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ELECTRON MICROSCOPY OF PHAGES LIBERATED BY MEGACIN A PRODUCING LYSOGENIC BACILLUS MEGATERIUM STRAINS

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Summary. Mitomycin C was added at fairly high concentration (5–10 μg/ml) to exponentially growing cultures of selected strains of *Bacillus megaterium*. Lysis of the bacteria followed, associated by liberation of phage and megacin A production. In contrast, a low concentration (0.5 μg/ml) of mitomycin induced only megacin A production. Electron microscopic examination of the lysates induced by 5–10 μg/ml of mitomycin in 19 strains of *B. megaterium* showed them all to contain phages; most of the strains proved polylysogenic. Their lysates contained distinct complete phages of different structures and dimensions. A few strains released defective phage particles. The significance of the electron microscopic findings is discussed in relation to megacinogeny.

Several papers have reported on an antibacterial substance designated megacin A, produced by *Bacillus megaterium* [1]. Megacins can be classified into at least three different groups [2]. Megacin A formed by 2–3% of *B. megaterium* strains isolated from soil samples exhibits a normal growth curve when cultivated in an adequate complete nutritional medium. No megacin A formation could be detected in the cultures unless the bacteria had been exposed to an agent disturbing DNA synthesis. On light ultraviolet irradiation of the growing cells, or addition of certain chemicals such as mitomycin C or nitrosoguanidine to their cultures, a marked lysis ensues, accompanied by megacin A production [3]. Megacinogeny is reminiscent of lysogeny, except that there is no infective phage, but an antibacterial substance is released during lysis of the cells. The resemblance of the two phenomena suggested that a prophage might be involved in both cases. Although it has been claimed that the two phenomena are related genetically [1, 4], no direct evidence could be obtained which supported this concept. On the other hand OZAKI *et al.* [5] succeeded in proving the enzymic nature of megacin A; the bacteriocin of strain 216 was shown to be identical with phospholipase A. Unfortunately, other strains produce too little megacin A for allowing its identification. There is, however, no doubt that different strains produce the same megacin A [1]. A turning point has recently been reached when a *Bacillus cereus* strain was found to be capable of producing a bacteriocin similar to megacin A; chemical evidence indicated that this bacteriocin is identical with phospholipase A [6]. This particular strain of *B. cereus*, designated W, is lysogenic for a highly specific phage which attacks all strains of *B. anthracis* [7]. Furthermore, another

prophage was also detected in strain W, controlling the liberation of a distinct phage, named *wx*. This particular phage did not propagate when tested on a great number of strains belonging to different species of the genus *Bacillus*. The only host which appeared sensitive to phage *wx* was a "cured" derivative, obtained from strain W of *B. cereus*, and not producing megacin A, *i.e.* phospholipase A [8]. Infection of these "cured" bacteria by phage *wx* converted the cells into phospholipase A producers. This was direct evidence of megacin A production being due to a certain prophage [9].

In the present study our aim was to examine lysates derived from megacinogenic strains of *B. megaterium* after induction, for the presence of either complete or defective phage particles.

Materials and methods

Bacterial strains. Out of a total of 18 megacin A producing strain, 10 were isolated from soil samples up to 1954 [10], and 8 further strains have recently been screened from about 150 isolates of *B. megaterium*. They were identified on the basis of the criteria established by SMITH *et al.* [11]. Strain No. 337 had been received from the collection of Dr. DEN DOOREN DE JONG [12]. Without induction this strain produced a "killing principle" [13], which was identified as a protein and designated as megacin Cx [14]. Strain MUT-C was a phage-resistant one, although highly sensitive to megacin A.

Medium OX. Trypton (Oxoid), 10 g; yeast extract (Oxoid), 2.5 g; K_2HPO_4, 1 g; NaCl, 5 g; distilled water, 1000 ml; pH 7.5. Solid medium was prepared by adding 1.5% agar to OX broth.

