Lysogenic Conversion to Phospholipase A Production
in *Bacillus cereus*

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SUMMARY

A double lysogenic *Bacillus cereus* strain w (*β, wx*) induced with mitomycin C liberates phages *β* and *wx*. In addition the lysis of the organism is associated with the accumulation of a bacteriocin which was identified as phospholipase A. When the bacteria were cured of prophage *wx* they lost the enzyme production ability; the w (*β*) bacteria, named for convenience as *cin⁻* bacteria, could be converted to phospholipase A production by *wx* phage infection. Phage *wx* and its mutant *wxc* were very slowly adsorbed by *cin⁻* bacteria in a liquid medium and did not propagate noticeably, but the presence of agar led to normal multiplication. The rate of lysogenic conversion was also enhanced by agar. The bulk of the enzyme produced by the convertant was isolated as a homomolecular protein and identified as phospholipase A.

INTRODUCTION

McCloy (1951) isolated and described a particular lysogenic strain of *Bacillus cereus* designated w. Strain w liberated a phage highly specific for *Bacillus anthracis*; this phage was named *β*. A recent investigation (Gaál & Ivánovics, 1972) of the susceptibility of a number of lysogenic strains of *B. cereus*, including strain w, to induction by mitomycin C (MC) showed that all the strains underwent mass lysis associated with phage liberation (Gaál & Ivánovics, 1972, Nagy & Gaál, 1974). In addition to phage released by the two strains w and v8, considerable amounts of bacteriocin were also found which accumulated in the cultures during the progress of lysis (Gaál & Ivánovics, 1972). The bacteriocins of two strains exhibited similar characteristics and a mode of action similar to that of the bacteriocin named megacin A produced by some strains of *Bacillus megaterium*. The literature on this bacteriocin has been reviewed (Ivánovics, 1965).

A recent development in the field of megacinogeny was the observation by Ozaki *et al.* (1966) that megacin A produced by strain 216 of *Bacillus megaterium* was identical with phospholipase A. It might be postulated that the bacteriocin produced by *Bacillus cereus* strain w after induction is also phospholipase A; this assumption was verified on a purified preparation of lysate (Gaál & Ivánovics, 1972).

Some observations indicated that the genetic control of megacinogeny, i.e. inducible phospholipase A production, might be governed by a plasmid (Ivánovics & Nagy, 1958). Prophage *β* contained in strain w of *Bacillus cereus* cannot account for phospholipase A production, and thus another plasmid, or perhaps a prophage, may be responsible for formation of the enzyme in this strain. That a plasmid might be involved was suggested by the fact that when w bacteria were grown in the presence of acridine orange, segregants appeared in the culture which were not able to produce bacteriocin. Moreover, these clones, referred to as *cin⁻* bacteria, were sensitive to the lysate of strain w, which produced distinct turbid plaques on them.

Plaque formation on cin^- bacteria was not prevented by antiserum to phage β, and therefore it was justified to assume the presence of a second phage in the lysate of strain w. This second phage was designated as wx, and its mutant, a clear plaque former, wxc (Gaál & Ivánovics, 1973). The aim of the present study was to elucidate the relationship between phage wx and bacteriocinogeny i.e. phospholipase A production by the bacteria.

<center>METHODS</center>

Bacterial strains. A doubly lysogenic *Bacillus cereus* strain w (β, wx), producing a bacteriocin, and its segregant w (β), designated for the sake of convenience as cin^- bacteria, were used in these experiments (Gaál & Ivánovics, 1973). The cin^- bacteria were used to assay phages wx and wxc. MUT-C of *Bacillus megaterium* was used as an indicator strain for the bacteriocin; and *Bacillus anthracis*, strain Davis, for assaying phage β.

Phage materials. A mixture of temperate phages β and wx was obtained by induction of culture w (β, wx) with mitomycin (MC) (Gaál & Ivánovics, 1973). Since the lysates thus obtained contained a low number of p.f.u. of each phage, they were concentrated by differential sedimentation and the virus particles were finally precipitated with polyethylene glycol as recommended by Yamamoto *et al.* (1970). The product resulted in a phage material containing both phages β and wx in concentrations of 10^6 and 10^9 to 10^{10} p.f.u./ml, respectively. Phage wxc was propagated in a soft agar layer seeded with cin^- bacteria. If necessary the lysates were purified as described above.

