

Association of a novel high molecular weight, serine-rich protein (SrpA) with fibril-mediated adhesion of the oral biofilm bacterium *Streptococcus cristatus*

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The surface of the oral plaque bacterium *Streptococcus cristatus* is decorated with a lateral tuft of fibrils. The fibrillar tuft functions in the adhesion of *S. cristatus* to heterologous bacterial species in the plaque biofilm. The tuft typically consists of a densely packed fringe of shorter fibrils 238 ± 19 nm long with longer, less abundant fibrils 403 ± 66 nm long projecting through the fringe of short fibrils. The two types of fibrils in the tufts of *S. cristatus* have been refractory to biochemical separation, complicating their characterization. A hexadecane partition assay was used to enrich for subpopulations of *S. cristatus* CR311 (type strain NCTC 12479) having distinct fibrillar morphotypes. Negative staining in the TEM revealed that cells of a hydrophobic subpopulation of *S. cristatus* (CR311var1) carried only the long fibrils (395 ± 32 nm). A hydrophilic subpopulation of *S. cristatus* (CR311var3) consisted of mixed morphotypes having no fibrils or remnant short fibrils (223 ± 49 nm). No long fibrils were observed on any cells in the CR311var3 subpopulation. The CR311var3 morphotype, unlike the wild-type strain and CR311var1, was not able to form corncocks with either *Corynebacterium matruchotii* or *Fusobacterium nucleatum*. Variant CR311var3 did not express the novel gene *srpA*, which encodes a high molecular weight (321,882 Da) serine-rich protein, SrpA. The SrpA protein contains two extensive repeat motifs of 17 and 71 amino acids and a gram-positive cell wall anchor consensus sequence (LPNTG). The unusual properties of SrpA most closely resemble those of Fap1, the fimbrial-associated adhesin protein of *Streptococcus parasanguis*. The association of long fibrils, high surface hydrophobicity, ability to form corncock formations, and expression of the *srpA* gene suggest that SrpA is a long fibril protein in *S. cristatus*.

Key words: corncocks; fibrils; SrpA; *Streptococcus cristatus*; tufts

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The surfaces of oral streptococci control the adhesive interactions that determine the specific ecologic niche of

these bacteria within the oral cavity. It is believed that many of these interactions are associated with appendages that dec-

orate the bacterial surface. Two classes of these structures, fibrils and fimbriae, have been described on different streptococcal

species (11). The majority of the streptococci carry short fibrils (40–400 nm), which are thin clumped structures that may be peritrichous or localized in tufts (8). A few streptococcal strains carry peritrichous fimbriae, with a width of 3–5 nm, which are longer (1–3 µm) and more flexible than fibrils.

The structure and function of one peritrichous fibril component on *Streptococcus gordonii* DL1 has been studied by McNab et al. (17), who showed that the sparse, peritrichous fibrils (61 nm long) are encoded by the *cshA* gene (18). The CshA polypeptide (259 kDa) contains 2508 amino acid residues and is apparently not glycosylated. The protein comprises a 41-residue amino-terminal signal sequence, a nonrepetitive region, and an extensive 101 amino acid sequence that repeats 13 times. A consensus carboxy-terminal cell wall anchor domain is responsible for covalent attachment of the fibrils to the peptidoglycan. The CshA polypeptide appears to be the structural and functional component of the fibrils. CshA is also associated with adhesion to fibronectin (19), other oral bacteria and mucosal surfaces of the mouse oral cavity (20). Recent studies have shown that the CshA-like fibrillar protein also occurs on the surfaces of other members of the mitis group of oral streptococci (6).

Fimbrial appendages have been found on *Streptococcus parasanguis* and *Streptococcus salivarius*. In the former species these peritrichous appendages contain a 200 kDa protein called Fap1 (30). It has been suggested that this protein is a structural subunit of one type of fimbriae produced by this organism. The Fap1 protein is glycosylated (3, 26) and every second amino acid residue is a serine. The protein contains 2552 amino acids, including a 50-amino acid N-terminal leader peptide, the consensus cell wall anchor sequence at the carboxy-terminus, and 1000 repeats of the sequence E/V/I-S (31). There appears to be a link between the fimbriae and adherence to salivary proteins (7).

The fibrils of *S. salivarius* are also peritrichous and consist of a glycoprotein assembled into flexible appendages 1.0 µm long and 3–5 nm wide (28, 29). In this glycoprotein the N-terminal unit contains the repeating motif A/X/T-E-Q/φ, where X represents a modified amino acid residue and φ, a blank cycle (16). This fimbrial glycoprotein is assembled into a filamentous structure that could not be dissociated. The common theme among these streptococcal appendages is the

relatively large size of their constituent protein, the presence of a number of novel repeat sequences in the protein, and their relative resistance to dissociation. In addition, at least two types of these proteins appear to be heavily glycosylated.

Our studies have focused on *Streptococcus cristatus*. This species was formerly known as *S. crista* (9, 27) and is a member of the mitis-oralis group of streptococci found in oral biofilms (22). In contrast to the peritrichous appendages on *S. parasanguis* and *S. salivarius*, the fibrils on *S. cristatus* are organized in localized tufts on the cell surface (8). We have shown in previous studies that the fibrillar tufts mediate binding of *S. cristatus* to several heterologous bacterial species, including the gram-positive aerobe *Corynebacterium matruchotii* (14) and the gram-negative anaerobe *Fusobacterium nucleatum* (15). This binding results in the formation of a unique bicellular arrangement called a ‘corn cob’ (22). These bicellular units are prominent in mature dental biofilms and are believed to constitute the mechanism responsible for ‘fixing’ the nonstreptococcal species to the plaque biofilm.

The fibrillar tufts of strains of *S. cristatus* appear to be complex organizations of two lengths of fibrils (10), teichoic acid (22) and polysaccharide (12). *S. cristatus* CR311 (type strain NCTC 12479) (9) represents a typical strain in which the tufts consist of a densely packed fringe of fibrils projecting approximately 250 nm from the cell surface (short tuft fibrils) and longer, sparser fibrils projecting through the short fibrils to a distance of 400 nm (long tuft fibrils). The long tuft fibrils tend to clump together towards the proximal end and separate or ‘splay’ out at the ends of the clumps. These fibrils are also often more prominent at the edges of the tufts (8). In this report a hexadecane partition assay was used to enrich for subpopulations of *S. cristatus* with a distinct tuft ultrastructure (tuft morphotypes). We examined the structure and properties of the surfaces of these subpopulation variants, establishing a relationship between the fibrils and the expression of a gene that encodes for an unusual, large molecular weight cell surface protein.

