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A SPECIFIC INHIBITOR OF MAGACIN A FROM *BACILLUS MEGATERIUM* PRODUCING MEGACIN A

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SUMMARY 1. A specific inhibitor of megacin A was found in the culture supernatants of *Bacillus megaterium* strain 216 producing megacin A and its mutants. This inhibitor was partially purified.

2. The inhibitor is heat labile but its chemical nature is still unknown.

3. It reversibly inhibits or slows down the turn-over rate of the phospholipase A activity of megacin A but it exerts no inhibitory effect on the phospholipase A of "Habu" (*Trimeresurus flavoviridis*) venom.

4. It seems to be important in giving the bacteria immunity against the megacin A they themselves produce.

INTRODUCTION

Bacillus megaterium strain 216 is sensitive to a high concentration of self-produced megacin A (Iványovics et al., 1954), while megacin A negative mutants are about 50 times more sensitive to megacin A than the parent (Holland and Roverts, 1964; Ozaki et al., 1966). The lower sensitivity of the parent might indicate that it is partially immune to megacin A.

Megacin A was identified as phospholipase A (phosphatide acylhydrolase, EC 3.1.1.4) in this laboratory (Ozaki et al., 1966). Subsequently, attempts were made to study the mechanism of immunity to megacin A. It was found (Ozaki et al., 1967) that protoplasts retained immunity when tested by protoplast-lysis (Iványovics, Alföldi and Nagy, 1959a) and that the immunity was highly specific for the phospholipase A activity of megacin A but not for that of heated "Habu" (*Trimeresurus flavoviridis*) venom.

For further studies on the mechanism of immunity, less sensitive mutants, if any, were needed. Accordingly, attempts were made to isolate mutants which were more and less sensitive than the parent strain 216 among bacteria surviving after induction of megacin A with N-methyl-N'-nitro-N-nitrosoguanidine (Marjai and Iványovics, 1964; Iványovics, 1965). In studies on the less sensitive mutants isolated,

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an inhibitor of megacin A was found in the culture fluid. The present paper describes results of studies on this inhibitor.

MATERIALS AND METHODS

1. *Bacterial strains used*

Bacillus megaterium strain 216, producing megacin A, was used as a source of megacin A. *B. megaterium* strain KM was very sensitive to megacin A and was used as an indicator for megacin A.

2. *Media*

The P-Y medium, used for growth of the strain 216, its mutants and strain KM, consisted of a dialyzate of polypeptone (Daigo Nutritive Chemicals Co., Japan) 10 g, a dialyzate of yeast extract (Daigo Nutritive Chemicals Co.) 5 g, NaCl 3 g, K_2HPO_4 0.73 g, KH_2PO_4 0.27 g and H_2O 1 liter (pH 7.2).

The nutrient agar, used for titrations of megacin and megacin inhibitor, consisted of P-Y medium containing 15 g agar per liter. In preparation of this, the polypeptone and yeast extract were not dialyzed. Overlay soft-agar medium contained 3 g instead of 15 g of agar per liter.

In some experiments, synthetic medium was used, consisting of glucose 5 g, glutamic acid 10 g, NH_4Cl 0.5 g, K_2HPO_4 7.3 g, KH_2PO_4 2.7 g, $FeCl_3$ 2.0 mg, $MnCl_2 \cdot 4H_2O$ 4 mg, $MgCl_2$ 0.2 g, Na_2SO_4 0.5 g and H_2O 1 liter (pH 7.2).

3. *Assay of megacin*

Titration of megacin activity by spot tests was carried out as described by Iványovics et al. (1957) with two exceptions: 1) *B. megaterium* strain KM was used as an indicator for megacin. 2) One unit of megacin activity is expressed as the activity of the highest dilution giving complete inhibition of growth of the indicator strain within the spot, and units of magacin are expressed as the dilution factor without dividing this by the volume (ml) of one loopful of fluid.

4. *Protoplasts*

A culture in the exponential growth phase was centrifuged and washed twice with saline, and cells were resuspended in 1 : 15 M phosphate buffer (pH 7.2) containing 15% (w/v) sucrose. The suspension was incubated at 30 C for 5 min with lysozyme (final concentration, 100 μg per ml). $MgCl_2$ was added to a final concentration of 0.004 M and incubation was continued for 30 min without shaking. The optical density at 550 mμ of the suspension was adjusted to approximately 0.6.

In some experiments, suspensions of washed protoplasts were used. After lysozyme treatment and stabilization by addition of $MgCl_2$, the suspension was centrifuged at 4,000 rev/min for 20 min and the supernatant was removed by aspiration. The dense portion above the pellet was resuspended in fresh buffered sucrose solution containing 0.004 M $MgCl_2$ leaving the pellet at the bottom.

5. *Protoplast-lysis*

Volumes of 5 ml of protoplast suspensions in model 14–302A cuvettes of a Coleman Junior spectrophotometer were mixed with 0.5 ml of buffered sucrose containing the test material. In the control, buffer was added in place of the test material. The cuvettes were incubated at 30 C. Every 10 min the cuvettes were inverted to mix their contents and their optical density at 550 mμ was read.

In studying the effects of the megacin inhibitor on protoplast-lysis by megacin, 3.0 ml of a more concentrated protoplast suspension in cuvettes were mixed with 2.0 ml of buffered sucrose containing a mixture of megacin and megacin inhibitor, which had been incubated at 37 C for 30 min.

The optical density of the reaction mixture was adjusted to approximately 0.5 at 550 mμ.

6. *Lecithin agar for measurement of indirect hemolysis*

A lecithin suspension was prepared by sonicating 200 mg of phosphatide in 40 ml of 0.005 M Tris buffer (pH 7.5) containing 0.15 M NaCl. Sodium chloride 9 g, agar 15 g and 0.005 M $CaCl_2$ were dissolved in 960 ml of 0.005 M Tris buffer (pH 7.5) in a boiling water bath. One ml of lecithin suspension was mixed with 24 ml of molten agar in a plate and the mixture was allowed to solidify.

