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Association of Probable Defective Phage Particles with Lysis by Bacteriophage AP50 in *Bacillus anthracis*

By ELISABETH NAGY AND G. IVÁNOVICS

Institute of Microbiology, Medical University, Szeged, Hungary

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INTRODUCTION

The main characteristics of a bacteriophage designated AP50 have been described previously (Nagy, 1974). This bacteriophage is unusual in its RNA and phospholipid content, and in its host specificity, the only sensitive species being *Bacillus anthracis* (Nagy, Prágai & Ivánovics, 1976). Only about one-third of the isolates of this pathogen could propagate AP50. The aim of the present study was to define factors involved in the plaque formation of AP50 in individual strains of *B. anthracis*.

METHODS

Bacteria. The majority of the *Bacillus anthracis* strains were isolated from veterinary specimens in Hungary about 50 years ago and maintained as spore suspensions. Their identity was confirmed recently (Ivánovics & Földes, 1958). These strains were fully virulent when first isolated, i.e. all were capsulogenic (C^+), but some of them have given rise to non-capsulogenic (C^-) mutants. A number of C^- mutants were obtained by the method of Sterne (1939) modified by Ivánovics (1962). Strains CN18-74 and CN35-18 were given by M. Sterne (Burroughs Wellcome, Beckenham, Kent), and strain Vollum and its non-proteolytic mutant, strain NPA, by H. Smith (University of Birmingham). Some isolates of *B. anthracis* Vollum C^- harbouring a defective prophage (designated by serial numbers and VC^-dl) were constructed (Ivánovics, 1967); these carry an incomplete genome of phage W (McCloy, 1951). A non-defective lysogenic isolate of strain Vollum C^- was constructed by lysogenizing it with phage Wβ; the isolate obtained was designated $VC^-(\beta)$. Strain Davis received from E. Meynell (University of Kent, Canterbury) proved to be a sensitive host for most anthrax phages but not AP50.

Phage AP50 *and its assay.* Phage AP50 was obtained as described by Nagy (1974). The lysate contained approximately 10^{10} p.f.u. ml^{-1} when assayed by an overlay technique with strain CN18-74. To test the sensitivities of the individual strains to AP50, serial dilutions of the phage were spotted on to the surface lawn of the organisms. The highest decimal dilution forming plaques was taken as the degree of the sensitivity of the strain.

Media. These were YP broth (yeast extract, peptone; Csiszár & Ivánovics, 1965) and YC (yeast extract, casein hydrolysate). The latter was used for detecting capsule production in a CO_2 atmosphere (Ivánovics, 1962).

Induction of strains. YP medium (10 ml) in 100 ml Erlenmeyer flasks was inoculated from an overnight broth culture and aerated by vigorous shaking in a water bath at 37 °C. The absorbance was measured by a Bausch & Lomb Spectronic 20 photometer at 620 nm. When the absorbance reached 0·25, mitomycin C (MC) was added to the culture which was then re-incubated. An MC concentration of 0·5 μg ml^{-1} caused lysis of most of the strains. When this MC concentration did not result in significant lysis within 4 h, the procedure was repeated with 5 μg MC ml^{-1}. Each lysate was assayed for the presence of phage plaques with strain Davis.

Adsorption of AP50. The rate of adsorption of AP50 was determined at 37 °C by the method of Adams (1959). The period of adsorption was not extended beyond 30 min because of the possibility of phage propagation.

Electron microscopy of the lysates. Lysates of cultures of 200 to 300 ml obtained by induction with MC were centrifuged and concentrated with polyethylene glycol (PEG) by the method of Yamamoto *et al.* (1970). The filtrates of the non-inducible strains were concentrated similarly. Concentrates were prepared for electron microscopy as described by Nagy & Ivánovics (1972); the JEM 100B electron microscope was operated at 80 kV.

