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STUDIES ON MEGACINOGENY IN BACILLUS CEREUS

II. BACILLUS CEREUS ISOLATES CHARACTERIZED BY PROPHAGE-CONTROLLED PRODUCTION OF MEGACIN A (PHOSPHOLIPASE A)

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Summary. Five out of a number of *Bacillus cereus* strains isolated from soil produced high titre specific bacteriocin (megacin A) in mitomycin C-induced cultures. In the course of cultivation with ethidium bromide, the strains gave off segregants not producing bacteriocin *(cin⁻)*. The lysate of two wild strains formed plaques on the corresponding *cin⁻* bacteria. The two phages (*wx*23 and *wx*26) were identical in antigenic structure with phage *wx* present in the lysate of *B. cereus* strain W, and converted *cin⁻* derivatives into cultures producing megacin A (phospholipase A). The phages produced plaques at 26°C but not at 37°C. In the lysates of the remaining three strains phages were not detected with biological and morphological methods; these cultures have been assumed to carry defective prophage genome. As the corresponding prophages are responsible for the determination of inducible phospholipase A production, phages named *wx* seem to form a separate group of *B. cereus* phages.

Strain *Bacillus cereus* W, isolated by McCloy [1], produces after UV or mitomycin C (MC) induction a bacteriocin identical in antibacterial spectrum with megacin A (phospholipase A) [2]. In the lysate of the strain two phages (*w* and *wx*) have been detected with appropriate indicator cultures [3]. Phage *wx* carries the information for phospholipase A production, thus it corresponds to a converting phage [4].

Among several dozens of *B. cereus* strains maintained in our collection, beside strain W, only one, V8, contained in its lysate a substantial amount of megacin A together with phage *w* [2]. As V8 is an old laboratory strain, contamination with *B. cereus* W strain could not be excluded. In the present work it has been studied (i) whether megacin A-producing cultures can be isolated from soil; (ii) whether these cultures contain a prophage responsible for megacin A production.

Materials and methods

Phages and bacterial strains. Phages isolated from *B. cereus* strains were designated with the registration number of the corresponding strain. As the phages were very similar in properties to phage *wx* of *B. cereus* W, the latter was regarded as the prototype of phages converting to phospholipase A production. Accordingly, the converting phages isolated from *B. cereus* strains 23 and 26 were named *wx*23 and *wx*26, respectively.

Plaque production by the phages was examined on a number of strains belonging to different species of the genus *Bacillus:* 40 *B. cereus*, 13 *B. thuringiensis*, 5 *B. licheniformis*, 4 *B. subtilis* (Marburg), 5 *B. aterrimus* and 5 *B. niger* [3]. For the detection of anthrax-specific phages, *B. anthracis* strains Sterne and Davis were used as indicators.

Culture media and phage buffer. Medium YP was prepared as described earlier [5]. Phage titration was carried out on $BPYe_1$ medium [3]. Megacin A (phospholipase A) was isolated from cultures grown in BCM medium prepared with 0.1% yeast extract and supplemented with thiamine (1 μg/ml) and glucose (0.2%) [4]. The concentrated phage material was suspended in the buffer of RABUSSAY *et al.* [6].

Preparation of phage material and titration of phages. MC-induced lysates of *B. cereus* wild strains were collected from plaques formed on the homologous *cin*⁻ cultures by scraping off the soft agar layer into 5 ml medium. After centrifugation the supernatant was filtered and mixed to a soft agar suspension of the corresponding *cin*⁻ bacteria and poured over solid agar plate as described in the preceding paper [7]. Incubation was done at 26°C for 24 hr, when necessary, the phage material was concentrated as described by YAMAMOTO *et al.* [8]. In this manner 10^8 p.f.u./ml phage material was produced.

Phage sensitivity of strains. The phages were dropped at 10^{-1}, 10^{-2} and 10^{-3} dilution onto soft agar layer containing the bacteria.

Cured isolates. The *cin*⁻ derivatives of bacteriocin-producing wild strains were obtained by culturing the latter in the presence of ethidium bromide until sporulation had occurred. Derivatives not producing bacteriocin were isolated from the spore suspension as described in reference [3].