Overlay soft agar consisted of NaCl 9 g, agar 3 g and 0.005 M Tris buffer (pH 7.5) in 1 liter. Then 3 ml of molten soft agar were mixed with washed rabbit red blood cells at 50 C, and poured over the lecithin agar as described in the " Experiment."

7. *Thin layer chromatography (TLC)*

Silica gel H (Merck) plates were used for TLC. Phospholipids were applied to the plates. The following solvents were employed for chromatography: solvent I, (chloroform-methanol-water, 65 : 25 : 4),

(Mangold, 1961), for separation of lysolecithin from lecithin, solvent II, (benzene-ethyl ether-ethyl acetate-acetic acid, 80 : 10 : 10 : 0.2) (Storry et al., 1967), for separation of free fatty acids from pbospholipids and lysophospholipids, solvent III, (phenol-water-acetic acid-ethanol, 8 : 2 : 1 : 1) (Higashi et al., 1966), for separation of lysolecithin and derivatives containing choline and solvent IV, (chloroform-acetone-methanol-acetic acid-water, 10 : 4 : 2 : 2 : 1) (Siakotos, 1965), for separation of each component of the phospholipids of *B. megaterium* strain KM.

Phospholipids and derivatives containing phosphorus were located by Dittmer's reagent (1964), substances containing choline with Dragendorff's reagent (Wagner et al., 1961), phosphatidyl ethanolamine with ninhydrin (Wagner et al., 1961) and free fatty acids with iodine (Mangold et al., 1960).

8. *^{14}C-labeled phosphatidylethanolamine*

B. megaterium strain KM was grown overnight in 1 liter of a modified synthetic medium, in which the concentrations of glucose and glutamic acid were reduced to 0.4 and 0.2% (w/v), respectively, and 2 mc of acetate-1-^{14}C were added per liter. The cells were centrifuged and washed once and then suspended in 20 ml of saline. Then 300 ml of chloroform-methanol (1 : 1) were added. The mixture was stood for 3 hr at room temperature, and then centrifuged and the supernatant was mixed with 120 ml of saline. The chloroform layer was separated and the chloroform was evaporated off. The residue was extracted with cold acetone. The residue was dissolved in chloroform and applied in a line on a TLC plate. After the development with the solvent IV, four bands were visualized by exposing strips at the edge of the plate to iodine vapor. The band corresponding to phosphatidylethanolamine was scraped off, extracted with chloroform-methanol (1 : 1) and concentrated. In this phosphatidylethanolamine preparation, the label was mainly in the carbon atoms of the fatty acids (Cronan and Wulff, 1969).

9. *Assay of phospholipase A activity*

The substrate solution was composed of 0.1% lecithin, 0.002 M $CaCl_2$, 0.1% sodium deoxycholate and 0.1 M Tris buffer (pH 7.5). Equal volumes of megacin preparation and substrate solution were mixed and incubated at 37 C for 30 min. The reaction mixture was treated with 4 volumes of chloroform-methanol (2 : 1). The chloroform layer was applied to TLC with solvent I.

In experiments on the liberation of free fatty acids, lecithin in the substrate solution was replaced by ^{14}C-labeled phosphatidylethanolamine (10^6 count/min/-ml). Each reaction mixture was composed of 50 μl of substrate solution and 50 μl of megacin preparation. The tubes were stoppered, and incubated at 37 C for 30 min, and 0.2 ml of chloroform-methanol (1 : 1) were added to each. The chloroform layer was removed and the methanol-water layer was extracted twice with a half volume of chloroform. The chloroform extracts were pooled and concentrated. A small amount of unlabeled palmitic acid dissolved in chloroform was added to the extract and the mixture was applied to TLC with solvent II. Spots of free fatty acids were located with iodine and scraped off. The radioactivity of the powder was determined using a Beckman liquid scintillation counter, model LS-150.

10. *Protein determination*

Protein was measured by the biuret method, or by measurement of the absorbancy at 280 mμ using a Hitachi spectrophotometer (Type EPU-2A).

11. *Determination of nucleic acids*

Orcinol (Mejbaum, 1939) and tryptophan-perchloric acid (Cohen, 1944) were used for determination of RNA and DNA, respectively. Component sugars were detected with phenol sulfuric acid reagent (Dubois et al., 1956).

12. *Chemicals*

N-methyl-N′-nitro-N-nitrosoguanidine (NG) was obtained from the K & K Laboratory (New York, U.S.A.). NG was dissolved in water just before use, and, if necessary, sterilized by passage through a Millipore filter. Lecithin was prepared from egg yolk phospholipids by alumina column chromatography as described by Singleton et al. (1965). Lysolecithin was prepared using phospholipase A in heated " Habu " venom by the method of Hanahan (1952) or using megacin A and TLC.

RNase (DNase free) and DNase (RNase free) were kindly given by Drs. F. Imamoto and N. Morikawa of this Institute. Sodium deoxycholate and DEAE cellulose were products of Matheson Coleman & Bell Co. and Brown Co., respectively. Six times recrystalized lysozyme was a product of Seikagaku Kôgyô. Trypsin and trypsin inhibitor were purchased from Worthington Biochemical Corporation

and Sigma Chemical Co., respectively. Purified pronase was donated by Seikagaku Kôgyô Co.

Phospholipase A of " Habu " (*Trimeresurus flavoviridis*) venom was prepared as follows: the venom solution dissolved in 0.02 M acetate buffer, pH 4.0, was heated at 100 C for 5 min, and centrifuged. The supernatant was adjusted to pH 5.0 and chromatographed on a CM-cellulose column equilibrated with 0.02 M acetate buffer, pH 5.0. The column was eluted with a concentration gradient of NaCl in the same buffer. The fractions eluted with 0.3–0.35 M NaCl were combined, dialyzed against distilled water and lyophilized. The lyophilized material was dissolved in 0.02 M acetate buffered saline, pH 5.0, heated again at 100 C for 10 min and chromatographed on a Sephadex G25 column to replace the solvent by 0.005 M Tris buffered saline, pH 7.5. Phospholipase A was eluted just after the void volume. The preparation was diluted 1 : 5 before use.

