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The Use of Transmission Electron Microscopy to Study the Composition of Pseudomonas fragi Attachment Material

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Abstract

Electron microscopy techniques were used to study the attachment matrix of Pseudomonas fragi ATCC 4973. Scanning electron microscopy (SEM) was used to demonstrate the presence of attachment fibrils of P. fragi adhered to stainless steel. Transmission electron microscopy (TEM) was used to examine thin sectioned cells stained with ruthenium red or alcian blue. Extracellular substances surrounding cells stained with ruthenium red or alcian blue had a mat- or spike-like morphology. This indicated that these substances were acidic mucopolysaccharides that may be involved in the attachment of P. fragi to food contact surfaces.

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Introduction

Bacterial attachment to food and food contact surfaces is of concern as it can result in the transmission of disease and in economic losses due to food spoilage. Pseudomonas fragi, a psychrotrophic food spoilage organism, has been shown to attach to stainless steel, rubber, and glass surfaces, the materials used most often in food and milk processing equipment, as well as to beef surfaces (Speers, et al., 1984; Zoltai, et al., 1981; Schwach and Zottola, 1982). Stone and Zottola (1985a) demonstrated with scanning electron microscopy (SEM) that P. fragi cells remained attached to stainless steel surfaces after suboptimum cleaning-in-place (CIP). This attachment phenomenon has been observed in both stationary and dynamic environmental growth conditions at 4° and 25°C with no major differences between the two conditions (Stone and Zottola, 1985b).

Numerous investigators have studied bacterial attachment of marine pseudomonads to solid surfaces, such as glass and plastic, utilizing transmission electron microscopy (TEM) and histochemical staining methodology. Luft (1964) was among the first to utilize ruthenium red to stain extracellular material around cell walls, and Pate and Ordal (1967) used this cationic dye to demonstrate surface filaments on myxobacteria. The capsular structure of Diplococcus pneumoniae and Klebsiella pneumoniae was investigated using ruthenium red by Springer and Roth (1973). Fletcher and Floodgate (1973) used ruthenium red and alcian blue to demonstrate the acidic polysaccharide exocellular exudate of a marine pseudomonad. Costerton, et al. (1978) reported that the glycocalyx of many adhering microorganisms was polysaccharide in nature. Hayat (1975) states that both alcian blue and ruthenium red are specific stains for acidic polysaccharide and acidic mucopolysaccharides. He stated that alcian blue and ruthenium red contain cationic groups which bind to polyanions.

The objectives of this work were two-fold. First, to demonstrate the attachment of P. fragi ATCC 4973 to stainless steel with SEM, and secondly, to utilize the histochemical stains, ruthenium red and alcian blue, and TEM techniques to characterize the attachment matrix of P. fragi.

Materials and Methods

SEM sample preparation

Pseudomonas fragi ATCC 4973 was cultured through one or two 24 h successive transfers and

used to inoculate trypticase soy broth (TSB) (Difco) at a 1% inoculum level. Two mL of this culture were placed in a vial containing one 6 mm X 6 mm stainless steel chip of the type most commonly used in the food industry (type 304 stainless steel with finish No. 4), and the culture was allowed to grow for 18-24 h at 21°C. The stainless steel chips were then removed and rinsed three times in a vial with 2 mL of 0.1 M cacodylate buffer (pH 7.0) for 1 min to remove organisms not firmly attached. The cells attached to the chips were fixed for SEM according to Hood and Zottola (1987) in a solution of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7:0) and 500 ppm ruthenium red (Electron Microscopy Sciences, Fort Washington, PA) for 4-18 h at 4°C. A second fixation was done in 2 mL of 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.0) and 500 ppm ruthenium red in water for 30-60 min at 21°C. The cells were dehydrated at room temperature (21°C) in an acetone series of 25, 50, 75, and 99% for 10 min each and 3 changes in 100% for 10 min each. The cells were critical point dried in a Bomar SPC/EX with CO_2 as the transition fluid. The stainless steel chips were then mounted on aluminum stubs with carbon paint and coated with gold-palladium in a Kinney vacuum evaporator, Model KSE-2AM. Observations of the cells attached to the chips were made using a Philips 500X scanning electron microscope at an accelerating voltage of 12 kV.