Assay of megacin A production. The cultures were shaken in 100 ml yeast extract-BCM medium in 1 litre Erlenmeyer flasks at 37°C on Gyrotory Shaker (New Brunswick Scientific Co.) at 350 r.p.m. until $OD_{620} = 0.25$ had been reached. Then 0.5 μg MC was added per ml medium and the culture was incubated until lysis had occurred. After centrifugation at 10 000 *g* and 4°C for 30 min in the MSE high speed centrifuge, to the clear supernatant ammonium sulphate was added to 0.7 saturation under continuous correction of the pH to 7.0. After the ammonium sulphate had completely dissolved, the liquid was refrigerated for 1 hr. The precipitate was centrifuged at 15 000 *g* and the deposit was dissolved in 2 ml of 0.75 M NaCl and the insoluble fraction was removed by centrifugation. Megacin A titre of the concentrates was determined as described elsewhere [2] on indicator strain *B. megaterium* MUT-C.

Phospholipase activity in the concentrated lysates was measured with the hydroxamate method [4] modified as follows. As substrate, L-α-lecithin (synthetic, dipalmitoyl, Fluka) was used at 1 μg/ml concentration in 1 ml assay system. On the basis of preliminary experiments, 3–4 units of enzyme were added to the system in 0.1 ml volume; to two other assay systems a half and a quarter of this amount were added. Hydrolysed ester bonds were expressed in μEq calculated with the formula used in previous experiments [4]. One unit of the enzyme corresponded to an amount breaking down one μEq ester bond at 37°C in 30 min. Specific enzyme activity calculated for 1 mg protein was measured by the method of LOWRY *et al.* [9].

Enzyme activity of the concentrates was identified by thin layer chromatography. Phosphatidyl ethanolamine (synthetic dipalmitoyl, A grade, Calbiochem) was used as substrate. Identification of phospholipase A activity was based on the method of GALLAI-HATCHARD and THOMPSON [10] modified as follows. The substrate was emulsified at 1 mg/ml in 0.05 M Tris buffer (pH 8.0) supplemented with sodium deoxycholate (250 μg/ml) and $CaCl_2$ (10 mM), in an MSE Ultrasonic Power Unit. All concentrates were tested in three assay systems containing the enzyme in gradually decreasing amounts. To 0.5 ml emulsion 2–3 units of enzyme concentrate were added in 0.1 ml volume; the second and third assay systems contained half and quarter of this amount, respectively. Incubation at 37°C lasted for 30 min, then chloroform extraction and other procedures were performed as described by GALLAI-HATCHARD and THOMPSON [10]. Chromatography was done in Silica gel G (Merck) thin layer (0.25 mm).

Lysogenic conversion. The lysate prepared from the parent strain was dropped undiluted and diluted to 10^{-1} and 10^{-2} onto soft agar layer inoculated with the homologous *cin*⁻ bacteria. The culture was incubated at 26°C for 24–48 hr, then the plaques were suspended in a few ml of saline and heated at 75°C for 10 min. Gradual dilutions of the heated spores were added in 0.1 ml volume to a mixture containing 1 ml *B. megaterium* MUT-C culture and 1 ml agar. After incubation at 37°C the ratio of colonies with and without clear zone was calculated. In liquid medium the time course of conversion was determined as described in [4], except that the system was incubated at 22–24°C. All experiments were performed in parallel in systems containing chloramphenicol (250 μg/ml).

Electron microscopic examination. Phages *wx*23 and *wx*26, propagated on soft agar and extracted, were centrifuged and filtered. The extract was concentrated as described by YAMAMOTO *et al.* [8] then purified with cyclic ultracentrifugation. *B. cereus* strains 22, 27

and 28, which failed to give plaques on the homologous *cin*⁻ strains, were induced with MC, filtered and concentrated. After ultracentrifugation the material was suspended in one hundredth of the original volume and mounted on carbon-impregnated Formvar-film placed on LKB grids. The preparations were stained with 2% uranyl acetate and examined in a JEOL 100 B electron microscope at 80 kV.

