

ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGES OF RUMEN BACTERIUM *Prevotella bryantii* TC1-1

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ABSTRACT

Sampling, inoculation and extraction procedures were elaborated for the isolation of bacteriophages from cattle rumen fluid. Several strains from four different species of rumen bacterial genus *Prevotella* were used as indicator organisms as well as *Escherichia coli*, *Salmonella* sp. and *Yersinia enterocolitica* strains. Only one strain, i.e. *P.bryantii* TC1-1 was successfully infected with filter sterilised rumen fluid inoculum containing rumen bacteriophages. Two plaque morphotypes were observed, both being turbid and rather small but clearly different in size. The preliminary transmission electron microscopy analysis showed that all observed bacteriophages have presumably icosahedral symmetry. The infection trials of other *Prevotella* strains with isolated bacteriophages from the strain TC1-1 were successful only with type strains of species *P.bryanti* and *P.brevis* respectively. Bacteriophage purification and long term storage procedures were elaborated.

Key words: microbiology / bacteriophage / bacteria / isolation / characterization / TEM / rumen

OSAMITEV IN OPIS BAKTERIOFAGOV VAMPNE BAKTERIJE *Prevotella bryantii* TC1-1

IZVLEČEK

Pripravili in opisali smo postopke vzorčenja, okužbe in ekstrakcije za osamitev bakteriofagov iz kravjega vampnega soka. Za indikatorske organizme smo uporabili več sevov iz štirih vrst vampnega bakterijskega rodu *Prevotella* ter seve vrst *Escherichia coli*, *Salmonella* sp. in *Yersinia enterocolitica*. Z vzorcem filtrsko steriliziranega vampnega soka, ki je vseboval vampne bakteriofage, smo uspešno okužili le en indikatorski sev, to je *P.bryantii* TC1-1. Opazili smo dve morfološki obliki plakov, obe motni in dokaj majhni. Začetni pregledi bakteriofagov s presevno elektronsko mikroskopijo so pokazali, da so vsi pregledani bakteriofagi najverjetneje ikozaedrično simetrični. Z bakteriofagi, izoliranimi iz seva *P.bryantii* TC1-1, smo uspešno okužili tudi tipski sev iste vrste in tipski sev vrste *P.brevis*. Preskusi okužbe drugih sevov iz rodu *Prevotella* ter ostalih indikatorskih organizmov so bili neuspešni. Pripravili smo postopke čiščenja in shranjevanja vampnih bakteriofagov.

Ključne besede: mikrobiologija / bakteriofagi / bakterije / osamitev / opis / TEM / vamp

INTRODUCTION

Several reports describing bacteriophages in crude ruminal fluid and a wide range of their morphological diversity have been published to date (Paynter *et al.*, 1969, Ritchie *et al.*, 1970, Klieve and Bauchop, 1988). Large numbers of bacteriophages have been found in the ruminal fluid shortly after feeding (Klieve *et al.*, 1993) and the bacteriophage densities up to $1,6 \times 10^{10} \cdot \text{ml}^{-1}$ of ruminal fluid were reported (Ritchie *et al.*, 1970, Klieve and Swain, 1993). However, the authors pointed out that the bacteriophage numbers alone are not indicative of the bacterial turnover and they concluded that at least lytic ruminal phages are of little importance for ruminal metabolism (Klieve *et al.*, 1989). It was shown by the same authors, however, that 25% of the analysed ruminal bacteria contained chromosomally stable lysogenic prophages and hence ruminal bacteriophages might play an important role in gene transfer phenomena *in vivo* or may be used as vehicles in the genetic engineering experiments, especially for ruminal bacteria where lack of genetic tools is apparent.

Bacteria from the genus *Prevotella* have been shown recently by molecular approaches to represent one of the two dominant bacterial populations and the most abundant Gram negative bacterial genus in the ruminal ecosystem (Withford *et al.*, 1998, Wood *et al.*, 1998, Tajima *et al.*, 1999, Ramšak *et al.*, 2000). Methods that do not demand time consuming and complicated isolation and cultivation steps (Amann *et al.*, 1995) were used in above mentioned studies. Several genes from ruminal *Prevotella* have been sequenced already (Peterka *et al.*, 2000) making one of the four presently recognised ruminal *Prevotella* species (Avguštin *et al.*, 1997) a plausible model organism for genetic manipulation. The knowledge of the genetic mechanisms and the tools that would make genetic engineering and gene transfer experiments in these organisms possible is still very scarce, however (Salyers *et al.*, 2000). Only few plasmids were described from ruminal *Prevotella* (Flint *et al.*, 1988, Avguštin, 1992, Ogata *et al.*, 1996) and only one transposon was successfully introduced in a limited number of ruminal *Prevotella* strains (Salyers *et al.*, 2000). It would be useful therefore, to search for other types of genetic elements, e.g. bacteriophages, that could be used for the mobilisation of genes. Lytic and temperate bacteriophages from ruminal bacterial strains from *P.brevis* have been described (Klieve *et al.*, 1991, Gregg *et al.*, 1994), however, it was shown, that the isolated bacteriophages had only a narrow host range (Klieve *et al.*, 1991). *P.brevis* also seems to be the least attractive species from the genus *Prevotella* to be chosen for a genetic model organisms due to its highly active nonspecific nuclease activity (Avguštin, 1992) and excessive slime production. The host strains for the described bacteriophages were shown also to belong to the genus *Bacteroides* rather than to the genus *Prevotella* on the basis of 16S rRNA sequence analysis (Avguštin *et al.*, 2000).