Preparation of thin sections of TEM

A P. fragi culture at a 10% inoculum level was grown in 1 L of TSB for 24 h in a reciprocal shaker bath at 21°C. Alternately, cells were grown on TSB containing 1.5% agar (TSA) for 24 h, removed with a sterile spatula, and suspended in cacodylate buffer (pH 7.0). Cells were harvested with centrifugation at 12,000 X g for 10 min at 4°C in a Sorvall RC2-B Superspeed centrifuge. The pellets were rinsed with 0.1 M cacodylate buffer, centrifuged, and then fixed in 4 mL of a solution containing 2.5% glutaraldehyde in 0.1 M cacodylate buffer and 500 ppm ruthenium red in water for 1-4 h at 4°C. The cells were then pelleted with centrifugation and resuspended in 2% osmium tetroxide in 0.1 M cacodylate buffer and 500 ppm ruthenium red for 30-60 min at 21° C. Identical fixations were done with 1% alcian blue (Electron Microscopy Sciences, Fort Washington, PA) in glutaraldehyde/cacodylate buffer and osmium tetroxide/cacodylate buffer at pH 6.5 (Behnke and Zelander, 1970). Control cells without stain were prepared by substituting 2 mL cacodylate buffer at pH 7.0 for the stains. The cells were pelleted and then dehydrated in an acetone series as described in the SEM procedure, and embedded in Spurr's epoxy (Spurr, 1969), see Table 1. The embedded cells were thin sectioned (gold) on an LKB Ultratome, placed on Formvar coated copper grids, post stained with uranyl acetate (3 min) and lead citrate (20 sec), and viewed using a Philips 300 TEM at 60 kV.

Negative stain preparations

Negative stains of P. fragi cells in late log phase (12-16 h) were made by placing a drop of culture in TSB on a Formvar coated copper grid and removing surplus fluid with filter paper, leaving a thin film to air dry within 5-10 min. A drop of a 1% (w/v) potassium phosphotungstic acid (PTA) at pH 7.0 was added to the grids and immediately removed with filter paper. The grids were then examined in the TEM as described above.

Table 1. Spurr's epoxy embedding resin formula

Component	g
Vinylcyclohexanedioxide	10
Digycidyl ether of polypropyleneglycol	6
Nonenylsuccinic anhydride	26
Dimethylaminoethanol	0.4
Cure schedule at 70°C (h)	8

Results and Discussion

SEM examination of P. fragi ATCC 4973 attached to stainless chips revealed fibrous fibrils of varied lengths and thicknesses extending from the cells to the corrugated surface of the chips and also to neighboring cell surfaces (Fig. 1A). These attachment matrices had the appearance of peritrichous fibrils around single cells while groups of cells had a matted network of fibrils between them. Cells were also observed adhered to the stainless steel chips without the aid of visible fibrils. Fraser and Gilmour (1986) have suggested that fibril formation results from the dehydration procedures during fixation causing a collapse of any extracellular material surrounding the cells. They proposed the use of cryo-SEM to prevent this shrinkage and to aid in determining whether fibrils are distinct attachment appendages or result from collapse of exopolymer material. Cells fixed with solutions containing ruthenium red had their ultrastructure preserved with greater detail of the cell surface (Fig. 1A) than cells prepared without ruthenium red (Fig. 1B), as observed in preliminary work (Schwach, 1982). This finding agrees with work by Garland et al. (1979) who reported that ruthenium red preserved mucous in a strand form on epithelial cells and resulted in attached microorganisms being preserved in higher numbers.

TEM examination of thin sectioned cells stained with ruthenium red or alcian blue showed regions of amorphous, electron dense material exterior to the cell wall. The ruthenium red stained cells exhibited a characteristic border "fringe" or reticulum (Fig. 2A) while the alcian blue stained cells had their matrix preserved in a "spike-like" pattern around the cells (Fig. 2B). However, some alcian blue stained cells had smooth electron dense borders around cells cut longitudinally whereas the spiked appearance was observed in transversely cut cells (Fig. 2B). Figure 2C shows an alcian blue stained cell exhibiting fibril-like appendages. The multilayered structure of the cell wall was evident in ruthenium red cells but, due to poorer contrast in alcian blue stained cells, the cell wall structure had to be enhanced with post staining. The unstained control cells had very low contrast in TEM (Fig. 2D), and only when post stained were faint mat-like substances observed around a few cells. The use of ruthenium red or alcian blue greatly enhanced these substances. The observations in this study are similar to those reported by Springer and Roth (1973) who studied the ultrastructure of bacterial capsules with ruthenium red. They attributed the spike-like and mat-like

