

Strains of *Actinomyces naeslundii* and *Actinomyces viscosus* Exhibit Structurally Variant Fimbrial Subunit Proteins and Bind to Different Peptide Motifs in Salivary Proteins

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Received 6 July 1998/Returned for modification 6 October 1998/Accepted 28 January 1999

Oral strains of *Actinomyces* spp. express type 1 fimbriae, which are composed of major FimP subunits, and bind preferentially to salivary acidic proline-rich proteins (APRPs) or to statherin. We have mapped genetic differences in the *fimP* subunit genes and the peptide recognition motifs within the host proteins associated with these differential binding specificities. The *fimP* genes were amplified by PCR from *Actinomyces viscosus* ATCC 19246, with preferential binding to statherin, and from *Actinomyces naeslundii* LY7, P-1-K, and B-1-K, with preferential binding to APRPs. The *fimP* gene from the statherin-binding strain 19246 is novel and has about 80% nucleotide and amino acid sequence identity to the highly conserved *fimP* genes of the APRP-binding strains (about 98 to 99% sequence identity). The novel FimP protein contains an amino-terminal signal peptide, randomly distributed single-amino-acid substitutions, and structurally different segments and ends with a cell wall-anchoring and a membrane-spanning region. When agarose beads with CNBr-linked host determinant-specific decapeptides were used, *A. viscosus* 19246 bound to the Thr₄₂Phe₄₃ terminus of statherin and *A. naeslundii* LY7 bound to the Pro₁₄₉Gln₁₅₀ termini of APRPs. Furthermore, while the APRP-binding *A. naeslundii* strains originate from the human mouth, *A. viscosus* strains isolated from the oral cavity of rat and hamster hosts showed preferential binding to statherin and contained the novel *fimP* gene. Thus, *A. viscosus* and *A. naeslundii* display structurally variant *fimP* genes whose protein products are likely to interact with different peptide motifs and to determine animal host tropism.

Colonization of host surfaces by commensal and pathogenic bacteria depends on bacterial adhesive and metabolic activities. *Actinomyces naeslundii* and *Actinomyces viscosus* form considerable portions of the microflora on oral surfaces in humans and in animal species like the rat and hamster, respectively. Adhesion of *A. naeslundii* (*A. naeslundii* genospecies 1 and 2 and *A. viscosus* serotype II, according to current taxonomy [28]) involves two distinct fimbriae, type 1 and type 2, while less is known about adhesive properties in *A. viscosus* (*A. viscosus* serotype I [28]) (6, 7, 20, 49). Type 1 fimbriae promote a protein-protein interaction with tooth-adsorbed salivary acidic proline-rich proteins (APRPs) and statherin (13, 15), while type 2 fimbriae bind to β -linked galactose and galactosamine structures on epithelial and bacterial cell surfaces (18, 34, 52, 56). Type 1 fimbriae are consequently thought to participate in early plaque development, and type 2 fimbriae are thought to participate in late plaque development and colonization of the oral mucosa (6, 7, 12, 18, 49, 50, 56). Thus, expression of type 1 and type 2 fimbriae and their variant binding types (20, 49) enables *A. naeslundii* to establish distinct intraoral colonization niches (6, 11, 18).

The genes encoding the major subunits of *A. naeslundii* type 1 and type 2 fimbriae have been cloned and sequenced from genospecies 2 (strain T14V) and genospecies 1 (ATCC 12104^T), respectively (58–60). The type 1 (*fimP*) and type 2 (*fimA*) fimbrial subunit genes encode structural FimP and FimA subunit proteins of 533 and 534 amino acids, respectively, that have 34% amino acid identity. Both subunit pro-

teins contain an amino-terminal signal peptide, seven conserved proline-containing regions, and a carboxy-terminal membrane-spanning domain in close proximity to a cell wall-anchoring LPXTG sequence (60). Moreover, the *fimP* gene is present with a cluster of six additional genes that are collectively involved in the biogenesis and function of type 1 fimbriae (61). To date, the precise genetic determinant responsible for APRP-binding activity has not been determined. In addition, the degree of structural variation within the type 1 major fimbrial subunit remains unknown.

APRPs and statherin are polymorphic multifunctional salivary proteins with an amino-terminal region for interactions with calcium and tooth surfaces (21, 26, 31, 42). The variants of APRPs are divided into two classes: allelic large APRPs (PIF-s, Db-s, Pa, PRP-1, and PRP-2), encoded by the *PRH1* and *PRH2* loci on chromosome 12p13.2 (4), and posttranslational variants, or small APRPs (PRP-3, PRP-4, PIF-f, and Db-f) (21). Moreover, statherin displays four structural variants due to alternative RNA splicing and posttranslational proteolytic cleavage (statherin, SV3, SV2, and SV4) (26). APRPs and statherin seem to be present in monkeys (43, 44), rabbits (48), rats (29), and hamsters (35), as well as humans, although the information is limited.