Media and buffers. YP consisted of a 'home-made' yeast extract peptone (Richter) (Csiszár & Ivánovics, 1965). For comparison, most experiments were carried out simultaneously with $BPYe_1$ medium, consisting of Difco ingredients supplemented with certain cations (Gaál & Ivánovics, 1973), BCM, a casamino acid medium fortified with 0·1 % yeast extract (Difco) (Ivánovics, Varga & Marjai, 1964). Dilutions were usually made in saline buffered with 20 % YP. Purified phage preparations were suspended in buffer as recommended by Rabussay, Zilling & Herrlich (1970).

Antiphage serum. Rabbits were immunized with purified phage wxc material (Gaál & Ivánovics, 1973). Since the phage material was contaminated with phage β, the antiserum also contained traces of antibody to this phage. The K value against phage wxc was 45 and the temperate phage wx was neutralized to the same extent.

Electron microscopy. Purified phage material was placed on carbon-impregnated Formvar films negatively stained with 2 % (w/v) uranyl acetate, and examined with a JEM–100B electron microscope.

Cultivation of bacteria, and titration of the phages and bacteriocin. Full details of these techniques are described in our previous papers (Gaál & Ivánovics, 1972, 1973).

Chemicals. Most chemicals were grade A, purchased from various firms. Mitomycin C, grade B, was a product of Calbiochem. Milliporefilters (Sartorius, Göttingen), pore size 0·45 μm, were used to filter phage materials.

Technique of phage conversion. When cultures of cin^- bacteria, grown either in YP or $BPYe_1$ media, attained an extinction of 0·25 at 620 nm (4×10^7/ml colony formers, i.e. 2×10^8/ml bacteria estimated on the basis of the average chain former), 0·5 ml samples were placed in several test tubes; samples of phage material were added (0·1 to 5 m.o.i.) and the total vol. was made up to 1·5 ml. The tubes were incubated in a 37 °C water bath and 0·25 ml samples were taken at intervals and rapidly centrifuged for 5 min. The sediments were resuspended in 1 ml of 1/50 dilution of antiphage serum wxc; in this way 99·9 % of the free phages were neutralized in 30 min. The bacteria were recentrifuged, taken

up in 2 ml of buffered saline, and serial decimal dilutions were prepared. One ml of melted YP agar at 47 °C was mixed with bacteria of the MUT-C bacteriocin indicator strain, and various dilutions (0·1 ml) of the samples were mixed with melted agar to which 0·1 ml (0·5 μg) MC had been added. The two were mixed and the total vol. of the system was made up to 2 ml, which was plated as an overlay on a YP agar plate. The ratio of the number of colonies producing a distinct halo to the number without halos was taken as the conversion rate.

Conversion was also estimated when a mixture of *cin⁻* bacteria and phage *wx* was smeared on agar plate and incubated at 37 °C; the bacteria were washed off with 1 ml of buffer, treated with antiphage serum, and plated as described above.

Preliminary concentration of the bacteriocin produced by a converted clone. An isolated colony surrounded by a distinct bactericidal halo in the MUT-C bacterial lawn was purified by restreaking. An overnight culture in 10 ml of BCM was added to 450 ml of BCM in a 2000 ml Erlenmayer flask and the culture was aerated at 37 °C in a 'Gyrotory' shaker (New Brunswick Sci. Co.) at 250 rev/min. When the bacterial growth attained an $E_{620} = 0·25$, mitomycin was added (0·5 μg/ml) to the culture, which was reincubated until mass lysis occurred at 100 min. This was followed by rapid cooling of the culture in a melting-ice bath. The residual bacteria were then sedimented in an MSE high speed 18 at 15000 *g* for 30 min. Ammonium sulphate was added to 75 % saturation to the supernatant fluid adjusted to pH 7·0 which was then stored at 4 °C overnight. The precipitate formed was separated by high-speed sedimentation, and the resultant pellets extracted in 4 ml of 0·02 M-tris-HCl (pH 7·2) and centrifuged at 15000 *g* for 15 min. The crystal-clear supernatant fluid was collected and the pellets again extracted in a similar manner three times. The combined extracts were dialysed against tris-HCl buffer. The total vol. of dialysate was 20 ml, containing 2 mg/ml protein its bacteriocin titre proved to be 2×10^4 unit/ml (Gaál & Ivánovics, 1972). This extract is referred to in the Results section as semi-purified bacteriocin preparation.

