# PHENOCOPY OF RESISTANCE TO PHAGE W IN BACILLUS ANTHRACIS

By

G. Iványovics and Judith Lantos

*Institute of Microbiology (Director : G. Iványovics), University Medical School, Szeged*

(Received March 5, 1962)

**Summary.** Liquid cultures of capsulogenic and non-capsulogenic strains of *Bacillus anthracis* were infected with mutant $\alpha$ or $\alpha$C of phage W. The lysis of cultures was followed by secondary growth of bacteria which was associated with an increase of the phage titre. The growth of the culture was terminated by a mass sporulation of bacteria. Individual spores which had formed in the presence of phage gave rise to either a colony of a plaque, but not to both. This indicated that a proportion of spores contained the phage genome in a labile (preprophage) form. The colonies developed from the spores were found to be highly sensitive to homologous phage, suggesting that the secondary growth consists of bacteria either sensitive to phage W or containing preprophage. Vegetative bacteria harvested during the secondary growth were not able to adsorb phage W, although they readily fixed a heterologous phage. The temporary loss of the specific receptor to phage W imitates a phage resistance, therefore the refractory state of bacteria to infection of phage W appears to be a phenocopy of phage resistance.

A lysogenic strain of *Bacillus cereus*, strain W, has been known to release a phage specific to *B. anthracis* [1]. McCloy named this temperate phage, phage W$\beta$ [2]. The "virulent mutant", W$\alpha$ (phage $\alpha$ in the following) does not form lysogenic complexes with the atypical asporogenic strain Davis of *B. anthracis* [2]. On the other hand, sporogenic strains of *B. anthracis* gave a low lysogenic response to phage $\alpha$ and by an adequate technique stable $\alpha$-lysogenic derivates could be obtained [3]. Thus phage $\alpha$ appeared to be a semitemperate rather than a virulent mutant of W$\beta$.

A recently isolated mutant of $\alpha$, designated $\alpha$C, appeared to be more virulent than phage $\alpha$ and it failed to establish itself as a stable prophage in *B. anthracis* [3]. In contrast, phage $\alpha$C yielded segregants of *B. anthracis* with defective prophage which did not liberate infective particles and the presence of the defective prophage was revealed only by the immunity pattern of the segregants [4]. It seems interesting that the genom of both phage $\alpha$ and $\alpha$C might be incorporated into the spores of *B. anthracis* in an unstable form possibly as a preprophage. Spores containing such a phage genom are lysed after germination instead of giving rise to colonies [3, 5].

It was remarkable that in spite of extensive attempts we have succeeded in one single case only in obtaining a bacterium isolate resistant to the semitemperate phages $\alpha$ and $\alpha$C [3]. This single resistant isolate did not prove to be lysogenic and did not adsorb the homologous phage [6]. The nature of this resistance appeared to be similar to that of T phages. Our aim was to study the resistance to phage W of *Bacillus anthracis*.

## Materials and methods

*Bacteria.* The virulent capsulogenic strain Vollum of *B. anthracis* designated $VC^+$ was used. The non-capsulogenic mutant of the same strain was designated as $VC^-$. The capsulogenic character was tested in carbon dioxide atmosphere [3].

For phage assays and for testing lysogeny of the isolated strain Davis of *B. anthracis* was used [2, 3].

*Phages.* The origin and characteristics of the phages used in the present studies have been described [3]. The studies were carried out with the semi-temperate phage $\alpha$ and its more virulent mutant $\alpha$C. Phage 27 *cr* of group $A_3$ isolated and characterized in our laboratory [7] was also used.

*Phage stocks* were obtained by propagation in strain Davis.

*Media.* Except for special cases the yeast extract peptone (YP) medium was used throughout. (For the details of this medium our previous papers should be consulted.) Medium YP was solidified by the addition of 1.5 per cent agar. As described previously, the cultivation of *B. anthracis* in the YC/BB system was performed in 25 per cent $CO_2$ atmosphere on YC agar containing $NaHCO_3$ [3].

*Buffer solution* (solution SMB) was prepared by adding 5 ml of 0.07 *M* phosphate buffer and 1 ml of a 1 per cent $MgSO_4 \cdot 7H_2O$ solution to 100 ml of saline.

*Cultivation in liquid media.* Fifteen ml YP medium was measured into a 100 ml Erlenmeyer flask and after inoculation it was incubated under rocking (68 rocks per minute with 7.5 cm amplitude) in a water bath of 35° C. The growth of the cultures was followed by turbidimetry as described earlier [7].