Results

Isolation of B. cereus strains and screening for megacin A production. The strains isolated from soil specimens were selected on the basis of colonial morphology. After needle-point inoculation onto agar plates the cultures were grown at room temperature for 12–16 hr then replicated by the velveteen stamp method and irradiated with UV light for 15 s. After incubation at 37°C, colonies with clear zone were reisolated and examined for morphological and biochemical characteristics as described by SMITH *et al.* [11] and for minimum nutrient requirement [12]. Dozens of isolates showing the characteristics of *B. cereus* were examined for bacteriocin production in the presence of MC. The majority produced bacteriocin in slight amounts; 5 strains producing more than 400 U/ml were studied further. Strain *B. cereus* W was used as control.

Segregants producing no bacteriocin. Derivatives were obtained from *B. cereus* isolates 22, 23, 26, 27 and 28 by culturing them in the presence of ethidium bromide [3]. These "cured" derivatives were designated as *cin*⁻ cultures.

Phages detectable in the lysates of B. cereus strains. The lysate of prototype strain *B. cereus* W in addition to converting phage *wx*, contains phage β which acts on *B. anthracis* strains [3]. Only two of the 5 *B. cereus* strains selected on the basis of bacteriocin production were shown to contain in their lysates converting phages (*wx*23 and *wx*26). The two phages were characterized by peculiar thermosensitivity. At 37°C they failed to produce plaques on the homologous *cin*⁻ bacteria. The optimum temperature for plaque production was 26°C. It was striking that plaque production was absent even at 26°C if the phages had been incubated at 37°C in soft agar layer together with the indicator culture. Accordingly, it may be assumed that phages *wx*23 and *wx*26 are thermosensitive, although their vegetative forms tolerated higher temperatures well: at 45°C for 60 min the phage lysates showed no decrease in titre.

In the lysates of three *B. cereus* strains slight amounts of certain phages were also detected; these failed to act on *B. anthracis* Davis, but produced plaques on *B. anthracis* Sterne (CN 18-74). These phages will be described in a subsequent paper.

Phages *wx*23 and *wx*26 exerted no action on various *Bacillus* strains either at 37°C or at 26°C.

On BPYe$_1$ medium inoculated with the homologous *cin*$^-$ strains, phages *wx*23 and *wx*26 produced turbid plaques about 1 mm in diameter with pin-point-sized clear centre. Both phages produced a few, seemingly clear plaques which, however, yielded no clear mutants on subcultivation.

Phages *wx*23 and *wx*26 were closely similar in antigenic structure as phages *wx* and *wxc* of *B. cereus* W. The K value for the immune serum prepared with phage *wxc* was 45 against the homologous phage; the corresponding values for phages *wx*23 and *wx*26 were 43 and 41, respectively. Subculturing

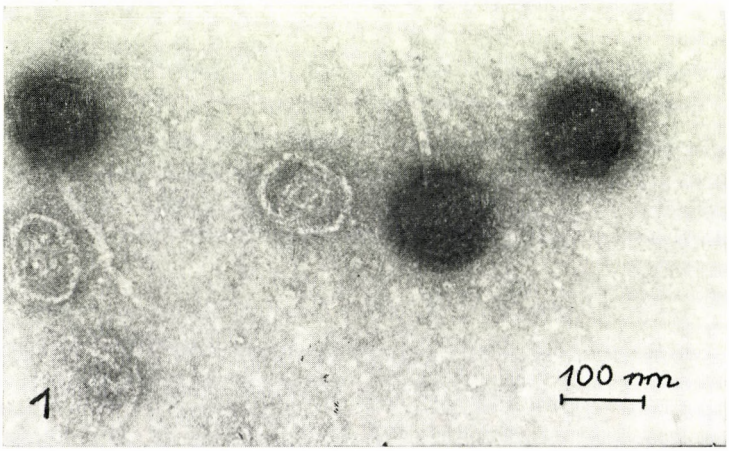

Fig. 1. Electron microscopic picture of phage *wx*23

of the plaques of phages *wx*23 and *wx*26 gave few plaque forming units. The plaques in soft agar were scraped off, suspended in culture medium and shaken vigorously by hand for 5 min. The extract was tested on the homologous *cin*$^-$ culture for the released number of plaque formers. The number of p.f.u. per plaque was 4×10^5 for *wx*23 and 10^5 for *wx*26.