Like that of *A. naeslundii* (13, 15), the adhesion of streptococci (14, 16, 23), *Candida albicans* (27), and *Porphyromonas gingivalis* (2) is promoted by APRPs and statherin. The binding sites for *A. naeslundii* LY7 (45) and *Streptococcus gordonii* Blackburn (16) reside in the Pro₁₄₉Gln₁₅₀ carboxy termini of APRPs. The patterns of binding to APRPs and statherin differ among bacterial species and individual strains of *A. naeslundii* (20, 49, 51). Thus, strains of *A. naeslundii* genospecies 1 and 2 from the human mouth show preferential binding to APRPs over statherin (18), while *A. viscosus* ATCC 19246, originating

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TABLE 1. Sequence identity among *fimP* subunit genes of *A. naeslundii* and *A. viscosus* with statherin- or APRP-binding specificities

Strain ^a	Binding specificity ^b		DNA identity ^c	Site of isolation ^d
	APRPs	Statherin		
<i>A. naeslundii</i>				
T14V	+++ (72)	+ (6)	Reference	Human periodontal pocket (7)
LY7	++ (56)	+ (1)	98.1	Human dental plaque (13)
P-1-K	++ (26)	+ (1)	98.6	Human dental plaque (18)
B-1-K	+++ (62)	+ (3)	98.6	Human buccal mucosa (18)
<i>A. viscosus</i> ATCC 19246				
	++ (35)	+++ (72)	83.7	Human cervicofacial actinomycosis (51)

^a The strains were identified by multivariate statistical analyses of phenotypic characteristics, serological reactions, and protein banding patterns of cell extracts analyzed by SDS-PAGE (18).

^b Binding to APRPs or statherin was scored as strong (+++), moderate (++), or weak (+) based on adhesion of radiolabeled bacteria to protein-coated hydroxyapatite beads. The figures within parentheses give the proportion (percentage of added cells) of bacteria attaching to protein-coated hydroxyapatite beads.

^c Nucleotide sequence identity (%) of the *fimP* genes (GenBank accession numbers are given in Materials and Methods) compared to the *fimP* gene of T14V reference strain (60). Ten regions of comparably high identity (83 to 97%; nucleotide sequences 1 to 210, 226 to 303, 361 to 501, 517 to 576, 595 to 741, 823 to 870, 949 to 1200, 1225 to 1266, 1297 to 1431, and 1456 to 1599) are interspersed with 10 regions of lower identity (38 to 78%; nucleotide sequences 211 to 225, 304 to 360, 502 to 516, 577 to 594, 742 to 822, 871 to 948, 1201 to 1224, 1267 to 1296, 1432 to 1455, and 1600 to 1617). The numbering starts from the bp 1 to 3 ATG start codon after alignment to the *fimP* gene of strain T14V.

^d Site of isolation, with a literature reference (in parentheses). The P-1-K and B-1-K strains were from different sites in the same individual but belonged to different ribotypes (19).

from a human with cervicofacial actinomycosis, displays preferential binding to statherin (51). DNA-DNA hybridization with specific *fimP* probes shows a genetic diversity among *fimP* genes encoding type 1 fimbriae (20, 49). Recently, recombinant *P. gingivalis* fimbrillin, which binds to statherin and to APRPs, was found to interact with Leu₂₉Tyr₃₀ and Tyr₄₁Thr₄₂Phe₄₃ of statherin (1). However, no further information is available concerning bacterial binding sites in statherin.

The aim of the present study was to characterize the type 1 fimbrial *fimP* subunit genes and host peptide recognition motifs associated with the different statherin- and APRP-binding specificities and to investigate the biological significance of this variation in binding specificity. We show that human oral isolates of *A. naeslundii* genospecies 2, with preferential binding to APRPs, contain structurally conserved *fimP* subunit genes, while *A. viscosus* ATCC 19246 contains a novel *fimP* gene which is associated with a carboxy-terminal ThrPhe recognition motif in statherin. The novel *fimP* gene and statherin-binding specificity were demonstrated in *A. viscosus* strains originating from the oral cavities of rat and hamster hosts.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and radiolabeling. *A. naeslundii* genospecies 2 P-1-K and B-1-K (18, 19), LY7 (from R. J. Gibbons, Forsyth Dental Center, Boston, Mass.), and T14V (from P. Kolenbrander, National Institutes of Health, Bethesda, Md.) and *A. viscosus* R28 (from M. Yeung, University of Texas Health Science Center, San Antonio [57]), 14476, 35451, and 35452 (all three from the Culture Collection of the University of Göteborg [CCUG]), and ATCC 19246 (51) were used in this study (Table 1). All strains were cultured overnight at 37°C in a nitrogen atmosphere with 5% CO₂ and 10% H₂ on Columbia II agar base plates (Becton Dickinson and Company, Cockeysville, Md.) supplemented with 30 ml of a human erythrocyte suspension per liter. For hydroxyapatite binding experiments, the bacteria were metabolically labeled by adding [³⁵S]methionine (200 μCi) (Tran ³⁵S-Label; ICN Pharmaceuticals Inc., Irvine, Calif.) to bacteria suspended in 100 μl of 10 mM phosphate-buffered saline, pH 7.2, prior to growth. For peptide binding experiments, strains LY7 and ATCC 19246 were metabolically labeled with 4 μCi of [³H]thymidine (ICN Pharmaceuticals) per ml, grown in Todd-Hewitt broth supplemented with 0.2% yeast extract (Difco Laboratories, Detroit, Mich.), and incubated in an atmosphere of 80% N₂, 10% CO₂, and 10% H₂ (BBL Gaspack; Becton Dickinson) at 37°C for 18 h (LY7) or 26 h (ATCC 19246).

Typing of Actinomyces. The strains originating from the human oral cavity (B-1-K, P-1-K, LY7, and T14V) and strain ATCC 19246 were characterized by the API products rapid ID 32 strep and ID coryne and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the whole-cell soluble proteins (18). The strains originating from the oral cavities of rat and hamster hosts (35452, R28, 35451, and 14476) were characterized by SDS-PAGE of whole-cell soluble proteins. All strains were then analyzed by whole-cell agglu-

ination by specific rabbit antisera 1 to 7 (40) (kindly provided by G. H. Bowden, University of Manitoba, Winnipeg, Canada).

