Identification of a 95 kDa putative adhesin from *Actinomyces* serovar WVA963 strain PK1259 that is distinct from type 2 fimbrial subunits

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The species *Actinomyces* serovar WVA963 is among the 20 bacteria most frequently isolated from human subgingival plaque. The interactions of this species with streptococci are inhibited by lactose, a function associated with type 2 fimbrial surface structures in *Actinomyces naeslundii*. Type 1 fimbriae mediate binding of cells to salivary proline-rich proteins. Specific polyclonal antisera against type 1 and type 2 fimbriae of *A. naeslundii* T14V revealed both types of fimbriae on *Actinomyces* serovar WVA963 strain PK1259. To investigate the role of type 2 fimbriae of strain PK1259 in *Actinomyces*–*Streptococcus* lactose-inhibitable coaggregations, spontaneous coaggregation-defective (*Cog*[−]) mutants that failed to coaggregate with streptococci were isolated; three were chosen for study. All three mutant strains synthesized type 1 fimbriae and a 59 kDa protein; mutant strains PK2415 and PK3092 synthesized type 2 fimbriae and a 57 kDa protein. In contrast, the *Cog*[−] strain PK2407 did not agglutinate with anti-type 2 antibodies or show the 57 kDa band, suggesting that the 57 kDa protein was the type 2 fimbrial subunit. Polyclonal antiserum raised against the *Actinomyces* serovar WVA963 strain PK2399, an antibiotic-resistant derivative of wild-type PK1259, blocked coaggregation between this strain and streptococci. Anti-PK2399 serum absorbed with mutant strain PK3092 bearing type 2 fimbriae retained its blocking ability. Surface sonicates of the parent and mutant strains were adsorbed to streptococcal cells and to lactose-agarose beads. Lactose eluates from both the streptococcal cells and the affinity beads were characterized by SDS-PAGE and corresponding immunoblots using anti-PK2399 serum absorbed with *Cog*[−] mutant PK3092. These blots revealed a 95 kDa putative adhesin in the parent strain PK2399 that was absent in the *Cog*[−] mutant strain PK3092. These results suggest the presence of a putative 95 kDa actinomyces adhesin distinct from the 57 kDa type 2 fimbrial subunit and that this adhesin mediates lactose-inhibitable coaggregation with streptococci.

Keywords: oral actinomyces, adhesin, fimbriae, coaggregation

INTRODUCTION

Actinomyces are among the initial colonizers during early plaque development (Nyvad & Kilian, 1987).

Besides their ability to bind to the tooth surface, they also interact with other genera of oral bacteria such as streptococci (Gibbons & Nygaard, 1970), veillonellae (Hughes *et al.*, 1988) or fusobacteria (Kolenbrander *et al.*, 1989) as part of dental plaque (Kolenbrander & London, 1993). Some of these coaggregations are mediated by a heat- and protease-sensitive actinomyces

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Abbreviation: PRP, proline-rich protein.

adhesin that binds to a cognate carbohydrate receptor on the streptococcal surface (McIntire *et al.*, 1978). Oral actinomyces synthesize two different types of fimbriae; type 1 fimbriae bind to proline-rich proteins (PRPs) (Gibbons *et al.*, 1988) present in saliva (Clark *et al.*, 1984), and type 2 fimbriae are involved in lactose-inhibitable intergeneric coaggregations with streptococci (Cisar *et al.*, 1983), as well as lactose-inhibitable binding to eukaryotic cells (Brennan *et al.*, 1984, 1986). Type 2 fimbriae are present on all *Actinomyces naeslundii* strains so far examined, but type 1 fimbriae are not produced by all these actinomyces (Cisar *et al.*, 1984; Johnson *et al.*, 1990; Yeung, 1992).

The genes encoding the fimbrial subunits of type 1 and type 2 fimbriae of *A. naeslundii* strains T14V and WVU45 have been cloned and sequenced (Donkersloot *et al.*, 1985; Yeung *et al.*, 1987; Yeung & Cisar, 1988, 1990). Both subunits are about 60 kDa in size and contain the cell surface binding motif LPxTG in the C-terminal region that is characteristic of many Gram-positive surface proteins (Schneewind *et al.*, 1992, 1995). The deduced amino acid sequences of FimA (subunit of type 2 fimbriae) of *A. naeslundii* WVU45 and that of FimP (subunit of type 1 fimbriae) of *A. naeslundii* T14V are 34% identical (Yeung & Cisar, 1990). The amino acid sequence homology between the FimA proteins of *A. naeslundii* WVU45 and *A. naeslundii* T14V is 70% (J. A. Donkersloot, personal communication; Yeung & Cisar, 1988). Studies by Cisar and colleagues (Cisar *et al.*, 1983, 1984) showed that *A. naeslundii* strains lacking FimA (type 2 fimbriae) have lost the ability to mediate lactose-inhibitable coaggregation with streptococci. However, it remains unclear if FimA possesses the lactose-sensitive adhesive function or if an accessory fimbrial protein is responsible (Cisar *et al.*, 1991). The presence of FimP on the cell surface was shown to be insufficient for adherence to salivary PRPs (Yeung, 1995). A minor protein at the tip of the type 1 fimbriae has been proposed to mediate the binding of *A. naeslundii* T14V to salivary PRPs (Nesbitt *et al.*, 1992).

This raised the question as to whether there may also be a distinct protein associated with FimA (type 2 fimbriae) or whether the FimA subunit itself provides the adhesive function. Examples of both possibilities have been documented for Gram-negative bacteria (Abraham *et al.*, 1987; Irvin *et al.*, 1989; Moch *et al.*, 1987; Uhlin *et al.*, 1985; Weiss *et al.*, 1988). In *Pseudomonas aeruginosa*, the adhesive function is located in the C-terminal region of the 15 kDa structural subunit of the type IV pili (Lee *et al.*, 1994). In the oral bacterium *Prevotella loescheii*, a distinct 75 kDa adhesin (London & Allen, 1990) is located at or near the tips of fimbriae (Weiss *et al.*, 1988). The extensively studied *Escherichia coli* PapG adhesin is found as part of a specialized structure at the tip of the P pili (Kuehn *et al.*, 1992). Recent studies have also shown that the FimH adhesin of type 1 pili in *E. coli* is assembled into a fibrillar tip structure that is distinct from the fimbrial subunit (Jones *et al.*, 1995).

To investigate the possibility that an actinomyces adhesin distinct from the type 2 fimbrial subunit may

mediate lactose-inhibitable coaggregations with streptococci, we chose *Actinomyces* serovar WVA963 strain PK1259 because it interacts only by lactose-inhibitable coaggregations with its streptococcal partners (Kolenbrander *et al.*, 1983). Spontaneously occurring mutants were selected for failure to coaggregate with streptococci and analysed and compared to the wild-type PK1259 for the presence of adhesins.

METHODS

Bacterial strains and culture conditions. All bacterial strains were of human origin and are listed in Table 1. The oral strain *Actinomyces* serovar WVA963 strain PK1259 was isolated from a subgingival site (Kolenbrander *et al.*, 1983). Actinomyces and streptococci were grown at 37 °C in CAMG medium (Maryanski & Wittenberger, 1975) containing (l⁻¹): tryptone (5.0 g); yeast extract (5.0 g); glucose (2.0 g); dibasic potassium phosphate (5.0 g); 0.5 ml Tween 80 (Sigma); and adjusted to pH 7.4. Cells were grown under anaerobic conditions [N₂/CO₂/H₂ (80:10:10, by vol.)] in Gas Pak systems (BBL Microbiology Systems). Veillonellae and fusobacteria were cultivated as described previously (Hughes *et al.*, 1988; Kolenbrander *et al.*, 1989). Bacterial cells used for coaggregation were grown overnight, washed three times and resuspended in coaggregation buffer (0.1 mM CaCl₂, 0.1 mM MgCl₂, 150 mM NaCl, 0.02%, w/v, sodium azide buffered with 1 mM Tris/HCl, pH 8.0).

Coaggregation assay. The coaggregation of the actinomyces strains with other oral bacteria was tested by the visual coaggregation assay (Kolenbrander & Andersen, 1990). Cells were grown overnight, harvested, washed, resuspended in coaggregation buffer and adjusted to a concentration of about 1 × 10⁹ cells ml⁻¹ [260 Klett units (red filter); Klett-Summerson]. Equal amounts of each cell suspension (100 µl) were mixed and scored for coaggregation. A score of 0 was given when, after mixing, no change in turbidity could be observed in the cell suspension; a maximum score of 4 was given when, after mixing, the coaggregated cells sedimented immediately, leaving a clear supernatant. Suspensions of each cell type individually showed no autoagglutination.

