Characterization of ϕ GA1, an inducible phage particle from *Brevibacterium flavum*

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(Received 26 May 1989; revised 25 September 1989; accepted 30 October 1989)

Eighteen related strains of *Arthrobacter*, *Brevibacterium* and *Corynebacterium* were used as indicator strains in an attempt to isolate corynephages from a large number of soils and from waste-water samples. Although no phages capable of producing plaques were isolated, one of the indicator strains used, *Brevibacterium flavum* ATCC 14067, was lysogenic for the inducible phage ϕ GA1. This phage was not observed to form plaques on any of the strains tested. ϕ GA1 is of B1-morphotype with a linear double-stranded DNA genome of 48.1 kb with cohesive ends; a restriction map is presented.

Introduction

Within the large heterogeneous group of coryneform bacteria a number of strains of *Corynebacterium* and *Brevibacterium* are extensively used for the industrial production of amino acids or nucleotides (Fukui *et al.*, 1982; Kinoshita, 1985). According to chemotaxonomic and physiological data, however, these bacteria form a cluster of closely related organisms, including some strains of *Arthrobacter*, which was called the glutamicum-ammoniogenes-cluster (Grund, 1987). For further characterization of this group of bacteria, probably representing only a single genus, phage typing (Korn *et al.*, 1978; Ackerman & Dubow, 1987) seemed to be an adequate approach. Several phages – for example for *Corynebacterium glutamicum* (Trautwetter *et al.*, 1987a), *C. lilium* (Trautwetter *et al.*, 1987b) or *Brevibacterium flavum* (Haroutunian *et al.*, 1986; Patek *et al.*, 1988) – have been described which usually were isolated from the surroundings of amino acid producing fermentation plants. Due to the considerable industrial interest in their host strains these phages are not readily available for taxonomic investigations. Thus we tried to isolate new phages from various natural habitats, although without success.

New phages, however, regularly appear in industrial

fermentations, causing considerable problems in maintaining amino acid production (Hongo *et al.*, 1972; Kashima *et al.*, 1976; Kato *et al.*, 1987). On the other hand, industrial micro-organisms are often natural sources for phage contamination due to lysogeny (Duchrow *et al.*, 1985; Sanders, 1987). Consequently, we tried to isolate temperate phages from potentially lysogenic 'amino acid producing' corynebacteria; here we describe the induction and physical properties of ϕ GA1 from *B. flavum* which had been reported to be lysogenic without further characterization of the respective phage (Momose *et al.*, 1976).

Methods

Bacteria and growth media. The following bacterial strains were used: *Arthrobacter albidus* DSM 20128, *Brevibacterium ammoniogenes* DSM 20305, 20306, ATCC 6872, *B. divaricatum* DSM 20297, ATCC 14020, 21792, *B. flavum* ATCC 14067, *B. lactofermentum* DSM 20412, 1412, ATCC 21086, *B. stationis* DSM 20302, *Corynebacterium callunae* DSM 20147, *C. glutamicum* DSM 20300, ATCC 21287, ATCC 21491, *C. lilium* DSM 20137, *C. spec.* ATCC 21857. ATCC strains were obtained from C. Blanco (Laboratoire de Microbiologie, INSA, 69621 Villeurbanne, France), DSM strains directly from the DSM (German Collection of Micro-organisms, Braunschweig). Bacteria were usually grown at 30 °C with LB (Miller, 1972), GPHF (Schneider *et al.*, 1987) or GPHF* (GPHF + 1 g NaCl l⁻¹, 1 g MgCl₂ l⁻¹, 0.05 g MnCl₂ l⁻¹) as broth or agar.

Isolation of bacteriophages. Twenty-eight soil samples from different locations in Germany, Spain, Brazil and Japan as well as samples from eight waste-water treatment plants (urban and meat- and milk-processing industry) were investigated for phages by direct plating and by an enrichment procedure (Adams, 1959), using the strains of amino

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Abbreviation: MC, mitomycin C.

acid producing corynebacteria listed above. For enrichment and plating LB and GPHF supplemented with different combinations of Mg^{2+} and Ca^{2+} (Hopwood *et al.*, 1985) were tried. Plates were incubated at 20 °C, 28 °C and 37 °C until a dense bacterial lawn appeared.

UV induction. Exponentially growing bacteria (OD_{578} 0.5; GPHF* broth culture) were collected by centrifugation, washed, resuspended in 10 ml water, and irradiated for 15 to 90 s (254 nm; 270 $\mu W\ cm^{-2}$; 30 cm distance). After 3 to 5 h cultivation at 32 °C bacteria were removed by centrifugation, the supernatant was filtered (membrane filter, 0.2 μm pore size) and used as a potential 'phage lysate'.

Mitomycin C (MC) induction. Exponentially growing bacteria were incubated with 0.01, 0.1, 0.5, 1.0 or 2.0 mg MC ml^{-1} . After 15 min, cells were collected by centrifugation, washed twice with GPHF*, resuspended in 25 ml GPHF* and cultured for 3–5 h. Finally, the bacteria were removed by centrifugation and the filtered supernatant was used as a 'phage lysate'.

