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Adherence, Coaggregation, and Hydrophobicity of *Streptococcus gordonii* Associated with Expression of Cell Surface Lipoproteins

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Streptococcus gordonii **Challis incorporated exogenous [^{3}H]palmitate into 13 polypeptides extractable from intact cells with sodium dodecyl sulfate. A 76-kDa surface-exposed polypeptide, implicated previously as a cell aggregation determinant, was shown to be one of these lipid-modified polypeptides. Differences in sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of lipopolypeptides were detected with mutants of *S. gordonii* that were altered in adherence, aggregation, coaggregation, or hydrophobicity. Lipid-modified polypeptides, tightly associated with the cell membrane, may be involved in the expression of cell surface properties of *S. gordonii* important for colonization of the human oral cavity.**

Viridans group streptococci adhere to saliva-coated surfaces (10) and coaggregate with other oral bacteria (19), properties that are thought to enable the streptococci to colonize the human oral cavity. Three species of oral streptococci (*Streptococcus gordonii*, *Streptococcus mutans*, and *Streptococcus sobrinus*) produce an antigenically related surface polypeptide with an apparent molecular mass of 185 to 210 kDa. In each species this polypeptide interacts with human salivary glycoproteins and may be involved in the attachment of streptococci to the tooth surface (2, 12, 24, 25). The polypeptide is believed to be an α-helical-coiled fibrillar molecule and to be held at the cell surface through its C-terminal region.

For *S. gordonii* and *S. sanguis*, early colonizers of the clean tooth surface (10), a variety of additional surface protein components have been implicated in cell adherence to the salivary pellicle (4, 9) and in cell aggregation reactions with saliva (23, 30), *Actinomyces* organisms (14, 20), platelets (3), or body fluids such as serum (27). Recently the gene encoding a cell surface-exposed polypeptide with a molecular mass of 76 kDa in *S. gordonii* was cloned, and the gene was insertionally inactivated in *S. gordonii* (17). Mutants that were deficient in production of the 76-kDa polypeptide were impaired in the abilities to aggregate in saliva or serum and to coaggregate with several species of *Actinomyces* (17). From nucleotide sequencing, the inferred amino acid sequence of about 250 amino acids at the N-terminal region of the 76-kDa polypeptide, termed SarA (15), was found to be 70% similar to the corresponding N-terminal region sequence of the AmiA polypeptide from *Streptococcus pneumoniae* (1). Twenty amino acids from the N-terminal methionine of the AmiA precursor, at the C-terminal end of the signal sequence, was the sequence Leu-Ala-Ala-Cys-Ser, which corresponds to the consensus cleavage site for precursors of bacterial lipoproteins (34, 35). On the basis of this it was suggested (11) that mature AmiA was a lipoprotein with a fatty-acylated N-terminal cysteine allowing membrane association or anchorage (11). The nucleotide sequence of *sarA* in *S. gordonii*, encompassing the N-terminal coding region of the polypeptide, is shown in Fig. 1. The deduced amino acid sequence revealed the prolipoprotein consensus sequence L-A-A-C-S at a point 20 amino acids from the N-terminal methionine of the SarA precursor (Fig. 1). Thus the signal sequences of the SarA and AmiA polypeptide precursors are of the same length, and the prolipoprotein cleavage sequences in the polypeptides are identical. The work described in the first part of this article set out to confirm that SarA in *S. gordonii* was a lipoprotein.

Incorporation of exogenous radioactive fatty acid into proteins is an established method for detecting lipoproteins in bacteria (22, 28, 34). Exponential-phase cells of *S. gordonii* DL1 (Challis) in TY-glucose medium at 37°C (18) were transferred to fresh warmed TY-glucose medium (1 ml) containing [9,10-^{3}H]palmitic acid (75 to 100 μCi; 40 to 60 Ci/mmol) (28) at an optical density at 600 nm of 0.1 (about 5×10^7 CFU/ml) and incubated for 2 h at 37°C. Cells were harvested by centrifugation (10,000 × *g* for 2 min at 20°C), washed once by suspension in deionized distilled water, and centrifuged as described above. To extract polypeptides, cells were suspended in 0.1 ml of sodium dodecyl sulfate (SDS) extraction buffer (50 mM Tris-HCl, pH 6.8, containing 2% [wt/vol] SDS and 0.1% [vol/vol] 2-mercaptoethanol) and incubated for 15 min at 70°C. The suspension was centrifuged (10,000 × *g* for 5 min at 20°C), and a portion of the supernatant (0.08 ml) was mixed with one-tenth volume of loading dye (0.01% [wt/vol] bromophenol blue in 70% [vol/vol] glycerol), heated for 5 min at 95°C, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (21). In some experiments, ^{3}H-labeled cell pellets were suspended in 0.1 ml of 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA. Glass beads (0.10- to 0.11-mm diameter, 0.1 ml) were added, and the cells were disrupted by vortex mixing them with the beads for 2 min. SDS extraction buffer (0.1 ml) was added, and the suspension was heated at 70°C for 10 min and centrifuged at 10,000 × *g* for 5 min. The supernatant fluid was collected and mixed with loading dye in preparation for gel electrophoresis.

