Different Type 1 Fimbrial Genes and Tropisms of Commensal and Potentially Pathogenic *Actinomyces* spp. with Different Salivary Acidic Proline-Rich Protein and Statherin Ligand Specificities

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Actinomyces spp. exhibit type 1 fimbria-mediated adhesion to salivary acidic proline-rich proteins (PRPs) and statherin ligands. *Actinomyces* spp. with different animal and tissue origins belong to three major adhesion types as relates to ligand specificity and type 1 fimbria genes. (i) In preferential acidic-PRP binding, strains of *Actinomyces naeslundii* genospecies 1 and 2 from human and monkey mouths displayed at least three ligand specificities characterized by preferential acidic-PRP binding. Slot blot DNA hybridization showed seven highly conserved type 1 fimbria genes (*orf1*- to *-6* and *fimP*) in genospecies 1 and 2 strains, except that *orf5* and *orf3* were divergent in genospecies 1. (ii) In preferential statherin binding, oral *Actinomyces viscosus* strains of rat and hamster origin (and strain 19246 from a human case of actinomycosis) bound statherin preferentially. DNA hybridization and characterization of the type 1 fimbria genes from strain 19246 revealed a homologous gene cluster of four open reading frames (*orfA* to *-C* and *fimP*). Bioinformatics suggested sortase (*orfB*, *orf4*, and part of *orf5*), prepilin peptidase (*orfC* and *orf6*), fimbria subunit (*fimP*), and usher- and autotransporter-like (*orfA* and *orf1* to *-3*) functions. Those gene regions corresponding to *orf3* and *orf5* were divergent, those corresponding to *orf2*, *orf1*, and *fimP* were moderately conserved, and those corresponding to *orf4* and *orf6* were highly conserved. Restriction fragment length polymorphism analyses using a *fimP* probe separated human and monkey and rat and hamster strains into phylogenetically different groups. (iii) In statherin-specific binding, strains of *A. naeslundii* genospecies 1 from septic and other human infections displayed a low-avidity binding to statherin. Only the *orf4* and *orf6* gene regions were highly conserved. Finally, rat saliva devoid of statherin bound bacterial strains avidly irrespective of ligand specificity, and specific antisera detected either type 1, type 2, or both types of fimbria on the investigated *Actinomyces* strains.

Adhesion of commensal and pathogenic bacteria to host tissue surfaces is a crucial event in colonization and infections (13, 19). Commensal bacterial species, which protect against pathogens by competing for host binding sites (47), may involve a diversity of adhesion types with multiple ecological niches (42, 45).

Actinomyces naeslundii and *Actinomyces viscosus* are dominant commensal *Actinomyces* spp. colonizing dental and mucosal surfaces of various animal hosts. They show extensive phenotypic and serologic variations (23). Human strains of *A. naeslundii* were recently grouped into genospecies 1 (*A. naeslundii* serotype I) and genospecies 2 (*A. naeslundii* serotypes II, III, and NV and *A. viscosus* serotype II) based on genetic relatedness (23). *A. viscosus* serotype I is the dominant species in the rat and hamster mouths. *Actinomyces* spp. have also been implicated in caries (34), periodontitis (24), and root canal infections (46), as well as in actinomycosis and septic infections (37).

The animal and tissue tropism of *A. naeslundii* and *A. viscosus* appears to involve a diversity of type 1 and type 2 fimbria adhesion types (8, 45, 49). Type 1 fimbriae, which mediate

binding to acidic proline-rich proteins (PRPs) and statherin, are more common on *A. naeslundii* genospecies 2 (an early plaque colonizer) than on genospecies 1 (a late plaque colonizer) (11, 16, 17). Moreover, while type 1 fimbriae on *A. naeslundii* of human origin bind ProGln in acidic PRPs, type 1 fimbriae on *A. viscosus* of rat and hamster origin bind ThrPhe in statherin (28). Type 2 fimbriae, which mediate binding to β-linked galactose structures, are highly prevalent on both *A. naeslundii* genospecies 1 and 2. Type 2 fimbriae involve at least four β-linked galactose specificities with different coaggregation and biological properties (16, 17, 45).

Biogenesis, assembly, and function of type 1 fimbriae of *A. naeslundii* strain T14V require the FimP subunit and additional proteins encoded by a cluster of seven genes (*orf1* to *-6* and *fimP*) (52). Recently, a type 2 fimbria gene cluster containing three or four genes (*ef-TU*, *orf977*, *fimA*, and *orf365*) was found in *A. naeslundii* strain T14V (51) (GenBank accession no. AJ401093). Structural variations in the major type 1 (FimP) and type 2 (FimA) subunit proteins correlate with different acidic-PRP and statherin and β-linked galactose adhesion types (17, 28). Allelic replacement of the *orf1* to *-4* and *fimP* genes of type 1 fimbriae and of the *orf365* and *fimA* genes of type 2 fimbriae abolish PRP adhesion and coaggregation by *A. naeslundii* strain T14V, respectively (51, 52).

Acidic PRPs and statherin are present in exocrine secretions, e.g., saliva (26) and nasal and bronchial secretions (9,

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38), of different animal species, e.g., humans (18, 38), monkeys (39, 40), and rabbits (43). Acidic PRPs, but not statherin, are also present in rats (2, 33) and hamsters (31). Acidic PRPs are highly polymorphic and multifunctional proteins that may determine host susceptibility and resistance to dental caries (3, 7, 26, 44, 53). While acidic PRPs promote avid adhesion of commensal species, such as *A. naeslundii* (14) and *Streptococcus gordonii* (15), statherin promotes the adhesion of potentially invasive species, such as *Porphyromonas gingivalis* (1) and *Candida albicans* (6, 21).

The aim of the present study was to investigate the structural and functional polymorphism of type 1 fimbriae on *Actinomyces* spp. with specificity for acidic PRPs and statherin. We found a diversity of *Actinomyces* spp. with different protein ligand specificities, type 1 fimbria genes, and tropisms. Those adhesion types typical of human commensal strains bound acid PRPs preferentially, while those typical of rat and hamster hosts and human infections bound statherin preferentially.

MATERIALS AND METHODS

Actinomyces **strains, typing, and culturing.** *A. viscosus* and *A. naeslundii* strains were isolated as previously described (16) or were from other sources. For *A. viscosus* strains, these sources were as follows: 19246, Culture Collection at the National Bacteriology Laboratory, Stockholm, Sweden; T6-1600, R28, and A828, the late M. Yeung, University of Texas Health Science Center, San Antonio, Tex.; 14476, 35452, and 35451, Culture Collection of the University of Göteborg (CCUG). *A. naeslundii* genospecies 2 strains M4356 and M4301 were from the late M. Yeung; *A. naeslundii* genospecies 1 strains 17534, ATCC 12104, 30267, 35334, and 29952 were from CCUG; *A. naeslundii* genospecies 2 strain PK1259 and genospecies 1 strains PK947 and PK606 were from P. Kolenbrander, National Institutes of Health, Bethesda, Md.