Chromosomal DNA isolation. Chromosomal DNA was isolated according to procedures described earlier (20).

PCR. PCRs were performed in 50-μl reaction volumes containing 1 μg of chromosomal DNA, 0.8 μM (each) primer, 1.6 U of *Taq* DNA polymerase (MBI Fermentas Ltd., Vilnius, Lithuania), 200 μM (each) dATP, dGTP, dCTP, and dTTP (deoxynucleoside triphosphate [dNTP] mixture; Boehringer Mannheim, Mannheim, Germany), 0.75 mM MgCl₂, and 0.02 μg of bovine serum albumin in PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.08% Nonidet P-40, pH 8.8). For strain ATCC 19246, the MgCl₂ concentration was increased to 1.5 mM. To digoxigenin label the DNA fragments, the dNTP mixture was replaced with DIGdNTP labeling mixture (Boehringer Mannheim) in the second PCR amplification.

Three PCR programs were used. PCR program 1 involved 1 cycle of denaturing at 94°C for 5 min, annealing at 59°C for 150 s, and elongation at 72°C for 150 s, followed by 33 cycles of denaturing at 94°C for 75 s, annealing at 59°C for 150 s, and elongation at 72°C for 150 s and a final extension at 72°C for 7 min. PCR program 2 involved denaturing at 94°C for 4 min followed by three cycles of denaturing at 94°C for 60 s, annealing at 60°C for 60 s, and elongation at 72°C for 60 s. This was then followed by additional cycles differing only in the annealing temperatures: 57°C (3 cycles), 54°C (3 cycles), 53°C (3 cycles), and 51°C (24 cycles). The final extension was at 72°C for 7 min. PCR program 3 involved 30 cycles of denaturing at 94°C for 60 s, annealing at 55°C for 90 s, and elongation at 72°C for 180 s.

PCR primer pairs. To amplify and sequence *fimP* genes and to generate DNA probes, the following primers were designed and used (the gene, strain origin, and primer locations are given within parentheses): no. 1, 5'-ACCTCTCCGG TGTGGACAA-3' (*fimP*; strain T14V [60]; forward primer, bp 550 to 569 counting from the bp 1 to 3 ATG start codon for all *fimP* genes); no. 5, 5'-ACAG AATGCACTCCCTCAA-3' (*fimP*; strain T14V; forward primer, bp -6 to 14); no. 27, 5'-TACGAGTGCACCAAGACCGC-3' (*fimP*; strain T14V; forward primer, bp 1147 to 1166); no. 36, 5'-TGGTAAGCAGACCTTCACGACTGA G-3' (*fimP*; strain 19246; forward primer, bp 1239 to 1263); no. 50, 5'-ACCTC GTTCTGACCGACGAT-3' (*fimP*; strain T14V; reverse primer, bp 758 to 739); no. 52, 5'-IGGIGCYTTIGTYTCLAC-3' (*fimP*; strain T14V; reverse primer, bp 1368 to 1351); no. 54, 5'-TGCTTGGCAACGTGACGGC-3' (*fimP*; strain T14V; reverse primer, bp 1598 to 1580); no. 61, 5'-GCGGTCTTGGTGCACCTCGTA-3' (*fimP*; strain T14V; reverse primer, bp 1166 to 1147); no. 22, 5'-CATCCCAAC AACACAGGAG-3' (upstream *fimP*; strain T14V; forward primer, bp 66 to 84 according to reference 60); no. 76, 5'-TCACCTCAGTGGCTGCCAGT-3' (downstream *fimP*; strain T14V; reverse primer, bp 1730 to 1711 according to reference 60); and no. 86, 5'-GGTACGAAGTGATGGGAGTAG-3' (*orf4*; strain T14V; reverse primer, bp 6672 to 6651 according to reference 61).

The *fimP* genes of strains B-1-K, P-1-K, and ATCC 19246 were generated from chromosomal DNA by first amplifying an internal *fimP* gene fragment, followed by a gene fragment upstream and one downstream from the internal fragment. The *fimP* genes of strains P-1-K and B-1-K were first amplified with primers 5 and 54 (PCR program 1), followed by primers 22 and 50 and primers 27 and 76 (both in PCR program 2). The *fimP* gene of strain ATCC 19246 was first amplified by primers 1 and 52 (PCR program 2), followed by primers 22 and 61 (PCR program 2) and 36 and 86 (PCR program 3). The *fimP* gene of strain LY7 was generated by amplifying two overlapping gene fragments with primers 22 and 50 and primers 1 and 76 (both in PCR program 2).

DNA probes corresponding to either the full-length *fimP* gene or a central portion of the gene, encoding preferential binding to APRPs (type 1:1 *fimP* gene) or to statherin (type 1:2 *fimP* gene), were generated for use in slot blot or Southern blot hybridization assays. A full-length and a central *fimP* DNA probe were generated from the *fimP* gene of strain T14V chromosomal DNA as previously described (20). A central type 1:2 *fimP* probe was amplified from the *fimP* gene of strain ATCC 19246 chromosomal DNA with primers 1 and 52 (PCR program 2).

