Characterization of the Corynebacteriophage CG33

By ANNIE TRAUTWETTER,¹* CARLOS BLANCO¹ AND SYLVIE BONNASSIE²

¹ Laboratoire de Microbiologie – Bât. 406, INSA, 20 avenue Albert Einstein, 69621 Villeurbanne Cedex, France

² Centre de Recherche de Biochimie et de Génétique cellulaires, CNRS, 118 route de Narbonne, 31062 Toulouse Cedex, France

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Bacteriophage CG33 was isolated from a strain of *Corynebacterium glutamicum* that had become contaminated during an industrial fermentation. CG33 was assigned to Bradley's group B since it had a polyhedral head 40 nm wide and a short non-contractile and striated tail 78 nm long. Adsorption to its host, *C. glutamicum* ATCC 13287, was enhanced in the presence of Ca²⁺. The latent period was 18 min at 34 °C; the burst size was 16 p.f.u. ml⁻¹. CG33 also formed plaques on *C. lilium* ATCC 15990 but at a low frequency. Its genome consisted of a linear double stranded DNA molecule of 13.4 kb with cohesive ends. A restriction map of the genome was obtained by using various endonucleases.

INTRODUCTION

Corynebacteria are pleomorphic asporogenous Gram-positive bacteria, and are widely distributed in nature (Bousfield & Gallely, 1978). Some are plant and animal pathogens (Vidaver, 1982; Carlson & Vidaver, 1982); other, non-pathogenic, strains are used in the production of amino acids and nucleotides (Yamada *et al.*, 1972; Yoshinaga & Nakamori, 1983) and for bioconversions (Fukui & Tanaka, 1982).

To date, little information is available concerning the genetics of the coryneform bacteria. This is perhaps a result of the belated interest in these organisms. Moreover, the transformation of Gram-positive organisms has proven rather difficult and this has slowed progress, (Sanchez *et al.*, 1986; Smith *et al.*, 1986; Katsumata *et al.*, 1984; Ozaki *et al.*, 1984). A few plasmid cloning vectors have been designed (Miwa *et al.*, 1985; Sandoval *et al.*, 1984; Yoshihama *et al.*, 1985; Martin *et al.*, 1987), but their usefulness for DNA transfer is limited by (i) the low yield obtained with the protoplast transformation technique (Martin *et al.*, 1987) and (ii) the absence of restriction-defective strains.

The existence of phages in corynebacteria has been known for many years (Hongo *et al.*, 1972; Kato *et al.*, 1984); a number of them are active on *Corynebacterium glutamicum* (Ozaki *et al.*, 1984; Patek *et al.*, 1985).

A cosmid vector that can be packaged *in vivo* and transduced through phage infection has been developed (Miwa *et al.*, 1985), whereas phage cloning vectors have not been described. However, such vectors would be of interest since the transformation of recombinant DNA into recipient cells does not require protoplast regeneration, and is therefore more efficient (Martin *et al.*, 1987). This step might be suitable for the amplification of cloned DNA and its adaptation to the restriction system of the recipient strain.

In order to construct a phage vector, we searched for phages carrying a DNA molecule of small size with few or unique sites for restriction endonucleases. We present in this paper a preliminary study of phage CG33 of *C. glutamicum*, describing its physiological and genetical properties.

Table 1. *Bacterial strains*

Strain	Strain no.
<i>Corynebacterium</i>	
<i>C. glutamicum</i>	ATCC 13287
<i>C. glutamicum</i>	ATCC 21491
<i>C. lilium</i>	ATCC 15990
<i>C. hydrocarboclastum</i>	ATCC 21131
<i>Corynebacterium</i> sp.	ATCC 21857
<i>Brevibacterium</i>	
<i>B. lactofermentum</i>	ATCC 21084
<i>B. flavum</i>	ATCC 14067, ATCC 21127, ATCC 21528
<i>B. divaricatum</i>	ATCC 14020, ATCC 21792
<i>B. ammoniagenes</i>	ATCC 6872
<i>B. helvolum</i>	ATCC 19390
<i>B. ketoglutamicum</i>	ATCC 15587
<i>Brevibacterium</i> sp.	ATCC 21860
<i>B. linens</i>	CNRZ 937, CNRZ 739, NCDO 739, ATCC 9174, ATCC 9175
<i>Arthrobacter</i>	
<i>A. simplex</i>	CCM 1652
<i>A. ramosus</i>	CCM 1646
<i>Arthrobacter</i> sp. SI55	Professor A. Gounot, University of Lyon private collection
<i>A. globiformis</i>	CCM 1650
<i>A. variabilis</i>	CCM 1565

METHODS

Bacterial strains and phage. The coryneform bacterial strains used in this work are listed in Table 1. Phage CG33 was isolated from a *Corynebacterium glutamicum* strain that had become contaminated during an industrial fermentation. The phage was routinely propagated on *C. glutamicum* ATCC 13287.

