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# Host-Derived Pentapeptide Affecting Adhesion, Proliferation, and Local pH in Biofilm Communities Composed of *Streptococcus* and *Actinomyces* Species▽

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**Salivary proline-rich proteins (PRPs) attach commensal *Actinomyces* and *Streptococcus* species to teeth. Here, gel filtration, mass spectrometry and Edman degradation were applied to show the release of a pentapeptide, RGRPQ, from PRP-1 upon proteolysis by *Streptococcus gordonii*. Moreover, synthetic RGRPQ and derivatives were used to investigate associated innate properties and responsible motifs. The RGRPQ peptide increased 2.5-fold the growth rate of *S. gordonii* via a Q-dependent sequence motif and selectively stimulated oral colonization of this organism in a rat model in vivo. In contrast, the growth of *Streptococcus mutans*, implicated in caries, was not affected. While the entire RGRPQ sequence was required to block sucrose-induced pH-decrease by *S. gordonii* and *S. mutans*, the N-terminal Arg residue mediated the pH increase (i.e., ammonia production) by *S. gordonii* alone (which exhibits Arg catabolism to ammonia). Strains of commensal viridans streptococci exhibited PRP degradation and Arg catabolism, whereas cariogenic species did not. The RGRPQ peptide mediated via a differential Q-dependent sequence motif, adhesion inhibition, and desorption of PRP-1-binding strains of *A. naeslundii* genospecies 2 (5 of 10 strains) but not of *S. gordonii* ($n = 5$). The inhibitable *A. naeslundii* strains alone displayed the same binding profile as *S. gordonii* to hybrid peptides terminating in RGRPQ or GQSPQ, derived from the middle or C-terminal segments of PRP-1. The present findings indicate the presence of a host-bacterium interaction in which a host peptide released by bacterial proteolysis affects key properties in biofilm formation.**

The resident polymicrobial flora of commensal or symbiotic bacteria constitutes a powerful innate defense (11, 12). Whereas infection by only 10 *Salmonella* organisms can be lethal to germfree mice, as many as $5 \times 10^6$ organisms are needed to kill mice with a normal intestinal microflora. However, apart from adhesion and pH-related proliferation, little is known about the host factors that govern the early colonization of commensal bacteria, as well as the microbial hierarchy of biofilm formation.

The human saliva innate defense involves an array of (poly)peptides, glycoproteins, and mucins, e.g., histatins (37, 38), proline-rich proteins (PRPs) (3, 22), scavenger gp-340 (14, 39), and MUC7 (6, 34), to control colonization and infections (7, 30). Acidic PRPs, which are polymorphic and multifunctional proteins (3, 22, 30), exhibit a C-terminal PQ dipeptide for bacterial binding (17, 18) and specific regions for interactions with dietary tannins (polyphenols) or calcium. Among a multiplicity of salivary receptors (16, 40), acidic PRPs attach early colonizing oral commensal viridans-type *Streptococcus* and *Actinomyces* species to teeth (20, 23) and contribute to a microbial ecology that accommodates tooth protective streptococci, such as *Streptococcus gordonii* (4, 43). Accordingly, whereas the prevalent allelic variants of acidic PRPs (e.g., PRP-1) coincide with caries resistance and adhesion of commensal bacteria, the less prevalent Db variant coincides with caries susceptibility and the adhesion of *Streptococcus mutans* (41), a caries-associated organism capable of producing high levels of acids from sucrose. In addition, small PRP-derived peptides are abundant in saliva and coincide also with caries-prone or -resistant subjects (2).

The oral commensal flora is partly composed of bacterial communities of viridans-type members of the genus *Streptococcus* (e.g., the early colonizing *S. gordonii*) and of members of the genus *Actinomyces* (e.g., *A. naeslundii*) (13, 27). Accordingly, *S. gordonii* and *A. naeslundii* may compete for PRP-1 adhesion sites, as well as nutrients. A neutral local plaque pH is of crucial importance to microbial ecology and health (9). Acid production from sucrose (pH decrease) promotes the growth of highly acidogenic and aciduric streptococci, such as *S. mutans*, and thereby development of caries. The catabolism of arginine to ammonia has therefore been suggested as a strategy adopted by oral commensal streptococci to maintain a neutral local pH at which they grow optimally (10).

Gram-positive bacteria, such as streptococci and staphylococci, use linear or cyclic peptides as signals in cell-to-cell

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communication (15, 31), e.g., the octapeptide pheromone regulating staphylococcal density and virulence (24). In the *S. gordonii* strain Challis of oral tissue origin, genes encoding peptide pheromone receptors are present (21). Recently, we showed that oral streptococci and actinomycetes proteolytically cleave PRP-1 and that *S. gordonii* strain SK12 produces two large fragments and a postulated RGRPQ peptide (32). Synthetic RGRPQ was found to desorb bound bacteria and to block a sucrose-induced pH decrease in dental plaque in situ (32). However, little is known about the functional role(s) of this and other salivary peptides in regulating biofilm formation or innate protection.

The aim of the present study was to investigate innate roles and determinants of the RGRPQ peptide in commensal biofilm formation and caries protection, as well as to chemically verify the release of the RGRPQ peptide from the PRP-1 polypeptide. The RGRPQ peptide influenced bacterial proliferation in vitro and in vivo, as well as adhesion and local pH in vitro. The present study shows an example of a host-bacterium interaction that results in the release of a peptide affecting three key components of biofilm formation: proliferation, adhesion, and local pH.

