The csp operon of *Streptococcus salivarius* encodes two predicted cell-surface proteins, one of which, CspB, is associated with the fimbriae

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A Tn917 mutant library was generated to identify genes involved in the biogenesis of *Streptococcus salivarius* fimbriae. A fimbria-deficient mutant was isolated by negative selection using an immunomagnetic separation technique with specific anti-fimbriae polyclonal antibodies (pAbs). The transposon was inserted in an ORF, called orf176, which encoded a protein of unknown function. The transposon prevented the transcription of orf176 as well as two genes located downstream, which are designated cspA and cspB and which form the csp operon. Sequence analyses of CspA and CspB revealed that both proteins possessed the classic cell-wall-anchoring motif (LPXTG) of Gram-positive bacterial surface proteins. Recombinant CspA (rCspA) and CspB (rCspB) proteins were generated in *Escherichia coli* and used to produce pAbs. Immunolocalization experiments showed that anti-rCspB, but not anti-rCspA antibodies specifically recognized *S. salivarius* fimbriae. Our results suggested that the csp operon encoded predicted cell-surface proteins, one of which, CspB, was associated with the fimbriae.

**INTRODUCTION**

Bacteria produce a vast array of surface structures, including fimbriae. Fimbriae are hair-like appendages that are composed of protein subunits with diameters ranging from 2 to 8 nm and that usually extend 1 to 2 μm from the cell surface (Low et al., 1996). They enable bacteria to establish microbial communities by recognizing specific receptors in their natural environments (Fernández & Berenguer, 2000; Klemm & Schembri, 2000). In some cases, a single bacterial species can express a wide variety of fimbriae with different binding specificities (Low et al., 1996).

The fimbriae of Gram-negative bacteria have been extensively studied, particularly those of *Escherichia coli* and *Salmonella* spp. (Mol & Oudega, 1996; Soto & Hultgren, 1999). In these organisms, fimbrial proteins are encoded by plasmid or chromosomal genes organized into operons. In most cases, the structural and regulatory genes involved in the biogenesis of fimbriae are contiguous. However, in some cases they are separated and constitute distinct operons (Thanassi & Hultgren, 2000). Unlike the fimbriae of Gram-negative bacteria, little information is available on the structure, composition and genetics of the fimbriae of Gram-positive bacteria. The fimbriae of *Actinomyces naeslundii* (Yeung, 2000) and *Streptococcus parasanguinis* (Wu & Fives-Taylor, 2001), two members of the indigenous oral flora, have been the most extensively studied.

Fimbriae have also been reported in *Streptococcus salivarius*, a pioneer species that colonizes the human oral mucosa (Handley et al., 1984). Recent work suggested that *S. salivarius* fimbriae are involved in the coaggregation of *S. salivarius* with the periodontopathogen *Prevotella intermedia* (Lévesque et al., 2003). Although fimbriae have been observed on up to 50 % of *S. salivarius* cells in the human oral cavity, no information is available on their biochemical composition and no molecular genetic studies have been done to investigate the genes involved in their biosynthesis and assembly (Handley et al., 1999). We purified *S. salivarius* fimbriae and determined a partial internal amino acid sequence for the fimbrillin. The internal amino acid sequence was composed of a repeated motif of two amino acids alternating with two modified residues: A/X/T-E-Q-M/Φ. We postulated that these residues (X and/or Φ) might be glycosylated amino acids, since carbohydrates are present in pure preparations of fimbrillin (Lévesque et al., 2001). To date, no effective methods have been described for the complete dissociation of *S. salivarius* fimbriae into subunits, suggesting that the fimbrial subunits may be covalently linked.

To identify the genes involved in the biogenesis of *S. salivarius* fimbriae, transposon mutagenesis was performed to generate

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*Abbreviations: IMS, immunomagnetic separation; pAbs, polyclonal antibodies; PTA, phosphotungstic acid.*

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mutants with altered fimbrial expression. In this paper, we report the isolation of a fimbria-deficient mutant of \textit{S. salivarius} and the identification of a fimbria-associated protein.

