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Mode of Action of Megacin

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SUMMARY: Megacin however highly bactericidal for sensitive organisms was not adsorbed by them. Its bactericidal action was markedly dependent on temperature. The viability of organisms exposed to megacin at 0° was not affected. When megacin was added to exponentially growing *Bacillus megaterium* there was cessation of growth followed by a gradual decrease in turbidity of the culture. The decrease in turbidity was, however, not associated with a total lysis of individual organisms; rather it was the consequence of the escape of the dense intracellular material from the organisms. Intracellular components, i.e. substances which absorbed in the ultraviolet region escaped from the bacteria into the medium while cell wall remained essentially intact. When *B. megaterium* suspended in a medium containing lactose was exposed to megacin, β -galactosidase appeared on the surface of bacteria.

Protoplast preparations made from *Bacillus megaterium* and *Micrococcus aurantiacus* (both sensitive to megacin) were converted into ghost-like structures on the addition of megacin. On the other hand, protoplasts made from insensitive species resisted megacin. Observations indicate that megacin causes a radical change in the osmotic barrier of sensitive organisms by attacking the cytoplasmic membrane. Data available suggest that megacin is either an enzyme which breaks down the osmotic barrier of sensitive cells, or is a substance capable of activating the intrinsic enzymes of cells which lead to an autolysis of cytoplasm.

Knowledge of the mode of action of megacin, an antibacterial principle obtained from *Bacillus megaterium* (Ivánovics & Alföldi, 1954), is scanty. In preliminary investigations, lysates of megacinogenic bacteria obtained in complex media were shown to exert a bactericidal action on organisms of sensitive strains. Furthermore, the addition of these lysates to cultures of *B. megaterium* growing exponentially resulted in a rapid cessation of the oxygen uptake (Ivánovics & Alföldi, 1955). In order to study the specificity of megacin action lysates containing megacin were partially purified and used instead of crude lysates. The purification of megacin has been made easier by the fact that the synthesis of megacin will take place in organisms growing in defined medium in the presence of an adequate concentration of manganese (Alföldi, 1957). Lysates produced in defined culture medium served for obtaining megacin concentrates of high potency. Experiments carried out with these concentrates give some information regarding the mode of action of this particular bacteriocin.

METHODS

Organisms. *Bacillus megaterium*, strain 216, and the phage resistant 'Mut-C' strain were the same as those described previously (Ivánovics & Alföldi, 1955; Ivánovics, Alföldi & Széll, 1957). Strain KM came from the laboratory of Dr C. Weibull (Central Bacteriological Laboratory of Stockholm City). The

other strains used in these investigations were from the collection of this Institute.

Culture media. Horse meat-extract agar containing 1 % (w/v) peptone and 1.5 % (w/v) agar. The defined culture medium was prepared as described by Alföldi (1957). Our previous work provides information concerning other culture media and methods (Ivánovics & Alföldi, 1955, 1957).

The titration and unit of megacin. One ml. of the young culture of the indicator strain 'Mut-C' (0.1 unit optical density equivalent to 0.07 mg. dry wt. organism/ml.) mixed with 1 ml. melted meat-extract agar was layered on agar plates. From the material containing megacin twofold serial dilutions were prepared and, by the aid of a platinum loop, 0.02 ml. amounts were placed on the plates, which were then incubated overnight at 37°. For more accurate titrations we prepared dilutions at closer intervals. In principle the highest dilution of which 0.02 ml. produced inhibition represents the unit of megacin. For the sake of convenience not this, but the fifty-fold amount, was taken as the unit, i.e. the reciprocal value of the titre expresses the number of megacin units/ml. When new lots of lysate or concentrate were assayed a concentrate of known activity was also included in the test.

Megacin concentrates. Megacin concentrates were obtained from *Bacillus megaterium*, strain 216, growing in a defined medium (Alföldi, 1957). The method of preparing megacin concentrates is outlined below. Two hundred ml. of defined culture medium in a 1 l. Erlenmeyer flask was inoculated with *B. megaterium*, strain 216. The inoculum was made from an overnight culture on a slope or YDC agar, the growth being suspended in 5 ml. saline; 1 ml. of this was used to inoculate 200 ml. medium. Cultivation was carried out with vigorous shaking (180 shakes/min., 4 cm. in amplitude) at 37°. When the culture attained an optical density of 0.5–0.6 unit (equiv. 0.55–0.75 mg. dry wt. organism/ml.), some of it was poured into a flat enamelled dish so that the layer of liquid was 2 mm. thick. The liquid was kept in violent motion and irradiated with a low-pressure ultraviolet lamp (230 erg./mm.²). The irradiated culture was transferred to a flask and re-incubated for another 4 hr. under the same conditions as before. During this period there was first some growth, followed by partial lysis. At the end of the incubation period the pH value of the culture was adjusted with 0.07 M-Na₂HPO₄ to pH 7.2 and the culture kept in an ice-box till the next day. The supernatant fluid obtained after centrifugation usually contained 40,000 units megacin/ml. To every 100 ml. of the solution we then added 30 ml. of 96 % (w/v) ethanol in water, followed by dialysis for 2 days in cellophan sacs against distilled water at 4°. The ethanol suppressed the growth of possible contaminants.