## MATERIALS AND METHODS

**Bacterial strains.** *Streptococcus* and *Actinomyces* strains were of defined origin (20, 27, 33) and grown overnight on Columbia-II-agar base plates (Becton Dickinson and Company, Cockeysville, MD), supplemented with 30 ml of a human erythrocyte suspension per liter, at 37°C in an atmosphere with 5% $CO_2$. Strains intended for hydroxyapatite adhesion tests were metabolically labeled by adding [$^{35}$S]methionine to the growth medium (33).

**Peptides.** The peptides RGRPQ, AGAPQ, AARPQ, GGRPQ, RGAPQ, RGRAA, RGRPA, RGRAQ, KAKVN, GQSPQ, and GRPQG were synthesized, purified by using high-pressure liquid chromatography, and characterized by mass spectrometry (Biomolecular Resource Facility, University of Lund, Lund, Sweden). AGRPQ was not possible to synthesize. ProGln (PQ) was from BACHEM AG, Switzerland, and the other peptides were from Sigma Genosys, United Kingdom. Hybrid peptides DzzEEKFLRRIGRFGPRGRPQ (z means L-phosphoserine) and DzzEEKFLRRIGRFGPGQSPQ were synthesized, purified by using high-pressure liquid chromatography, and characterized by mass spectrometry (Mimotopes Pty Ltd., Clayton, Victoria, Australia). The peptides were dissolved in sterile water, the pH was adjusted to 6.8 to 7.0, and aliquots were frozen at −20°C. The RGRPQ peptide for in vivo testing was from Thermo Electron GmbH, Germany.

**Proliferation.** Bacterial cells were cultured consecutively in a chemically defined medium (44) at 37°C in an atmosphere with 5% $CO_2$. The second culture, used as an inoculum (1%, 1/100 μl [inoculum/medium]), was grown for 14 h. The inoculum (an $A_{550}$ of 0.03 corresponding to $8 \times 10^7$ cells/ml) was added to a minimal growth medium (supplemented with peptide or control) obtained by dilution of the chemically defined medium (with all 20 naturally occurring free amino acids) eight times and filter sterilization in sterile 96-well microtiter plates (Millipore). The wells were incubated at 37°C in a 5% $CO_2$ atmosphere. Bacterial numbers were recorded every hour by measuring the absorbance at 550 nm ($A_{550}$) using a Spectra MAX 340 spectrometer (Molecular Devices, Sunnyvale, CA). The absorbance values which were used to calculate the generation time, were linearly proportional to the number of bacteria measured by viable counting.

**pH increase (ammonia production).** An overnight bacterial culture was washed once in 10 ml of sterile water, adjusted to $4.8 \times 10^9$ cells/ml with water, and kept on ice for a minimum of 30 min before use (46). The bacterial cell suspension (90 μl) was mixed with test peptide and sterile water to a final volume of 100 μl and a final peptide concentration of 0.5 mM. The test and control suspensions were incubated at 37°C aerobically, and the pH was measured every 30 min for the 330-min test period by using a pH electrode.

**Sucrose-induced pH decrease (acid production).** To measure bacterial metabolic responses to peptides in the presence of sucrose, bacteria were grown on agar plates, harvested, and washed once in 10 ml of starvation solution [20 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol-HCl, 35 mM NaCl, 62 mM KCl, 2 mM $MgSO_4$, 1 mM $K_2HPO_4$ (pH 7.0)]. After centrifugation, the cell pellet was suspended in 10 ml of the same solution, followed by incubation at 37°C for 1 h in an atmosphere with 5% $CO_2$ for starvation (42). Pelleted bacteria were resuspended in KCl solution (50 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$) to $2 \times 10^9$ cells/ml and kept on ice until use. Bacterial cells (45 μl) were mixed with the test peptide, and KCl solution was added to give a final volume of 97 μl at a final peptide concentration of 2 mM (*S. gordonii* SK12) or 5 mM (*S. mutans* Ingbritt). The pH was measured immediately as the starting pH (0 min). Sucrose solution (3 μl, 1 M) was added to each well, the mixtures and a control cell suspension were incubated at 37°C aerobically, and the pH was measured every 10 min during the initial 30-0min test period and every 30 min during the subsequent 120-min test period by using a pH electrode.

**Adhesion and adhesion inhibition.** The adhesion of $^{35}$S-labeled bacteria to hybrid peptides (19) coated onto hydroxyapatite beads was measured essentially as described previously (5-mg beads, $5 \times 10^8$ cells/ml, and 1 h of adhesion) (33). Adhesion to PRP-1 for the adhesion inhibition experiments was performed similarly except for the use of 8-mg beads, $2 \times 10^8$ cells/ml, and 30 min of adhesion. Inhibition of adhesion was done by preincubation of cells with peptides for 20 min at room temperature prior to the adhesion assay.