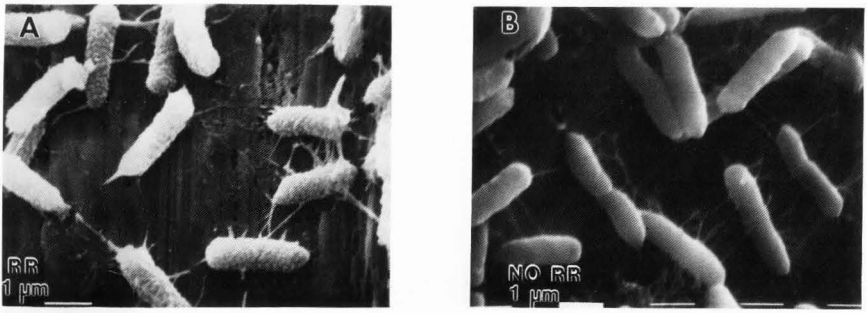

Fig. 1 (A) SEM micrograph of P. fragi ATCC 4973 attached to a stainless steel chip fixed with ruthenium red (RR) in fixatives. (B) cells fixed without RR.

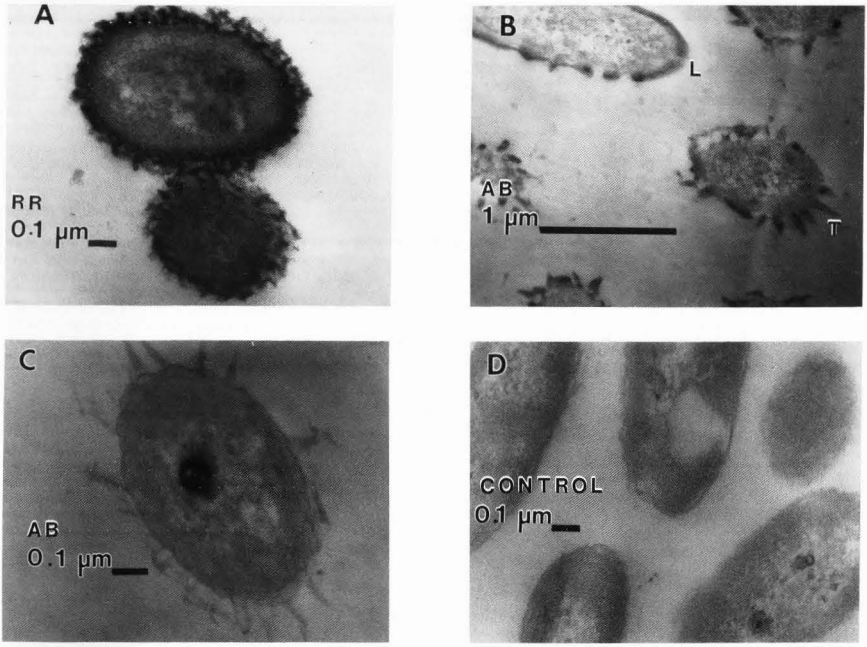

Fig. 2 TEM micrographs of P. fragi ATCC 4973. (A) Cells stained with ruthenium red (RR) showing mat-like border. (B) Cells stained with alcian blue (AB) demonstrating smooth electron dense borders in cells cut longitudinally (L) and the spiked appearance of transversely cut cells (T). (C) Cell stained with AB exhibiting thinner, fibril-like appendages. (D) Unstained control cells.

appearances of the stained material to dehydration and embedding procedures. They found that alcohol dehydration causes some crosslinking of fibrils whereas dehydration with epoxies kept fibrils separated. The spike-like appearance of tangently-cut sections were attributed to the "tips" of the fibrils being sectioned. Because little or no extracellular material was observed in the unstained controls, the ruthenium red and alcian blue may have aided the fixation of the polymers surrounding the cells (Garland et al., 1979).

Because ruthenium red and alcian blue are reported to preferentially stain acidic mucopolysaccharides in cells and tissues (Hayat, 1975), the attachment of P. fragi may be attributed to a matrix of acidic mucopolysaccharide surrounding the cells.