From the solution of low electrolyte content thus prepared, the active principle was readily adsorbed by the anion-exchange cellulose Ecteola (Peterson & Sober, 1956), while no adsorption took place on acid-cellulose CM-W (Peterson & Sober, 1956). Ecteola (2.5 g.) was added to 500 ml. dialysed megacin solution and the suspension was stirred for 1 hr. at room temperature. After centrifugation the supernatant fluid was carefully poured off and the sediment resuspended in 50 ml. buffered 1 % (w/v) NaCl. The pH

value of the saline was adjusted to pH 7 with phosphate buffer 0.07M; 5 ml. buffer/100 ml. saline. Elution was carried out by stirring at room temperature for 1 hr. The liquid containing the megacin was separated from the cellulose preparation by centrifugation.

Depending on the starting materials, etc., in different experiments the eluates contained 200,000 to 300,000 units megacin/ml. In six attempts at concentration the yield was 40–56 % of the original megacin. The nitrogen content of the concentrates varied between 50 and 90 μ g. N/ml. Since the nitrogen content of dialysed lysates in individual lots was *c.* 60 μ g. N/ml. thus roughly fivefold purification was achieved during concentration. After being sterilized by passage through sintered glass bacterial filters the concentrates could be kept unchanged for several months in an ice-box. The concentrates did not contain proteolytic enzymes; no effect on gelatin films observed even after prolonged standing.

Preparation of protoplasts. *Bacillus megaterium* was grown from a heavy inoculum from an overnight agar slope in a defined medium (Alföldi, 1957) with vigorous shaking for 6–7 hr. at 37°. Cultures containing equiv. 1–1.5 mg. dry wt. organism/ml. were centrifuged and the bacteria washed twice with buffered saline. The bacteria were then resuspended in medium PM which contained 0.02 M-phosphate buffer (pH 7.2) + 0.2 M sucrose + 0.01 M-MgSO₄·7H₂O. Crystalline lysozyme was added (100 μ g./ml.) to the suspension which was then incubated at 37°. A stable protoplast suspension was obtained within a few minutes. Protoplasts were prepared in a similar manner from *Sarcina flava*, *Micrococcus aurantiacus* and *B. subtilis*. These organisms were harvested from a horse-meat peptone broth after growth at 37° for 8 hr. In the case of *B. subtilis*, a lysozyme concentration of 300 μ g./ml. was used for making the protoplasts. From Gram-negative bacteria only penicillin-induced 'protoplasts' were studied, prepared according to the technique of Lederberg (1956).

Buffered saline solution. 0.9 g. NaCl/100 ml. distilled water + 5 ml. 0.07 M-phosphate buffer (pH 7.2).

Assay of β -galactosidase. This was done by the method of Landman (1957) with *o*-nitrophenyl- β -D-galactoside.

Crystalline ribonuclease. This was prepared after McDonald (1955) and kindly given by Professor B. F. Straub (Budapest).

Lysozyme was prepared from egg white by isoelectric point crystallization.

Trypsin ('1:250') was bought from Difco Laboratories, U.S.A.

Polymyxin B sulphate. (Chas. Pfizer and Co. Inc. U.S.A.); 1 mg. equivalent to 10,000 units was used in these experiments.

RESULTS

Adsorption and bactericidal effect of megacin

Some of the known antibiotics of polypeptide character such as polymyxin B are highly surface-active and are therefore readily adsorbed in a non-specific manner by bacteria. Their bactericidal effect (due to surface-activity; Bliss, Chander & Schoenbach, 1949) is only slightly influenced by temperature. With

this in mind it seemed of interest to do similar experiments with megacin concentrates. One of our megacin concentrates (4000 units/ μg . N) had exactly the same stalagmometric value as the solvent alone. On the other hand, polymyxin B (100 μg ./ml.) markedly decreased the surface tension of saline. Further, megacin was not significantly adsorbed by a thick suspension (equiv. 10 mg. dry wt. organism/ml.) of megacin-sensitive *Bacillus megaterium* KM.