Nucleotide sequencing. The PCR product was electrophoretically analyzed on 1% agarose gels with 0.3 μg of ethidium bromide/ml in $1\times$ Tris-borate-EDTA (TBE) buffer. The PCR fragment was then purified with phenol chloroform and ligated to the pGEM-T vector with T4 DNA ligase (Promega Corp., Madison, Wis.) according to the manufacturer's instructions. The ligation mixture was transformed into *Escherichia coli* JM 109 competent cells (Promega), and transformants containing the appropriate clone were identified by growth on Luria-Bertani agar plates containing carbenicillin (50 $\mu\text{g}/\text{ml}$), 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal; 40 $\mu\text{g}/\text{ml}$), and isopropyl- β -D-thiogalactopyranoside (IPTG; 0.1 mM). Plasmid DNA was isolated with the plasmid Maxi kit (Qiagen GmbH, Hilden, Germany), and the sizes of DNA inserts were subsequently confirmed by *SalI* and *NcoI* (Promega) cleavage.

Sequencing reactions, employing the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham-Life Science, Cleveland, Ohio), were performed according to the manufacturer's instructions. The T7 and SP6 sequencing primers (Pharmacia Biotech, Uppsala, Sweden) and additional internal synthetic primers specific for cloned DNA were used.

Southern blot analysis. Chromosomal DNA (8 μg) from strain ATCC 19246 was digested with *Bss*HIII and *Bam*HI, and DNAs from B-1-K, P-1-K, LY7, and T14V were digested with *Bam*HI prior to separation on 0.7% agarose gels. Southern blotting was performed with nylon membranes (Hybond-N⁺; Amersham International plc, Amersham, United Kingdom). Nylon membranes containing bacterial DNA were probed with *fimP* type 1:1 full-length (LY7, B-1-K, and P-1-K) and *fimP* central type 1:2 (ATCC 19246) DNA probes. Prehybridization was performed at 70°C for 4 h in a solution of $5\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.02% *N*-lauroylsarcosine, 0.1% SDS, and 1% blocking reagent (Boehringer Mannheim), and hybridization was done at 70°C overnight in prehybridization solution containing the probe (20). The membranes were then washed twice in $2\times$ SSC-0.1% SDS buffer at room temperature for 5 min and twice in $0.5\times$ SSC-0.1% SDS buffer at 70°C for 15 min. DNA hybridized to probe DNA was detected with a digoxigenin DNA detection kit (Boehringer Mannheim) as described by the manufacturer.

Slot blot hybridization assay. Chromosomal DNA (3 μg) was loaded on nylon membranes (Hybond N⁺) with a slot blot manifold filter system (Milliblot-S; Millipore, Västra Frölunda, Sweden) as described previously (3). Essentially, hybridization with *fimP* type 1:1 full-length DNA probe and central type 1:1 and type 1:2 DNA probes and washing and detection conditions were as described for Southern blot hybridization except that hybridization and the second stringent washing were done at 80°C. The degree of hybridization was scored from 0 to 6 by comparison with a standardized scale based on densitometric measures (20) (GS-700 imaging densitometer and Molecular Analyst software; Bio-Rad, Hercules, Calif.): 0, <0.01; 1, 0.01 to <0.04; 2, 0.04 to <0.10; 3, 0.10 to <0.16; 4, 0.16 to <0.22; 5, 0.22 to <0.27; and 6, \geq 0.27.

Computer analysis. Complete open reading frames of *fimP* nucleotide sequences, deduced amino acid sequences, and hydrophobicity profiles (30) were analyzed with the Wisconsin Package (version 9.0) from the Genetics Computer Group (University of Wisconsin, Madison).

Isolation of APRPs and statherin. Freshly collected parotid saliva from one individual homozygous for allelic APRP variants (PRP-1 and PIF-s) was separated on a DEAE-Sepharose column (15 by 1.6 cm; Pharmacia) with a linear gradient of 25 mM to 1.0 M NaCl in 50 mM Tris-HCl, pH 8.0. The peak containing APRPs and statherin was concentrated by ultrafiltration on a Centriprep 10 concentrator (Amicon Inc., Beverly, Mass.) and separated by gel filtration (HiLoad 26/60 Superdex S-200 Pregrade; Pharmacia) in 20 mM Tris-HCl-0.5 M NaCl, pH 8.0. Each of the resolved PRP-1-PIF-s, PRP-3-PIF-f, and statherin protein peaks was finally purified on a Macrorep high Q column (15 by 1.6 cm; Bio-Rad) with a linear gradient of 25 mM to 1.0 M NaCl in 50 mM Tris-HCl, pH 8.0. The identities and purities of APRPs and statherin were confirmed by SDS-PAGE, native alkaline electrophoresis, NH₂-terminal amino acid sequencing, and bacterial binding properties.

Peptide synthesis. Synthetic decapeptides mimicking the carboxy-terminal portion of statherin and with a core of six Asp and various Glu residues were synthesized (Quality Controlled Biochemicals Inc., Hopkinton, Mass.). The amino and carboxy termini were unmodified. High-performance liquid chromatography with reversed-phase and anion-exchange columns confirmed that the purity of the peptides exceeded 95%. The synthetic decapeptides mimicking the carboxy-terminal portions of APRPs were characterized as previously described (16). A Glu₆ hexapeptide was used as a control (Sigma Chemical Co., St. Louis, Mo.).