Phage curing. Exponentially growing bacteria were incubated with 0.02 mg ethidium bromide ml^{-1} . After 30 min cells were washed and grown as after MC induction and finally plated on GPHF agar without divalent cations but supplemented with 12 g trisodium citrate l^{-1} to obtain single colonies. This medium was used in order to prevent relysogenization of potentially cured bacteria. Overnight cultures (1 ml) were grown from single colonies and then used to inoculate 150 μl cultures in microtitre plates (96 wells per plate). In the early exponential growth phase (OD_{620} 0.2–0.3) 25 μl 'phage lysate' was added, resulting in a m.o.i. of approximately 1–2. (The m.o.i. could only be evaluated by calculation from the amount of phage DNA prepared from the 'phage lysate'.) The OD_{620} of the culture was measured with a Titertek Multiskan MC photometer.

Handling of DNA. DNA preparation, manipulation and hybridization with biotinylated probes was done as described by Schneider *et al.* (1987) and Schneider & Kutzner (1989).

Physical mapping of the $\phi GA1$ genome. Due to the lack of sufficient amounts of DNA from free phages, total DNA was prepared from UV-induced cultures of *B. flavum* ATCC 14067 120 min after induction (see Fig. 2). Samples (1–1.5 μg) of the total DNA were digested with various combinations of restriction enzymes; after agarose gel electrophoresis and transfer to nitrocellulose filters, the fragments were hybridized with biotinylated DNA of the free phage (see Fig. 4) or with pHS1 and pHS2. These are recombinants of *Bam*HI digested pUC13 with the *Bgl*II-C fragment (pHS1) and the *Bgl*II-D + I fragments (pHS2) of $\phi GA1$. The resulting restriction map was supported by selected digests of DNA from free phages.

Electron microscopy. From 1:51 of phage lysate obtained by the induction methods described above, phages were collected by high-speed centrifugation (90 min, 25000 r.p.m., 70 Ti-rotor, Beckman Ultracentrifuge L8-M). The resulting pellet was resuspended in 20 ml 0.1 M-ammonium acetate (pH 7.5) and filtered through a membrane filter (0.2 μm pore size); phage were washed twice and pelleted by low- and high-speed centrifugations. The final phage-pellet was resuspended in 5 ml 0.01 M-ammonium acetate (pH 7.5) and phage were collected by high-speed centrifugation. Finally, this pellet was resuspended in 100 μl 0.01 M-ammonium acetate (pH 7.5) and used for electron microscopy. For negative staining phosphotungstic acid (PTA; 2%, w/v in 2%, w/v ammonium acetate, pH 6.8) was mixed with the phage suspension (1:1) and applied onto a Formvar-coated copper grid (300 mesh). The liquid was carefully removed by absorbing it into a piece of filter paper; the remaining film was air-dried before observation in a Zeiss EM-109 electron microscope at 80 kV acceleration voltage.

Results

Isolation of phages

In spite of the numerous combinations of samples, indicator strains, media and incubation temperatures no plaques were observed. In addition, none of the 18 strains, all of which had been used in UV- and MC-induction experiments, could be demonstrated to carry an inducible prophage by the formation of plaques, although at least one of these strains was later shown to be lysogenic.

Lysogeny of *B. flavum* ATCC 14067

After induction of a large culture of *B. flavum* ATCC 14067 (1:51) with UV or MC, but not without induction, and subsequent concentration by ultracentrifugation a small amount of phage was detected. (i) By electron microscopy, phage particles were observed which had a morphology similar to that of other bacteriophages of amino acid producing corynebacteria (Kato *et al.*, 1984; Haroutunian *et al.*, 1986; Trautwetter *et al.*, 1987b; Patek *et al.*, 1988): they were of morphotype B1 (Ackermann & Eisenstark, 1974) with an isometric head 50 nm in diameter and a tail 350 nm long (not shown). (ii) Phage DNA preparation yielded 1–5 μg DNA per litre of culture induced. Digestion with several restriction endonucleases showed that this represented a DNA clone with a size of about 48 kb, which only appeared in induced cultures. More detailed studies (see below) confirmed that this DNA was the genome of the so-far unknown phage $\phi GA1$.

Search for an indicator strain by phage curing

After ethidium bromide treatment, 750 single colonies were tested for $\phi GA1$ sensitivity by the microtitre-plate method. Whereas most of these strains showed no differences in growth with or without phage suspension, four strains were phage-sensitive, showing lysis after a latent period of 2.5 h (Fig. 1). Hybridization of total DNA prepared from the phage-sensitive cultures (without addition of 'phage lysate') with the $\phi GA1$ -specific probes pHS1 and pHS2 (see Fig. 3) showed, however, that these strains were not cured of the $\phi GA1$ prophage (experiment similar to that shown in Fig. 2; data not shown). When these phage-sensitive strains were used as indicators in plate tests with $\phi GA1$, no plaques were obtained. Furthermore, the phage sensitivity was a very unstable trait which was lost when the culture was refrigerated for 1–2 d or after 1–2 subcultivations. So far, transient phage sensitivity cannot be explained and no indicator strain is yet known for $\phi GA1$.