RESULTS

1. *Isolation of mutants of strain* 216 *with high and low sensitivity to megacin*

A culture (10 ml) of *Bacillus megaterium* strain 216 was grown until its optical density reached 0.1. Then N-methyl-N'-nitro-N-nitrosoguanidine (NG) was added to a final concentration of 20 μg per ml and the mixture was incubated further at 37 C for 10 min. Then the culture was divided into two 5 ml portions which were treated as follows:

1) One hundred 0.05 ml aliquots were taken from one 5 ml portion. Each was diluted with 5 ml of warm medium containing 1 mg per ml of pronase to minimize the bactericidal effect of megacin on highly sensitive, non-induced mutants. These mixtures were incubated until growth of survivors after megacin induction reached the stationary phase. Then, 0.1 ml of each culture was diluted with 10 ml of fresh medium and mixtures were incubated at 37 C. When optical density of each culture reached 0.1, induction was carried out again by treatment with NG for 10 min and 0.05 ml of each culture was diluted with 5 ml of medium containing pronase and incubated at 37 C. This procedure was repeated once more. Each of the 100 cultures was plated on agar and colonies were tested for megacin production. For this, replica plates were made. The colonies on them were killed with chloroform vapor, covered with soft agar containing about 10^7 bacteria of *B. megaterium* strain 216. After incubation the inhibition zones were examined. Colonies producing little megacin were picked up and their sensitivities to megacin A were examined. Thus, more sensitive strains than the parent were obtained. Among the large number of mutants obtained, strains 400 and 462 were chosen as representative sensitive mutants.

2) The other 5 ml of culture, after induction with NG, were stored in the incubator and survivors, which grew in the presence of a high concentration of megacin A, were streaked on plates. Thus less sensitive mutants were isolated. All colonies were picked up and their sensitivities to megacin A were examined. Strains 253, 265 and 272 were chosen as representative mutants with low sensitivity to megacin.

2. *Sensitivities of isolated mutants to megacin A*

The sensitivities to megacin A of the strains isolated were tested in two ways: by spot tests and by tests of protoplast lysis.

1) Measurement of sensitivity by spot tests

The results are shown in Table 1. Strains 400, 462 and 261 were very sensitive and strains 265, 253 and 272 were not very sensitive to megacin A.

2) Measurement of sensitivity by protoplast lysis

Protoplasts of strain 216 and its mutants were unstable at 37 C but stable at 30 C, so experiments were carried out at 30 C. As shown in Figs. 1a, b, c, the results obtained by protoplast lysis agreed with these obtained by spot tests. Strains 253, 262 and 272 (less sensitive mutants found by spot tests) were less sensitive and strains 400 and 462 (more sensitive mutants found by spot tests) were more sensitive to megacin A on protoplast lysis than the parent

TABLE 1. *Sensitivity of mutants to megacin A*

Strain \ Megacin dilution	5^{-1}	5^{-2}	5^{-3}	5^{-4}	5^{-5}	5^{-6}	5^{-7}	5^{-8}
B. meg. KM (megacin non-producer)	4	4	4	4	4	4	4	2
B. meg. 216 (parent strain)	4	4	4	2	0	0	0	0
More sensitive mutant #400	4	4	4	4	4	2	0	0
#462	4	4	4	4	4	2	0	0
#261	4	4	4	4	2	0	0	0
Less sensitive mutant #265	4	2	0	0	0	0	0	0
#253	4	2	0	0	0	0	0	0
#272	4	2	0	0	0	0	0	0

4: Complete inhibition of growth 2: Slight inhibition 0: No inhibition

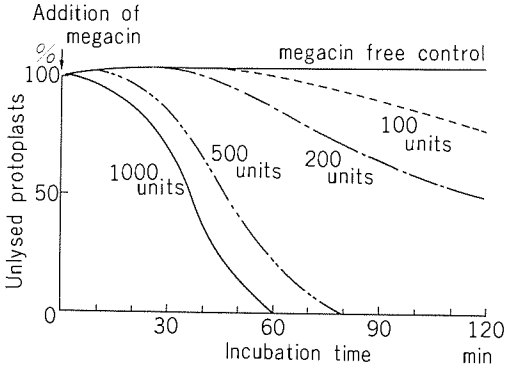

FIGURE 1a. *Protoplast-lysis of B. megaterium 216 (parent strain) by megacin.*

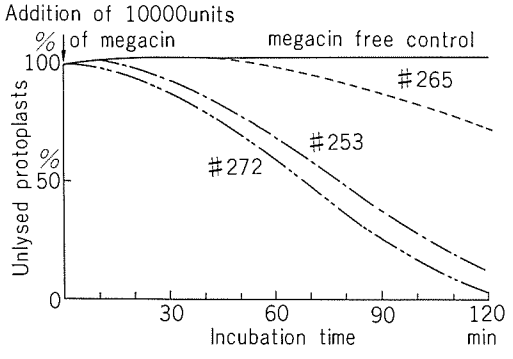

FIGURE 1b. *Protoplast-lysis of less-sensitive mutants by megacin.*

strain. Strain KM is exceptional but results by protoplast lysis can be explained that its protoplasts are more stable at 37 C than those

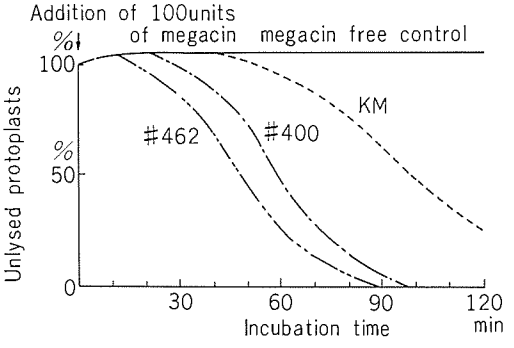

FIGURE 1c. *Protoplast-lysis of more-sensitive mutants by megacin.*

of the mutants.

To exclude the possibility of liberation of some factor from the cells which might affect the reaction of megacin with phospholipids of the protoplast membrane during digestion of the cell walls by lysozyme, similar experiments were performed with washed protoplasts suspensions. The results were similar to those described above.