Induction of megacin A. A liquid culture was aerated by shaking in a water bath at 37°C, and its growth was monitored by a Bausch and Lomb Spectronic 20 optical densitometer. When bacteria were induced to megacin A production, 0.5 μg/ml of mitomycin C (MC) was added to the culture. Further technical details of the induction and of the titration of megacin have been described previously [15].

Titration of megacin A. Preparation of the indicator plate has been described previously [15]. Several twofold dilutions of the lysate were prepared in saline solution containing 20% OX broth. By means of a loop, 0.02 ml amounts of the serial dilutions were placed onto the surface of the indicator plates. One unit of megacin A/ml was taken as the reciprocal of the highest dilution which gave either a clear zone or lysed at least half of the bacteria.

Induction of the prophage. One volume of a 18 hr broth culture of the strain under investigation was added to ten volumes of freshly prepared OX medium and incubated on a shaker to a titre of 5×10^6 cells per ml. Then mitomycin C was added to a concentration of 5–10 μg per ml and incubation was continued. After 3–4 hr the suspension clarified somewhat and was centrifuged first at 6000 *g* and then at 100 000 *g* in a Hans Janetzky VAC-60 apparatus. The pellet was resuspended in as little saline as possible and centrifuged again at 6000 *g*.

Electron microscopy. The purified phage preparation was dialyzed against distilled water and placed on carbon-impregnated Formvar-film stained with 1% uranyl acetate and examined in a JEM-7 electron microscope at 80 kV.

Results

Liberation of phage particles by megacinogenic strains of B. megaterium. Although maximum production of all megacinogenic A strains could be induced by the addition of 0.5–1 μg/ml of MC, phage liberation could be induced only by MC at a concentration of 5–10 μg/ml. Altogether 4 apperently morphologically distinct, and 2 defective phages were detected in the lysates of 18

individual strains of megacin A producing *B. megaterium*. Table I shows the distribution of the different phages produced by the individual strains. Electron microphotographs of the preparations made of concentrated lysates of a number of *B. megaterium* strains revealed that all of them contained either complete bacteriophages of different structure and dimension, or defective phage particles (Table 1, Figs 1–5). Different strains of *B. megaterium* could release from one to five kinds of phage. Strain 704 produced five different bacteriophages. This was apparently a polylysogenic culture carrying several genomes of morphologically complete as well as defective phages. This was consistent with the date of HENNEBERRY and CARLTON [16]. They isolated several circular DNA molecules from the bacterial genome of the *B. megaterium* strain 216 which they believed to be the genomes of some temperate phages. It should be noted that polylysogeny is frequently found among different strains and species of microorganisms (RAUTENSTEIN [17]).

Ultrastructure of the phages produced by B. megaterium. Bacteriophages released by different strains of *B. megaterium* after induction are morphologic-

Table I

B. megaterium phages obtained by mitomycin C induction

Strains	Designation of phage					Total	Megacin A, unit/ml
	1	2	3	4	5		
216		+	+	+		3	40 000
508			+			1	100
575			+			1	200
588		+	+	+		3	400
594				+		1	100
604		+	+			2	100
637		+				1	200
638		+		+		2	300
659		+				1	100
689	+	+	+			3	200
701	+	+		+	+	4	200
704	+	+	+	+	+	5	400
717		+	+	+	+	4	100
743				+	+	2	400
791	+	+				2	400
807[a]	+	+	+			3	100
807		+	+			2	400
842				+		1	400
337		+		+		2	0

ally different. *B megaterium* produced phages belonging to practically every morphological group classified by BRADLEY [18] and TIKHONENKO [19]; they were designated as phages 1, 2, 3, 4, 5. Phages 1–4 were complete phages particles, while phage 5 appeared to be a defective one having a tail without a head.

Phage 1 has a head measuring 120 nm in diameter and a tail 270 nm in length, terminating in an easily discernible base plate. The phage has a contractile tail sheath. The contracted sheath is shortened by about half its length and is approximately 140 nm. The base plate of phage 1 has a complex structure and is much wider than the tail itself (Fig. 1).

