The Role of Exopolysaccharides in Adhesion of Freshwater Bacteria

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Cultures of two strains of freshwater bacterial isolates adhered readily to inert glass surfaces exposed in the growth medium. The process of microbial film formation could be followed by a new staining technique based on congo red, a dye specific for carbohydrate material. In conjunction with a chemical assay for total carbohydrate, the association of extracellular polysaccharides with attached cells was demonstrated. Under optimal growth conditions, the involvement of exopolysaccharide in the adhesion process appeared to follow the initial attachment of bacterial cells, leading to the formation of microcolonies enmeshed in polysaccharides. A non-polysaccharide-producing mutant attached to glass slides in numbers similar to the wild-type bacteria, but did not form microcolonies. Growth conditions such as glucose or Ca^{2+} limitation which affected polysaccharide synthesis in the wild-type prevented microcolony formation, but not cell attachment. It is proposed that exopolysaccharide production is involved in the development of the surface films, but possibly not in the initial adhesion process. In those strains which do produce polysaccharide, the cells which attach develop into microcolonies.

INTRODUCTION

Bacteria can adhere to almost any surface in any environment in which they are present. Various cell-surface structures have been observed and thought to be associated with the attachment process (Corpe *et al.*, 1976). Specific structures such as fimbriae (Rosenberg *et al.*, 1982) and pili (Weiss, 1973) or the holdfasts of *Caulobacter* spp. and other stalked bacteria (Corpe, 1970*a*) may be involved in attachment to specific substrates. However, in aqueous environments adhesion is believed to be mediated by extracellular polymers (Fletcher & Floodgate, 1973; Geesey *et al.*, 1977).

The involvement of extracellular polymers in bacterial attachment has been documented for both freshwater (Jones *et al.*, 1969; Sutherland, 1980) and marine bacteria (Floodgate, 1972; Corpe, 1970*b*; Marshall *et al.*, 1971*a, b*). Analysis of bacteria isolated from these environments has shown the polymers produced to be largely composed of acidic polysaccharides (Fletcher, 1980; Sutherland, 1980). The extent to which the polysaccharides are involved in the adhesion process is, however, open to question. Some reports suggest roles both in the initial, reversible phase of adhesion (Corpe, 1970*a*; Fletcher, 1979) and in the later, irreversible phase (Marshall *et al.*, 1971*a*; Fletcher & Floodgate, 1973; Zobell, 1943). Brown *et al.* (1977) presented evidence suggesting that excess polymer production may even prevent adhesion, although trace amounts of polysaccharide might be required initially. Although the association of exopolysaccharide (EPS) with attached bacteria has been demonstrated by both electron microscopy (Geesey *et al.*, 1977; Dempsey, 1981) and light microscopy (Zobell, 1943; Allison & Sutherland, 1984), there is little direct evidence to suggest that EPS participates in the initial stages of adhesion, despite its synthesis by many species in the adherent population. Involvement of EPS may be concerned

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Abbreviations: EPS, exopolysaccharide; SEM, scanning electron microscopy.

not with adhesion, but with the development of the microbial film (Wardell *et al.*, 1983). In this paper, we propose that, for the bacterial strains tested, EPS is not involved in the attachment process *per se*, but in the formation of microcolonies and thus in the synthesis of a microbial surface film.

METHODS

Bacteria and culture conditions. The two wild-type Gram-negative bacteria used in this study, S61 and R1a, were isolated from the surface of chemically clean glass slides suspended in a fast-flowing stream near Edinburgh for 24 h. The non-mucoid derivative of S61, S61NM, was isolated after mutagenesis as described below. Unless otherwise stated, all growth experiments were performed at 30 °C in yeast extract (YE) medium with the following composition (g l⁻¹): Na₂HPO₄, 1.0; KH₂PO₄, 0.3; K₂SO₄, 0.1; NaCl, 0.1; MgSO₄·7H₂O, 0.02; CaCl₂, 0.01; FeSO₄, 0.001; yeast extract (Oxoid), 1.0; casein hydrolysate (Difco), 1.0. Glucose was added as carbon source (1%, w/v) after autoclaving as a separate solution. Calcium- and magnesium-deficient media were prepared by omitting their salts.

