Ruthenium Red Staining Reveals Surface Fibrils and a Layer External to the Cell Wall in *Streptococcus salivarius* HB and Adhesion Deficient Mutants

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Ruthenium red staining revealed both the long and short classes of cell surface fibril in thin sections of *Streptococcus salivarius* HB, indicating that the fibrils contained polyanionic polymers, probably polysaccharides. Also visible was a 16.2 ± 2.2 nm thick ruthenium red staining layer (RRL) outside the 16.7 ± 2.2 nm thick cell wall. The fibrils could not be seen after conventional glutaraldehyde and osmium fixation. The RRL was protease resistant and was not involved in septum formation. Loss of the fibrils after protease treatment coincided with a decrease of 54% in cell surface hydrophobicity, indicating that cell surface hydrophobicity was due partly to fibrils and partly to the RRL. There was no correlation between the lengths of fibrils as measured on whole cells after negative staining and on thin sections of ruthenium red stained cells. The thickness of the RRL was the same in three adhesion deficient mutants – strains HB-7, HB-V5 and HB-V51 – with various fibril lengths. However, a completely bald mutant, HB-B, had a significantly thicker RRL than *S. salivarius* HB, although it was unable to adhere to buccal epithelial cells, and it could not co-aggregate with *Veillonella parvula* V1. The RRL therefore did not contain adhesins.

INTRODUCTION

The cell surface of *Streptococcus salivarius* carries a number of structurally and functionally distinct classes of fibrils, peritrichously distributed over the cell. Ultrastructural analysis of adhesion deficient mutants, using negative staining, has shown that strain HB carries long (178 nm) protease resistant fibrils, and three classes of shorter, protease sensitive fibrils – 91, 72 and 63 nm long respectively (Handley *et al.*, 1984; Weerkamp *et al.*, 1986). The 91 nm fibrils (antigen B) are responsible for co-aggregation with *Veillonella parvula* V1, and the 72 nm fibrils (antigen C) are responsible for adhesion to host surfaces. The functions of the 178 nm and 63 nm fibrils are unknown (Weerkamp *et al.*, 1986).

Previously, we studied adhesion deficient mutants of *S. salivarius* HB by ultrastructural analysis of methylamine tungstate stained preparations (Weerkamp *et al.*, 1986). This technique of negative staining is considered to give a reproducible image with excellent resolution, within the limits presented by biological specimens. However, negative staining does not reveal details of the underlying structure of the surface layers and gives no information on their chemical composition.

Ruthenium red staining has been widely used in electron microscopy to locate acidic polysaccharide like material. The technique was studied in detail by Luft (1971) and used in a number of subsequent studies on surface structure and composition, often describing adhesive components (Costerton & Irvin, 1981; Fletcher & Floodgate, 1973; Brooker, 1979; Jones *et al.*,

Abbreviations: RRL, Ruthenium red staining layer; BEC, buccal epithelial cells; TA, teichoic acid; LTA, lipoteichoic acid.

1969). Ruthenium red complexes in a catalytic reaction with carboxyl or hydroxyl groups in the presence of osmium tetroxide to give an electron dense precipitate. Polymers carrying a high charge density, usually acidic polysaccharides, are stained in this way (Luft, 1971). Brooker (1979) also reported that the neutral polysaccharide dextran, on the surface of *Leuconostoc mesenteroides*, is also stained by ruthenium red. Polysaccharides are not detected after conventional fixation. When *S. salivarius* cells were fixed in osmium and glutaraldehyde and post-stained (Gibbons *et al.*, 1972), they were found to carry a fairly thin amorphous layer called a 'fuzzy coat' outside the cell wall, with only a suggestion of fibrils radiating outwards from the cell wall. Structures equivalent to clearly defined fibrils seen by negative staining (Handley *et al.*, 1984) were not seen.

The work reported in this paper describes the surface ultrastructure of *S. salivarius* HB and four adhesion deficient mutants, after ruthenium red staining.

METHODS

Organisms and culture methods. *S. salivarius* HB and the mutant strains HB-7, HB-V5 and HB-V51 were kindly provided by Dr A. H. Weerkamp (University of Groningen, The Netherlands). Strain HB carries three different lengths of fibril – 178 nm (unknown function), 91 nm (antigen B or co-aggregation adhesin) and 73 nm (antigen C or host associated adhesion factor). Strain HB-7 carries only the 91 nm fibrils, HB-V5 carries the 178 nm and 73 nm fibrils and strain HB-V51 carries 63 nm fibrils of unknown function. These measurements had been established by us previously (Weerkamp *et al.*, 1986). Strain HB-B was a spontaneous variant which occurred in a chemostat culture grown under glucose limitation at a slow dilution rate (0.148 h^{-1}). It had lost the ability to adhere to buccal epithelial cells (BEC) and could not co-aggregate with *V. parvula* V1.