Selection for Cog⁻ mutants. Strain PK2399, a spontaneous antibiotic-resistant derivative of PK1259, was used to obtain spontaneous Cog⁻ mutants by selecting those that failed to coaggregate with their streptococcal partners as described previously (Kolenbrander, 1982). Strain PK2399 was obtained by successive plating of strain PK1259 on agar containing 1000 µg streptomycin (Sigma) or 500 µg spectinomycin (Sigma) (Table 1). A suspension (10⁹ cells ml⁻¹) of strain PK2399 was mixed with a dense cell suspension (10¹⁰ cells ml⁻¹) of the appropriate streptococcal partner and centrifuged at 400 g for 2 min to remove coaggregates. The supernatant fluid was collected and additional partner cells were added; the procedure was repeated until no visible aggregates were seen. The resulting mutant-enriched supernatant fluid was plated on antibiotic-containing media. Colonies were selected and tested for coaggregation with the streptococcal partner in microtitre wells (Kolenbrander, 1982). Isolates that appeared coaggregation-negative with the appropriate partner were purified on antibiotic media and retested to confirm the Cog⁻ phenotype. All Cog⁻ strains were tested for coaggregation with the other streptococcal reference strains as well as with other strains of oral bacteria.

Electron microscopy. Strains of *Actinomyces* serovar WVA963 were prepared for negative staining by the method of Handley

Table 1. Organisms used in this study

Strain	Characteristics	Reference/source
<i>Actinomyces</i> serovar WVA963 strain PK1259	Wild-type, reference strain for actinomyces coaggregation group F	Kolenbrander <i>et al.</i> (1983)
PK2399	Parent, double-antibiotic-resistant derivative of PK1259*	This study
PK2407	Cog ⁻ mutant of PK2399 selected with C104	This study
PK2415	Cog ⁻ mutant of PK2399 selected with C104	This study
PK3092	Cog ⁻ mutant of PK2399 selected with 34	This study
<i>Actinomyces naeslundii</i> T14V	Expresses both type 1 and type 2 fimbriae	Cisar <i>et al.</i> (1988)
<i>Actinomyces naeslundii</i> 5519	Expresses only type 1 fimbriae	Cisar <i>et al.</i> (1988)
<i>Actinomyces naeslundii</i> 147	Expresses no fimbriae	Cisar <i>et al.</i> (1988)
<i>Streptococcus gordonii</i> DL1	Reference strain for coaggregation group 1	Kolenbrander & Andersen (1986)
<i>Streptococcus oralis</i> H1	Reference strain for coaggregation group 2	Kolenbrander & Andersen (1986)
<i>Streptococcus oralis</i> 34	Reference strain for coaggregation group 3	Kolenbrander & Andersen (1986)
<i>Streptococcus oralis</i> C104	Reference strain for coaggregation group 3	Kolenbrander & Andersen (1986)
<i>Streptococcus oralis</i> J22	Reference strain for coaggregation group 4	Kolenbrander & Andersen (1986)
<i>Streptococcus</i> SM strain PK509	Reference strain for coaggregation group 5	Kolenbrander & Andersen (1986)
<i>Streptococcus gordonii</i> PK488	Reference strain for coaggregation group 6	Kolenbrander & Andersen (1986)
<i>Veillonella atypica</i> PK1910	Reference strain for veillonellae coaggregation group I	Hughes <i>et al.</i> (1988)
<i>Fusobacterium nucleatum</i> PK1594	Coaggregation partner of all actinomyces strains used in this study	Kolenbrander <i>et al.</i> (1989)

* Resistant to 1000 µg streptomycin ml⁻¹ and 500 µg spectinomycin ml⁻¹.

et al. (1984, 1985). Cells were incubated statically for 48 h, collected by centrifugation at 1500 g, and washed three times in distilled water. The final pellet was resuspended to give a milky suspension. A drop of this suspension was placed on a Formvar-coated copper grid (400 mesh; Agar Aids) which had been carbon-coated in a Bio-Rad E6200 Turbo Coater and plasma glowd in a Fisons Instruments PT7150 RF Plasma Etcher. Bacteria were negatively stained with 1% (w/v) methylamine tungstate (EM Scope). Micrographs of fimbriae were taken on a Hitachi 600 electron microscope at 100 kV, at magnifications of 60000 and 80000.

Agglutination of whole cells with PRP-coated latex beads. PRPs bound to polystyrene latex beads (Polysciences) were prepared as described by Gibbons *et al.* (1988) and Hsu *et al.* (1994). As a control, bovine serum albumin was bound to latex beads. To test binding to PRPs, 100 µl PRP-coated latex beads was mixed with 5 µl bacterial cell suspension (1×10^9 cells ml⁻¹) in flat-bottom microtitre wells (Flow Laboratories). Agglutination was scored after overnight incubation at 4 °C.

Immunological testing for type 1 and type 2 fimbriae on *Actinomyces* serovar WVA963 strains. IgG of rabbit antiserum R59 against type 1 fimbriae and IgG of rabbit antiserum R55 against type 2 fimbriae of *A. naeslundii* T14V were kindly provided by J. Cisar (Cisar *et al.*, 1988). Agglutination of cells by these antibodies was tested by mixing 25 µl antibody (100–0.1 µg protein ml⁻¹, final concentration) with an equal amount of *Actinomyces* serovar WVA963 cells (5×10^8 cells

ml⁻¹ in coaggregation buffer) in V-shaped microtitre plates (Dynatech Laboratories). Agglutination was scored after overnight incubation at 4 °C. Cells of *A. naeslundii* T14V were included as a positive control.

Anti-PK2399 serum. Antiserum was raised in New Zealand White rabbits by injection of 0.5 ml containing 5×10^8 cells of *Actinomyces* serovar WVA963 strain PK2399, suspended in coaggregation buffer (without sodium azide) with an equal amount of Freund's complete adjuvant. Preimmune serum was withdrawn and the injection schedule was started with an intradermal inoculation. Boost injections without Freund's adjuvant were administered intravenously in the marginal ear vein at 9 d intervals thereafter. Two test bleeds followed by two production bleeds, 5 and 25 ml, respectively, were taken at 18 d intervals and two additional production bleeds were taken at 3 d intervals just prior to exsanguination on day 80. Antisera were screened for the ability to agglutinate strain PK2399 and to block coaggregation between strain PK2399 and its streptococcal partners, strains 34 and C104.

Antiserum absorption with Cog⁻ mutants. Antiserum raised against the parent strain was repeatedly absorbed with 0.6 g wet weight of cells of a Cog⁻ mutant until the antiserum failed to agglutinate the Cog⁻ mutant strain. For 3 ml antiserum, this usually required about 10–15 absorptions with 0.6 g Cog⁻ mutant cells in each absorption. Mutant-absorbed antiserum was tested for its ability to inhibit coaggregation between the parent actinomyces strain PK2399 and its streptococcal

partners, strains 34 and C104. The protein concentration of absorbed antiserum was between 6 and 35 mg ml⁻¹.

Blocking of coaggregation with antiserum. Bacterial cell suspensions were adjusted to 160 Klett units (about 5×10^8 cells ml⁻¹) in coaggregation buffer. A 50 µl aliquot of actinomyces cell suspension was mixed with 10 µl of undiluted or diluted antiserum (diluted in modified coaggregation buffer containing 100 mM Tris, pH 8.0, and 0.2%, w/v, BSA) in a silanized glass tube (10 × 75 mm) and incubated for 30 min at room temperature. These tubes were prepared by coating glass tubes with Sigmacote (Sigma), rinsing three to four times with distilled water, and drying. After adding 50 µl streptococcal partner cells and vortexing, the coaggregation was scored. A score of 0 was given when, after mixing of two partners, no change in turbidity could be observed; a maximum score of 4 was given when, after mixing the cells, the coaggregated cells sedimented immediately, leaving a clear supernatant. Controls containing preimmune sera did not agglutinate actinomyces and did not block coaggregations. Also, the modified coaggregation buffer used to dilute the antiserum did not inhibit coaggregation.

