R-plasmid RP1 promotes adhesion of gram-negative bacteria to medical prostheses and glass

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Summary. The presence of R-plasmid RP1 increased the adhesion of chemostat-grown iron- and carbon-limited Proteus mirabilis to the surfaces of various medical prostheses and to glass. Similar results were obtained with iron-limited Pseudomonas aeruginosa and anaerobically-grown Escherichia coli. Changes in the surface properties of P. mirabilis indicated that the R-plasmid-mediated increase in negative charge was one of the factors that promoted adhesion.

Introduction

Adhesion of bacteria to surfaces may be an important initial event in the pathogenesis of infectious diseases (Vosbeck and Mett, 1983) and adherent bacteria may be less sensitive to natural host defences (Costerton and Marrie, 1983) and to antibiotics (Gwynn et al., 1981) than are bacteria suspended in body fluids. Bacteria that adhere to prostheses may serve as foci of infection (Christensen et al., 1983) and antibiotic-resistant organisms are a particular problem (Eykyn, 1984).

R-plasmid RP1 mediates changes in the outer membrane of Pseudomonas aeruginosa (Kenward et al., 1978) and in the physicochemical surface properties of Escherichia coli (Klemperer et al., 1980) and Proteus mirabilis (Onaolapo and Klemperer, 1986). Its effects on adhesion to various inert surfaces have, therefore, been investigated with cells grown in continuous culture at approximate doubling times of 2 h and 7 h, which lie well within the range of doubling times reported for bacteria growing in body fluids (Maw and Meynell, 1968; Hooke et al., 1985).

Because cell-envelope properties vary with the environment (Brown and Williams, 1985), the adhesion of several phenotypes was investigated in this study. Iron-limitation may be important in vivo because it is an important defence against infection (Griffiths, 1983). Phenotypic changes may be particularly marked in the presence of plasmids, because these place a major synthetic burden on cells (Levin, 1980), particularly when they are energy-limited and grown in anaerobic conditions (Tempest, 1978). In this study iron- and carbon-limited cells have been investigated in aerobic conditions, and carbon-, phosphate- and potassium-limited cells in anaerobic conditions.

Materials and methods

Bacterial cultures

Proteus mirabilis PB13, a motile, but non-swarming strain isolated from a urinary-tract infection, was supplied by Dr B. W. Senior, Ninewells Hospital, Dundee. Pseudomonas aeruginosa PA01 (ATCC 15692) and Escherichia coli K12 W3110 (originally from Dr P. A. Meacock, Leicester University) were also used. R-plasmid RP1 from Professor E. J. L. Lowbury, The Accident Hospital, Birmingham, was transferred to these strains (Meynell and Meynell, 1970).

Stock cultures were maintained on Nutrient Agar (NA) (Oxoid) and the presence of the plasmid checked by plating on NA containing ampicillin 25 µg/ml for P. mirabilis, carbenicillin 200 µg/ml for Ps. aeruginosa and kanamycin sulphate 15 µg/ml for E. coli. The plasmid was stable under all conditions tested. Cells, with (R⁺) and without (R⁻) RP1, were grown at 37°C in 50-ml chemostats (Gilbert and Stuart, 1977).

Media

Simple salts media that provided at least a ten-fold excess of ingredients, unless otherwise stated, were prepared from Analar grade chemicals in double-distilled water.

For P. mirabilis, the medium contained 40 mM glucose, 0-2 mM (NH₄)₂SO₄, 0-2 mM MgSO₄, 0-005 mM FeSO₄, 30 mM KCl, 0·1 mM NaCl, 0-06 mM nicotinic acid and 100 mM KH₂PO₄/(NH₄)₂HPO₄ buffer, pH 7-0, and had an osmolality equivalent to 0·85% saline. For glucose-
limited cultures, glucose was reduced to 3 mM, and for iron-limited cultures, iron was omitted and the phosphate buffer passed once, before use, through a column of Chelex 100 ion exchange resin (Bio-Rad Laboratories, Watford). For Ps. aeruginosa, iron was not added and the medium contained 40 mM glucose, 40 mM (NH₄)₂SO₄, 0.4 mM MgSO₄, 0.62 mM KCl, 5 mM NaCl, 3.2 mM KH₂PO₄, 60 mM 3-N-morpholinopropane sulphonic acid (MOPS) buffer, pH 7.8.