Media and cultures. Strains were grown in LB medium (Miller, 1972) (5 g NaCl l⁻¹, 5 g yeast extract l⁻¹, 10 g biotrypticase l⁻¹). LB medium supplemented with 5 g NaCl l⁻¹, 2 g glucose l⁻¹ and 10 mM-CaCl₂ was used as the basal medium for phage plating. Cultures were grown with shaking at 26 °C for *Brevibacterium linens* CNRZ 918, 30 °C for *Arthrobacter* species and *B. linens* ATCC 9175 and ATCC 9174, and 34 °C for the other coryneform strains.

Phage titration and lytic spectrum. These were determined by the soft agar overlay method described by Adams (1959). An approximate value was first obtained by spotting phage dilutions on plates seeded with *C. glutamicum*. The precise titre was determined by mixing appropriate amounts of bacteria and phages in soft agar.

Optimum ionic conditions for CG33 adsorption. An early log phase culture (30 ml) of *C. glutamicum* ATCC 13287 was infected with CG33 (m.o.i. 0.01) and incubated in the presence or absence of various concentrations of MgSO₄ or CaCl₂. The adsorption process was followed by removing 1 ml samples at 10 min intervals, and determining the residual free phage after immediate filtration through a Millipore HA (0.45 µm) filter. Adsorption during incubation in LB medium was studied simultaneously.

One-step growth curves. These were determined by the method of Adams (1959). CG33 was added at an m.o.i. of 0.01 to 1 ml of an exponentially growing culture of *C. glutamicum* with 10 mM-CaCl₂. After 15 min of adsorption at 34 °C without shaking, the phage-host mixture was diluted 1000-fold in LB medium. Incubation was continued at the same temperature but with vigorous shaking. Phage production was assayed at intervals of 20 min for 150 min by mixing with *C. glutamicum* cells in soft agar.

Preparation and purification of phage lysate. *C. glutamicum* was grown with shaking to an OD₅₇₀ of 0.3–0.5 and infected with CG33 (m.o.i. 0.02) in the presence of 10 mM-CaCl₂. After adsorption (30 min, 34 °C), the infected culture was diluted 10-fold in LB supplemented with 10 mM-CaCl₂. Incubation was continued under aeration until lysis occurred (7–8 h). After removing the cell debris by centrifugation, phages in the lysate were concentrated by precipitation with 10% (w/v) polyethylene glycol 6000 and 0.5 M-NaCl overnight at 4 °C (Yamamoto & Alberts, 1970) and purified first by step gradient and then by equilibrium CsCl gradient centrifugation according to Maniatis *et al.* (1982).

Electron microscopy. A drop of CG33 purified as described above was placed on a copper grid coated with formvar and stained with 2% (w/v) uranyl acetate for 30–40 s. The microscope used was a Philips EM300.

SDS-PAGE. Purified phage samples containing about 125 µg protein were heated (5 min, 100 °C) in the presence of 2.3% (w/v) SDS and 5% (v/v) β-mercaptoethanol. The denatured proteins were fractionated on 12.5% (w/v) polyacrylamide gels (Laemmli, 1970) and then stained first with Coomassie brilliant blue R (Laemmli, 1970) and then with silver (Oakley *et al.* 1980).

Phage DNA purification. This was done as for bacteriophage λ (Maniatis *et al.*, 1982).

DNA restriction analysis. The digestion of phage DNA with restriction endonucleases was done according to the manufacturer's instructions (Boehringer; Amersham). The restriction DNA fragments were analysed on agarose gels.

RESULTS AND DISCUSSION

Morphology of CG33

When observed under the electron microscope, CG33 looked like phages of group B of Bradley's classification (Bradley, 1967), since it had a polyhedral head 40 nm wide, and a short non-contractile and striated tail 78 nm long and 7 nm wide (Fig. 1). Little information is available concerning phages of coryneform bacteria. To date, the best-studied corynebacteriophages active on the saprophytic strains of corynebacteria are those resulting from phage contaminations that occurred in Japan during industrial fermentation processes involving glutamic-acid-producing bacteria (Hongo *et al.*, 1972). The data collected on 12 phages infecting either *Brevibacterium* or *Corynebacterium* strains established that these phages belonged to Bradley's group B and had a polyhedral head 40–70 nm wide and a relatively long tail (150–300 nm) (Hongo *et al.*, 1972; Ozaki *et al.*, 1984; Patek *et al.*, 1985). Compared with these phages, CG33 is relatively small.