In this paper we describe the isolation, initiative characterization and the preliminary results of the host range experiments of new bacteriophages, isolated from the ruminal bacterium *P.bryantii* TC1-1.

MATERIAL AND METHODS

The crude rumen fluid obtained from a fistulated black-and-white Holstein cow was used as a source of the bacteriophages. The rumen fluid was obtained as described elsewhere (Ramšak *et al.*, 2000) and than transferred as soon as possible to the anaerobic glove box, where it was filter sterilised through a series of membrane filters (Sartorius) with 0.45 and 0.2 μm pores, respectively. The indicator bacterial strains of rumen origin used in this study were *P.ruminicola* 23 and TC18, *P.bryantii* B14 and TC1-1, *P.brevis* GA33 and *P.albensis* M384. Their characteristics and origins were described elsewhere (Avguštin *et al.*, 1997). They were grown anaerobically on M2 medium (Hobson, 1969) under O_2 free CO_2 as described in Bryant's

modifications of the Hungate's anaerobic technique (Bryant, 1972) and on solid M2 agar medium (2% w/v of agar) or soft M2 medium (0.65% w/v of agar) in an anaerobic glove chamber (Scholzen Technik, Kriens, Switzerland) at 38° C under a mixture of O₂ free CO₂ and H₂ (19:1). Other indicator strains of nonruminal origin were strains from species *Escherichia coli*, *Salmonella* sp. and *Yersinia enterocolitica* and grown aerobically on LB medium. The bacteriophage plaques were observed and photographed under a stereo microscope Leica MZ8 using the Kodak color photograph film (asa 100). The plaques were eluted in 1 ml of the dilution medium (M2 without rumen fluid) and stored in elution buffer A (dilution medium, 10 mM CaCl₂). Preparations for transmission electron microscopy (TEM) were made by grinding bacteriophage plaque in 0.1 M phosphate buffer (PBS) pH 7, incubating the sample on formvar- and carbon-coated grid for 5 min, rinsing with distilled water, and stained with 1% uranyl acetate. Grids were examined by Philips CM.100 transmission electron microscope. Images were recorded by Bioscan CCD camera Gatan, using DigitalMicrograph software.

RESULTS AND DISCUSSION

Filter sterilised rumen fluid was used as the inoculum of the indicator bacterial strains. The bacterial cultures chosen as the indicator organisms were the strains of the ruminal species *P.ruminicola*, *P.brevis*, *P.bryantii* and *P.albensis* and they were grown over night in M2 medium in Hungate tubes and than transferred to the anaerobic glove box. 0.75 ml of each bacterial culture was mixed with 4 ml of soft M2 agar and 0.2 ml of filter sterilised rumen fluid. The mixtures were poured into the petri dishes and allowed to solidify. Following the incubation of the infected bacterial strains over night in the anaerobic glove box, the petri dishes were examined for the appearance of plaques. The plaques were detected only in the case of the indicator organisms *P.bryantii* TC1-1 (Fig. 1) and were found to be rather small (diameter less than 1 mm), and turbid. The discovered plaques could be grouped into at least two groups according to the diameter (i.e. small and large). The plaques were found to become progressively turbid with the prolonged incubation.

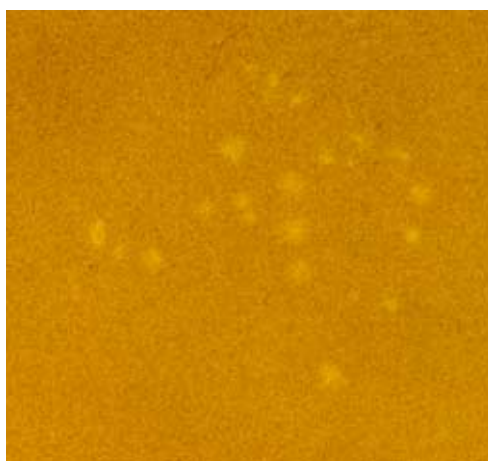


Figure 1. Stereo microscopy photograph showing two morphotypes of bacteriophage plaques on a lawn of bacterial cells *P.bryantii* TC1-1.

