Evaluation of Bacterial Glycocalyx Preservation and Staining by Ruthenium Red, Ruthenium Red-Lysine and Alcian Blue for Several Methanotroph and Staphylococcal Species

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EVALUATION OF BACTERIAL GLYCOCALYX PRESERVATION AND STAINING BY RUTHENIUM RED, RUTHENIUM RED-LYSISE AND ALCIAN BLUE FOR SEVERAL METHANOTROPH AND STAPHYLOCOCCAL SPECIES

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Abstract

The cationic reagents, ruthenium red (RR), alcian blue (AB) and ruthenium red-lysinse were compared for ultrastructural preservation and staining of the bacterial glycocalyx. En bloc RR, AB and RR-lysinse procedures, and a glutaraldehyde/OsO4 fixation without cationic reagent, were evaluated by transmission electron microscopy (TEM) for several methanotroph and staphylococcal species. The glutaraldehyde/OsO4 fixation was totally ineffective in preserving or staining the glycocalyx material in all species studied. The RR procedure was more effective than the glutaraldehyde/OsO4 fixation but was generally less so than the RR-lysinse procedure. By the RR-lysinse procedure, extensive layers that were often filamentous or fibrous were observed in the methanotrophs, and extended fibrous elaborations were seen in the staphylococci. By the AB procedure, staphylococci glycocalyx material consisted of condensed curved structures or net-like features not directly comparable to the RR-lysinse images. In the methanotrophs, filamentous or fibrous layers were often comparable between AB and RR-lysinse. Thus, ultrastructural observation of the bacterial glycocalyx in all species studied was improved by use of RR-lysinse, whereas the use of AB showed species variation.

Key Words: alcian blue, ruthenium red, ruthenium red-lysine, bacterial glycocalyx, polysaccharide, staphylococci, methanotroph, en bloc reagent, staining, transmission electron microscopy.

Introduction

Extracellular polysaccharides are a major component of structures external to the outer surface of microbial cells (Sutherland, 1990). If these structures are of bacterial origin and lie outside the integral elements of the outer membrane of gram-negative cells and outside the peptidoglycan of gram-positive cells, then by definition we are dealing with the bacterial glycocalyx (Costerton et al., 1981). The term glycocalyx was first used in this context by Bennett (1963). In general, there are two types of bacterial glycocalyx (Costerton et al., 1981). One is composed of globular glycoproteins, often in regular arrays. These are also known as S layers (Sleytr, 1972). The other consists of fibrous matrices of polysaccharide with variation in thickness and other characteristics such as flexibility and strength of association with the bacterial cell body (Costerton et al., 1981; van Iterson, 1984). The polysaccharide is highly polymeric in nature and consists of numerous anionic moieties of variable composition (Cosierion et al., 1981; van Iterson, 1984). The bacterial glycocalyx is also highly hydrated, as much as 99% water (Sutherland, 1972). These properties make effective observation of the bacterial glycocalyx by TEM difficult and often subject to uncertainty of interpretation. The aldehydes and osmium tetroxide generally are considered to have a low affinity for polysaccharide (Hayai, 1981). Insufficient stabilization by these chemical fixatives may result in observation of glycocalyx material not preserved. An alternative interpretation is that due to a lack of innate electron density and an inability to stain with conventional reagents (Roth, 1977), any preserved glycocalyx material is not visible in the TEM. During dehydration, the step-wise removal of water from the highly hydrated bacterial glycocalyx (Sutherland, 1972) can cause deformation or condensation (Roth, 1977; Springer and Roth, 1973). This adverse modification may be decreased or minimized by reagents with multiple attachment sites or by cross-linking agents (Costerton et al., 1981). Lectins (Molina et al., 1988; Oreifici et al., 1986) and specific antibodies (Molina et al., 1987; Progulske and Holt, 1980) are two possible cross-linking approaches. Another approach is the use of cationic reagents.
traditional for specific or semi-specific staining of poly-
ions by light microscopy, such as ruthenium red (RR) and alcian blue (AB). Ruthenium red was first used to
semi-specifically stain pectin (Jensen, 1962). This was
extended to include acidic mucopolysaccharides, includ-
ing acidic protein polysaccharides or glycoaminoglycans
(Luft 1971a, 1971b). Many other substrates react with
RR. The generalities concluded by Luft (1971a, 1971b,
1976) were that reaction of RR is favorable with poly-
ions of high charge density. Neither high charge density
alone, nor a high polymeric character alone, makes a fa-
vorable reaction. A relationship to the number of ioniz-
able reaction. A relationship to the number of ioniz-
ing carboxylic acid groups of a polymer is possible.
Neutral polysaccharides, polypeptides or proteins are
generally unreactive (Luft, 1971a). Ruthenium red alone
has been applied to study bacterial outer layers (Pate and
Ordal 1967; Springer and Roth, 1973; Titus et al., 1982;
Fassel et al., 1990, 1992). For some bacterial species,
considerable additional outer material of various descrip-
tions are observed. In other cases, condensation or col-
lapse, perhaps due to dehydration artifacts of the glycocy-
alyx, is still reported (Costerton et al., 1981; Molinari et
al., 1988). In general, where a high proteinaceous
component (Buckmire and Murray, 1973; McCowan et
al., 1979; Costerton et al., 1974) or a glycoalyx well
anchored to many attachment sites (Costerton et al.,
1981) occurs, an extended structure is observed. Also,
cross-linking antibodies applied in conjunction with RR
have improved observation of extended fibrillar struc-
tures (Mackie et al., 1979; Patterson et al., 1975).