**Desorption (reversal of aggregates of bacteria and PRP-1–beads).** Aggregation of bacteria by PRP-1-coated latex beads was carried out as described previously (32), except for the use of bacterial suspensions of $6 \times 10^9$ cells/ml. Bovine serum albumin-coated latex beads served as a control. Desorption of strain T14V bound to PRP-1-coated beads was measured by adding peptide (5.06 mM) to preformed aggregates of bacteria and PRP-1–beads and visual scoring of the aggregates after 1 min of incubation at scores from 0 to 5 (5, no visible aggregates; 4, small aggregates in unclear solution; 3, moderate aggregates in unclear solution; 2, moderate aggregates in clear solution; 1, large aggregates in clear solution; and 0, clumping of aggregates in clear solution). Aggregation of bacteria by PRP-1-coated beads in the absence or presence of RGRPQ peptide used the same scoring but in the reversed order (e.g., 0 marks no visible aggregates and 5 indicates clumping of aggregates in clear solution; see Fig. 4A).

**Identification of RGRPQ released by bacterial proteolysis of the PRP-1 polypeptide.** *S. gordonii* SK12 was cultured on agar plates and incubated in starvation solution at 37°C for 1 h in 5% $CO_2$. After centrifugation, pelleted cells ($2 \times 10^9$ cells/ml, 75 μl) were incubated with PRP-1 (5 mg/ml) in M-DIL buffer (0.43% NaCl, 0.042% KCl, 0.1% $Na_2HPO_4$, 0.1% $KH_2PO_4$, 1% glycerophosphate disodium salt, 0.024% $CaCl_2$, 0.01% $MgCl_2 \cdot H_2O$) for 2 h. After centrifugation, the supernatant (200 μl) was subjected to gel filtration (Superdex-peptide-column HR 10/30; Pharmacia) in 20 mM Tris-HCl–500 mM NaCl (pH 8.0). The flow rate was 0.5 ml/min, and the absorbance was monitored at 214 nm. Purified PRP-1 (50 μg) and synthetic RGRPQ peptide (100 mM, 5 μl) were run in parallel as reference substances.

**Mass spectrometry and Edman degradation.** Nano-electrospray mass spectrometry was carried out with a Q-Tof instrument (Micromass/Waters) operated as described previously (25) and Edman degradation with a Procise Ht sequencer instrument (Applied Biosystems) operated according to the manufacturer's recommendations. For mass spectrometry, 1 to 2 μl of the peptide fraction (500 μl) was used without prior desalting and mixed with an equal volume of spraying solvent (60% acetonitrile containing 1% acetic acid). For N-terminal sequence analysis, 60 μl of the fraction was applied to a Biobrene precycled glass fiber filter disc.

**PRP degradation.** PRP degradation was measured essentially as described previously (32). Briefly, PRP-1 (0.2 mg/ml) was coincubated with bacteria ($2 \times 10^9$ cells/ml) at 37°C for 20 h, followed by native alkaline polyacrylamide gel electrophoresis. The degree of cleavage was scored from 0 to 5 by densitometry (0 = 0 to 10% [negative activity], 1 = 10 to 20% [low activity], 2 = 20 to 40% and 3 = 40 to 60% [moderate activity], 4 = 60 to 80%, and 5 = 80 to 100% [high activity] loss of acidic PRP-1).

**In vivo rat model.** Sprague-Dawley rats (Møllegard Breeding Laboratories, Ejby, Denmark), 21 days old, were desalivated at day 1 by surgery as described previously (8). The rats were desalivated at day 1 and infected at days 2 and 3 by swabbing the oral cavity after dipping the swab in a bacterial suspension of *S. gordonii* SK12 and *S. mutans* NG8 ($2 \times 10^9$ and $1 \times 10^9$cells, respectively) supplemented with peptide (5 mg/ml) or control. Each animal was given orally RGRPQ peptide (4 mg/day) or sterile water four times daily ($4 \times 100$ μl, 10 mg of peptide/ml) from days 4 to 21. At day 9, all rats were reinfected with *S. gordonii* with peptide (5 mg/ml) or control. The animals received a ground diet supplemented with 56% sucrose and sterile-distilled water. The rats were screened for bacterial growth at days 4, 11, 18, and 21 by using oral swabs, and subsequent dilutions plated on MS (*S. gordonii*) and MSB (*S. mutans*) agar plates were used to establish the bacterial numbers (CFU). All animal experiments were approved