Slika 1. S stereo mikroskopsko lupo narejen fotografski posnetek, ki prikazuje oba morfotipa bakteriofagnih plakov na trati indikatorskega organizma *P.bryantii* TC1-1.

The observed plaques were extracted from the solidified medium and eluted into several different buffers suitable for bacteriophage storage. The elution buffer A was found to be the

most suitable, making possible the subsequent infection of the indicator organisms with the eluate. The isolated bacteriophages were also stored in the elution buffer A, covered with few drops of chloroform, at 4° C. So prepared and stored bacteriophages remained infective for at least two months.

Using 200 µl of the filter sterilised eluate containing bacteriophages, the indicator bacterial organisms were infected again as described above. This time, bacteriophage containing plaques of similar morphological properties were observed also on indicator organisms *P.bryantii* B₁₄ and *P.brevis* GA33. Indicator organisms that are not of ruminal origin i.e. *E. coli*, *Salmonella* sp. and *Y.enterocolitica* were also tested, however, no plaques were observed.

The numbers of observed plaques are given in Table 1. The maximal numbers are still low and for further manipulations it would be of utmost importance to optimise the elution, storage and infection conditions in order to raise the number of plaques on the lawn of indicator bacteria. The isolated bacteriophages obviously express a certain amount of host specificity, however, they are also able to infect only a moderately related strain from the ruminal species *P.brevis* (GA33), which was recently described as the member of the true ruminal *Prevotella* supercluster (Ramšak *et al.*, 2000). The originally infected strain TC1-1 is classified as a member of the species *P.bryantii*, which is also a member of the true *Prevotella* supercluster, but represent a rather distant line of descent within the supercluster and is fairly distant to the *P.brevis*. It is interesting that both species belong to extensive slime producers, which is until now of an unknown structure and chemical composition. It seems that the slime does not cover the surface receptors and thus prevent the bacteriophage adsorption. The effect of the incubation temperature on the efficiency of the inoculation and bacteriophage production and release was investigated by lowering the incubation temperature gradually from 38° C to 27° C within 16 hrs, too. The growth of the indicator bacterial organisms seemed to be unaffected, however, the plaques were not observed and we can conclude that the infection or possibly some other stage of the whole process is temperature sensitive.

Table 1. The number of observed bacteriophage plaques on M2 agar plates containing different indicator organisms

Preglednica 1. Število opaženih bakteriofagnih plakov na M2 agarških ploščah z različnimi indikatorskimi organizmi

| Indicator organism Indikatorski organizem | Maximal number of plaques observed on one M2 soft agar plate containing the indicator organism Največje število opaženih plakov na M2 mehkih agarških ploščah z indikatorskim organizmom |
|--|---|
| <i>P.bryantii</i> TC1-1 | < 48 |
| <i>P.bryantii</i> B ₁₄ | 3 |
| <i>P.brevis</i> GA33 | 4 |
| <i>P.ruminicola</i> 23 | 0 |
| <i>P.ruminicola</i> TC18 | 0 |
| <i>P.albensis</i> M384 | 0 |
| <i>E.coli</i> | 0 |
| <i>Salmonella</i> sp. | 0 |
| <i>Y.enterocolitica</i> | 0 |

The eluted bacteriophages were examined also by transmission electron microscopy. The bacteriophages were found in samples obtained directly from plaques observed on the bacterial lawn as well as in the samples of the inoculated indicator organisms grown in liquid M2 medium.

The preliminary examination showed that the bacteriophages may be of icosahedral symmetry with the average diameter of approximately 120 nm. No tails were observed in this initial experiments, however, they may be broken off due to rough physical treatment of the samples. The bacteriophages attached to lateral sites of the collapsed *P.bryantii* TC1-1 cell, and harboured within the cell are shown on Fig. 2 (A and B).