Ruthenium red was coupled with the diamine, lys-
ine, and glutaraldehyde in another approach that makes
use of the cross-linking concept. Jacques and Graham
(1989), by inclusion of lysine in a prefixation with
glutaraldehyde and RR, stabilized and visualized exten-
sive glycocalyces for several bacterial species. This
procedure has since been applied to ruminal bacteria
(Akin and Riggsby, 1990). Clostridium difficile (Davies
and Borriello, 1990) and Streptococcus suis (Jacques
et al., 1990). It is based on the development of a glutaral-
dehyde-lysine fixation by Boyles (Boyles, 1982, 1984;
Boyles et al., 1985). Lysine, which is positively
charged at physiological pH, forms large polymers with
glutaraldehyde. Cross-linkages of various lengths and
directions with anionic sites of the glycocalyx could
form stable configurations resistant to later stages of
processing (Boyles, 1982, 1984; Boyles et al., 1985).
Where the RR molecule exactly fits in this scheme, i.e.,
acting to stabilize or stain, is not yet established.

Another cationic reagent, alcian blue, was charac-
Alcian blue is thought to bind negatively charged mole-
ties primarily through electrostatic or ionic linkages
(Behnke and Zelander, 1970; Luft, 1971a; Scott et al.,
1964). It has been used to stain acidic mucopolysaccha-
rside (Luft, 1971a; Kiernan, 1990). For light micros-
copy, it reacts specifically only with sulphate-ester
groups at pH 1.0 and with both carboxyl and sulfate
groups at pH 2.5 (Kiernan, 1990; Jones and Reid, 1973).

Figure 1. Transmission electron micrographs for
Methylomonas albus BG8 (A, C, D, E) and for Methyo-
latinum trichosporium Ob3b (B, C, D, F) are shown by the
glutaraldehyde/OsO₄ fixation (A, B), the ruthenium red
procedure (C), the alcian blue procedure (D) and the
ruthenium red-lysine procedure (E, F).

(A) Methylomonas albus BG8 by the glutaraldehyde/
OsO₄ fixation, shows cup-like features (arrows) in LR
White resin.

(B) Methylisinus trichosporium Ob3b by the glutarala-
dehyde/OsO₄ fixation, shows limited (arrow) and spike-
like structures (curved arrow).