Suspensions of *Bacillus megaterium* were exposed to megacin at different temperatures, and samples taken at intervals for plating to determine the colony count. It can be seen from Fig. 1 that whereas the viability of organisms exposed to megacin at 0° was not affected, there was a progressive decrease in viability with increase in temperature and with increase in time of exposure to megacin.

Decrease of turbidity of suspensions by the action of megacin

When megacin was added to exponentially growing cultures of *Bacillus megaterium* shaken at 37°, their turbidity gradually decreased. The results of a typical experiment carried out with strain KM is shown in Fig. 2. It can be

Fig. 1

Fig. 2

Fig. 1. Bactericidal effect of megacin at different temperatures. A young broth culture of *Bacillus megaterium*, strain KM, was distributed into test tubes previously brought to the required temperature. Megacin was added to give a final concentration of 200 units/ml. Initially the number of colony formers was 1.55×10^6 /ml. At intervals appropriate dilutions were made with ice-cold broth and 0.1 ml. of suitable dilutions plated on meat extract agar. ●, at 0°; ○, at 12°; ◐, at 29°; ◑, at 40°.

Fig. 2. Effect of megacin on turbidity of cultures of *Bacillus megaterium*, strain KM, grown in meat-extract broth or in defined medium. Megacin (2000 units/ml.) was added to the cultures which were then re-incubated with gentle shaking at 37°. ● = in defined medium; ○ = in meat-extract broth.

seen that the addition of megacin to cultures in meat-extract broth led to a rapid cessation of growth, while with the cultures in the defined medium there was some growth before the decrease in turbidity. The rate of decrease in turbidity was highly dependent on the medium used and also on the initial optical density, i.e. the age of the culture. Similar observations were made with strain 'Mut-C', a phage-resistant mutant of *B. megaterium*. However, in this case the decrease in turbidity of cultures was less marked. When young cultures of *B. megaterium* strains were mixed with megacin and then poured into test tubes so that there was a deep layer of culture and these were incubated statically and thus under semi-anaerobic conditions, the results were variable. Sometimes lysis occurred in the control broth cultures in the absence of added megacin while, on the other hand, megacin did not always cause a decrease in the turbidity of cultures grown in a defined medium.

The addition of megacin to killed bacterial cultures of *Bacillus megaterium* did not cause a decrease in turbidity. The turbidity of heat-killed organisms (60° for 30 min.) and the turbidity of cultures sterilized by chloroform remained unaltered after adding high concentrations of megacin. Megacin concentrates did not exhibit a clearing effect when spotted on the surface of a soft agar layer containing a suspension of killed *B. megaterium* KM. This technique, used by Murphy (1958) for assaying a phage-associated enzyme of *B. megaterium*, did not reveal any lytic action of megacin concentrate on the killed organisms.

Cultures of *Bacillus megaterium* exposed to megacin were examined at intervals under oil immersion in a phase-contrast microscope. Before the addition of megacin, the cytoplasm of the organisms was very dense and showed a dark greyish violet structure. As the time of exposure to megacin increased, the homogeneity of the cytoplasm gradually decreased and distinct granules appeared in the interior. This change was associated with shrinkage of the contour of the organism. Finally, when a very marked decrease in the optical density of the culture had taken place, the organisms either seemed empty or only large dark granules were seen in their interior.

When counts were made in a haemocytometer the number of empty organisms was found to be identical with the number of organisms present at the time of addition of megacin to the culture (Table 1). This experiment indicated that the change in turbidity of the culture under the action of megacin was not due to a total lysis of the organisms but rather to the escape of optically dense intracellular materials from the organisms. This leakage of cell contents was confirmed by taking samples at intervals, removing solid materials by centrifugation and then measuring the absorption of the supernatant fluids at 260 m μ . It can be seen from Fig. 3 that there was a steady increase in the u.v.-absorbing capacity of the supernatant fluids. An escape of cell material was also observed when megacin was present in a concentration so dilute that there was no marked decrease of turbidity within a short time. This was made apparent by assays of the adaptive enzyme β -galactosidase in suspensions harvested from media containing lactose instead of glucose. Megacin appeared to disrupt the osmotic barrier in bacteria and thus allow the

o-nitrophenyl- β -D-galactoside to reach the enzyme. In the control suspension (without megacin) the value of β -galactosidase remained permanently very low during the whole period of the experiment and served as a blank in photometric estimates of the *o*-nitrophenol split by the β -galactosidase.

Microscopic and chemical investigations of cultures containing megacin suggested that there was a marked change in the osmotic barrier of organisms. The decrease in turbidity of the culture was, however, only an apparent lysis.