Assay of phospholipase A. A colorimetric assay based upon the colour reaction of acyl esters with iron hydroxamate (Brown & Bowles, 1966) was used. A sample solution of egg yolk lecithin purified according to Singleton *et al.* (1965) was evaporated under a stream of N_2. The lipid was emulsified in 0·05 M-tris-HCl buffer, pH 8, containing 10 mM-CaCl₂ and 0·1 M-NaCl, supplemented with sodium deoxycholate (250 μg/ml) and Triton X-100 (Serva) (1 μl/ml) to give a lecithin concentration of 2 mg/ml. The emulsion was homogenized in an MSE ultrasonic power unit. Several twofold dilutions of each eluate fraction were made and 0·1 ml of each of these dilutions was mixed with 1 ml of the lipid emulsion. The assay systems were incubated at 37 °C for 30 min. The iron hydroxamate solution was prepared and the procedure was carried out as described by Augustyn & Elliott (1969). The extinction developed was measured at 525 nm in a Unicam SP 800 photometer.

As the phospholipase A hydrolyses only one of the two acyl ester bonds, the final product of the reaction is lysolecithin. At any moment the extinction is comprised of the extinctions of the unsplit lecithin and the lysolecithin formed. The amount of acyl ester bonds, in μEq, was calculated using the following formulae:

$$E_0 = 2 . E_e . \gamma,$$
$$E_t = 2 . E_e . \gamma - E_e . c_t,$$

where: E_0 = extinction value at time 0; E_e = extinction value per ester group; E_t = extinction value at time *t*; γ = lecithin (μmol) in the assay system; c_t = lysolecithin formed (μmol) at time *t*.

Table 1. *The size of the structural elements of the phages*

	wxc	β
Diam. of head	733*	600
Diam. of tail	100	133
Length of tail	1535	2333

* All measurements are angstroms.

The enzyme activity was calculated for an assay system containing 1 to 2 μm protein. The unit of enzyme activity was defined as the amount of enzyme catalysing the splitting of one μEq of ester bonds at 37 °C in 30 min.

The identification of phopholipase A activity. Some of the peak fractions eluted from the DEAE-cellulose column were used for the identification of the activity of the enzyme by thin-layer chromatography. The method was as described previously (Gaál & Ivánovics, 1972) except that the spots were developed with iodine vapour, followed by starch solution treatment of the silica gel layer.

Gel electrophoresis. The polyacrylamide gels were prepared in the usual way (Davis, 1964). A disc measuring 60 × 10 mm contained 7·5 % of polyacrylamide (Cyanogum 41) in 0·75 M-acetic acid and 0·12 M-KOH buffer, pH 4·3, containing 7 M-urea, 0·14 M-acetic acid and 0·35 M-β-alanine, pH 4·5, were used in the electrode vessels. The combined fractions of the peak eluted from the DEAE-cellulose column were dialysed and concentrated with Carbowax 20000. 100 μl samples of the preparation containing 100 to 125 μg protein were layered on top of the column. One of the tubes was stained with 0·1 % Amido black. An unstained column was sliced into 16 equal pieces. Each slice was extracted with 1 ml of saline containing 20 % YP; extracts were assayed for antibacterial activity.

Protein estimation. The method of Lowry *et al.* (1951) was used.

RESULTS

Some characteristics of phages β, wx *and the mutant* wxc

The plaque morphologies and the host ranges of *wx* and *wxc* have already been described (Gaál & Ivánovics, 1973). Phage β did not form plaques on *cin⁻* bacteria but it did on *Bacillus anthracis*, strain Davis. In contrast, the only host for phages *wx* and *wxc* was *cin⁻* bacteria of strain w. When a few hundred *wxc* phages were plated with *cin⁻* bacteria, confluent lysis occurred. The extract of the soft agar layer contained both β and *wxc* phages the latter being predominant. Phages β and *wxc* differed in size and shape (Table 1 and Fig. 1). The phage β tail exhibited a definite cross striation; it was constricted at the neck. The distal end of the tail showed no clear structural definition but appeared to terminate in an end plate, with short lobate projections. Phage *wxc* had a hexagonal head and a short tail without definite structure. Phage material grown on *cin⁻* bacteria was examined as to the distribution of the two phages in a sucrose density gradient. Fig. 2 shows the distribution of the phages in the individual fractions. The peak values were in different fractions. Phage *wxc* is heavier than phage β. This is in accordance with the difference in the head sizes. If the phage heads are considered as regularly spherical, the ratio of their masses is 1·22:1·0.