(C) By the ruthenium red procedure, Methylomonas
albus BG8 (cell indicated by large black arrow) cup
features (arrow) with good definition are seen in a
complete surrounding layer. For Methylisinus trichospo-
orium Ob3b (cell indicated by large white arrow) round
bead features (white arrowhead), thick filaments (curved
arrow) and filamentous or fibrous material (arrow) are
observed.

(D) By the alcian blue procedure for Methylomonas
albus BG8 (cell indicated by large black arrow), both the
cup layer (arrow) and material within the cup (curved
arrow) are retained. For Methylisinus trichosporium
Ob3b (cell indicated by large white arrow) an inner
electron dense layer (long arrow) is surrounded by an
less dense outer fibrous layer (arrow).

(E) By the ruthenium red-lysine procedure for Methyl-
omonas albus BG8, a complete cup layer (arrow) with
fibrous material and elaborate external material (curved
arrow) between cells are observed.

(F) By the ruthenium red-lysine procedure for Methyl-
isinus trichosporium Ob3b, a dense thick innermost layer
(long arrow), a gradation of material of varying electron
density and a less dense fibrous outermost material
(arrow) suggest complex layers.

All magnification bars are 0.5 µm.

In electron microscopy, it has been used in bacterial
studies to demonstrate outer material in Eikenella strains
(Progulske and Holt, 1980), Pseudomonas fragi (Herald
and Zottola, 1988) and several methanotrophs (Fassel et

In this study, the use of RR and AB in previously
reported procedures (Fassel et al., 1990; 1991) are com-
pared to the RR-lysine approach based on Jacques and
Graham (1989). The comparison is carried out for sev-
eral species of methanotrophs Methylomonas albus BG8,
Methylisinus trichosporium Ob3b, Methylcystis parts
OBBP, and Methylcystis species strain Lake Washing-
ton. They have been shown to play an important role in
cycling of carbon in natural environments (Whittenbury
and Dalton, 1981). Work with a totally unrelated group
of organisms was desired to evaluate the utility of these
approaches with other bacterial species. Species of the
gram positive coagulase negative staphylococci were se-
lected. Staphylococcos species studied are Staphylocco-
cus aureus ATCC 25923, Staphylococcus epidermidis
RP62 and Staphylococcus hominis SP2. These species
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Figure 2. Transmission electron micrographs for *Methylocystis* species strain Lake Washington and *Methylocystis paris* OBBP are shown for the ruthenium red procedure (A), the alcian blue procedure (B) and the ruthenium red-lysine procedure (C, D).

(A) By the ruthenium red procedure, an electron dense fibrous layer (arrow) surrounds cells of *Methylocystis paris* OBBP.

(B) By the alcian blue procedure, material in a fibrous layer (arrow) surrounds cells of *Methylocystis* species strain Lake Washington.

(C, D) By the ruthenium red-lysine procedure, an extensive fibrous layer (arrow) is seen for (C) *Methylocystis* species strain Lake Washington and (D) *Methylocystis paris* OBBP. Some fibrous and/or filamentous material is observed near cells (curved arrow) of *Methylocystis paris* OBBP.

All magnification bars are 0.5 µm.

are widespread potential opportunistic pathogens and infectious agents (Edmiston *et al*., 1989). Through improved visualization of the glycocalyces of both groups of bacterial species, our environmental and medical studies will be facilitated. We hope other laboratories will also find this useful in their applications.

**Material and Methods**

**Cell Culture**

Methanotrophic species studied are *Methylomonas albus* BG8, *Methylosinus trichosporium* Ob3b, *Methylocystis paris* OBBP, and *Methylocystis* species strain Lake Washington. They were grown as suspension cultures at 30 °C in nitrate mineral salts medium under 50:50 air: methane atmospheres until mid-to late log phase, 36 hours (Whittenbury and Dalton, 1981). Staphylococcal species are *Staphylococcus aureus* ATCC 25923, *Staphy-
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_Lococcus epidermidis_ RP62 and _Staphylococcus hominis_ SP2. These organisms were recovered from frozen storage (-70 °C) and plated on blood agar plates for test of viability. After 24 hrs the organisms were inoculated to trypticase soy broth and incubated for 18 hrs at 35 °C.