*Phage assay* was made in agar overlay.

*Preparation of spore suspension.* Bacteria appropriately spored in liquid medium were centrifuged and washed with saline. The sediment was resuspended in 0.05 *M* phosphate buffer (pH 7.0) and digested at 47 °C in a water bath for a few hours. This was followed by washing, and centrifugation in a horizontal centrifuge at 100 *g* for 7 min., and the supernatant was heated as described earlier [3].

*Examination of the spores.* 0.1 ml of an appropriate dilution of heat-treated spore material was plated on YP agar. Simultaneously, a similar amount of the same material mixed with a suspension of strain Davis was layered in soft agar. The number of colony and plaque-formers was determined. The phage sensitivity and lysogeny of the colonies developed was determined by cross-streaking as described previously [3].

*Phage ultrafiltrates.* Cultures in the secondary phase were centrifuged and the supernatant filtered through an ultrafilter of 35 to 20 m$\mu$ pore diameter (Membran Filter Göttingen "mittel") at 15—20 atm. nitrogen pressure. The filtrates thus obtained were in every case free of phages.

## Results

*Changes in the bacterium—phage population.* Cultures of *B. anthracis* strain Davis infected with $\alpha$ or $\alpha$C phages were completely lysed after a few hours of reincubation. No secondary growth occurred in the lysate on further incubation, thus no resistant bacteria could be isolated from strain Davis. A different phenomenon was observed when exponentially growing cultures (0.15 to 0.25 o. D. = 60 to $120 \times 10^6$ bacteria per ml) of $VC^+$ or $VC^-$ were infected (multiplicity of infection, 0.5 to 1) with phage $\alpha$ or $\alpha$C, and reincubated. The turbidity of the infected cultures increased for about 2 hours. This period was followed by a sudden mass lysis of cultures in the 4th to 5th hour of incubation. Up to the 10th to 12th hour of the experiment, the turbidity remained on the same very low level. A gradual increase of turbidity started this time, to last until the cultures had reached their maximum optical density values in 24 to 30 hours. The course of events was essentially the

same when using $VC^+$ or $VC^-$ strains of *B. anthracis* with either phages *a* or *a*C. Fig. 1 gives a graphic presentation of the process.

The titre of the phage material obtained in strain $VC^+$ and $VC^-$ cultures was always higher than that obtained in strain Davis. The difference was particularly great if the samples were taken during the secondary growth. Thus the mean value and the probable error of the titre of phage *a*C in samples taken from infected $VC^+$ cultures at the beginning or the middle of secondary

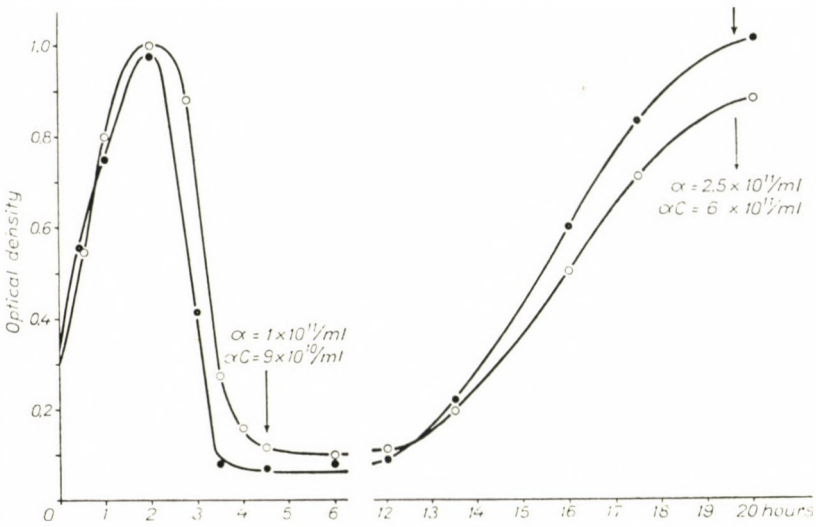


*Fig. 1.* Growth curves of cultures of strain $VC^-$ infected with phage *a* and phage *a*C, respectively

● infected with phage *a*C; ○ with phage *a*

The figures show the plaque forming units/ml in samples taken at the time indicated by arrows.

growth in 10 individual experiments was $2.41 \times 10^{11} \pm 0.6 \times 10^{11}$/ml, while the mean titre of 6 lysates obtained in strain Davis amounted to $2.38 \times 10^{10} \pm \pm 0.45 \times 10^{10}$/ml.