Phages *wx* and *wxc* displayed an unusual, and hitherto unreported feature. Where *cin⁻* bacteria were infected with either *wx* or *wxc* particles at 0·1 to 2·0 m.o.i. in liquid nutrient media and aerated by shaking, the phages did not propagate. Several liquid media, including
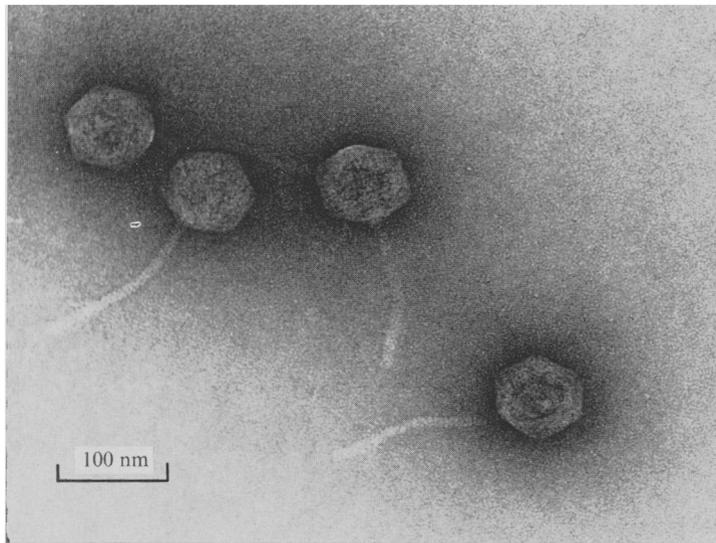
Phage *wxc*
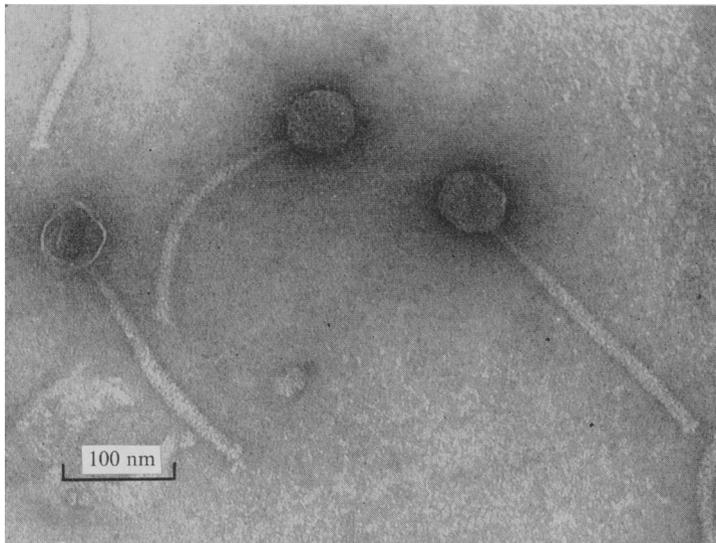
Phage *β*

Fig. 1. Electron microscope examination of phages *wxc* and *β*.