Cells were handled as pellets until all fixation/wash procedures were completed. Then, they were enrobed in 4% agar and handled as 1 mm³ blocks.

**Glutaraldehyde/OsO₄ fixation**

Fixation was in 2.5% glutaraldehyde in buffer for 2 hours, followed by washes of 3X 10 minutes each. Postfixation was in 1% (w/v) OsO₄ in buffer for 2.5 hours, followed by washes of 3X 10 minutes duration each. The buffer for these solutions was 0.1 M cacodylate pH 7.0-7.3. Dehydration was in a graded ethanol series of 10%, 25%, 50%, 70%, 95%, 100% anhydrous, and 100% anhydrous. Infiltration and embedment was in Spurr resin, Standard Medium Mixture (Polysciences)

_Figure 3._ Transmission electron micrographs for _Staphylococcus aureus_ ATCC 25933 is shown by the glutaraldehyde/OsO₄ fixation (A), the ruthenium red procedure (B), the alcian blue procedure (C) and the ruthenium red-lysine procedure (D). For _Staphylococcus aureus_ ATCC 25933:

(A) no glycocalyx material is seen by a glutaraldehyde/OsO₄ fixation. This is also observed for _Staphylococcus hominis_ SP2 and _Staphylococcus epidermidis_ RP62 (electron micrographs not shown).

(B) By the ruthenium red procedure, sparse fibrous material (arrow) appears irregularly around cells.

(C) By the alcian blue procedure, curved irregular electron dense structures (arrow) occur between and are frequently continous with cells. A net-like structure of fine strands (curved arrow) also is observed.

(D) By the ruthenium red-lysine procedure, fibrous material (arrow) is extensively elaborated and surrounds cells.

All magnification bars are 0.5 µm.
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Figure 4. Transmission electron micrographs for *Staphylococcus hominis* SP2 (A, C, E) and *Staphylococcus epidermidis* RP62 (B, D, F) are shown by the ruthenium red procedure (A, B), the alcian blue procedure (C, D) and the ruthenium red-lysine procedure (E, F). (A, B) By the ruthenium red procedure, *Staphylococcus hominis* SP2 shows some fibrous material (arrow). *Staphylococcus epidermidis* RP62 shows considerably more glycolcalyx material (arrow) around and between cells. (C, D) By the alcian blue procedure for both species, curved electron dense (arrow) structures occur that are frequently continuous with cells (shown for *Staphylococcus hominis* SP2 in C). A fine netlike structure (curved arrow) is continuous with cells of *Staphylococcus hominis* SP2 (C). It is shown completely surrounding a cell of *Staphylococcus epidermidis* RP62 (D). (E, F) By the ruthenium red-lysine procedure, the fibrous material completely surrounds each cell of *Staphylococcus hominis* SP2 (E). The fibrous material (arrow) is more extended and elaborated for *Staphylococcus epidermidis* RP62 (F). Additional extended material is seen between cells for both species (E and F).

All magnification bars are 0.5 µm.

Data Sheet #127), except for *Methylophanes albus* BG8, where an LR White embedment proves more effective. This processing followed London Resin Company Limited, London, England; Data Sheet 305A. Thin sections were cut on a Porter-Blum MT-II using a diamond knife, and post-stained in 25% ethanolic 2% uranyl acetate and Reynolds lead citrate. Sections were studied on a Hitachi H-600 at 75 kV.