Table 1. *The turbidity and haemocytometer count of Bacillus megaterium, strain KM, culture in broth after the addition of 2000 units megacin/ml.*

Time (min.)	Optical density units	Count/ml.*
0	0.120	1.97×10^7
20	0.100	2.16×10^7
50	0.000	1.00×10^7

* 1/10 dilutions of samples were made with saline containing 0.01% crystal violet + 0.1% formalin and counted in a Buerker chamber.

Fig. 3. Escape of u.v.-absorbing material from *Bacillus megaterium*, strain KM, in the presence of megacin. Megacin (2000 units/ml.) was added to a culture growing exponentially in defined medium and re-incubated, with gentle shaking, at 37°. Samples were taken at intervals, cooled and centrifuged. The u.v.-absorbing capacity of supernatant fluids was measured between 240 and 280 mμ. (a) Absorption curves of individual samples; ○, 30 min.; ●, 45 min.; ○, 60 min.; ●, 90 min.; ●, 120 min. (b) ○ = optical density values of suspension at 590 mμ; ● = extracellular fluid at 260 mμ at different times.

In fact, the cell wall was left uninjured or only slightly injured, indicating that the primary action of megacin was exerted not on the cell wall itself but on deeper layers of the organisms. This increased the permeability of the osmotic barrier.

Megacin action on protoplasts

Very stable preparations of protoplasts were made by lysozyme treatment of strains KM and 'Mut-C' of *Bacillus megaterium*. The optical densities of preparations were measured in the presence of various concentrations of megacin (Fig. 5). By microscopic observation, it was found that the presence of megacin caused the transformation of protoplasts into 'ghosts'; the latter appeared before there was a significant change in the optical density of the suspension. This result again supported the view that the megacin acted on the osmotic barrier of the cells.

There is now good evidence which indicates that the action of megacin concentrate is specific and due to its bacteriocin content. Autolysates as well

Fig. 4

Fig. 5

Fig. 4. The appearance of β -galactosidase in suspensions of *Bacillus megaterium*, strain KM, in the presence of megacin. Organisms were grown in defined medium but with glucose replaced by lactose, collected by centrifugation and washed twice with saline. The washed organisms were resuspended to equiv. 1 mg. dry wt./ml. Megacin (200 units/ml.) was added to suspensions preheated to 37° and kept at this temperature. At intervals, 0.1 ml. samples were taken and added to 2 ml. *o*-Nitrophenyl- β -D-galactoside-containing assay medium. After 5 min. β -galactosidase activity was stopped by adding 2 ml. ethanol (96%, w/v). The intensity of colour developed in the cell-free fluid was estimated at 420 $m\mu$ in a Beckman photometer.

Fig. 5. The change in optical density of protoplast suspensions prepared from *Bacillus megaterium*, strain KM, in the presence of various concentrations of megacin. Megacin was added to the protoplast suspension pre-heated and incubated at 37° . ●—, Control; ○—, 400 units megacin/ml.; ●···, 2×10^3 units/ml.; ○---, 4×10^3 units/ml.; ●—, 1×10^4 units/ml.

as extracts obtained by lysozyme treatment from washed non-induced organisms of megacinogenic strain 216 of *Bacillus megaterium* did not transform protoplasts into ghosts. On the other hand, when cultures of strain 216 were u.v.-irradiated and re-incubated, the appearance of the principle responsible for protoplast destruction paralleled the increase in the antibacterial action of the cell extract, i.e. the elaboration of megacin. The megacin concentrates used in the above experiments were devoid of any proteolytic activity. Our protoplast preparations resisted treatment with trypsin ('1:250') at 100 µg./ml. No change in the structure of protoplasts was seen under the microscope following the addition of crystalline ribonuclease (100 µg./ml.) or a megaterium phage suspension of high potency.

The specificity of megacin action

Megacin has a very narrow antibacterial spectrum which includes only strains of *Bacillus megaterium* and some coloured cocci. A small proportion of strains of *B. anthracis* and *B. subtilis* tested appeared to be slightly sensitive to crude lysates of megacinogenic strains (Ivánovics, Alföldi & Abraham, 1955). Whether this latter action can be considered specific or not needs further

Table 2. *Conversion of 'protoplasts' into ghosts by megacin or polymyxin B*

To suspensions of 'protoplasts', megacin (400–2000 units/ml.) or polymyxin B (100 µg./ml.) was added and incubated at 37° for 30 min.