It should be noted that coaggregation blocking was determined immediately after adding partner cells to the 30 min preincubated actinomyces/antiserum mixture. Coaggregations were detectable immediately after partner cell addition and were often enhanced by mixing the vessel contents, whereas agglutination was not detectable until several hours after the initial mixing and was scored without additional mixing. Agglutination was routinely scored after overnight incubation at 4 °C. In each coaggregation-blocking experiment, an agglutination control consisting of the parent strain PK2399 and a non-partner streptococcus, *Streptococcus gordonii* DL1, was included; no agglutination was detected in any of the coaggregation-blocking experiments.

Surface preparations from actinomyces strains using mild sonication. Cells were grown overnight in 1 litre screw-capped bottles. Cells were harvested and washed three times in PBS (1.7 mM KH₂PO₄, 5 mM Na₂HPO₄ and 0.15 M NaCl, pH 7.4). After a final wash in 20 mM Tris/HCl (pH 8.0), cells were resuspended in ice-cold 20 mM Tris/HCl (pH 8.0) at a final concentration of 0.2–0.3 g wet weight ml⁻¹. In an ice-bath, a glass tube containing 3 ml cell suspension was exposed to mild ultrasonication with a microdisruptor KT40 (maximum power is 50 W; Kontes) for two 1 min periods. No cell breakage was observed by microscopic examination of the sonicated cells. All subsequent procedures were done at 4 °C. The sonicated cells were pelleted by centrifugation for 20 min at 27000 g. The supernatant was centrifuged two additional times at 35000 g for 40 min and then filtered through a low protein binding filter (0.22 µm pore diameter; Millipore). The protein concentration of the supernatant fluids was determined by the Micro-Bradford assay (Bio-Rad) using bovine serum albumin as standard. A typical protein concentration of the sonicated surface preparations was 0.3–0.6 mg ml⁻¹.

SDS-PAGE and immunoblotting. The actinomyces surface preparations were boiled three times for 5 min under reducing conditions (10%, v/v, β-mercaptoethanol) in SDS-PAGE loading buffer (0.125 M Tris/HCl, pH 6.8; 4%, w/v, SDS; 20%, v/v, glycerol; 0.004%, w/v, bromophenol blue) (Laemmli, 1970). A sample of usually 5 µg protein per lane was separated on precast 4–20% Tris/glycine gels (Novex). Separated proteins were stained with the ISS Pro-Blue staining kit (Integrated Separation Systems). For immunoblots, the gels were blotted (Towbin *et al.*, 1979) onto nitrocellulose paper (no. 00770, 0.20 µm pore size; Schleicher & Schuell) in a Mini-Protein II Transfer Apparatus (Bio-Rad) or Western Transfer

Apparatus (Novex) in buffer containing 134.4 mM glycine, 17.5 mM Tris/base and 20% (v/v) methanol. The molecular masses of actinomyces proteins were determined by comparing the distance migrated with prestained molecular mass standards (Bio-Rad): phosphorylase A, 106 kDa; bovine serum albumin, 80 kDa; ovalbumin 49.5 kDa, carbonic anhydrase, 32.5 kDa; soybean trypsin inhibitor, 27.5 kDa; and lysozyme, 18.5 kDa. Proteins blotted to nitrocellulose were incubated with IgG of rabbit antiserum R59 against type 1 fimbriae of *A. naeslundii* T14V or with antiserum R55 against type 2 fimbriae of *A. naeslundii* T14V or with Cog⁻-mutant-absorbed rabbit antiserum against either parent *Actinomyces* serovar WVA963 strain PK2399 or against surface sonicates of the parent strain bound to lactose-agarose beads (1:300–1:1000 dilution containing between 10 and 35 µg protein ml⁻¹). Bands were visualized by a goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad). Immune complexes were visualized with alkaline-phosphatase-conjugated anti-rabbit IgG (Bio-Rad) and a dye indicator system supplied by Promega.

Adsorption of actinomyces surface sonicates to lactose-agarose beads. The adsorption of mild sonicates of actinomyces cells to lactose-agarose affinity beads was slightly modified from the method of Hughes *et al.* (1992). Surface sonicates were obtained by using cells (12–22 g wet wt) from 8 to 16 l culture medium. After a final wash in 20 mM Tris/HCl (pH 8.0), cells were resuspended in 20 mM Tris/HCl (pH 8.0) to a final concentration of 0.5–0.7 g cells wet weight ml⁻¹ in the presence of 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride and 50 mM iodoacetic acid. Aliquots of about 15 ml cell suspension were placed in an ice-bath and exposed twice for 1 min to mild ultrasonication as described above. A typical protein concentration of a sonic surface preparation was in the range of 2–4 mg ml⁻¹.

After addition of 0.02% (w/v) sodium azide and 1 mM CaCl₂, the supernatant was mixed with 1.5 ml prewashed (two washes with 10 ml 20 mM Tris/HCl, pH 8.0) lactose-agarose beads (Sigma) in a polypropylene tube. The surface sonicate/bead mixture was rotated end-over-end overnight at 4 °C. The lactose-agarose beads were washed six times with adsorption buffer (20 mM Tris/HCl, pH 8.0; 1 mM CaCl₂; 0.02%, w/v, sodium azide). Bound proteins were eluted by incubation with 2–4 ml 100 mM lactose in 20 mM Tris/HCl (pH 8.0), 2 mM EDTA and 0.02% (w/v) sodium azide for 6 h at 4 °C. The final wash prior to the lactose elution as well as the material eluted with lactose were dialysed overnight against 20 mM Tris/HCl (pH 8.0), 2 mM EDTA and 0.02% (w/v) Na₂S₂O₃. The dialysed samples were concentrated from 2 ml to about 50 µl in Centricon-10 centrifugal concentrators (Amicon). These concentrated samples were very dilute and had protein concentrations in the background range of the Micro-Bradford assay (less than 2 µg ml⁻¹). In control experiments, surface sonicates were adsorbed to mannose-agarose beads (Sigma) which were chemically prepared by the manufacturer in the same way and treated here in an identical manner to the lactose-agarose beads.

Adsorption of surface sonicates to streptococcal partner cells. Cells from a 3 l culture of *Streptococcus oralis* 34 (4.5 g wet wt) were harvested by centrifugation and washed three times in PBS and finally with 20 mM Tris/HCl (pH 8.0). Surface sonicate obtained from 12 g wet weight of actinomyces cells was suspended in about 25–30 ml adsorption buffer and was incubated overnight with 4.5 g streptococcal partner cells by rotating end-over-end in the presence of protease inhibitors. Cells were subjected to five cycles of washing with adsorption buffer and centrifugation for 10 min at 10000 g. Proteins were

eluted from the streptococcal cells by incubation with 5 ml 100 mM lactose in 20 mM Tris/HCl, 2 mM EDTA and 0.02% (w/v) NaN_3 for 6 h. The lactose eluate was dialysed, concentrated by ultrafiltration with a Centricon-10 unit as described above and analysed by an SDS-PAGE/immunoblot procedure. The protein concentration of the concentrated washes was at background level (less than $2 \mu\text{g ml}^{-1}$) of the Micro-Bradford assay (Bio-Rad).

Antiserum against actinomyces surface components bound to lactose-agarose affinity beads. The surface sonicate obtained from 20 g wet weight cells (16 l culture) of strain PK2399 was filtered through a low protein binding filter (0.22 μm pore diameter; Millipore) and adsorbed to 1 ml lactose-agarose beads as described above. After overnight adsorption, the lactose-agarose beads were washed six times with adsorption buffer without sodium azide and kept frozen in 0.5 ml aliquots. A 0.5 ml aliquot of the agarose beads was mixed with Freund's complete adjuvant and injected subcutaneously into a New Zealand White rabbit on day zero. Boost injections were performed every 3 weeks using 0.5 ml beads without addition of Freund's adjuvant. Test and production sera were taken every 21 d until exsanguination on day 120. Antisera were tested for their ability to agglutinate strain PK2399 and to block coaggregation between strain PK2399 and *S. oralis* strains 34 and C104.

RESULTS

Selection of Cog^- mutants of *Actinomyces* serovar WVA963 strain PK1259

Spontaneous mutants of the double-antibiotic-resistant strain PK2399 that failed to coaggregate with the streptococcal partners of the parent and wild-type strains were selected. Of the reference strains from the six streptococcal coaggregation groups (Table 1), only those representing groups 3, 4 and 5 coaggregated with wild-type PK1259 and parent strain PK2399, and these interactions were lactose-inhibitable (Table 2). With the

intent of obtaining different kinds of Cog^- mutants, two different selections for Cog^- mutants were carried out by using two streptococcal strains representing coaggregation group 3. The two strains *S. oralis* 34 and C104 representing streptococcal coaggregation group 3 were chosen as partners. From these two selections, a total of 35 Cog^- mutants was obtained. All mutants failed to coaggregate with all streptococcal reference strains; however, all still interacted with *Fusobacterium nucleatum* PK1594 (Table 2). Five Cog^- mutants, represented by strain PK2407, also did not coaggregate with *Veillonella atypica* PK1910. The other 30 mutants represented by strains PK2415 and PK3092 still coaggregated with *V. atypica* PK1910. Five mutants represented by PK2407 and an additional five mutants represented by PK2415 were selected with *S. oralis* C104; the other 25 mutants represented by PK3092 were selected with *S. oralis* 34.