**METHODS**

\textbf{Bacterial strains and culture conditions.} All \textit{S. salivarius} strains were grown in a Hogg-Jago lactose (HJL) medium (Marciset \& Mollet, 1994) and incubated at 37 C, or at 30 C when they harboured pTV1-OK. A37 is a hyperfimbriated mutant isolated from \textit{S. salivarius} ATCC 25975 by positive selection for resistance to 0-5 mM 2-deoxyglucose (Brochu et al., 1993; Gauthier et al., 1990) and was grown in HJL broth supplemented with 0-5 mM 2-deoxyglucose. \textit{E. coli} strains were grown aerobically at 37 C in Luria–Bertani (LB) medium, or at 30 C in LB-kanamycin broth when they harboured pTV1-OK. When needed, antibiotics were added as follows: 50 \mu g ampicillin ml\(^{-1}\) or 30 \mu g kanamycin ml\(^{-1}\) for \textit{E. coli}, and 10 \mu g erythromycin ml\(^{-1}\) or 500 \mu g kanamycin ml\(^{-1}\) for \textit{S. salivarius}.

\textbf{Transposon mutagenesis.} Plasmid pTV1-OK is a thermosensitive delivery vector for Tn917 mutagenesis (Gutierrez et al., 1996). It harbours the \textit{aph}3-3 gene, which confers kanamycin resistance on both \textit{E. coli} and Gram-positive organisms, and the Tn917 transposon, which confers erythromycin resistance on Gram-positive organisms. Plasmid pTV1-OK was obtained by transformation of \textit{E. coli} HB101 and selection at 30 C on LB-kanamycin agar. The transformation was selected at 30 C on HJL-kanamycin agar. Transformants were negatively stained and observed with a transmission electron microscope as described below to ensure that the streptococcal cells still produced fimbriae. Tn917 mutagenesis was performed as described by Gutierrez et al. (1996) with the following modifications: a single transformed colony was inoculated into 10 ml HJL-kanamycin broth and grown overnight at 30 C. The culture was diluted 1:1000 in HJL supplemented with erythromycin at a sublethal concentration of 0-04 \mu g ml\(^{-1}\) and incubated at 30 C for 3 h. Independent pools of Tn917 insertion mutants were generated by a temperature shift to 42 C with overnight incubation.

\textbf{Immunomagnetic separation (IMS) procedure.} Immunomagnetic beads [2-8 \mu m diameter, 6-5 \times 10^6 beads ml\(^{-1}\) (Dynabeads M-280; Dynal)] with covalently linked sheep anti-rabbit immunoglobulin G (IgG) were used. The beads (approx. 6-5 \times 10^6) were washed twice with sterile PBS (pH 7-4) supplemented with 0-1% (w/v) bovine serum albumin (BSA), separated by magnetic force, and then resuspended to the original volume in PBS–BSA. They were then coated with capture antibodies by incubating them for 3 h at 4 C with specific anti-fimbriate polyclonal antibodies (pAb HL-72) (Levesque et al., 2001) diluted 1:50 in PBS–BSA. The mixture was rotated at 50 r.p.m. to prevent settling of the beads. The pAb-coated magnetic beads were stored at 4 C and washed before use as described by the manufacturer. The \textit{S. salivarius} transposon mutant library was washed twice with sterile PBS (pH 7-4) and sonicated on ice (2 x 20 s, 20% duty cycle, power level 3; Branson Sonic Power Co.) in PBS (pH 7-4) to break up streptococcal chains. A 15 \mu l volume of pAb-coated beads was mixed with 1 ml of the \textit{S. salivarius} transposon mutant library (approx. 2 \times 10^10 cells) and incubated at 4 C overnight with rotation at 50 r.p.m. Beads with attached bacteria were discarded by magnetic force. The supernatant was recovered and incubated again at 4 C overnight with a 15 \mu l volume of pAb-coated beads. The isolation of \textit{S. salivarius} Tn917 insertion mutants with altered fimbrial expression required eight rounds of IMS. The final supernatant was spread on HJL agar supplemented with 10 \mu g erythromycin ml\(^{-1}\) and the plates were incubated at 37 C for 48 h. Clones with altered fimbrial expression were identified by colony immunoblot using pAb HL-72 and observed by transmission electron microscopy as described below.