To test the effectiveness of different extraction methods, bacteria were collected after incubation with [^{3}H]palmitic acid, washed with water, and suspended and incubated in one of the following solutions (0.1 ml): SDS extraction buffer (10 min at 70°C); 0.1 M NaOH in water (30 min at 0 to 4°C); 0.1 M Na_2CO_3 in water (pH 11.5) (30 min at 0 to 4°C); 1% (wt/vol) sodium lauroyl sarcosinate in 10 mM Tris-HCl

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TGA ATG GAG ATA TCG ATG AAG AAA GGT AAA ATC CTT GCA CTT GCG
                    M   K   K   G   K   I   L   A   L   A  10

GGA GTT GCT CTC TTG GCT ACT GGC GTT CTT GCA GCG TGC TCA AAT
 G   V   A   L   L   A   T   G   V   L   A   A   C   S   N  25

TCT ACA TCA AAC TCA TCA AAC TCA TCA AGC TCT GGC GCA GAT CAA
 S   T   S   N   S   S   N   S   S   S   S   G   A   D   Q  40

GTT TTT AAC TAT ATC TAC GAA GTT GAT CCA GAA AAC TTG AAT TAT
 V   F   N   Y   I   Y   E   V   D   P   E   N   L   N   Y  55
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FIG. 1. Nucleotide sequence and deduced N-terminal amino acid sequence of the *sarA* gene product. A Shine-Dalgarno sequence (ribosome binding site) upstream of the Met initiation codon ATG is shown with a broken underline. The amino acids underlined at positions 20 to 24 represent a prolipoprotein cleavage sequence, and the arrow marks the signal peptidase cleavage site.

buffer containing 1 mM EDTA (20 min at 20°C) (14); or 1% (vol/vol) Tween 20 in water (20 min at 20°C). Suspensions were then centrifuged at 12,000 × *g* for 10 min at 4°C, and supernatants were removed and neutralized where necessary with 1 M HCl. Portions (0.1 ml) were mixed with SDS extraction buffer (0.05 ml), and loading dye was added in preparation for gel electrophoresis. After electrophoresis, proteins were stained with Coomassie blue R250 or silver nitrate (26). Gels were prepared for fluorography by fixing them for 30 min in 25% (vol/vol) methanol in water, incubating them for 30 min in Amplify (Amersham Corp., Arlington Heights, Ill.), and drying them onto Whatman 3MM paper under vacuum. Dried gels were exposed to Kodak X-Omat K film at −80°C. Molecular masses of polypeptides were estimated from their migration distances by reference to a plot relating migration distances for marker proteins to their log molecular masses.

When [^{3}H]palmitate was added to exponentially growing cultures of *S. gordonii* Challis, radioactivity was incorporated into a subset of *S. gordonii* proteins solubilized from cells with SDS extraction buffer. On autoradiographs of SDS-PAGE gels, 13 polypeptide bands were visible, and these had molecular masses of 78, 76, 70, 63, 58, 44, 42, 39, 36, 34, 31, 30, and 24 kDa (Fig. 2, lane 1). The 36- and 31-kDa bands were the most strongly labeled and therefore may be the most abundant. Covalent attachment of ^{3}H-labeled fatty acid is likely because radioactivity remained associated with the polypeptides after heating in SDS with 2-mercaptoethanol and their subsequent resolution by SDS-PAGE. An identical pattern of labeled polypeptides was obtained for extracts prepared by SDS solubilization of cells broken with glass beads, showing that cell disruption was not necessary for the radioactively labeled polypeptides to be solubilized. An identical pattern of labeled proteins was obtained for cells grown in brain heart infusion medium (14) (data not shown).

To ascertain whether the SarA polypeptide (76 kDa) was a lipoprotein, the [^{3}H]palmitate labeling pattern of proteins in the aggregation-deficient strain of *S. gordonii* (SarA$^-$) (17) was compared with the pattern in the isogenic parent strain Challis. The mutant did not produce the 76-kDa labeled polypeptide; otherwise, there were no differences in radioactive protein profile (Fig. 2, lane 2). These results, in conjunction with the sequence data in Fig. 1, confirm that the SarA polypeptide is lipid modified, most likely by glyceryl substitution of N-terminal cysteine (34).