Material and methods

Bacterial strains and culturing conditions

S. cristatus CR311 was originally isolated from a human periodontal abscess (8) and has been designated the type strain for this species (NCTC 124790) (9). *S. cristatus* CR311var1 and *S. cristatus*

CR311var3 were isolated after serial enrichment in a hexadecane partition assay (2). *S. cristatus* CC5A was isolated from human dental plaque corn cobs by Mouton et al. (22) and has been used as a prototype strain in studies of corn cob formation (14, 15).

The CR311 strains were grown in tryptone soy broth containing 0.1% yeast extract (TSBY) or on tryptone soya agar containing 0.1% yeast extract (TSOY) in a CO₂ atmosphere at 37°C overnight. *S. cristatus* CC5A was grown in brain heart infusion (BHI; BD, Sparks, MD) or Todd-Hewett (TH; BD, Sparks, MD) broth in an atmosphere containing 5% CO₂ at 37°C. *C. matruchotii* NCTC 14266 was grown in BHI broth for 48 h with gentle shaking at 37°C. *F. nucleatum* ATCC 10953 was grown in BHI supplemented with 0.2% yeast extract (BD, Sparks, MD), 0.05 liter cysteine (Fischer Scientific, Fairlawn, NJ) and 0.5% sodium bicarbonate under anaerobic conditions (Gas Pack; BBL Microbiology Systems, Cockeysville, MD) at 37°C. All strains used in this study are listed in Table 1.

Hexadecane enrichment assay

For the basic hexadecane assay (24) cells were grown overnight in TSB broth in a static culture, washed in Sorenson’s phosphate buffer (pH 7.2) and the optical density adjusted to 0.5 at 440 nm. Cell suspensions (3 ml) were partitioned with 200 µl hexadecane (BDH chemicals). A hydrophilic variant *S. cristatus* CR311var3 was isolated from the buffer phase by growth on BHI blood agar. The hydrophobic variant *S. cristatus* CR311var1 was obtained from the hexadecane phase. The bacterial surface ultrastructure of the subpopulations was then examined by electron microscopy of negatively stained preparations.

Electron microscopy

Specimens were prepared for electron microscopy on carbon coated formvar grids (400 mesh) in a Bio-Rad E6200 Turbo Coater (Hercules, CA). All grids were made hydrophilic in a plasma glow unit (Plasma Barrel Etcher PT7150, Fisons, Loughborough, UK). The bacteria were negatively stained using 1 or 2% methylamine tungstate (8). The grids were photographed using a Phillips Fei Technai Biotwin TEM. To determine the average lengths of the tuft fibrils, negative images were printed in TIF format at actual negative size with the nm scale bar.

Table 1. Bacterial strains and plasmids used in this study

Strain or Plasmid	Description	Source/Reference
Strains		
<i>S. cristatus</i> CR311	wild-type strain; type strain; fibrillar tufts	Laboratory collection
<i>S. cristatus</i> CC5A	wild-type strain; fibrillar tufts	Laboratory collection
<i>S. cristatus</i> Psha	wild-type strain; fibrillar tufts	Laboratory collection
<i>S. cristatus</i> Pshb	wild-type strain; fibrillar tufts	Laboratory collection
<i>S. cristatus</i> CR3	wild-type strain; fibrillar tufts	Laboratory collection
<i>S. cristatus</i> CR311var1	hydrophobic subpopulation of CR311	This study
<i>S. cristatus</i> CR311var3	hydrophilic subpopulation of CR311	This study
<i>S. oralis</i> CN3410	wild-type strain; fibrillar tufts	Obtained from H. Jenkinson (13)
<i>S. parasanguis</i> FW213	fimbriate strain; expresses <i>fapI</i>	Obtained from P. Fives-Taylor (7)
<i>C. matruchothii</i> NCTC 14266	corncob partner	Laboratory collection
<i>F. nucleatum</i> ATCC 10953	corncob partner	Laboratory collection
<i>Escherichia coli</i> JM109	host strain for all plasmids	Promega
Plasmids		
pB15-23	pCR-Script SK ⁺ contains 21 kb <i>SaI</i> fragment from <i>S. cristatus</i> B15 locus (Amp ^r)	(5)
pB15-18	subclone of pB15-23 contains 10.9 kp <i>EcoRI</i> — <i>Sau3A</i> fragment (Amp ^r)	This study
pB15-2.2	subclone of pB15-18 contains 2249 bp <i>EcoRI</i> – <i>HindIII</i> fragment cloned into pGem-T (Amp ^r)	(5)
pB15-1	subclone of pB15-18 contains 1017 bp <i>HindIII</i> fragment (Amp ^r)	This study
pB15-8	subclone of pB15-18 contains 7622 bp <i>HindIII</i> — <i>Sau3A</i> fragment (Amp ^r)	This study
Zap Express TM	lambda cloning vector	Stratagene
pBK-CMV	phagemid vector derived from ZAP Express TM (Kan ^r)	Stratagene
pY-1	contains a fragment, with an additional 3895 bp adjacent to the <i>Sau3A</i> end of pB15-23; cloned into pBK-CMV (Kan ^r)	This study
pW-2	contains a fragment with an additional 550 bp adjacent to pY-1 cloned into pBK-CMV (Kan ^r)	This study

Measurements were taken from the bacterial surface (delineated by the dark area).

Corncob assay

S. cristatus wild-type and mutants *C. matruchothii* and *F. nucleatum* strains were grown for 18 h and 48 h in BHI broth, and were washed and suspended in cold 0.15 M sodium chloride solution for the corncob assay. The assay was performed as described by Lancy et al. (14, 15). Corncob preparations were viewed by light microscopy (phase contrast) at 100× magnification using oil emersion with the semiphotomat set to darkfield and 100 ASA.

srpA gene sequence

A portion of the insert of plasmid pB15-23 (5) containing sequence downstream from the previously described ABC (adenosine triphosphate-binding-cassette) transport locus of *S. cristatus* CC5A (GenBank Accession Number U96166), was subcloned into three new plasmids, designated pB15-2.2, pB15-1 and pB15-8, to facilitate sequencing of this region (Table 1). The insert DNA from pB15-2.2 and pB15-1 was sequenced by the primer walking method. In order to sequence the relatively large repetitive insert of pB15-8, a set of nested deletions was made using the Exo-SizeTM deletion kit (NEB, Beverly, MA). To obtain the sequence adjacent to the pB15-23 DNA insert, *S. cristatus* CC5A genomic DNA

was digested to completion with *Bam*HI and *Sac*I and was ligated to similarly digested ZAP ExpressTM vector (Stratagene, La Jolla, CA). This library was then screened with a probe sequence made using a *Bam*HI-*Sau3A*I fragment derived from the 3'-end of pB15-8. A positive plaque yielded the phagemid pY-1, which contained an additional 3895 bp of sequence downstream from pB15-8. To obtain the sequence adjacent to the pY-1 insert, *S. cristatus* CC5A genomic DNA was digested to completion with *Bam*HI and *Hind*III and ligated to similarly digested ZAP ExpressTM vector. This Zap library was screened with a *Hind*III-*Sac*I DNA fragment derived from the 3'-end of pY-1. A positive plaque yielded phagemid pW-2, which was used to obtain an additional 550 bp of sequence. Automated cycle sequencing reactions were conducted by the Genetics Core Facility at the University of Pennsylvania using an Applied Biosystems, Inc. 373 A sequencer with Dye Primer chemistry (Foster City, CA). The *srpA* gene sequence was deposited in GenBank under Accession Number U96166.

