Bacteriocinogenesis in *Bacillus megaterium*

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SUMMARY: A strain of *Bacillus megaterium* has been found under certain conditions to liberate a bacteriocin-like agent which has been termed 'megacin'. The conditions necessary for induction of megacin formation in this strain are reminiscent of those leading to induction of bacteriophage in other (lysogenic) strains of the same species. Megacin, however, differs from bacteriophage in not giving rise to any further production of antibacterial principle, after acting on bacterial cells. Induction of megacin formation by ultraviolet irradiation is dependent on the composition of the medium. Only in a complete medium is megacin produced, although certain preliminary stages in the induction process and a concomitant, reversible photobiological effect can take place in minimal nutrient medium.

We have reported recently (Ivánovics & Alföldi, 1954, 1955) that within *Bacillus megaterium* species mutual antagonism between strains is a frequent phenomenon which can be attributed to a water-soluble antibacterial substance of protein character designated by us as megacin. In some strains megacin production is greatly enhanced by small doses of ultraviolet (u.v.) light. After growth for 90-120 min. mass lysis ensues with liberation of megacin into the medium. On account of its mode of formation and its properties we classed megacin with the bacteriocins, yet it differs essentially from the known bacteriocins, e.g. colicin and pyocin (Fredericq, 1953; Jacob, Siminovitch & Wollman, 1952; Jacob, 1954) in that it acts on all strains of its own species and not only on some individual 'sensitive' ones. Nevertheless, the antibacterial spectrum of megacin is very narrow; besides certain strains of *Bacillus subtilis* and *B. anthracis*, megacin acts only on the pigment-forming non-pathogenic micrococci, e.g. *Micrococcus aurantiacus*, *M. cinnabareus* (Ivánovics, Alföldi & Ábrahám, 1955).

Lysogenesis, as studied extensively in *Bacillus megaterium* (Lwoff, 1953a), and megacin formation are strikingly analogous in several ways. In order to determine whether these two phenomena are physiologically related, the conditions necessary for induction of megacin formation by *B. megaterium*, strain 216, were studied in greater detail.

MATERIALS AND METHODS

The experimental techniques used were the same as those described in our recent publication (Ivánovics & Alföldi, 1955).

Megacin-forming strain. A *Bacillus megaterium* strain isolated, and designated as no. 216, by us (Ivánovics & Alföldi, 1955) was used in these studies.

Complete nutrient media. These were prepared as described earlier (Ivánovics & Alföldi, 1955), namely yeast extract and acid-hydrolysed casein (YC), yeast

extract peptone medium (YP), and yeast extract plus enzymically digested casein (YDC) medium. This latter medium was earlier designated by us as medium-9. The agar media were prepared by adding 15 g. of agar to each litre of the liquid media mentioned above.

Defined medium. A lactate ammonium chloride nutrient (Friedlein, 1928) was used as basal medium. *Bacillus megaterium* strain 216 develops extremely slowly in this medium alone; yet it makes a useful medium when supplemented with 1 % glucose and 0.05 % ammonium glutamate (FGG medium). Glucose was sterilized separately by filtration and added to the nutrient immediately before use.

Cultivation, and induction with u.v. light, of strain 216. The organisms were grown at 35° in 100 ml. Erlenmeyer flasks containing 20 ml. of the nutrient solution, with gentle shaking. The inoculum consisted of 0.2 ml. of bacterial suspension of optical density 0.40 prepared from a 24 hr. YDC or FGG agar culture of the strain. The optical densities of bacterial suspensions and cultures were measured with an 'Oriphot' (Elektromos Mérő-eszközök Gyára, Budapest) microphotometer with a photoelectric vacuum cell. Measurements were carried out in test tubes 14.5–15.0 mm. in diam., using an orange colour filter (610 m μ .).

The source of the u.v. light was a 'Hanau' high-pressure mercury lamp used at a distance of 25 cm. under the conditions described earlier (Ivánovics & Alföldi, 1955). The energy emitted by the lamp at 25 cm. distance in the optical axis amounted to 7.3×10^5 erg./cm.², of which 3.3×10^5 erg. fell to the spectral range below 3500 Å. The bacterial suspensions were poured in Petri dishes in a layer not more than 2–3 mm. thick and irradiated with u.v. light while the vessel was gently swirled by hand.