\textbf{Electron microscopy.} Ten-microlitre samples of bacterial suspensions (approx. 8 \times 10^7 cells ml\(^{-1}\)) were applied to carbon-coated Formvar copper grids (Canemco) and negatively stained with 1% (w/v) phosphotungstic acid (PTA) (pH 7-0) for 10 s. The grids were air-dried prior to examination with a JEOL 2000 transmission electron microscope operating at 80 kV. For immunogold labelling experiments, 10 \mu l samples of bacterial suspensions (approx. 8 \times 10^7 cells ml\(^{-1}\)) were applied to carbon-coated Formvar copper grids. After 30 min, the grids were blocked with PBS (pH 7-2) containing 1% (w/v) IgG-free and protease-free BSA (Jackson ImmunoResearch Laboratories) for 30 min. The grids were then incubated with specific rabbit pAbs diluted in blocking buffer for 1 h and rinsed in PBS (pH 7-2) and distilled water. The grids were incubated for 1 h with 12 nm colloidal gold-labelled donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) diluted 1:40 in blocking buffer and rinsed in PBS (pH 7-2) and distilled water. The samples were negatively stained and examined as described above.

\textbf{DNA manipulations.} Routine DNA manipulations were performed as described by Sambrook et al. (1989). Large-batch preparations of plasmid DNA were obtained using a Qiagen Plasmid Maxi kit. Genomic DNA was isolated from streptococci using a Puregene DNA Isolation kit (Gentra Systems). For the Southern blot experiments, the 3-6 kb \textit{KpnI}–\textit{StuI} and 1-0 kb \textit{EcoRV} fragments from pTV1-OK used as Tn917 and vector probes, respectively, were labelled with [\textit{32P}]dATP by the random priming DNA labelling technique (Sambrook et al., 1989). Based on the nucleotide sequence of Tn917 (Shaw \& Clewell, 1985), the oligonucleotide primers Tn917-L (\textit{5′}-TGTCCACCGTCAAGTAAAATG-\textit{3′}) and Tn917-R (\textit{5′}-GAACATATTTTGGCGGACG-\textit{3′}) were designed for the extremities of the transposon. Inverse PCR using the Tn917-L and Tn917-R primers was performed to recover the Tn917 insertion site from mutant D37 chromosomal DNA. Inverse PCR was carried out in a 100 \mu l volume containing 10 \mu l 10× PCR buffer (Amersham Biosciences), 5 \mu l 10× deoxynucleoside triphosphate mix (2 mM each dATP, dCTP, dGTP and dTTP), 1 \mu l each primer stock solution (50 \mu M), 5 \mu l template DNA (200 ng EcoRI-restricted and self-ligated mutant D37 chromosomal DNA) and 2-5 \mu l Taq DNA polymerase (Amersham Biosciences). A single 785 bp PCR product was amplified using a three-step profile: 1 min denaturation at 94 C, 1 min annealing at 60 C and 2 min extension at 72 C for a total of 35 cycles. The 785 bp product was sequenced with the Tn917-L and Tn917-R primers. Isolation of DNA regions contiguous to the insertion site was carried out by chromosome walking using appropriate synthesized oligonucleotides. DNA sequencing
was performed by the DNA sequencing service of Université Laval. Computer-assisted DNA and protein analyses were performed using the Genetics Computer Group Sequence Analysis software package, Version 9.1 (Devereux et al., 1984).

