Bacteriophages of *Streptococcus equi*

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**SUMMARY**

Bacteriophages were isolated from twelve lysogenic strains of *Streptococcus equi*. Based on sensitivity data and antiserum neutralization tests, the phage isolates were divided into two distinct but related groups. All twelve phage changed the colonial morphology of *S. equi* from mucoid to matt. Possible phage-mediated effects on *S. equi* virulence are discussed.

**INTRODUCTION**

Temperate bacteriophages have been found in streptococci of Groups A, B, C, D, G, H, and N (Krause, 1957; Fox & Wittner, 1965; Brailsford & Hartman, 1968; Russell, Norcross & Kahn, 1969; Colon, Cole & Leonard, 1971; Parsons et al. 1972; Keogh & Shimmin, 1974); however, most research has been with the human pathogen, *Streptococcus pyogenes* (Kjems, 1958a; Maxted, 1955, 1964; Fischetti, Barron & Zabriskie, 1968; Fischetti & Zabriskie, 1968). Work with Group C streptococci phages has largely been limited to B/563, the virulent phage (now designated CI) isolated by Alice Evans (Evans, 1934; Maxted, 1964; Fox & Wittner, 1965; Cohen, Gross & Harrell, 1975); and to human Group C (*S. equisimilis*) temperate bacteriophages (Maxted, 1964; Fox & Wittner, 1965; Wannamaker, Almquist & Skjold, 1973; Cohen et al. 1975; S. E. Read, personal communication). No work has yet been reported on temperate bacteriophages specific for *S. equi*, the Group C streptococcus that causes strangles in horses (Stableforth, 1959; Mahaffey, 1962).

Freeman (1951), Groman (1953) and Matsuda & Barksdale (1967) established the concept of phage conversion in relation to virulence in *Corynebacterium diphtheriae* in showing that toxin production is determined by the presence of a phage. Similarly, Zabriskie (1964) has shown that the erythrogenic toxin of Group A streptococci that cause scarlet fever is due to temperate bacteriophages.

This work was performed to demonstrate and classify temperate bacteriophage of *S. equi*, as the initial step of an investigation into the possible role of bacteriophage in virulence of the organism and its possible value in improved vaccine production.

**METHODS**

*Media.* The media used for bacterial and phage growth were modifications of Friend & Slade’s (1966) MM media consisting of BBL brain heart infusion agar (BHIA) supplemented with 0.2% (w/v) yeast extract, 10 µg/ml of DL tryptophan and 10⁻⁴ M-CaCl₂. Soft agar (SA) used in phage plating contained brain heart infusion broth (BHIB), 0.75% (w/v) agar, 10⁻⁴ M-CaCl₂, and 1.25% (w/v) glucose. MM medium was also used in broths,
Difco BHIB being substituted for BHIA. Phage dilutions were made in BHIB. Strains were also propagated on blood agar plates made from Gibco tryptose phosphate broth without dextrose, 1.5% (w/v) agar, and 5% (v/v) defibrinated sheep blood. For morphology tests, powdered hyaluronidase (300 units/mg) was obtained from the Nutritional Biochemical Corporation, Cleveland, and was added to blood agar plates at a concentration of 10 mg % (w/v), as described by Wilson (1959). All incubations were at 37 °C in a Wedco CO2 incubator (5% CO2).

Strains. Streptococcus equi strains e9, e10a, e10b, e11, e12, e22, e24, e27, e29, e32, and e34 were isolated from submaxillary lymph node abscesses in cases of strangles in New York State and New Jersey; strain e22 was cultured from the hock joint of a horse with metastatic strangles; and e21 was cultured from a nasal swab of a pony. Strain e13 was lyophilized in the culture collection of the Department of Veterinary Microbiology, Cornell University, and its origin was unknown. Strains were identified as S. equi both by capillary precipitation with antibody to group C streptococcal antigen, and biochemically by production of acid from salicin, but not from lactose, sorbitol or trehalose.

A strain of Bacillus subtilis 168 (CU184) was provided by Dr S. A. Zahler. Temperate bacteriophages were obtained by spontaneous release from overnight broth cultures of S. equi (see below). Attempts to induce bacteriophage from strains e21 and e23 with Mitomycin C were made using the method of Parsons et al. (1972).

Preparation of phage stocks. Overnight broth cultures were centrifuged for 30 min at 2000g, and then filtered through an HA membrane filter (0.45 μm pore size; Millipore Corporation, Bedford, Massachusetts).

Agar layer method of phage assay. A modification of the soft agar (SA) overlay technique described by Adams (1959) was used to identify and measure phage lysate titres. Indicator strains were grown overnight in broth, transferred (10%, v/v, inoculum) to fresh broth and incubated for 2 h before being plated with SA. Lysates were spotted on lawns; positive lysates were titred by serial dilutions.

Plaque purification and preparation of high titre phage stocks. Plaque purification was performed according to Adams (1959), as was the plate method for preparing high titre phage stocks. Lysates of at least 10⁸ p.f.u./ml were recovered.