Phage 2 belongs to a group of phages with contractile sheath. It is however, much smaller than phage 1. The head of the phage is only 60 nm in diameter and the tail is 110 nm long. The tail ends in a small base plate (Fig. 2).

Phage 3 belongs to the group with non-contracting tail. Its head is a hexagon of 55 nm in diameter and its non-contracting tail shows a well defined striation. The base plate is rather small (Fig. 3).

Phage 4 particles have a head 65 nm in diameter and a very short tail. The tail actually consists of a base plate connected by a very short neck with the head (Fig. 4).

Phage 5 resembles the extended tail of a T-even bacteriophage. Its particles are actually headless tails ending in a small base plate with thin fibres (Fig. 5). Thus, the particles of this phage have all the structural elements of the normal phage tail. Structures of this kind are well-known to be produced by ISHII [20] and pyocin isolated by TAUBENECK [21] and extensively studied by TIKHONENKO *et al.* [22] and by POPOVA *et al.* [23]. Thus, all strains of *B. megaterium* release phage particles as shown by electron microscopy. It should be added that the presence of biologically inactive defective particles can be detected only by electron microscopy.

Of all the described phages the most frequent was phage 2 which was produced by 14 strains out of 19, and particles bearing a morphological likeness to phage 5 were released by 10 out of 19 strains (Table II).

All strains except strain 337 produced megacin A. It is not known whether there is a connection between megacin A and the defective phages released by *B. megaterium* after induction.

Fig. 1. A particle of phage 1 with extended tail sheath; bacterial flagella indicated by arrow. Uranyl acetate (× 250 000)
Fig. 2. Particles of phage 2 with extended an contracted tail sheaths. Uranyl acetate (×250 000)
Fig. 3. A particle of phage 3 with non-contracting tail. Uranyl acetate (×250 000)
Fig. 4. Particles of phage 4, with short tail indicated by arrow. (× 250 000)
Fig. 5. Particles of defective phage 5 structurally resembling the tail of T-even phages. Uranyl acetate (× 250,000)