Strains were characterized by using the Analytical Profile Index products Rapid ID 32 strp and ID coryne (bioMérieux, Marcy l'Etoile, France), by whole-cell protein patterning by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and by whole-cell agglutination with *Actinomyces*-specific rabbit antisera (G. H. Bowden, University of Manitoba, Winnipeg, Canada) (16, 28, 36).

Actinomyces strains were cultured overnight on Columbia II agar base plates (Becton Dickinson Microbiology Systems, Cockeysville, Md.) supplemented with a human erythrocyte suspension (30 ml/l) at 37°C in a nitrogen atmosphere with 5% CO_2 and 10% H_2 or in an atmosphere with 5% CO_2 or in candle jars. Bacteria were metabolically labeled with [^{35}S]methionine (200 μCi; Amersham, Little Chalfont, United Kingdom) for adhesion experiments.

Hydroxyapatite assay. Binding of [^{35}S]methionine-labeled bacteria (5×10^8 cells/ml) to salivary protein PRP-1 and coating of hydroxyapatite beads (5 mg) (Fluka, Chemie AG, Buchs, Switzerland) with saliva were done as previously described (7, 14). Inhibition experiments with ArgGlyArgProGln and lactose were performed with 10^8 bacterial cells/ml and 4 mg of hydroxyapatite beads.

Saliva sampling. Parotid saliva was collected from healthy human subjects (7). Whole rat saliva was collected from Sprague-Dawley rats (body weight, 400 to 425 g). The rats were anesthetized, placed in a forward supine position, and stimulated by pilocarpine or pilocarpine and isoproterenol (5 mg/kg of body weight for each) followed by collection of whole saliva for 15 min. The saliva samples were kept on ice (22). The saliva-mediated adhesion patterns were similar irrespective of the type of saliva stimulation.

Acidic PRPs and statherin. Acidic PRPs and statherin were isolated from human parotid saliva as previously described (28).

Vectors and bacterial strains for DNA library construction. Phagemid vector λTriplEx and *Escherichia coli* strain XL1-Blue (*endA1 gyrA96 hsdR17 lac* negative *recA1 relA1 supE44 thi-1* [F' *lac*^{q}Z ΔM15 *proAB* Tn*10*]) were used to generate a genomic DNA library from *A. viscosus* strain 19246. *E. coli* strain BM25.8 (*supE44 thi* Δ[*lac-proAB*] [F' *traD36 proAB*$^+$ *lac*^{q}Z ΔM15] λ*imm*^{434} (Kan^r)P1 (Cam^r) *hsdR*($r_{k12}{}^-$ $m_{k12}{}^-$)) *was used for converting the λTriplEx vector to a plasmid pTriplEx vector (Clontech, Palo Alto, Calif.).*

Genomic DNA library construction, screening, and sequencing. Genomic DNA was isolated from *A. viscosus* strain 19246 as previously described (17), partially digested with *Mbo*I, ligated to an *Eco*RI adapter, gel purified, and size fractionated. The 4- to 8-kb DNA fragments were ligated to the λTriplEx vector

to construct a DNA library with a titer of 6.2×10^5 PFU/ml and an average insert size of 5.5 kb (Clontech). The library was amplified in *E. coli* XL1-Blue and screened by plaque DNA hybridization using a *fimP*-specific probe (λTriplEx library user manual; Clontech). Positive clones were converted from phagemid λTriplEx to plasmid pTriplEx in *E. coli* BM25.8 according to Clontech's instructions. Plasmid DNA was isolated by the JETstar kit (Genomed Inc.), and insert size was determined by *Eco*RI digestion. Clones with proper insert sizes were sequenced using vector primers (Clontech) and the BigDye terminator cycle sequencing ready reaction kit (Perkin-Elmer). Clones with overlapping sequences were subjected to further sequencing by primer walking. The sequences were established from at least two clones and from both strands. In this way, clones pTL1-8, pTL21-1, pTL15-5, pTL31-1, and pTL35-1 were identified and characterized (see Fig. 2). Clones pTL36-2 and pTL37-1 were identified using a probe derived from the 5' end of *orfA* in clone pTL35-1. The nucleotide sequences were then assembled into a 9,439-bp type 1 fimbria-related gene cluster.

Bioinformatics. Nucleotide and amino acid sequence analyses were performed using the Sequence Analysis Software Package, version 9.1 (Genetics Computer Group, University of Wisconsin, Madison); bioinformatics tools available at National Center for Biotechnology Information website http://www.ncbi.nlm.nih.gov/, European Bioinformatics Institute website http://www.ebi.ac.uk/index.html, and Expert Protein Analysis System website http://www.expasy.ch/; and the FramePlot, version 2.3, program (4, 20).

Slot blot DNA hybridization. Slot blot DNA hybridization was performed under high-stringency conditions (hybridization and stringency washing at 80°C) as previously described (28). The degree of hybridization was scored from 0 to 6 by comparison with a standardized scale based on densitometric measures (GS-700 imaging densitometer and Molecular Analyst software; Bio-Rad, Hercules, Calif.) in which 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 = ≥0.27 (17).

Restriction fragment length polymorphism (RFLP). *Actinomyces* chromosomal DNA (5 or 8 μg) was digested separately with *Bam*HI, *Pvu*II, *Bss*HII, *Acc*I, *Stu*I, or *Sal*I and separated on 0.7% agarose gels. The restriction fragment length variations were detected by a *fimP* gene probe designed from *A. naeslundii* strain T14V by Southern blot hybridization as previously described (28). The hybridization data matrix was processed into an *Actinomyces* phylogenetic tree by the PAUP3.1.1 package (phylogenetic analysis using parsimony; Sinauer Associates) with a bootstrap value of 1,000.

DNA probes and primers. DNA probes were generated and labeled with digoxigenin by PCR as previously described (17).

The following primer sequences were used to generate *fimP*- and *orfA*-specific probes from plasmids containing strain 19246 DNA for library screening: a 822-bp *fimP* gene probe (forward, 5'-ACCCTCTCCGGTGTGGACAA-3'; reverse, 5'-IGGIGCYTTIGTYTCIAC-3') and a 470-bp *orfA* probe (5'-AAGAT GCGCCATGTCAACC-3' and 5'-TGACCGTTGTTCACGAATCC-3').