The strains were stored at -70°C in glycerol and were grown up for each experiment on Brain Heart Infusion agar (BHI) with 5% (v/v) horse blood. Liquid cultures were then grown in BHI broth with 0.3% yeast extract for 18 h at 37°C in a candle jar. (A jar in which a candle has been burned until extinguished in order to lower the amount of oxygen available.)

Ruthenium red staining. A modification of the method used by Luft (1971) was used. A solution of ruthenium red (Johnson & Matthey Chemicals) was made up in water to a final concentration of 10 mg ml^{-1} (1500 p.p.m.). Treated or untreated cells were washed three times in 0.2 M-sodium cacodylate buffer (pH 7.3) and fixed initially in a mixture of 0.5 ml 3.6% (v/v) glutaraldehyde, 0.5 ml 0.2 M-cacodylate buffer (pH 7.3) and 0.5 ml ruthenium red stock solution for 1 h at room temperature. After three washings in 0.2 M-cacodylate buffer, cells were fixed in a mixture of 0.5 ml 4% (w/v) osmium tetroxide in distilled water, 0.5 ml 0.2 M-cacodylate buffer (pH 7.3) and 0.5 ml ruthenium red stock solution. The cells were fixed overnight in this mixture, as the intensity of the ruthenium red staining was found to be markedly greater than when fixed for 3 h as recommended by Luft (1971). Controls were prepared by substituting distilled water for the ruthenium red stock solution. Cells were then washed three times in 0.2 M-cacodylate buffer, dehydrated in a graded series of ethanol and embedded in either Spurr resin (Agar Aids) or LR white resin (London Resin Co.). Sections were cut on a Reichert OMU4 microtome and sections were photographed on a Hitachi H600 electron microscope.

Cell surface hydrophobicity. The method used by Rosenberg *et al.* (1980) was modified as reported by Handley *et al.* (1987). Briefly, cells were washed three times in 0.066 M Sörensons phosphate buffer (pH 7.2) and resuspended to an OD_{440} of 0.5. Samples of cells (3.0 ml) were blended for 60 s on a vortex mixer after 200 μl hexadecane (BDH) had been layered on top. The tubes were left to stand for 15 min for phase separation to occur and the OD_{440} of the aqueous phase was measured. Hydrophobicity was calculated from six replicates as the percentage decrease in optical density of the original bacterial suspension due to cells partitioning into the hexadecane layer (Handley *et al.*, 1987).

Protease treatment. *S. salivarius* strains were grown in BHI broth plus 0.3% yeast extract overnight and washed in water three times. Protease treatment (protease type XIV from *Streptomyces griseus*; Sigma) was done as described by Handley *et al.* (1984) at a final concentration of 0.5 mg ml^{-1} in Sörensons phosphate buffer at pH 7.2.

Measurements. The width of the cell wall and ruthenium red staining layer (RRL) were all measured on micrographs at a magnification of $\times 114000$. Thin pen lines were drawn at the internal and external perimeters of the two layers and the distances between them measured with a graticule divided into tenths of a millimetre. When 20 identical micrographs were measured for cell wall thickness, the measurement error was found to be $\pm 0.8\text{ nm}$. The widths of the fibrillar layers after ruthenium red staining were measured from a line drawn around the outer perimeter of the fibrils to a line drawn at the base of the RRL (see Results). All mean measurements are quoted with the standard deviations, which includes the measurement error. All measurements from ruthenium red stained sections were made on 30 different micrographs. The measurements of fibril lengths after negative staining were made on 10 different micrographs (Weerkamp *et al.*, 1986).

Statistics. The relationship of the thickness of both the wall and the RRL within batch measurements, between batch measurements and between strains, was analysed using the nested analysis of variance design (Winer, 1971). To test for differences between strains the variance ratio of variability between batches to variability between strains was calculated (Handley *et al.*, 1987).