3. *Production of megacin and the megacin inhibiting factor during growth of the less sensitive mutant* 265

The megacin titer was estimated in the supernatants of shaking cultures of the less sensitive mutant, 265. Megacin activity was found in the exponential phase but it disappeared in the

stationary phase, as shown in Fig. 2. The disappearance of megacin activity suggested that some factor accumulated in the supernatant in the stationary phase, which inhibited megacin activity.

This inhibitory activity was assayed in supernatants devoid of megacin activity. Serial 1 : 5 dilutions of the megacin preparation were prepared in saline containing 0.005 M Tris buffer, pH 7.5, and were mixed with an equal volume of each supernatant to be tested. In the control series culture supernatant was replaced by saline containing 0.005 M Tris buffer, pH 7.5. After incubation at 37 C for 30 min, megacin activity was assayed by spot tests. The inhibitory titer was expressed as the difference between the megacin units in the control and the experimental sample. The results shown in Fig. 2 indicate that the disappearance of megacin activity was due to an inhibitory factor produced, and the inhibitory titer was greatest in the early stationary phase.

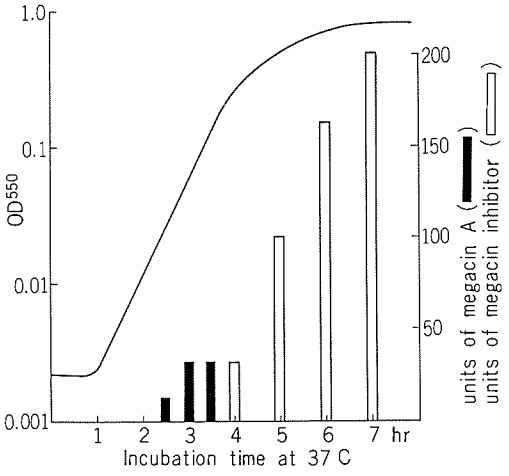

FIGURE 2. *Production of megacin inhibitor during bacterial growth.*
Strain: less-sensitive mutant #265.

To compare the productions of the inhibitory factor by various strains, supernatants of overnight cultures of the strains listed in Table 2 were assayed for inhibitory activity. The less sensitive mutants produced most inhibitor, followed by the parent strain 216 while the more sensitive mutants produced least inhibitory activity. Strain KM produced no inhibitory activity.

4. *Heat lability of the megacin inhibitor*

The inhibitory factor was stable on heating at 50 C for 30 min, but it was destroyed on heating at 70 C for 5 min (no table shown).

Culture supernatant which was devoid of megacin activity as shown in Fig. 2 was found to gain megacin activity on heating at 70 C for 5 min. Since megacin is fairly heat stable (Ivánovics et al., 1959b), the results suggests

TABLE 2. *Inhibition of megacin A by supernatants of cultures of various strains*

Culture supernatant \ Megacin dilution	5^{-1}	5^{-2}	5^{-3}	5^{-4}	5^{-5}	5^{-6}	5^{-7}	5^{-8}
Control (0.005 M Tris buffer, pH 7.5)	4	4	4	4	4	2	0	0
B. meg. KM	4	4	4	4	4	2	0	0
B. meg. 216	4	4	2	0	0	0	0	0
More sensitive mutant #400	4	4	4	2	0	0	0	0
#462	4	4	4	2	0	0	0	0
#261	4	4	4	2	0	0	0	0
Less sensitive mutant #265	4	2	0	0	0	0	0	0
#253	4	2	0	0	0	0	0	0
#272	4	2	0	0	0	0	0	0

Indicator : *B. megaterium* KM
4 : Complete inhibition of growth 2 : Slight inhibition 0 : No inhibition

that the inhibitory factor is not an inactivator but a reversible inhibitor.

5. *Partial purification of the megacin inhibitor*

To 2 liters of the supernatant of a culture of the less sensitive mutant 265 in the early stationary phase which contained no megacin activity, ammonium sulfate was added to 0.8 saturation. The resulting precipitate was dialyzed against saline containing 0.005 M Tris buffer, pH 7.5. Preliminary studies showed that megacin in the dialyzed solution was eluted from a DEAE cellulose column with up to 0.4 M NaCl in buffer while the megacin inhibitor was not. Therefore, the dialyzed solution was applied to a DEAE cellulose column (1 × 15 cm). The column was thoroughly washed with 4 liter of buffered 0.4 M NaCl until megacin activity could no longer be detected in the eluate. Then it was eluted with a linear gradient of 0.4 to 0.9 M NaCl in 1 liter of buffer at a flow rate of 150 ml per hr and fractions of 8 ml were collected.

As shown in Fig. 3, the megacin inhibitor was eluted two fractions later than the peak of the O.D. at 260 mμ. There was no shoulder in spectrum of the fraction with maximal inhibitory activity either at 260 or 280 mμ.

6. *Chemical nature of the megacin inhibitor*

The partially purified preparation of inhibitor obtained by DEAE cellulose chromatography was dialyzed against saline containing 0.005 M Tris buffer, pH 7.5, and used in the following analyses.

1) Color reactions and effects of nucleases

The absorption spectrum of the inhibitor was measured in a Shimazu multipurpose recording spectrophotometer, model MPS 50 L. It had a peak (O.D.=0.56) at around 260 mμ but no shoulder at around 280 mμ (O.D.=0.29).

The inhibitor gave a strong orcinol reaction, and a very faint tryptophan-perchloric acid reaction but no biuret reaction. It contained pentose as indicated by the absorption maximum at 480 mμ observed after the phenol-sulfuric acid reaction. These data show that the main component was RNA. Similar RNA was found in a preparation obtained from the supernatant of a culture in synthetic medium by the same procedure. Therefore, this RNA was not derived from the P-Y medium.