Count of colony-formers. Samples (0.1 ml.) of an appropriate dilution of culture were plated on the surface of the agar medium.

Bacterial cell count. The cultures were diluted with aqueous methyl violet solution (0.02 %, w/v), and the numbers of cells and chains were counted in a Buerker haemocytometer.

Megacin titration. A highly sensitive variant of the 'mutilate' strain of *Bacillus megaterium* (Pasteur Institute, Paris) was used as the indicator strain. One ml. of a suspension of the strain, optical density 0.20, was mixed with an equal amount of melted YP agar and layered upon a plate 10 cm. diam. prepared from the same agar. Serial dilutions of the lysate were then dropped on the plates, and the megacin titres determined. The highest dilution of lysate capable of giving a partial inhibition of growth varied from 1:10,000 to 1:40,000 in individual experiments carried out under different conditions. The megacin content of the lysate in a control culture grown in YDC medium and induced with u.v. radiation under optimal conditions was taken to be 100 % and all other values referred to it.

Stained preparations. Bacteria on slides were kept for 10 min. in Bouin's fixing solution, washed, treated with tannic acid, and stained with methyl violet (10 sec., 1 %, w/v, solution) according to Gutstein (1926).

RESULTS

Induction in complete medium

Growth of induced bacteria. Young, 3–4 hr., cultures were exposed to u.v. radiation for 30 sec. An example of the behaviour of cultures after induction with u.v. radiation is represented in Fig. 1. At about 90 min. after irradiation, the culture began to lyse. The antibacterial titre of the lysate was found to be 1:25,000.

Fig. 1. Growth after u.v. irradiation of culture of *Bacillus megaterium*, strain 216, grown in YDC medium. In this and all other diagrams the time of irradiation is zero time. Irradiation for 30 sec.; A, optical density; B, bacterial count; C, chain count under the microscope; D, number of colony-formers on YDC agar.

In this and similar experiments it was observed that the bacteria gradually increased in number until lysis occurred. Nevertheless, bacteria plated out on the surface on nutrient agar in their early phase of growth after irradiation were still capable of forming colonies.

These experiments indicate that the lysis of irradiated bacteria does not begin until after the first generation following irradiation.

The morphology of induced cells. The finer cytologic structures, e.g. the nuclear structure, have been disregarded in our investigations, cell integrity alone being considered at this stage. Cell size and the condition of cell walls

in stained or unstained, living preparations, were examined with the phase-contrast microscope.

In young cultures of our strain, as in *Bacillus megaterium* strains in general, the septa are formed before the cells are fully developed in size. Thus the bicellular form is typical (Pl. 1, fig. 1). Not infrequently, the cells are linked by cell-wall remnants to form slack chains. After induction, the septate cells soon decrease in number and, correspondingly, the individual cells increase in size; they become more and more elongated, yet division does not cease, as is clearly indicated by the rising number of cells estimated by direct count (Fig. 1, curve *B*). On comparing the morphological details with the number of cells in induced cultures it appears obvious that the rise in density during growth after irradiation is made up of two components: elongation and division of the cells. After 60 min. some individual cells no longer stain evenly, so that the cell wall is not everywhere visible (Pl. 1, fig. 2). The cells become more and more varied in their morphology, and the proportion of abnormal cells increases. Besides faintly staining cellular remnants, a few cells more or less disintegrated are found (Pl. 1, figs. 3-5), but perfectly normal forms still occur.

These observations do not necessarily mean that the lesions in induced cells are primarily lesions of the cell walls. Under the phase-contrast microscope, vacuoles are seen in the cytoplasm in steadily increasing numbers, and it appears rather that bacterial disintegration is preceded by increased permeability of the cell wall. Support for this view is afforded by the observation that on applying Gutstein's (1926) staining method the tannic acid, used as a mordanting agent, penetrates the cytoplasm allowing the cell to be uniformly stained (see Pl. 1, figs. 2, 3).