Northern and Southern blotting

For Northern blots, RNA was obtained from *S. cristatus* CC5A using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA 10 µg was loaded onto a 1% agarose gel containing 1 × MOPS and 6% formaldehyde as described (25). Southern blots

were prepared and run as described previously (4). Genomic DNA from *S. cristatus* strains CC5A, Psha, Pshb, CR3, *S. parasanguis* FW213, *Streptococcus oralis* CN3410 and *F. nucleatum* ATCC 10953 was isolated as described (4) and digested with *Hind*III. Probe DNA was made by amplifying the 5'-end of *srpA* from strain CC5A using the PCR primers SRP003 (5'-CCGGATCCTCTCCAGAAACAAGTGGTCGT-3') and SRP004 (5'-CCGAATCTAGTGGTTCAGGGGAAGGAA-3'). Probe DNA was labeled with α[³²P]dATP (6000 Ci/mmol; Amersham, Piscataway, NJ) using a random primer labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Polymerase chain reaction (PCR)

DNA primers SRP003 and SRP004 were used to amplify the 5'-end of *srpA* from *S. cristatus* strains. Approximately 2 µg of genomic DNA was used as the template. Conditions were 2 min at 94°C followed by 30 cycles of 1 min at 94°C, 2 min at 60°C and 3 min at 72°C. Amplified products were examined on an 8% agarose gel stained with ethidium bromide. Molecular size was estimated by comparison with a commercial 100 bp ladder.

DNA fingerprinting

Genomic DNA was digested with *Eco*RI and the fragments separated on an 8%

agarose gel. DNA bands were visualized by staining with ethidium bromide.

Protein analysis

Proteins were examined on 8% SDS-polyacrylamide gels as described by Correia et al. (4). Samples were prepared by boiling whole washed cells in solubilizing solution for 10 min. Some samples were treated with 1 mg/ml of trypsin (Sigma, St. Louis, MO) for 1 h at 37°C prior to gel electrophoresis. Protein bands were visualized by staining with Coomassie Brilliant Blue. Molecular size markers were from Bio-Rad.

Statistics

Fibril length was determined from measurements made on 10–20 cells per strain or variant. The Student *t*-test was used to compare the mean lengths of fibrils.

Results

Isolation of hydrophobicity mutants of *S. cristatus* and characterization of surface fibrils

Based on the possibility that the long fibrils in the tufts of *S. cristatus* give the bacterial surface its predominantly hydrophobic character (10), a hexadecane partitioning method was used to enrich for subpopulations that lacked the longer tuft fibrils (2). When wild-type strain *S. cristatus* CR311 was partitioned between hexadecane and buffer, hydrophobic and hydrophilic subpopulations were recovered from each phase, respectively. The bacteria recovered from the hexadecane partitioning assay were fingerprinted by comparing genomic DNA restriction endonuclease fragments and total soluble protein patterns (data not shown).

Examination of the *S. cristatus* subpopulations, recovered from the hexadecane partitioning assay, in the TEM revealed alterations in the surface fibril composition. As shown in a negatively stained preparation in Fig. 1A, the parent strain CR311 carries short fibrils arrayed within the tufts. The average length of the short fibrils is 238 ± 18.9 nm. The long fibrils are also distributed throughout the tufts but clusters can often be seen located in the tuft farthest away from the septum (Fig. 1B). The long fibrils average 403 ± 68.7 nm in length. They were sparser than the short fibrils and projected through the more densely packed short fibrils. Even though the cells were washed, the fibrils sometimes had associated