Table 1. *Characteristics of Bacillus anthracis strains*

Group	Strain designation	Sensitivity* to AP50 at: 26 °C	37 °C	Adsorption of AP50 at 37 °C (%)	Induction by MC* Absorbance Max.	Min.	Max./Min.	Defective phage in lysate
A	CN18-74	8	8	85	1·00	0·40	2·5	+
	CN35-18	8	8	95	0·75	0·15	5·0	+
	A68 C^-	6	6	79	0·95	0·32	3·0	+
	A71a C^+	6	6	57	0·84	0·34	2·5	NT
	A71a C^-	6	6	60	0·88	0·37	2·3	+
	A78 C^-	6	6	70	1·00	0·30	3·3	+
	A81 C^+	6	6	63	0·90	0·28	3·2	NT
	A81 C^-	6	6	63	0·84	0·28	3·0	+
	A91 C^+	5	5	59	0·68	0·08	8·5	NT
	A91 C^-	5	5	69	0·74	0·10	7·4	+
	A92 C^+	6	6	70	0·85	0·25	3·4	NT
	A92 C^-	6	6	72	0·82	0·28	3·3	+
	A96 C^-	6	6	70	0·80	0·10	8·0	+
B	A66 C^+	4	0	60	0·88	0·40	2·2	+
	A72 C^+	6	0	83	0·82	0·38	2·1	+
	A74a C^+	6	0	72	0·90	0·40	2·2	+
	A75 C^+	3	0	77	0·80	0·31	2·5	NT
	A76 C^+	6	0	51	0·92	0·41	2·2	NT
	A93 C^+	4	0	34	0·89	0·40	2·2	+
C	Vollum C^+	0	0	0	0·85	0·75	1·1	NT
	Vollum C^-	0	0	0	0·80	0·75	1·06	−
	NPA C^-	0	0	0	0·65	0·52	1·2	−
	A87 C^-	0	0	0	1·10	1·00	1·1	−
	A88 C^+	0	0	0	0·65	0·58	1·1	NT
	A89 C^+	0	0	0	0·90	0·83	1·08	NT
	A94 C^+	0	0	0	0·85	0·78	1·08	−
D	5VC^- *dl*	0	0	46	†0·52	0·18	2·9	+
	6aVC^- *dl*	3	3	94	†0·50	0·15	3·3	+
	6bVC^- *dl*	0	0	50	†0·42	0·10	4·2	+
	7VC^- *dl*	3	3	92	†0·46	0·12	3·8	+
	10VC^- *dl*	0	0	43	†0·50	0·07	7·1	+
	11VC^- *dl*	3	3	95	†0·55	0·10	5·5	+

NT, Not tested. * See Methods. † Induction with 5 μg MC ml^{-1}.

RESULTS AND DISCUSSION

The strains tested, which included both wild-type C^+ and non-capsulogenic C^- strains, were classified on the basis of their sensitivity to phage AP50 into four groups (Table 1). Phage AP50 formed plaques on all strains of group A at both 26 and 37 °C. Included in this group were two isolates of Sterne (CN18-74, CN35-18) which have been studied previously (Nagy & Ivánovics, 1972; Nagy, 1974). Group B consisted of strains that were not able to propagate the phage at 37 °C but gave plaques at 26 °C after 48 h, although these were very turbid. The strains belonging to group C did not plaque with AP50. The variation in the sensitivities of C^+ and C^- strains could not be related to the genetic alleles of capsule production. Six defective lysogenic isolates comprised group D, although there were marked differences between the sensitivities of the strains to phage AP50. Three of them were moderately sensitive, while three did not propagate the phage; with the latter, the adsorption rate was between 43 and 50%.

The groups exhibited definite differences in the adsorption of phage AP50, in parallel with their phage sensitivities (Table 1). With group C the adsorption rate was always 0%.

One lysogenic derivative of strain Vollum C^- [$VC^-(\beta)$] was neither sensitive to phage AP50 nor capable of adsorbing it. This result suggested that normal prophage W(β) does