At the end of the first phase, *i. e.* at the time of maximum lysis, the phage titre was considerable. Nevertheless, this value usually still increased during secondary growth. This process is demonstrated in Fig. 1 and Table I. During secondary growth the phage titre usually starting from $10^{11}$/ml approximated and occasionally reached $10^{12}$/ml.

According to our observations, both the capsulogenic and the non-capsulogenic mutants of strain Vollum exhibited 2 phases of growth when infected with either *a* or *a*C phages. The first phase terminated with lysis and was followed by secondary growth. During the secondary growth phase usually an increase in the phage titre occurred.

**Table I**

*Number of plaque forming units/ml in cultures of VC⁻ and VC⁺ infected with phage α and αC, respectively*

| Sample taken at | Host cell and phage | | | |
|---|---|---|---|---|
| | VC⁻ | | VC⁺ | |
| | α | αC | α | αC |
| 1st period*..... | $1.6\times10^{11}$ | $1.6\times10^{11}$ | $1\times10^{11}$ | $8\times10^{10}$ |
| 2nd period .... | $1.6\times10^{11}$ | $3\times10^{11}$ | $8\times10^{11}$ | $4.5\times10^{11}$ |

* 1st period = at maximum lysis; 2nd period = at about the middle of secondary growth

**Table II**

*Colony and plaque formers of spore materials obtained from the secondary growth of VC⁺ in the presence of phage αC*

| N° of exp. | P. f. u./ml in the culture[1] | Plating of the spore material | | | Proportion of plaque and colony formers %[4] |
|---|---|---|---|---|---|
| | | Number of colony formers/ml | | Plaque formers per ml | |
| | | on YP agar[2] | YC/BB culture[3] | | |
| 1. | $1.6\times10^{11}$ | $3.1\times10^{6}$ | $2.9\times10^{6}$ | $2.9\times10^{5}$ | 8.6 |
| 2. | $1.2\times10^{11}$ | $3.3\times10^{6}$ | $4.1\times10^{6}$ | $9.1\times10^{4}$ | 2.7 |
| 3. | $1\times10^{12}$ | $1.5\times10^{6}$ | $6.2\times10^{6}$ | $5.6\times10^{5}$ | 27.0 |
| 4. | $3.9\times10^{10}$ | $5.0\times10^{6}$ | $4.1\times10^{6}$ | $9.6\times10^{5}$ | 16.0 |

[1] The numbers indicate the plaque forming units/ml in the sample taken to obtain spore material.

[2] Colonies formed on YP agar appeared to be rough.

[3] Colonies developed in the presence of $CO_2$ were all mucoid in appearance.

[4] Estimated number of colonies on YP agar by the formula:

$$\frac{100\times\text{plaque formers}}{\text{plaque} + \text{colony formers}}$$

*Morphological changes in the phage infected cultures.* The cytological changes in phage infected VC⁺ and VC⁻ cultures were followed by phase-contrast microscopy. In the first phase, that is after the infection, proliferation and later extensive lysis of the cells was seen. At the time of maximum lysis a moderate number of cells was demonstrable in the samples. In addition to the few intact bacteria, a great amount of destroyed, empty cell wall residues, amorphous debris, granules, etc., were found. The intact bacteria were generally single; no chains could be seen. Empty cell wall residues or amorphous debris sticking to the ends of the intact single bacteria were frequently found.

During the period of lysis the above-described morphology was seen for a few hours. The beginning of secondary growth was readily demonstrable

by microscopy. It was characterized by a gradually increasing number of bacteria followed by the formation of longer and longer chains. At the middle of the secondary growth the culture consisted mainly of chains of 16 to 24 members. The length of the chains then exhibited a further increase. No spores were found in the newly formed chains at the beginning, while later, after 15 to 20 hours, some of the cells developed spores. The number of spores gradually increased and in the 30th to 36th hour of the experiment practically every cell contained a spore. Though the vegetative part of the spore-bearing cells was successively autolysed, the spores remained sticking together in chains of 4 to 8 members.

It has to be emphasized that the above-described morphology was the same for both the $VC^+$ and the $VC^-$ strains. Cultures of $VC^+$ were examined by different staining methods for the presence of capsule; no capsule formation was seen.