Linking of peptides to CNBr-agarose beads. Linkage of peptides to CNBr-activated Sepharose 6MB agarose beads (Pharmacia) was performed as described by the manufacturer. Essentially, the activated beads were incubated for at least 20 min in 1 mM HCl and washed with HCl on a sintered glass filter. The peptides, dissolved in coupling buffer (0.1 M NaHCO₃-0.5 M NaCl, pH 8.3),

were added to the beads on a molarity basis and rotated end over end for 2 h at room temperature. The beads were sedimented by low-speed centrifugation (300 \times g for 15 min) and washed with coupling buffer. Reversed-phase high-performance liquid chromatography on the supernatants confirmed linking of peptides. Any remaining CNBr groups were blocked with glycine (0.2 M) followed by five washes in coupling buffer alternated with five washes in acetate buffer (0.1 M sodium acetate-0.5 M NaCl, pH 4.0) on a sintered glass filter. The prepared beads were stable for at least 1 week in coupling buffer at 4°C.

Binding of bacteria to peptides linked to agarose beads. Samples containing 7 mg of the peptide-linked agarose beads were aliquoted into each well of a microtiter plate and washed three times in KCl buffer supplemented with 0.5% albumin. Following washings in buffered KCl and resuspension in buffered KCl supplemented with albumin, aliquots of 125 μl of a suspension (16×10^4 cpm/ml; 6×10^8 bacteria/ml) of strains ATCC 19246 and LY7 were added to each well, and the mixture was rotated end over end for 1 h at room temperature. Triplicate samples were done. After the samples were washed with KCl-albumin buffer, the number of bacteria remaining attached to the beads was estimated by scintillation counting.

Hydroxyapatite assay. Adherence of [³⁵S]methionine-labeled bacteria (5×10^4 to 15×10^4 cpm/ml; 5×10^8 bacteria/ml) to purified proteins (6 $\mu\text{g}/\text{ml}$) adsorbed onto hydroxyapatite beads (Fluka, Chemie AG, Buchs, Switzerland) was measured as described previously (13).

Nucleotide sequence accession numbers. The GenBank accession numbers for the *fimP* gene sequences are AF106035 (strain B-1-K), AF107020 (strain LY7), AF107019 (strain P-1-K), AF106034 (strain ATCC 19246), and M32067 (strain T14V) (60).

RESULTS

Highly conserved *fimP* subunit genes in *A. naeslundii* isolates with preferential binding to APRPs. To investigate the degree of structural diversity in *fimP* genes among *A. naeslundii* genospecies 2 strains, *fimP* genes were amplified by PCR and sequenced from strains LY7, P-1-K, and B-1-K (Table 1). Each *fimP* gene had an open reading frame of 1,602 nucleotides and a putative ribosome binding site with similarity to the *E. coli* consensus sequence (33) 9 nucleotides upstream of the ATG start codon. No internal repeat sequences were observed, and each *fimP* gene contained a high G/C ratio (67 mol%). The respective *fimP* gene had between 98.1 and 98.6% nucleotide sequence identity to the *fimP* gene of *A. naeslundii* T14V (60) (Table 1). Nucleotide substitutions were randomly distributed throughout the individual *fimP* genes. Thus, the *fimP* genes from isolates with the same APRP-binding specificity were highly conserved.

A novel *fimP* subunit gene in *A. viscosus* ATCC 19246 with preferential binding to statherin. To investigate the genetic basis for preferential binding of *A. viscosus* ATCC 19246 to statherin, the *fimP* gene was amplified by PCR and sequenced from strain ATCC 19246. The *fimP* gene contained 1,608 nucleotides and a putative ribosome binding site similar to the *E. coli* consensus sequence 9 nucleotides upstream of the ATG start codon. This *fimP* gene had a G+C content of 63.5 mol%, and no internal repeat sequences were identified. The *fimP* gene from strain ATCC 19246 had 83.7% nucleotide sequence identity to the *fimP* gene of *A. naeslundii* T14V (60) (Table 1). Interspersed with 10 regions of comparably high sequence identity (ranging from 83 to 97%) were regions of lower sequence identity (ranging from 38 to 77%). In addition, evenly distributed single-nucleotide substitutions were present. Thus, *A. viscosus* ATCC 19246 exhibits a genetically variant *fimP* gene conferring preferential binding to statherin.

In Southern blot hybridization of restriction enzyme-digested chromosomal DNA from strains ATCC 19246 (*Bss*HIII and *Bam*HI digestion), LY7, P-1-K, and B-1-K (*Bam*HI digestion) with *fimP* DNA probes, hybridization to single DNA fragments occurred (data not shown). Thus, the *fimP* genes were present as single gene copies.

Predicted amino acid sequence for the novel *FimP* subunit protein associated with statherin-binding specificity. The *fimP* gene of *A. viscosus* ATCC 19246 encodes a protein product



FIG. 1. Amino acid sequence alignment of the FimP major subunit proteins of *A. naeslundii* T14V (upper sequence) and *A. viscosus* ATCC 19246 (lower sequence). Identical (.), conserved (:), and similar (.) amino acids are indicated. Boldface indicates a putative N-terminal signal peptide with a cleavage site (↓) and a C-terminal LPXTG sequence. The seven shaded boxes indicate domains, each incorporating a proline residue and reported to be conserved between the FimP and FimA subunit proteins (60).

with 535 amino acid residues. The N terminus contains a putative signal peptide containing a 22-amino-acid hydrophobic segment (residues 9 to 30) preceded by an 8-amino-acid stretch harboring three basic amino acids (Fig. 1). The structural arrangement of amino acids at the C terminus of the signal peptide suggests a probable cleavage site between the two alanine residues at positions 30 and 31 (55). Cleavage would generate a mature fimbrial subunit protein of 505 residues (53.56 kDa). The major structural portion of FimP is dominated by hydrophilic amino acid residues consistent with an isoelectric point of 4.7, contains four cysteine residues, and is rich in Thr (13.4%). The FimP protein ends with a cell wall-anchoring LeuProLeuThrGly sequence followed by a membrane-spanning domain composed of 17 hydrophobic amino acids and a short charged tail of 5 basic amino acids (Fig. 1).