The following primer sequences were used to generate *orf1*- to -6- and *fimP*-specific full-length probes for slot blot hybridization (and RFLP) from plasmids pMY261A-100 and pMY1113 containing type 1 fimbria genes from *A. naeslundii* strain T14V (kindly provided by the late M. Yeung) (52): *orf1* probe, 5'-TCAC GATGCAGATGACCTTC-3' and 5'-TTGAGGGAGTGCATTGCTGT-3'; *orf2* probe, 5'-ACTCCCTGAGCTACACCTG-3' and 5'-GAGGTGAAGGTGCCA TCAC-3'; *orf3* probe, 5'-TCCCAGTCATCACGTCGCCC-3' and 5'-GTACTC GTTGTGCCAGAC-3'; *orf4* probe, 5'-CTACTCCCATCACTTCGTGACC-3' and 5'-CTTCATCCAGGTCTGCAT-3'; *orf5* probe, 5'-ATGCAGACCTGGAT GAAG-3' and 5'-GGTGTGGGTGAACGAAC-3'; *orf6* probe, 5'-CGGTC GTCCTGGTGGTGAC-3' and 5'-AAGCGCTGAAGAGCTGCCA-3'; *fimP* probe, 5'-ACAGCAATGCACTCCCTCAA-3' and 5'-TGCTTGGCAACGTGA CGGC-3'.

Antisera. Synthetic peptides were generated from the deduced amino acid sequences of FimP (DRLDKRIKKEALTPV) and Orf1 (TGKDSDT RPDHDVAC) proteins from *A. naeslundii* T14V (Innovagen AB, Lund, Sweden). The synthetic peptides were used for immunization of rabbits and generation of FimP- and Orf1-specific antisera (Agri Sera AB, Umeå, Sweden). The type 2:1 fimbria-specific antiserum R70-3 was kindly provided by J. O. Cisar, National Institutes of Health.

Immunofluorescence staining. Immunofluorescence staining of whole bacterial cells was performed essentially as described previously (32, 41). Briefly, whole cells (10^9 cells/ml) were applied to an objective slide (Novakemi AB), incubated separately with FimP (1:200) and type 2:1 (1:800) antisera (and the corresponding presera dilutions) in 10 mM phosphate-buffered saline, pH 7.2–0.05% Tween 20, washed, and incubated with a secondary goat anti-rabbit immunoglobulin G (IgG)-fluorescein isothiocyanate conjugate (Sigma). Fluorescence signals were detected using a microscope (model DMRBE; Leica AB, Stockholm, Sweden) and scored 0 to 4; scores 0 and 1 indicate no distinguishable

TABLE 1. Ligand specificity, fimbria genes, and tropism of *Actinomyces* spp.

Species	Strain[g]	Adhesion type	Binding to[a]:		DNA hybridization[b] for:							Fluorescence signal score[c] with:		Origin[d] (reference or source[f])	Coagg.[e] group
			PRP-1	Statherin	orf3	orf2	orf1	fimP	orf4	orf5	orf6	FimP	Type 2:1 antisera		
Preferential PRP binding															
A. naeslundii genospecies 2	Strains (n = 9)	PRP ≫ statherin	43.2 ± 14.8	7.4 ± 13.6	5	6	5	6	6	3	6	3	0	Human mouth (16)	
	LY7	PRP ≫ statherin	56.4	11.9	5	6	4	6	6	3	6	3	0	Human mouth (16)	F
	T14V	PRP ≫ statherin	53.4	24.5	6	6	6	6	6	4	6	4	0	Human mouth (16)	A
	M4356	PRP > statherin	53.0	31.0	6	6	6	6	6	4	6	2	0	Monkey mouth (50)	
	M4301	PRP > statherin	38.8	28.6	6	6	6	6	6	5	6	2	4	Monkey mouth (50)	
A. naeslundii genospecies 1	Pn-19-N	PRP ≫ statherin	68.1	7.5	6	6	6	6	6	0	6	3	3	Human mouth (16)	
	Pn-6-N	PRP ≫ statherin	62.5	6.9	3	5	5	4	6	0	5	2	2	Human mouth (16)	
	Strains (n = 4)	Low avidity to PRP	13.3 ± 7.8	0.5 ± 0.5	3	5	5	5	6	1	6	0	4	Human mouth (16)	C/D
Preferential statherin binding															
A. viscosus	Strains (n = 6)	Statherin ≫ PRP	23.7 ± 16.2	55.2 ± 6.5	0	4	4	3	6	1	6	3	2	Rat or hamster mouth (50)	
	ATCC 19246	Statherin ≫ PRP	28.0	59.7	0	4	4	3	5	0	5	2	2	Human actinomycosis (ATCC)	
Statherin-specific binding															
A. naeslundii genospecies 1	Strains (n = 5)	Low avidity to statherin	3.7 ± 1.5	26.3 ± 7.7	1	0	0	0	5	0	5	0	4	Human blood, synovial fluid (CCUG)	
	ATCC 12104	Low avidity to statherin	4.0	25.7	1	0	0	1	6	0	6	0	4	Human sinus (ATCC)	B

[a] Adhesion of bacteria to a fixed concentrations (5 to 6 μg/ml) of PRP-1 and statherin used to coat hydroxyapatite beads. Statherin-specific binding was assayed at 25 μg of proteins/ml due to no binding at the low protein amount. The numbers of adhering bacteria are given as percentages of added bacteria. Mean values ± standard deviations are given.

[b] Slot blot DNA hybridization under high-stringency conditions with full-length DNA probes designed from the type 1 fimbria genes (*orf1* to 6 and *fimP*) in *A. naeslundii* T14V.

[c] Immunofluorescence staining of whole bacterial cells with FimP and type 2:1 fimbria-specific antisera. Scores 0 and 1, no or weak staining, respectively; scores 2, 3, and 4, moderate-, high-, and very high-intensity staining, respectively (see Materials and Methods).

[d] The sources of the strains are given in Materials and Methods.

[e] Coaggregation (coagg.) groups A, B, C or D, and F represent the coaggregation properties found in *Actinomyces* isolates from the human mouth.

[f] ATCC, American Type Culture Collection.