**Northern blots.** Total RNA was extracted from streptococci using the RNeasy kit (Qiagen), according to the manufacturer’s protocol, and separated on 1% (w/v) agarose/formaldehyde gels (Sambrook et al., 1989). Northern hybridization procedures were performed as described by Lortie et al. (2000). Immobilized RNA was hybridized with DNA probes labelled with [γ-32P]dATP by the random priming DNA labelling technique (Sambrook et al., 1989). The oriT and csp probes were amplified by PCR from S. salivarius ATCC 25975 genomic DNA using the following primers: D37-8 (5′-GGAGAAACATATGGAATACAC-3′) and D37-9 (5′-TTTAAGATGTATTATGCGAATGC-3′) for oriT, and D37-5 (5′-CTACGACTTCTGAATGGC-3′) and D37-13 (5′-AATAGAGGCATGTCTTTCG-3′) for csp.

**Cloning into the expression vector.** The full-length coding regions of cspA and cspB were first amplified by PCR using the following primers: D37-14 (5′-AAAGAATTCTGCTGATATGG-3′); with a 2 bp substitution creating an NdeI site and D37-15 (5′-GGCCCTTGGCTGATATGG-3′); with a 2 bp substitution creating an NdeI site for cspA, and D37-24 (5′-ATCGATATGTTAAGACATCAAG-3′); with a 2 bp substitution creating an NdeI site and D37-25 (5′-AAAGAATTCTGCTGATATGG-3′); with a 2 bp substitution creating a BamHI site for cspB. PCR products were then cloned into pCR2.1-TOPO (Invitrogen) as described by the manufacturer.

**Expression of recombinant proteins.** E. coli BL21(DE3)(pSS301) (1446 bp amplified fragment of cspA in pCR2.1-TOPO) and pSS307 (2576 bp amplified fragment of cspB in pCR2.1-TOPO) were used to transform E. coli DH10B. The 1413 bp NdeI fragment of pSS301 and the 2563 bp NdeI fragment of pSS307 were subcloned in-frame downstream from the hexa-His sequence in the T7 expression vector pET28a (+) (Novagen) precut by the same enzymes and transformed into E. coli DH10B. The nucleotide sequences of the inserts were confirmed by sequence analysis. Plasmids designated pSS305 and pSS308 were then transformed into E. coli BL21(DE3) to express the hexa-His-tagged CspA and hexa-His-tagged CspB recombinant proteins, respectively.

**RESULTS**

**Construction of a Tn917 mutant library of S. salivarius**

To identify genes involved in the biogenesis of S. salivarius fimbriae, we generated a transposon mutant library using the Tn917 delivery vector pTV1-OK (Gutierrez et al., 1996). Approximately 100 randomly selected erythromycin-resistant (Emr) mutants were replicated on medium supplemented with kanamycin to determine the percentage of Tn917 insertion mutants that had lost the plasmid backbone. We found that 90% of the selected Emr clones were kanamycin-sensitive (Kms), while 10% were kanamycin-resistant (Kmr). This suggested that 90% of the Emr clones had integrated the transposon into the chromosome (Emr) and had lost the plasmid backbone (Kms). Southern blot analyses of chromosomal DNA from 15 randomly selected Emr/Kms mutants digested with Stul (Stud cuts once in pTV1-OK) and probed with Tn917 DNA indicated that transposition was random and resulted in a single chromosomal insertion (data not shown). When analysed by Southern blotting, the Stud-digested chromosomal DNA of ten randomly selected Emr/Kms clones reacted with the Tn917 and vector probes, indicating that replication fusion had occurred (data not shown). Cointegrated structures have already been reported for transposons of the Tn3 family, to which Tn917 belongs (Brown & Evans, 1991; Heffron, 1983). The transposon mutant library of S. salivarius ATCC 25975 was used to identify fimbria-related gene(s) by isolating mutants with altered fimbrial expression.