YP and BPYe₁ and others not dealt with in the Methods section were tried before and after supplementing with amino acids and different cations at various concentrations and varying the pH in the range of 5·5 to 8·5. The results of these experiments led us to conclude that the presence of agar may play a decisive role, for in media containing 0·2 to 0·7 % agar the phages propagated in *cin⁻* bacteria. This problem seems all the more complex, when one considers the factors involved in the kinetics of phage multiplication. The question is under detailed study, the results of which will be published later.
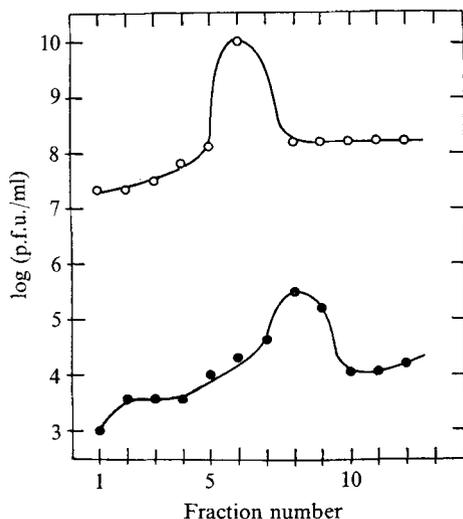
Fig. 2. Distribution of the phages *wxc* and β on a 30 to 60 % (w/v) linear sucrose gradient in 0·02 M-tris-HCl buffer for 120 min at 80000 g at 20 °C in a VAC centrifuge. The sucrose density gradient column contained 4 ml on which was placed 0·2 ml of purified phage material. The tube was punctured and 24 successive drops were collected and assayed. O—O, p.f.u. of phage *wxc*; ●—●, phage β; assayed with *cin⁻* and Davis bacteria, respectively.

Table 2. *Conversion rates in liquid and agar media* *

Medium	KCN (0·01 M)	m.o.i.	Approximate percentage conversion rates after an incubation of (h)						
			0	2	3	4	6	8	16
Liquid	—	5	2–3	5–6	.	20	40	70	100
Liquid	+	5	1–2	2–3	.	10	20–23	40–50	100
Agar	—	5	3–4	60	100	.	.	.	.
Agar	+	5	2–3	45	100	.	.	.	.
Liquid	—	2	1	5	.	.	20	.	.
Agar	—	2	2	80	100	.	.	.	.
Agar	+	2	2	10	.	.	90	.	.

* The values presented in the Table are the pooled data of few different experiments. Either the range of the conversion ratio or the approximate average value is given.

Conversion of cin⁻ bacteria to phospholipase A bacteriocin-producers

The surface of a nutrient agar plate was inoculated with two drops of a *cin⁻* suspension (about 1×10^7 bacteria), evenly dispersed in a spot about 2 cm in diam. and dried. The bacterial inoculum was covered with 0·1 ml of *wx* phage material containing 2×10^6 p.f.u./ml. After incubation for 20 h the bacterial growth was washed off and examined for colonies producing bacteriocin. Practically all colony formers produced bacteriocin. When the inoculum was treated with a 1:100 dilution of phage, however, only 5 to 10 % of the bacteria had been converted. The growth exposed to phage *wx*, consisting mainly of spores, was suspended in saline, followed by heating at 65 °C for 20 min; the number of colonies with halo formation was the same as without heating. It is apparent in view of the above experiment that the bacteriocinogeny was due to the lysogenic state of the spores and not to phage contamination.

As regards both quantitative and kinetic aspects of the lysogenic conversion, the conflicting results shown in Table 2 posed some puzzles. One explanation might be that the
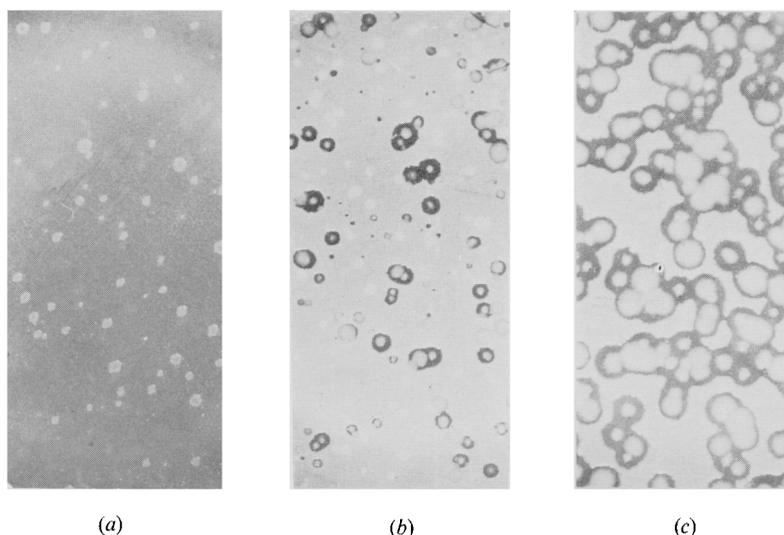
Fig. 3. Lysogenic conversion of *cin⁻* bacteria exposed to phage *wx* for different times. Phage-infected cells were mixed with bacteriocin-sensitive MUT-C bacteria and layered in soft agar and incubated. (*a*) o min; (*b*) 2 h; (*c*) 3 h.