*Analysis of the secondary population.* Characterization of the bacteria grown secondarily with simultaneous phage proliferation was attempted through the analysis of the spore material. Heated spore suspensions obtained from the secondary cultures were plated on YP plates, YC/BB cultures and in cultures with Davis indicator. YC/BB cultures were incubated in 25 per cent $CO_2$ atmosphere.

A considerable number of experiments was performed on 4 different occasions under the conditions described above. The results of these experiments performed with strain $VC^+$ and phage $\alpha C$ are summarized in Table II.

A proportion of the spores was found to yield infective centres in the indicator organism. The plaques formed were structureless, clear and measured 3 mm in diameter. Thus they were characteristics for mutant $\alpha$ [2, 3]. The proportion of spore-forming plaques was different in the individual experiments. Colonies obtained from YP and YC/BB cultures in different experiments were examined for both phage sensitivity and lysogeny. Table III contains information on the isolated colonies from YP and YC/BB cultures obtained in the experiments included in Table II.

The results of the four experiments permit the following conclusions. In spite of the high phage content of the cultures, only part of the spores contained phage genom. Among the 104 and 123 colonies examined in the four experiments, none was found to be lysogenic or resistant to the phage. This means that the spores of the secondary growth developed at a high phage concentration, were neither lysogenic nor immune to the phage. In spores producing infective centres no stable prophage was present and the phage genom was in its labile state known to be characteristic of this system [3,5]. We have to emphasize that the plaques formed (Table II) could not have originated from contamination, as no plaques were found on plating with streptomycin-resistant Davis strain in streptomycin agar.

**Table III**

*Analysis of individual colonies developed from spores on YP plates and YC/BB cultures*

(Material of the four individual experiments summarized in Table II)

| N° of exp. | Behaviour of individual colonies when tested for lysogeny and sensitivity* | |
|---|---|---|
| | Colonies of YP agar | Colonies in YC/BB culture |
| 1. | Lysogenic 0/30<br>Sensitive 30/30 | 0/30<br>30/30 |
| 2. | Lysogenic<br>Sensitive | 0/15<br>15/15 |
| 3. | Lysogenic 0/17<br>Sensitive 17/17 | 0/6<br>15/15 |
| 4. | Lysogenic 0/6<br>Sensitive 16/16 | |

* Proportion of colonies tested. When an equal number of colonies was tested both for lysogeny and sensitivity, the same colonies were examined for both characteristics.

Essentially the same phenomenon was observed with the non-capsulogenic strain when infected with phages *a* or *a*C. Results of two such experiments are presented in Table IV.

**Table IV**

*Colony and plaque formers of spore materials obtained from the secondary growth of VC⁻ in the presence of either phage a or phage aC*

| N° of exp. | Phage | Colony formers/ml | Plaque formers/ml | Proportion of plaque formers % |
|---|---|---|---|---|
| 1. | *a* | $2.7 \times 10^6$ | $8.8 \times 10^5$ | 25 |
| | *a*C | $3.1 \times 10^6$ | $2.5 \times 10^1$ | 0.001 |
| 2. | *a* | $5.8 \times 10^5$ | $5.5 \times 10^7$ | 99 |
| | *a*C | $3.0 \times 10^7$ | $1.6 \times 10^6$ | 5.3 |

As demonstrated, only a low proportion of spores of strain $VC^-$ contained the genom of phage *a*C in one experiment, while in the other the relations were similar to those found for the $VC^+$ strain (see Table II). On the other hand, spores isolated from the secondary growth in the presence of phage *a* contained phage genom very frequently. This phage genom was, however, in a labile state; none of the 54 colonies developed and examined separately was found to be lysogenic, while each of them was phage sensitive. We have to mention, however, that on plating the spores developed in the presence

of phage *a*, intact colonies were obtained only at high dilutions of the spore material, and even then some of the colonies were eroded, due to contamination from the lysed spores. When, however, smears were prepared from the area between colonies, the presence of phage was frequently demonstrated. These observations also suggest that individual spores which had formed in the presence of phage give rise to either a colony or a plaque [3, 5]. It can thus be stated that the spore material of secondary growth developed in the presence of phage consists of either sensitive or phage carrier population. It should, however, be stressed that the bacteria have grown for a considerable period before sporulation in the presence of a high concentration of phage, so that they must have possessed some resistance to phage in their vegetative phase.