Electron microscopy. The lysate of *B. cereus* strains 23 and 26, tested on homologous *cin*$^-$ cultures, showed the presence of plaque formers. Electron micrographs (Figs 1 and 2) prepared from the concentrates of the two phages revealed B-type phages according to BRADLEY's classification [13]. The size of phages *wx*23 and *wx*26 is shown in Table I. The phages were similar in morphology to the converting phage and its clear mutant of *B. cereus* W, but they differed somewhat from the latter in size (the head of *wxc* is 73 nm in diameter and the length of its tail is 153 nm [4]). In the concentrates of phages *wx*23 and *wx*26 a number of tailless empty phage heads were seen (Fig. 1). This observation and results showing the low ratio of plaque formers in the phage

population, are in correlation and indicate a low degree of tolerance of the phages to mechanical injury.

In the concentrated lysate of the remaining three *B. cereus* strains 22, 27 and 28, repeated electron microscopic examinations failed to detect intact or defective phage structures. This was in agreement with the failure of bio-

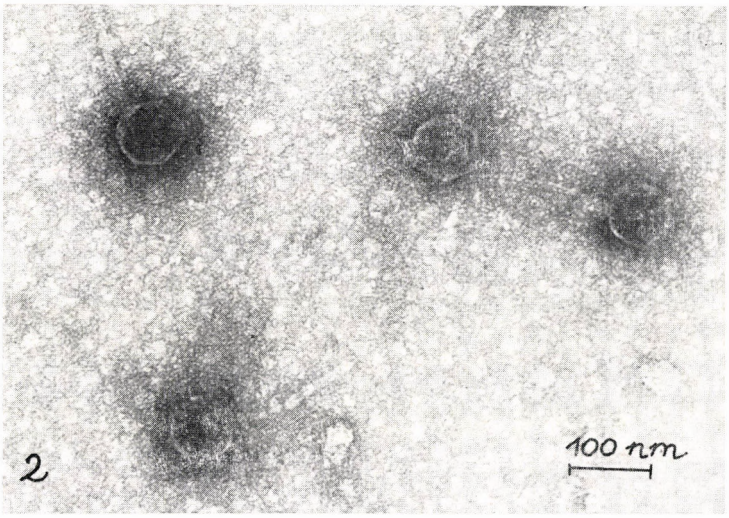

Fig. 2. Electron microscopic picture of phage *wx*26

Table I

Size of the structural elements of phages wx23 and wx26

Structural element, nm	*wx*23	*wx*26
Diameter of head	100	64
Length of tail	210	160

logical tests to show plaque formers on homologous *cin*⁻ strains and with the fact that lysates of these strains caused no lysogenic conversion.

Converting capacity of the lysates of B. cereus cultures. As shown in Table II, all *cin*⁻ derivatives including that of *B. cereus* W, were tested against all lysates. Plaque formation and conversion were highly strain-specific in case of phages *wx* and *wx*23. Phage *wx*26 exhibited a less specific behaviour as it

Table II

Characteristics of crude lysates of B. cereus strains

B. cereus strain	Bacteriocin U/ml*	Phage p.f.u./ml*	Plaque production and lysogenic conversion by crude lysates of the cured strains					
			22 *cin*⁻	23 *cin*⁻	26 *cin*⁻	27 *cin*⁻	28 *cin*⁻	W *cin*⁻
22	800	0	—	—	—	—	—	—
23	2000	3×10^5	—	p.c.	—	—	—	—
26	2000	8×10^5	p.c.	—	p.c.	—	p.c.	—
27	3200	0	—	—	—	—	—	—
28	2000	0	—	—	—	—	—	—
W	3200	2×10^5	—	—	—	—	—	p.c.