Organism	Sensitivity to megacin	Conversion into ghost by	
		Megacin (400–2000 units/ml.)	Polymyxin B (100 µg./ml.)
<i>Bacillus megaterium</i> , strain KM	+	+	+
<i>B. megaterium</i> , strain Mut-C	+	+	+
<i>B. megaterium</i> , strain 216	+	+	+
<i>Micrococcus aurantiacus</i>	+	+	+
<i>Bacillus subtilis</i> , 302	—	—	—
<i>B. subtilis</i> , 304	—	—	—
<i>Sarcina flava</i>	—	—	—
<i>Escherichia coli</i> B*	—	—	+
<i>Salmonella typhosa</i> O*	—	—	+
<i>S. abony</i> *	—	—	+
<i>Shigella schmitzii</i> *	—	—	+

* Penicillin-induced spherical bodies.

investigation. Megacin concentrates of high activity now offer an opportunity to investigate the specificity of megacin action on protoplast preparations made from different organisms (Table 2). Some of our 'protoplast' preparations were made by the penicillin technique (Lederberg, 1956). While we designate all our preparations as 'protoplast', that is only for the sake of simplicity, since we accept the distinction between penicillin- and lysozyme-formed 'protoplasts' (Brenner *et al.* 1958). For comparison, a solution of polymyxin B was also included in these experiments.

Protoplasts made from strains of species sensitive to the bactericidal action

of megacin were converted, without exception, into ghosts by this bacteriocin. Polymyxin B also effected the destruction of protoplasts of these strains and its action included the spherical bodies ('protoplasts') of Gram-negative bacteria. It is interesting to note that protoplasts of *Bacillus subtilis* and of *Sarcina flava* resisted polymyxin B and megacin.

In a quantitative experiment with protoplasts of *Bacillus megaterium*, strain KM, the lowest concentration of polymyxin B which brought about conversion of protoplasts into ghosts was 50–100 μg . (500–1000 units/ml.) while the effective limiting concentration of megacin was 200 units/ml. The megacin concentrate used in this experiment contained 2000 units megacin/ μg . N.

DISCUSSION

The experiments described above indicate that megacin caused a radical change in the osmotic barrier of sensitive cells. Escape of intracellular components into the medium was a consequence of megacin action either on cells or on protoplasts of sensitive organisms. As this effect was not observed on heat- or chloroform-killed organisms this suggests a primary action on the function of the osmotic barrier, followed by the death of sensitive organisms. The fact that megacin did not lyse killed organisms, nor attack the cell wall of living ones distinguishes its action from that of the enzyme associated with megaterium phage (Murphy, 1958) which dissolves the cell wall of *Bacillus megaterium*. The available evidence does not allow more precise definition of the actual point of attack of megacin.

The very narrow antibacterial range of megacin (Ivánovics, Alföldi & Abraham, 1955) points to a highly specific action of megacin on sensitive organisms. The specificity of antibacterial action is in a good accordance with the results of experiments made with protoplasts. Only protoplasts prepared from *Bacillus megaterium* and *Micrococcus aurantiacus* were attacked by megacin; those of *Sarcina flava* and *B. subtilis*, which are not sensitive to megacin, were spared. Although 'spherical bodies' of Gram-negative bacteria induced by penicillin cannot be considered as true protoplasts (Brenner *et al.* 1958) they give ghost-like structures when treated with polymyxin; megacin apparently left these spheres untouched. The action of megacin is reminiscent of the enzyme plakin (Amano, Kato & Shimizu, 1952) isolated from horse platelets. Plakin converts protoplasts of *B. megaterium* into ghosts (Amano *et al.* 1956). It should be stressed, however, that its action is not so highly specific as that of megacin: its antibacterial action and its action in converting protoplasts into ghosts is not confined to *B. megaterium* but affects several of the genus *Bacillus*.

We have failed so far to establish the enzymic character of megacin by enzyme kinetic studies. That its bactericidal action is highly dependent on temperature cannot be considered as evidence for a primary enzyme function, for this would also be the case if an autolytic process in cells or protoplasts were enhanced by megacin. One may wonder about the biological significance of the production of this particular bacteriocin. Megacinogeny is reminiscent of lysogeny except for the liberation of mature phage particles or

the production of defective phage (Ivánovics, Alföldi & Lovas, 1957). This question has already been discussed (Ivánovics & Alföldi, 1957) and we cannot exclude a possible synthesis of non-organized phage material governed by a highly degraded prophage. Jacob & Fuerst (1958) described the enzyme endolysin, elaborated by mutants of lysogenic *Escherichia coli* K 12 which were made highly defective artificially. Endolysin is claimed to be an enzyme which attacks cell-wall constituents of several Gram-negative bacteria. So far, we have failed to obtain from typically lysogenic strains of *Bacillus megaterium* defective mutants capable of producing megacin instead of phage particles.

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