*Phage adsorption by the secondary population.* In order to elucidate the mechanism of secondary growth in the presence of a large phage population it has been examined whether the vegetative bacteria in secondary growth were able to adsorb heterologous or homologous phages. For this purpose bacteria from a secondary growth of strain $VC^+$ grown in the presence of phage *a*C were repeatedly washed to remove phages and their capacity to adsorb homologous or heterologous phages was examined. As a heterologous phage an $A_3$ phage (strain *27cr)* was used.

From a culture in secondary growth before spore formation (0. D. = = 0.4 to 0.5), which contained about $10^{11}$ to $5\times10^{11}$ plaque forming units per ml a sample was taken and centrifuged in a cooled angle head centrifuge for 10 to 15 minutes at 2100 r. p. m. The sediment was washed 4 to 5 times with excess SBM solution and finally resuspended in SBM. The suspension contained approximately $160\times10^6$ cells and $10^5$ to $10^6$ phage particles per ml. Phage *a*C or $A_3$ was added to aliquots of the washed bacterial suspension and incubated for 30 minutes at 37° C. The bacteria were centrifuged in the cold and the phage content of the supernatant was determined. Control experi-

**Table V**

*Adsorption of phage aC and $A_3$ by bacteria of strain $VC^+$ grown in the absence (control) and presence of phage aC*

| N° of exp. | Bacterial suspension | Phage | M. o. i. | Phage added p. f. u./ml | Phage found p. f. u./ml | Adsorption % |
|---|---|---|---|---|---|---|
| 1. | Control | *a*C | 0.22 | $4.6\times10^7$ | $6.5\times10^5$ | 98.6 |
| | Secondary growth | *a*C | 0.25 | $4.9\times10^7$ | $4.1\times10^7$ | 13.3 |
| 2. | Secondary growth | *a*C | 0.20 | $3.7\times10^7$ | $3.6\times10^7$ | 0.0 |
| 3. | Control | $A_3$ | 1.0 | $2.0\times10^8$ | $1.1\times10^7$ | 94.7 |
| | Secondary growth | $A_3$ | 1.3 | $2.5\times10^8$ | $2.6\times10^7$ | 86.4 |
| 4. | Control | $A_3$ | 0.3 | $6.6\times10^7$ | $1.4\times10^7$ | 78.8 |

ments were carried out with bacteria grown in the absence of phage and washed with SBM solution.

As demonstrated in Table V, the secondary growth obtained in the presence of phage αC did not adsorb the homologous phage (in one experiment the moderate rate of adsorption was within the limits of experimental error). In contrast, heterologous phage $A_3$ was adsorbed to approximately the same extent by both bacterial suspensions.

Numerous experiments were performed with strain $VC^-$ under the same conditions. In these experiments, however, the bacteria were washed only twice with SBM, while the residual phage was inactivated by heat treatment (70° C for 20 minutes). The amount of adsorbed phage exhibited great variations in the individual experiments, probably because of differences in the effectiveness of removing cell debris. The cell debris resulting from autolysis are known to adsorb phage α [6]. Omitting the detailed description of each individual experiment we may summarize the results of 12 different experiments as follows.

Bacteria from the secondary growth (in the presence of either α or αC phages) failed to adsorb homologous phages, or the amount of adsorbed phage was remarkably lower than that in the control experiment. Phage $A_3$ was, however, readily adsorbed by the bacteria from the secondary growth. The mean values of adsorption are presented in Table VI.

The mean values of phage adsorption by the secondary growth varied greatly in the individual experiments. One third of the suspensions obtained from the secondary growth did not adsorb phage in demonstrable amounts, while the same suspensions adsorbed 60 to 80 per cent of the heterologous phage ($A_3$).

*Attempts to influence phage adsorption by lysates.* The experiments presented above have shown that the secondary bacterium growth obtained in

**Table VI**

*Mean values of adsorption of phage α, αC and $A_3$ by bacteria of strain $VC^-$ grown in the absence (control) and presence of phage α and αC*

(Summary of results of 12 individual experiments)

| Phage used in adsorption test | Phage adsorption in % by different bacterial suspensions | | |
|---|---|---|---|
| | Control | Secondary growth in phage αC | Secondary growth in α |
| αC ..... | 90 | 30 | n. t. |
| α..... | 95 | n. t. | 28 |
| $A_3$..... | 94 | 70 | 73 |

n. t. = not tested.