RESULTS

Ruthenium red staining of S. salivarius HB and its adhesion deficient mutants

Fibrils on the cell surface of *S. salivarius* HB were revealed in thin sections by ruthenium red staining as a dense confluent layer of short fibrils with longer fibrils projecting through (Fig. 1*a*). At the base of the fibrillar fringe a more electron dense RRL was seen. This was consistently found on every cell of all strains examined. The fibrils differed in density from cell to cell in a population, sometimes revealing just the wall and the RRL, with patches carrying no fibrils, but with an intact RRL entirely covering the cells (micrographs not presented). Mutants HB-V5 (Fig. 1*b*), HB-7 (Fig. 1*c*), HB-V51 (Fig. 2*a*) and HB-B (Fig. 2*b*) also carried the RRL outside the cell wall on all cells. The RRL had a loose amorphous outer edge which could be seen more clearly when few or no fibrils were present on a strain (Figs 1*c* and 2*a, b, d*). In order to look for possible differences between mutants, other than fibril length, the thickness of the RRL was measured for all strains. Two batches of each strain were grown simultaneously in BHI broth with 0.3% yeast extract and stained with ruthenium red, before thin sectioning all at the same time. Samples were also negatively stained. Cells were embedded in LR white resin.

The thickness of the RRL was measured on 30 micrographs for each strain. Statistical analysis showed that the RRL on HB-B (18.0 ± 2.2 nm) was significantly thicker ($P = 0.000018$) than the RRL (16.2 ± 2.2 nm) on HB. This was true for both batches of cells. There was no significant batch to batch variation in the RRL thickness of either strain, and no other differences were found in RRL thickness when statistical comparisons were made between HB and HB-7 and HB-V51. HB-V51 RRL thickness was compared to that of HB-V5 RRL, the strain from which it was derived (Weerkamp *et al.*, 1987). The mean wall thickness for strain HB was 16.7 ± 2.2 nm and there were no significant differences between wall thickness when the mutants were compared with HB (30 micrographs from each of two batches were measured).

When the lengths of fibrils were compared after negative staining and after ruthenium red staining no consistent correlations emerged. Lengths of fibrils in ruthenium red stained preparations were measured from the end of the fibrils to the base of the RRL. Fig. 1(*d*) is a negatively stained preparation of HB-7 showing that the cell surface is smooth; this was so for all cells in all strains. In contrast, the outer surface of the RRL is very uneven (Figs 1*c* and 2*a, b, d*): therefore it is thus probable that negative staining with methylamine tungstate penetrates through the RRL. In addition, polysaccharides may not be stainable with negative stains as they lack the necessary bulky side chains of amino acids for exclusion of the stain (Tzaphlidou & Chapman, 1986). The perimeter seen after negative staining is therefore probably the surface of the cell wall itself.

After ruthenium red staining the short fibrillar fringe on strain HB was found to have shrunk by 22% when compared to measurements taken on fibril length after negative staining on the same batch of cells grown in BHI broth for 18 h (Table 1). This shrinkage factor was consistent, and similar values were obtained in three separate experiments (three growth batches, each stained and fixed separately) on strain HB. In contrast no shrinkage occurred in the fibrillar fringe of HB-7 or HB-V5 as the lengths of the short fibrils after ruthenium red staining were similar to the measurements on the same batch of cells after negative staining (Table 1). The fibrillar fringe of HB-V51 was 12% longer by ruthenium red staining than by negative staining (Table 1). The reasons for shrinkage and apparent extension after ruthenium red staining are not known.

The RRL was not involved in septum formation (Fig. 2*d*) and only appeared on the surface of the new wall after the cells had separated. This was observed for all cells in all strains.

When *S. salivarius* HB was fixed in glutaraldehyde and osmium and post-stained with uranyl acetate and lead citrate, a discontinuous, very loose layer, with a variable width, was observed

Fig. 1. The sectioned cells in (a)–(c) were all stained with ruthenium red, but were not post-stained. (a) Strain HB showing long fibrils projecting through the more densely distributed short fibrils with the electron dense RRL underneath. Arrows indicate wall thickness. (b) HB-V5 also shows long fibrils projecting through a dense fringe of shorter fibrils. (c) HB-7 carries sparse short fibrils only, with the RRL underneath, outside the cell wall. (d) HB-7 negatively stained with 1% methylamine tungstate. (e) A section of strain HB after conventional fixation in glutaraldehyde and osmium, and post-staining with uranyl acetate and lead citrate, showing a loose, discontinuous layer outside the cell wall. Bar markers represent 100 nm.