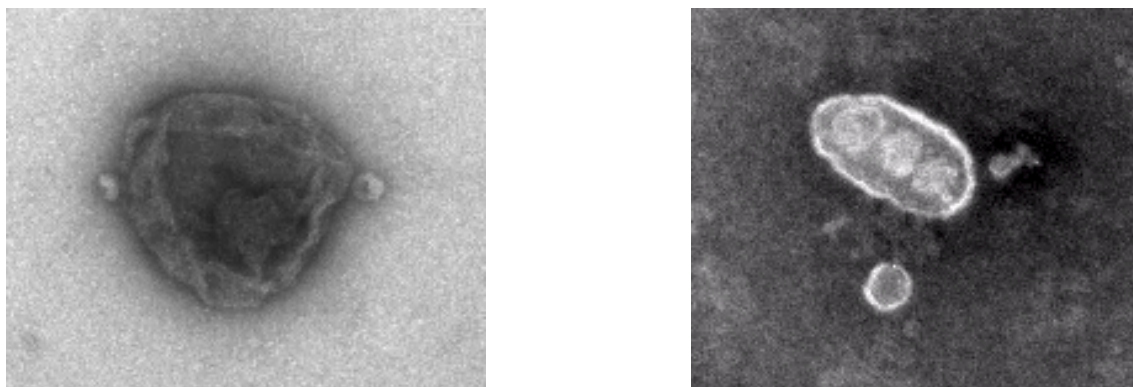


Figure 2. A: transmission electron microscopy (TEM) photograph showing a collapsed *P.bryantii* TC1-1 cell with two bacteriophages on lateral sides of the cell. B: TEM showing a transverse section of a *P.bryantii* TC1-1 cell harbouring three viral particles and an additional particle outside of the cell.

Slika 2. A: s presevnim elektronskim mikroskopom (TEM) narejen fotografski posnetek sesedle se celice *P.bryantii* TC1-1 z bakteriofagoma na obeh straneh celice. B: TEM fotografski posnetek prečnega prereza celice *P.bryantii* TC1-1, s tremi bakteriofagi in enim bakteriofagom izven celice.

The bacteriophages of ruminal bacteria from the genus *Prevotella* are increasingly interesting due to the lack of other mobile genetic elements, that would make gene transfer experiments involving this important gut bacteria possible. The observation that up to 70% of bacterial isolates from the rumen sample, grown on nonselective medium, can be identified as members of genus *Prevotella* (VanGylswyk, 1990), was recently confirmed also by molecular ribotyping of specific PCR products (Wood *et al.*, 1998). It became apparent through such molecular studies, that ruminal prevotelas indeed represent one of the most important bacterial populations within the rumen and that they might well be the candidates for the gene transfer and genetic manipulation model systems (Peterka *et al.*, 2000). However, a successful broad range gene transfer system must be elaborated first, but the apparent lack of plasmids and transposons in prevotella strains makes this task a great deal harder. Bacteriophages are one of the possible alternatives. As have been reasoned before (Klieve *et al.*, 1989), at least the lytic bacteriophages do not seem to be of great importance for the bacterial turnover in the rumen and are also rather unsuitable without extensive manipulation for genetic experiments. The bacteriophages of the strain *P.bryantii* TC1-1 however show some indications, that they might be of the temperate nature. In this case they could likely be exploited as genetic vehicles and used straightforward or incorporated into some other, larger mobile element, that would allow the gene transfer to occur. The fact that they were successfully used for the infection of only moderately related bacterium *P.brevis* GA33 underlines their potential.

POVZETEK

Opisali smo postopke vzorčenja, okužbe in osamitve bakteriofagov iz kravjega vampnega soka. Z vzorcem filtrsko steriliziranega vampnega soka, ki je vseboval vampne bakteriofage, smo uspešno okužili le en indikatorski sev, to je *P.bryantii* TC1-1. Opazili smo dve morfološki obliki plakov, obe motni in dokaj majhni, s podaljševanjem inkubacijskega časa pa so plaki postajali vedno bolj motni. Zniževanje inkubacijske temperature je preprečilo pojavljanje plakov na bakterijski ruši, čeprav so indikatorski organizmi na videz rasli enako dobro kot pri 38° C. Bakteriofage smo uspešno izolirali iz plakov, ki so nastali na poltrdnem M2 gojišču z zraslim indikatorskim organizmom, sevom *P.bryantii* TC1-1, in z njimi okužili tudi tipski sev iste vrste in tipski sev vrste *P.brevis*. Obe vrsti sodita v enega najpomembnejših vampnih bakterijskih rodov, t.j. *Prevotella*, kar je pokazala tudi vrsta v zadnjem času opravljenih molekularno bioloških preiskav vampnega mikrobnega ekosistema. Poskusi okužbe drugih sevov iz rodu *Prevotella* ter ostalih indikatorskih organizmov so bili neuspešni. Začetni pregledi bakteriofagov s presevno elektronsko mikroskopijo so pokazali, da so vsi pregledani bakteriofagi najverjetneje ikozaedrično simetrični. Dosedanji rezultati dopuščajo možnost, da so opisani bakteriofagi temperentni. Takšni mobilni genetski elementi so še posebno zanimivi zaradi po eni strani primernosti vampnih prevotel za genetsko manipulacijo in po drugi strani očitnega pomanjkanja primernih genetskih orodij potrebnih za izvajanje takšnih postopkov.

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