[g] Strains, individual strains grouped together based on virtually identical reactivity patterns.
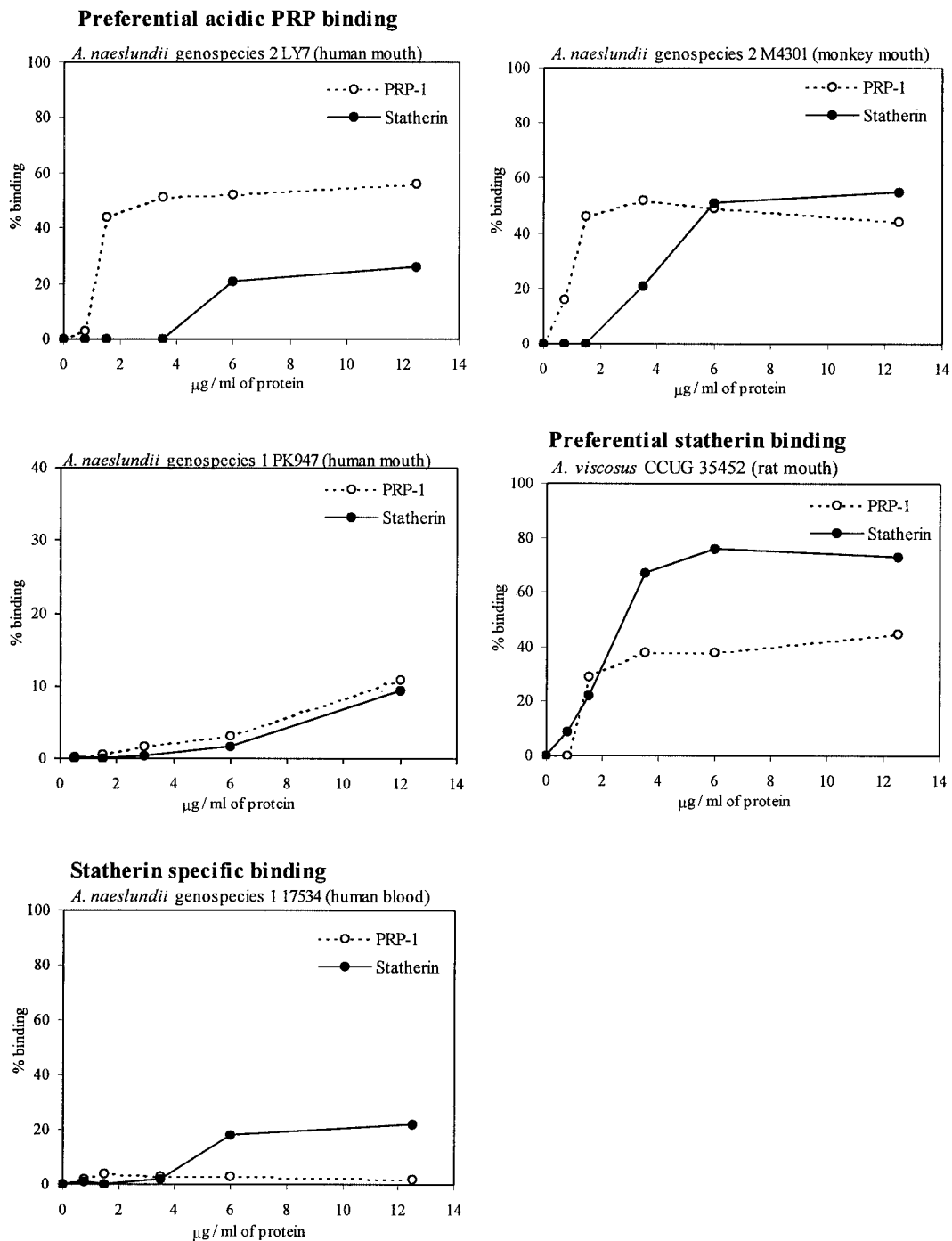
FIG. 1. Binding of *Actinomyces* strains to PRP-1 and statherin used to coat hydroxyapatite beads.

cells and bare fluorescence and cells without a distinguishable cell envelope but with faint fluorescence, respectively; scores 2, 3, and 4 indicate cells with a dark central region and a well-defined cell envelope with greenish fluorescence with moderate, high, and very high intensity, respectively, after subtraction of the presera reactivity.

Western blots of *Actinomyces* cell sonicates. *Actinomyces* cells from a 3-day culture were harvested, washed, and suspended in sterile water to a concentration of 2×10^{10} cells/ml and sonicated four times for 15 s each using a B-30 Sonifier cell disruptor (Optilab-BO Philip Instrumentation AB, Stockholm, Sweden). Proteins were then precipitated with acetone, dissolved in SDS sample

buffer (0.0625 M Tris, 10% glycerol, 2% SDS, 5 mM dithiothreitol, 0.01% pyronin), boiled, and frozen and thawed twice. After electrophoresis on 4 to 20% Tris-glycine gels (Bio-Rad) in electrophoresis buffer (0.1% SDS, 25 mM 2-amino-2-[hydroxymethyl]-1,3-propanediol, 192 mM glycine), pH 8.3, at 15 mA for 1.5 h, the proteins were transferred to polyvinylidene difluoride Immobilon-P transfer membranes with a pore size of 0.45 μm (Millipore Corporation, Bedford, Mass.). Membranes were incubated with FimP- and Orf1-specific antisera and presera (all diluted 1:800), washed, and incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit IgG antisera (1:2,000; P0448; Dako). Antibody binding was detected using Supersignal West Pico chemiluminescent
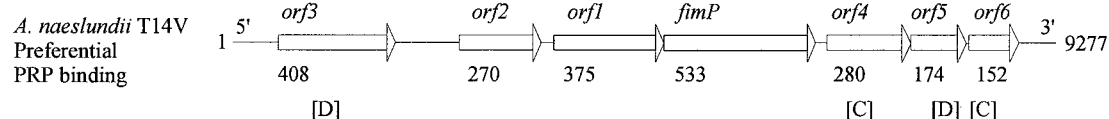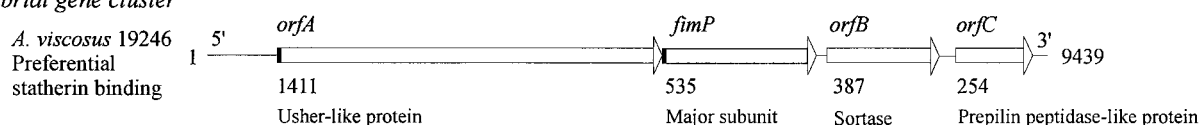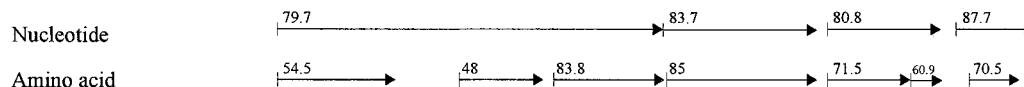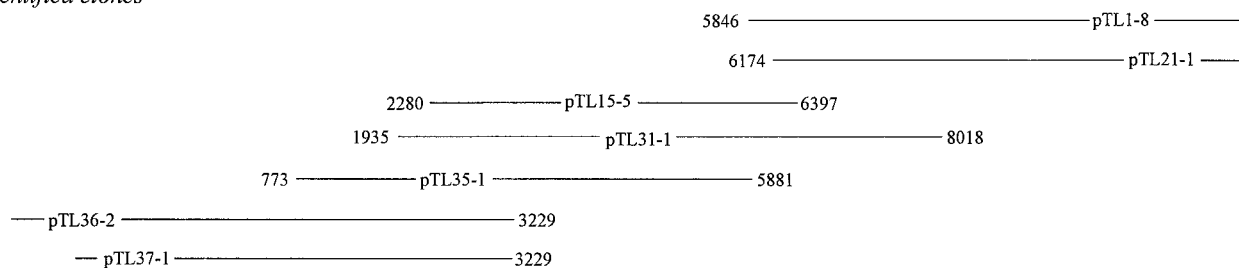
FIG. 2. Type 1 fimbria genes in *A. viscosus* strain 19246 and *A. naeslundii* strain T14V with preferential statherin and acidic-PRP binding, respectively. Bars with arrowheads, ORFs and direction of transcription; black boxes, signal peptides. The numbers of amino acids in the deduced proteins are given below each bar. The most conserved (C) and diverse (D) genes among *Actinomyces* strains with different ligand specificities are indicated. Plasmids (clones) used for sequencing and assembly of the *A. viscosus* strain 19246 type 1 fimbria gene cluster are indicated (the corresponding base positions in the type 1 gene cluster of strain T14V are given).