Comparison of the variant FimP subunit proteins. The novel FimP protein of *A. viscosus* ATCC 19246 had 80.6% amino acid sequence identity to the 533-amino-acid-residue FimP protein of *A. naeslundii* T14V. The FimP proteins from *A. naeslundii* LY7, P-1-K, and B-1-K showed 97.4 to 98.7% amino acid sequence identity to FimP of *A. naeslundii* T14V. The *A. viscosus* ATCC 19246 FimP protein showed 10 regions

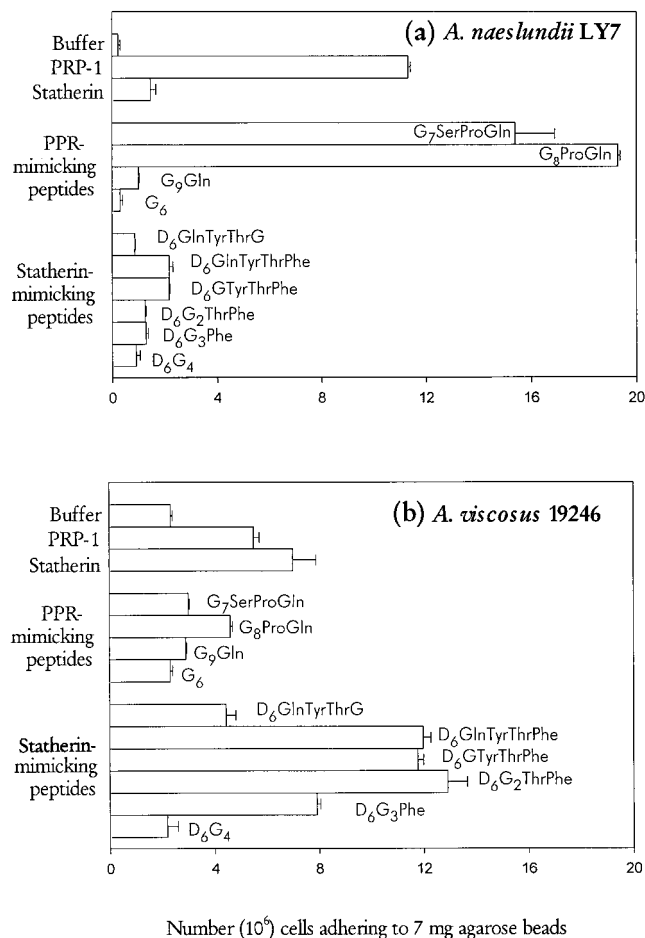


FIG. 2. Adhesion of *A. naeslundii* LY7 (a) and *A. viscosus* ATCC 19246 (b) to statherin or PRP-1 (0.6 nmol/7 mg of beads) and to statherin- or PRP-1-mimicking decapeptides (17.4 nmol/7 mg of beads) covalently linked to agarose beads. The bars represent the means (plus standard errors) of two independent experiments in which triplicate samples were assayed.

of high sequence identity ($\geq 85\%$) to the FimP protein of *A. naeslundii* T14V, and 7 of those regions contained proline residues (Fig. 1), which are considered to be conserved in FimP and FimA proteins (60). Four cysteine residues were present in both FimP proteins. Interspersed between the high-sequence-identity regions were regions of lower amino acid sequence identity ($\leq 52\%$), including a 19-amino-acid region (residues 102 to 120) with only 21% identity.

The variant FimP subunit proteins are associated with Thr-Phe and ProGln recognition motifs in statherin and APRs. To investigate the peptide recognition motif in statherin for *A. viscosus* ATCC 19246, radiolabeled bacteria were bound to agarose beads with covalently linked pure PRP-1, statherin, or synthetic decapeptides mimicking the carboxy termini of the two proteins (Fig. 2 and 3). *A. viscosus* ATCC 19246 and *A. naeslundii* LY7 bound preferentially to statherin and PRP-1, respectively, although binding to the reciprocal protein was observed (Fig. 2). Binding to fixed and varying amounts of synthetic peptides (Figs. 2 and 3) distinguished similar characteristics for the recognition motifs in statherin and PRP-1. First, *A. viscosus* ATCC 19246 and *A. naeslundii* LY7 bound most abundantly to the C-terminal segments of statherin (Thr₄₂Phe₄₃) and PRP-1 (Pro₁₄₉Gln₁₅₀), respectively. Second,