Electron microscopy of surface structures

Electron microscopic examination of the wild-type PK1259 revealed peritrichous fimbriae on the cell surface (Fig. 1). The parent and all Cog^- mutant cells appeared morphologically identical in shape and fimbrial structures (data not shown) to those found on the wild-type. The fimbriae appeared as flexible structures of varying length with an overall mean diameter of 3.30 nm (± 0.05 nm). Each population examined was composed of cells with different densities of fimbriation, with some cells carrying densely distributed fimbriae or more sparsely distributed fimbriae. No clear substructure was visible at higher magnifications. It was not possible to distinguish two structural types of fimbriae on any of the cell types examined. There was no correlation of fimbrial size, length, width or number per cell with coaggregation phenotype.

Table 2. Intergeneric coaggregation of *Actinomyces* serovar WVA963 strains with several streptococci, *V. atypica* PK1910 and *F. nucleatum* PK1594

The method for assigning coaggregation scores has been described by Kolenbrander & Andersen (1990). A score of 4 is the maximum. Scores printed in bold indicate coaggregations that are reversed by adding lactose (60 mM final concentration). Coaggregation groups are in parentheses.

<i>Actinomyces</i> serovar WVA963 strains	Coaggregation score							
	Streptococcal coaggregation group reference strain							PK1910* PK1594†
	DL1 (1)	H1 (2)	34 (3)	C104 (3)	J22 (4)	PK509 (5)	PK488 (6)	
PK1259 (wild-type)	0	0	4	4	4	3	0	3 3
PK2399	0	0	4	4	4	3	0	3 3
PK2407 (Cog^- mutant) type 2 ⁻	0	0	0	0	0	0	0	0 3
PK2415 (Cog^- mutant) type 2 ⁺	0	0	0	0	0	0	0	3 3
PK3092 (Cog^- mutant) type 2 ⁺	0	0	0	0	0	0	0	3 3

* *Veillonella atypica* PK1910.

† *F. nucleatum* PK1594.

Fig. 1. Electron micrograph of fimbriae of *Actinomyces* serovar WVA963 wild-type PK1259 negatively stained with 1% methylamine tungstate. The actinomyces cell is just visible at the left edge of the micrograph. Bar, 100 nm.

Table 3. Agglutination of parent and Cog⁻ strains of *Actinomyces* serovar WVA963 with IgG against fimbriae of *A. naeslundii* T14V

Values are the lowest protein concentration ($\mu\text{g ml}^{-1}$) to cause agglutination of bacterial cells.

Strain	Antibody	
	Anti-type 1*	Anti-type 2†
PK1259	12	6.2
PK2399	6.2	6.2
PK2407	3.1	> 100‡
PK2415	3.1	6.2
PK3092	3.1	6.2
T14V	0.11	0.2

* Immune rabbit IgG (R59) directed against type 1 fimbriae (Cisar *et al.*, 1988).

† Immune rabbit IgG (R55) directed against type 2 fimbriae (Cisar *et al.*, 1988).

‡ Highest concentration tested ($100 \mu\text{g ml}^{-1}$) did not show any agglutination.

Functional characterization of fimbriae

The ability of actinomyces cells to bind to PRP-coated latex beads was evaluated. All tested strains, wild-type strain PK1259, parent strain PK2399 and Cog⁻ mutant strains PK2407, PK2415 and PK3092, agglutinated with PRP-coated latex beads in a manner indistinguishable from the control strain *A. naeslundii* T14V. There was no agglutination with latex beads coated with bovine serum albumin.

Further, the agglutination of parent and mutant strains of *Actinomyces* serovar WVA963 was tested with IgG against type 1 and type 2 fimbriae of *A. naeslundii* T14V. All strains tested (Table 3) agglutinated with R59 IgG

Fig. 2. Immunoblots of surface proteins of wild-type and mutant strains of *Actinomyces* serovar WVA963 and *A. naeslundii* T14V. Proteins were separated by 4–20% Tris/glycine SDS-PAGE, transblotted and incubated with either (a) anti-type 1 ($5.5 \mu\text{g protein ml}^{-1}$) or (b) anti-type 2 ($0.85 \mu\text{g protein ml}^{-1}$) to develop lane 2 and $5.0 \mu\text{g protein ml}^{-1}$ to develop lanes 3–9) IgG serum made against fimbriae purified from *A. naeslundii* T14V. The amount of protein loaded per lane in (a) was $5 \mu\text{g}$ in lanes 2–4 and $10 \mu\text{g}$ in lanes 5–9. The amount loaded in (b) was $2.5 \mu\text{g}$ in lane 2, $5 \mu\text{g}$ in lanes 3 and 4, and $9 \mu\text{g}$ in lanes 5–9. Lane 1 contains the prestained molecular mass standards (molecular masses are 106, 80, 49.5, 32.5, 27.5 and 18.5 kDa). *A. naeslundii* T14V strains: lane 2, wild-type T14V (type 1⁺, type 2⁺); lane 3, mutant 147 (type 1⁻, type 2⁺); and lane 4, mutant 5519 (type 1⁺, type 2⁻). *Actinomyces* serovar WVA963 strains: lane 5, parent PK2399; lane 6, Cog⁻ mutant PK3092; lane 7, Cog⁻ mutant PK2415; lane 8, Cog⁻ mutant PK2407; lane 9, wild-type PK1259.

against type 1 fimbriae of *A. naeslundii* T14V, indicating the presence of type 1 fimbriae on these strains. Type 2 fimbriae were detected on all strains except the Cog⁻ strain PK2407. In comparison to the control strain *A. naeslundii* T14V, a 30-fold higher IgG concentration was generally necessary to observe agglutination of the *Actinomyces* serovar WVA963 strains (Table 3). However, even at the highest concentration tested ($100 \mu\text{g ml}^{-1}$), anti-type 2 IgG did not agglutinate cells of the Cog⁻ mutant PK2407, indicating the absence of type 2 fimbriae on this strain.

Table 4. Blocking of coaggregation by Cog⁻ mutant absorbed anti-PK2399 serum

The absorbed antisera were against parent PK2399 and absorbed with Cog⁻ mutant PK3092 (absorbed antiserum A-3092) or Cog⁻ mutant PK2407 (absorbed antiserum A-2407). The strains listed are coaggregation partner strains of *Actinomyces* serovar WVA963 strain PK2399. —, Complete blocking, coaggregation score of 0; ±, partial blocking, coaggregation score of 1 or 2; ++, no blocking, maximum coaggregation score of 3 or 4. ND, Not done.

Antiserum dilution (final)	Coaggregation					
	Absorbed antisera preincubated with cells of parent strain PK2399 before addition of partner cell types					
	A-3092			A-2407		
	<i>S. oralis</i> C104	<i>S. oralis</i> 34	<i>F. nucleatum</i> PK1594	<i>S. oralis</i> C104	<i>S. oralis</i> 34	<i>F. nucleatum</i> PK1594
1:6	—	—	++	—	—	++
1:11	—	—	++	++	++	++
1:22	—	—	++	++	++	ND
1:33	—	—	++	++	++	ND
1:55	—	—	++	++	++	ND
1:110	±	±	++	++	ND	ND
1:220	++	++	++	++	ND	ND
1:330	++	++	++	++	ND	ND
Buffer (control)	++	++	++	++	++	++
Preimmune 1:6 dilution	++	++	++	++	++	++

Fig. 3. Comparison of the binding of parent strain surface sonicate to lactose-agarose and mannose-agarose beads. This immunoblot was developed with antiserum A-3092 (see legend to Table 4). Lanes: 1, prestained molecular mass standards (106, 80, 49.5, 32.5, 27.5 and 18.5 kDa indicated by line ticks on the left); 2, 5 µg surface sonicate of parent strain PK2399; 3, boiled lactose-agarose beads prior to adsorption of surface sonicate; 4, boiled lactose-agarose beads after the first wash with adsorption buffer; 5, boiled lactose-agarose beads after six washes with adsorption buffer; 6, boiled lactose-agarose beads after two elutions of bound proteins with 100 mM lactose in each elution buffer; 7, boiled mannose-agarose beads prior to adsorption of surface sonicate; 8, boiled mannose-agarose beads after the first wash with adsorption buffer; 9, boiled mannose-agarose beads after six washes with adsorption buffer; 10, boiled mannose-agarose beads after two elutions with 100 mM lactose.