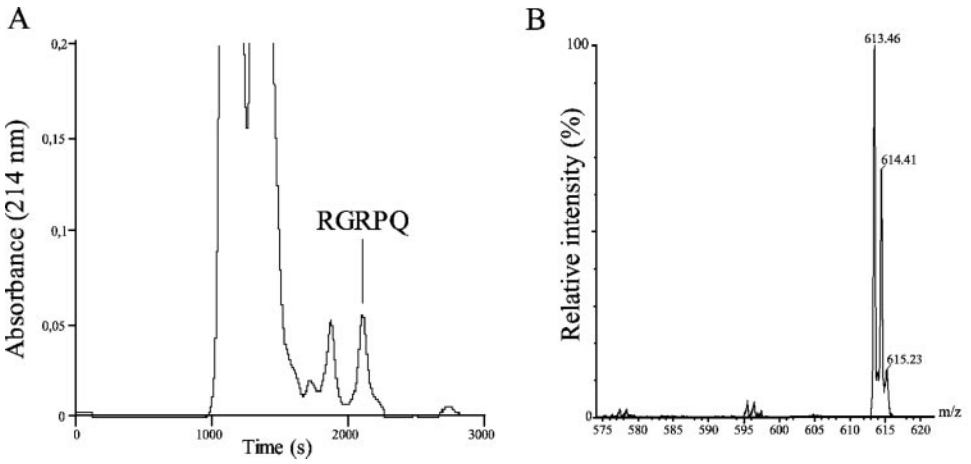


FIG. 1. Identification of a pentapeptide, RGRPQ, derived from proteolysis of PRP-1 by *S. gordonii* SK12. (A) Isolation of the RGRPQ peptide (marked) by gel filtration of the PRP-1 proteolysis mixture. Synthetic RGRPQ was used as a reference to localize the RGRPQ peptide. (B) Nano-electrospray mass spectrometry of the RGRPQ peptide recovered from gel filtration reveals the expected monoisotopic mass 613.46 Da. (C) Edman degradation of the fraction from gel filtration reveals the sequence RGRPQ.

by the local ethical committee of the Karolinska Institutet at Huddinge University Hospital, Huddinge, Sweden.

**Statistics.** Data are summarized as means and standard errors of the mean (SEM) or as median values. Unpaired *t* tests are used for two-group comparisons of peptide measurements referred to in results. For the analyses of the measurements of CFU displayed in Fig. 3, the Wilcoxon rank sum test was used for comparisons between pairs of groups at each time point. For an overall comparison between groups, a derived variable of the total number of CFU across the different time points within each rat is used. All tests are two-sided, and the significance level was set at $P < 0.05$.

## RESULTS

**Proteolytic release of RGRPQ from the PRP-1 polypeptide.** Release of the RGRPQ peptide from the PRP-1 polypeptide after proteolysis by *S. gordonii* SK12 was shown in three modes. First, a peptide component eluted in the same position as synthetic RGRPQ upon gel filtration of the proteolysis products (Fig. 1A). Second, the purified peptide revealed the monoisotopic mass 613.46 Da, which corresponds to the singly protonated RGRPQ peptide (Fig. 1B). Third, its sequence, RGRPQ, was demonstrated by Edman degradation (Fig. 1C).

**Stimulation of proliferation of *S. gordonii* in vitro by RGRPQ and related peptides.** We investigated the ability of RGRPQ and Ala substituted peptides to stimulate the growth rate or proliferation of *S. gordonii* in a minimum growth medium containing free amino acids (1:20 peptide versus free amino acids) (Table 1 and Fig. 2A). A 2.5-fold-increased growth rate occurred for *S. gordonii* at 150 μM RGRPQ compared to the minimum growth medium (the generation time was reduced from 4.65 to 1.85 h), whereas *S. mutans* was not affected (data not shown). The growth rate effect by RGRPQ on *S. gordonii* was dose dependent at peptide concentrations between 25 and 250 μM and optimal at 150 μM.

Pentapeptides ending in a Q residue increased the growth rate efficiently, whereas those without a C-terminal Q residue did not (mean $A_{550}$ of 0.060 versus 0.043, $P < 0.0001$). Ala substitutions (i.e., RARPQ and AARPQ), but not a glycine substitution (i.e., GGRPQ), located in the N-terminal segment reduced the activity, suggesting an extended and C-terminal Q

TABLE 1. Influence of RGRPQ-related peptides on growth and $NH_3$ production (*S. gordonii*), acid production (*S. mutans*), and adhesion inhibition/desorption (*A. naeslundii*)

| Peptide[a] | *S. gordonii* SK12 | | *S. mutans* | *A. naeslundii* T14V | |
|---|---|---|---|---|---|
| | Growth/fold increase[b] | $NH_3$ production[c] | Ingbritt acid production[d] | Adhesion inhibition (%)[e] | Desorbing activity[f] |
| +RGRPQ | 2.5 | 7.7 | 6.2 | 74 | 4 |
| −RGRPQ (control) | 1.0 | 6.2 | 4.5 | 0 | 0 |
| RGAPQ | 2.7 | 7.8 | 5.0 | 75 | 4 |
| RGRAQ | 2.5 | 7.2 | 4.8 | 60 | 4 |
| AGAPQ | 2.3 | 6.1 | 5.5 | 55 | 4 |
| RARPQ | 1.9 | | | 56 | 2 |
| AARPQ | 1.8 | 5.9 | 4.8 | 21 | 3 |
| AGRPA | 1.4 | | | 13 | 0 |
| RGRPA | 1.2 | 7.7 | 4.5 | 7 | 1 |
| RGRAA | 1.1 | 7.3 | | 13 | 2 |
| GGRPQ | 2.7 | 6.1 | 5.0 | 74 | 4 |
| GQGPPQ | 2.6 | | | 24 | 0 |
| GRPQG | 2.4 | | | 8 | 0 |
| GQSPQ | 2.3 | 6.4 | | 46 | 3 |
| KAKVN | 1.3 | 6.5 | | 10 | 0 |
| RGQRP | 1.8 | | | 13 | 0 |

[a] Effect of RGRPQ on various bacterial properties compared to buffer solution (−RGRPQ, control). Data are means from triplicate (column 2) or duplicate (column 3) measurements of single experimental runs or from single measurements of repeated runs (columns 4 to 6). All assays were repeated at least three times with similar results. SEM values for repeated experimental runs are given in footnotes *b* to *f* for each assay.

