Transfection of the Anaerobe *Bacteroides thetaiotaomicron* with Phage DNA

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The properties of a *Bacteroides thetaiotaomicron* phage, β_1 , are described. Phenol-extracted phage β_1 DNA transfects Ca^{2+} -treated *B. thetaiotaomicron* cells and transfection is not limited to a particular stage in the growth cycle. An obligatory step in the transfection procedure is a 33-fold dilution in broth, allowing replication of the infected cells. Prolonged incubation of treated cells with DNA prior to dilution in broth results in a large decrease in phage titre. The application of this system to the investigation of the genetics of obligate anaerobes is discussed.

INTRODUCTION

Studies on obligate anaerobes are at present hampered by the lack of a genetic system (Morris, 1976). As the factors governing competence for transfection and transformation are often very similar (Riva & Polsinelli, 1968), studies on transfection in the obligate anaerobe *Bacteroides thetaiotaomicron* were undertaken in order to define the conditions required for the uptake of DNA. Mandel & Higa (1970) described the calcium treatment of *Escherichia coli* cells which allowed transfection by λ DNA. This method has been successfully applied to various nucleic acids of viral (Sjöström, Lindberg & Philipson, 1972; Taketo, 1974; Bursztyn *et al.*, 1975), plasmid (Cohen, Chang & Hsu, 1972) and bacterial origin (Lindberg, Sjöström & Johansson, 1972; Cosloy & Oishi, 1973).

Strains of *B. thetaiotaomicron* were formerly recognized as subspecies of *Bacteroides fragilis* but the species rank has recently been restored as a result of DNA homology studies (Cato & Johnson, 1976). As this is also the first report of a *B. thetaiotaomicron* phage, the characteristics of the phage are briefly described.

METHODS

Bacteria and bacteriophage. The bacteriophage host was *B. thetaiotaomicron*, isolated from a human faecal sample. The bacterium was identified as *B. thetaiotaomicron* and this was confirmed by Dr L. V. Holdeman, V.P.I. and S. U. Anaerobic Laboratory, Blacksburg, Virginia, U.S.A. The phage, β_1 , was isolated from sewage from an anaerobic digester by conventional methods (Adams, 1959).

Phage techniques. Brain-heart infusion (BHI) broth and agar were used for phage and bacterial propagation (Burt & Woods, 1976). Exponential phase cultures were infected with phage at a multiplicity of infection of 0.01 and lysis occurred within 2 to 3 h. Concentration and purification of phage lysates was achieved by two cycles of differential centrifugation at 6000 g for 10 min and at 57 500 g for 90 min. Single step growth curves, adsorption kinetics and inactivation by antiserum, ultraviolet light (u.v.) and heat were determined by the methods of Adams (1959).

Preparation of DNA. Phage DNA was extracted by a modified procedure of Sjöström *et al.* (1972). Sodium dodecyl sulphate was added to the concentrated phage suspension to a final concentration of 1% (w/v) and the suspension was incubated at room temperature for 30 min. DNA was extracted by the water-saturated phenol technique. The DNA had a u.v. absorption ratio (260/280 nm) of 1.8 to 2.0.

Assay for infectious DNA. Competent bacteria were prepared by the method of Mandel & Higa (1970). An overnight culture was diluted 200-fold in BHI broth and incubated for 5 h, to an extinction of 0.85 at 600 nm. The cells were cooled quickly, harvested by centrifuging at 4 °C and resuspended in an equal volume of cold 0.1 M-MgCl₂. The cells were then recentrifuged and resuspended in half of the original volume of cold 0.1 M-CaCl₂, and kept at 0 °C for 20 min. The cells were finally centrifuged and resuspended in one-tenth of the original volume of 0.1 M-CaCl₂. Phage DNA (0.1 ml) in standard saline citrate buffer (0.15 M-NaCl/0.015 M-sodium citrate, pH 7.0) was added to 0.2 ml cells and kept at 0 °C for 15 min. The cell/DNA mixture was given a heat pulse of either 2 min at 42 °C or 10 min at 37 °C, diluted into 10 ml prewarmed broth, and incubated for 2 to 4 h before plating for plaque-forming units (p.f.u.) by the double agar layer technique (Adams, 1959).