Re-incubation, in a new medium, of cells irradiated in YDC nutrient. After u.v. irradiation, cultures grown for various lengths of time were cooled rapidly and centrifuged between 8 and 12°. Thereafter, the bacteria were suspended in fresh nutrient solution to allow growth to continue. If the sediment was resuspended and re-incubated in complete medium such as YDC, YC, YP, or horse meat-extract peptone broth, after a definite period of growth the culture underwent mass lysis with production of megacin. Lysis was not inhibited even when the transfers of cells into fresh media were made immediately after the irradiation of the culture. Thus under these circumstances there was no evidence for reversibility of the induced state, such as was found when irradiated cells were transferred to the surface of a solid medium.

It was quite otherwise when irradiated cells were transferred, after the lapse of different periods of time, to the defined FGG nutrient medium (Fig. 2). For 45 min. the change in the culture treated with u.v. radiation was completely reversible. After longer periods of time the transferred cells were found to be already disintegrated, yet in the lysate there were only small amounts of megacin. This suggests that lysis of cells and megacin production are not strictly parallel processes. Synthesis of active megacin is apparently not complete until the very end of the residual growth period, and is contingent upon adequate conditions in the medium.

Fig. 2. Behaviour of bacteria, grown in YDC medium and irradiated with u.v. light, after their transfer to FGG medium. *Bacillus megaterium*, strain 216, grown in YDC, irradiated for 30 sec., and reincubated. 10 ml. samples taken at various times thereafter. The bacteria were washed and reincubated in FGG medium. The figures by the curves indicate the amounts of megacin produced (% of max.).

Induction in FGG (defined) nutrient medium

Characteristics of irradiated cultures. In FGG medium seeded with the standard inoculum (grown on FGG agar), maximal growth varied from 0.50 to 1.00 optical density in different experiments.

Young cultures exposed to u.v. radiation (10 sec.) had usually not lysed after 24 hr. of reincubation. Occasionally, however, a very slight reduction in density was observed within 3 hr. When this occurred a small amount of megacin, less than one-tenth of the amount produced under optimal conditions in YDC medium, was found in the culture. In other cases, when practically no reduction in optical density was observed, only traces of megacin were formed in FGG medium.

Although in the defined medium u.v. irradiation was followed neither by appreciable lysis nor by megacin production, some photobiological effect was nevertheless apparent when the cells were plated on to the surface of an agar medium. As can be seen in Fig. 3 the optical density of the culture

grown in FGG medium and irradiated, reached a maximum at about 200 min. and thereafter remained constant. Cell division ceased about 90 min. after irradiation, which means that the further rise in optical density was due solely to elongation of the cells. It was notable that when the irradiated bacteria

Fig. 3. Young culture of *Bacillus megaterium*, strain 216, grown in FGG medium, irradiated and allowed to continue growing. A, optical density; B, total bacterial count estimated in the haemocytometer; C, individual chain count; D, E, number of colony formers obtained on FGG and on YDC agar, respectively.

were plated immediately on to nutrient agar, only 0.3 % of the colony-formers originally present had yielded colonies in YDC medium, as compared with 2.4 % in FGG agar. However, this effect gradually disappeared; when plated out 90 min. later the colony counts slightly exceeded their initial values.

Subculture in YDC medium of bacteria irradiated in FGG medium. The experiment shown in Fig. 4 demonstrates that both lysis and megacin production ensued when cells cultured and irradiated in FGG solution were transferred to complete yeast extract plus casein digest medium. The figure shows the effect of transfer at times up to only 90 min. after irradiation, but closely similar

results were observed when bacteria, irradiated in FGG medium, were transferred to complete nutrient solution as long as 5 hr. after the u.v. treatment. Thus, although in the defined medium irradiated bacteria ceased multiplying and growing after 200 min., they retained their 'induced' state several hours.

Fig. 4. Behaviour of cells induced in FGG medium after their transfer to YDC medium. Young culture of *Bacillus megaterium*, strain 216, grown in FGG medium, irradiated for 10 sec. and resuspended in 10 ml. of YDC medium and reincubated. The figures on the curves indicate the amount of megacin produced as percentage of maximum. Arrows show transfer, after irradiation, from FGG to YDC medium.