P. fragi ATCC 4973 has been reported to possess perifichous fimbriae (Fuerst and Hayward, 1969). In order to demonstrate the presence or absence of fimbriae on our culture, negative staining with PTA was employed. Electron dense borders were seen around cells stained with PTA while the flagella were outlined in a thin layer of PTA (Fig. 3A). One would expect that if fimbriae were present, they would also be outlined in a thin layer of PTA. Groups of cells exhibited spike-like appendages stretching between cells similar to those observed in the alcian blue stained cells (Fig. 3B). It is possible that any fimbriae present were obscured by the polysaccharide material surrounding the cells as demonstrated with histochemical staining. Fimbriae also could have been coated with the polysaccharide and formed the spiked-like appendages seen in the thin sections. Because negative staining of P. fragi ATCC 4973 failed to demonstrate the presence of fimbriae, it is possible that, under the conditions of this study, our culture did not produce fimbriae. Duguid et al. (1966) found that fimbriae production in Enterobacteriaceae can be influenced by culture conditions such as substrate, incubation temperature, growth stage, and number of serial transfers.

Conclusions

This study suggested that the attachment matrix of P. fragi is composed of an acidic polysaccharide or mucopolysaccharide material. Although fimbriae have been reported on this species, none were observed in our culture under the culture conditions used indicating that their role, if any, in attachment to stainless steel was overshadowed by the polysaccharide material surrounding the cells.

Future work is needed to remove the matrix from cells and further identify its composition. This could possibly aid in the formulation of detergents or sanitizers to remove cells attached to food contact surfaces, and the development of an alternative methodology for the determination of attachment.

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References

Behnke O, Zelander T (1970). Preservation of intercellular substances by the cationic dye alcian blue in preparative procedures for electron microscopy. J. Ultrastruct. Res. 31:424-438.

Costerton JW, Geesey GG, Cheng KJ (1978). How bacteria stick. Sci. Amer. 238:86-95.

Duguid JP, Anderson ES, Campbell I (1966). Fimbriae and adhesive properties in Salmonellae. J. Pathol. Bacteriol. 92:107-138.

Fletcher M, Floodgate GD (1973). An electron-microscopic demonstration of an acidic polysaccharide involved in the adhesion of a marine bacterium to solid surfaces. J. Gen. Microbiol. 58:325-334.

Fraser TW, Gilmour A (1986). Scanning electron microscopy preparation methods: their influence on the morphology and fibril formation in Pseudomonas fragi (ATCC 4973). J. Appl. Bacteriol. 60:527-533.

Fuerst JA, Hayward AC (1969). Surface appendages similar to fimbriae (pili) on Pseudomonas species. J. Gen. Microbiol. 58:227-237.

Garland CD, Lee A, Dickson MR (1979). The preservation of surface-associated microorganisms prepared for scanning electron microscopy. J. Microscopy 116:227-242.

Hayat MA (1975). Positive Staining for Electron Microscopy. Van Nostrand Reinhold Company, New York.

Hood SK, Zottola EA (1987). An electron microscopic study of the adhesive properties of Lactobacillus acidophilus. J. Food Sci. 52:791-794.

Luft JH (1964). Electron microscopy of cell extraneous coats as revealed by ruthenium red staining. J. Cell Biol. 23:54A-55A.

Pate JL, Ordal EJ (1967). The fine structure of Chondrococcus columnaris. III. The surface layers of Chondrococcus columnaris. J. Cell Biol. 35:37-51.

Schwach TS (1982). Use of electron microscopy to study bacterial attachment to food and food contact surfaces. M.S. Thesis, University of Minnesota, St. Paul, MN.

Schwach TS, Zottola EA (1982). Use of scanning electron microscopy to demonstrate microbial attachment to beef and beef contact surfaces. J. Food Sci. 47:1401-1405.

Speers JGS, Gilmour A, Fraser TW, McCall RD (1984). Scanning electron microscopy of dairy equipment surfaces contaminated by two milk-borne microorganisms. J. Appl. Bacteriol. 57:139-145.

Springer EL, Roth IL (1973). The ultrastructure of the capsules of Diplococcus pneumoniae and Klebsiella pneumoniae stained with ruthenium red. J. Gen. Microbiol. 74:21-31.

Spurr AR (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultra. Res. 26:31-43.

Stone LS, Zottola EA (1985a). Effect of cleaning and sanitizing on the attachmemt of Pseudomonas fragi to stainless steel. J. Food Sci. 50:951-956.

Stone LS, Zottola EA (1985b). Relationship between the growth phase of Pseudomonas fragi and its attachment to stainless steel. J. Food Sci. 50:957-960.