**Ruthenium red en bloc procedure**

Prefixation was in 0.2% glutaraldehyde and 0.15% RR in buffer for 30 minutes. Fixation immediately followed in 1% glutaraldehyde and 0.05% RR in buffer for 2 hours. The washes were 3X 10 minutes each with 0.05% RR included in the wash buffer. Post-fixation was in 2% OsO4 and 0.05% RR in buffer for 2.5 hours. The washes were 3X 10 minutes each with 0.05% RR included in the wash buffer. The buffer for these solutions was 0.1 M cacodylate pH 7.0-7.3. Dehydration in the graded ethanol series included 0.05% RR through the 70% stage. All remaining steps were carried out as above.

**Ruthenium red-lysine en bloc procedure**

Prefixation was in 0.075% RR, 2.5% glutaraldehyde and 75 mM lysine in buffer for 20 minutes. Fixation was in 0.075% RR and 2.5% glutaraldehyde in buffer for 2 hours (Jacques and Graham, 1989). This was followed by washing in buffer 3X 10 minutes each. Postfixation was in 1% OsO4 for 2 hours followed by 3X 10 minutes each washes. The buffer for these solutions was 0.1 M cacodylate pH 7.0-7.3. Further processing was identical to the glutaraldehyde/OsO4 fixation procedure above.

**Results**

By the glutaraldehyde/OsO4 fixation, in the absence of en bloc reagent, glycolcalyx material is absent or limited for all species examined. In general, improvement is seen with cationic reagents.

In Figure 1 *Methylophanes albus* BG8 and *Methylosinus trichosporium* OB3b are shown. For *Methylophanes albus* BG8 (Figure 1A), accessory cup features (arrow) are observed external to the outer membrane of the gram-negative cell wall for a glutaraldehyde/osmium tetroxide fixation in LR White resin. These cup features are not preserved in Spurr resin without cationic reagents ruthenium red or alcian blue as previously shown (Fassol et al., 1990, 1992). For *Methylosinus trichosporium* OB3b (Figure 1B), a few spike-like structures (curved arrow) and suggestion of some limited material (arrow) are observed external to the outer membrane irrespective of embedding resin. This figure and all following electron micrographs are from Spurr resin embeddings.

Figure 1C shows the ruthenium red procedure. The *Methylophanes albus* BG8 cell is indicated by a large black arrow. Cup features (arrows) are retained with good definition. As previously reported (Fassol et al., 1990), cups are seen in a gap-less layer surrounding the outer membrane. The *Methylosinus trichosporium* OB3b cell in Figure 1C is indicated by large white arrow. Features seen with ruthenium red include round bead-like structures (white arrowhead), thickened electron dense filaments (curved arrow), and some fibrous material (arrow). To aid description of glycolcalyx, the term filamentous is used where individual strands or filaments can be easily identified. The term fibrous is used for outer material where separate and distinct strands or individual filaments are not easily discerned.

By the alcian blue procedure (Figure 1D) for *Methylophanes albus* BG8 (cell indicated by large black arrow), fibrous material (curved arrow) appears in cups (arrow). For *Methylosinus trichosporium* OB3b (cell indicated by large white arrow), two layers of different electron density are observed. A dense inner layer (long arrow) is completely surrounded by a more amorphous or fibrous outer layer (arrow), as detailed previously (Fassol et al., 1992).
By the ruthenium red-lysine procedure, several observations similar to the above are seen for *Methylomonas albus* BG8 (Figure 1E). A well defined and complete cup layer is preserved (arrow). Fibrous material appears within cups. Additionally, more elaborate material frequently extends between cells (curved arrow). For *Methyllosinus trichosporium* OB3b (Figure 1F) by the ruthenium red-lysine procedure, an electron dense inner material (long arrow) is frequently seen. A gradation of electron density in the other glycolaxy material occurs with frequent suggestion of an outermost fibrous layer (arrow) around many cells.

