension was centrifuged and the supernatant was used to assay the hyaluronidase activity.

The effect of hyaluronidase treatment on biofilm formation was visualized by scanning electron microscopy. Biofilms were grown in TSB supplemented with HA as described above, except that a polystyrene disk (Nunc) was immersed in each well before inoculation. After 18 h, biofilms were treated with hyaluronidase for 18 h. The disks were removed, rinsed with distilled H₂O, and fixed with 2.5% glutaraldehyde in 0.1 M Sørensen buffer. Dehydrated samples were obtained through a series of ethanol rinses and dried at the critical point with liquid CO₂.

Hydrophobicity assay. Surface hydrophobicity of *S. intermedius* NCTC 11324 and the isogenic mutant was measured by the hexadecane affinity method, as described by Westergren & Olsson (1983). Cells grown in TSB and TSB plus HA (1 µg ml⁻¹) were collected at early exponential phase, washed twice with phosphate-buffered saline (PBS, Sigma), pH 7.2, and resuspended to an OD₄₅₀ of 1.0. Volumes of 1.2 ml bacterial suspensions were mixed with 200, 250 or 300 µl hexadecane. The OD₄₅₀ in the aqueous phase was then measured, and the relative hydrophobicity was calculated. Each assay was performed in triplicate in three independent experiments.

Adhesion to epithelial cells. Adherence studies were performed using biotinylated bacteria and an established epithelial cell line (PE/

CA-Pj41, clone D2, European Collection of Cell Cultures, ECACC) from oral mucosa. The cells were grown in Iscove's Modified Dulbecco's Medium (Sigma) supplemented with 2 mM glutamine (Invitrogen Life Technologies), 10% fetal bovine serum (Invitrogen Life Technologies) and 1% penicillin-streptomycin-fungizone (PSF, Invitrogen Life Technologies). Cells were seeded in 24-well culture plates and incubated at 37 °C in 5% CO₂ to establish a confluent monolayer.

Biotinylation of *S. intermedius* was performed according to the modified method described by Fallgren *et al.* (2001). Briefly, *S. intermedius* wild-type and Hyl⁻ were grown for 10 h (OD₆₀₀ 0.800) in TSB, harvested by centrifugation (6000 r.p.m. for 10 min), and washed twice with PBS, pH 7.2. The bacterial OD₆₀₀ was adjusted to approximately 1. Equal volumes of bacteria and biotin solution (EZ-Link-Sulfo-NHS-LC-biotin, Pierce; 0.2 mg ml⁻¹ in PBS) were incubated for 2 h at room temperature. The streptococcal cells were washed three times with PBS.

The confluent cells were washed twice and fixed on tissue-culture wells by incubation for 30 min at 37 °C with 4% glutaraldehyde in PBS, then washed twice with PBS. Uncovered plastic surfaces were blocked with 3% BSA (Sigma) in PBS for 1 h at 37 °C. The wells were washed twice with PBS, and 100 µl biotinylated bacterial cell suspensions in PBS (OD₆₀₀ 0.7) were added to each well before incubation for 1 h at 37 °C. Unbound bacteria were then removed with PBS containing 0.05% Tween 20. NeutrAvidin (Pierce; 100 µl of 1/1500 dilution in PBS) was added to each well before further incubation for 1 h at 37 °C. The plates were then washed twice with PBS, and 100 µl ImmunoPure TMB substrate (Boule Nordic) was added to each well. The colour was allowed to develop for 5 min, and the reaction was stopped with 2 M H₂SO₄. The A₄₅₀ was measured in a Synergy HT Multi-Detection microplate reader (Biotek). Each assay was performed in triplicate in three independent experiments.

Statistical analysis. Student's *t* test was used to analyse the data normally distributed. *P* values of <0.05 were considered significant.

RESULTS

Growth and expression of hyaluronidase in the presence of HA

At stationary phase, cell density (OD₆₀₀) of the *S. intermedius* Hyl⁻ mutant was only approximately 22% of the wild-type in MM with HA as sole carbohydrate source (wild-type OD₆₀₀ 0.848, SE 0.142; Hyl⁻ mutant OD₆₀₀ 0.193, SE 0.097). No difference was observed between the wild-type and Hyl⁻ in MM supplemented with glucose (data not shown). The wild-type and Hyl⁻ had similar growth rates in TSB with or without addition of HA (data not shown).

Real-time RT-PCR was used to assess hyaluronidase gene transcription. *S. intermedius* hyaluronidase was upregulated in TSB supplemented with HA by a mean factor of 3.1 (SE range 1.16–8.3) compared to cells grown in the absence of HA.