substrate (no. 34080; Pierce, Rockford, Ill.) and Cronex medical X-ray film 4 (Sterling Diagnostic Imaging, Inc.).

RESULTS

Acidic PRP and statherin ligand specificities among commensal and potentially pathogenic *Actinomyces* strains. A panel of *Actinomyces* strains with different animal and tissue tropisms were screened for binding to acidic-PRP-1- and statherin-coated hydroxyapatite beads (Table 1). The following protein ligand specificities were detected. (i) In preferential acidic-PRP binding, oral strains of *A. naeslundii* genospecies 2 of human ($n = 11$) and monkey ($n = 2$) origin bound avidly to acidic PRPs but deviated in their relative, although weak, capacities for binding to statherin and oral strains of *A. naeslundii* genospecies 1 bound either avidly ($n = 2$) or weakly ($n = 4$) to acidic PRPs. (ii) In preferential statherin binding, *A. viscosus* strains from rat or hamster plaque ($n = 6$) and a case of human actinomycosis bound statherin avidly and preferentially. (iii) In statherin-specific binding, strains of *A. naeslundii* genospecies 1 from septic and other human infections displayed low-avidity binding only to statherin ($n = 6$). The different ligand specificities were confirmed by determining the binding of representative strains to acidic PRP-1 and statherin at various concentrations used to coat hydroxyapatite surfaces (Fig. 1).

Type 1 fimbria genes among *Actinomyces* strains with different ligand specificities and tropisms. To investigate the type 1 fimbria genes involved in generating different *Actinomyces* ligand specificities, bacterial DNA was hybridized with DNA probes specific to the type 1 fimbria genes (*orf1* to *-6* and *fimP*) of *A. naeslundii* strain T14V with preferential acidic-PRP binding (Table 1). Three major gene patterns, on the basis of ligand specificity, were detected: (i) *A. naeslundii* genospecies 1 and 2 strains with preferential acidic-PRP binding hybridized strongly with all DNA probes, except that genospecies 1 strains virtually lacked hybridization with *orf5* and hybridized moderately with *orf3*; (ii) *A. viscosus* strains with preferential statherin binding hybridized strongly with the *orf4-* and *orf6-*specific probes, moderately with the *orf2*, *orf1*, and *fimP* probes, and weakly, if at all, with the *orf3* and *orf5* probes; and (iii) the human *A. naeslundii* septic strains with statherin-specific binding hybridized strongly only with the *orf4* and *orf6* probes. Thus, the *orf4* and *orf6* gene regions are highly conserved among *Actinomyces* spp. with different ligand specificities and tropisms.

Type 1 fimbria genes in *A. viscosus* strain 19246 with preferential statherin binding. To further investigate the type 1 fimbria genes related to preferential statherin binding, we cloned and sequenced a *fimP*-containing 9,439-bp gene cluster from *A. viscosus* strain 19246 (Fig. 2; GenBank accession no. AF106034). The 9,439-bp fragment contained four open read-

ing frames (ORFs) (*orfA*, *fimP*, *orfB*, and *orfC*) and displayed an overall 81.3% nucleotide sequence identity to the type 1 fimbrial gene cluster (*orf1* to *-6* and *fimP*) from *A. naeslundii* strain T14V, with preferential acidic-PRP binding. The predicted *orfA*, *fimP*, *orfB*, and *orfC* were homologous to *orf3-1*, *fimP*, *orf4-5*, and *orf6*, respectively, and had a high G+C content (>60%), typical of the genus *Actinomyces*.

Sequence analysis of ORFs encoding type 1 fimbria with preferential statherin binding. *orfA* contained a putative ribosome-binding site 10 bp upstream of an ATG start codon and showed a 79.7% sequence identity to *orf3*, *orf2*, and *orf1* (Fig. 2). *orfA* encoded a hydrophilic 1,411-residue protein with a putative N-terminal signal peptide (residues 1 to 45) and a cell wall-anchoring LPLSG domain (residues 1371 to 1375) followed by a transmembrane helix. The C-terminal region of OrfA displayed a 39 to 41% sequence similarity to regions of the FimP subunit protein. OrfA showed a 35 to 40% sequence similarity to (i) usher-like proteins, (ii) autotransporters (IgA protease and pullulanase), (iii) extracellular organelle assembly proteins (K88 minor fimbrial subunit and major pilin protein), and (iv) glycosidases (muramidase, neuraminidase, and pullulanase).

fimP encoded a 535-residue FimP subunit protein with an N-terminal signal peptide, a hydrophilic protein core with four cysteine residues, and a C-terminal LPLTG domain followed by a transmembrane domain (28). FimP displayed a 35 to 40% sequence similarity to the type 2 fimbria FimA subunit protein, *Streptococcus dysgalactiae* M-like and IgG binding proteins, *Neisseria meningitidis* transferrin binding protein, and *Haemophilus influenzae* adhesin.

