Properties of Sphaeroplasts of a Halotolerant Achromobacter Strain and Their Infection with Bacteriophage Deoxyribonucleic Acid

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SUMMARY

A halotolerant collagenolytic strain of *Achromobacter* was converted to sphaeroplasts by growth in the presence of penicillin in sucrose-supplemented medium. The sphaeroplasts could be stabilized by sucrose and CaCl₂ and supported phage growth. Sphaeroplasts formed from bacteria incubated for 90 min were competent for transfection with DNA isolated from phage φ3. A linear relationship between infective units and DNA concentration was obtained. Prolonged incubation of sphaeroplasts with DNA caused a drop in phage titre.

INTRODUCTION

Halotolerant, collagenolytic Achromobacter strains have been implicated in leather decay (Thomson, Woods & Welton, 1972) and the genetic system of collagenase production is being studied. Conditions for transformation and transfection are often similar (Bott & Wilson, 1967). Therefore, prior to the investigation of a transformation system, the properties of sphaeroplasts of one of the collagenolytic strains and their infection by phage DNA were examined.

Sphaeroplasts are osmotically sensitive spherical bodies with partially degraded, non-rigid walls. Wall rigidity is due to a mucopeptide which forms the innermost layer in the wall of Gram-negative bacteria (Weidel, Frank & Martin, 1960). Depolymerization of this layer, necessary for conversion to sphaeroplasts, can be accomplished with penicillin (Lederberg, 1956, 1957; McQuillen, 1958) which inhibits mucopolymer synthesis (Park, 1968), with lysozyme (Gebicki & James, 1958), or with glycine (Brown, Drummond & North, 1962). Sphaeroplasts of *Escherichia coli* and *Proteus mirabilis* can be stabilized by spermine or spermidine (Tabor, 1962; Van Rensburg, 1969a). Infection of protoplasts and sphaeroplasts by isolated bacteriophage DNA with the production of mature phage has been reported in a number of bacteria (Iliashenko, 1964; Pitout & Van Rensburg, 1969; Riggs & Rosenblum, 1969; Tichy & Landman, 1969; Van Rensburg, 1969b; Streips & Walker, 1971).

METHODS

**Media.** Nutrient broth (Difco) supplemented with 0.4 M-NaCl was used. For double-agar-layer phage assays, the bottom layer was salt agar which consisted of (g): Bacto-tryptone (Difco), 13; NaCl(0.4 M), 23.4; glucose, 1.5; and Oxoid agar no. 3 (BDH Ltd, Poole, Dorset), 11, in 11 distilled water. Phage agar top layer contained in 11 distilled water (g): Bacto-tryptone, 10; NaCl, 23.4; glucose, 3; and Oxoid agar no. 3, 6. Sphaeroplast medium consisted of the salt medium supplemented with 0.5 M-sucrose and 0.2% (w/v) MgSO₄·7H₂O. The transfection medium consisted of (g): nutrient broth, 10; Bacto-yeast extract, 1; glucose,
0.5; K$_2$HPO$_4$, 2; KH$_2$PO$_4$, 1; NaCl, 23.4; and 0.5 m-sucrose, in 1 l distilled water. Lysozyme (1 mg/ml, BDH Ltd), tris buffer (0.1 M and 1.0 M, pH 8.0) and 2% (w/v) ethylenediaminetetraacetic acid (BDH Ltd) were used in sphaeroplasting experiments. Incubation was at 37 °C.

**Bacteria and bacteriophages.** Achromobacter sp. 2, a halotolerant collagenolytic bacterium isolated from cured hides (Thomson *et al.* 1972), was used. A bacteriophage, χ3, which was isolated after spontaneous liberation by *Achromobacter* sp. 2, was used.

**Penicillin sphaeroplasts.** These were prepared by diluting 10 ml of an overnight culture in 100 ml of sphaeroplasting medium. Penicillin (500 to 5000 units/ml) was added and the culture incubated with aeration. Samples were removed at intervals, examined microscopically and counted on a haemocytometer. For photography, a drop of sphaeroplast culture was mounted on a microscope slide in 2% (w/v) aqueous methyl cellulose containing a drop of Gram’s safranin.