Wild-type *S. intermedius* planktonic cells showed approximately four times more hyaluronidase activity in TSB supplemented with HA than in TSB alone (Fig. 1). The hyaluronidase accumulated during growth up to early stationary phase, whereupon the level dropped. *S.*

Fig. 1. Hyaluronidase activity of *S. intermedius* wild-type and Hyl⁻ measured during 24 h growth in TSB supplemented or not with HA. Hyaluronidase activity was normalized to cell number. The data are means and standard errors from three independent experiments.

intermedius Hyl⁻ showed no hyaluronidase activity in either the presence or absence of HA (Fig. 1). It was interesting to investigate whether biofilm cells are also able to produce hyaluronidase. Measurement of hyaluronidase activity in biofilm cells after 18 h biofilm formation showed an activity corresponding to 351 U ml⁻¹ (SE 39) in TSB supplemented with HA and 123 U ml⁻¹ (SE 28) in TSB not supplemented with HA; the differences were statistically significant.

Hyaluronidase promotes biofilm cell detachment and disaggregation

The possible involvement of hyaluronidase and HA in biofilm formation by *S. intermedius* was investigated using the microtitre plate assay and culture media supplemented or not with HA. In TSB supplemented with HA the Hyl⁻ mutant formed 31 % more biofilm after 36 h than the wild-type. In non-supplemented TSB a non-significant reduction was observed in the wild-type compared to the Hyl⁻ mutant (data not shown). Total growth, assessed as the sum of planktonic and biofilm cells, was not significantly different in any case (Fig. 2). Treatment of biofilms for 16 h with hyaluronidase reduced the biofilm mass by about 66 %, concomitant with 48 % increase in the planktonic fraction (Fig. 2). Hyaluronidase added at the time of inoculation reduced the *S. intermedius* wild-type and Hyl⁻ biofilm formation to almost the same levels as those observed with hyaluronidase added after 18 h (data not shown). Hyaluronidase treatment of established biofilms for 3 h had no significant effect on the biofilm mass (data not shown). Measurement of the hyaluronidase activity in the wells of the wild-type showed an activity of 270 U ml⁻¹ (SE 10), a value approximately the same as the amount added in the hyaluronidase detachment assay. We used scanning electron microscopy to detect possible

Fig. 2. Growth of *S. intermedius* wild-type and Hyl⁻ in TSB supplemented with HA. Cell density of biofilm (solid bars) and planktonic cells (speckled bars) in the wells after 18 h (initial biofilm, dark grey bars), 36 h without hyaluronidase treatment (initial biofilm plus 18 h incubation, black bars) and 36 h with hyaluronidase treatment (initial biofilm plus 18 h with hyaluronidase treatment, light grey bars) monitored as OD₆₀₀. Control wells not supplemented with HA and hyaluronidase were included (open bars). Biofilm cells were washed and resuspended to the same volume as the planktonic cells. The results are means and standard errors from three independent experiments.

alterations in biofilm architecture by hyaluronidase treatment. Wild-type *S. intermedius* formed very dense biofilms in TSB medium supplemented with HA. When the biofilm was treated with hyaluronidase only a thin layer of cells remained attached to the surface (Fig. 3).

Expression of hyaluronidase does not influence cell-surface hydrophobicity

Since hyaluronidase may be cell-bound through the anchor domain containing the LPXTG motif, we determined whether inactivation of *hyl* altered cell hydrophobicity compared to the wild-type when grown in TSB supplemented or not with HA. Cell hydrophobicity was determined by adsorption to hexadecane and calculated as the percentage loss in OD₄₅₀ relative to that of the initial bacterial suspension. Inactivation of the hyaluronidase-encoding gene in *S. intermedius* resulted in no significant differences in hydrophobicity compared to the wild-type at early exponential phase irrespective of HA supplementation or not (unsupplemented medium: wild-type 39 %, SE 1.49 and Hyl⁻ 33 %, SE 1.62; HA-supplemented medium: wild-type: 36 %, SE 0.66 and Hyl⁻: 37 %, SE 0.29).

Inactivation of *hyl* does not affect adherence of *S. intermedius* to epithelial cells.

We hypothesized that hyaluronidase could act as an adhesin and mediate binding of *S. intermedius* to epithelial cells. For this purpose we compared the wild-type and

Fig. 3. Scanning electron microscopy of *S. intermedius* wild-type biofilms grown for 36 h in TSB supplemented with HA, not treated (left picture) or treated with hyaluronidase for 18 h (right picture). Scale bars, 20 µm.