For *Methylocystis* species strain Lake Washington and *Methylocystis paris* OBBP by a glutaraldehyde/OsO₄ tetroxide fixation, observation of glycolaxy is limited to remnants of spikes, or strands, or is completely absent (electron micrographs not shown), as was seen for *Methyllosinus trichosporium* OB3b. By the ruthenium red procedure (Figure 2A), a matted fibrous layer (arrow) is found for some cells, as shown here for *Methylocystis paris* OBBP. For other cells, abundant filamentous material is often seen. Additionally, for some cells of *Methylocystis* species strain Lake Washington, discrete bead-like structures were observed (Fassel et al., 1992). By the alcian blue procedure, a fibrous layer (arrow) completely surrounds cells of *Methylocystis* species strain Lake Washington (Figure 2B) as is similarly found in cells of *Methylocystis paris* OBBP (Fassel et al., 1992).

By the RR-lysine procedure, cells are also surrounded by an extensive fibrous layer (arrow) for *Methylocystis* species strain Lake Washington (Figure 2C) and *Methylocystis paris* OBBP (Figure 2D). For the latter, filamentous or fibrous material (curved arrow) is also seen frequently in a mass between cells. An electron translucent region, possibly artifact, between the fibrous layer and the cell wall is noted for some cells.

For *Staphylococcus aureus* ATCC 25923 (Figure 3A) and the other staphylococci species studied, no suggestion of glycolaxy material is seen by a glutaraldehyde/OsO₄ fixaion. In *S. aureus* (Figure 3B-3D), by the ruthenium red procedure (Figure 3B) sparse fibrous material (arrow) occurs irregularly around cells. Where there is a grouping of cells, more material is frequently seen on the outermost cells. By the alcian blue procedure (Figure 3C), curved irregular electron dense structures (arrow) appear continuous with cells and extend between cells. A netlike structure that appears to consist of fine strands (curved arrow) is occasionally seen. In contrast, by ruthenium red-lysine (Figure 3D), an elaborate fibrous material (arrow) surrounds cells and extends between cells.

By the ruthenium red procedure for *Staphylococcus hominis* SP2 (Figure 4A), some fibrous material (arrow) is seen. For *Staphylococcus epidermidis* RP62 (Figure 4B), more fibrous material (arrow) is observed. By the alcian blue procedure, for *Staphylococcus hominis* SP2 (Figure 4C) and *Staphylococcus epidermidis* RP62 (Figure 4D), curved electron dense structures (arrow) are seen. A netlike structure (curved arrow) is shown continuous with some *Staphylococcus hominis* SP2 cells (Figure 4C) and completely surrounding a cell of *Staphylococcus epidermidis* RP62 (Figure 4D). By the ruthenium red-lysine procedure for *Staphylococcus hominis* SP2 (Figure 4E), fibrous material (arrow) surrounds the cell body as a more complete layer. In addition, more elaborate material extends between cells. For *Staphylococcus epidermidis* RP62 (Figure 4F), elaborate and extensive fibrous material surrounds cells and is maintained between cells (arrow).

**Discussion**

The bacterial glycolaxy is defined as structures containing polysaccharide of bacterial origin and lying outside the integral elements of the outer membrane of gram-negative cells and outside the peptidoglycan of gram-positive cells (Costerton et al., 1981). It is rich in highly varied and diverse polymeric polysaccharides (Costerton et al., 1981; van Iterson, 1984) and is also a highly hydrated structure containing 99% water (Sutherland, 1972). These characteristics make optimizing their observation for transmission electron microscopy challenging. One approach is the use of cationic reagents, RR and AB, in en bloc procedures with chemical fixatives. A previous successful method utilizing RR or AB improved observation of glycolaxy material over a glutaraldehyde/OsO₄ fixation for several species of methanotrophs. This material bore a resemblance to freeze etch images without cationic reagent or chemical fixative (Fassel et al., 1990; 1992). The use of a cross-linking reagent, the diamine lysine, with glutaraldehyde was developed by Boyles (Boyles, 1982, 1984; Boyles et al., 1985) and applied to fixation of actin filaments, extracellular matrix and the mammalian glycolaxy. This approach was combined with the use of RR by Jacques and Graham (1989) and applied to the bacterial glycolaxy by themselves and several authors (Akin and Rigby, 1990; Davies and Borriello, 1990; Jacques et al., 1990).