Phage sensitivities for determination of different bacteriophage. To eliminate strain variation in sensitivity testing, lysogens of strain e21 were isolated for each phage. These strains were tested for lysogeny by (overnight) passage through broths containing homologous antisera and re-isolation of the same phages, as confirmed by serum neutralization studies. Lysogenic derivatives of strain e21 were used as indicator strains for the purified lysates (10⁸ p.f.u./ml) of the different phage isolates which were spotted on seeded lawns.

Adsorption rates. Adsorption experiments were carried out according to Adams (1959), as modified by Kjems (1958a). Single infection conditions existed, the ratio of bacterial chains to p.f.u.s being approx. 1000 : 1.

Serology. Guinea pigs were inoculated in opposite footpads with 0.2 ml of a 1 : 1 mixture of phage and Freund's adjuvant. After 2 weeks, 6 subcutaneous injections, each of 0.5 ml of phage lysate, were given during the next 2 weeks. Phage titres ranged from 1 to 5 x 10⁸ p.f.u./ml. Guinea pigs for production of negative control antisera were similarly inoculated with BHIB. Animals were bled 1 week after the last inoculation.

Antiphage sera (1 : 60 dilution) were assayed as outlined by Adams (1959). Incubation was at 37 °C in a Universal Oscillating shaker.

Electron microscopy. A lysate of P9 was centrifuged at 40000g for 90 min. Pellets were suspended in 0.25 ml of 1% (w/v) ammonium acetate, resulting in approx. tenfold con-
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Fig. 1. Electron micrograph of *S. equi* bacteriophage (P9) grown on *S. equi* (e21).

centration of the original lysate. The suspensions were negatively stained on Formvar coated copper grids with neutral 3 % (w/v) potassium phosphotungstate (PTA), and were examined in a Phillips electron microscope (Model 201) operated at 60 kV.

RESULTS

Bacteriophage isolation

Phage were isolated from 12 strains of *Streptococcus equi* and named according to their origin. Thus phage P9 was isolated from *S. equi* strain e9. Strains e21 and e23 were sensitive to all 12 phages. These two indicators released no detectable phage even after attempted induction with Mitomycin C.

Colonial morphology

After 24 h incubation on blood agar plates, strains e21 and e23 were mucoid; all other strains were matt, although mucoid and glossy variants could be isolated. Colonial morphology was verified by growth on blood agar plates containing hyaluronidase, which prevented hyaluronic acid capsule formation and caused both mucoid and matt colonies to change to glossy (Wilson, 1959).

Lysogenic derivatives of strain e21 were invariably matt after 24 h incubation. The designation e21m(P9) refers to a lysogenic derivative of strain e21 carrying phage P9.

Plaque morphology

On sensitive strain e21, all phage produced plaques with a clear area 2 mm in diam., surrounded by a more turbid halo about 4 to 5 mm in diam. On lysogenic derivatives of strain e21, there was a similar sized plaque, but the halo was nearly absent.
Table 1. Sensitivity of 12 lysogenic derivatives of S. equi (e21) to 12 bacteriophage isolated from strains of S. equi

<table>
<thead>
<tr>
<th>Lysogenic derivative of S. equi*</th>
<th>P9</th>
<th>P10a</th>
<th>P10b</th>
<th>P12</th>
<th>P22</th>
<th>P24</th>
<th>P27</th>
<th>P34</th>
<th>P11</th>
<th>P13</th>
<th>P29</th>
<th>P32</th>
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<td>e21m(P9)</td>
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<td>e21m(P10a)</td>
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<td>e21m(P10b)</td>
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<td>e21m(P24)</td>
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<td>e21m(P27)</td>
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<td>e21m(P34)</td>
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<td>e21m(P11)</td>
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<td>e21m(P13)</td>
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<td>e21m(P29)</td>
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<td>e21m(P32)</td>
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* Lysogenic derivatives were obtained by harvesting viable S. equi (e21) from plaques produced by each of the 12 bacteriophage.
† +, sensitive to phage lysate; -, no sensitivity.

Phage morphology

The S. equi phages observed conformed to Bradley's (1967) class B bacteriophages, having long, non-contractile tails. The relatively small hexagonal heads appeared to have octahedral symmetry (Fig. 1).

Phage sensitivities

When the twelve lysogenic derivatives of strain e21, each carrying a different S. equi bacteriophage, were cross-tested for sensitivity to the twelve phages, two major groups emerged (Table 1).

Serology

As shown in Fig. 2, the antiserum against P9 neutralized phages P9, P10a, P10b, P12, P22, P24, P27, and P34 but not P11, P13, P29, and P32. Fig. 3 shows that antiserum to P13 neutralized phages P11 and P13 completely, P29 and P32 weakly, and the other eight phage not at all.

Adsorption of phage P10b

Phage P10b adsorbed equally well to strains e21 and e21m(P11), but did not adsorb to a strain of Bacillus subtilis 168 (Table 2).