Fig. 1. Effect of 'phage lysate' on ethidium bromide treated host strains. *B. flavum* was treated with ethidium bromide and subsequently tested for sensitivity to a *B. flavum* 'phage lysate' prepared by UV induction as described in the text. The figure depicts one of the four out of 750 single colonies tested which exhibited sensitivity to the phage lysate added to exponentially growing (m.o.i. 1–2) cultures as indicated by the arrow. ●, Growth without addition of phage lysate; □, strain sensitive to addition of phage lysate, revealing a latent period of 2.5 h; ■, strain not sensitive to phage lysate.

Fig. 2. Induction of ϕ GA1. Total DNA of *B. flavum* was prepared (lane 1) 0, (lane 2) 50, (lane 3) 110, (lane 4) 170 and (lane 5) 230 min after UV-induction: (a) 1 μ g of this DNA was digested with *Bgl*II, separated by agarose gel electrophoresis and (b) hybridized with biotinylated pHS1 (see also Fig. 3). The right-hand lane in both (a) and (b) contains molecular markers (*Hind*III-digested phage λ). An obvious increase of phage DNA is observed at 110 min, correlating very well with the increase in phage DNA that occurs in the supernatant 140 min after induction (data not shown).

Latent period

The experiments described above had indicated a latent period of ϕ GA1 of 2.5 h. This was supported by phage-DNA preparations from different culture supernatants;

phage DNA could only be obtained 140 min or more after induction, either by UV or by MC (data not shown). Similarly, an obvious increase of intracellular phage-DNA was observed reproducibly 110 min after induction (Fig. 2).

Fig. 3. Restriction map of ϕ GA1. The seven restriction endonucleases used were as follows: A, *Apa*I; B, *Bgl*II; K, *Kpn*I; S, *Sac*I; Sc, *Sca*I; Sm, *Sma*I; X, *Xba*I. The *Bgl*II fragments cloned in pHS1 and pHS2 are indicated by the names of the recombinant plasmids. The last line of the diagram shows the *Bgl*II + *Sac*I fragments discussed in Fig. 4.

Physical characterization of the ϕ GA1-genome

The ϕ GA1 genome was mapped by analysis with seven restriction endonucleases which cut the DNA up to ten times (Fig. 3). Mapping was supported by hybridization with the phage-specific probes pHS1 and pHS2 (Fig. 3). When total DNA from induced cultures was used for mapping by hybridization with biotinylated ϕ GA1 DNA, a circular restriction map was obtained. Attempts to support this finding by selected single and double digests of the DNA of the free phage (which could only be obtained in very low amounts) clearly demonstrated that the phage genome is linear (48.1 kb) with cohesive ends (Fig. 4). Induced cells presumably contain relatively large amounts of replicative phage DNA which is ligated at the cohesive ends.

Discussion

In this paper we describe ϕ GA1 as an inducible phage from *B. flavum* ATCC 14067; tailed virions of the B1

morphotype, containing a linear double-stranded DNA genome of 48.1 kb with cohesive ends, are released 140 min after induction with UV or MC. In spite of these properties, typical for tailed phages (*Siphoviridae*), ϕ GA1 was not observed to form plaques on any of the 18

Fig. 4. Total DNA (1.5 μ g) from an induced culture of *B. flavum* was digested with *Bgl*II + *Sac*I (lane 1) and hybridized with biotinylated DNA from free phage particles (lane 2). The resulting fragment pattern was compared with phage DNA restricted with the same enzymes (lane 3). The fragments are labelled as in the last line in Fig. 3. Lane 3: as the DNA was not heat-treated prior to agarose gel electrophoresis, part of the cohesive end-fragments stick together ($E + I = \text{cos}$), whereas the rest can be observed as the single fragments E and I; together with E', the E fragment ran as a double band on this gel. Lane 2: the two single cos fragments E and I are missing; the total DNA after induction probably includes phage DNA mainly in replicative forms (circular or concatemeric) with both ends ligated.

strains tested as potential indicators. Kato *et al.* (1987) and Patek *et al.* (1985) discussed similar findings: phage particles containing DNA were observed after induction with MC of different amino acid producing corynebacteria. No lytic effects could be demonstrated with these particles which were thus called 'defective phages' or 'phage-like particles'. As no restriction maps or other comparable features of these particles are available, it is not known whether they are related to ϕ GA1. Furthermore, more elaborate tests than plaque formation (such as lysogenization with a genetically marked derivative of ϕ GA1) are necessary to show whether ϕ GA1 is able to complete its life-cycle after induction from a temperate state by infection of a new host cell, or whether it is also to be regarded as defective.

We thank Dr C. Blanco (Villeurbanne) for supplying several bacterial strains, Dr D. Kramer (Darmstadt) for his help with the electron microscope studies, A. Lieke (Darmstadt) for excellent photographic assistance, and Dr E. Grund (Bielefeld) for his expertise on the taxonomy of the coryneform bacteria. H.S. was supported by an undergraduate grant from the Hermann Schlosser Stiftung which is gratefully appreciated.

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