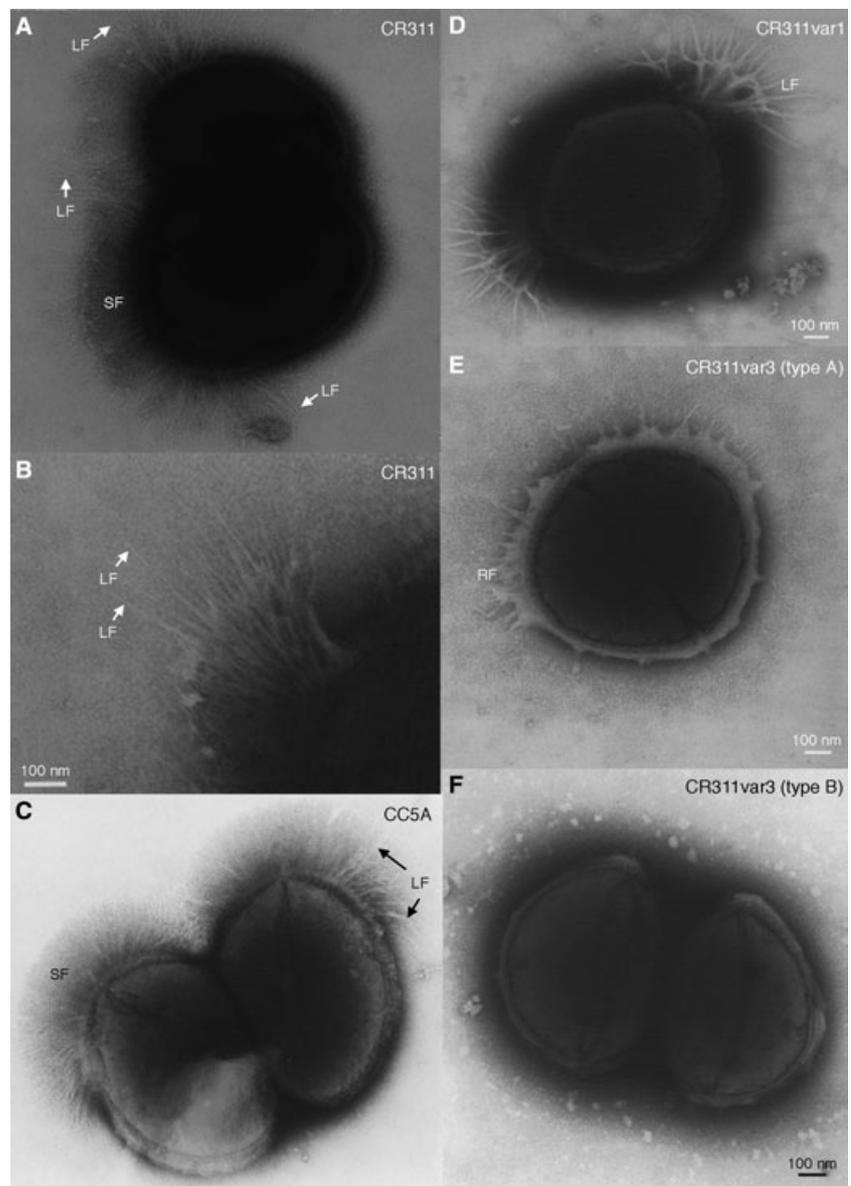


Fig. 1. Negatively stained preparations of wild-type and fibrillar tuft variants of *S. cristatus*. A) Wild-type CR311 showing the organization of the short (SF) and long (LF) fibrils. B) Enlargement of the long fibrils in the fibrillar tufts of CR311. C) Wild-type CC5A showing both short and long fibrils. D) CR311var1 showing only the long fibrils that splay out at the ends. E) CR311var3 (type A) showing the remnant tuft fibrils (RF) only. F) CR311var3 (type B) having no fibrils. Magnification bar = 100 nm.

extraneous electron transparent material (Fig. 1B). A negative stain of *S. cristatus* CC5A shows the same pattern of long and short tuft fibrils (Fig. 1C), indicating that this general fibril arrangement is characteristic of the species.

A hydrophobic variant (CR311var1) that only carried long fibrils was isolated from the hexadecane phase (Fig. 1D). These fibrils average 395 ± 32.4 nm in length (Table 2). The length of the fibrils on CR311var1 was not statistically significantly different from the length of the

long fibrils on the wild-type strain. The long fibrils clumped during staining to give a branched appearance but the ends of the long fibrils consisted of very fine fibrils.

One hydrophilic variant (CR311var3) was isolated from cells partitioning into the buffer phase and lacked both the short and long fibrils. However, examination of overnight cultures of CR311var3 revealed four distinct morphologic types. Most of the cells showed sparse, thin, lateral fibrils that did not appear to be like the

Table 2. Average lengths of tuft fibrils on *S. cristatus* CR311 and fibril variants and surface hydrophobicity

Strain	Average length of short fibrils (nm)	Average length of long fibrils (nm)	Average length of remnant fibrils (nm)	Percent hydrophobicity
CR311	238 ± 18.9	403 ± 68.7		91.6 ± 4.1
CR311var1		395 ± 32.4		92.9 ± 7.1
CR311var3			223 ± 48.8	55.7 ± 21.9

lateral tufts that project from cell surface crenations (CR311var3 type A). These cells lacked the long tuft fibrils but had sparse remnants of the lateral short fibrils in association with surface crenations in the position of the tuft (Fig. 1E). Other members of the population (CR311var3 type B) had no polar fibrils and showed no obvious surface crenation (Fig. 1F). A small percentage of the cells in the population appeared to have possible remnants of short tuft lateral fibrils in combination with fine polar fibrils or only the fine fibrils (data not shown). The remnant lateral short fibrils average 223 ± 48.8 nm in length. They are not statistically significantly different from the short tuft fibrils on the wild-type strain. Thus, the fibril phenotype of CR311var3 appears to be variable. In cultures of this variant there are typically 40% cells with the remnant lateral fibrils (Fig. 1E), 37% cells having no fibrils (Fig. 1F), 14% cells with sparse fine polar fibrils and 9% cells with fine polar and remnant lateral fibrils.

Surface properties of the fibril variants

Both *S. cristatus* CR311 and CR311var1 formed cornicobs with *C. matruchotii* (Fig. 2). In contrast, *S. cristatus* CR311var3 did not form cornicobs with *C. matruchotii*. Similar results were obtained in coaggregation experiments with *F. nucleatum* (data not shown). The data obtained with the *S. cristatus* CR311var3 mutant confirmed that the long fibrils are required for this species to form cornicobs. Electron microscopy in previous studies showed that members of this species bind to *C. matruchotii* and *F. nucleatum* through the tuft fibrils (14, 15). Table 2 compares the hydrophobicity of the wild-type strain CR311 with that of the two structural variants. The results are from an average of six replicate experiments carried out for each strain. The results confirm that strains that have the long fibrils (CR311 and CR311var1) are significantly more hydrophobic than CR311var3 strains, which carried only remnants of short tuft fibrils.

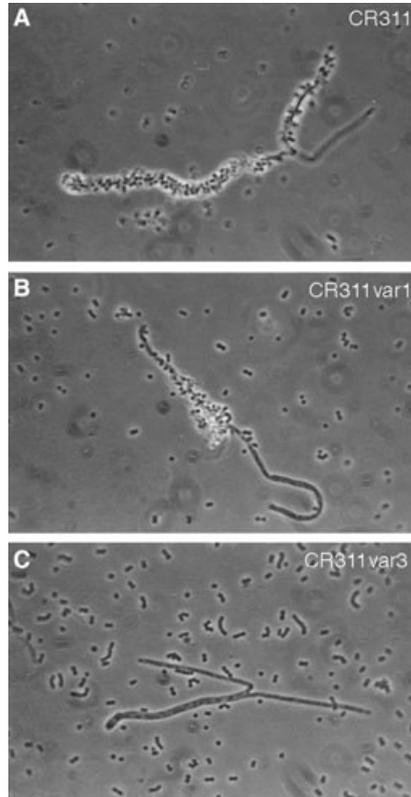


Fig. 2. Phase contrast microscopy of cornicob formation between *S. cristatus* fibril variants and *C. matruchotii*. A) Wild-type CR311. B) CR311var1. C) CR311var3. Magnification ×100.

Correlation between the loss of short fibrils and expression of a novel serine-rich protein in *S. cristatus*

A previously characterized transposon insertion mutant, *S. cristatus* CC5A-B15, exhibited a 70% reduction in binding to *F. nucleatum* relative to the wild-type strain CC5A (4). The transposon insertion was mapped to an ABC transport locus comprising four open reading frames (ORF) (5). The fibrillar tufts of this mutant have a disordered, irregular appearance, in contrast to the relatively ordered arrangement of the fibrils on the wild-type strain. The short fibrils could not be clearly discerned on the mutant.