Atomic absorption spectroscopy. The concentration of Ca²⁺ and Mg²⁺ in YE media deficient in these two cations was measured by atomic absorption spectroscopy. The sample (20 µl) was dried in a furnace, ashed at 950 °C and atomized at 2850 °C. Absorbance was measured at 285.2 nm for Mg²⁺ and 422.7 nm for Ca²⁺, on a Varian A4 1475 spectrophotometer with a GTA 95 tube atomizer.

Mutagenesis. Washed, exponential-phase cells were resuspended in 10 ml 0.1 M-phosphate buffer (pH 7.0) containing 30–60 µg *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) ml⁻¹ and incubated for 30 min. Cells were pelleted by centrifugation at 30000 *g* for 20 min, washed and resuspended in YE medium. After overnight incubation, bacteria were serially diluted and plated onto solid medium. Mutants were selected on the basis of their non-mucoid colonies. One such mutant, a non-capsulate variant (S61NM), was used subsequently.

Quantitative estimation of EPS. Bacteria were grown in 1 litre volumes of the appropriate medium contained in 2 litre Erlenmeyer flasks at 30 °C and shaken at 120 r.p.m. After incubation, they were removed by centrifugation at 30000 *g* for 30 min. EPS was precipitated from the supernatant by the addition of 2 vols cold (–40 °C) acetone. Precipitated polymer was dissolved in distilled water and reduced in volume by rotary evaporation. Purification was achieved by centrifuging the polymer solution (200000 *g*; 3 h) and dialysing the supernatant fluid against running tap water (48 h) and distilled water (24 h). Samples were then lyophilized and weighed.

Attachment assay. Visualization of cells and associated polymer was based on a previously described procedure (Allison & Sutherland, 1984). Glass slides (75 × 25 mm) suspended in shake flasks of each culture were removed at regular intervals and fixed with 10 mM-cetylpyridinium chloride. The slides were stained with a mixture of saturated congo red solution and 10% (v/v) Tween 80 solution, counterstained with 10% (w/v) Ziehl carbol fuchsin and finally air-dried at 37 °C. This allowed the association of an exopolysaccharide matrix with attached cells to be clearly demonstrated by light microscopy.

For estimation of attached cell numbers, identical glass slides were removed and rinsed with a sterile salts solution (YE salts). Dry, sterile cotton wool swabs were used first, followed by swabs moistened with YE salts, to remove the attached bacteria from the slides. Both swabs were vortexed in 10 ml YE salts. This bacterial suspension was serially diluted in YE salts and plated on YE agar. After incubation for 24–48 h at 30 °C, the viable cells could then be counted; the numbers therefore slightly underestimated the attached microbial population (Paul & Loeb, 1983). The mechanical shear generated by the swabbing was sufficient to remove all attached cells. To ensure complete removal of the attached bacteria, swabbed surfaces were examined microscopically.

To study attachment under non-growth conditions bacteria from batch culture were used; they were harvested, washed with YE salts and resuspended to a suitable concentration. Chemically clean sterile glass slides were then immersed for varying periods of time, and the number of attached cells was estimated as before.

Scanning electron microscopy (SEM). Chemically clean sterile glass coverslips (13 mm diameter) were suspended in shake-flask cultures, removed at regular intervals and rinsed in YE salts. Fixation for 2 h in 0.1M-cacodylate buffer, pH 7.2, containing 2.5% (v/v) glutaraldehyde and 3% (w/v) tannic acid was followed by two 20 min washes in distilled water. Further fixation in 2% (v/v) osmium tetroxide in 0.1 M-cacodylate buffer for 2 h was again followed by two 10 min distilled water washes. Cells were dehydrated by immersion in 10% (v/v) acetone for 5 min, followed by transfer to 20%, 40%, 60%, 80% and 90% acetone, and eventually to three changes of pure acetone. The samples were subjected to critical-point drying using acetone and CO₂ as the transitional fluid, then sputter coated with gold (2 min at 20 mA deposition). Specimens were observed in a Cambridge Stereoscan 250 microscope at an accelerating voltage of 40 kV.