**Screening the transposon library for fimbria-deficient mutants**

To enrich the library of S. salivarius mutants with altered fimbrial expression, we developed an IMS procedure using pAbs directed specifically against S. salivarius fimbriae. Colony immunoblot experiments conducted with over 4000 S. salivarius mutants obtained by IMS resulted in the isolation of three mutants with altered fimbrial expression: C34 (Emr/Kms), D11 (Emr/Kms) and D37 (Emr/Kms). Transmission electron microscopy observations clearly showed fimbriae extending from the cell surfaces of mutants C34 and D11, but in very small amounts compared to the wild-type strain, whereas mutant D37 was completely devoid of fimbriae. As shown in Fig. 1, immunolabelling with pAb HL-72 stained fimbriae extending from the cell surface of wild-type cells (Fig. 1a), while no organized...
structures resembling fimbriae were recognized by these pAbs on mutant D37 cells (Fig. 1b). Mutant D37 was selected for further investigation.

**Recovery of the Tn917-interrupted gene**

The presence of Tn917 in the chromosome of mutant D37 was confirmed by Southern blot analysis of StuI-digested chromosomal DNA using the Tn917 DNA probe. Hybridization with the vector probe confirmed that the plasmid had integrated into the chromosome of this mutant (data not shown). Repeated subculturing in selective media confirmed that D37 was stable. To recover the interrupted gene, chromosomal DNA from D37 was digested with EcoRI and self-ligated. Inverse PCR performed using the Tn917-L and Tn917-R primers, which are complementary to the left and right ends of the transposon, respectively, allowed the insertion site to be isolated. An ORF, named orf176, was found to be interrupted by the insertion of Tn917 and the plasmid backbone at the TATTA target site (Fig. 2).

orf176 was predicted to encode a 176 aa protein with an estimated molecular mass of 20.4 kDa and an isoelectric point (pI) of 4.39. In silico structural prediction analyses suggested that the protein was cytoplasmic. BLAST searches of current databases did not reveal any significant identity with bacterial fimbrial or non-fimbrial proteins. A potential ribosome-binding site was identified 6 bp upstream from the predicted start codon of orf176 (Fig. 2b). A promoter sequence (TTGCTC-N18-TACACT) was also identified 50 bp upstream from the ATG start codon by its similarity to the consensus $-35$ and $-10$ sequences (Lisser & Margalit, 1993). The TRTG motif ($-16$ region) found in several *Bacillus subtilis* and other Gram-positive bacteria promoters (Voskuil & Chambliss, 1998) was also present. A terminator-like structure (factor-independent terminator) was located 32 bp downstream from the stop codon of orf176 (Fig. 2b).

**DNA sequence analysis of the chromosomal regions adjacent to orf176**

To investigate the possibility that the insertion of a large DNA fragment could have affected the expression of contiguous genes, DNA regions flanking orf176 were isolated using direct and inverse PCR approaches. The organization of the ORFs located upstream and downstream from orf176 is shown in Fig. 2(a). Nucleotide sequence analysis of the 1.2 kb portion upstream from orf176 revealed the presence of an ORF oriented in the same transcriptional direction.
The deduced amino acid sequence of this ORF had significant levels of identity with the DNA topoisomerase I of *Enterococcus faecalis* (Bidnenko et al., 1998) (E value 1e⁻³¹) and the topoisomerase III of *B. subtilis* (Kunst et al., 1997) (3e⁻¹⁴) and *E. coli* (Perna et al., 2001) (3e⁻¹¹). This ORF was therefore designated *topA*.