The RNA was susceptible to RNase (DNase free) as shown by the increase in $O.D._{260}$ of the cold 5 per cent TCA soluble fraction after the incubation at 37 C for 30 min with RNase, but no decrease in the inhibitory activity was detected after treatment with RNase. DNase (RNase free) had no effect on the inhibitor. Megacin was not inhibited by RNA's extracted with a phenol-cresol mixture (Kirby, 1956) either before or after pretreatment at 37 C with 0.1% sodium laurylsulfate for 5 min or with 1 mg/ml trypsin for 30 min. Thus it seems that the RNA, which is the main component of the inhibitor preparation, has no relation to the inhibitory activity.

2) Effect of trypsin

As described above the megacin inhibitor was destroyed by heating at 70 C at neutral pH and the main component of the partially purified preparation, RNA, had no relationship to the inhibitory activity. Thus the megacin inhibitor was suspected to be a protein. Accordingly the effect of trypsin on it was investigated.

A preparation of megacin inhibitor was

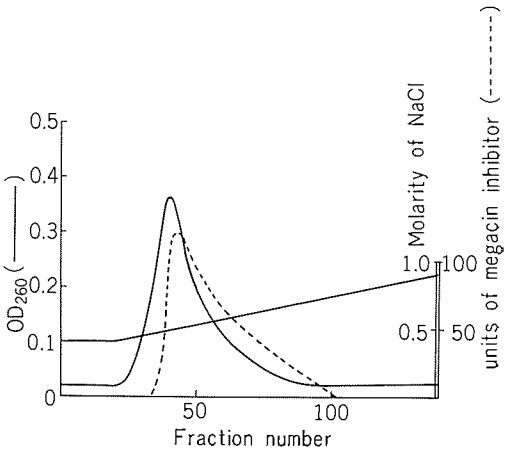

FIGURE 3. *DEAE Cellulose Chromatography of megacin inhibitor.*

treated with 3 mg of trypsin in 3.0 ml of 1 : 15 M phosphate buffer at pH 7.5 and 37 C. After 30 min, the reaction was stopped by addition of 6.8 mg of trypsin-inhibitor dissolved in 1.5 ml of the same buffer. Five control mixtures with the following components were run at the same time: (1) untreated megacin inhibitor, (2) trypsin without megacin inhibitor, (3) trypsin and trypsin inhibitor without megacin inhibitor, (4) trypsin inhibitor without trypsin or megacin inhibitor, (5) none (buffer control). The final volumes were adjusted to 4.5 ml with buffer. Aliquots of each sample were mixed with an equal volume of serial 1 : 3 dilutions of megacin preparations. The mixtures were incubated at 37 C for 30 min and spot tests were made.

As shown in Table 3, the activity of megacin inhibitor was slightly increased and not destroyed by trypsin. Moreover, trypsin caused a slight, but definite inactivation of megacin A, in contrast to the results of Iványovics et al. (1959b).

3) Sucrose density gradient ultracentrifugation

To see whether the megacin inhibitor is closely associated with RNA the partially purified preparation was subjected to ultracentrifugation in a sucrose density gradient. Linear density gradients of 5 to 20% sucrose in 25 ml containing 0.5 M NaCl and 0.005 M Tris buffer pH 7.5 were placed in three tubes of a Spinco SW 25 rotor. The samples tested were 1) 3.0 ml of buffered 0.5 M NaCl containing 300 units of megacin inhibitor, 2) 2.0 ml of medium containing 300 units of megacin inhibitor which had been incubated with 1.0 ml of medium containing 30 μg of RNase at 37 C for 60 min and 3) 3.0 ml of medium containing 300 units of megacin. Ultracentrifugation was carried out at 24,000 rev/min for 48 hr in a Spinco model L ultracentrifuge. The results in Figs. 4a and b show that the inhibitor was not associated with RNA.

TABLE 3. *Effect of trypsin on megacin inhibitor*

Additions	Megacin titer
None	3^6
Megacin inhibitor	3^2
Megacin inhibitor+trypsin +trypsin-inhibitor	3
Trypsin	3^4
Trypsin+trypsin-inhibitor	3^6
Trypsin-inhibitor	3^6

Incubations were carried out in 1 : 15 M phosphate buffer, pH 7.5. For details see text.

In further studies on the chemical nature of the megacin A inhibitor, attempts were made to measure its UV spectrum, but no UV absorption could be detected even in the fraction exhibiting maximum activity.

7. *Behavior of the megacin inhibitor in other assay procedures for megacin A*

As described in the previous report (Ozaki et al., 1966), megacin A activity can be assayed indirectly by measurement of hemolysis due to formation of lyso-compounds from phosphatides and directly by measurement of protoplast-lysis of a megacin-sensitive strain. These two assay methods were employed to examine the effect of the megacin inhibitor on megacin A.

1) Effect of the inhibitor on protoplast-lysis

Volumes of 3 ml of protoplast suspension in sucrose medium were introduced into three tubes with the megacin sensitive strain 400 at a final O.D. of 1.0 at 550 mμ. Tube A (control) contained 3 ml of 15% sucrose in 1 : 15 M phosphate buffer, pH 7.5. Tube B contained 3.0 ml of sucrose solution containing 100 units of megacin A previously incubated with 150 units of megacin inhibitor at 37 C for 30 min. Tube C contained 3.0 ml of sucrose solution containing 100 units of megacin A. All the tubes were incubated at 30 C and every 10 min the tubes were mixed by inversion and their optical densities were measured in a Coleman Junior spectrophotometer. As shown in Fig. 5, there was no decrease in optical density in tube B indicating that megacin A activity was

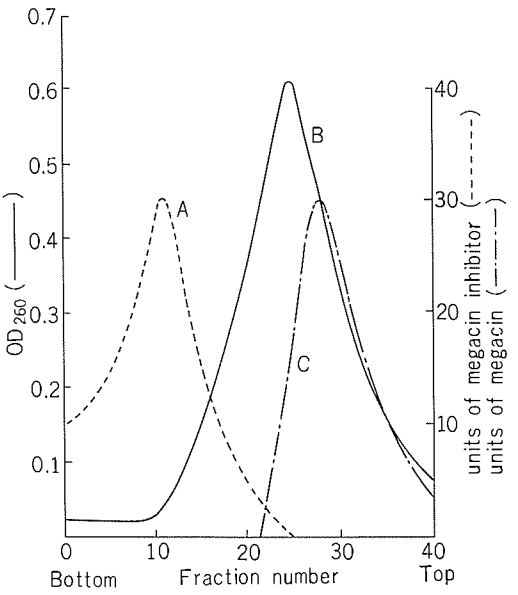

FIGURE 4a. *Sucrose Density Gradient Ultracentrifugation of megacin inhibitor or megacin.*
curves A (.....) *and B* (——) *are for tube* (*1*).
curves C is for tube (3), *which is superimposed in this figure.*