the presence of a large phage population either did not adsorb the homologous phage at all or the adsorption was extraordinarily moderate. It seemed as if the decrease or disappearance of the W phage receptor had been in connection with a soluble enzymatic principle from the large amount of phage or bacterium lysate. To test this possibility, cultures in the secondary growth phase were centrifuged and the supernatant filtered through a collodion membrane. The phage-free filtrates were mixed to exponentially growing $VC^-$ bacteria. Samples of the mixture were centrifuged after 30 or 60 minutes and the adsorption of phage $\alpha$ by the sedimented cells was examined. This treatment did not influence phage adsorption, thus no decrease in the receptor substance of the bacteria could be demonstrated on treatment with phage-free lysates. In this experiment we have failed to demonstrate the presence of a soluble substance rapidly destroying the receptor.

## Discussion

The conditions of equilibrium of phage—bacterium populations have been studied with appropriate accuracy in a few instances only. The conditions valid for lysogenic systems were poorly understood before the recognition of the phenomenon of lysogenesis. Recently, the fundamental studies of LWOFF [8] have yielded valuable informations in this field.

The phenomenon described in connection with the phage of *Streptococcus cremoris* deserves special mention. As described by HUNTER [9], cultures of that coccus are partially lysed by a specific phage and the latter attains very high titres in the system. Cocci isolated from lysates of high phage content did, however, not prove to be lysogenic and they exhibited an unaltered phage sensitivity. This particular state of equilibrium between bacteria and phages was difficult to understand on the basis of the experimental data available.

A special sort of equilibrium of phage-bacterium population was found in our experiments with virulent and semitemperate mutants of phage W. After infection of *B. anthracis* cultures with these phages mass lysis ensues which is followed by a secondary growth of the surviving bacteria. The population of the secondary growth appeared to be resistant to the homologous phage, though only a proportion of bacteria were found to carry phage genom in a labile form. These labile complexes did not form colonies but were lysed after the germination of the spores. Colonies grown from spores liberated from phages did not differ in their phage sensitivity from those of the original bacteria. Thus, the phage sensitivity of the bacterium clone that had survived phage infection was recovered.

It seemed remarkable that the phage adsorption of the cells grown secondarily in the presence of a large phage population differed considerable

from that of the original bacteria. Unfortunately, the further examination of this problem was limited by technical difficulties. It was, however, apparent that bacteria from the secondary growth did not adsorb the homologous phage or the adsorption of phage was considerably diminished. It is difficult to find the cause of the disappearance of the phage receptor or, more accurately, of its missing function, on the basis of the experiments presented above. There are, however, several possible explanations. There might be a certain inhibitor that interferes with the adsorption of the phage to the receptor. This would, however, be in contrast with the observation that washed suspensions also failed to adsorb phage. It could be supposed that certain receptor destroying substances might be liberated either by the phages or by the lysed bacteria. Our experiments, however, did not support such a supposition. Most probably the conditions of receptor synthesis might be disturbed after the first lytic phase. Thus, though the genetic background for receptor production might be intact, the receptor defect brought about by environmental conditions results in missing infection which was followed by spore production. The colonies developed from such spores in a phage-free environment are sensitive to phage and this progeny would not differ from the normal bacterium. Thus the phenomenon seems to imitate phage resistance. This non-inheritable receptor deficiency might be regarded as the phenocopy of phage resistance.

## LITERATURE

1. McCLOY, E. W.: J. Hyg., **49**, 114 (1951).
2. McCLOY, E. W.: J. gen. Microbiol., **18**, 198 (1958).
3. IVÁNOVICS, G.: J. gen. Microbiol. **28**, 87 (1962).
4. IVÁNOVICS, G.: to be published.
5. McCLOY, E. W.: Proc. 6th Int. Congr. Microbiol., **3**, 49 (1953).
6. LANTOS, J., IVÁNOVICS, G.: Acta microbiol. Acad. Sci. hung. **8**, 379 (1961).
7. LANTOS, J., VARGA, I., IVÁNOVICS, G.: Acta Microbiol. Acad. Sci. hung., **7**, 31 (1960).
8. LWOFF, A.: Bact. Rev., **17**, 269 (1953).
9. HUNTER, G. J. E.: J. Hyg., **45**, 307 (1947).

Address of the authors:

GYÖRGY IVÁNOVICS, JUDIT LANTOS

Institute of Microbiology, University Medical School, Beloiannisz tér 10, Szeged, Hungary