Bacteriology. — *Further Observations on the Bacteriophage of Bacillus megatherium.* By L. E. DEN DOOREN DE JONG. (Communicated by Prof. A. J. KLUYVER).

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When I last wrote about the bacteriophage of *Bac. megatherium* [1]) I took for granted that when the spores of *Bac. megatherium* were heated to temperatures destructive to the bacteriophage in vitro (e.g. 10′ at 90° C), the spores would be rid of a possibly existing bacteriophage infection. Hence when it was found that the cultures of certain strains of *megatherium,* after being subjected to a careful pasteurization for 10′ or 15′ at 90° C or 100° C, and then filtrated, still contained a very active bacteriophage for certain *megatherium* mutilates, this fact was looked upon as an absolute proof of the bacteriophage not being an autonomic ultra microbe, but a product of the living bacterial cell.

Several investigators in our country [2]) and also COWLES [3]) have fully confirmed the correctness of the above mentioned experiments. They suggested the possibility of the spores transmitting their thermoresistant properties to foreign substances which they had included at the time of their spore formation, as an explanation of the phenomena. Hence according to them the phage in the bac. spore would pass into another unknown but more resistant form, since from a physical point of view it is not thinkable that during the process of pasteurization a temperature lower than the surrounding one could be maintained inside the spore. Although there are various objections to this hypothesis, still the fact that so little is known of the factors determining the thermoresistance of the spores, makes it desirable for us to test its value by further experiments.

Firstly experiments were done to find out whether strains of spore forming bacteria which had never been found to possess the property of phage production, could be brought to do so by allowing the bacteriophage to act upon them during the process of spore formation. The positive result of these experiments which were begun in the middle of 1930, does not necessitate our accepting D'HERELLE's theory of the

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[1]) The bacteriophage of *Bac. megatherium*; a product of the living bacterial cell. Proceedings Kon. Akad. v. Wetensch. **33**, N°. 1 (1930), 51 and Ned. Tijdschr. v. Hyg. Microb. en Serol. **4**, 255 (1930); Studien über Bacteriophagie I Zbl. Bakter. I, Orig. **120** p.l. (1931).

[2]) See on this subject Ned. Tijdschr. v. Hyg. Microb. en Serol. **5**, 54 (1931) and C. R. RITSEMA VAN ECK, diss. Leyden 1931.

[3]) PH. B. COWLES, J. Bact. **22**, 119 (1931).

autonomic ultramicrobial nature of the phage. After further consideration it seemed quite possible to find an explanation of the phenomena in a further development of my view of the subject. It seems to me quite reasonable to think of the phage which has been brought into the bacteria cell, as only acting as a stimulus to the latent power of phage formation in that cell. In other words the phage found after the process had taken place, would owe its origin to the bacteria cell itself, and not at all to the phage, which had provided the stimulus to phage formation. In the meantime COWLES' article mentioned above appeared in August 1931. He too came to the conclusion that non-bacteriophagic strains of several spore forming micro-organisms (*Bac. megatherium, Cl. tertium* and more especially *Bac. anthracis* and *Bac. subtilis*) could be acted upon by diverse homologous bacteriophages and altered in such a way that the lytical principle could be demonstrated in filtrates of cultures which had been obtained from heated spores. But his spores which we may look upon as artificially "infected" were never infected to such a degree as the spores of the *megatherium* strains which I had used and which following my line of thought might be looked upon as naturally infected. All the colonies of these cultures contrary to the artificially infected *subtilis* strain produced active filtrates and heating them up to temperatures which the spores could only just bear was never able to destroy their phage producing powers. On the other hand pasteurization of the artificially infected *anthracis* strains up to 95° C. was able to destroy the phage producing powers of those cultures. COWLES came to the following conclusion: "This does not necessarily mean that the lytic agent as such survives the heating process but it does mean that the recovering of a lytic principle from a pasteurized culture is not conclusive proof of the spontaneous generation of bacteriophage".

The results of these experiments led me to take up a more elaborate study of the question of artificial "infection". I have not confined myself to the *Bac. megatherium* as I did in the past, but have used other species of the genus *Bacillus*. Unlike COWLES I always confined myself to the use of *megatherium* bacteriophages, first taking the phage of *megatherium* N⁰. 899 and using the two types 899 O and 899 ⊙. A dissemination of filtrates of *Bac. megatherium* 899 like those of 333, on spreadings of almost all of the susceptible *megatherium* mutilates, gave rise to two kinds of plaques, the normal round hole form of plaque, and a second form with a small bacteria colony in the centre (called dot plaques). If the culture is allowed to go on incubating [1]), a concentric ring of bacteria material is formed. As far as we could see the two types of 899 never interchanged. Hence it seemed to me important, moreover, to find out whether both types of phage only led to the production of its own type when used to activate the spores of phage free strains.

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[1]) Zbl. Bakter. 1 Orig. **120** p. 19 (1931).

A. Experiments with the 899 O phage.

This phage was obtained by putting strain 899 in an ERLENMEYER bulb with 1 % peptone water and incubating for two days at 30° C., after which the culture was filtered through a Seitz filter. The filtrate obtained was disseminated in various degrees of dilution from 10^{-1} to 10^{-6} c.c. on spreadings of a specially susceptible *megatherium* mutilate viz. 338*b*′ [1]). The plates obtained were then incubated at 37° C. Within 24 hours they showed a mixture of two kinds of plaques which when obtained from the weaker solutions (usually 10^{-5} or 10^{-6}) were situated so far from each other that they could be pricked away with the agar on which they were lying, without the danger of mutually infecting each other.