Morphology of the induced cells. The changes observed in bacteria irradiated and subcultured in defined medium can be summarized as follows. During the first 90 min. the individual rods did not differ in outward appearance before and after irradiation; after 90 min. irradiated cells gradually became elongated, but not even in preparations made 260 min. later was any disintegration or lysis observed. This morphological picture corresponds with findings represented in Fig. 3.

DISCUSSION

As we have already described (Ivánovics & Alföldi, 1955) some of the *Bacillus megaterium* strains are bacteriocinogenic. This fact opens up new possibilities for the study of what is called lethal biosynthesis. Some such individual

strains lyse gradually when irradiated with u.v. light and subcultured. This is associated with the accumulation in the lysate of a considerable quantity of the bacteriocin designated by us as megacin (Ivánovics & Alföldi, 1954). Our present experiments show that after u.v. treatment 97–99 % of the cells of our inducible *B. megaterium* strain lyse. This is about the same proportion as that observed in the lysogenic strains of this species.

So far we have failed to demonstrate any lysogenic property in *Bacillus megaterium*, strain 216. In the lysate obtained after induction we were unable to detect even a single infective phage particle, although lysate corresponding to 10^8 cells had been plated out on various indicator strains of *B. megaterium* sensitive to temperate phages. Furthermore, no phage particles were seen in lysates of strain 216 under the electron-microscope (Ivánovics, Alföldi & Lovas, to be published). No infective phages were found either in lysates obtained after u.v. irradiation or in any cultures of our megacinogenic strain grown on liquid or solid media. We think it essential to stress this point, because the behaviour of our strain following irradiation with u.v. light is in many respects reminiscent of the behaviour of the typically lysogenic strain 899 (1) of *B. megaterium* (Lwoff, Siminovitch & Kjølgaard, 1950; Lwoff, 1953*b*). But while after induction this lysogenic strain 899 (1) produces phage, strain 216 produces megacin. Our strain also differs markedly from the strain 91 (1) studied by Lwoff & Siminovitch (1952) which lyses after irradiation but whose lysate contains neither phage nor bacteriocin.

The experiments undertaken so far do not enable us to answer the question whether megacin is a non-reproductive product of some prophage which may be carried by strain 216 and may govern its hereditary character. The similarities observed between the lysogenic *Bacillus megaterium*, strain 899 (1), and the bacteriocinogenic strain 216 in their induction, support this hypothesis. On the induction of lysogenic *B. megaterium*, strain 899 (1), by u.v. light Lwoff *et al.* (1950), as also Delaporte & Siminovitch (1952), observed first division, then elongation, and finally lysis of cells occurring in the second generation following irradiation. The cells of our bacteriocinogenic strain divide for half an hour after irradiation without any essential changes in shape or size. In the second generation following irradiation the cells have already become elongated, although cell division still occurs. The cells are twice their original size, but at the same time gradual disintegration begins. In the first generation the process of induction is still reversible if the cells are plated on the surface of nutrient agar. In inducible lysogenic bacteria this is a well-known phenomenon (Lwoff, 1953*b*). With our strain the process is also reversible when the irradiated cells are transferred to a defined medium.

On the evidence presented it would appear that lysogeny and megacinogeny are somehow interconnected, although we do not yet know how.

We wish to thank Dr M. R. Pollock for advice and for correcting the manuscript.

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EXPLANATION OF PLATE

Culture of *Bacillus megaterium*, strain 216, grown on YDC medium, irradiated for 30 sec. and re-incubated. Stained preparations made at various times. Fig. 1, before irradiation; 2, 60 min. after; 3, 90 min. after; 4, 120 min. after; 5, 150 min. after irradiation. Cell-wall staining after Gutstein. $\times 1800$.

(Received 23 July 1956)

G. IVÁNOVICS & L. ALFÖLDI—BACTERIOGINOGENESIS IN *B. MEGATERIUM*. PLATE 1

(Facing p. 530)