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Identification and characterization of a salivary-pellicle-binding peptide by phage display

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ABSTRACT

Objective: Dental biofilms are associated with oral diseases, making their control necessary. One way to control them is to prevent initial bacterial adherence to the salivary pellicle and thereby eventually decrease binding of late colonizing potential pathogens. The goal of this study was to generate a salivary-pellicle-binding peptide (SPBP) with antifouling activity towards primary colonizing bacteria. In order to achieve this goal we aimed to: (i) identify novel SPBPs by phage display; (ii) characterize the binding and antifouling properties of the selected SPBPs.

Methods: A library of 2×10^9 phages displaying a random sequence of 12-mer peptides was used to identify peptides that bound selectively to the *in vitro* salivary pellicle. Three rounds of panning resulted in the selection of 10 pellicle-binding phages, each displaying a novel peptide sequence. The peptides were synthesized and their binding to the *in vitro* salivary pellicle was characterized in the presence and absence of calcium ions and Tween-20. The antifouling property of hydroxyapatite (HA) and saliva-coated HA discs treated with and without SPBPs were evaluated against *Streptococcus gordonii*.

Results: Ten unique SPBPs were identified using the phage display. One of these peptides, SPBP 10 (NSAAVRAYSPPS), exhibited significant binding to the *in vitro* salivary pellicle which was neither influenced by calcium ions, nor affected by up to 0.5% Tween-20. Its antifouling property against *S. gordonii* was significantly higher on the treated surfaces than on untreated surfaces.

Conclusions: Use of the phage display library enabled us to find a specific SPBP with antifouling property towards *S. gordonii*.

1. Introduction

In the oral cavity, the salivary proteins and peptides are selectively adsorbed to the enamel surface to form a proteinaceous film called the acquired salivary pellicle.[1,2] This pellicle is composed of peptides and (glycosylated) proteins, such as statherin, histatins, proline-rich proteins and mucins. The pellicle also contains lipids. Together these constituents make the pellicle dynamic in structure and function. Some

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domains of the salivary pellicle-forming proteins serve as receptors for adherence of early colonizing bacteria during dental-biofilm formation.[3,4]

The development of a dental biofilm is a multi-step process which begins when oral Streptococcal species, the early colonizers, recognize and adhere to moieties of proteins in the salivary pellicle.[5] One of the early colonizers, *Streptococcus gordonii*, binds to the salivary amylase as part of the salivary pellicle *via* the amylase binding protein A (ApbA) moiety on its cell wall.[6] When the pellicle is left untreated, the early colonizers aggregate with late-colonizing bacteria which finally will result in the development of a mature biofilm.

S. gordonii co-aggregates with other primary colonizer such as *Actinomyces naeslundii* and secondary colonizers such as *Porphyromonas gingivalis* which are both implicated in periodontitis.[7–9] *S. gordonii* also promotes adherence of opportunistic yeast pathogen such as *Candida albicans*.[10] Besides periodontal infection, dental biofilms are also suggested to be associated with systemic diseases and preterm births, underlining the need for their prevention and control.[11,12] Biofilms can be controlled in various ways, *e.g.* by preventing bacterial adherence to the enamel surface, or by removing established biofilms.

Oral care products currently used to control dental biofilms include antibacterial agents such as ethanol, sodium lauryl sulfate, triclosan, cetylpyridinium chloride, chlorhexidine digluconate, and nisin. Although *in vitro* studies with these antimicrobial agents on dental biofilm models showed antimicrobial efficacy, they did not completely remove the biofilms.[13]

Synthetic combinatorial technologies have been used to develop new antimicrobial peptide libraries with potential antibiofilm activity. Such a library was used to identify a decapeptide (KKVVFKVKFK), named KSL, which has a broad range microbicidal activity, and also inhibits biofilm development.[14,15] Phage display is an alternative, established method for identifying novel peptides that have the selectivity to target biomolecules, such as enzymes, cell-surface receptors and biomaterials such as hydroxyapatite (HA) and titanium.[16–18]

The goal of this study was to generate a salivary-pellicle-binding peptide with antifouling activity towards bacteria. In order to achieve this goal we aimed to: (i) to identify peptide sequences with specific selectivity to the *in vitro* salivary pellicle using the phage display library; (ii) to determine the binding characteristics of the selected peptides and evaluate their antifouling property against *S. gordonii*.