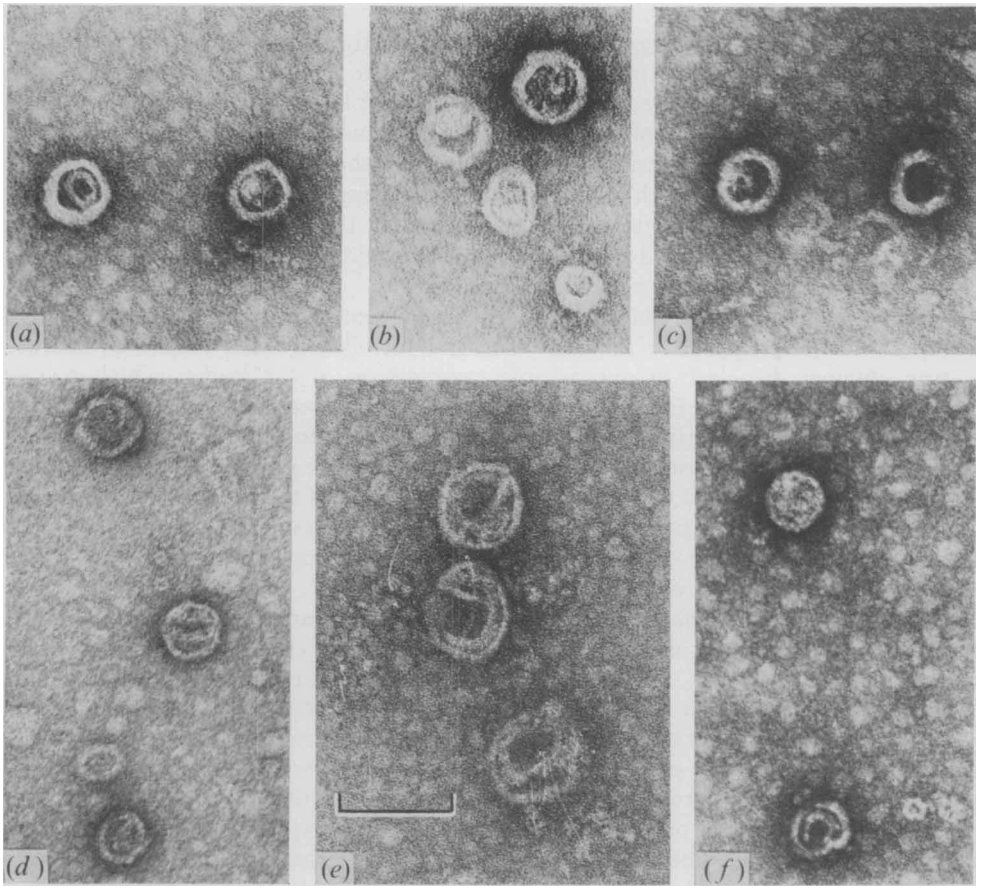

Fig. 1. Electron micrographs of concentrated lysates of *B. anthracis* strains of group A [(*a*) A71a C$^-$, (*b*) A81 C$^-$, (*c*) A91 C$^-$, (*d*) A92 C$^-$], group B [(*e*) A66 C$^+$] and group D [(*f*) 7VC$^-$ *dl*]. Negative staining with 2% (w/v) uranyl acetate. Bar marker represents 100 nm.

not elicit receptor production corresponding to the sensitivity to AP50, but one or more of its fragments do (see group D).

When incubated in the presence of MC the individual strains attained a maximum absorbance within 60 to 120 min, and thereafter the absorbance of the cultures dropped to a minimum at 240 min. This lysis was marked in group A, but was less evident in group B at an MC concentration of 0·5 μg ml^{-1}. In order to express the induction of lysis of the strains numerically, the ratios of the maximum to minimum values of the growth curves were calculated (Table 1). Addition of 0·5 μg MC ml^{-1} elicited marked lysis in most strains of groups A and B, but the values of this ratio varied considerably; the lowest was 2·1 for strain A72 C$^+$. In spite of this, the lysis of this strain was estimated to be about 90% using a calibration curve (Nagy & Ivánovics, 1972). For the members of group C, which were neither sensitive to phage AP50 nor capable of adsorbing it, the ratios were near 1·0. Thus, for Vollum C$^-$ the ratio indicated a cell decrease of only 10%. In group D, only an MC concentration of 5 μg ml^{-1} could elicit mass lysis. This was in accordance with the data described earlier by McCloy (1958) and Ivánovics (1967) who showed that lysogenic or defective lysogenic strains with phage W resist lysis by ultraviolet irradiation. In contrast, the strains of group C were not more liable to lysis when the MC concentration was increased. Thus, the slight decrease in absorbance following the peak shown by the strains of group C may be due to an aggregation of chain-formers or partial autolysis of bacteria, or both.

Altogether 21 strains were selected for electron microscopic examination; 17 of these were inducible with MC. All lysates of the inducible strains tested contained structures resembling defective phage particles. In every preparation, round or partially-deformed empty bacteriophage heads were seen, varying in diameter from 53 to 80 nm (Fig. 1). None of the lysates showed other morphological elements, such as tail-like structures or filaments. These electron microscope observations were similar to those for the lysates of the Sterne strains (CN18-74, CN35-18) which are very sensitive to phage AP50, and which were previously also found to contain defective phage particles in their lysates (Nagy & Ivánovics, 1972). None of the concentrated filtrates of the four strains belonging to group C showed any structures resembling defective phage particles.