Fig. 2. All sectioned cells were stained with ruthenium red, but were not post-stained. (a) Strain HB-V51 showing very sparse fibrils attached to the continuous RRL outside the cell wall. (b) HB-B has no fibrils, but carries a RRL. (c) HB after protease treatment showing a RRL with a loosened edge. (d) HB-7 showing two septa. The RRL is not involved with septum formation and only occurs at the cell surface. Bars represent 100 nm.

Table 1. Comparison of the lengths of the short fibrils after negative staining and ruthenium red staining

Measurements after ruthenium red staining were done on micrographs of thin sections; measurements after negative staining were done on micrographs of whole cells.

Strain	Length (nm)	
	Ruthenium red staining*	Negative staining†
HB	68.0 ± 6.9	87.1 ± 5.0
HB-V5	74.9 ± 3.6	73.6 ± 10.7
HB-7	91.3 ± 7.7	90.0 ± 3.0
HB-V51	73.2 ± 4.9	64.4 ± 4.6

* Measurements represent the mean of the fibril length measured on 30 separate micrographs, all from one batch of cells. Measurements were taken from the end of the fibrils to the base of the RRL.

† Measurements taken on 10 separate micrographs of the same batch of cells as used for ruthenium red staining.

outside the cell wall of all cells in a population (Fig. 1*e*). The cell wall thickness (16.2 ± 1.6 nm; mean of two batches of 10 replicate measurements) was not significantly different from the cell wall thickness measured after ruthenium red staining.

Protease treatment and hydrophobicity measurements

S. salivarius HB was treated with 0.5 mg protease ml⁻¹ at 37 °C for up to 3 h. Samples of cells were simultaneously negatively stained, fixed and stained with ruthenium red and tested for changes in hydrophobicity. We previously reported that the short fibrils are protease sensitive and the long fibrils are protease resistant after 2 h treatment at this concentration (Handley *et al.*, 1984). However, after 3 h treatment the long fibrils were removed, intact, from the cell surface by the longer exposure to protease. Fig. 2(*c*) shows the surface of strain HB after 3 h of protease treatment, still with an intact RRL. All protease treated cells carried a RRL which at 17.3 ± 1.3 nm was not significantly different from the thickness of the RRL before protease treatment (16.2 ± 2.2 nm). The edge of the RRL appeared to be slightly looser after protease treatment (Fig. 2*c*) which would account for the slightly thicker measurement for the RRL.

The cell surface hydrophobicity of strain HB before protease treatment was $94.2 \pm 1.5\%$, but after treatment it dropped to $43.4 \pm 5.3\%$ (mean of two batches, six replicates each), a drop of 54%.

Chemostat variant HB-B

S. salivarius strain HB-B arose spontaneously in a glucose limited chemostat after prolonged growth of strain HB at a low dilution rate (0.148 h⁻¹). This mutant was recognized by its inability to adhere to BEC and to co-aggregate with *V. parvula* V1. In addition, it had a very low cell surface hydrophobicity ($27.9 \pm 6.8\%$) after growth in BHI for 18 h as compared to HB (94.2 ± 1.5) a difference of 70.4%. Similar variants often appeared at low growth rates. HB-B was completely bald by negative staining, and carried an RRL outside the wall. However, the RRL was significantly thicker than that of HB.

DISCUSSION

Fixing and ruthenium red staining of *S. salivarius* HB and its mutants preserved both long and short fibril classes and revealed a homogeneous layer outside the cell wall which is the same thickness as the wall itself. The mean value for the wall thickness after ruthenium red staining was 16.7 ± 2.2 nm, which is quite thin. The thinnest wall so far reported for a Gram-positive organism is 15.17 nm for *Butyrivibrio fibrisolvens* (Cheng & Costerton, 1977). The *B. fibrisolvens* wall thickness was quoted after glutaraldehyde and osmium fixation and post-staining. The ruthenium red stained sections in this study were not post-stained to maximize the contrast produced by ruthenium red, but they were fixed in glutaraldehyde and osmium. Ruthenium red

staining did not itself alter the wall thickness. (It should be noted that the wall layer was measured – see arrows in Fig. 1a – between the inner edge of the RRL and an electron dense band, which was assumed to belong to the plasma membrane.)