Nucleotide sequence analysis of the 4.2 kb portion downstream from *orf176* revealed the presence of two ORFs transcribed in the same direction as *orf176* (Fig. 2). No ORFs of significant size were detected on the reverse strand. The first ORF was 147 bp downstream from *orf176*. It was predicted to encode a 459 aa protein with an estimated molecular mass of 46.2 kDa and a pI of 4.20. The first 31 aa were predicted to compose a signal peptide. The putative leader sequence cleavage site of the precursor protein was predicted between the alanine and the aspartic acid at positions 31 and 32, respectively. A cell-wall-anchoring motif (LPXTG) of Gram-positive bacterial surface proteins (Fischetti et al., 1990) followed by a hydrophobic domain and a charged tail were also identified at the C terminus of the protein. This ORF was thus named *cspA* since the encoded polypeptide was predicted to be a cell-surface protein. BLAST searches revealed significant identity with *Streptococcus agalactiae* (Tettelin et al., 2002) (E value 2e⁻¹²). The second ORF, named *cspB*, was 17 bp downstream from *cspA* and was predicted to encode an 846 aa protein with an estimated molecular mass of 90.6 kDa and a pI of 4.35. The first 42 aa were predicted to compose a signal peptide. Thus, the putative leader sequence cleavage site of the precursor protein encoded by *cspB* was predicted to be between the alanine and the aspartic acid at positions 42 and 43, respectively. An LPXTG motif followed by a hydrophobic domain and a charged tail were also identified at the C terminus of the protein. BLAST searches of current databases revealed significant identity with cell-surface proteins of Gram-positive bacteria that belong to two families of...
adhesins ($E$ values ranging from $9 \times 10^{-32}$ to $8 \times 10^{-4}$). The first family includes members of the antigen I/II family of oral streptococci (Jenkinson & Demuth, 1997): SspA and SspB of *Streptococcus gordonii*; PAc, SpaP and Sr of *Streptococcus mutans*; and SpaA of *Streptococcus sobrinus*. The second family includes the aggregation substances of enterococci (Weaver, 2000): Asa373, Asc10 and Asa1 of *E. faecalis*; and Ash701 of *Enterococcus faecium*.

Potential ribosome-binding sites were located 9 and 5 bp upstream from the predicted start codons of *cspA* and *cspB*, respectively (Fig. 2b). A promoter sequence (TTGAGT-$N_{16}$-TATCAT) was located 37 bp upstream from the ATG start codon of *cspA*. There were no potential promoter sequences or apparent transcription-termination sequences between *cspA* and *cspB*, suggesting that they were cotranscribed. A terminator-like structure (factor-independent terminator) was found 68 bp downstream from *cspB*.

**Transcriptional studies**

Northern blot analyses of total RNA isolated from *S. salivarius* ATCC 25975 and fimbria-deficient mutant D37 using the orf176 coding region as a DNA probe allowed the detection of a single 0.6 kb mRNA transcript in wild-type cells (data not shown). The size of this transcript was consistent with the transcription of orf176 into a monocistronic mRNA. Since orf176 was interrupted by the insertion of Tn917 as well as the plasmid backbone in the chromosome of mutant D37, the 0.6 kb orf176 transcript was not detected in these cells (not shown). To assess the effect of the cointegrated structure on the transcription of the genes located downstream, Northern blots were also conducted using a *csp* DNA probe. The probe allowed the detection of a single 4 kb mRNA transcript in wild-type cells (Fig. 3, lane 1). The size of this transcript was consistent with the cotranscription of *cspA* and *cspB*. Interestingly, no mRNA transcript was detected with the *csp* probe in the fimbria-deficient mutant D37 cells (Fig. 3, lane 2).

**Immunolocalization of CspA and CspB**

The fact that the *csp* operon (i) was located downstream from the Tn917-interrupted gene, (ii) contained genes encoding predicted cell-surface proteins and (iii) was not transcribed in the fimbria-deficient mutant D37 suggested that this operon could encode fimbria-related proteins. We therefore used pAbs raised against the rCspA and rCspB proteins to determine whether there was an association between CspA and CspB and *S. salivarius* fimbiae.

As shown in Fig. 4, immunogold labelling with pAb KL-46 raised against rCspA did not stain the fimbiae (indicated by the arrow) extending from the surface of wild-type cells. Immunogold labelling with pAb KL-46 was also conducted using whole cells of A37, a hyperfimbriated mutant of *S. salivarius* ATCC 25975. As with the wild-type cells, no labelling was observed with A37 even though the mutant produced many more fimbiae than the parental strain (data not shown). Dot blot experiments conducted using fimbiae

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**Fig. 3.** Northern blot of total RNA isolated from *S. salivarius* ATCC 25975 (lane 1) and mutant D37 (lane 2) hybridized with the *csp* DNA probe. The arrow indicates the *cspA*-*cspB* 4 kb transcripts. The bands at 1.5 and 2.9 kb correspond to non-specific hybridization to 16S and 23S rRNA, respectively.