Growth characteristics of CG33

C. glutamicum ATCC 13287 was shown to be a suitable host for CG33. Titres as high as 8×10^9 p.f.u. ml⁻¹ were routinely obtained. The plaques were clear and about 2 mm in diameter with an irregular periphery.

The effect of several cations (Mg²⁺, Ca²⁺, Cu²⁺, Mn²⁺, Zn²⁺, Ni²⁺ and K⁺) on the efficiency of plaquing (e.o.p.) was assayed. Even in the absence of any added cation, CG33 multiplied on its host, although it formed small (1 mm) or even pin-hole plaques. Among all the cations examined, Ca²⁺ and Mg²⁺, separately or together, produced a small increase in the e.o.p. of CG33, and gave rise to plaques 2 mm in diameter. The difference in the size of the plaques obtained with or without Mg²⁺ or Ca²⁺ confirmed the role of these cations in CG33 adsorption. Thus, when *C. glutamicum* was infected without any additional cations, the phage progeny liberated around the lysed cells had more difficulty in adsorbing to the surrounding ones and therefore resulted in smaller plaques.

To clarify the characteristics of CG33 infection, adsorption experiments were done in the presence of Mg²⁺ or Ca²⁺. The addition of Mg²⁺ or Ca²⁺ accelerated adsorption and increased the final number of adsorbed phages (Table 2). The optimum Ca²⁺ concentration was 10 mM: it produced about 85% adsorption in 30 min and 95% in 60 min, with no further decrease in free phage after 60 min. The addition of 50 mM-Mg²⁺, the optimum Mg²⁺ concentration for adsorption, together with 10 mM-Ca²⁺, did not improve the rate of adsorption (Table 2). The adsorption experiments enabled us to distinguish the roles of Ca²⁺ and Mg²⁺ with regard to CG33 adsorption, whereas their effects on the e.o.p. were similar. This is not contradictory since the effect of cations on the e.o.p. was examined separately by plaque formation after incubation overnight. So, it was likely that after several hours contact, all the added phages were able to adsorb to host cells and to give plaques. Therefore, the optimum conditions for the adsorption of CG33 in LB medium were 10 mM-CaCl₂ at 34 °C for 30 min.

The phage growth parameters were determined from the results of four independent experiments. The latent period of CG33 was 18 ± 2 min at 34 °C. The rise period lasted 42 ± 2 min. The apparent minimum burst size, defined as the ratio of p.f.u. ml⁻¹ in the latent period to that at the plateau, was 16 ± 4 p.f.u. ml⁻¹. However, the actual burst size may be higher since a significant proportion of CG33 could adsorb to cells in the absence of Ca²⁺. Moreover, it was

Fig. 1. Electron micrograph of CG33 negatively stained with 2% (w/v) uranyl acetate. Bar, 50 nm.

Table 2. *Adsorption of phage CG33 on C. glutamicum ATCC 13287*

Values are the percentage of free phage. Adsorption took place in LB medium at 34 °C. The phage/host cell input ratio at the time of infection was 0.01. Samples were immediately filtered through 0.45 µm filters and titrated. A control, containing only phages, incubated for 2 h under the same conditions, showed no decrease in the number of viable phage. Results are the mean of at least five experiments.

Time of sample removal (min)	Cation concn (mM)					
	None	Mg ²⁺			Ca ²⁺ (10)	Mg ²⁺ (50) + Ca ²⁺ (10)
		10	50	100		
0	100	100	100	100	100	100
15	62	62	40	63	39	35
30	44	37	32	39	14	14
40	31	34	19	26	9	7
50	30	16	17	25	5	3
60	24	14	16	19	4	3

more than likely that the titration of phages on *C. glutamicum* resulted in an underestimate due to a proportion of the rods being arranged at an angle to each other, giving V formations of more-complex angular arrangements (Rogosa *et al.*, 1974; Veldkamp, 1970), which could result in one plaque appearing from the infection of two joined cells by two phages.

CG33, from its growth characteristics, appeared similar to the corynebacteriophages described by Hongo *et al.* (1972) since it needed Ca²⁺ for adsorption. Nevertheless, its growth cycle was faster and its burst size smaller.