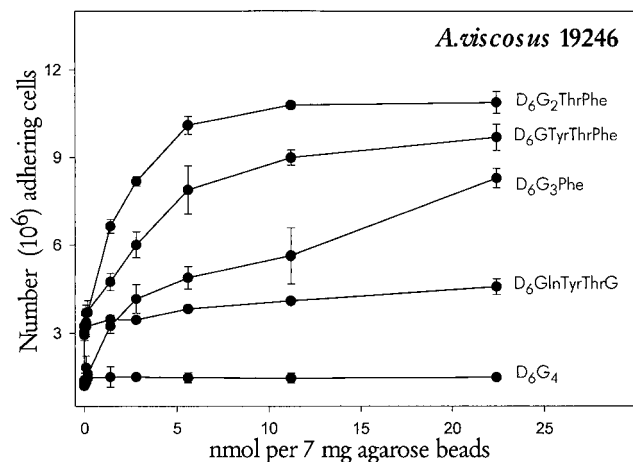


FIG. 3. Adhesion of *A. viscosus* ATCC 19246 to varying amounts of statherin-mimicking decapeptides covalently linked to agarose beads. Triplicate determinations were used in two independent experiments. The data are presented as means \pm standard errors.

addition to the synthetic peptides of residue 41 (Tyr) and 148 (Ser), respectively, reduced, rather than enhanced, binding by bacteria. In addition, substitution of Gly for Phe at residue 43 of statherin dramatically diminished binding by strain ATCC 19246, which displayed partial binding activity to peptides harboring only the Phe₄₃ residue of statherin.

The variant FimP proteins are typical of strains of *Actinomyces* originating from the oral cavity of human versus rat and hamster hosts. To investigate the biological significance of the variant FimP proteins, *Actinomyces* strains of different animal origins were tested for binding specificity and the presence of variant *fimP* genes (Table 2). Oral isolates of *A. viscosus* from rats and hamsters displayed preferential binding to statherin, and chromosomal DNA from these strains hybridized with

DNA probes specific to the *fimP* gene encoding statherin-binding fimbriae. In contrast, chromosomal DNA from isolates of *A. naeslundii* from the human mouth with preferential binding to APRPs hybridized exclusively with a DNA probe specific to the *fimP* gene encoding APRP-binding fimbriae. Thus, the variant FimP proteins may determine the animal tropism for *A. naeslundii* and *A. viscosus*.

DISCUSSION

In the present study, we show genetically variant *fimP* subunit genes in *A. viscosus* and *A. naeslundii* that correlate to different ThrPhe and ProGln recognition motifs in statherin and APRPs and to different animal host tropisms. Similar to other surface proteins of gram-positive bacteria, the novel statherin-binding FimP protein contains a signal peptide and ends with cell wall-anchoring and membrane-spanning domains. Our findings extend the present knowledge of surface-associated proteins for bacterial adherence and colonization, and *Actinomyces* spp. forming distinct fimbriae may serve as a model to study the assembly and function of adhesive organelles in gram-positive bacteria.

The statherin-binding FimP protein of *A. viscosus* ATCC 19246 contains features typical of surface-associated proteins of gram-positive bacteria (9, 37), including (i) an amino-terminal signal peptide and (ii) a carboxy-terminal portion with a cell wall-anchoring (LPXTG motif) and a putative membrane-anchoring domain. The FimP protein lacks both tandem repeats and a distinct Pro-rich wall-spanning domain, which are typical features of the M and Sfb proteins of *Streptococcus pyogenes* (22, 53) and the collagen binding adhesin of *Staphylococcus aureus* (39). Similar to FimA (60), the FimP proteins contain seven proline-containing regions and four cysteine residues. The proline residues may maintain extended peptide conformations (36) or mediate noncovalent protein-protein interactions (10, 38, 41), while the cysteine residues may form disulfide bridges stabilizing functional domains for subunit-subunit interactions or adhesion activity (24).

TABLE 2. Presence of variant FimP subunit proteins in *A. naeslundii* and *A. viscosus* strains of different animal host origins

Strain ^a	Binding specificity ^b		Hybridization with <i>fimP</i> DNA probes ^c			FimP protein ^d		Origin ^e
	APRPs	Statherin	Type 1 full-length probe	Type 1:1 central probe	Type 1:2 central probe	Type 1:1	Type 1:2	
<i>A. naeslundii</i> T14V	+++ (72)	+ (6)	5	4	0	Yes	No	Human periodontal pocket (7)
<i>A. naeslundii</i> LY7	++ (56)	+ (1)	4	3	0	Yes	No	Human dental plaque (13)
<i>A. naeslundii</i> P-1-K	++ (26)	+ (1)	4	4	0	Yes	No	Human dental plaque (18)
<i>A. naeslundii</i> B-1-K	+++ (62)	+ (3)	5	4	0	Yes	No	Human buccal mucosa (18)
<i>A. viscosus</i> ATCC 19246	++ (35)	+++ (72)	3	0	5	No	Yes	Human actinomycosis (51)
<i>A. viscosus</i> 35452	++ (44)	+++ (73)	4	0	5	No	Yes	Rat mouth (CCUG)
<i>A. viscosus</i> R28	++ (56)	+++ (81)	4	0	5	No	Yes	Rat mouth (57)
<i>A. viscosus</i> 35451	++ (38)	+++ (70)	4	0	4	No	Yes	Hamster mouth (CCUG)
<i>A. viscosus</i> 14476	++ (37)	+++ (80)	4	0	4	No	Yes	Hamster mouth (CCUG)

^a The isolates were identified by multivariate statistical analyses of phenotypic characteristics, serological reactions, and protein banding patterns of cell extracts analyzed by SDS-PAGE (18).

^b Binding to APRPs or statherin was scored as strong (+++), moderate (++), or weak (+) based on adhesion of radiolabeled bacteria to protein-coated hydroxyapatite beads. The figures within parentheses give the proportion (percentage of added cells) of bacteria attaching to protein-coated hydroxyapatite beads.