**Osmotic sensitivity and stabilization of sphaeroplasts.** Duplicate 10 ml samples of sphaeroplasts were collected by centrifugation at 2000 g for 20 min. The pellets were resuspended in 10 ml volumes of 0.5 m-sucrose, deionized water, CaCl$_2$ (10$^{-2}$ M), MgCl$_2$ (10$^{-2}$ M), NaCl (10$^{-3}$ M, 0-4 M), spermidine (10$^{-4}$ M, 10$^{-3}$ M, 10$^{-4}$ M, 10$^{-3}$ M), nutrient broth and transfection medium. Extinction at 660 nm was measured after 10 min in an EEL colorimeter (Evans Electroselenium Ltd).

**Phage techniques.** Phage lysates were prepared by the modification (Adams, 1959) of the double-agar-layer method of Hershey, Kalmanson & Bronfenbrenner (1943). Methods for obtaining high titres of phage and for phage purification were those of Adams (1959).

**Phage growth.** After incubation with penicillin for 5 h a sphaeroplast culture was diluted 1:10 in sphaeroplastic medium containing 1000 units penicillin/ml. Phage was added at a titre of 10$^4$ plaque-forming units (p.f.u.)/ml to both sphaeroplast and bacterial cultures. Samples were removed at 10 min intervals and diluted in 0.4 M-NaCl. Samples (0.1 ml) were added to 0.1 in1 volumes of indicator bacteria in 2.5 ml of top layer agar at 45 °C prior to pouring on salt agar plates. Plaques were counted after 24 h of incubation.

**Preparation of phage χ3 DNA.** A modification of the phenol extraction method of Mandell & Hershey (1960) was used. An equal volume of water-saturated phenol was added to purified phage (1 × 10$^{11}$ p.f.u./ml) in saline-citrate buffer (0.15 M-NaCl) and 0.01 M-sodium citrate, pH 7.0). The tube was rotated on an angled turntable at 45 rev./min for 15 min. The mixture was chilled at 4 °C, centrifuged at 2000 g for 5 min and the phenol layer removed with a Pasteur pipette. The phenol extraction was repeated three times and the final product dialysed at 4 °C for 24 h against three changes of the saline-citrate buffer. The concentration of DNA was determined by ultraviolet absorption at 260 nm.

**Transfection.** Competence for transfection was determined by inoculating 10 ml of an overnight culture into 100 ml of nutrient broth followed by incubation with aeration. At 30 min intervals 3 ml samples of bacteria were removed and converted to penicillin sphaeroplasts. After 5 h, aeration of the sphaeroplasts was stopped and after a further 7 h of incubation they were centrifuged at 2000 g for 15 min and resuspended in transfection medium (1 × 10$^8$/ml). To 0.5 ml volumes of sphaeroplast suspension, phage DNA was added to a final concentration of 20 µg/ml. The mixture was incubated for 2 h and assayed for infective centres. Dilutions for the assay were made in transfection medium to prevent lysis of sphaeroplasts. For all further transfection experiments, bacteria were incubated for 120 min prior to conversion to sphaeroplasts. In control experiments, sphaeroplasts and DNA alone were each assayed for infective centres.
RESULTS

**Penicillin sphaeroplasts**

Incubation with penicillin caused the rod-shaped cells to swell terminally forming pear-shaped bodies (Fig. 1). After 5 h of incubation these intermediate forms were converted to sphaeroplasts. One hundred per cent conversion to sphaeroplasts was obtained with 500 to 1000 units penicillin/ml but higher concentrations produced aberrant forms. Sphaeroplasts were not produced by treatment with lysozyme (1 mg/ml) + EDTA (2%, w/v) in tris buffer, nor with glycine (2%, w/v).

**Stability of sphaeroplasts**

Sphaeroplasts were stabilized when suspended in transfection medium, nutrient broth + 0.4 M NaCl, sucrose (0.5 M) and CaCl$_2$ (10$^{-2}$ M). NaCl (0.4 M, 10$^{-2}$ M) and MgCl$_2$ (10$^{-2}$ M) provided little support (Table 1). Spermidine did not stabilize sphaeroplasts and suspension in water caused rapid lysis.