Total carbohydrate assay. Chemically clean, sterile glass coverslips (18 × 18 mm) were suspended in shake flask cultures and removed at regular intervals. After being rinsed three times in distilled water, the coverslips were crushed and assayed for adsorbed carbohydrate using the phenol/sulphuric acid method (Dubois *et al.*, 1956). D-Glucose was used as standard. Coverslips suspended in cell-free medium were treated under identical conditions as a control.

Fig. 1. Polysaccharide adsorption to a glass surface. Glass coverslips were suspended in shake-flask cultures of freshwater isolates S61 (●), R1a (□) and a non-mucoid mutant, S61NM (■) at 30 °C, 120 r.p.m. After removal and careful rinsing of the coverslips, adherent polymer was assayed as described in Methods. A control containing uninoculated medium (○) was used for comparison.

RESULTS

Attachment of freshwater isolates

The bacteria used in this study were capsulate (strain S61) or slime-producing (R1a). EPS production was maximal for both strains in the late-exponential and early-stationary phase. Using chemically clean glass coverslips as the test surface, the association between adsorbed carbohydrate material and attached cells was assayed at regular intervals (Fig. 1). With both strains, carbohydrate material accumulated gradually over the initial 5–6 h exposure, then increased rapidly over the remaining 10 h. A slight non-specific adsorption of glucose from the culture medium occurred in the control.

The accumulation of cells and associated polysaccharide can be seen in Fig. 2. After 9 h exposure (Fig. 2a), strain S61 formed a microbial aggregate surrounded by a copious matrix of polysaccharide material. This increased both in area and depth after 11 h, causing loss of resolution after 13 h (Allison & Sutherland, 1984). Microcolony formation appeared to develop on the surface in a planar manner until a critical size was reached, whereupon subsequent development was away from the surface. SEM observations (Fig. 3) revealed the bacteria firmly anchored to the substratum, probably by secreted adhesive mucilage. After 15 h the cell monolayer developed outwardly from the surface, becoming a complex biofilm.

The carbohydrate material associated with attached cells of strain R1a (Fig. 2b) did not stain as intensely as that surrounding strain S61. This was probably a reflection of the differing composition and structure of the two polymers, which affects the staining by congo red (Wood, 1980). Bacteria were again seen to be enmeshed in a polymeric matrix. After 10 h, a compact microcolony was observed enclosed in a film of polymer (Fig. 2b). After a further 3 h growth and development the microcolony was larger, but still compact and enveloped by polymer.

Attachment of the non-mucoid variant of strain S61, S61NM, was observed under identical test conditions. Carbohydrate formation on the test surface showed a similar trend to that found earlier with another isolate which did not produce polysaccharide (Allison & Sutherland, 1984). Only trace amounts of polysaccharide could be seen during the time course of the experiment (Fig. 2c). Microcolony formation was not observed, the cells being arranged individually, in pairs, or occasionally in groups of three or four. Cell counts did, however, indicate attachment levels similar to those seen with the parent strain (Fig. 4). This result would imply that when EPS is produced, cells recovered from a surface are present due to both attachment and

Fig. 2. Attachment and microcolony formation of freshwater isolates to chemically clean glass slides suspended in shake flasks at 30 °C, as described in the text. (a) Strain S61 after 9 h; (b) strain R1a after 10 h; (c) non-mucoid S61 NM after 10 h. Magnification approx. $\times 1100$.

Fig. 3. Scanning electron micrograph of strain S61 attached to glass by adhesive polymer. The bacteria were grown in YE medium and allowed to adhere to suspended coverslips. After 15 h growth, the coverslips were removed and processed. Polymeric material can be observed attaching cells to each other and to the surface. Bar, 2 μ m.

Fig. 4. Comparison of attachment of strain S61 (○) and a non-mucoid mutant of it, S61 NM (●). Glass slides were suspended in shake flasks of YE medium and removed at regular intervals for cell counts. Each point represents the mean of three independent readings, plus or minus the standard deviation.

Fig. 5

Fig. 6

Fig. 5. EPS adsorption to a glass surface. This was performed as in Fig. 1, using strain S61 grown in YE medium deficient in Ca^{2+} and Mg^{2+} ions (●), Ca^{2+} ions (□) or Mg^{2+} ions (■). A control containing uninoculated medium was again used for comparison (○).