Since these results indicated that an acylated protein might be involved in determining streptococcal cell surface properties, alterations in the production of [^{3}H]palmitate-labeled proteins were looked for in another cell surface mutant of *S. gordonii*. Strain OB74 is a more hydrophobic derivative of *S. gordonii* Challis and coaggregates better with oral *Actinomyces* species (16). Cells of strain OB74 were previously shown to overproduce surface-exposed polypeptides with approximate molecular masses of 45, 43, and 33 kDa (16). When cells of strain OB74 were incubated with [^{3}H]palmitate and proteins were solubilized with SDS extraction buffer and subjected to SDS-PAGE, a 44-kDa band (corresponding to a doublet) was found to be present in increased intensity and a new labeled polypeptide band was visible at 33 kDa (Fig. 2, lane 3). Thus surface polypeptides previously identified as being overexpressed in the more hydrophobic mutant strain are lipid-modified proteins.

These experiments strongly suggested an association between expression of lipoproteins and cell surface properties in *S. gordonii* on the basis of comparison of wild-type and isogenic mutant strains. Such associations would be difficult to assign to independently isolated strains of streptococci. For example, *S. sanguis* ATCC 10556, *S. sanguis* 133.79 (3), and *S. sanguis* FW213 (4), all commonly used strains, had

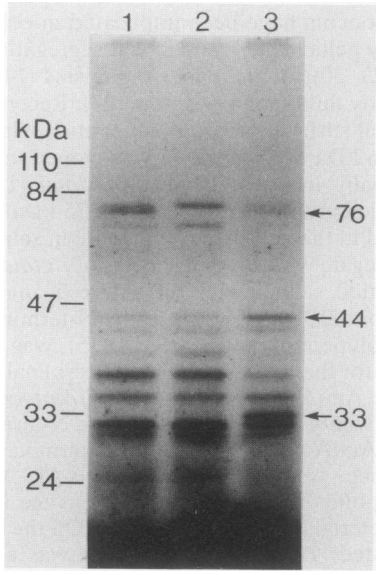

FIG. 2. SDS-PAGE (10% [wt/vol] acrylamide) patterns (revealed by fluorography) of [^{3}H]palmitate-labeled polypeptides solubilized with SDS extraction buffer from intact cells of *S. gordonii* Challis (lane 1), d1b (lane 2), and OB74 (lane 3). Positions of molecular mass markers (in kilodaltons) are indicated for reference. Bands at 76, 44, and 33 kDa are referred to in the text.

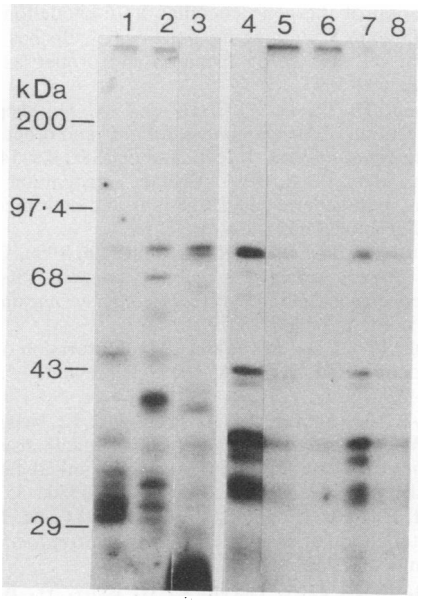

FIG. 3. SDS-PAGE (10% [wt/vol] acrylamide) patterns (revealed by fluorography) of [^{3}H]palmitate-labeled polypeptides solubilized with SDS extraction buffer from intact cells of *S. sanguis* ATCC 10556 (lane 1), *S. sanguis* 133.79 (lane 2), *S. sanguis* FW213 (lane 3), and *S. gordonii* Challis (lane 4). Lanes 5 through 8 are fluorograph patterns of labeled polypeptides solubilized from cells of *S. gordonii* Challis with 0.1 M NaOH (lane 5), 0.1 M Na_2CO_3 (lane 6), 1% (wt/vol) sodium lauroyl sarcosinate (lane 7), and 1% (vol/vol) Tween 20 (lane 8). Positions of molecular mass markers (in kilodaltons) are indicated.

widely differing lipoprotein profiles (Fig. 3, lanes 1 to 4), their only similarity being that all extracts contained a labeled 78-kDa polypeptide.

All the well-characterized lipoproteins of procaryotes contain *N*-acyl glyceride cysteine at the N terminus (34). It seems likely that the lipoproteins detected by [^{3}H]palmitate labeling in *S. gordonii* would be of the similar glyceride-cysteine type, but only for SarA, which has been shown to contain the prolipoprotein consensus sequence, is this certain. It is possible that a lipopolypeptide could contain fatty acid directly acylated to cysteine within the body of the polypeptide (33). Alternatively, a ^{3}H-labeled polypeptide might carry a glycosylated phospholipid at the C terminus (6).