The outer edge of the RRL was not as well defined as the edge of the wall. Although it is electron dense, the RRL had a looser, less homogeneous appearance than the cell wall. The fuzzy coat previously described by Gibbons *et al.* (1972) on *S. salivarius* is the layer outside the cell wall which can be seen after conventional osmium and glutaraldehyde fixation. The fuzzy coat was also seen in this study in the absence of ruthenium red and it appeared as a very loose, patchy fibrous layer (Fig. 1e) with an indeterminate width. Glutaraldehyde fixes proteins and osmium tetroxide fixes primarily lipids, so any polysaccharide outside the cell wall would not be detected by conventional glutaraldehyde and osmium fixation. Ruthenium red is therefore revealing components not seen by conventional fixation.

The fibrils are likely to be different in composition from the RRL as protease removes both short and long fibrils, leaving the RRL intact, although with an apparently loosened edge. The fibrils consist of glycoproteins (Weerkamp & Jacobs, 1982), and antigens B and C have different relative proportions of protein, hexoses and amino sugars. Ruthenium red is probably staining the polysaccharide component present in the fibrils, although this is predominantly in the form of neutral sugar (Weerkamp & Jacobs, 1982).

It is not yet known whether the RRL should be considered to be part of the cell wall. It is not involved with septum formation and remains at the cell surface as the septum grows inwards. However, the outer membrane of Gram-negative cells is thought not to be involved with septum formation (Burdett & Murray, 1974) and yet the outer membrane is considered to be part of the cell wall. The cell walls of Gram-positive bacteria are consistently described as homogeneous layers, containing peptidoglycan and teichoic acid (TA), usually ranging from 20–50 nm thick (Shockman & Barrett, 1983), although the exact location of TA is not clear. Polysaccharide containing layers have not been previously observed outside the cell wall, presumably because ruthenium red staining has not been included in the fixation procedures. We have found that a RRL is common in oral streptococci, and *S. sanguis* strain 311, which has a lateral tuft of fibrils, also carries a thick 37 ± 6 nm RRL layer (Hesketh *et al.*, 1987). Strains of *S. milleri* and *S. salivarius* (a Lancefield K⁻ strain) with peritrichous fimbriae, and *S. sanguis* strains with peritrichous fibrils, also carry a similar layer outside the wall (P. S. Handley unpublished data). Brooker (1979) found that different *L. mesenteroides* strains show a variety of different, very complex, surface layer patterns, outside the cell wall after ruthenium red staining, and that growth in different media influences the appearance of the ruthenium red staining layers. It is therefore possible that surface layers containing polymers with a high charge density (usually polysaccharides) are more common than previously thought on the surfaces of Gram-positive bacteria.

In support of this suggestion are the many biochemical observations showing that neutral and acidic polysaccharides covalently linked to the peptidoglycan are common in Gram-positive bacteria (Shockman & Barrett, 1983; Rogers *et al.*, 1980) and are considered to be components of the cell wall. For example, in Group B streptococci both the Group B polysaccharide and the type III specific polysaccharide are covalently bound to the peptidoglycan and are considered to be part of the cell wall (de Cueninck *et al.*, 1982). If the RRL of *S. salivarius* is found to be covalently bound to peptidoglycan then it may be considered as a structural wall component.

The composition of the *S. salivarius* RRL is unknown, although some surface polysaccharides have been identified in other studies. The Lancefield Group K cell wall polysaccharide antigen of *S. salivarius* has been isolated and contains galactose, glucose, rhamnose and a trace of glucosamine (Montague & Knox, 1968). The structural location of the Group K antigen is unknown, but it may be located in the RRL, because strains HB-V5, HB-V51 and HB-7 all produce the Group K antigen (Weerkamp *et al.*, 1987) and they all carry the RRL. It is not known whether HB-B or protease treated cells still carry the Group K antigen.

Lipoteichoic acid (LTA), a negatively charged polymer of polyglycerophosphate is present in *S. salivarius* HB and LTA exposure at the cell surface is inversely related to the fibril density. In another bald strain, HB-C12, more LTA was exposed than in strain HB (Weerkamp *et al.*, 1987).

This observation indicates that LTA may be present in the RRL.

It is not known why the RRL of strain HB-B is thicker than the same layer for HB. Preliminary studies on the RRL thickness for HB-B grown at different dilution rates in a glucose limited chemostat show that the thickness of this layer varies with growth rate, a variation not shown by HB. The RRL is therefore capable of phenotypic changes. Whether biochemical changes coincide with structural changes is presently under study.

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