[b] Bacterial growth was estimated by measurements of cell numbers (Abs550) after culture in a minimum growth medium in the presence or absence (control) of 150 μ M peptide. The fold increase in cell numbers at stationary phase (12 h) is given for each peptide. SEM = ±(0.026 to 0.056)-fold increase.

[c] $NH_3$ production was indirectly measured by the pH unit obtained after incubation of an *S. gordonii* SK12 cell suspension in the presence or absence (control) of 0.5 mM peptide for 330 min. The resultant pH unit is given for each peptide and the control. SEM = ±(0.047 to 0.24) pH units.

[d] Final pH after incubation of *S. mutans* with 30 mM sucrose in the presence or absence (control) of 30 mM peptide for 150 min. The final pH units are given for each peptide. SEM = ±(0.085 to 0.49) pH units.

[e] Percent inhibition of the adhesion of *A. naeslundii* T14V to PRP-1-coated hydroxyapatite beads by preincubation of the bacteria with 10 mM peptide or control (0% inhibition). Strain T14V binds avidly to PRP-1 in the absence of peptide (mean 87% adhering of added bacteria). SEM = ±(0.5 to 6.23)%.

[f] Degree of desorption of aggregates of bacteria and PRP-1-coated latex beads in the presence or absence (control) of 5.06 mM peptide. The degree of desorption was measured using a 0 to 5 score scale (ranging from the clumping of aggregates in clear solution to no visible aggregates, respectively). SEM = ±(0.0 to 0.33) score units.

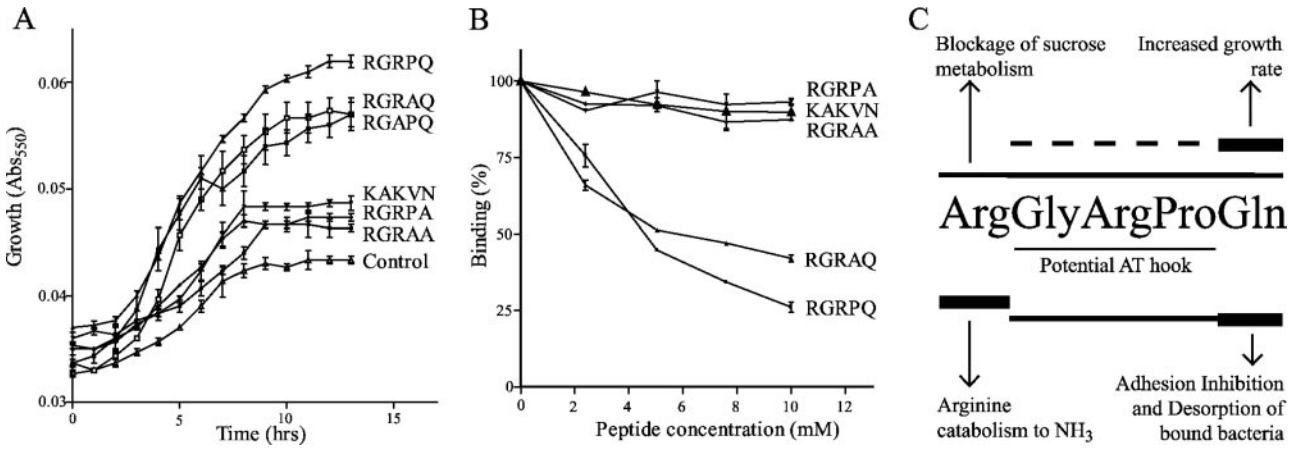


FIG. 2. Illustration of RGRPQ innate properties and tentative motifs responsible for each property. (A) Effect of RGRPQ derivatives on the growth of *S. gordonii* SK12 when cultured in a minimum growth medium in the presence or absence (control) of peptide. The bacterial cell numbers were estimated from measuring the $A_{550}$ of the cell culture. The mean absorbance values and the SEM from one experimental run with triplicate determinations are shown. (B) Effect of RGRPQ derivatives on the adhesion (percent binding bacteria out of total added) of *A. naeslundii* T14V to PRP-1-coated hydroxyapatite beads. (C) RGRPQ innate properties and tentative RGRPQ motifs for each property. Lines indicate each tentative motif with suggested key determinants marked by a broadened boldface line. The proliferation XXXQ motif is marked by a dotted line to emphasize its unique nature compared to the XXXQ adhesion motif. The potential AT hook marks the GRP motif present in some transcriptional factors.

motif (e.g., XXXQ). Moreover, PRP-1-derived peptide sequences with a terminal or internal Q terminus, such as GQSPQ (C terminus) and PQGPPQ (repetitive segment) or GRPQG (internal segment), also stimulated the proliferation of *S. gordonii* effectively (Table 1).

**Stimulation of proliferation of *S. gordonii* in vivo by the RGRPQ peptide.** The ability of the RGRPQ peptide to selectively stimulate oral colonization of *S. gordonii* in vivo was evaluated by using a rat colonization model (Fig. 3). Rats were coinoculated orally with *S. gordonii* SK12 and *S. mutans* NG8 and orally administrated peptide (4 mg/day, $n = 14$ rats) or placebo (sterile water, $n = 14$ rats). The RGRPQ peptide, but not placebo, promoted strong selective colonization of *S. gordonii* in vivo, *whereas S. gordonii* in the absence of peptide was strongly suppressed under the favorable conditions provided for *S. mutans* by the desalivation and 56% sucrose diet. The Wilcoxon rank-sum $P$ values between RGRPQ peptide and placebo were $P = 0.0001$ at day 4 and $P = 0.0006$ for day 11 and $P = 0.0003$ for the total counts summed across time points.