The covalent attachment of lipid to protein is suggestive of a protein normally being found in the vicinity of the cytoplasmic membrane. Several extraction methods were therefore used to try to release [^{3}H]palmitate-labeled polypeptides from cells of *S. gordonii*. Bacteria were incubated with different agents to distinguish between those proteins that might be associated peripherally with the cytoplasmic membrane from those that are integral membrane-bound proteins. Treatment of plasma membranes with Na_2CO_3 removes peripherally bound proteins, and this procedure discriminates between peripheral and integral membrane proteins (7). Similarly, polypeptides loosely associated with cell membranes are usually soluble in 0.1 M NaOH, whereas integral membrane proteins remain NaOH insoluble (31). Neither NaOH nor Na_2CO_3 at 0 to 4°C was effective in solubilizing [^{3}H]palmitate-labeled polypeptides from intact cells of *S. gordonii* Challis (Fig. 3). Neither treatment removed labeled fatty-acyl groups from the proteins, and the ^{3}H-labeled polypeptides were extractable from cells with SDS extraction buffer after incubation of cells with alkali (data not shown). It has been shown elsewhere that only about 50% of the total [^{3}H]palmitate incorporated into the *Bacillus licheniformis* penicillinase lipoprotein is released even after hydrolysis with 0.1 M NaOH for 2 h at 37°C (22).

Incubation of *S. gordonii* cells with the detergent sodium lauroyl sarcosinate at ambient temperature removed about half the total radioactive lipoproteins (Fig. 3, lane 7), while incubation with Tween 20 was less effective and resulted in about 10% solubilization of the lipoproteins (Fig. 3), assessed by densitometer scanning of autoradiographs. Equal numbers of cells were extracted in each case, so the fluorographs of SDS-PAGE gels in Fig. 3 are quantitative and qualitative indicators of lipoproteins solubilized by the various treatments. All the [^{3}H]palmitate-labeled polypeptides resisted extraction with alkali, which suggested that they were integral membrane proteins. Disruption of the lipid bilayer, with SDS or with sodium lauroyl sarcosinate, appeared to be necessary for their solubilization.

Colonization and virulence determinants of oral streptococci, and of group A streptococci and enterococci, have been shown to involve structural proteins that emanate from the cell surface with C-terminal anchors (29). This article identifies several lipoproteins present on the surface of *S. gordonii* that may also be important for cell surface properties. These lipoproteins, identified by [^{3}H]palmitate-labeling, may include adhesins which recognize components of saliva, serum, or molecules on the surfaces of other cells, such as *Actinomyces* cells. Although no adhesin function has been demonstrated yet for SarA lipoprotein (15, 17) recent reports suggest that two *S. sanguis* adhesins are lipoproteins. A fimbrial protein gene cloned from *S. sanguis* FW213 (5) and a putative salivary adhesin gene *ssaB* (8) cloned from *S. sanguis* 12 both encode polypeptides of about 34 kDa that are 87% similar. There is evidence that the polypeptides are involved in adherence of *S. sanguis* to saliva-coated surfaces and that SsaB may have a role in coaggregation with *Actinomyces* cells (8). Inspection of the inferred amino acid sequences of these proteins (5, 8) reveals that each carries the prolipoprotein consensus cleavage site L-x-y-C-S (34, 35) at the C-terminal end of the putative signal sequence. These polypeptides are likely to be lipoproteins and in this respect are similar to SarA. However, they showed no significant overall sequence similarity over the first 250 amino acids to the sequence of SarA (about 15% identity by using an ALIGN program).

Among streptococci, MalX and AmiA proteins in *S. pneumoniae* (11) and the MelE protein in *S. mutans* (32) have been suggested to be lipoproteins on the basis of deduced amino acid sequences. Each of these proteins is coded for by a gene that is part of an operon encoding protein components of a putative transport system. The *ami* operon in *S. pneumoniae* is structurally similar to the *opp* operon in *Salmonella typhimurium*, which encodes five polypeptide components of a periplasmic binding protein-dependent oligopeptide uptake system (13). It has been demonstrated that the *ami* system in *S. pneumoniae* is probably involved in the uptake of oligopeptides (1). However, while the AmiA polypeptide is proposed to be the functional equivalent of the periplasmic OppA protein in *S. typhimurium*, mutations in *amiA* do not affect oligopeptide uptake (1). The physiological functions of these streptococcal lipoproteins presently are ill defined. The results in this article suggest that, as well as possibly being involved in

uptake processes, lipoproteins in oral streptococci may be important for adherence-related functions.

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