orfB displayed a putative ribosome-binding site 12 bp upstream of a GTG start codon and 80.8% sequence homology to the corresponding regions of *orf4* and two-thirds of the 5′ end of *orf5*. A 177-bp segment corresponding to the 3′ end of *orf5* was deleted in *orfB*. The 387-residue OrfB protein contained two transmembrane domains (residues 52 to 74 and 301 to 323) and an Arg-Gly-Asp (RGD) tripeptide (residues 30 to 32). OrfB had a 42% sequence similarity to Orf365 of type 2 fimbriae and a 182-residue region with an LITC motif (residues 256 to 259) and 39% sequence similarity to *Staphylococcus aureus* sortase (30, 48).

orfC displayed a putative ribosome-binding site 6 bp upstream of an ATG start codon and 87.7% sequence homology to *orf6* and a 0.3-kb fragment downstream to *orf6*. The 254-residue OrfC protein displayed similarities to integral membrane proteins, and its N-terminal 139 residues resembled a PAP2 (type 2 phosphatidic acid phosphatase) domain, a sequence motif shared by bacterial and mammalian acid phosphatases. OrfC displayed 40% sequence similarity to *Pseudomonas putida* type 4 prepilin-like protein leader peptide-processing enzyme and *Mycobacterium* lipoprotein signal peptidase.

Antiserum detection of predicted ORFs. To detect the presence of predicted ORFs and posttranslational processing of the type 1 fimbrial proteins, whole-cell sonicates from *A. viscosus* strain 19246 and *A. naeslundii* strain T14V were analyzed in Western blots using FimP- and Orf1 (C-terminal OrfA domain)-specific antisera (Fig. 3). The FimP antiserum detected high-molecular-weight (hmw) components and 65- and 45-kDa proteins in both strains, indicating polymerized (hmw), native
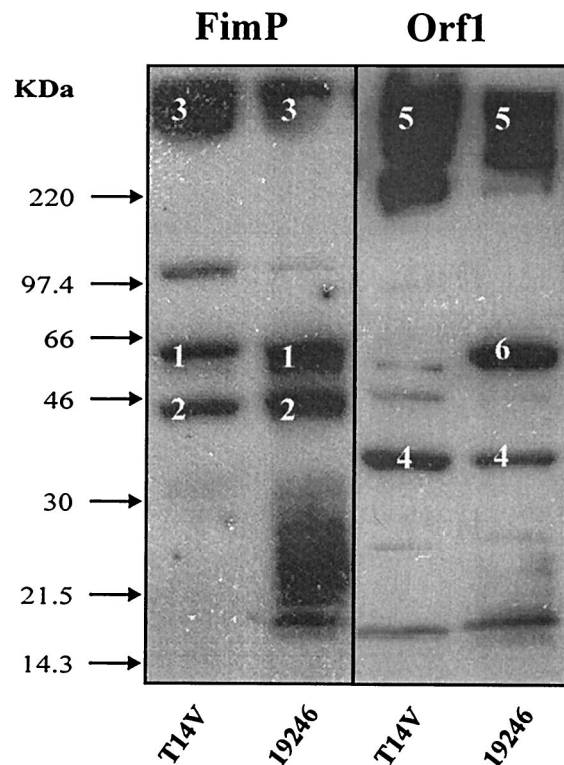

FIG. 3. Detection of FimP- and Orf1-like proteins in *A. viscosus* strain 19246 and *A. naeslundii* strain T14V by specific antisera. Whole-cell proteins were separated by SDS-PAGE, blotted to membranes, and reacted with FimP- and Orf1-specific antisera. Marked proteins are native (1), truncated (2), and polymerized (3) FimP and native (4) and polymerized (5) Orf1 in strain T14V and truncated (4 and 6) and polymerized (5) OrfA in strain 19246. Arrows, molecular weight markers.

(65-kDa) and truncated (45-kDa) FimP proteins, respectively. The Orf1 antiserum detected hmw components in both strains, suggesting the presence of Orf1 (and OrfA) in polymerized fimbriae. In addition, the Orf1 antiserum detected a 39-kDa protein in both strains, indicating proteolytic processing of OrfA to a Orf1-like 39-kDa protein in strain 19246, as well as a 65-kDa protein in strain 19246, indicating larger OrfA fragments.

Type 1 and type 2 fimbria patterns on *Actinomyces* spp. with different ligand specificities. Immunofluorescence staining with type 1 (FimP) and type 2:1 fimbria-specific antisera showed that the investigated strains carried either type 1, type 2, or both types of fimbriae on their surfaces (Table 1). The type 2:1 antisera detected type 2 fimbriae on all strains except for *A. naeslundii* genospecies 2 strains that harbor a structurally different FimA subunit protein (17). The type 1 (FimP) antisera detected type 1 fimbriae on all strains with high-avidity binding to acidic PRP or statherin but not on those strains with low-avidity binding to statherin or acidic PRPs. Thus, FimP and type 2:1 antisera may detect some, but not all, heterogeneity in ligand binding mediated by structural variations in type 1 and type 2 fimbriae.