Immunological characterization of type 1 and type 2 fimbriae of *Actinomyces* serovar WVA963 strain PK1259

To investigate the relationship of these putative fimbrial subunits with the subunits of type 1 and type 2 fimbriae of *A. naeslundii* strains T14V (type 1⁺, type 2⁺), 5519 (type 1⁺, type 2⁻) and 147 (type 1⁻, type 2⁻), surface preparations were made, the proteins were separated by SDS-PAGE, and the type 1 and type 2 subunits were identified on immunoblots by their reactions with R59 anti-type 1 and R55 anti-type 2 sera, respectively (Fig. 2). Anti-type 1 sera reacted strongly with a band of about 59 kDa in the parent strain T14V (Fig. 2a, lane 2) and strain 5519 (Fig. 2a, lane 4). This band was absent in the type 1 negative strain 147 (Fig. 2a, lane 3). Also absent in lane 3 were immunoreactive proteins of higher molecular size. For comparison, all five of the *Actinomyces* serovar WVA963 strains (Fig. 2a, lanes 5–9) produced type 1 immunoreactive proteins at 59 kDa, and all synthesized high molecular size immunoreactive proteins, indicating the presence of type 1 homologous fimbriae on these strains (Cisar *et al.*, 1991; Yeung *et al.*, 1987). As expected, the band at 59 kDa reacted much more weakly than with the *A. naeslundii* strains, since a 30-fold higher concentration of anti-type 1 serum was needed to agglutinate these strains as compared to the homologous *A. naeslundii* T14V strains (Table 3).

Likewise, surface preparations of the reference strains were reacted with anti-type 2 serum (Fig. 2b). A strongly staining band at about 57 kDa was evident in strain

T14V (Fig. 2b, lane 2), as well as numerous high molecular size proteins, as has been reported by Donkersloot *et al.* (1985) and Yeung & Cisar (1988). Neither of the reference type 2⁻ mutants (Fig. 2b, lanes 3 and 4) produced the 57 kDa protein. Since the anti-type 2 serum reacted about 30-fold more strongly with *A. naeslundii* T14V than with the *Actinomyces* serovar WVA963 strains (Table 3), a fourfold higher protein concentration of the latter strains was applied to the gel in order to detect immunoreactive proteins. Four of the *Actinomyces* WVA963 strains (Fig. 2b, lanes 5, 6, 7 and 9) possessed an immunoreactive protein at 57 kDa, but Cog⁻ mutant PK2407 did not synthesize this protein (Fig. 2b, lane 8), and it did not produce any high molecular size type 2 immunoreactive proteins (Fig. 2b, upper portion of lane 8).

Characterization of antiserum raised against whole cells of parent *Actinomyces* serovar WVA963 strain PK2399

Agglutination of whole cells of PK2399 by the polyclonal anti-PK2399 serum indicated a titre of 1:13 000. Two aliquots of the antiserum were absorbed, respectively, with either Cog⁻ strain PK3092 (class I) or Cog⁻ strain PK2407 (class II). Strain PK3092 synthesized type 2 actinomyces fimbriae, but strain PK2407 lacked these fimbriae. Anti-PK2399 serum absorbed with PK3092 (A-3092) agglutinated the parent at a 1:1024 dilution of antiserum but did not agglutinate class II mutant cells. However, anti-PK2399 serum absorbed with PK2407 (A-2407) agglutinated the parent and Cog⁻ strain PK3092 at a 1:1024 dilution of antiserum but not PK2407 at any dilution. These results suggested that the surface epitopes of class II mutant strain PK2407 were different from the class I mutant strain PK3092 and parent strain PK2399.

Anti-PK2399 serum absorbed with the parent strain PK2399 failed to block coaggregation of PK2399 and its streptococcal partners. PK3092-absorbed anti-PK2399 serum (A-3092) completely blocked the coaggregation between the parent strain PK2399 and its streptococcal partners at a 1:55 dilution (Table 4). PK2407-absorbed anti-PK2399 serum (A-2407) showed about a 10-fold weaker ability to block coaggregation. None of the absorbed antisera blocked coaggregation between the parent strain PK2399 and *F. nucleatum* PK1594, suggesting that the blocking of the streptococcal-actinomyces coaggregations was due to specific antibody recognition of lactose-binding molecules on the actinomyces surface.

Lactose-agarose beads as affinity matrix for enrichment of lactose-binding proteins in surface sonicates

Lactose-agarose beads were used as an affinity matrix to enrich for lactose-binding proteins released by mild sonication of actinomyces cells. To exclude nonspecific interactions between actinomyces surface components and the agarose beads, mannose-agarose beads were

chosen as a control matrix, since mannose did not inhibit coaggregation. A surface sonicate of the parent strain PK2399 (Fig. 3, lane 2) was adsorbed to either lactose-agarose beads or mannose-agarose beads. Proteins released by boiling from both lactose-agarose (Fig. 3, lanes 3–6) and mannose-agarose beads (Fig. 3, lanes 7–10) during several steps after the adsorption of the surface preparation were detected by an immunoblot using absorbed serum A-3092.

Neither lactose-agarose beads (Fig. 3, lane 3) nor mannose-agarose beads (Fig. 3, lane 7) showed any immunoreactive bands before mixing with the surface preparation. After mixing the lactose-agarose beads with the surface preparation and one wash with buffer, the lactose-agarose beads showed an enrichment of high molecular mass material (above the highest molecular mass standard at 106 kDa) (Fig. 3, lane 4), but the mannose-agarose beads (Fig. 3, lane 8) showed no enrichment compared to the starting surface sonicate (Fig. 3, lane 2). Comparison of the surface sonicate (Fig. 3, lane 2) with proteins remaining bound to six-times-washed lactose-agarose beads and released by boiling revealed a considerable enrichment of high molecular mass proteins (above 106 kDa) and a decrease in all other immunoreactive proteins (Fig. 3, lane 5). Further, these high molecular size proteins could be eluted by incubating the lactose-agarose beads with 100 mM lactose for 6 h at 4 °C as shown by their absence in boiled beads after lactose elution (Fig. 3, lane 6). In contrast, the mannose-agarose beads did not retain high molecular mass proteins from the surface sonicates (Fig. 3, lane 9). Washing the mannose-agarose beads with 100 mM lactose did not remove any additional proteins from the mannose-agarose beads (Fig. 3, lane 10), suggesting that the faster migrating ladder of bands (perhaps lipoteichoic acid) were bound to agarose beads non-specifically. Thus, lactose-agarose beads specifically enriched high molecular mass actinomyces surface proteins.

SDS-PAGE and immunoblot analysis of actinomyces surface proteins from parent and Cog⁻ mutant strains enriched by lactose-agarose beads

Freshly harvested cells from 8 l culture medium (about 10 g cells wet wt) were used to affinity-purify lactose-binding surface proteins. The routine lactose-agarose bead enrichment analysis of the parent included Pro-blue (Fig. 4a, lanes 1–5) and/or silver staining (Fig. 4a, lanes 6 and 7) of SDS-PAGE of surface sonicates (Fig. 4a, lane 2), the first buffer wash (Fig. 4a, lane 3), a concentrated sixth buffer wash (Fig. 4a, lanes 4 and 6; one half of entire concentrated sample loaded onto gel), and a concentrated 100 mM lactose elution (Fig. 4a, lanes 5 and 7; one-quarter of entire sample from original 8 l culture loaded onto gel). Little or no difference was observed between the starting surface sonicate preparation and the first buffer wash, which probably reflected trapped surface proteins in the bead matrix. After six washes, very little protein was detectable by Pro-blue staining (Fig. 4a, lane 4). Some high molecular