multiplicity of phage infection affected the rate and velocity of conversion. The conversion rate was 6 to 8 % only when the m.o.i. was 0·1 in the liquid medium with a 20 h incubation; this observation leads us to conclude that the bacteria which became lysogenic did not burst spontaneously. The slow rate of conversion was also unexpected when there was an abundance of phage in the liquid medium. In contrast, when the *cin⁻* bacteria were exposed to phage infection on agar medium, the whole bacteria population was converted within 3 h (see Fig. 3). The presence of KCN did not influence the results significantly; the logical interpretation of this finding is that the phage adsorption, rather than replication of virus particles, is delayed in the liquid medium. The conversion to bacteriocinogeny failed when phage *wxc* was used; the clear plaque formers apparently killed the *cin⁻* bacteria.

Investigation of converted clones

An individual liquid culture consisting of several clones with halos was induced on addition of MC. The lysis of the cultures was intense and occurred within about the same time as for wild type bacteria w. The lysate of the converted clones gave 10^8 p.f.u./ml, compared with only 10^6 p.f.u./ml for the original strain w. In contrast the lysate of w contained about 25 to 30 % more bacteriocin than that of the converted strain. When the lysate was made from the original strain w, 50 % of the phospholipase A was precipitated on adjustment of the pH to 4 (Gaál & Ivánovics, 1972), whereas under the same conditions only an insignificant amount of the activity was precipitated from the lysate of converted clones. In the earlier investigation (Gaál & Ivánovics, 1972), the phospholipase A produced by the wild type strain was assayed by semiquantitative methods, based on haemolysis by the lysolecithin formed. It was essential that a more exact method be used for identification of the phospholipase A produced by the converted clones. For the isolation of the phospholipase A and its identification as a bacteriocin, a sample of the concentrate described in the Methods section was fractionated on a DEAE-cellulose column. Fig. 4 shows the protein profile and the activity distribution. One part of the protein with no activity was washed off
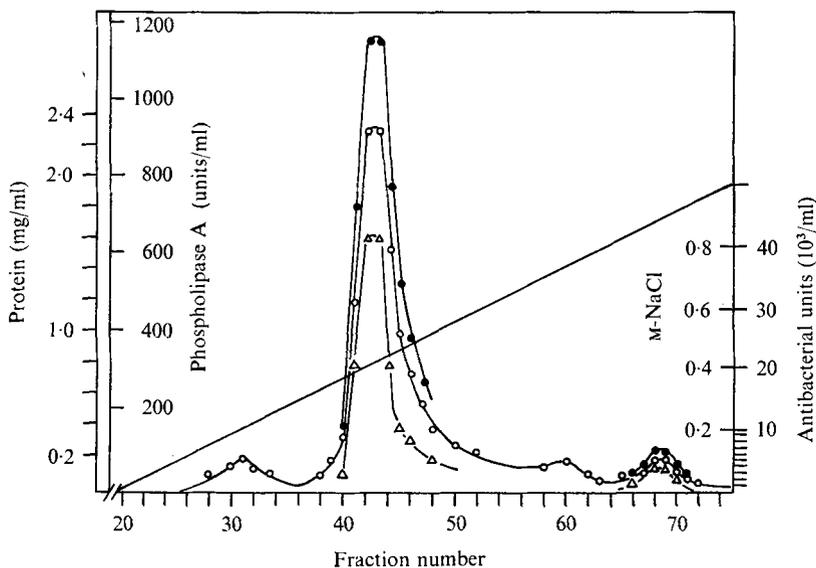
Fig. 4. Elution profile of bacteriocin from DEAE-cellulose column. Bed dimensions: 15 × 200 mm. Sample: 30 mg protein in 15 ml concentrate of the bacteriocin from a converted clone. When 20 ml of 0·02 M-tris-HCl, pH 7·2, was used as starting buffer, 8·5 mg inactive protein was washed off. The absorbed protein was eluted with increasing NaCl in buffer. Two ml fractions were collected: ●—●, phospholipase A activity; △—△, antibacterial titre; ○—○, protein material absorbing at 280 nm.