Two general types of bacterial glycolaxy have been described. One is composed of S layers of globular glycoproteins often in regular arrays (Sleytr, 1972). The other is a complex fibrous matrix (Costerton et al., 1981). Both are represented in the species of the present study.

Many gram-negative species have regular arrays of wine glass-, globet- or cup-shaped subunits that are considered by Sleytr (1972) as special types of S layers. Examples include *Flexibacter polymorphus* (Ridgway, 1977), several species of *Chromatium* (Remens et al., 1970; Hageage and Gherna, 1970, 1971) and *Methylomonas albus*. The cup layer of *Methylomonas albus* strain BG8, fits the S layer definition of Sleytr (1972), and was identified by negative staining and freeze etch electron microscopy by Haubold (1978) and in thin section by Wilkinson (1971).

By TEM, the cup layer was not observed in Spurr
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embedments without RR (Fassel et al., 1990), AB (Fassel et al., 1992) or RR-lysine. It was seen without these reagents in LR White. However, the cups lacked strong definition as individual structures and were often not in a complete layer around the outer membrane (Fassel et al., 1990). Further, the improvement in the cup layer by RR is not increased by either AB or the use of lysine. The appearance of the cup layer by RR compares directly with the images obtained by freeze etch, where cationic reagents or chemical fixatives were not used (Fassel et al., 1990). Based on the Spurr observations alone, one cannot differentiate between the improved preservation of the cups or the alternative possibility of improved staining of otherwise invisible cup features. However, the inferior appearance of cups in LR White (Fassel et al., 1990), their improvement with RR in either resin (Fassel et al., 1990) and the agreement with the freeze etch cup layer (Haubold, 1978; Fassel et al., 1990) suggest an improvement in preservation of the cup layer by RR processing. This improvement also agrees with the suggestions of a cup layer composition that includes acidic mucopolysaccharides, acidic protein polysaccharides or glycoprotein.

Additionally, fibrous material appears within cups by the AB or RR-lysine procedure. Elaborate material that frequently extends between cells is seen by RR-lysine processing. For Methylosinus albus BG8, RR alone is inadequate for observation of this glycocalyx material. Perhaps this composition is sufficiently different from the cup layer that RR alone is insufficient to effect its preservation or staining.

Glycocalyces of the remaining three methanotrophs appear to fall under the fibrous matrix type. In previous work, freeze etch was compared to a glutaraldehyde/OsO₄ fixation without cationic reagent. For Methylosinus trichosporium Ob3b and the two Methylocystis species, large filamentous and complex layers were revealed outside the outer membrane of these gram-negative cells by freeze etch after ultra-rapid freezing (Fassel et al., 1990; 1992). When fixed by a glutaraldehyde/OsO₄ procedure, spike-like structures or a complete lack of any glycocalyx material was observed. This can be interpreted as suggestive of collapse and/or condensation perhaps due to an ethanol dehydration artifact (Bayer and Thuro, 1977) that is minimized or avoided by ultra-rapid freezing and by freeze etch. An alternative explanation is this material lacks innate electron density and fails to stain by conventional procedures (Roth, 1977). Perhaps some combination of both dehydration artifact and inability to stain may occur.

When cells of Methylosinus trichosporium Ob3b and the two Methylocystis species were processed in a procedure with AB extensive layers that were filamentous or fibrous were observed. For Methylosinus trichosporium Ob3b, this glycocalyx material appears as two layers of distinct electron density. This was interpreted as suggesting a segregation of different polyanions (Fassel et al., 1992). By the RR procedure for these three species, although some fibrous layers were observed, a variety of other features have consistently been seen for some cells. This includes round bead-like structures, thick filaments, and filamentous layers (Fassel et al., 1990, 1992; Titus et al., 1982).