Fig. 6. Effect of Ca^{2+} and Mg^{2+} deficiency on cell attachment to glass surfaces. Strain S61 was grown in YE medium deficient in Mg^{2+} (■), Ca^{2+} (□) or both (●), and in normal YE medium (○). Each point represents the mean of three independent readings, plus or minus the standard deviation.

multiplication. If however a non-mucoid mutant (as strain S61NM), producing only trace amounts of EPS (D. G. Allison, unpublished results), is studied, microcolony formation will not be observed. Multiplication will occur, but without EPS production, colony formation will not take place. The bacteria will not remain on the surface, due to insufficient polymer in which to embed. The majority will therefore be present on the surface due only to attachment. As similar numbers of attached bacteria were observed for both the mucoid and non-mucoid strains, this may possibly suggest the involvement of some other surface component in adhesion which would otherwise be masked by polysaccharide production.

Effect of Ca^{2+} and Mg^{2+} on attachment

YE medium deficient in either Ca^{2+} , Mg^{2+} or both was used to study the effect of these ions on attachment. Using strain S61, the results for carbohydrate accumulation are seen in Fig. 5. In media deficient in both cations, considerably less carbohydrate material was produced on the coverslip compared to media containing both ions in excess. Fewer cells were attached (Fig. 6), and there was no visible appearance of associated carbohydrate material until the 11–12 h stage. Microcolony formation began to occur, but was not extensive. Analysis of the deficient media by atomic absorption spectroscopy revealed trace amounts of Ca^{2+} and Mg^{2+} (4 and 8.29 p.p.m. respectively). Omission of Ca^{2+} alone from the medium appeared to have little significant effect on the production of adhesive polysaccharide (Fig. 5), and cells adhered in a similar manner and numbers to those in normal YE medium (Fig. 6). In Mg^{2+} -deficient medium the carbohydrate response was intermediate between that observed with Ca^{2+} deficiency alone and that seen with deficiency of both cations. The number of cells becoming attached was similar in both cases (Fig. 6). Despite the observation that less polymer was associated with the attached cells in medium deficient in both Ca^{2+} and Mg^{2+} , more polymer was finally produced relative to cell dry weight in the bulk aqueous phase than occurred in normal YE medium: after 15 h the yields of polymer [$\text{mg EPS (mg cell dry wt)}^{-1} \text{ ml}^{-1}$] were YE medium, 0.027; YE medium minus Ca^{2+} , 0.291; YE medium minus Mg^{2+} , 0.860; YE medium minus both cations, 1.147 (means of three independent readings).

Fig. 7. Effect of carbon substrate concentration on adherence to glass slides. Strain S61 was grown under glucose excess (1%, w/v, O) and glucose limitation (0.065%, ●), harvested, washed and resuspended to similar concentrations under non-growth conditions in YE salt solution at 30 °C, 120 r.p.m. Suspended glass slides were sampled over 5 h. Each point represents the mean of three independent readings plus or minus the standard deviation.

Effect of glucose limitation on attachment

Glucose was growth limiting for strain S61 at a concentration of 0.065%. The amount of carbohydrate material associated with attached cells was one-tenth of that found in normal YE medium containing 1% (w/v) glucose. Consequently, small microcolonies similar to those shown in Fig. 2(b) were observed only after 13 h growth. Cell counts (Fig. 7) revealed that the number of viable bacteria attached was greater with cells which had been grown under glucose limitation than with those grown in glucose excess. Having been resuspended in non-growing conditions, the bacteria recovered from the surface were due entirely to attachment and not multiplication.

DISCUSSION

The accumulation of attached bacteria through the production of non-specific polymers has been described by Marshall *et al.* (1971*a*) as a three-stage process. The first, or reversible stage, is the initial attraction of cells to the surface. Electrostatic forces are generally thought to be involved. The second phase is attachment, and the third growth. During these two latter phases, exopolymers are believed to play an important role in permitting cell adhesion to occur (Fletcher & Floodgate, 1973; Marshall, 1976). Once single cells have attached firmly, growth and division occur, leading to microcolony formation (Wardell *et al.*, 1983). Electron micrographs of attached populations have clearly demonstrated the presence of a reticulum of polymeric fibrils, forming an enveloping matrix (Eighmy *et al.*, 1983; Geesey *et al.*, 1977; Costerton, 1980). These fibrils appear to facilitate cell-cell and cell-surface bridging interactions, anchoring cells both to each other and to the surface.