***Actinomyces* strains with preferential acidic-PRP and statherin binding represent phylogenetically different lin-**

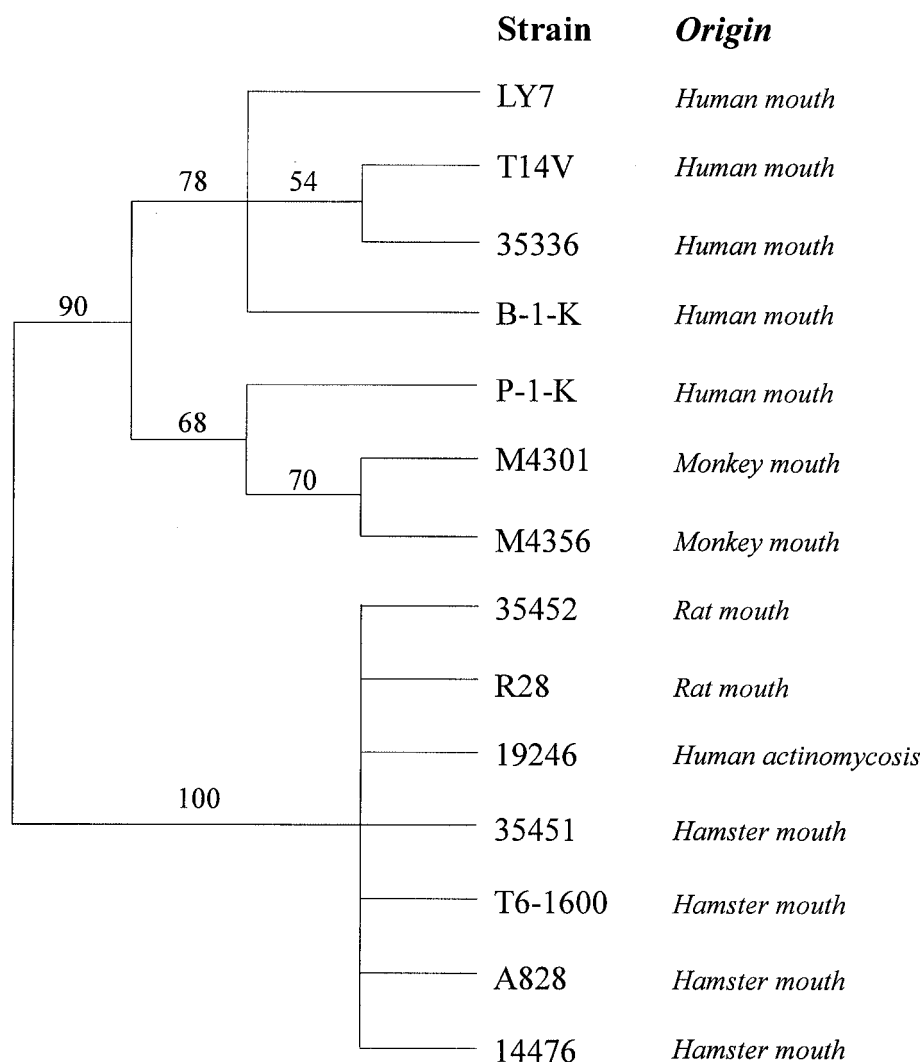| Strain	*Origin*
LY7	*Human mouth*
T14V	*Human mouth*
35336	*Human mouth*
B-1-K	*Human mouth*
P-1-K	*Human mouth*
M4301	*Monkey mouth*
M4356	*Monkey mouth*
35452	*Rat mouth*
R28	*Rat mouth*
19246	*Human actinomycosis*
35451	*Hamster mouth*
T6-1600	*Hamster mouth*
A828	*Hamster mouth*
14476	*Hamster mouth*

FIG. 4. Clustering of oral strains of *Actinomyces* strains of human or monkey and rat or hamster origin by RFLP analyses using *fimP* as a probe. Confidence probability values are shown at major branches.

eages. To investigate the evolutionary relatedness of strains with different ligand specificities and tropisms, *Actinomyces* strains were subjected to RFLP analysis with a *fimP* gene probe designed from strain T14V (Fig. 4). The RFLP dendrogram revealed clustering of oral human and monkey strains with preferential acidic-PRP binding, while rat and hamster strains (and strain 19246 from a human case of actinomycosis) with preferential statherin binding clustered together.

Rat and human saliva exhibit high *Actinomyces* binding irrespective of ligand specificity. Both *A. naeslundii* strain T14V (human mouth) and *A. viscosus* strain 35452 (rat mouth) bound avidly to rat and human saliva (Fig. 5a). ArgGlyArg-ProGln, a type 1 fimbria inhibitor (27), but not lactose, a type 2 fimbria inhibitor, partially blocked the binding of strain T14V to rat and human saliva, indicating type 1 fimbria-mediated binding properties. Neither of the two inhibitors blocked the binding of strain 35452, confirming its variant type 1 fimbria binding specificity.

We next investigated the capacity of rat and human saliva to bind septic rat or hamster and human *Actinomyces* strains with variant ligand specificities (Fig. 5b). Both rat and human saliva displayed high binding capacity of *Actinomyces* strains irrespective of ligand specificity. Human saliva even bound oral strains of rat or hamster origin better than those of human origin. Statherin was detected in human, but not in rat, saliva using statherin-specific antisera in Western blot experiments (data not shown).

DISCUSSION

This paper shows a diversity of acidic-PRP and statherin adhesion types among commensal and potentially pathogenic *Actinomyces* strains. Those adhesion types typical of human commensal strains bound acidic PRPs preferentially, while those typical of rat and hamster hosts and human infections bound statherin preferentially. Binding to statherin (originally detected in *A. viscosus* strain 19246 from a human case of cervicofacial actinomycosis [12]) may be involved in human infections. *A. naeslundii* 12104, isolated from a human case of
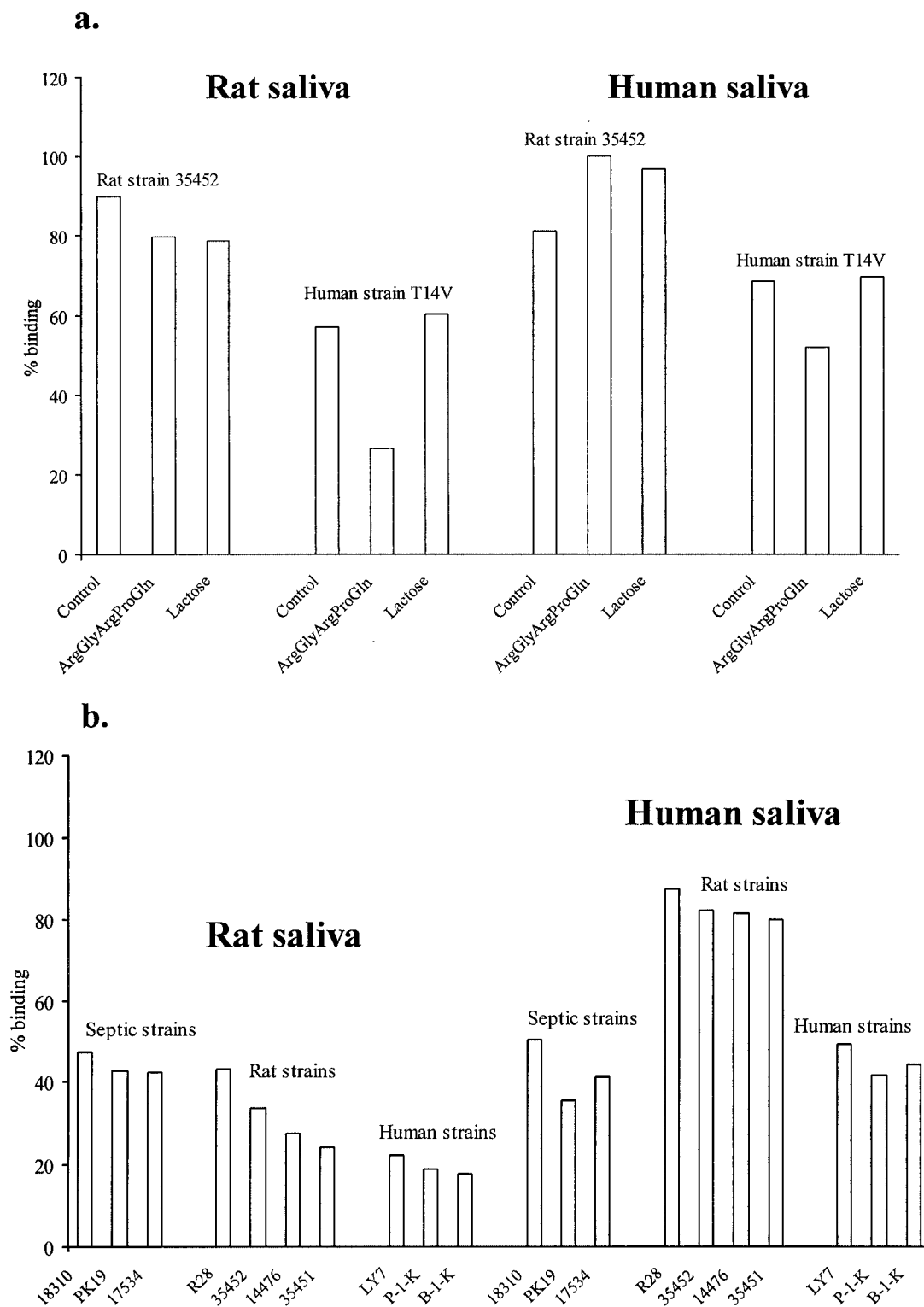
FIG. 5. Adhesion of *Actinomyces* strains to rat and human saliva used to coat hydroxyapatite beads. (a) Adhesion without (control) and with addition of the type 1 fimbria inhibitor (ArgGlyArgProGln) and type 2 fimbria inhibitor (lactose); (b) adhesion of *Actinomyces* strains from the rat and human mouths and from septic infections in humans.