The use of lysine with RR, resulted in images that were markedly similar to the AB fibrous layer surrounding the two Methylocystis species. However, there was a clear zone between the cell outer membrane and the fibrous layer. This may be suggestive of an artificial cell shrinkage, an area of washout, lack of stain or other artifact. Where cells were observed with a fibrous layer by RR processing, or for all cells by AB processing, this clear zone was not seen. However, a few cells on occasion had evidence of shrinkage that was internal to the outer membrane. Also, for Methylocystis parisi by RR-lysine a more massive congregation of filamentous or fibrous material was noted between cells. For Methylosinus trichosporium Ob3b by RR-lysine, the gradation of electron density in glycocalyx material may again suggest a separation of polyanions. The clear zone was not seen for this species. This may suggest a greater vulnerability to this type of artifact by the Methylocystis species. On the basis of the observation of bead-like structures and thick filaments by RR and their absence by RR-lysine or AB, one could conclude these features are perhaps due to collapse of more filamentous or fibrous material, and their insufficient stabilization or stain reaction with RR. However, the bead-like structures have been observed by scanning electron microscopy (SEM) in the absence of RR or other cationic reagent (Fassel et al., 1990; Titus et al., 1982). There is some suggestion of this structure by freeze etch (Fassel et al., 1990). Alternatively, their lack of observation by RR-lysine or AB might mean an insufficient stabilization or other damage that results in loss of their structural integrity. Perhaps, the composing polymers of the beads are split apart and the structures broken to merge visually with other fibrous or filamentous material.

It was desirable to study the effects of these procedures on glycocalyx preservation or staining on a different group of bacteria. For the staphylococcal species studied, there was no evidence of a glycocalyx by the glutaraldehyde/OsO₄ fixation even in species known for production of external polysaccharide as a slime layer. By RR some fibrous material was observed for all species studied. A considerable increase in the amount of fibrous material in extended configuration around and between cells was seen in RR-lysine processed cells for all species. For SP2, an incomplete layer close to the cell body by RR was replaced by a more complete layer surrounding the cell by RR-lysine. This suggests an improvement in preservation and/or staining of this glycocalyx, possibly by a more efficient cross-linkage and stabilization. For RP62, the difference between RR and RR-lysine processing is less obvious with considerable material observed for both.

A very different image was seen by AB processing for all species of staphylococci studied. Curved electron dense structures and net-like structures may be
the result of condensations or distortions of glycocalyx material for these cells. This is very likely due to insufficient stabilization and subsequent collapse. The lack of agreement with the extended configurations seen with RR-lysine also suggests the inability of AB to interact as effectively with the components of the staphylococci compared to RR-lysine procedures. The additional cross-linking ability of the lysine procedure does not appear superior to the interaction of alcian blue with these glycocalyxes. For the staphylococci, the most abundant and extended glycocalyces were generally similar and extended glycocalyces were observed for cells by RR-lysine procedures. The lack of agreement with the extended configurations seen with RR or RR-lysine appears somewhat genera/species dependent. Differences in complex polyanionic polysaccharide composition of the glycocalyx for the different bacteria could account for these observations.

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Cation reagent effect on the bacterial glycocalyx


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Discussion with Reviewers

T. Zelander: Having in mind the highly hydrated condition of the glyocalyx material, it occurred to me, that the spinning of unfixed bacteria to a pellet might influence the distribution and appearance of the glyocalyx material as seen in some of the pictures. Did you consider this possible influence or have I totally misunderstood your paragraph on methods?
Authors: We cannot conclusively rule out any effect of the centrifugation on the glycocalyces. However, of the two types of glycocalyces we feel the S layers to be less likely effected. The fibrous/filamentous matrices may be the more susceptible in the extended nature of their layers. Previous freeze etch comparisons for the methanotrophs with fibrous/filamentous matrices suggested a good correspondence between freeze etch and images obtained by the alcian blue procedure (Fassel et al., 1992). Therefore, we feel confident with our methods.