All strains of *B. anthracis* which were able to propagate phage AP50 were thought to harbour a defective prophage on the basis of their inducibility by MC. Further evidence for this was obtained by electron microscopic examination of the lysates which revealed structures resembling phage fragments. Such a structure may well be helper genome for the multiplication of phage AP50. Strains artificially converted to be defective lysogens with phage W merit special interest since the origin of their defective genome is known. Although phage AP50 can be adsorbed by all of these strains only half of them were moderately sensitive, while the rest did not propagate AP50 at all. This suggests that the character of the gene fragments carried by the sensitive strains may be important in respect of their supporting the propagation of phage AP50.

Lysogenic strains of *B. anthracis* liberating infective phage particles are not rare in nature. Buck *et al.* (1963) collected more than a hundred *B. anthracis* isolates from several laboratories and found that 18% liberated infective phages of varying host specificities. It is striking that in our material no lysogenic strains, but only defective lysogens were found. The proportion of the latter exceeded that of the lysogenic strains of Buck *et al.* (1963).

In other bacterial species phage genome is established as essential for the replication of bacteriophages. As early as 1963, the coli phage P4 was discovered as a satellite, the propagation of which depended on prophage P2 as a helper (Six, 1963). When M13, a filiform coli phage undergoes propagation, a satellite or defective mini phage appears in the progeny; the length of this is one-fifth to one-half of that of the normal M13 and it contains single-stranded DNA. The mini particles replicate only upon coinfection with normal M13 (Hewitt, 1975). Another example of cooperation of phage genomes is that which exists between phage mutant $T_1 am23$ and λ. Phage λ allows the replication of this amber mutant in a non-permissive host (Christensen, 1976). The propagation of RNA phage AP50 in *B. anthracis* may also depend on helper genome that is probably a defective phage fragment.

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REFERENCES

ADAMS, M. H. (1959). *Bacteriophages*. New York: Interscience.

BUCK, C. A., ANACKER, R. J., NEWMAN, F. S. & EISENSTARK, A. (1963). Phage isolated from lysogenic *Bacillus anthracis*. *Journal of Bacteriology* **85**, 1423–1430.

CHRISTENSEN, J. R. (1976). *Red* system of bacteriophage complements the growth of bacteriophage T_1 gene 4 mutant. *Journal of Virology* **17**, 713–717.

CSISZÁR, K. & IVÁNOVICS, G. (1965). Transduction in *Bacillus subtilis*. *Acta microbiologica Academiae scientiarum hungaricae* **12**, 72–89.

HEWITT, J. A. (1975). Miniphage – a class of satellite phages to M13. *Journal of General Virology* **26**, 87–94.

IVÁNOVICS, G. (1962). The pathogenicity of *Bacillus anthracis* lysogenic with mutants of phage W. *Journal of General Microbiology* **28**, 87–101.

IVÁNOVICS, G. (1967). Defective lysogene *Bacillus anthracis* Stämme. *Acta microbiologica Academiae scientiarum hungaricae* **14**, 71–75.

IVÁNOVICS, G. & FÖLDES, J. (1958). Problems concerning the phylogenesis of *Bacillus anthracis*. *Acta microbiologica Academiae scientiarum hungaricae* **5**, 89–109.

McCLOY, E. W. (1951). Studies on a lysogenic Bacillus strain. I. A bacteriophage specific for *Bacillus anthracis*. *Journal of Hygiene, Cambridge* **49**, 114–125.

McCLOY, E. W. (1958). Lysogenicity and immunity to Bacillus phage W. *Journal of General Microbiology* **18**, 198–220.

NAGY, E. (1974). A highly specific phage attacking *Bacillus anthracis* strain Sterne. *Acta microbiologica Academiae scientiarum hungaricae* **21**, 257–263.

NAGY, E. & IVÁNOVICS, G. (1972). Defective lysogeny in *Bacillus anthracis* Sterne strains. *Acta microbiologica Academiae scientiarum hungaricae* **19**, 69–76.

NAGY, E., PRÁGAI, B. & IVÁNOVICS, G. (1976). Characteristics of phage AP50, an RNA phage containing phospholipids. *Journal of General Virology* **32**, 129–132.

SIX, F. W. (1963). A defective phage depending on phage P_2. *Bacteriological Proceedings*, 138.

STERNE, M. (1939). The use of anthrax vaccines prepared from avirulent uncapsulated variant of *Bacillus anthracis*. *Onderstepoort Journal of Veterinary Science and Animal Industry* **13**, 307–312.

YAMAMOTO, K. R., ALBERTS, B. M., BENZINGER, R., LAWHORNE, L. & TREIBER, G. (1970). Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. *Virology* **40**, 734–744.