Fig. 2. SDS-PAGE of the structural proteins of CG33. The acrylamide gel (12.5%, w/v) was stained successively with Coomassie blue and silver. Lane 1, molecular mass markers: α_2 macroglobulin (170.0 kDa), phosphorylase B (97.0 kDa), glutamate dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa) and trypsin inhibitor (20.1 kDa). Lane 2, molecular mass markers: bovine albumin (68.0 kDa), ovine albumin (45.0 kDa), chymotrypsin (25.0 kDa) and cytochrome *c* (12.5 kDa). Lane 3, purified CG33.

Host spectrum

Among the 25 strains examined (Table 1), only two were sensitive to CG33; its usual host *C. glutamicum* ATCC 13287 and, at a very low efficiency, *C. lilium* ATCC 15990 (6.4×10^{-7}). When plaques obtained on the latter strain were picked and propagated again on the same strain, high titre phage stocks were produced, suggesting the presence of a restriction-modification system in *C. lilium*. Evidence of the existence of restriction-modification systems in corynebacteria was provided by Katsumata *et al.* (1984). Thus, CG33 could be used to select restriction-defective strains of *C. lilium*, which would be helpful for transformation.

Structural polypeptides of CG33

The analysis of the polypeptides of CsCl-purified CG33 by SDS-PAGE is shown in Fig. 2. The capsid of CG33 contained 10 polypeptides whose molecular masses ranged from 14.5 to 48 kDa. Six proteins (14.5, 16.5, 18.5, 27.0, 32.0 and 35.0 kDa) appeared in very small amounts, whereas four (30.0, 42.0, 45.0 and 48.0 kDa) apparently constitute major structural phage components. If CG33 were similar to coliphage λ (Murialdo & Siminovitch, 1971), the most abundant polypeptide (30.0 kDa) could represent the principal component of the phage head, and the second most abundant (42.0 or 48.0 kDa) the main component of the tail.

Genome organization of CG33

We concluded that CG33 nucleic acid was a double stranded DNA molecule since it could be digested by restriction endonucleases (Wells & Neuendorf, 1981). The genome size was 13.4 kb as determined by summing the size of the restriction fragments produced by each enzyme. CG33 DNA was not cleaved by *Cla*I, *Eco*RI, *Kpn*I, *Sac*I, *Sma*I, *Sph*I, *Xho*I or *Bam*HI. The restriction map obtained appeared circular (Fig. 3). Nevertheless, there were some indications, such as the presence of restriction fragments in small amounts in DNA digests, that the genome was linear.

Fig. 3. Restriction map of the CG33 chromosome. The approximate location of the 'cos' site is shown.

Fig. 4. Mapping of the 'cos' sequence of CG33. Lanes EE', PP', NN', SS', BB' and HH' represent CG33 DNA digested with *EcoRV*, *PvuII*, *NruI*, *ScaI*, *BglII* and *HindIII*, respectively. Samples in lanes E, P, N, S, B and H were loaded directly on to an agarose gel; samples in lanes E', P', N', S', B' and H' were loaded after heating for 15 min at 72 °C in 6% (w/v) saccharose and 44 mM-EDTA, and rapidly cooling on ice. In each case heating produced new fragments (A₁, A₂ etc.) while one restriction fragment (A, B etc.) disappeared or was reduced in intensity.

The circularization of CG33 DNA is apparently a result of annealing of cohesive ends since the circular configuration is unstable and suppressed by heating. CG33 DNA digests were heated at 72 °C for 15 min, cooled rapidly on ice, and loaded on agarose gels. For each digest, heating resulted in the disappearance of one restriction fragment and the appearance of two new fragments (Fig. 4). The size of these new fragments equalled that of the disappearing one, thus enabling us to localize the 'cos' sequence on the CG33 DNA map (Fig. 3): it was found in the *HindIII*-D, *NruI*-C, *PvuII*-B, *BglII*-B and *ScaI*-A fragments.

Inasmuch as the CG33 chromosome is rather small, is made of double stranded DNA, possesses a single cleavage site for *EcoRV* and is not digested by numerous common restriction enzymes, this phage constitutes a suitable candidate for the development of a phage cloning vector. Such a vector, introduced by transfection into recipient cells, would be useful for the amplification of cloned DNA before subcloning in plasmid vectors, especially since until now, protoplast transformation of corynebacteria in the absence of restriction-defective strains was fairly difficult.

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