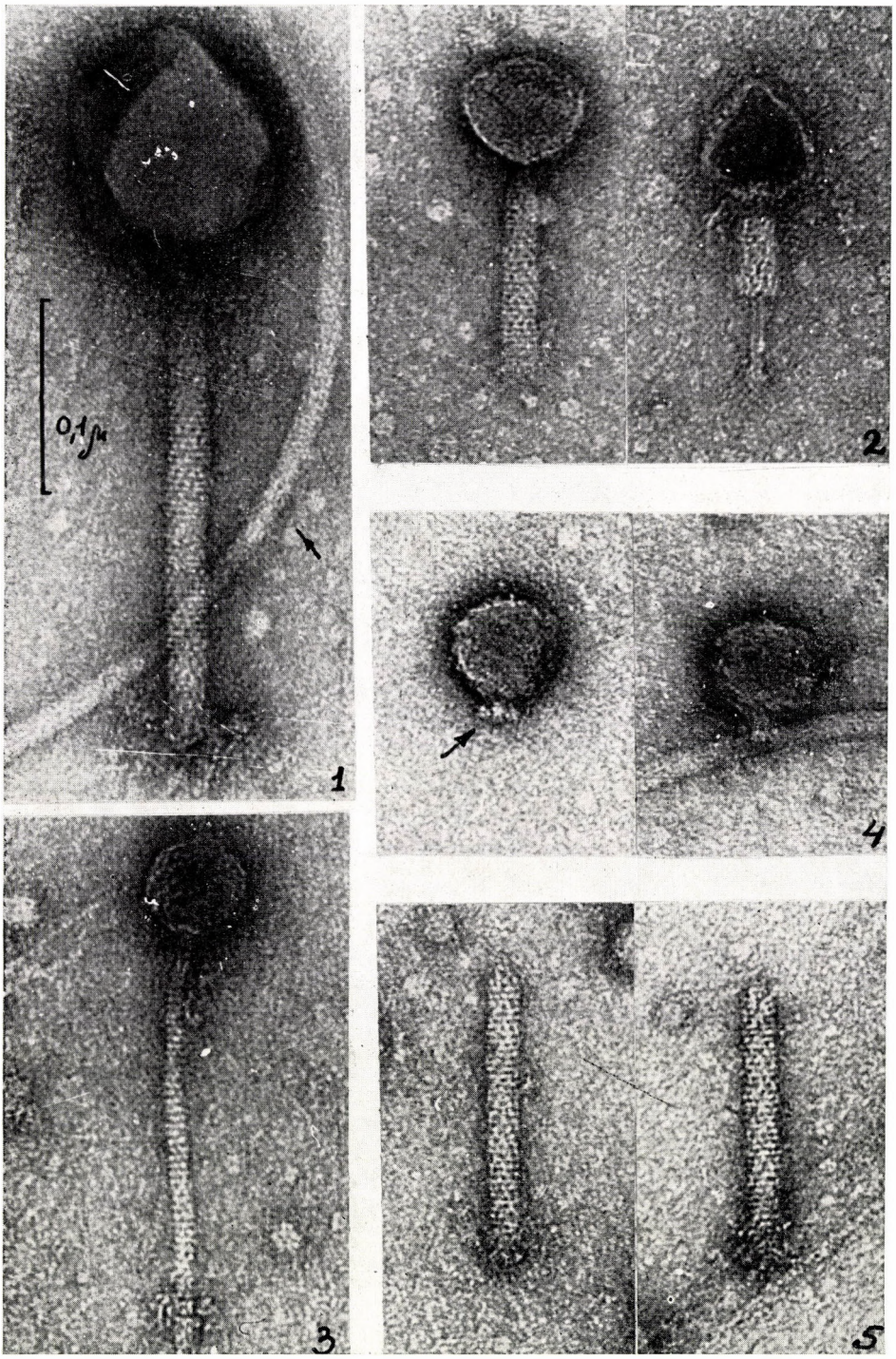
0,1 μ
1
2
3
4
5

Table II

Dimensions of B. megaterium phages obtained by mytomycin C induction

Phage No.	Dimension of head, nm	Dimension of tail, nm
1	120	270
2	60	110
3	55	210
4	65	—
5	—	160

Discussion

The presence of phages has been revealed by electron microscopy in the lysates of all the strains investigated. They, except No. 337, produced megacin A and the addition of mitomycin C induced a marked lysis accompanied by an accumulation of megacin A in the culture. Strain No. 337 was not lysed significantly, but the culture ceased growing at the middle of the exponential phase, and the arrest of growth was probably due to the presence of MC at high concentration (5 μg/ml). No traces of megacin A were found in the supernatant of the culture of No. 337; this strain is known to produce megacin Cx [14] instead of megacin A. Nevertheless, electron microscopy revealed two phages (PH 2 and PH 5) in its supernatant.

It is difficult to conclude to any correlation between megacin A production and the phages liberated by induction. The morphological features of phages are not related to any biological characteristics. The present results indicate that all the investigated *B. megaterium* strains harbour a few prophages. They have been detected by electron microscopy, while earlier attempts to reveal any phage released by strain 216 had failed [24]. Thus, when the bacteria were exposed to light ultraviolet irradiation and a preparation was made with Kellenberger's technique for electron microscopy, although the gradual lysis of the bacteria could be followed, neither mature phage particles nor structures reminiscent of some incomplete form of phage were found around the cells during different stages of the lysis. Investigations are in progress to isolate and propagate individual phages liberated by megacinogenic strains of *B. megaterium* in order to study whether any of these phages is capable of converting a negative strain to a megacinogenic one.

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