the column with buffer in fractions 0 to 20. The peak consisting of fractions 40 to 50 contained the bacteriocin and phospholipase A activities. It was striking that a second, small activity peak was present in fractions 66 to 72. This suggested that the bacteriocin is identical with phospholipase A, although two different enzymes may occur in the converted clone lysate. Fig. 5 shows the results of polyacrilamide gel electrophoresis of the main peak from the DEAE-cellulose column. The main band gives bacteriocin activity, the extract of the upper light band was inactive in the bacteriocin test. The specificity of phospholipase A activity was identified on a thin-layer chromatogram (Fig. 6). The reference spots of lecithin and lysolecithin were in the same positions in the chromatogram as the substrate and its product in the assay system.

A sample of the main active fraction was diluted with 0·05 M-acetate buffer, pH 5, in a test-tube and submerged in a boiling water bath for 5 min. When assayed for bacteriocin activity the heated and control samples gave identical titres. The thermal stability of the enzyme suggested that it was phospholipase A_2 (van den Bosch et al. 1965). The small active peak of the eluate was subjected to a detailed investigation. Whether this protein phospholipase A splits another ester bond or has a different isoelectric point is still undecided.

DISCUSSION

Since the discovery that Corynebacterium diphtheriae acquires by phage infection a hereditary ability to synthesize toxin (Freeman, 1951), several examples of lysogenic conversion have become known. It is not our intention to discuss lysogenic conversion, except to mention one example where lysogeny apparently caused an alteration in lipid metabolism. Duval-Iflah (1974) found that lysogenic conversion of Staphylococcus pyogenes suppressed lipase
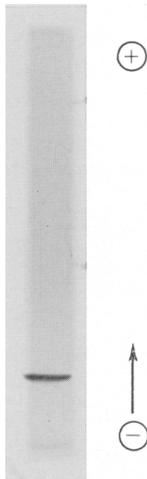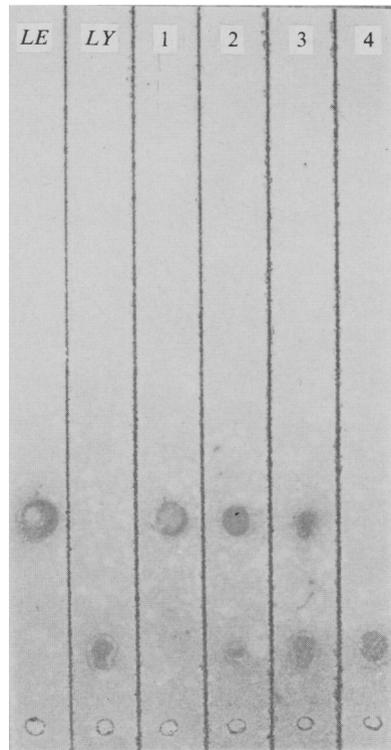
Fig. 5

Fig. 6

Fig. 5. Acrylamide gel electrophoresis of the peak fraction.

Fig. 6. Phospholipase A activity identified with thin-layer chromatography; LE = lecithin; LY = lysolecithin; Serial twofold dilution was made of the peak fraction and each dilution was added to the enzyme systems. 1 designates the highest dilution (1:8).

activity. In our case the lysogeny did not suppress enzyme activity, but governed the production of an enzyme involved in lipid metabolism. As regards its control of phospholipase A production, phage *wx* showed a high degree of specificity; not a single strain from one hundred belonging to several species of the genus Bacillus was capable of supporting the propagation of either phage *wx* or *wxc* (Gaál & Ivánovics, 1973).

Another particular feature of phages *wx* and *wxc* is a requirement for agar micelles, which are probably needed for the stage of adsorption to the bacteria. This is still only our conception; further study is necessary.

A great many wild strains of *Bacillus cereus* were screened for the production of bacteriocin (apparently phospholipase A) after induction with mitomycin C, but only a few exceptional strains gave lysates of high titre (unpublished observations).

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