^c The *fimP* type 1:1 full-length DNA probe was generated from the entire *fimP* type 1 fimbrial gene of *A. naeslundii* T14V, and the *fimP* central type 1:1 (APRP-binding specificity) and type 1:2 (statherin-binding specificity) DNA probes were generated from the central segments of the *fimP* type 1 fimbrial genes of *A. naeslundii* T14V and *A. viscosus* ATCC 19246, respectively (see reference 20 and Materials and Methods). The DNA probes were labeled with digoxigenin and used in slot blot hybridization to probe chromosomal DNA from *Actinomyces* spp. under high-stringency conditions. The degree of hybridization was scored from 0 to 6 by comparison with a scale based on densitometric measures: 0, 0.01; 1, 0.01 to <0.04; 2, 0.04 to <0.10; 3, 0.10 to <0.16; 4, 0.16 to <0.22; 5, 0.22 to <0.27; and 6, \geq 0.27.

^d Presence of the different FimP subunit proteins based on binding specificity and *fimP* DNA probe hybridization pattern.

^e Host and tissue origins of the strains, with references (parentheses).

The present findings show that structurally variant FimP proteins on *A. viscosus* and *A. naeslundii* recognize statherin and APRPs differently. The structural variation resulting from both single-amino-acid substitutions and prominent structural changes reduces the amino acid identity between the two FimP proteins to about 80%. Both single-amino-acid substitutions, as shown for S fimbriae of *E. coli* (17), and prominent structurally different amino-terminal domains, as shown for the tip-localized minor G adhesin of P fimbriae of *E. coli* (24), are known to alter binding specificity. The single-amino-acid substitutions changing binding specificity of S fimbriae reside in the fimbrial subunit and not in the minor SfaS adhesin (17), and structural differences in the subunit of mannose-binding fimbriae of *E. coli* affect the minor FimH adhesin specificity (32). However, since only a limited number of genes are involved in type 1 fimbriae biogenesis and function (61), and since the *E. coli* K88 and K99 major fimbrial subunits contain a carbohydrate or protein binding domain (5, 25), we suspect the FimP proteins to contain the statherin- or APRP-binding domain.

Another finding of the present study is the highly conserved nature of *fimP* genes from isolates with preferential APRP binding. Higher-than-97% nucleotide and amino acid sequence identity was observed among *Actinomyces* isolates originating from the human mouth. This is a novel finding, since the major subunit genes of P fimbriae of *E. coli* isolates from urinary tract infections display extensive genetic diversity as a result of antigenic variation (8, 54). The conserved nature of the *fimP* fimbrial genes may be a unique feature of *Actinomyces* spp., which are commensal bacteria colonizing the human mouth from birth (11). Early colonization by bacteria could induce an immunological tolerance toward surface-associated bacterial proteins generally considered to be immunodominant. One may speculate that FimP proteins from *A. viscosus* strains with preferential binding to statherin would also have highly conserved sequences.

The present findings provide evidence for a carboxy-terminal Thr₄₂Phe₄₃ motif in statherin targeted by the novel FimP protein of *A. viscosus* ATCC 19246. Among peptides mimicking the carboxy terminus of statherin, those containing Thr₄₂Phe₄₃ promoted high bacterial binding activity while residues 41 (Tyr) and 40 (Gln) did not contribute to binding. Moreover, substitution of Phe₄₃ markedly diminished binding activity, and peptides harboring only Phe₄₃ showed partial binding activity. Interestingly, the carboxy-terminal Pro₁₄₉Gln₁₅₀ motif in APRPs targeted by *A. naeslundii* LY7 displays similar characteristics (45). The dual binding activity of statherin and APRPs could be explained either by mimicking recognition motifs or the actual presence of ProGln and Phe in statherin and APRPs, respectively. Furthermore, since a diversity of *fimP* gene structure seems to exist in *Actinomyces* (20) and since both *A. naeslundii* genospecies 1 and 2 seem to harbor variant APRP-binding specificities (18), a further diversity of recognition motifs may exist among *Actinomyces* strains.

Taken together, the present findings suggest that variation in binding specificities may determine the ecological niches for isolates of *Actinomyces* spp. Significantly, in this study we have observed a relationship between genetic variation of *fimP* genes, altered binding specificity for host proteins, and tissue origin of bacterial isolates. While the APRP-binding FimP protein is a typical characteristic of *A. naeslundii* strains originating from different sites in the human mouth, the statherin-binding FimP protein seemed to be present in *A. viscosus* strains from the oral cavities of the rat and the hamster. Although this implies variation in type 1 fimbrial binding specificity in animal tropism, it should be noted that *A. viscosus*

ATCC 19246 originates from a human case of cervicofacial actinomycosis (51) and that *A. naeslundii* contains serologically defined subpopulations of possibly deviating adhesive phenotypes (20, 28, 49). Interestingly, recent studies with *E. coli* have suggested that single-amino-acid substitutions in the mannose-binding FimH adhesin may change commensal phenotypes to pathogenic types by shifting their ecological niches (46, 47). Thus, structural variations in type 1 fimbriae could affect the specificity of *Actinomyces* spp. for different animal hosts and ecological niches as well as being involved in various skin or soft tissue infections.

ACKNOWLEDGMENTS

This study was supported by grants 10906 and 11302 from the Swedish Medical Research Board, the Marabou Research Foundation, Sweden, and by grants DE8601 and DE7009 from the National Institutes of Health, Bethesda, Md.

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