Additional sequence obtained downstream of the CC5A-B15 Tn916 insertion locus revealed an unusually large ORF of 10,146 bp (Fig. 3). This ORF (positions 6301–16446; [for numbering refer to Accession Number U96166]) is encoded on the opposite strand from the ABC cassette operon that was previously shown to affect interbacterial binding. This large ORF is designated *srpA* for the potential serine-rich protein it encodes. The *srpA* ORF is flanked by direct repeats of 732 bp, which themselves show homology to a transposase encoded by Tn5382 from *Enterococcus faecalis* (GenBank Accession Number AF173641). Abutting DR1a (positions 6207–6248) and 3' to the *srpA* coding sequence is a prokaryotic factor-independent terminator as determined by the method of Brendel & Trifonov (1). An ORF of 1329 bp that does not show a similarity with any genes in the nonredundant databases was found upstream of *srpA* and within the boundaries marked by the direct repeats.

Transcription of *srpA* in *S. cristatus* CC5A was examined by Northern blotting (Fig. 4A). An approximately 11.0 kb transcript was detected, indicating that *srpA* was monocistronic. A lower molecular weight hybridization positive band was also detected and most likely represents transcript degradation due to the exceptionally large size of the mRNA for the SrpA protein. Southern blot analysis showed that DNA sequence homologous to the *srpA* sequence of strain CC5A could be detected in a number of tufted *S. cristatus* strains including PshA, Pshb and CR3 as well as in a tufted strain of *S. oralis* (CN3410) (Fig. 4B). No homology was detected with DNA from *S. parasanguis* FW213 that contains the *fap1* gene.

The *srpA* ORF potentially encodes a protein (SrpA) of 321,882 Da (Fig. 5) that comprises 3382 amino acids, 1346 of which (39.8%) are serines. The serines alternate with nonserine residues (with the exception of a recurring NNQN motif) over approximately 75% of the protein. The serine repeat sequence has three distinct patterns. Closest to the carboxyl terminal is a 17-amino acid unit (SASTSMSNVASISAS[E, V, Q, A, I]), repeated 35 times (not consecutively as there are some truncated repeats) containing the infrequently used amino acid methionine. The amino acid in the 17th position of this repeat is variable. Adjacent to the methionine-type repeat is a 71-amino acid unit that is repeated 22 times. The order of serines in this repeat is disrupted by the polar amino acids NNQN

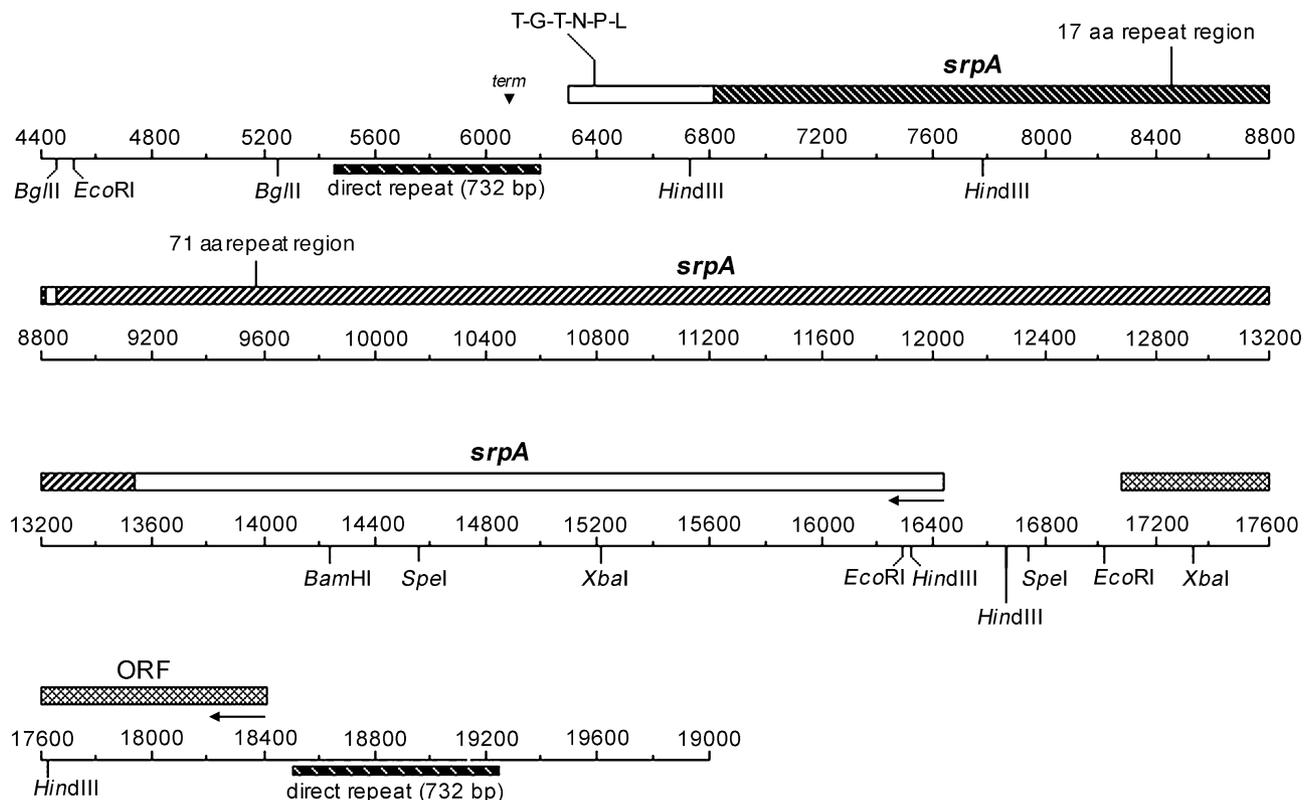


Fig. 3. Physical map of the region of the *S. cristatus* CC5A genome containing the *srpA* ORF. The map shows 14.6 kb of DNA immediately downstream from the binding-deficient locus of *S. cristatus* CC5A-B15 sequenced previously (5). Two ORFs are depicted by filled bars above the scale. The ORFs reside within a region bounded by a 732 bp direct repeat. Arrows show the direction of transcription. The scale is in bp and numbering continues from the upstream sequence previously published. The complete contiguous 19,841 bp sequence is in the GenBank file (Accession Number U96166).

and is a characteristic feature of the larger serine repeat unit. Some of the NNQN-type repeat units contain insertions or deletions within the repeat pattern. Some of the alternating serine residues appear in regions with no obvious pattern. One of these regions comprises the amino-terminus of the protein. A standard signal peptide sequence (23) is found at the amino-terminus. At the carboxy-terminus, SrpA has the gram-positive cell wall (peptidoglycan) anchor consensus sequence LPNTG preceding a typical hydrophobic domain and charged tail found in gram-positive surface proteins (23). Codon usage for *srpA* was not unusual relative to previously sequenced *S. cristatus* ORFs.