**Fig. 4.** Immunolocalization of CspA on the *S. salivarius* ATCC 25975 cell surface by electron microscopy. Bacterial cells were successively incubated with pAb KL-46 and 12 nm colloidal gold-conjugated donkey anti-rabbit IgG and then negatively stained with 1% PTA (pH 7.0). Fimbriae that are not labelled by the antibodies are indicated by the arrow (see text). Bar, 100 nm.
purified from glass bead extracts (Lévesque et al., 2001) did not react with the anti-rCspA pAbs (data not shown). These results suggested that CspA was not associated with the fimbrial structure extending outside the cell wall.

Immunogold labelling with pAb KL-49 raised against rCspB stained the fimbriae extending from the surface of wild-type (Fig. 5a) and hyperfimbriated mutant A37 (Fig. 5b) cells. As can be seen in Fig. 5, the hyperfimbriated mutant cells showed more extensive labelling of fimbriae than the wild-type cells. No immunolabelling by pAb KL-49 of the cells showed more extensive labelling of fimbriae than the wild-type cells. No immunolabelling by pAb KL-49 of the fimbria-deficient mutant D37 was observed (Fig. 5c). When dot blot experiments were conducted using purified fimbriae, the results showed that anti-rCspB pAbs reacted with the fimbrial preparation, confirming that CspB was associated with S. salivarius fimbriae.

**DISCUSSION**

Bacteria produce a variety of surface structures and appendages that are involved in host colonization. In the oral cavity, bacterial adherence to salivary components, the oral mucosa and other bacteria is often mediated by fimbriae (Hamada et al., 1998; Handley et al., 1999; Lamont & Jenkinson, 2000). However, little is known about the structure, composition and genetics of the fimbriae of Gram-positive bacteria. The difficulty in studying these fimbriae is largely due to their resistance to extraction from the cell surface coupled with possible covalent links between the fimbrial subunits, making them impossible to dissociate into individual subunits for SDS-PAGE analysis. In this study, we sought to identify the genes involved in the biogenesis of S. salivarius fimbriae. We thus devised an original immunogenetically based strategy that enabled us to isolate the fimbria-deficient mutant D37.

The interrupted gene of mutant D37 was recovered by inverse PCR and the gene was sequenced in the wild-type strain. Inactivation of orf176 in S. salivarius generated a fimbria-deficient phenotype. Since the structural and regulatory genes involved in the biogenesis of fimbriae are often clustered within a single chromosomal region (Low et al., 1996; Mol & Oudega, 1996; Thanassi & Hultgren, 2000), DNA regions flanking orf176 were isolated and sequenced. orf176 was located between topA, which encodes a putative topoisomerase, and the csp operon containing two genes (cspA and cspB), which encode predicted cell-surface proteins.

Interestingly, the csp operon was not transcribed in the fimbria-deficient mutant D37. This may suggest that transcription of the csp operon was linked to orf176 expression and that the Orf176 protein acted as its transcriptional regulator. However, in silico structural prediction analyses of Orf176 failed to assign the classic helix–turn–helix motif to a portion of the polypeptide. The absence of this DNA-interacting motif and the fact that Orf176 did not share significant identity with any proteins in current databases, including unfinished microbial genomes, suggested that Orf176 might be a novel, fairly rare regulatory protein.

Although transcription of orf176 generated a single monocistronic transcript, we cannot exclude the possibility that the insertion of a large fragment of DNA (at least two copies of Tn917 along with the plasmid backbone) might affect the architecture of this chromosomal region, thus altering the transcription of the csp operon. Gene knockout of orf176 combined with complementation experiments will be required to discriminate between these hypotheses. However, until now, all our attempts to inactivate orf176 in the wild-type strain by allelic exchange or to complement the mutant strain have proven unsuccessful due to the resistance of S. salivarius to genetic manipulation.