**Multiplication of bacteriophages in sphaeroplasts and bacteria**

Immediately after phages were added to sphaeroplasts there was a decrease in phage titre (Fig. 2). No decrease was observed when phages were added to bacteria. The decrease obtained with sphaeroplasts was presumably due to the release of non-infective vegetative phages by premature lysis of the sphaeroplasts which were removed from the stabilizing medium and diluted in 0.4 M NaCl prior to plating on salt agar (Hayes, 1968).
Fig. 2. Phage multiplication in sphaeroplasts (○—○) and bacteria (●—●). Phages at a titre of $1 \times 10^4$ p.f.u./ml added to sphaeroplasts and bacteria ($1 \times 10^8$ ml).

Fig. 3. Development of competence for transfection. Concentrations of sphaeroplasts ($1 \times 10^8$/ml) and DNA (20 μg/ml) and time of incubation of sphaeroplasts with DNA (60 min) were kept constant. Abscissa represents time of incubation of bacteria in nutrient broth before conversion to sphaeroplasts.

Table 1. Stability of sphaeroplasts

Ten ml volumes of penicillin-induced sphaeroplasts were centrifuged at 2000 g for 20 min and resuspended in 10 ml of the different solutions. Extinction at 660 nm after 10 min was determined in an EEL colorimeter.

<table>
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<tr>
<th>Suspending medium</th>
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<tr>
<td>Sphaeroplast medium</td>
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<td>Transfection medium</td>
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<td>Spermidine, 10$^{-2}$ M</td>
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<td>Deionized water</td>
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Sphaeroplasts and transfection of Achromobacter

Fig. 4. Relationship between concentration of α3 phage DNA added and infective units produced after 90 min of incubation. Bacteria were grown in nutrient broth for 120 min before conversion to sphaeroplasts. Sphaeroplasts were incubated with DNA for 60 min. Volume of incubation mixture and sphaeroplast concentration were kept constant.

Fig. 5. Release of phage by sphaeroplasts treated with α3 phage DNA at 20 μg/ml. Bacteria were grown in nutrient broth for 120 min before conversion to sphaeroplasts. Abscissa represents time of incubation of sphaeroplasts with phage DNA.

Transfection

Optimal competence for infection by phage DNA was obtained when bacteria were incubated for 120 min before conversion to sphaeroplasts (Fig. 3). Over the concentration range 0 to 50 μg DNA/ml a linear relationship between DNA concentration and infective centres was obtained (Fig. 4). Length of incubation of competent sphaeroplasts with DNA affected the phage titre (Fig. 5). The optimal time of incubation was 60 min.

Discussion

The spherical bodies produced by penicillin treatment correspond to sphaeroplasts on grounds of morphology, osmotic sensitivity and phage infection. Stages in the development of penicillin sphaeroplasts were similar to those reported by McQuillen (1960) for Bacillus megaterium. The aberrant forms produced by penicillin concentrations higher than 1000 units/ml appear to be due to end-to-end adhesion of cells with partial or total degradation of adjacent cell walls. Phage multiplication in sphaeroplasts indicates the retention of receptor sites for bacteriophages. As these sites are contained in the lipoprotein and lipopolysaccharide layers (Oram & Reiter, 1968) this confirms that penicillin affects only the mucopeptide layer (Park & Strominger, 1957).

The initial rapid increase in phage titre (Fig. 5) suggests the rapid uptake of DNA by sphaeroplasts. The decrease in effective transfection after 60 min may be due to the α3 DNA being partially degraded by host nucleases. Green (1966) reported the denaturation of foreign DNA by defensive cellular nucleases.

Conversion of bacteria to sphaeroplasts results in the fixation of the competent state (Iliashenko, Dityatkin & Danileichenko, 1968). As competence for transfection can be associated with competence for transformation (Bott & Wilson, 1967), competent sphaeroplasts of Achromobacter are being used to investigate transformation in this strain.

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