Fig. 4. Comparison of actinomyces proteins eluted from lactose-agarose beads by 100 mM lactose. (a) Protein stain of surface sonicate of the parent strain PK2399 adsorbed to lactose-agarose beads. Lanes 2–5 were stained with Pro-blue; lanes 6 and 7 were silver stained. Lanes: 1, prestained molecular mass standards (106, 80, 49.5, 32.5, 27.5 and 18.5 kDa); 2, 5 µg surface sonicate of parent strain PK2399; 3, 5 µg protein released with the first adsorption buffer wash; 4 and 6, protein released into buffer after six washes with adsorption buffer and concentrated 40-fold; 5 and 7, protein eluted with 100 mM lactose and concentrated 40-fold. Arrowheads indicate the positions of 57 and 95 kDa proteins. (b) Immunoblot of surface sonicates of the parent and Cog[−] mutant strains released from lactose-agarose beads by eluting with 100 mM lactose. The immunoblot was incubated with A-3092 (see legend to Table 4). Lanes 1–5 were loaded with identical samples of the parent strain PK2399 as listed in Fig. 4(a). Lane 6, 40-fold concentrated surface sonicate proteins of class II Cog[−] mutant PK2407 eluted from lactose-agarose beads with 100 mM lactose. Lane 7, 40-fold concentrated surface sonicate proteins of class I Cog[−] mutant strain PK3092 eluted with 100 mM lactose. The arrowhead indicates the position of the 95 kDa protein.

mass proteins, greater than 106 kDa, and some low molecular mass peptides, less than 30 kDa, were faintly visible by silver staining (Fig. 4a, lane 6). The concentrated lactose-released proteins (Fig. 4a, lanes 5 and 7), however, revealed bands that were readily detectable with Pro-blue (Fig. 4a, lane 5) in the high molecular mass range (greater than 106 kDa) (and a detectable band at 57 kDa). The silver-stained lane (Fig. 4a, lane 7) of a

different experiment showed an additional band with a molecular mass of 95 kDa.

The immunoblot (Fig. 4b, lanes 2–5) of an identical gel of parent strain samples (Fig. 4a, lanes 2–5) as well as the concentrated lactose-released proteins from the two classes of Cog[−] mutant surface sonicates (Fig. 4b, lanes 6 and 7) was developed with absorbed serum A-3092. The results showed the adsorption and elution of lactose-binding proteins from the lactose-agarose beads (Fig. 4b, lanes 2–5). The PK2399 eluate (Fig. 4b, lane 5) contained a 95 kDa protein as well as higher molecular mass proteins. The higher molecular sizes may be composed of fimbriae-adhesin polymers of various lengths. Interestingly, the 95 kDa band could also be seen in the concentrated lactose elution of the class II Cog[−] mutant strain PK2407 (Fig. 4b, lane 6), which was lacking the 57 kDa type 2 fimbrial monomer (see Fig. 2b, lane 8). Only one additional higher molecular size band of approximately twice the size of the 95 kDa protein was observed (Fig. 4b, lane 6). The concentrated lactose wash of the class I Cog[−] mutant PK3092 (Fig. 4b, lane 7) did not reveal any protein bands of 95 kDa or larger size. Since the PK3092-absorbed antiserum (A-3092) used in this immunoblot blocked coaggregation (Table 4), the 95 kDa protein it recognized might be the adhesin involved in the lactose-inhibitable coaggregations between actinomyces and streptococci. The observation that the Cog[−] mutant PK2407 bears this 95 kDa putative adhesin agrees with the finding that PK2407 cells removed most of the coaggregation-blocking antibodies from anti-parent serum (Table 4).

Antiserum raised against parent PK2399 surface sonicates adsorbed to lactose-agarose beads

Parent surface sonicate components that were bound to lactose-agarose beads (Fig. 4b, lane 5) were used to obtain antibodies directed against the putative adhesin and its high molecular size complexes. The antiserum was absorbed with class I mutant PK3092 cells, and, at a dilution of 1:55, it completely blocked the parent PK2399 coaggregation with its partner *S. oralis* 34. Even undiluted antiserum had no effect on the lactose-noninhibitable actinomyces–fusobacteria coaggregation. This antiserum was called L-A-3092, and its coaggregation-blocking ability was the same as that of the A-3092 serum (complete blocking at 1:55 dilution; see Table 4).

SDS-PAGE and immunoblot analysis of actinomyces surface proteins enriched by adsorption to *S. oralis* 34 as affinity matrix

To investigate a possible role in mediating lactose-inhibitable coaggregation with streptococci, preparations enriched for the 95 kDa protein from actinomyces surface sonicates were tested for the ability to adsorb to streptococcal partner cells. Cells of *S. oralis* 34 were mixed with the surface sonicate proteins of 12 g wet weight of actinomyces cells and rotated end-over-end for 16 h. After washing the streptococcal cells five times

Fig. 5. Comparison of actinomyces proteins bound to streptococcal partner *S. oralis* 34 and eluted with 100 mM lactose. Immunoblot of surface sonicates of *Actinomyces* serovar WVA963 strains eluted from *S. oralis* 34 with 100 mM lactose. The immunoblot was incubated with antiserum L-A-3092. Lanes: 1, prestained molecular mass standards (106, 80, 49.5, 32.5, 27.5 and 18.5 kDa) (the pointer to the left indicates the position of the 106 kDa standard); 2, 5 µg surface sonicate of wild-type strain PK1259; 3, 5 µg proteins released with the first adsorption buffer wash; 4, protein released into buffer after five washes with adsorption buffer and concentrated 40-fold; 5, 40-fold concentrated surface sonicate proteins of wild-type strain PK1259 eluted with 100 mM lactose; 6, 40-fold concentrated surface sonicate proteins of parent strain PK2399 eluted with 100 mM lactose; 7, 40-fold concentrated surface sonicate proteins of Cog⁻ mutant PK2407 eluted with 100 mM lactose; 8, 40-fold concentrated surface sonicate proteins of Cog⁻ mutant strain PK3092 eluted with 100 mM lactose; 9, 2 µg surface sonicate of *S. oralis* 34. The arrowhead on the right indicates the position of the 95 kDa band.

with buffer, the bound proteins were eluted with 100 mM lactose. Fig. 5 shows molecular mass standards (Fig. 5, lane 1), surface sonicates of the wild-type strain PK1259 (Fig. 5, lane 2), the first buffer wash (Fig. 5, lane 3) and the fifth and final wash before the lactose wash (Fig. 5, lane 4). The immunoblot was developed with L-A-3092 serum, which was very immunoreactive with high molecular mass material in surface sonicates (Fig. 5, lane 2). By comparison, the immunoreactivity of A-3092 serum with surface sonicates (see Fig. 3, lane 2 and Fig. 4b, lane 2) is primarily with lower molecular size material (below 80 kDa). This would be expected for an antiserum raised against putative lactose-sensitive adhesin and adhesin-fimbriae complexes bound to the lactose-agarose beads. Loosely bound high molecular size material was removed from the streptococcal cells by the buffer washes (Fig. 5, lanes 3 and 4).

An enrichment of the 95 kDa putative adhesin was observed with lactose eluates of the wild-type strain PK1259 (Fig. 5, lane 5), parent strain PK2399 (Fig. 5, lane 6) and the Cog⁻ mutant strain PK2407 (Fig. 5, lane 7). Prominent in the lanes of the wild-type and parent was the immunoreactive high molecular size material

that was absent in the lane of the Cog⁻ mutant strain PK2407 (Fig. 5, lane 7). Bands of about the size of adhesin dimers were again visible in the lactose eluates from the streptococcal surface (Fig. 5, lane 7) as they were in the lactose eluates from the lactose-agarose beads (see Fig. 4b, lane 6). No immunoreactive material was present in the lactose-released proteins of the class I Cog⁻ mutant strain PK3092 (Fig. 5, lane 8) or from cells of the streptococcal partner *S. oralis* 34 (Fig. 5, lane 9) that were treated identically with buffer and lactose.

DISCUSSION

This study was initiated to identify the actinomyces adhesin on *Actinomyces* serovar WVA963 that mediates lactose-inhibitable coaggregations with streptococcal partners. *Actinomyces* serovar WVA963 strain PK1259 (wild-type) was chosen since all of its known coaggregations with streptococci are lactose-inhibitable (Kolenbrander *et al.*, 1983; Kolenbrander & London, 1992). Two classes of spontaneously occurring Cog⁻ mutants were selected. Mutant strain PK3092 (class I) synthesized type 2 fimbriae, which have been shown to be involved in lactose-inhibitable coaggregations with streptococci (Cisar *et al.*, 1983, 1984), but class II mutants (e.g. PK2407) lacked these fimbriae. Since neither mutant class coaggregated with streptococci, it was assumed that the lactose-sensitive adhesin was absent from these mutants. We have shown here that a 95 kDa protein on the surface of the parent is the putative adhesin that mediates these lactose-inhibitable coaggregations with streptococci. Furthermore, it appears that the class II mutant produced this protein but was unable to present the putative adhesin properly on the cell surface. These results suggest that the putative adhesin is a protein distinct from the type 2 fimbrial subunit which is about 57 kDa in this oral actinomyces.