**Effects on local pH by the RGRPQ peptide.** We next investigated potential mechanisms for the ability of the RGRPQ peptide to block a sucrose-induced pH decrease in oral biofilms in situ (32) (Table 1). When added to *S. gordonii* SK12 (which exhibits the catabolism of arginine to ammonia), peptides with an N-terminal arginine increased the pH, whereas peptides without this characteristic did not (mean pH 7.28 versus 6.35, $P < 0.0001$). Moreover, peptides with Ala substitutions located in the C-terminal segment or at the internal Arg residue affected the pH marginally, if at all, suggesting the N-terminal Arg as the responsible motif for ammonia produc-

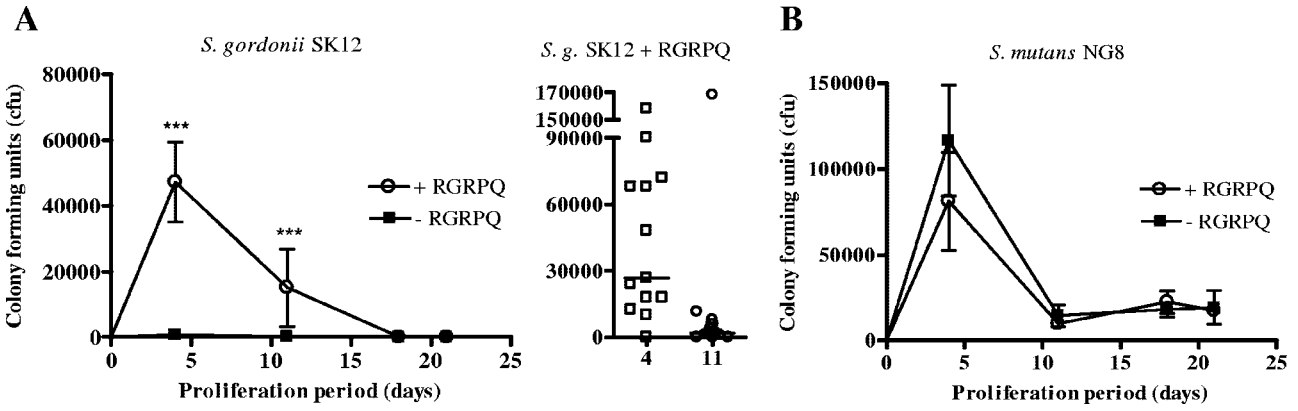


FIG. 3. (A and B) Promotion of colonization of *S. gordonii* SK12 (A), but not of *S. mutans* NG8 (B), by oral administration of RGRPQ peptide (○) compared to control (sterile water ■) in a rat model. Rats ($n = 14$ peptide group, $n = 14$ control group) were coinfected with *S. gordonii* SK12 and *S. mutans* NG8 at the start of the study (day 0). Bacterial numbers (in CFU) are presented as means of the 14 animals in each group, and bars show the SEM. Asterisks indicate statistically significant differences at given time points (***, $P < 0.001$) based on comparisons between pairs of groups at each time point using the Wilcoxon rank-sum test. Also shown in panel A are the median and the individual *S. gordonii* CFU values for the RGRPQ-treated rats at the 4- and 11-day time points.

TABLE 2. Proteolysis of PRP-1 by commensal and cariogenic streptococci

| Species | No. of strains | No. of strains with PRP-1 proteolysis[a] | | | | Arg catabolism[b] | Acidogenicity score[c] |
|---|---|---|---|---|---|---|---|
| | | High | Mod | Low | Neg | | |
| Strictly commensal | | | | | | | |
| *S. gordonii* | 7 | 6 | 0 | 0 | 1 | + | 1 |
| *S. sanguinis* | 5 | 3 | 0 | 2 | 0 | + | 1 |
| *S. parasanguinis* | 5 | 3 | 0 | 0 | 2 | + | 1 |
| *S. crista* | 1 | 1 | 0 | 0 | 0 | + | 1 |
| Potentially cariogenic | | | | | | | |
| *S. oralis* | 11 | 0 | 0 | 7 | 4 | − | 2 |
| *S. mitis* | 10 | 0 | 0 | 1 | 9 | − | 2–3 |
| Highly cariogenic | | | | | | | |
| *S. mutans* | 5 | 0 | 0 | 0 | 5 | − | 3 |
| *S. sobrinus* | 1 | 0 | 0 | 0 | 1 | − | 3 |

[a] Number of strains with different degrees (high, moderate or low) of proteolysis or no proteolysis (negative).
[b] Active (+) or inactive (−) in the formation of ammonia from arginine (13).
[c] High (score = 3), moderate (score = 2), and low (score = 1) ability to produce organic acids from sucrose (13).

tion. In contrast, incubation of peptides with *S. mutans* Ingbritt (which lacks catabolism of arginine) did not increase the pH (data not shown). Moreover, the RGRPQ peptide blocked pH decrease (an indirect measure of acid production) in both *S. mutans* Ingbritt and *S. gordonii* SK12 after challenge with sucrose (Table 1). Other peptide sequences displayed markedly lower blockage of acid production compared to RGRPQ for *S. mutans* (mean pH 5.1 versus 6.1, $P = 0.0145$) and for *S. gordonii* (mean pH 5.6 versus 6.3, $P = 0.0387$). Thus, the ability of the full-length RGRPQ peptide to block a sucrose-induced pH decrease in oral biofilms in situ may be a function of both ammonia production and the blockage of acid production by viridans streptococci.