2. Materials and methods

2.1. Collection of saliva

Unstimulated human whole saliva samples were collected from 10 healthy volunteers, in tubes pre-cooled on ice. The saliva samples were vortexed for 2 min to reduce its viscosity and subsequently centrifuged (40 min, 3000 × *g*, 4 °C) to remove insoluble cellular debris. The clarified human whole saliva (CHWS) was stored in aliquots at −20 °C.[19]

2.2. Bacteria and chemicals

Escherichia coli ER2738 was maintained on Luria Bertani (LB) medium agar plates containing 10 g/l Bacto-Tryptone (Difco, Detroit, USA), 5 g/l yeast extract (Difco, Detroit, MI, USA), 10 g/l NaCl (Sigma–Aldrich, St. Louis, MO, USA), 15 g/l agar (BD, Sparks, USA) and 45 μM tetracycline (Sigma–Aldrich) under aerobic conditions at 37 °C. *S. gordonii* ATCC 35105 was maintained on brain heart infusion (BHI) agar plates containing 37 g/l BHI (BD Bioscience, USA) and agar (BD) under anaerobic (10% CO_2, 10% H_2, and 80% N_2) conditions at 37 °C. Titration and amplification of eluted phages was done using LB agar plates supplemented with 0.2 mM isopropyl-β-D thiogalactopyranoside (Sigma–Aldrich) and 0.1 mM 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal, Sigma–Aldrich).

2.3. In vitro salivary pellicle on hydroxyapatite

Prior to surface panning with the phage display peptide library, a mixture of 1 mg of HA (surface area = 40 m^2/g, Biorad, Macro-Prep ceramic hydroxyapatite, Type I, 20 μM, Hercules, CA, USA) with 0.5 ml CHWS was kept in suspension by end-over-end rotation at 37 °C for 2 h. The mixture was centrifuged at 14,000 × *g* at room temperature (RT) for 15 min, the supernatant was removed by pipetting and the saliva-coated HA sediment was washed three times with saliva buffer (2 mM K_2HPO_4, 50 mM KCl, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$, pH 6.8).

The saliva-coated HA for peptide binding assay was prepared by incubating 100 mg of HA with 3 ml of CHWS. The mixture was continuously mixed by end-over-end rotation at 4 °C for overnight, centrifuged and the saliva-coated HA was washed with saliva buffer as mentioned above.

2.4. Surface panning of in vitro salivary pellicle using phage display peptide library

The Ph.D.-12 Phage Display Peptide Library from New England Biolabs (Ipswich, MA, USA) was used to select peptides binding to HA coated with an *in vitro* formed salivary pellicle. The phage library consisted of approximately 2×10^9 independent clones with each phage displaying a unique 12-mer peptide. 1 μl of the phage library, representing 2×10^9 phages was added to 1 mg of saliva-coated HA suspended in 200 μL of saliva buffer and mixed continuously by end-over-end rotation at RT for 20 min. The suspension was centrifuged at 10,000 × *g* for 1 min and the supernatant containing unadsorbed phages was discarded. The pellet was washed five times to remove non-specifically bound phages. Washing was done with 1 ml saliva buffer containing 0.1% Tween-20 (v/v), mixed end-to-end for 5 min and centrifuged at 10,000 g for 1 min at RT. The phages adsorbed to the salivary pellicle were eluted with 1 mL of 0.2 M glycine–HCl, pH 2.2, supplemented with 1 mg/ml BSA, incubated at RT for 10 min and neutralized with 150 μl of 1 M Tris–HCl, pH 9.1. The low pH of the Glycine-HCl buffer weakens the interaction between the phage displaying peptide and the salivary pellicle target molecule, which facilitates the elution of the phages.[20]

The eluted phages were amplified by infecting 20 ml of 100 fold diluted overnight culture of *E. coli* in LB broth and