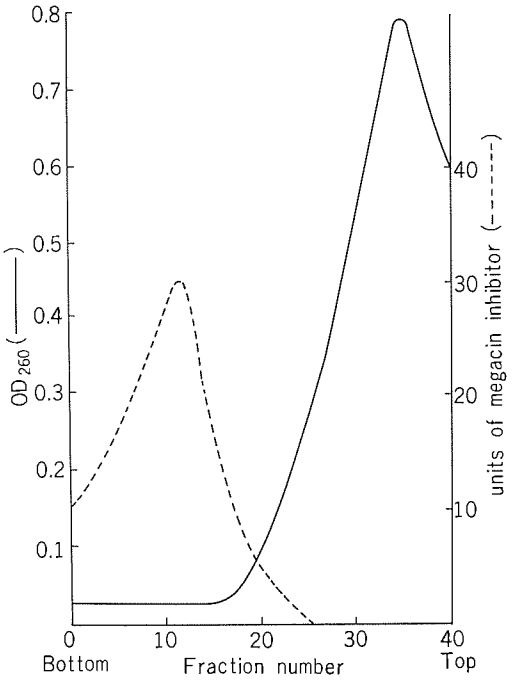

FIGURE 4b. *Sucrose Density Gradient Ultracentrifugation of megacin inhibitor after RNase treatment.*

completely inhibited by the inhibitor.

2. Effect of the inhibitor on lysolecithin formation

i) Megacin A is known to be a kind of phospholipase A (Ozaki et al., 1966). Accordingly the effect of the megacin inhibitor on the formation of lysolecithin from egg yolk lecithin was examined by measurement of hemolysis.

Two holes were made on a lecithin agar plate (see " Materials and Methods "). Into one was put 0.2 ml of saline containing 400 units of megacin inhibitor and into the other 0.2 ml of saline. The plate was kept overnight in the cold. Then 0.2 ml of saline containing 200 units of megacin A was put into each hole and the plate was incubated at 37 C overnight. Then it was covered with a thin layer of overlay blood agar medium (see " Materials and Methods ") and kept for 5 hr at room temperature.

The results are shown in Fig. 6. No hemolysis due to formation of lysolecithin was seen around the hole containing megacin inhibitor.

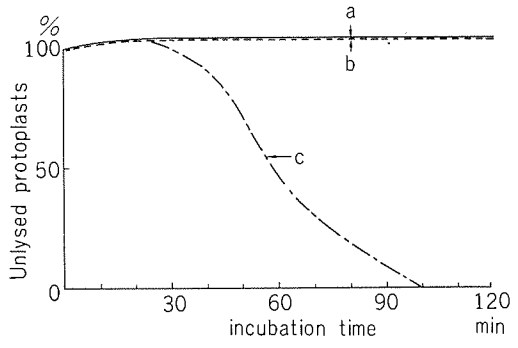

FIGURE 5. *Effect of megacin inhibitor on protoplast-lysis by megacin.*
Curve a : control without megacin
b : 100 units of megacin treated with 150 units of megacin inhibitor added at 0 min
c : 100 units of megacin added at 0 min
Strain : more sensitive mutant #400

ii) To investigate the inhibition of lyso-

lecithin formation more precisely, the following experiments were performed. Two series of 0.2 ml volumes of serial 1 : 5 dilutions of megacin A preparation in 0.005 M Tris buffered saline, pH 7.5, were prepared. To one series were added 0.2 ml aliquots of megacin inhibitor preparation which had been dialyzed against buffered saline. To the other series were added 0.2 ml of buffered saline. After incubation at 37 C for 30 min, the megacin activity remaining was assayed (1) by spot tests, and (2) by adding 0.1 ml of the contents of each tube to 0.1 ml of substrate solution (see "Materials and Methods") containing lecithin. After incubation at 37 C for 30 min, 4.0 ml of chloroform-methanol (2 : 1) and 1.0 ml of saline were added to each tube with mixing. Each chloroform layer was concentrated in a rotary evaporator and subjected to TLC with solvent I. Lecithin and lysolecithin were located with Dittmer's reagent.

In the spot test, complete inhibition of growth was seen with dilutions of up to $1 : 5^5$ in the control with megacin and with dilutions of up to $1 : 5^2$ in the series with megacin plus inhibitor. The data on TLC are shown in Fig. 7a and b. Lysolecithin was detected at dilutions of up to $1 : 5^4$ of the control with megacin and of up to $1 : 5^2$ of the series with megacin plus inhibitor. No spots other than lecithin and lysolecithin could be detected on