Zoltai PT, Zottola EA, McKay LL (1981). Scanning electron microscopy of microbial attachment to milk contact surfaces. J. Food Prot. 44:204-208.

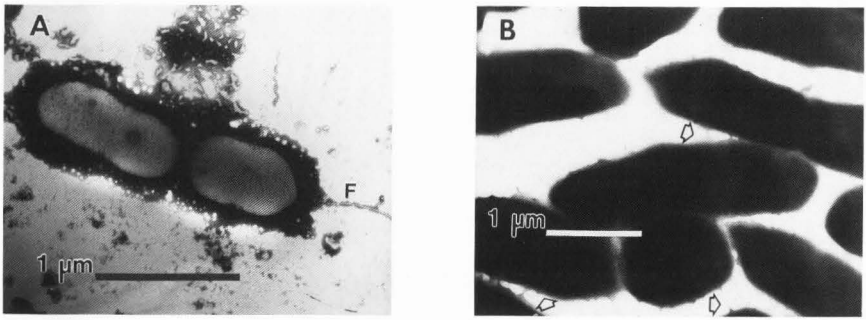

Fig. 3. TEM micrographs of P. fragi negatively stained cells. (A) PTA stained cells with heavy border and flagella (F) outlined in a thin layer of PTA. (B) PTA stained cells with material stretching between cells.

Discussion with Reviewers

P.Stanley: During preparation for TEM, the cells were rinsed in cacodylate buffer before fixation. Was any significant portion of the outer cell wall removed during this rinse? Since the outermost wall layers are responsible for attachment, one would want to be sure none were inadvertently removed in a study of this type.

Authors: Cacodylate buffer (0.1 M, pH 7.0) was used in the SEM rinsing procedures without any obvious removal of attachment fibrils from the cell surface and, although no investigation was performed to determine its effect upon the cell wall, it appeared to have little or no effect upon the removal of the outer cell wall, as the ruthenium red and alcian blue stains illustrated. Glauert (1975, in "Fixation, Dehydration and Embedding of Biological Specimens," North Holland Publishing Company, Amsterdam, 16-17) reports that cacodylate performs with similar results as phosphate buffers, except that it does not support cell growth and the arsenic may also act as a fixative.

Reviewer 1: How many cells were observed without fibrils? Does this relate to your observation concerning longisections?

Authors: As noted in the text, some cells were observed attached to stainless steel without the presence of fibrils. Cells without fibrils usually occurred as single cells rather than in large groups of cells. Although fibrils were absent, they still had the characteristic rough cell surface as depicted in Figure 1. No effort was made to enumerate the proportion of cells without fibril formation, but a majority of the cells observed had fibrils. It is possible that these cell differences may aid in explaining the observation of smooth borders around longitudinally cut cells in Figure 2B rather then the fact that they are longisections, but this was not investigated.

P. Stanley: Two lines of evidence suggest that proteins could be involved in attachment. First, the exopolymer of a sediment bacterium (Mittelman and Gessey, Astr. Ann. Mtg. ASM. 1983. Q5) and outer layers of a marine pseudomonad (Forsberg et al., J. Bacteriol. 104:1338. 1970) were shown to contain approximately 15% protein. Second, proteases improved removal of biofilms from R-O membranes (Whittaker, et al., App. Environ. Microbiol. 48:395. 1984). Did the authors examine the outer surface of P. fragi for proteins which might have been involved in attachment?

Authors: The ruthenium red stain is specific for acidic mucopolysaccharides which Hayat (1985) stated are "invariably associated with protein." Also, the mucopolysaccharides themselves may have amino groups present. In the investigation presented above, no attempt was made to determine if proteins were involved in the attachment process; however, studies are currently being performed to characterize the material responsible for attachment.

P. Stanley: Cells used for TEM analysis were grown in broth or on colonies on agar, whereas cells for SEM analysis grew while attached. Is there evidence that the outer layers of bacteria remain constant under these various growth conditions making the SEM-TEM comparisons valid?

Authors: Cells were grown on the agar surface and then removed in order to simulate growth during attachment to solid surfaces and broth cultures were used to examine the cell surface in a liquid medium. In general, no differences were observed in the thin sections from agar grown and broth cultures. Previous work in our laboratory (Stone and Zottola, 1985b) has shown that attachment to stainless steel with fibril formation occurred within 30 minutes of contact time in broth culture. This would suggest that the cells possess an attachment matrix while in broth culture. However, fibril formation increased with increasing contact time.