Penicillin sphaeroplasts. Sphaeroplasts were prepared by the methods of Thomson & Woods (1973). Microscopic examination after incubation with penicillin revealed that 95 to 100 % of the cells had been converted to sphaeroplasts. After the penicillin treatment, 25 µg protamine sulphate ml⁻¹ was added, and the sphaeroplasts were stored on ice. Equal volumes of DNA and sphaeroplasts were mixed and incubated for 10 to 60 min prior to assaying for p.f.u.

RESULTS

Properties of phage β₁

Phage β₁ is specific for the anaerobe *B. thetaiotaomicron* and has a hexagonal head, tail (144 nm) and a base plate but no visible fibres (Fig. 1*a*). Electron micrographs of concentrated phage lysates indicated the presence of many defective phage particles without tails, which either contained phage DNA or were empty, and appeared to be of two different sizes (Fig. 1*a, b*). The ratio of defective phage to whole phage, determined by counting 17 electron micrographs, was 99:1. The viable phage particles banded in a single peak with a density of 1.44 g ml⁻¹ and were separated from the defective phage particles by caesium chloride equilibrium centrifugation at 130000 g for 23 h (Fig. 2). Electron micrographs from the p.f.u. peak (fractions 13 to 20) showed mostly whole phage (Fig. 1*c*) but electron micrographs from the protein peak (fractions 19 to 22) only showed defective particles (Fig. 1*d*). Few free phage tails were observed in electron micrographs. Stability studies assessed by p.f.u. assays in different buffers showed no loss in viability at 4, 20 or 37 °C. Thermal inactivation studies at 60 and 70 °C showed no loss in viability after 1 h at 60 °C but 100 % inactivation after 5 min at 70 °C. The burst size of the phage was found to vary from 16 to 290 in seven different experiments. The phage had a neutralization constant with antiserum of 123.5 min⁻¹, an adsorption constant at 37 °C of 4 × 10⁻¹⁰ ml min⁻¹ and a u.v. inactivation constant of 0.01 s⁻¹ (Adams, 1959). The phage contained double-stranded DNA, which had a *T_m* of 83 °C and a GC ratio of 33.4 mol %.

Carrier state

Turbid plaques were observed occasionally and the resistant colonies were isolated from the centre of a turbid plaque. A phage-resistant clone, which produced phage, was obtained and the stability of this association was investigated by determining the persistence of phage in progeny from six consecutive colonial subcultures. After each of six clonings, 40 colonies were tested for phage production and phage resistance. The number of colonies after each cloning which produced phage spontaneously varied from 25 to 90 % and the number of colonies resistant to phage varied between 49 and 100 %. The carrier state was eliminated by a single passage through phage antiserum. This indicates that the carrier state is not true lysogeny as lysogens continue to produce phage after antiserum treatment. The phage was not induced by u.v. irradiation, mitomycin C (Calbiochem, B grade), temperature or ageing.

Fig. 1. Electron micrographs of *B. theta*taomicon phage. (a) Intact phage and defective phage. Bar marker represents 100 nm. (b) Concentrated phage lysate showing excess of defective phage. Intact phage particles are indicated by arrows. Bar marker represents 100 nm. (c) Intact particles from p.f.u. peak (fractions 13 to 20) after density gradient centrifugation (see Fig. 2). Bar marker represents 500 nm. (d) Defective particles from protein peak (fractions 19 to 22, see Fig. 2). Bar marker represents 500 nm.

Fig. 2. Fractionation of intact and defective phage particles by CsCl density gradient centrifugation. ▲, Infectivity (p.f.u. ml⁻¹); △, extinction at 280 nm; ○, density (g ml⁻¹).

Table 1. *Identification of the infective principle*

Phage β_1 DNA was treated as described in the Table; 0.1 ml was then added to 0.2 ml Ca²⁺-treated cells at 0 °C and the mixture was incubated at 42 °C for 2 min. The cell/DNA mixture was diluted into 10 ml BHI broth and incubated for 4 h prior to plating by the soft agar layer technique. Final DNA concentration 6.5 μ g ml⁻¹.