A possible connection between the putative cell wall associated SrpA protein, fibrillar tuft, and corn cob formation prompted us to look for *srpA* DNA in *S. cristatus* corn cob-deficient mutants. PCR was used to determine whether *srpA* DNA could be detected in variants CR311var1 and CR311var3 (Fig. 6). PCR primers designed to amplify the 5'-end of the *srpA* ORF were used to avoid the extensive repeat sequence

throughout most of the ORF. Amplified product of the expected size (820 bp) was obtained with template DNA from the wild-type strain CR311 and the short fibril-deficient variant CR311var1. However, no PCR product was obtained with DNA from the long fibril-deficient variant CR311var3, indicating that the *srpA* gene was not present or was extensively altered in *S. cristatus* CR311var3.

Total protein from the wild-type and variant strains of CR311 was examined on 8% SDS-polyacrylamide gels for the presence of polypeptide bands of exceptionally high apparent molecular weight. A single, high molecular weight polypeptide band was detected in extracts from *S. cristatus* CC5A and CR311 (Fig. 7). This polypeptide was absent in total protein preparations from the long fibril-deficient mutant CR311var3. A tryptic digest of the high molecular weight CR311 polypeptide contained a single 230 kDa peptide as predicted from the deduced amino acid sequence (data not shown). This relative resistance to trypsin was expected based on the extensive serine-rich repeats in the amino acid sequence of SrpA.

Discussion

Mouton et al. (22) made the original observation that *S. cristatus* contained characteristic tufts of fimbriae (now called fibrils), which were proposed to function in adhesion to *C. matruchoitii*. These studies influenced numerous investigations, leading to the conclusion that streptococcal species carrying these fibrils play a prominent role in the interactions among diverse bacteria in the dental biofilm. Studying the structure and composition of these fibrils may offer some unique insights into the ecology of the plaque biofilm.

Electron microscopy of negative stained preparations revealed that *S. cristatus* has two types of fibrils, long (ca. 400 nm) and short (ca. 240 nm), within the fibril tuft (2, 8). It had been difficult to segregate these two types of fibrils, by either biochemical or genetic methods, to determine function. The ability of the long fibrils to impart a hydrophobic character to the surface of the streptococci was previously shown by hexadecane partitioning and the binding of colloidal gold particles to the

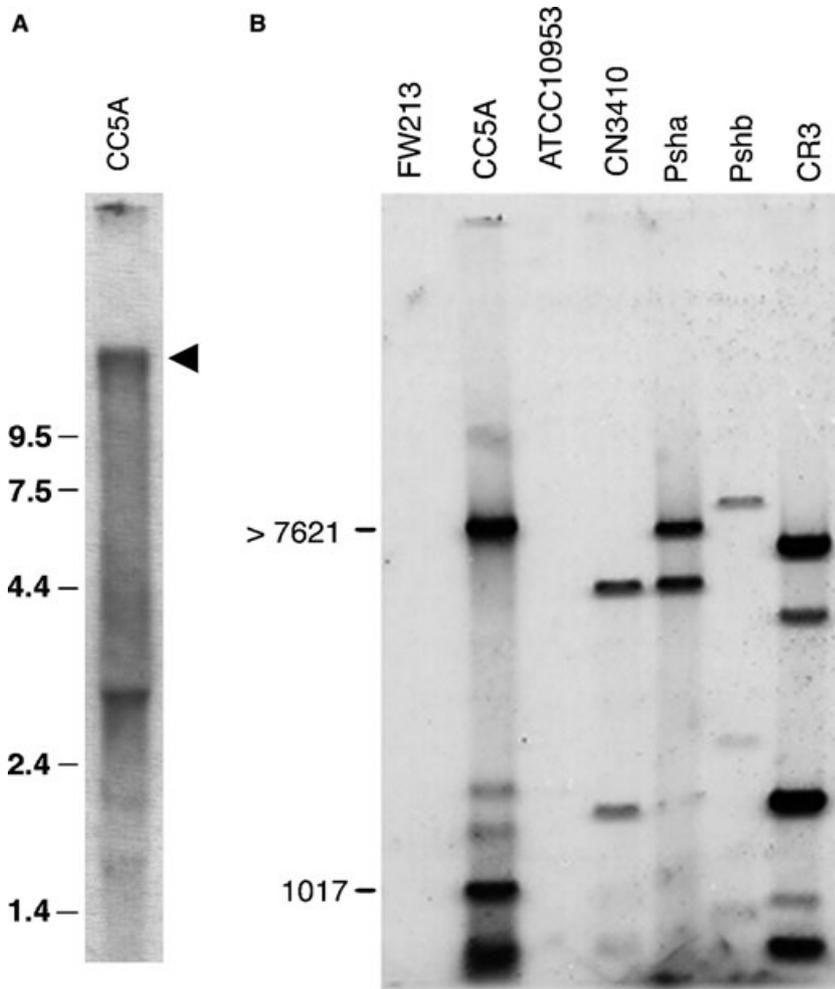


Fig. 4. Genetic analysis of the *srpA* ORF. A) Northern blot showing the *srpA* transcript from *S. cristatus* CC5A. Total RNA from wild-type CC5A was hybridized with a DNA sequence amplified by PCR from the 5'-end of the *srpA* gene. Molecular size standards are shown in kb. The arrow marks the position of a 11 kb hybridization positive band. B) Southern blot of the distribution of *srpA* DNA in various *Streptococcus* strains. Total genomic DNA was obtained from *S. cristatus* strains CC5A, CR3, Psha and Pshb; *S. oralis* CN3410; *S. parasanguis* FW213; and *F. nucleatum* ATCC 10953. The DNA samples were digested to completion with *Hind*III and the fragments hybridized with a fragment of DNA amplified from the 5'-end of the CC5A *srpA* ORF. The positions of molecular size markers are shown in bp.

tips of the fibrils (10). This apparent relationship between surface hydrophobicity and fibril composition was exploited in the present study to enrich for variants in the wild-type population that contained the different fibril arrangements. We found that fibril expression is a highly variable phenotype (summarized in Fig. 8). In general, all of the cells in cultures of the wild-type strain CR311 carry tufts of fibrils with no fibril-less (type B) cells or cells with fine polar fibrils (type D) detectable. The fine polar fibrils are very difficult to detect and it is possible that they may only have been present on a few cells in the wild-type population before hexadecane enrichment was carried out. Cultures of the

hydrophobic variant CR311var1 contain only long fibrils and no type B cells. However, cultures of the hydrophilic variant CR311var3 exhibited a mixed morphologic phenotype such that approximately equal numbers of cells carried remnant short fibrils (type A) or no fibrils (type B) (ca. 40% in each category). The length of the remnant short fibrils was not statistically different from that of the short fibrils present on strain CR311. Variant CR311var3 can be considered to be devoid of long fibrils. Although short fibrils were detected on CR311var3 they were very sparse, indicating that the majority of dense short fibrils in the tuft had been lost. The recovery of phenotypically distinct

morphotypes suggests that there may be more than one structural gene for fibrils in *S. cristatus*. The multiplicity of fimbrial (fibril) genes among different species of oral streptococci, including *S. parasanguis*, *S. gordonii* and *S. salivarius*, supports this complexity of fibril structure and function. It is possible that this bacterium may undergo phase variation.