Antiserum raised against the parent strain and absorbed with the class I mutant PK3092 (A-3092) blocked lactose-inhibitable coaggregation with streptococci but not the noninhibitable coaggregation with fusobacteria, indicating its specificity for lactose-inhibitable coaggregations (Table 4). In contrast, class II mutant PK2407 absorbed antiserum (A-2407) lost nearly all of its blocking ability presumably because of the presence of the putative adhesin on PK2407 (Table 4). Thus, we used the class I mutant absorbed antiserum to identify the putative actinomyces lactose-sensitive adhesin in immunoblots.

Comparison of native surface sonicates of parent and mutants did not reveal any differences by SDS-PAGE or immunoblots incubated with A-3092 serum. However, the putative adhesin was detected in lactose eluates obtained from these surface sonicates after adsorption to lactose-agarose affinity beads. The putative adhesin appeared as a 95 kDa band in the parent (Fig. 4b, lane 5) and class II mutant (Fig. 4b, lane 6) but was absent in the class I mutant (Fig. 4b, lane 7). In addition, the parent strain contained a large amount of high molecular mass material (Fig. 4b, lane 5) which presumably consisted of adhesin-fimbriae complexes of various sizes. This high

molecular mass material was absent in the class II mutant (Fig. 4b, lane 6), which is consistent with the absence of type 2 fimbriae on this strain. The class II mutant contained a single additional band of higher molecular size which has the size of an adhesin dimer. For comparison, a tetramer of the 75 kDa lactose-sensitive adhesin has been reported for the oral strain *P. loescheii* PK1295 (London & Allen, 1990).

To obtain an antiserum against the putative adhesin, surface sonicate of the parent was adsorbed to lactose-agarose beads and, after extensive washing, was used as the antigen. After adsorption with class I mutant cells, this antiserum (L-A-3092) exhibited the same coaggregation-blocking ability as the mutant-absorbed anti-parent serum (A-3092). This specific blocking of lactose-inhibitable coaggregations indicated its usefulness in identifying the putative lactose-sensitive adhesin. The interaction of surface sonicates with streptococcal partner cells when used as an affinity matrix was investigated with L-A-3092. Immunoblots of lactose eluates revealed a 95 kDa band in wild-type, parent and class II mutant cells, but not in class I mutant cells. Thus, both an artificial specific affinity matrix like lactose-agarose beads and the natural streptococcal surface receptor could bind the 95 kDa lactose-sensitive protein. The bound protein remained adherent through five buffer washes, which suggests that the binding is specific.

It appears that the putative adhesin is present in very small numbers per cell, since 15–20 g wet weight of actinomyces cells was needed to obtain sufficient surface protein for enrichment by affinity chromatography. The lactose eluate had to be concentrated about 40-fold and about one-quarter of the concentrate was loaded per lane for SDS-PAGE. In another oral bacterium, *P. loescheii* PK1295, there are only about 400 molecules per cell for its lactose-sensitive adhesin (Weiss *et al.*, 1988) and this same organism presents only 310 molecules of a second adhesin on its cell surface (Weiss *et al.*, 1988). In *E. coli*, the ratio of 1 adhesin molecule per 1000 pilin subunits for the P pili has been reported (Lindberg *et al.*, 1987). Presumably, adhesins with high affinity for their cognate receptors do not need to be in high numbers per cell to be effective in maintaining the union of the two cell types.

The presence of a biochemically functional adhesin on the cell surface of *Actinomyces* serovar WVA963 strain PK2407 in the absence of type 2 fimbriae is comparable to the presence of the P pilus adhesin (PapG) on the surface of *E. coli* mutants that lack a P pilus (Lindberg *et al.*, 1987; Uhlin *et al.*, 1985). Also, *E. coli* mutants producing type 1 adhesin (FimH) in the absence of type 1 pili retain the mannose-sensitive haemagglutination but are unable to bind to epithelial cells (Hultgren *et al.*, 1990). Recently, an adhesin, PilC, was described for *Neisseria gonorrhoeae* that is distinct from the pilus subunit PilE of type IV pili in *N. gonorrhoeae* (Rudel *et al.*, 1995). Likewise, we have identified an adhesin on the class II mutant PK2407 that can remove coaggregation-blocking antibodies but cannot mediate coaggregation. The putative adhesin could be released from mutant

PK2407 by mild sonication and bound to streptococcal cells. The lactose-sensitive function relevant to coaggregation was demonstrated by the binding of the 95 kDa putative adhesins of parent and mutant to streptococci and subsequent elution by lactose. Thus, we propose that the 95 kDa putative adhesin is distinct from the 57 kDa type 2 fimbrial subunits. The results also indicate that to mediate coaggregation it must be presented properly on the type 2 fimbriae. Additional support for two distinct proteins comes from our characterization of the class I mutant PK3092, which produces type 2 fimbriae but has no detectable adhesin.

REFERENCES

- Abraham, S. N., Goguen, J. D., Sun, D., Klemm, P. & Beachey, E. H. (1987). Identification of two ancillary subunits of *Escherichia coli* type 1 fimbriae by using antibodies against synthetic oligopeptides of *fim* gene products. *J Bacteriol* **169**, 5530–5535.
- Brennan, M. J., Cisar, J. O., Vatter, A. E. & Sandberg, A. L. (1984). Lectin-dependent attachment of *Actinomyces naeslundii* to receptors on epithelial cells. *Infect Immun* **46**, 459–464.
- Brennan, M. J., Cisar, J. O. & Sandberg, A. L. (1986). A 160-kilodalton epithelial cell surface glycoprotein recognized by plant lectins that inhibit the adherence of *Actinomyces naeslundii*. *Infect Immun* **52**, 840–845.
- Cisar, J. O., Curl, S. H., Kolenbrander, P. E. & Vatter, A. E. (1983). Specific absence of type 2 fimbriae on a coaggregation-defective mutant of *Actinomyces viscosus* T14V. *Infect Immun* **40**, 759–765.
- Cisar, J. O., David, W. A., Curl, S. H. & Vatter, A. E. (1984). Exclusive presence of lactose-sensitive fimbriae on a typical strain (WVU45) of *Actinomyces naeslundii*. *Infect Immun* **46**, 453–458.
- Cisar, J. O., Vatter, A. E., Clark, W. B., Curl, S. H., Hurst-Calderone, S. & Sandberg, A. L. (1988). Mutants of *Actinomyces viscosus* T14V lacking type 1, type 2, or both types of fimbriae. *Infect Immun* **56**, 2984–2989.
- Cisar, J. O., Barsumian, E. L., Siraganian, R. P., Clark, W. B., Yeung, M. K., Hsu, S. D., Curl, S. H., Vatter, A. E. & Sandberg, A. L. (1991). Immunochemical and functional studies of *Actinomyces viscosus* T14V type 1 fimbriae with monoclonal and polyclonal antibodies directed against the fimbrial subunit. *J Gen Microbiol* **137**, 1971–1979.
- Clark, W. B., Wheeler, T. T. & Cisar, J. O. (1984). Specific inhibition of adsorption of *Actinomyces viscosus* T14V to saliva-treated hydroxyapatite by antibody against type 1 fimbriae. *Infect Immun* **43**, 497–501.
- Donkersloot, J. A., Cisar, J. O., Wax, M. E., Harr, R. J. & Chassy, B. M. (1985). Expression of *Actinomyces viscosus* antigens in *Escherichia coli*: cloning of a structural gene (*fimA*) for type 2 fimbriae. *J Bacteriol* **162**, 1075–1078.
- Gibbons, R. J. & Nygaard, M. (1970). Interbacterial aggregation of plaque bacteria. *Arch Oral Biol* **15**, 1397–1400.
- Gibbons, R. J., Hay, D. I., Cisar, J. O. & Clark, W. B. (1988). Adsorbed salivary proline-rich protein 1 and statherin: receptors for type 1 fimbriae of *Actinomyces viscosus* T14V-J1 on apatitic surfaces. *Infect Immun* **56**, 2990–2993.
- Handley, P. S., Carter, P. L. & Fielding, J. (1984). *Streptococcus salivarius* strains carry either fibrils and/or fimbriae on the cell surface. *J Bacteriol* **157**, 64–72.
- Handley, P. S., Carter, P. L., Wyatt, J. E. & Hesketh, L. M. (1985). Surface structures (peritrichous fibrils and tufts of fibrils) found on *Streptococcus sanguis* strains may be related to their ability to