Treatment	No. of p.f.u. ml ⁻¹	% of control
Control	1.5×10^4	100
No DNA	0	0
No cells	0	0
Cells untreated with Ca ²⁺	0	0
Sphaeroplasts	0	0
DNA preincubated with 2 μ g DNAase ml ⁻¹ for 5 min at 37 °C	0	0
DNA+cells incubated with 2 μ g DNAase ml ⁻¹ after 2 min heat pulse	1.3×10^4	87
DNA preincubated with 20 μ g RNAase ml ⁻¹ for 30 min at 37 °C	1.2×10^4	80
DNA preincubated with 20 μ g pronase ml ⁻¹ for 30 min at 37 °C	1.0×10^4	67
Control (no protamine sulphate)	5.6×10^4	
Ca ²⁺ -treated cells with 25 μ g protamine sulphate ml ⁻¹	8.6×10^4	

Transfection

Phage β_1 DNA was shown to be the infective agent in the *B. thetaiotaomicron* transfection system (Table 1). Infectivity was lost when the DNA was preincubated with DNAase, but RNAase and pronase had little effect. However there was 13% reduction in transfection when the cell/DNA mixture was treated with DNAase after the 2 min heat pulse treatment. Transfection only occurred when cells were treated with 0.1 M-CaCl₂ and did not occur with penicillin sphaeroplasts. Protamine sulphate, which enhances transfection of *E. coli* sphaeroplasts (Benzinger, Kleber & Huskey, 1971), did not affect transfection in *B. thetaiotaomicron*.

The number of p.f.u. increased with phage DNA concentrations between 1.6 and 6.5 μ g ml⁻¹ (Table 2). Further increases in DNA concentration did not markedly increase the number of p.f.u.

Competence for transfection throughout the growth cycle of *B. thetaiotaomicron* was determined. No relationship was found as cells at various growth stages were equally competent. However, the degree of competence varied greatly between different cultures of the *B. thetaiotaomicron* strain.

Table 2. *Effect of DNA concentration on the number of p.f.u.*

Various amounts of DNA were distributed into tubes containing 0.2 ml Ca^{2+} -treated cells at 0 °C. Mixtures were heated for 2 min at 42 °C, diluted into 10 ml BHI broth and incubated for 4 h prior to plating by the soft agar layer technique.

DNA concn ($\mu\text{g ml}^{-1}$)	Titre (p.f.u. ml^{-1})
1.6	8.9×10^8
3.25	1.5×10^9
6.5	9.4×10^9
13	9.3×10^9

Fig. 3

Fig. 4

Fig. 3. Kinetics of appearance of p.f.u. after dilution of the cell/DNA mixture. A cell/DNA mixture was heated at 42 °C for 2 min, diluted in 10 ml BHI broth and incubated at 37 °C. Samples were removed at intervals and assayed for p.f.u. Final DNA concentration 10 $\mu\text{g ml}^{-1}$.

Fig. 4. Kinetics of transfection and viability of Ca^{2+} -treated cells at 37 and 42 °C. Cell/DNA mixtures were incubated at 37 °C (▲) or 42 °C (●), sampled at intervals, diluted into 10 ml BHI broth, incubated at 37 °C for 4 h and assayed for p.f.u. Cells treated with Ca^{2+} at 0 °C were incubated at 37 °C (Δ) or 42 °C (○) and samples were removed at intervals, diluted and plated to determine cell viability. Final DNA concentration 13 $\mu\text{g ml}^{-1}$.

No transfection was observed when the cell/DNA mixture was plated immediately after the heat pulse treatment. Transfection was only detected after the cell/DNA mixture was diluted 33-fold into BHI broth and incubated for 2 to 4 h before plating (Fig. 3). No p.f.u. (detectable at a level of 5 p.f.u. ml^{-1}) were produced when the cell/DNA mixture was diluted 0- to 12-fold. Because the conditions now favour continued cycles of phage replication, it must be emphasized that the p.f.u. do not represent primary infections as the time interval of incubation is greater than the minimum latent period of intact phage adsorbed to these bacteria.

As the transfection procedure usually involves chilling at 0 °C and a heat pulse of either 10 min at 37 °C or 2 min at 42 °C, the effect of temperature on transfection was investigated. Transfection occurred at 0 °C without a heat pulse, and heating the cells for 2 min at 42 °C did not affect the number of p.f.u. However, further incubation of the Ca^{2+} -treated cell/DNA mixture at 42 °C for 10 min or 37 °C for 50 min resulted in a marked decrease in the number of p.f.u. (Fig. 4). This is probably due to the lethal effects of Ca^{2+} at high concentrations, as the viable cell count decreased simultaneously with the decrease in p.f.u. No loss in the viability of the cells was observed when cells and DNA were mixed in the absence of Ca^{2+} .