* Lysate of individual strains grown in BCM + yeast extract medium. Intensive gyration (in Gyrotory Shaker, New Brunswick, 350 r.p.m.) in the presence of 0.5 μg/ml of MC

p. = plaque formation on individual *cin*⁻ strains

c. = conversion of *cin*⁻ strains

— = neither plaque formation on, nor lysogenic conversion of, the cured strains

0 = no plaque formation on homologous *cin*⁻ strain

produced plaques on, and conversion of, not only the homologous but also two other *cin*⁻ bacteria.

The time course of conversion by phages *wx*23 and *wx*26 is seen in Table III. Homologous *cin*⁻ bacteria in the exponential phase of growth were infected with 5 m.o.i. and the degree of conversion was determined at 22°C

Table III

Conversion rate (per cent) and number of colony formers (10^7/ml) in $PBYe_1$ medium in presence and absence of chloramphenicol

m.o.i.: 5 per colony formers

System		Assay for	CA	Time in minutes at 22°C					
bacterium	phage			0	15	30	60	120	600
23 *cin*⁻	*wx*23	conversion	—	0	8	30	40	90	100
			+	0	0	0	1	2	2
23 *cin*⁻		colony formers	—	1.2	2.0	2.5	5.0	5.5	8.2
			+	1.0	1.2	1.3	1.0	1.5	1.2
26 *cin*⁻	*wx*26	conversion	—	1	10	45	70	90	100
			+	0	0	0	2	2	2
26 *cin*⁻		colony formers	—	1.2	1.5	2.0	2.5	4.2	8.0
			+	1.6	2.0	2.0	2.0	2.0	2.0

CA = chloramphenicol: — nil, + 250 μg/ml

in the presence and absence of chloramphenicol. The number of colony forming units was determined in parallel experiments. Exponential phase bacteria showed 100% conversion in 5 hr, whereas with chloramphenicol the conversion rate was only 1–2%. On the basis of experiments with phage *wx* [7] it was evident that phages *wx*23 and *wx*26 converted the homologous *cin*⁻ bacteria more rapidly than did *wx* its *cin*⁻ culture. In the presence of chloramphenicol none of these phages converted the cultures to phospholipase A production (Table III). In agreement with previous findings [7], it would appear that the receptor for the two phages, similarly as the receptor for *wx*, is produced only in actively growing bacteria and no adsorption occurs in resting cells.

Examination of concentrated lysates of B. cereus strains and demonstration of the identity of bacteriocins with phospholipase A. The concentrates contained 10 000–60 000 units of bacteriocin (megacin A). As seen in Table IV, the degree of concentration varied from strain to strain.

Table IV shows the phospholipase A content and the specific activity of the concentrates. All concentrates were subjected to thin layer chromatography in order to confirm the presence of phospholipase A. Fig. 3 shows the cleavage of the concentrated lysate of *B. cereus* strain 27 on phosphatidyl ethanolamine. As lysophosphatidyl ethanolamine was not available, the lysate

Table IV

Bacteriocin ("megacin A") and phospholipase A activity of concentrates obtained from lysates of B. cereus strains

Strain	Protein, mg/ml	Megacin A titre, U/ml	Specific megacin A activity	Phospholipase A activity, U/ml	Specific enzyme activity
22	1.62	10 000	6 300	480	295
23	1.30	10 000	7 700	440	338
26	2.75	30 000	11 000	1440	525
27	4.10	60 000	14 500	2120	515
28	1.00	20 000	20 000	600	600
W	3.74	60 000	16 000	1440	385

from *B. cereus* W was used as a control of phospholipase A activity. The concentrated lysates of the five strains examined and of *B. cereus* W were similar in the order of specific activity for phospholipase A.