Hyl⁻ mutant. Adhesion of Hyl⁻ was similar to the wild-type, suggesting that hyaluronidase is not directly involved in adhesion of *S. intermedius* to epithelial cells.

DISCUSSION

In this study addition of HA to the growth media was an attempt to create a simple model reflecting the importance of HA and hyaluronidase in *S. intermedius* colonization. Both HA and hyaluronidase influenced *S. intermedius* biofilm formation, while total growth remained constant and similar in the wild-type and Hyl⁻. Interestingly, we found that despite lacking hyaluronidase the Hyl⁻ mutant formed more biofilm than the wild-type strain in TSB supplemented with HA. It seems therefore that hyaluronidase does not act as an adhesin to promote biofilm formation. Hydrophobic interactions with surfaces have been shown to be involved in adhesion, which is the first step of biofilm formation. Similar hydrophobic interactions were found in the wild-type and Hyl⁻ mutant, indicating that hyaluronidase may not be involved in initial adhesion and subsequent biofilm formation.

It has been shown that polysaccharide-degrading enzymes of microbial or phage origin may cause localized destructions, with possible weakening of the biofilm community structure and subsequent loss of both cells and macromolecules (Sutherland, 2001). Recently, the oral pathogen *Actinobacillus actinomycetemcomitans* was shown to secrete a hydrolytic enzyme that cleaves poly-β-1,6-*N*-acetyl-D-glucosamine, a polysaccharide adhesin of diverse species, leading to biofilm dispersal (Itoh *et al.*, 2005; Kaplan *et al.*, 2003). We suggest that hyaluronidase could act similarly, breaking down adhesins or extracellular matrix of *S. intermedius* biofilm and thus promote detachment. Supporting this possibility, we found that exposure of HA-grown biofilms to hyaluronidase for 18 h strongly

reduced the biofilm mass. These results, which were confirmed by scanning electron microscopy, indicate that HA formed part of the extracellular structure of the biofilm. It is well known that the composition of biofilms is influenced by the types of nutrients in the environment (Bowden & Li, 1997). In group A streptococci several genes are differentially expressed in HA-enriched media (Zhang *et al.*, 2007). We cannot exclude, therefore, that the ability of *S. intermedius* to hydrolyse HA may influence expression of factors positively or negatively associated with biofilm formation.

We studied the possible involvement of *S. intermedius* hyaluronidase in adhesion to epithelial cells, since attachment to epithelial cells may be an initial event in periodontal infection. The *S. intermedius* hyaluronidase could presumably be involved in bacterial adhesion in two ways: by functioning as an adhesin or by degrading the HA cell-surface coatings, thereby allowing direct contact between the bacterium and specific receptors on the cell surface (King *et al.*, 2004). Our data do not support such functions since no difference was found between the Hyl⁻ strain and the wild-type as to adhesion to epithelial cells. However, we have not investigated whether the epithelial cells used in this study actually produce HA. The present model might differ from conditions observed in sites in which exogenous HA (e.g. from saliva) may be present.

S. intermedius wild-type was able to utilize HA as a sole carbon source. The Hyl⁻ mutant on the other hand was incapable of using HA as energy source. In the oral cavity, the gingival crevice shows 28.2 mg HA ml⁻¹ (Smith *et al.*, 1995), against 0.46 mg ml⁻¹ in whole unstimulated saliva (Pogrel *et al.*, 1996). The higher concentration of HA in the gingival crevice may be an important ecological factor in *S. intermedius* distribution around the margins of the teeth. We showed by real-time RT-PCR that hyaluronidase is upregulated in TSB supplemented with HA. The results

were confirmed by biochemical methods. The ability to sense the environment and mount an appropriate adaptive transcriptional response may be of crucial importance for *S. intermedius* colonization and pathogenicity.

The establishment of biofilms by bacteria protects them from host defences and antibiotics. Biofilm formation is a dynamic process which involves initial attachment of cells to the surface, production of extracellular polymers, maturation, and dispersion of single cells from the biofilm. Approaches to combat biofilms may be to promote biofilm dispersal. We showed in the present study that hyaluronidase promotes *S. intermedius* detachment. It is possible that *S. intermedius* secretes hyaluronidase to detach from the surface or from other bacteria in an attempt to colonize other tissues. A better understanding of the mechanisms involved in *S. intermedius* biofilm disruption by hyaluronidase could lead to improved strategies for treatment and prevention of diseases caused by this bacterium.

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