DISCUSSION

The current investigation has shown that Streptococcus equi bacteriophage can be isolated with ease. Growth in phage antisera has established the lysogenic nature of this host-phage relationship, which also confers immunity to superinfection.

Adaptation of all of the phages to a common host (strain e21) eliminated potential difficulties such as phage restriction by bacterial nucleases (Glover, 1972) and thereby facilitated phage comparisons.

Based on sensitivity testing and serological relationships, the twelve phage were assigned
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Fig. 2. Neutralization of *S. equi* bacteriophage by antiserum to bacteriophage P9. A 1:60 dilution of antiserum was used for each neutralization attempt. Percent viable bacteriophage at each time was calculated from the number of bacteriophage at t₀. ●——●, P11, 13, 29, 32; ▲——▲, P12; ▲——▲, P22; ○——○, P10a; △——△, P10a; ■——■, P34; □——□, P24; ■——■, P9; ○——○, P27.

Fig. 3. Neutralization of *S. equi* bacteriophage by antiserum to bacteriophage P13. A 1:60 dilution of antiserum was used for each neutralization attempt. Percent viable bacteriophage at each time was calculated from the number of bacteriophage at t₀. ●——●, P9, 10a, 10b, 12, 22, 24, 27, 34; ○——○, P32; ▲——▲, P29; ■——■, P11; △——△, P13.

Table 2. Adsorption of phage P10b to non-lysogenic (e21) and lysogenic (e21m[P11]) strains of *S. equi* and to *B. subtilis*

<table>
<thead>
<tr>
<th>Strain</th>
<th>% unadsorbed bacteriophage (at t = 5 min)</th>
<th>Adsorption constant K (ml/min)</th>
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<tbody>
<tr>
<td>e21</td>
<td>11.25%</td>
<td>2.18 × 10⁻⁹</td>
</tr>
<tr>
<td>e21m(P11)</td>
<td>11.5%</td>
<td>2.06 × 10⁻⁹</td>
</tr>
<tr>
<td><em>B. subtilis</em> 168</td>
<td>100%</td>
<td>0</td>
</tr>
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</table>

to at least two distinct groups. The following phages behaved identically in the tests recorded here: P9, P10a, P10b, P12, P22, P24, P27, and P34; and are thus designated group I.

Phages P29 and P32 were feebly inactivated by antiserum produced against P13, and are co-immune with P13. Phage P13 thus constitutes group IIA, while phages P29 and P32 comprise group IIB.
Phage PII belongs to serological group IIA, but it is hetero-immune with the other members of the group. Cells lysogenic for PII are not sensitive to group I phages, but cells lysogenic for group I phages are lysed by PII. Two possible explanations for this include PII coding for an antigenic change in the cell wall of e21 prohibiting group I phages from adsorbing (Robbins & Uchida, 1962); or PII coding for a restriction enzyme that destroys group I phages (Glover, 1972). The fact that P1ob (and presumably other group I phages) adsorbs to e21m(PI1) and e21 at similar rates indicates the latter possibility is most probable. Phage PII constitutes group IIC.

After 24 h incubation, S. equi phages are capable of changing the mucoid strain e21 to matt. However, it is not yet clear what S. equi virulence change (if any) accompanies this morphological change.

Colonial morphology in this work has been interpreted in the light of Wilson's (1959) classic study on Group A streptococci, where he showed that matt colonies after 24 h incubation were mucoid when younger (8 to 12 h). Mucoidness was due to hyaluronic acid encapsulation, a factor believed to be of importance in virulence; and matt colonies contained only disintegrated capsules. Also, he showed that glossiness indicated total lack of capsular formation, and that M antigen production was 'only indirectly and imperfectly related to the colony form'. The matt form has also been taken to indicate the presence of M antigen whose connection with virulence has similarly been debated (Mahaffey, 1962; Woolcock, 1975).

The temperate bacteriophages from S. equi might thus affect virulence via phage-mediated M protein production as suggested by Cleary, Johnson & Wannamaker (1975) for Group A streptococci. Also, one cannot exclude the possibility of finding more than one type of S. equi M protein, each conferring different virulence characteristics, although historically only one serotype of S. equi has been found. This possibility would be consistent with the well documented failures of vaccination against strangles (Mahaffey, 1962; Woolcock, 1974).

Plaque morphology on strain e21 invariably proved to be a small clear area surrounded by a more turbid, wider halo. Kjems (1958b) showed that this was due to phage hyaluronidase activity on host hyaluronic acid, although Fox & Wittner (1965) could demonstrate neither the enzyme nor its induction in association with human group C phage particles. It is possible that S. equi hyaluronidase may be an additional virulence factor.

Sincere thanks are due to Dr S. A. Zahler, Dr M. Kemen, and Dr S. G. Campbell for advice and encouragement, and to Miles Frey for the electron micrograph.

REFERENCES


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