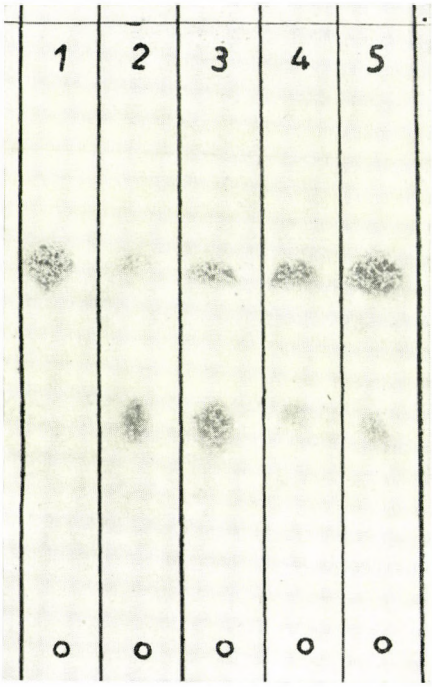

Fig. 3. Thin layer chromatography for identification of phospholipase A activity of the concentrates. Solvent, chloroform : methanol : water (65 : 25 : 4 v/v). Detection: iodine vapour followed by starch solution treatment of the silica gel layer. 1. L-phosphatidyl ethanolamine (dipalmitoyl). 2. Action of concentrate on *B. cereus* W strain. 3–5. Serial twofold dilution was made of the basal dilution of the concentrate obtained from strain 27. Lower spots indicate lyso-phosphatidyl ethanolamine

Discussion

Part of several dozen of *B. cereus* strains isolated from soil produced megacin A (phospholipase A). Five strains showing the presence of high titre bacteriocins in their lysates after MC induction were selected for further studies. These cultures exhibited multiple lysogenicity: in addition to the prophage responsible for megacin A production they carried another prophage differing from phage β of *B. cereus* W. Under the effect of ethidium bromidie, all five strains gave off segregants not producing phospholipase A. In the lysates of three of the strains, phages converting to phospholipase A production could not be detected; accordingly, it may be assumed that, although the gene segment giving information for phospholipase A production was present, the prophage was defective in these cultures. The phages shown in the other two strains differed in size from converting phages of *B. cereus* W; their relationship to the latter, however, was proved by a similar antigenic

structure and high host specificity. It is therefore justified to regard *B. cereus* phages associated with phospholipase A production as a characteristic group of bacterial viruses and to use the term *wx* for their designation. Phages belonging to this group may differ in size and thermosensitivity. While the virions of the phages showed an identical degree of heat tolerance, phages *wx*23 and *wx*26 formed plaques only at temperatures lower than 37°C (optimum at 26°C). The relationship of the converting phages isolated in our studies is evident also from the fact that in resting cells the receptors for them are absent, whereas in dividing cells an unidentified precursor serves as the receptor. This hypothetical precursor loses its adsorbing capacity after incorporation into the cell.

REFERENCES

1. McCloy, E. W.: J. Hyg. (Lond.) **40,** 114 (1951).
2. Gaál, V., Ivánovics, G.: Z. allg. Mikrobiol. **12,** 393 (1972).
3. Gaál, V., Ivánovics, G.: Acta microbiol. Acad. Sci. hung. **20,** 210 (1973).
4. Ivánovics, G., Gaál, V., Prágai, B.: J. gen. Virol. **24,** 345 (1975).
5. Csiszár, K., Ivánovics, G.: Acta microbiol. Acad. Sci. hung. **12,** 73 (1965).
6. Rabussay, D., Zilling, W., Herrlich, P.: Virology **41,** 91 (1970).
7. Gaál, V., Ivánovics, G.: Acta microbiol. Acad. Sci. hung. **23,** 277 (1976).
8. Yamamoto, K. R., Alberts, B. M., Benzinger, R., Lawhorne, L., Treiber, G.: Virology **40,** 734 (1970).
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.: J. Biochem. **193,** 265 (1951).
10. Gallai-Hatchard, J. J., Thompson, R. H. S.: Biochim. biophys. Acta (Amst.) **98,** 128 (1965).
11. Smith, N. R., Gordon, R. E., Clark, F. E.: Aerobic mesophilic sporeforming bacteria. U.S. Dept. of Agriculture, Miscellaneous Publication No. 559 (1946).
12. Knight, B. C. J. G., Proom, H.: J. gen. Microbiol. **4,** 508 (1950).
13. Bradley, D. E.: Bact. Rev. **31,** 230 (1967).

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