**PRP degradation and ammonia production are typical properties of commensal viridans streptococci.** A panel of oral *Streptococcus* species of defined arginine catabolism and cariogenic properties (13) was tested for their ability to cleave PRP-1 (and the potential release of RGRPQ) by incubation with PRP-1 and native alkaline gel electrophoresis (Table 2). A high degree and frequency of PRP-1 proteolysis occurred among strains of strictly commensal streptococci (e.g., *S. gordonii* and *S. sanguinis*) with arginine catabolism and low acidogenicity, whereas the opposite was observed for highly (e.g., *S. mutans*) and potentially (e.g., *S. mitis*) cariogenic streptococci without arginine catabolism but with high acid production (Table 2).

**Adhesion inhibition and desorption of *A. naeslundii* T14V by the RGRPQ peptide.** We tested RGRPQ and structural analogues thereof for their ability to inhibit the adhesion of *A. naeslundii* T14V to PRP-1 coated onto hydroxyapatite beads, and to detach this strain from PRP-1-coated latex beads (Table 1 and Fig. 2B). High-to-moderate inhibitory or desorbing activity occurred with peptides ending in a Q residue, whereas peptides without a Q residue had virtually no activity (mean inhibition of 54% versus 11%, $P < 0.0001$, mean desorption of 2.6 versus 0.3, $P < 0.0001$). In addition to Ala substitutions at the C-terminal Q residue, substitution of the P or G residues (RGRAQ and RARPQ/AARPQ, respectively) reduced the inhibitory activity. Moreover, the previously delineated dipeptide PQ lacked inhibitory and desorbing activity (data not shown), and the GGRPQ peptide showed equal activity to RGRPQ. Thus, an XXXQ adhesion motif with a dependence on both glycine and proline (i.e., GXPQ) may be at work.

***Actinomyces* strains using the same PRP-1 binding sites as *S. gordonii* are inhibited by RGRPQ.** Oral commensal (e.g., *S. gordonii* and *A. naeslundii*), as well as pathogenic, bacteria may compete for PRP-1 binding sites. We therefore investigated the ability of the RGRPQ peptide to selectively inhibit the adhesion of a panel of *A. naeslundii* genospecies 2 ($n = 10$) and *S. gordonii* ($n = 5$) strains (including strain SK12) with avid binding to PRP-1 coated onto hydroxyapatite beads. The RGRPQ peptide blocked the adhesion of half of the *A. naeslundii* strains ($n = 5$), but none of the *S. gordonii* strain (Fig. 4). We next used hybrid peptides terminating in PRP-1 epitopes (RGRPQ and the C-terminal GQSPQ) to delineate potential PRP-1-binding sites for *A. naeslundii* T14V (inhibitable by RGRPQ), *A. naeslundii* LY7 (noninhibitable by RGRPQ), and *S. gordonii* Blackburn (noninhibitable by RGRPQ) (Fig. 4). Whereas the inhibitable *A. naeslundii* T14V and *S. gordonii* Blackburn bound avidly to both peptides, *A. naeslundii* LY7 showed avid binding only to the GQSPQ peptide. Thus, the RGRPQ peptide selectively inhibits *Actinomyces* strains that use the same PRP-1 binding sites as *S. gordonii*, which may differ by having a low-affinity binding mode insensitive to free peptides.

## DISCUSSION

The present study shows an example of host-microbe symbiosis or commensalism in which *S. gordonii*, an early colonizing oral commensal bacterium, uses proteolysis of salivary PRP-1 to generate a pentapeptide, RGRPQ, which affects key components of biofilm formation (i.e., adhesion, proliferation, and local pH). The RGRPQ peptide stimulates the proliferation of *S. gordonii* and stabilizes a neutral pH for optimal growth of this organism; it also desorbs competing *Actinomyces* bacteria from PRP-1. This suggests the RGRPQ peptide as a host-derived factor guiding the microbial hierarchy of oral biofilm formation and potentially as a prebiotic molecule. Moreover, the stimulation of *S. gordonii* colonization in rats by the RGRPQ peptide suggests effects on biofilm formation in vivo. Such effects could be beneficial to oral ecology or point to applications to support the implantation of *S. gordonii* to interfere with cariogenic microorganisms (28, 45) or to apply vaccinations with *S. gordonii* vectors (35). Other small peptide candidates to combat infectious diseases are the tripeptide inhibiting streptococcal infections in vivo (5), the P1025 peptide inhibiting *S. mutans* adhesion and colonization (26), and the antifungal peptides derived from histatins or MUC7 (6, 36).