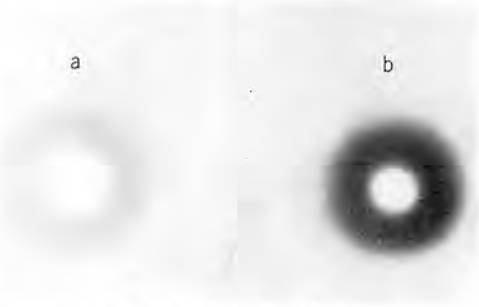

FIGURE 6. *a: With megacin inhibitor*
b: Without megacin inhibitor

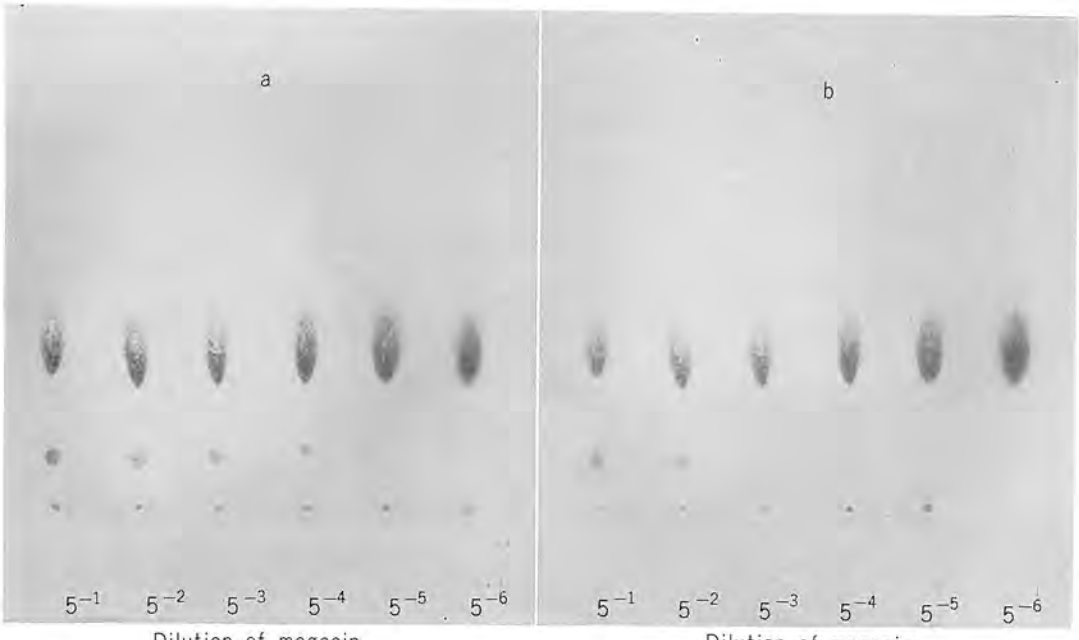

FIGURE 7. *Effect of the megacin inhibitor on lysolecithin formation*
a: megacin alone *b: megacin plus megacin-inhibitor*

either plate. There was a difference of one dilution between results on the control series with megacin in spot tests and TLC, but otherwise data obtained by the spot tests and TLC coincided well.

iii) No spots other than lecithin and lysolecithin were detected on the TLC plate of the series of megacin plus inhibitor, or in samples of megacin at dilutions of $1:5^3$ and $1:5^4$. These samples were chloroform extracts and other chloroform-insoluble materials might have been present. Thus these data do not exclude the possibility that lysolecithin formed by phospholipase A was broken down by the megacin inhibitor to compounds containing choline, since the latters would be soluble in methanol.

To test this possibility, quantitative studies on the liberation of free fatty acids were made using ^{14}C-labeled phosphatidylethanolamine as substrate (see " Materials and Methods "), with more than 90% of the ^{14}C in the fatty acid portion. Experimental procedures were similar to those in the previous experiment (see " Materials and Methods "), using 2,400 units of megacin and 400 units of megacin inhibitor. In the spot test, the control series with megacin caused complete inhibition at the dilutions tested ($1:2$–$1:2^5$), while in the series with megacin plus inhibitor, complete inhibition was observed with dilutions of $1:2$ and $1:2^2$. After incubation with substrate solution, the reaction mixtures were treated as described in the " Materials and Methods," and free fatty acids were estimated.

As shown in Fig. 8, the radioactivity in the substrate was liberated as free fatty acids in the control series with high concentrations of megacin. Thus the fatty acids of the substrate are liberated by phospholipase A activity. In the series with megacin plus inhibitor, no liberation of free fatty acids was observed with dilutions of megacin of $1:2^3$–$1:2^5$, where megacin activity in spot tests was undetectable and no formation of lysolecithin was observed in the previous experiment. If the megacin inhibitor is a kind of lysophospholipase liberating other fatty acids from lyso-compounds, the amount of free fatty acids in the samples at corresponding dilutions to the control series with megacin should be double.

At dilutions of $1:2$ and $1:2^2$, where megacin activity was detectable both by spot tests and by formation of lysolecithin, as described above, measureable amounts of free fatty acids were liberated. However, the amounts were smaller than those calculated from curve A of the control series with megacin (Fig. 8) for the value of megacin units minus inhibitor units.

All the above data suggest that the megacin inhibitor inhibits, or slows down the turn-over rate of the phospholipase A activity of megacin A. They also show that the methods for determining megacin inhibitory activity are inadequate.

iv) To see whether lyso-compounds are broken down by megacin inhibitor in different ways from lysophospholipase, lysolecithin, prepared by digestion with megacin A and purified by TLC, was incubated with megacin inhibitor

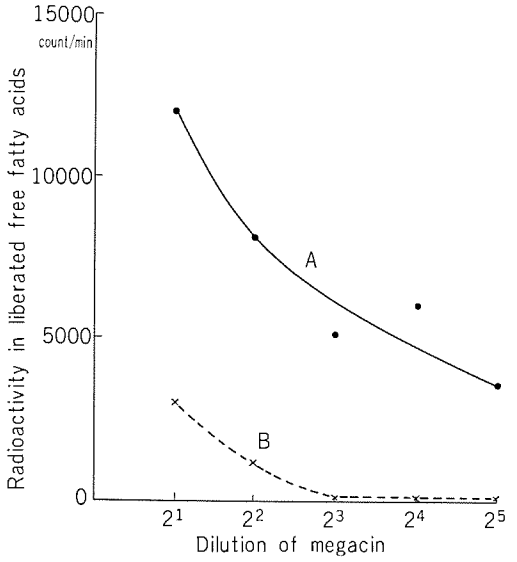

FIGURE 8. *Effect of megacin inhibitor on phospholipase A activity of megacin A.*
curve A (●——●); *megacin*
curve B (×——×); *megacin treated with* 400 *units of megacin inhibitor.*

alone. In this experiment, lysolecithin containing 10 μg phosphorus in 50 μl was used in place of lecithin as substrate. The reaction mixture consisted of 50 μl of substrate solution and 50 μl of the inhibitor preparation, and after incubation at 37 C for 30 min, 1.0 ml of chloroform-methanol (1 : 1) was added to facilitate evaporation. The mixture was concentrated by evaporation, and then the samples and the control were subjected to TLC with the solvent III. Spots of lysolecithin were found but no other spots containing choline or free choline detectable with Dragendorff's reagent.