The cellular location of CspA and CspB, two predicted cell-surface proteins, was examined by immunolabelling experiments using pAbs directed against purified CspA and CspB recombinant proteins. Although CspA was not associated with the fimbrial structure extending outside the cell wall, we could not rule out the possibility that it was associated with the cell-wall-embedded portion of the fimbriae, a portion that is not accessible for detection by antibodies. CspA might also be an accessory protein that is not integrated into the fimbrial structure, but is needed for the biogenesis of the fimbriae. In A. naeslundii T14V, six accessory fimbrial proteins, as well as the fimbrillin, are required for the correct assembly of type 1 fimbriae (Yeung, 2000; Yeung & Ragsdale, 1997).

Immunolocalization experiments with anti-rCspB pAbs showed that CspB was associated with the fimbriae. Although CspB was not the S. salivarius fimbrillin as it did not possess the A/X/T-E-Q-M/Φ repeated motif previously reported (Lévesque et al., 2001), this protein was also a fimbrial subunit since gold particles were observed attached to the fimbriae. In E. coli type 1 fimbriae, fimbrillin as well as other fimbrial subunits are interspersed at intervals along the fimbriae (Klemm & Krogfelt, 1994; Krogfelt & Klemm, 1988). In addition to its role in the structural organization of fimbriae, CspB may also have adhesin functions. Indeed, it was shown to share identity with several proteins of the streptococcal antigen I/II family. Members of the antigen I/II family are multifunctional adhesins that mediate interactions between oral streptococci and other oral bacteria, cell matrix proteins and human salivary glycoproteins (Jenkinson, 1994; Jenkinson & Demuth, 1997; Jenkinson & Lamont, 1997). Consistent with this hypothesis, we recently demonstrated that fimbriated S. salivarius cells coaggregated with P. intermedia while fimbria-deficient mutant D37 cells were unable to interact with this periodontopathogen (Lévesque et al., 2003).

The CspA and CspB proteins contained a cell-anchoring motif at the carboxyl terminus, suggesting that they are probably C-terminal-linked surface proteins. Indeed, all C-terminal-linked surface proteins from Gram-positive bacteria have a specific sorting signal composed of similar arrangements of amino acids (Fischetti, 2000; Navarre & Schneewind, 1999). CspA and CspB are the first putative C-terminal-linked surface proteins to be identified in...
S. salivarius. The only surface protein identified in S. salivarius is β-D-fructosyltransferase, which catalyses the polymerization of the fructose moiety of sucrose in fructans (Rathsam et al., 1993). However, this protein does not possess the LPXTG motif and its anchoring mechanism has not yet been determined (Rathsam & Jacques, 1998). Based on the general model for C-terminal-linked surface proteins of Gram-positive bacteria, these proteins are cleaved between the threonyl and glycyl residues of the LPXTG motif and the newly liberated carboxy terminus of threonine is anchored to cell-wall peptidoglycan (Navarre & Schneewind, 1994, 1999; Schneewind et al., 1995). Since CspB is associated with the fimbriae, it is more probable that the carboxy terminus of threonine, rather than being anchored to the cell-wall peptidoglycan, is covalently linked to another fimbrial subunit, as proposed by Yeung et al. (1998) for A. naeslundii fimbriae and Ton-That & Schneewind (2003) for Corynebacterium diphtheriae fimbriae. This covalent linkage between fimbrial subunits would also account for the inability to dissociate S. salivarius, A. naeslundii and C. diphtheriae fimbriae into individual subunits.

This work represents the first molecular genetic study undertaken to identify the genes involved in the biogenesis of S. salivarius fimbriae. Further studies will be needed to define the mechanisms used by Gram-positive bacteria to assemble fimbrial structures and regulate their synthesis.

Fig. 5. Immunolocalization of CspB on S. salivarius ATCC 25975 (a), hyperfimbriated mutant A37 (b) and fimbria-deficient mutant D37 (c) cell surfaces by electron microscopy. The bacterial cells were successively incubated with pAb KL-49 and 12 nm colloidal gold-conjugated donkey anti-rabbit IgG and then negatively stained with 1 % PTA (pH 7·0). Bars, 100 nm.
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