- coaggregate with other oral genera. *Infect Immun* **47**, 217–227.
- Hsu, S. D., Cisar, J. O., Sandberg, A. L. & Kilian, M. (1994). Adhesive properties of viridans streptococcal species. *Microb Ecol Health Dis* **7**, 125–137.
- Hughes, C. V., Kolenbrander, P. E., Andersen, R. N. & Moore, L. V. H. (1988). Coaggregation properties of human oral *Veillonella* spp.: relationship to colonization site and oral ecology. *Appl Environ Microbiol* **54**, 1957–1963.
- Hughes, C. V., Andersen, R. N. & Kolenbrander, P. E. (1992). Characterization of *Veillonella atypica* PK1910 adhesin-mediated coaggregation with oral *Streptococcus* spp. *Infect Immun* **60**, 1178–1186.
- Hultgren, S. J., Duncan, J. L., Schaeffer, A. J. & Amundsen, S. K. (1990). Mannose-sensitive haemagglutination in the absence of piliation in *Escherichia coli*. *Mol Microbiol* **4**, 1311–1318.
- Irvin, R. T., Doig, P., Lee, K. K., Sastry, P. A., Paranchych, W., Todd, T. & Hodges, R. S. (1989). Characterization of the *Pseudomonas aeruginosa* pilus adhesin: confirmation that the pilin structural protein subunit contains a human epithelial cell-binding domain. *Infect Immun* **57**, 3720–3726.
- Johnson, J. L., Moore, L. V. H., Kaneko, B. & Moore, W. E. C. (1990). *Actinomyces georgiae* sp. nov., *Actinomyces gerencseriae* sp. nov., designation of two genospecies of *Actinomyces naeslundii*, and inclusion of *A. naeslundii* serotypes II and III and *Actinomyces viscosus* serotype II in *A. naeslundii* genospecies 2. *Int J Syst Bacteriol* **40**, 273–286.
- Jones, C. H., Pinkner, J. S., Roth, R., Heuser, J., Nicholes, A. V., Abraham, S. N. & Hultgren, S. J. (1995). FimH adhesin of type 1 pili is assembled into a fibrillar tip structure in the *Enterobacteriaceae*. *Proc Natl Acad Sci USA* **92**, 2081–2085.
- Kolenbrander, P. E. (1982). Isolation and characterization of coaggregation-defective mutants of *Actinomyces viscosus*, *Actinomyces naeslundii*, and *Streptococcus sanguis*. *Infect Immun* **37**, 1200–1208.
- Kolenbrander, P. E. & Andersen, R. N. (1986). Multigeneric aggregations among oral bacteria: a network of independent cell-to-cell interactions. *J Bacteriol* **168**, 851–859.
- Kolenbrander, P. E. & Andersen, R. N. (1990). Characterization of *Streptococcus gordonii* (*S. sanguis*) PK488 adhesin-mediated coaggregation with *Actinomyces naeslundii* PK606. *Infect Immun* **58**, 3064–3072.
- Kolenbrander, P. E. & London, J. (1992). Ecological significance of coaggregation among oral bacteria. *Adv Microb Ecol* **12**, 183–217.
- Kolenbrander, P. E. & London, J. (1993). Adhere today, here tomorrow: oral bacterial adherence. *J Bacteriol* **175**, 3247–3252.
- Kolenbrander, P. E., Inouye, Y. & Holdeman, L. V. (1983). New *Actinomyces* and *Streptococcus* coaggregation groups among human oral isolates from the same site. *Infect Immun* **41**, 501–506.
- Kolenbrander, P. E., Andersen, R. N. & Moore, L. V. H. (1989). Coaggregation of *Fusobacterium nucleatum*, *Selenomonas flueggei*, *Selenomonas infelix*, *Selenomonas noxia*, and *Selenomonas sputigena* with strains from 11 genera of oral bacteria. *Infect Immun* **57**, 3194–3203.
- Kuehn, M. J., Heuser, J., Normark, S. & Hultgren, S. J. (1992). P pili in uropathogenic *Escherichia coli* are composite fibres with distinct fibrillar adhesive tips. *Nature* **356**, 252–255.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lee, K. K., Sheth, H. B., Wong, W. Y., Sherburne, R., Paranchych, W., Hodges, R. S., Lingwood, C. A., Krivan, H. & Irvin, R. T. (1994). The binding of *Pseudomonas aeruginosa* pili to glycosphingolipids is a tip-associated event involving the C-terminal region of the structural pilin subunit. *Mol Microbiol* **11**, 705–713.
- Lindberg, F., Lund, B., Johansson, L. & Normark, S. (1987). Localization of the receptor-binding protein adhesin at the tip of the bacterial pilus. *Nature* **328**, 84–87.
- London, J. & Allen, J. (1990). Purification and characterization of a *Bacteroides loescheii* adhesin that interacts with procaryotic and eucaryotic cells. *J Bacteriol* **172**, 2527–2534.
- McIntire, F. C., Vatter, A. E., Baros, J. & Arnold, J. (1978). Mechanism of coaggregation between *Actinomyces viscosus* T14V and *Streptococcus sanguis* 34. *Infect Immun* **21**, 978–988.
- Maryanski, J. H. & Wittenberger, C. L. (1975). Mannitol transport in *Streptococcus mutans*. *J Bacteriol* **124**, 1475–1481.
- Moch, T., Hoschützky, H., Hacker, J., Kröncke, K.-D. & Jann, K. (1987). Isolation and characterization of the α -sialyl- β -2,3-galactosyl-specific adhesin from fimbriated *Escherichia coli*. *Proc Natl Acad Sci USA* **84**, 3462–3466.
- Nesbitt, W. E., Beem, J. E., Leung, K.-P. & Clark, W. B. (1992). Isolation and characterization of *Actinomyces viscosus* mutants defective in binding salivary proline-rich proteins. *Infect Immun* **60**, 1095–1100.
- Nyvad, B. & Kilian, M. (1987). Microbiology of the early colonization of human enamel and root surfaces in vivo. *Scand J Dent Res* **95**, 369–380.
- Rudel, T., Scheuerpflug, I. & Meyer, T. F. (1995). *Neisseria* PilC protein identified as type-4 pilus tip-located adhesin. *Nature* **373**, 357–359.
- Schneewind, O., Model, P. & Fischetti, V. A. (1992). Sorting of protein A to the staphylococcal cell wall. *Cell* **70**, 267–281.
- Schneewind, O., Fowler, A. & Faull, K. F. (1995). Structure of the cell wall anchor of surface proteins in *Staphylococcus aureus*. *Science* **268**, 103–106.
- Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**, 4350–4354.
- Uhlen, B. E., Norgren, M., Båga, M. & Normark, S. (1985). Adhesion to human cells by *Escherichia coli* lacking the major subunit of a digalactoside-specific pilus-adhesin. *Proc Natl Acad Sci USA* **82**, 1800–1804.
- Weiss, E. I., London, J., Kolenbrander, P. E., Hand, A. R. & Siraganian, R. (1988). Localization and enumeration of fimbria-associated adhesins of *Bacteroides loescheii*. *J Bacteriol* **170**, 1123–1128.
- Yeung, M. K. (1992). Conservation of an *Actinomyces viscosus* T14V type 1 fimbrial subunit homolog among divergent groups of *Actinomyces* spp. *Infect Immun* **60**, 1047–1054.
- Yeung, M. K. (1995). Construction and use of integration plasmids to generate site-specific mutations in the *Actinomyces viscosus* T14V chromosome. *Infect Immun* **63**, 2924–2930.
- Yeung, M. K. & Cisar, J. O. (1988). Cloning and nucleotide sequence of a gene for *Actinomyces naeslundii* WVU45 type 2 fimbriae. *J Bacteriol* **170**, 3803–3809.
- Yeung, M. K. & Cisar, J. O. (1990). Sequence homology between the subunits of two immunologically and functionally distinct types of fimbriae of *Actinomyces* spp. *J Bacteriol* **172**, 2462–2468.
- Yeung, M. K., Chassy, B. M. & Cisar, J. O. (1987). Cloning and expression of a type 1 fimbrial subunit of *Actinomyces viscosus* T14V. *J Bacteriol* **169**, 1678–1683.

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