For E. coli, the medium contained 20 mM glucose, 22.2 mM NH₄Cl, 0.2 mM MgSO₄, 0.002 mM FeSO₄, 5.6 mM KH₂PO₄, 1.8 mM Na₂HPO₄ and MOPS buffer 50 mM, pH 7.6. For glucose-limited cultures, glucose was reduced to 3 mM, for phosphate-limited cultures KH₂PO₄ was reduced to 0.1 mM and Na₂HPO₄ replaced by 1.8 mM NaCl and for potassium-limited cultures KH₂PO₄ was reduced to 0.03 mM.

P. mirabilis and Ps. aeruginosa cultures were aerated by passing O₂ through a sinter at the base of the chemostat at 35 ml/min; E. coli was grown anaerobically by passing O₂-free N₂ through the medium at 40 ml/min.

Measurement of adhesion

Cultures of R⁺ and R⁻ cells were established in chemostats and then mixed by the transfer of 1–10% of each culture to the other. At the same time pieces of sterile prostheses or glass slides were added to the chemostat chambers as required. Cultures were harvested after about 50 generations. Surfaces of prostheses or slides were rinsed twice with 10 ml volumes of saline to remove loosely attached cells and were then swabbed vigorously. Swabs, and the objects studied, were shaken vigorously in 5 ml of saline in a vortex mixer. The walls of the chemostat were washed twice with 50 ml volumes of saline, swabbed vigorously and the swabs shaken vigorously as before. To determine the proportion of R⁺ cells in a mixture, samples were counted on NA and NA plus antibiotic. The results were compared with counts of bacteria suspended in the medium. Single-culture chemostats were treated similarly.

Measurement of surface charge

Electrostatic interaction chromatography as described by Pederson (1981) was used to measure the relative surface charge. Cells were harvested, washed twice and resuspended in various concentrations of NaCl in 0.01M phosphate buffer to E₄₇₀ 5.0. A 0.1-ml volume of each suspension was applied to the top of a column prepared in a Pasteur pipette, from QAE sephadex (anion exchanger; functional group quaternary ammonium) or CM sephadex (cation exchanger; functional group sulphonic acid) (Pharmacia Fine Chemicals, AB Uppsala, Sweden; bead size 40–120 μm). Elution was performed at once, with 2 ml of suspending medium; the eluate was collected and the E₄₇₀ measured. Results are expressed as percentage absorbance, i.e., E₄₇₀ eluate as a percentage of E₄₇₀ initial suspension.

Results

Adhesion of mixed cultures to surfaces

The proportion of P. mirabilis R⁺ cells adherent to various medical prostheses and to glass was always 10 to 100 times greater than that in the surrounding liquid culture (fig. 1). Sampling accuracy was high, but there was considerable fluctuation in results when samples were taken on different occasions. It was, therefore, not possible to establish with certainty whether there were significant variations in adherence by cells grown in different conditions, although, generally, iron-limited R⁺ cells adhered in greater numbers than did carbon-limited ones. Growth rate, in contrast, seemed to have little effect.

Similar results were obtained with iron-limited Ps. aeruginosa. After 75 doublings (D = 0.1 h⁻¹), the percentage of R⁺ cells in the medium had fallen to 0.1% but the percentage attached to dacron used for arterial grafts and to pieces of urinary catheter (silicone rubber) was in the range 60–75%. E. coli was not tested with medical prostheses. However, anaerobically-grown carbon-, phosphate- and potassium-limited cells all showed a similar differential adhesion to glass, the R⁺ : R⁻ ratio being eight to eighty times higher than in the liquid, a ratio similar to that found for adhesion to glass by P. mirabilis.

Adhesion of pure cultures of P. mirabilis to surfaces

Comparison of pure cultures which had the same count of suspended cells (fig. 2) indicated differences in adherence between R⁺ and R⁻ cells, although the differences were not as great as in mixed cultures.