The results presented here, however, indicate that EPS is not directly involved in the initial attachment process, but possibly in the development of the microbial film as proposed by Wardell *et al.* (1983). Strain S61, a Gram-negative capsulate bacterium, adhered to and grew on

glass slides over a 15 h sampling period; associated with the attached cells was a film of carbohydrate-containing material. Similar results were observed for strain R1a, a slime-forming bacterium. In comparison, the attachment ability of a non-mucoid acapsulate mutant of strain S61 was not impaired, as it attached as well as the wild-type strain, but without microcolony formation. Pringle *et al.* (1983) described similar results. A freshwater strain of *Pseudomonas fluorescens* produced a mucoid mutant which showed lower levels of attachment than the non-mucoid wild-type. There were no major detectable differences in the outer membrane profiles of the two *P. fluorescens* types. This was also true of the strains used in this study (D. G. Allison, unpublished results). This would suggest that EPS synthesis is not a prerequisite for bacterial adhesion.

The importance of Ca^{2+} and Mg^{2+} to bacterial attachment has been well documented (Fletcher & Floodgate, 1976; Marshall *et al.*, 1971a). Previous studies have shown the integrity of the cell-polysaccharide association to be dependent on the presence of these two cations (Fletcher & Floodgate, 1976). When grown in the absence of significant amounts of the two cations, strain S61 produced more polymer in the surrounding medium than when both cations were present in excess, but the amount of polymer adsorbed to the substratum was minimal. Cell attachment still occurred, but without the formation of microcolonies. This would indicate that these cations, and in particular Mg^{2+} , play an important part in the regulation of polysaccharide synthesis and its attachment to the cell in strain S61, although their precise role remains unclear. Webb (1948) found that reduced concentrations of Mg^{2+} in the growth medium caused *Clostridium welchii* to increase the yield of EPS. However, both Tempest *et al.* (1965) and Corpe (1964) demonstrated that Mg^{2+} was essential for maximal synthesis of EPS. The reduced ability to synthesize polysaccharide was thought to be due to lower levels of enzyme precursors for polymer synthesis. Alteration of the outer membrane due to lack of divalent cations may reduce the number of attached cells. Anwar *et al.* (1983) observed that in Mg^{2+} -depleted cultures of *Pseudomonas aeruginosa*, changes in the outer membrane occurred. The tertiary structure of the polysaccharide in the presence of multivalent cations may be important for the maintenance of the biofilm, although limitation of the ions did not restrict the ability of the bacterial cells to adhere.

When bacteria were grown under glucose-limiting conditions, only trace amounts of carbohydrate could be detected associated with the attached cells (data not presented). Attachment of bacteria to glass in a non-growth medium was greater for cultures pregrown under glucose limitation compared to those pregrown in glucose excess (Fig. 7). These results were similar to those seen for the non-mucoid mutant in that small microcolonies were observed, but the actual number of viable cells attached to the surface was greater. Preformed EPS does not appear to enhance attachment. Involvement of EPS in the attachment process appears to be limited to microbial film formation. Once the cells became attached to the surface, growth and division occurred. Associated with this was EPS synthesis leading to the development of microcolonies enmeshed in a polysaccharide matrix. The microcolonies enlarged, coalescing and eventually producing a layer of cells completely covering the glass surface.

Brown *et al.* (1977) presented evidence to suggest that large amounts of polymer might inhibit rather than aid attachment. They suggested that specific receptors on the cell surface might interact with organic material initially adsorbed on the surface of the substratum (Baier *et al.*, 1968). Polysaccharide production would therefore saturate the receptors, making them unavailable for adhesion.

The results presented here would suggest that, for the strains tested, EPS produced in the culture fluid is not directly involved in the attachment process. Instead, the role of the polymer could be concerned with the development of a microbial film. Once attachment occurs, growth and division on the surface can take place associated with EPS synthesis. Only when polysaccharides were produced did microcolony formation occur. The actual mechanism of the initial attachment is still not fully understood, but a specific interaction between a cell surface receptor and the substratum surface is believed to be responsible.

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