sinusitis (10), and potentially invasive *Porphyromonas gingivalis* and *Candida albicans*, bind also to statherin. Moreover, statherin blocks adhesion of *C. albicans* to epithelial cells (21). In human saliva, statherin may therefore protect against potentially invasive strains recognizing statherin-mimicking epithelial cell surface ligands. The avid adhesion of statherin-binding strains to rat saliva despite its lack of statherin and the weak adhesion of human septic strains to statherin argue also for an involvement of yet-uncharacterized statherin-mimicking molecules.

The present findings also suggest a correlation between ligand specificity and presence of type 1 fimbria genes. (i) While *A. naeslundii* genospecies 2 strains with preferential acidic-PRP binding harbored highly conserved type 1 fimbria genes (*orf1* to *-6* and *fimP*), genospecies 1 strains displayed divergent *orf5* and *orf3* genes. (ii) Strains of *A. viscosus* with preferential statherin binding seemed to carry a homologous gene cluster of four genes (*orfA*, *fimP*, *orfB*, and *orfC*). Additional arguments for two major evolutionary lines of type 1 fimbriae came from RFLP analyses based on *fimP*, clustering human and monkey and rat and hamster strains into phylogenetically different groups. (iii) The human septic (and 12104) strains with statherin-specific binding exhibited only conserved *orf4* and *orf6* genes. The conserved *orf4* and *orf6* gene regions may encode intracellular sortase-, usher-, or chaperone-like functions, while the divergent *orf5* and *orf3* gene regions may encode surface-localized functions subject to selective external pressures.

The present findings may indicate a diversity of *A. naeslundii* and *A. viscosus* subpopulations that have evolved specific ligand binding properties to fit different ecological niches. Notably, *A. naeslundii* and *A. viscosus* harbor taxonomically distinct subpopulations with huge phenotypic and serologic variation (23). Moreover, reference strains of *Actinomyces* coaggregation groups A, B, C or D, and F, which exhibit type 2 fimbria-mediated coaggregations with different streptococci (25), belonged to different acidic-PRP and statherin adhesion types. Accordingly, the type 1 (FimP) and type 2 (FimA) antisera detected either type 1, type 2, or both fimbriae on all investigated strains. In this respect, it is noteworthy that studies with *E. coli* type 1 fimbriae suggested that variations in ligand specificity may transform commensal phenotypes to pathogenic ones by changing their ecological niches (42).

The present findings provide some information on the biogenesis and assembly of adhesive proteins in gram-positive bacteria. The conserved *orfB* gene encodes a sortase homolog, as evidenced by its LITC motif and 39% sequence similarity to the *S. aureus* sortase (a transpeptidase anchoring LPXTG-containing proteins to the cell walls of gram-positive bacteria) (30, 48). Many gram-positive bacteria contain sortase homologs (35), and inactivation of sortase in *S. aureus* and *Streptococcus gordonii* prevented bacterial adhesion and infections (5, 29). Furthermore, inactivation of *orf365*, a homolog of *orfB* encoding sortase, abolished type 2 fimbria polymerization and FimA surface localization in *Actinomyces* (51), suggesting that the OrfB sortase guides the LPXTG-containing OrfA and FimP proteins during type 1 fimbria biogenesis and assembly.

It is likely that the prepilin peptidase-like OrfC protein cleaves off the signal peptides from FimP and OrfA during their secretion and that FimP and OrfA interact with the OrfB

sortase. OrfA, corresponding to Orf3, Orf2, and Orf1, displays sequence similarities to ushers, autotransporters, and extracellular organelle assembly proteins and contains a C-terminal FimP-homologous region. Hypothetically, OrfA could provide a platform for fimbria assembly. The C-terminal FimP-homologous region of OrfA (Orf1-like region) could be cleaved off from OrfA and integrated into polymerized fimbriae, as suggested by the observation that the Orf1-specific antisera detected truncated Orf1-like proteins and polymerized fimbriae in strain 19246. Integrated in fimbriae, OrfA-derived proteins, either the Orf1-like C-terminal or the Orf3-like N-terminal domains, could possess adhesive properties. Notably, the *orf3* gene region correlated with binding specificity. Finally, the *fimP* and *fimA* gene structures also correlate with binding specificity (17, 28), and therefore the structural subunit proteins are alternative adhesin candidates.

In conclusion, fimbria-mediated adhesion of *Actinomyces* spp. may provide a powerful model to (i) characterize proteins involved in biogenesis and assembly of adhesive organelles in gram-positive bacteria and (ii) understand the role of commensal bacteria and adhesion types in health and chronic infectious diseases.

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