Physical segregation of these morphotypes allowed us to examine fibril function. The long fibrils appear to be important for corn-cob formation. Both *S. cristatus* CR311 and CR311var1 adhered to other oral bacteria and formed indistinguishable corn-cobs. Both the quantity of the corn-cobs and the density of streptococci attached to *C. matruchotii* were similar in each case. This suggests that the loss of the short fibrils does not adversely affect binding of *S. cristatus* to *C. matruchotii*. In contrast, attachment of CR311var3 to *C. matruchotii* was significantly reduced relative to that observed with the wild-type strain. Thus, it appears that the loss of the long fibrils is linked to the loss of corn-cob formation.

Our studies provide evidence that a novel gene for a high molecular weight serine-rich protein is associated with the long fibrils, and therefore corn-cob formation, in *S. cristatus*. According to best estimates this SrpA protein is greater than 300 kDa. Its unusually large apparent molecular weight hampered its characterization even on low percentage SDS-polyacrylamide gels. The SrpA protein is marked by two relatively long stretches of amino acids that repeat multiple times. Also, almost half of the protein comprises serine. The SrpA protein is physically similar but neither genetically nor serologically related to the Fap1 fimbrial-associated protein of *S. parasanguis* (31). There is no similarity between either the nucleotide or the deduced amino acid sequences of Fap1 and SrpA. Fap1 is a bit smaller than SrpA (2552 vs. 3382 amino acids), contains two extensive amino acid repeat regions, and its amino acid composition is 43% serine. Even more fascinating is that most of the serine residues in SrpA are arranged in dipeptides as in Fap1. Both proteins contain the gram-positive cell wall anchor motif LPNTG. Similar, but not identical, properties have been reported for other very large streptococcal surface proteins such as CshA (18–21) and *S. salivarius* cell wall associated proteins (17, 30), which appear to be involved in colonization. The physical similarities between SrpA and these other surface appendage-associated proteins suggest a

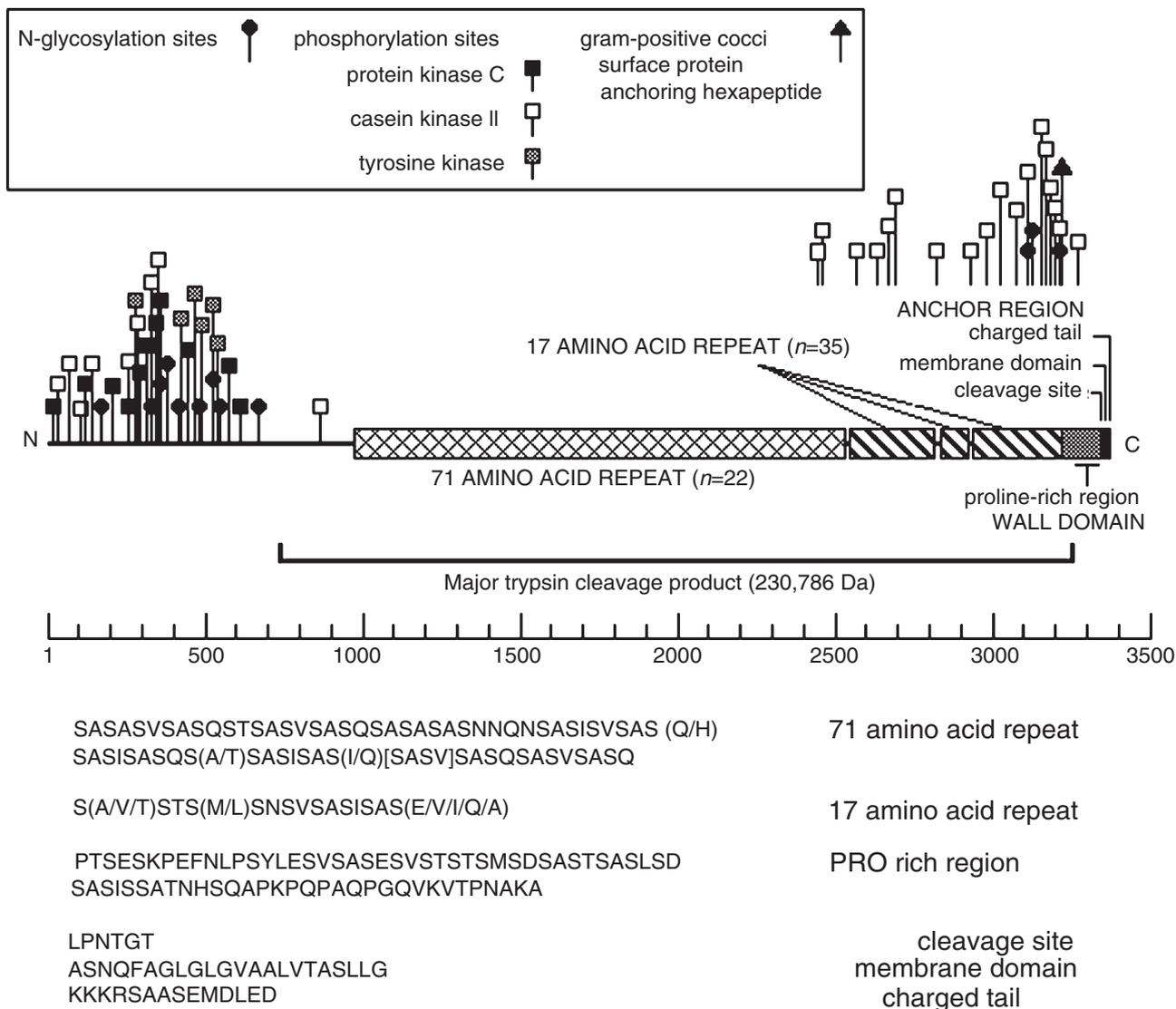


Fig. 5. Schematic representation of the SrpA protein from *S. cristatus* CC5A. Organization of the predominant features of the protein are shown. Theoretical glycosylation and phosphorylation sites were determined by computer analysis. Amino acid sequences of the repeat regions and carboxy terminal features of gram-positive wall-anchored proteins were deduced from the nucleotide sequence deposited in GenBank. Sites of amino acid variation within the repetitive sequences are bound by parentheses. Brackets indicate where deletions occur in some of the repeats.