The result clearly excludes the possibility that megacin inhibitor decomposes lyso-compounds further to some surface inactive substance, and all the data described above demonstrated that megacin inhibitor inhibits the phospholipase A activity of megacin A or slows down its turnover rate.

8. *Specificity of inhibitory activity*

Next, the possibility that megacin inhibitor inhibits all phospholipase A's was examined. The immunity retained in the protoplasts of the parent strain 216, showed high specificity to phospholipase A of megacin A but not to that of heated " Habu " venom. Thus this problem had to be examined on an enzymic level.

Two series of 5 μl volumes of serial 1 : 5 dilutions (5^{-1}–5^{-5}) of a purified phospholipase A preparation of heated " Habu " venom (see " Materials and Methods ") were set up. Volumes of 100 μl containing 400 units of megacin inhibitor were added to one series and 100 μl of 0.005 M Tris buffered saline, pH 7.5, to the control series. Tubes were incubated at 37 C for 60 min. Then 100 μl of the contents of each tube were incubated with 100 μl of substrate solution containing of ^{14}C-labeled phosphatidylethanolamine at 37 C for 30 min with shaking. The amount of fatty acids liberated was estimated as described above.

As shown in Fig. 9, the inhibitor did not inhibit the phospholipase A activity of " Habu " venom. Thus a high specificity of the inhibitor for megacin was demonstrated.

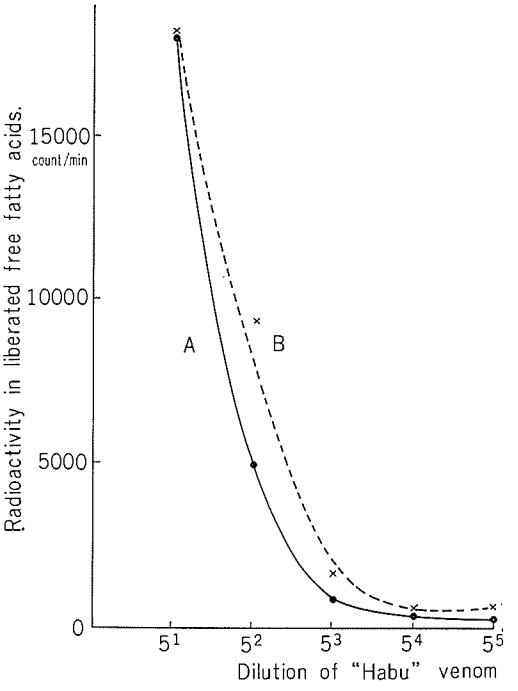

FIGURE 9. *Effect of megacin inhibitor on phospholipase A activity of " Habu " venom.*
curve A (●——●); *" Habu " venom*
curve B (×——×) *" Habu " venom treated with 400 units of megacin inhibitor.*

DISCUSSION

Megacin A is an exceptional bacteriocin in the following two respects: 1) it is an enzyme, hydrolyzing phospholipids to lysophospholipids and 2) it causes lysis of some bacteria. *Bacillus megaterium* strain 216 and some strains derived from it, which all produce megacin A were relatively insensitive to this megacin A. Some of the strains derived from strain 216 were more sentitive than the parent and some were less sensitive. These differences in sensitivity to megacin A seem to indicate that these strains have different degrees of immunity to megacin. Megacin A is a kind of phospholipase A, so the mechanism of immunity should be demonstrated most clearly on the enzyme level. The data described here show that bacteria with im-

munity to megacin produce a megacin inhibitor. It reversibly inhibits megacin or slows down the turn-over rate of the phospholipase A activity cf megacin A rather than inactivating it.

As described in the previous report (Ozaki et al., 1967), the immunity was retained by protoplasts and this immunity showed high specificity for the phospholipase A of megacin A but not for that of heated " Habu " venom. As suggested in the previous report, some fatty acids of phospholipids in the cytoplasmic membranes may have a low affinity for megacin A but not for the phospholipase A of heated " Habu " venom. This possibility and the possible effect of the inhibitor on the affinity have still to be examined.

The chemical nature of the megacin inhibitor is uncertain. It may be a protein because it is destroyed by heating at 70C for 5 min but it is resistant to trypsin. The main component of the partially purified preparation was RNA, but this was shown by ultracentrifugation to have no relationship to the inhibitory activity.

Megacin A is a phospholipase A, so the method used for its determination in this study (spot tests) is not accurate. Therefore, the method used for assay of megacin inhibitor is also inaccurate. For example, as shown in this paper, a mixture of megacin A and its inhibitor, in which megacin activity was retained as judged by spot tests, exhibited much reduced enzymic activity. Nevertheless, biological assay methods such as spot tests for megacin activity and for inhibitory activity seem useful methods of assay.

Genetic analyses to see whether the gene of the inhibitor is chromosomal or episomal have not yet been made. However, from the results in Fig. 2, it seems likely that the presence of a low concentration of megacin A in the medium leads to the induction of inhibitor production.

Very recently Guterman and Luria (1969), and Foulds and Shemin (1969) reported the production of a colicin inhibitor and inactivator, respectively. The former reported that lipopolysaccharide excreted by excreter type mutants derived from indicator strains inhibited colicin B. Thus this inhibitor is quite different from ours. The latter workers reported that an inactivator of bacteriocin was produced by some mutants of *Serratia marcescens* which inactivated the bacteriocin they produced. This inhibitor seems rather similar to ours. However, their inactivator seems to be a very heat-labile protease and irreversibly inactivates not only self-produced bacteriocin but also colicins K and E_2. This is in contrast to the megacin inhibitor which inhibits megacin A activity reversibly and specifically inhibits the phospholipase A activity of megacin A, not inhibiting that of heated " Habu " venom, which belongs to the same class of enzymes.

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