Effect of R-plasmid RP1 on the surface charge of P. mirabilis

More P. mirabilis R⁺ cells than R⁺ cells adsorbed to QAE sephadex indicating that the former are relatively more negatively charged than the latter in these conditions (fig. 3). There was no difference in the adsorption of R⁺ and R⁻ cells to CM sephadex.

Discussion

The increase in adhesion associated with plasmid RP1 is in contrast to the action of the multiresistant plasmid described by Denoya et al. (1986), which decreased adherence of Klebsiella pneumoniae to a
cerebrospinal fluid shunt. Plasmid RP1 is an Inc P plasmid and the relatively high percentage of $R^+$ cells in surface growth from mixed cultures is probably partly due to surface transfer (Bradley et al., 1980). However, plasmid RP1 also promotes the adherence of cells in pure culture, which suggests a direct effect on surface properties.

Differences in adhesion between $R^+$ and $R^-$ cells were most easily demonstrated after growth in iron-limited conditions, although results of samples
Fig. 3. Surface charge of iron-limited $R^-$ (□) and $R^+$ (■) *P. mirabilis*, measured by adherence to QAE sephadex (anion exchanger). Bars indicate SD except where they were too small to be recorded.

taken on different occasions varied, possibly because accumulations of adherent bacteria were dislodged into the liquid from time to time as a result of continuous mixing (Costerton and Marrie, 1983). Adhesion to inert surfaces has been used as a model for adherence to tissues (Harber et al., 1983) and the effect of the plasmid on anaerobically-grown cells suggests that adhesion may be important in the maintenance of $R^+$ cells in the gut.

The accumulation of bacteria on a surface is the net result of several factors (Berkeley et al., 1980) of which hydrophobic interactions have often been considered to be of prime importance (Dahlbäck et al., 1981). However, although plasmid RPl codes constitutively for sex pili (Bradley, 1983), which might be expected to increase host cell hydrophobicity (Tewari et al., 1985), $R^+$ cells of *P. mirabilis* are less hydrophobic than $R^-$ cells when grown in continuous culture in the same conditions as those reported here (Onaolapo and Klemperer, 1986). Hydrophobic interactions do not therefore, appear to account for the initial stages of adhesion. Cells in the fluid in a continuous culture system are growing exponentially. Once they have adhered to a surface and become embedded in a microcolony, conditions will be different and some cells may effectively be stationary. Stationary-phase $R^+$ cells of *P. mirabilis* are more hydrophobic than $R^-$ cells, particularly when they are iron-limited.

Solid surfaces are normally negatively-charged when immersed in water (Marshall, 1980) and increased adherence of *Neisseria meningitidis* and *Mycoplasma pneumoniae* was found under conditions in which the net negative charge was reduced (Feldner et al., 1983; Criado et al., 1985). The increase in negative charge of $R^+$ cells, both of *E. coli* (Klemperer et al., 1980) and of *P. mirabilis*, whether chemostat-grown or in stationary phase (Onaolapo, 1986), at first appears anomalous. However, adhesion was measured during growth, in a medium which included an excess of magnesium ions and it has been suggested that an increase in negative charge might provide increased binding sites for divalent cations, which act as a bridge between the bacterial cell and a surface. Such a mechanism might explain the adherence of oral streptococci and *Shigella* spp. to surfaces (Nesbitt et al., 1982; Kabir et al., 1985). Similarly, plasmid-
mediated, charge-dependent adherence of Yersinia enterocolitica to hydroxyapatite was enhanced by the addition of calcium ions (Lachica and Zink, 1984). It is well known that surface growth is particularly important in nutrient-poor environments and the chemiosmotic theory has been used to explain why it is energetically advantageous for organisms to be attached to a surface (Ellwood et al., 1982). It would, therefore, seem to be particularly advantageous for cells carrying a plasmid to adhere efficiently to surfaces. The virulence plasmid, col V, K-94, enhances the attachment of E. coli to sand and glass in chlorinated water (Hicks and Rowbury, 1986).

We thank Drs S. H. Silverman and R. Leeming, The General Hospital, Birmingham, for supplying medical prostheses. J. A. Onaolapo acknowledges with thanks a Commonwealth Scholarship.

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