similarity in function such that it is likely that the *srpA* gene also codes for a fibril-related protein. It is known that protease treatment of the streptococci results in removal of the long fibrils and correspondingly reduced cell surface hydrophobicity, cornucob formation and the other properties that appear to be associated with these structures (13). A relationship between the *srpA* gene and the long fibrils is also supported by PCR analysis, which showed that a homologous sequence could not be amplified from DNA from the fibril-deficient variant CR311var3. Furthermore, the SrpA protein was not detected in whole cell lysates of this variant, indicating that the gene is not expressed. The loss of the

gene or gene expression, accompanied by the absence of the long fibrils, reduction in hydrophobicity, inability to form cornucobs with *C. matruchotii* and loss of coaggregation with *F. nucleatum*, further strengthens this relationship between the long fibrils and the SrpA protein. It is possible that the loss of *srpA* expression indirectly affects transport or assembly of the long fibrils. However, the putative repeat structure and exceptionally large size of the SrpA protein is more consistent with a structural role for the protein.

The putative structure of the SrpA protein supports the presence of N-linked glycosylation sites. However, we do not have direct evidence that the native SrpA

protein is modified with carbohydrate. Previous studies have shown that the surface of *S. cristatus* is decorated with a Ruthenium red-staining material that may be polysaccharide (12). Based on the finding that polysaccharide is present in the fibrillar tufts, it has been speculated that the fibrils of *S. cristatus* CR311 are composed of a glycoprotein (10, 13). This idea would also be consistent with reports that the Fap1 protein of *S. parasanguis* (3, 26) and the fibril elements of *S. salivarius* (16) are modified with carbohydrate.

Thus, the similarity of our data with *S. cristatus* to those of other studies (17, 18, 28–30) strengthens the possibility that these relatively large streptococcal surface

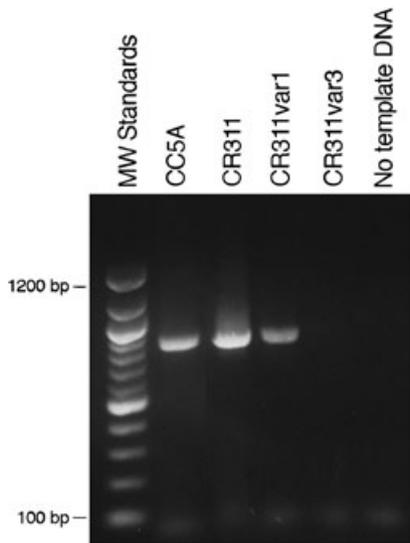


Fig. 6. PCR analysis of *srpA* gene sequences in wild-type and fibril variants of CR311. Total DNA from CC5A, CR311, CR311var1 and CR311var3 was amplified in PCR using primers made against the 5'-end of the *srpA* gene. PCR products were separated on an agarose gel and stained with ethidium bromide. A 100 bp DNA ladder was used as molecular size markers.

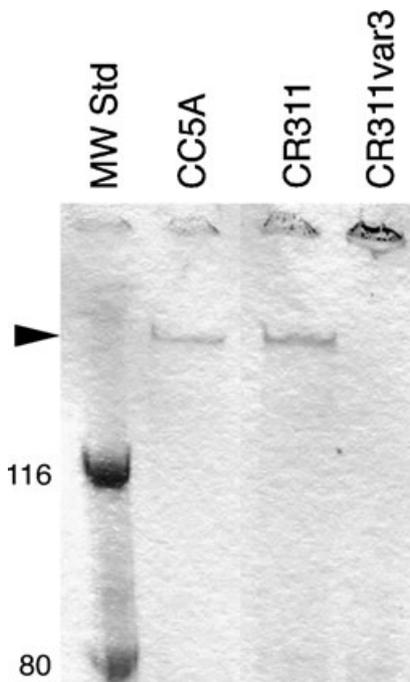


Fig. 7. SDS-polyacrylamide gel analysis of high molecular weight proteins in wild-type and fibril variants of CR311. Proteins from whole cell lysates of CC5A, CR311 and CR311var3 were separated on an 8% SDS-polyacrylamide gel and detected by staining with Coomassie Blue. Molecular weight markers are in kDa. The arrow marks the position of the putative SrpA protein.

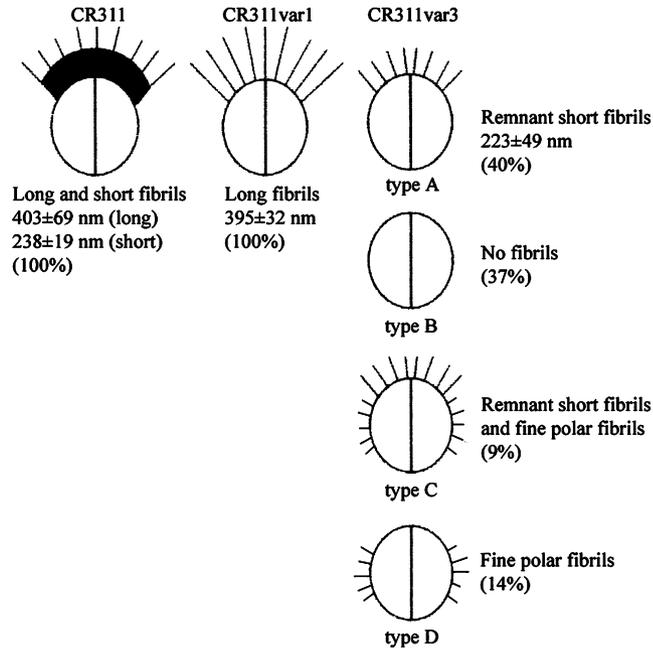


Fig. 8. Population distribution of the various *S. cristatus* CR311 fibrillar tuft morphological variants. Percent values represent the relative distribution of each fibril type in stationary phase cultures.

proteins, with extensive repeat structure and adhesion associated properties, represent a family of fibril/fimbrial proteins important for oral biofilm formation. The present study was performed using planktonic phase bacteria grown under nutrient-rich conditions. It is important now to extend these studies using bacteria grown under biofilm-forming conditions to examine the expression of the *srpA* gene and fibril formation.

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