DISCUSSION

Phage β_1 , used in the transfection studies, is interesting as an excess of defective particles of two different sizes were observed in phage lysates, even after repeated cloning. The reason for the production of defective particles at a high frequency is not known and is part of a further study being undertaken to elucidate the β_1 phage-host relationship. However, the defective particles are unlikely to be due to instability of the whole phage, as the phage, assessed by p.f.u., was very stable and few free phage tails were observed in electron micrographs. The defective particles are not likely to be another spontaneously produced phage without a tail as concentrated filtrates of uninfected or u.v.-induced bacterial cultures had no observable phage particles. Phage were only obtained when sensitive bacteria were infected with the β_1 phage.

Mosig (1963) reported the production of small T_4 particles, which had a lower buoyant density in CsCl than the average particles and were not viable. These particles contained short DNA molecules which prevented normal assembly (Chao, Chao & Speyer, 1974). The smaller 'heads' of phage β_1 may arise in a similar manner. Serological and other studies are in progress to determine whether the different structures visible in electron micrographs have the same origin.

The pseudolysogeny we have observed with phage β_1 is similar to that reported by Keller & Traub (1974) for a *Bacteroides fragilis* phage. Since there is an alteration between phage-resistant and phage-sensitive phenotypes, the phage carrier state may be due to the continuous development of phage-sensitive bacteria from a phage-resistant population.

Competence for transfection in *B. thetaiotaomicron* required that the cells were suspended in a reaction medium containing high concentrations of Ca^{2+} but was not limited to a particular stage in the growth cycle. Taketo (1974) found a similar effect with *E. coli* cells, but Sjöström *et al.* (1972) found that competence in *Staphylococcus aureus* was limited to a short period in early exponential phase. Although the competent cells were prepared by a standard growth method, the number of transfectants varied greatly when the same DNA was used in different experiments. This appears to be a common feature of some transfection systems as Földes & Trautner (1964) observed large variations in p.f.u. with *Bacillus subtilis* between experiments using the same DNA concentrations. Benzinger & Kleber (1971) found that only 1 out of 15 penicillin sphaeroplast preparations of *E. coli* was optimally competent. A further example of the sensitivity of the cell/DNA mixtures in transfection was reported by Földes & Trautner (1964) who showed that in the transfection of *Bacillus subtilis* extreme care had to be taken in the handling of cell/DNA mixtures.

Transfection occurred at 0 °C and the heat pulse, although effective, was not essential for DNA uptake. Cohen *et al.* (1972) found that no uptake of R plasmid DNA by *E. coli* occurred unless the cells were given a heat pulse. Burt & Woods (1976) reported that R plasmid transfer from *E. coli* to *Bacteroides* spp. was possible after heat treatment of the recipients for 20 min at 50 °C.

A feature of the transfection system was the necessity for both the 33-fold dilution of the cell/DNA mixture and the subsequent 2 to 4 h growth period. The reasons for these requirements are not known but Romig (1962) found a similar requirement for replication of infected *Bacillus subtilis* cells before plaques were detected. The 20-fold dilution step may be necessary to reduce the concentration of Ca^{2+} below a certain level for transfection.

The thermosensitivity of the transfection system can be partly explained by a loss of viability of the Ca^{2+} -treated cells, although the number of transfectants decreased more rapidly than the number of viable cells. It is unlikely that nuclease activity contributes to this thermosensitivity as treatment of the cell/DNA mixture with DNAase after the 2 min heat pulse only resulted in a 13 % decrease in the number of p.f.u.

This is the first report of phage DNA uptake in a *Bacteroides* species. In other bacterial species, Ca^{2+} is necessary for the uptake of bacterial DNA resulting in the transformation

of antibiotic resistance and auxotrophic markers (Lindberg *et al.*, 1972; Cosloy & Oishi, 1973). Since spontaneous genetic transfer has not been described in *Bacteroides* species (Burt & Woods, 1976), the transfection procedure described may be utilized to develop a transformation system.

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