The present findings also suggest specific RGRPQ motifs involved in affecting adhesion, proliferation, or pH (Fig. 2C). Whereas the blockage of a sucrose-induced pH-decrease in *S. gordonii* and *S. mutans* required the entire RGRPQ sequence, proliferation by *S. gordonii* and adhesion inhibition of *Actinomyces* seemed to involve differential XXXQ motifs with highly crucial Q residues. Their differential natures are illustrated by

A

| Species | PRP-adhesion -RGRPQ | +RGRPQ |
|---|---|---|
| *A. naeslundii* gsp 2 | | |
| strain T14V | 5 | 0 |
| strain (n=4) | 4-5 | 1-2 |
| strain LY7 | 5 | 5 |
| strain (n=4) | 5 | 5 |
| *S. gordonii* | | |
| strain Blackburn | 5 | 4 |
| strain (n=6) | 4 | 4 |

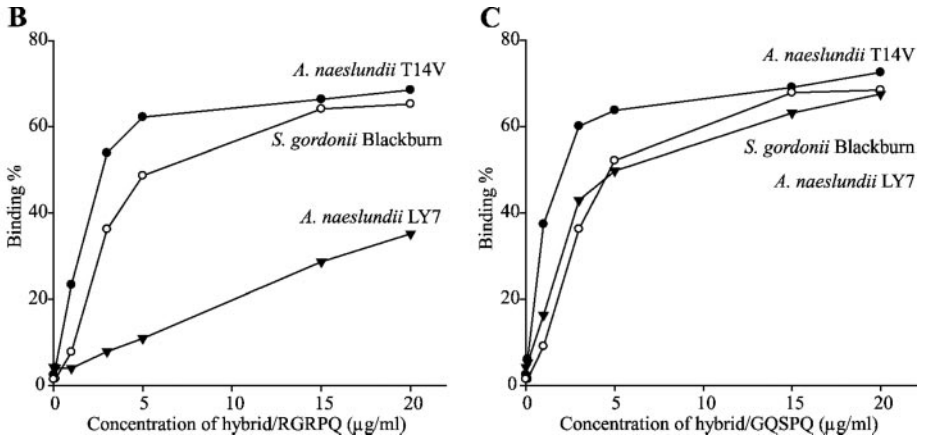


FIG. 4. (A) Selective inhibition of the adhesion of certain *A. naeslundii* genospecies 2 strains by the RGRPQ peptide. Adhesion was determined by measuring the aggregation of the bacteria with PRP-1-coated latex beads in the presence (+RGRPQ) or absence (−RGRPQ) of peptide. Score 5 indicates clumping of aggregates in clear solution, and score 0 indicates no aggregates (see Materials and Methods). (B and C) Adhesion of *A. naeslundii* and *S. gordonii* strains to hybrid peptides with a RGRPQ or GQSPQ terminus. Adhesion (percent binding bacteria of total added bacteria) of $^{35}$S-labeled *A. naeslundii* strains T14V and LY7 and *S. gordonii* Blackburn to a hybrid/RGRPQ peptide (B) or a hybrid/GQSPQ peptide (C) coated onto hydroxyapatite beads. The hybrid peptides are designed with the calcium binding 15-amino-acid N-terminal segment of statherin and a proline residue followed by RGRPQ or GQSPQ, derived from the middle or C-terminal segments of PRP-1, respectively.

the fact that proliferation allowed both an internal Q residue and broader sequence variations. Although the G and P residues of the RGRPQ peptide may play direct or indirect roles in bacterial binding, a more powerful SMD and QSAR approach is necessary to resolve how amino acids 2 to 4 (i.e., GRP segment) contribute to activity. In contrast, the production of ammonia—as inferred from a pH increase in strains with Arg catabolism—seemed to depend on the simple R residue at the N terminus. Hypothetically, the RGRPQ peptide may inhibit sugar metabolism (sucrose-induced pH decrease) in *S. gordonii* and *S. mutans* through general mechanisms, whereas proliferation, adhesion, and a pH increase may involve species-specific receptors or characteristics (e.g., Arg catabolism). Although further studies are required to explore the modes of action of the RGRPQ peptide, it is noteworthy that ERGMT and ARNQT pentapeptides are part of a signaling system in *Bacillus subtilis* that uses unspecific oligopeptide permeases and intra- or extracellular receptors to affect gene expression. Although the *S. gordonii* strain Challis contains genes for peptide signaling (21) and the GRP segment of the RGRPQ peptide is an AT-hook motif found in some transcriptional factors (1), further studies are required to explore the possibility of the RGRPQ peptide mimicking natural peptide signals between oral viridans streptococci. If so, the RGRPQ peptide could induce a more rapid growing bacterial phenotype. However, it cannot be excluded that the RGRPQ peptide acts as a nutrient or stimulates the uptake of amino acids for growth or inhibits the uptake of sugars in the blockage of acid production.

Bacterial proteolysis of salivary PRPs may, similar to casein degradation by lactococci (29), release a network of peptides that favors oral commensal bacteria and inhibits pathogens. In this respect, it is noteworthy that the PRP-1 polypeptide, from which the RGRPQ peptide is released, may constitute a caries resistance factor or allele, whereas the proposed caries susceptibility Db allele encodes a protein with a 21-amino-acid insert close to the RGRPQ cleavage site. It is striking that the RGRPQ peptide carries information for proliferation, adhesion, and pH, all of which are key components in biofilm formation. Consequently, future studies on host-bacterium commensalism related to salivary PRPs or other innate polypeptides may uncover disease mechanisms and suggest strategies to interfere with biofilm formation and protect against bacterium-related diseases.

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