Then we took a bit of agar with an O plaque on it, and containing of course a little bacteria material necessary to increase the lytic principle, and inoculated it in 1 % peptone water and incubated for two days at 30° C. The culture fluid obtained was again filtered through a SEITZ filter. Its purity and concentration was tested and determined by disseminating the filtrate in varying degrees of dilution on 338*b*′ spreadings. In the greater number of cases the filtrate so obtained was found to contain the pure phage, that was in this case 899 O, which at a temperature of 37° C. in 338*b*′ always formed plaques with an average diameter of 2 m.m. It was sometimes necessary to repeat the process as one always ran the risk in digging out a large plaque, of taking a small undeveloped plaque of the other species with it.

We used the same strains for these experiments that we used in our previous research work on this subject, viz. *Bac. megatherium* 333, 334, 335, 336, 337, and 338. With the exception of 333 these strains were known to lack the power of producing a perceptible bacteriophage.

These six strains were inoculated in duplo on slanting 1 % peptone agar in tubes which were then put straight away into an incubator at a temperature of 37° C. Six hours later a thin transparent layer of bacteria could be seen on the peptonagar in all the tubes. Microscopic examination showed that no spore formation had taken place among the various spore forming bacteria. After that one of the two series of tubes was put in an incubator at 30° C. so as to facilitate spore formation. Then the other series of cultures was taken and 0.3 c.c. of the filtrate 899 O (able to produce per c.c. 20×10^7 plaques in spreadings of 338*b*′ when titrated), was added to each of the six tubes. Care was taken to cover the whole of the bacterial culture with the fluid. This series of cultures was then incubated at 30° C. The next day the bacterial layer in most of the tubes was found to be perceptibly more or less affected [2]). Still after the lapse

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[1]) For further details on this mutilate confr. Studien über Bakteriophagie II pp. 16 and 17.

[2]) I knew from former experiments that the spreadings of several *megatherium* strains e.g. 334, 335 and 337 if examined in very young sporeless condition, may form small plaques. (See Studien über Bakteriophage II, note on page 15).

of a few days a considerable development of spore forming bacteria was found to have taken place in most cases, and these could be reasonably supposed to have been influenced by the phage.

A week after the filtrate had been added to the bacterial cultures, a fair amount of bacterial substance from each of the two series of tubes was inoculated in culture tubes containing 5 c.c. of peptone water. Then all the twelve inoculated tubes were pasteurized while entirely immersed in water for 10′ at 90° C., as soon as possible after the inoculation had been carried out. Great care was taken to keep the temperature within the tubes at 90° C. during the 10′. Then they were cooled down quickly and placed in a temperature of 30° C., or 37° C., to give the bacteria the opportunity of multiplying, so as to facilitate their transference. Two days afterwards a little bacterial substance from each tube with the exception of 335 which had not survived the pasteurization, was inoculated into an ERLENMEYER flask containing 1 % peptone water. These cultures were incubated at 30° C. for two days and then filtrated. Then each of the 10 filtrates, five of which being filtrates of *megatherium* strains which had not been in contact with the 899 O and the other five having been in contact with the 899 O, was tested in various degrees of dilution on spreadings of the mutilate 338*b*′.

The filtrates derived from the strains which had not been treated with the phage 899 led to the following results:

Filtrate 333: When diluted 10^{-3} thousands, when diluted 10^{-5} hundreds of plaques averaging 0.5 m.m., belonging to the O and the ⊙ types.
Filtrate 334: When diluted 10^{-1} no plaques.
Filtrate 336: ,, ,, ,, ,, ,, .
Filtrate 337: ,, ,, ,, ,, ,, .
Filtrate 338: ,, ,, ,, ,, ,, .

These results were absolutely in agreement with former observations.

The filtrates derived from strains which had been brought into contact with the 899 O phage before pasteurization, led to quite different results:

Filtrate 333: When diluted to 10^{-1} only a few hundreds of plaques with an average diameter of 0.5 m.m. belonging to the O type and also to the ⊙ type [1]). Plaques of 2 m.m. were not met with.
Filtrate 334: *When diluted to 10^{-1} a hundred plaques with a diameter averaging 2 m.m., all of the O type.*
Filtrate 336: *When diluted to 10^{-2} a hundred plaques with an average diameter of 2 m.m., all of the O type.*

[1]) It is noticeable that the 333 strain when treated with phage 899 always formed its own phage (plaques of 0,5 m.m.) and never a mixture of the two phages. Also that comparing it with the filtrate of the untreated strain, the titer of its own 333 phage had become less.

Filtrate 337 : *When diluted to 10^{-1} a hundred plaques with an average diameter of 2 m.m. all of the* O *type.*

Filtrate 338 : When diluted to 10^{-1} no plaques.

B. Experiments with the 899 ⊙ *phage.*

We then made a pure culture of the phage 899 ⊙ in absolutely the same way as was done with the phage described under *A*. This too was found to lead to the formation of plaques on spreadings of 338*b'* and at 37° C., plaques with an average diameter of 2 m.m. The titer of the filtrate used had to be 8×10^{7}. The six *megatherium* strains were here too inoculated on slanting jellied 1 % peptone agar, incubated at 37° C., and then after six hours covered with 0.3 c.c. of the filtrate 899 ⊙. In some of the tubes the influence of the phage was noticeable, but still after the lapse of a week a considerable growth of the spore formers had taken place everywhere ; then a fair amount of bacteria material was taken from each of the tubes and inoculated in tubes containing 5 c.c. 1 % peptone water. These suspensions were then pasteurized with the same precautions for 10' at 90° C., and then incubated at 30° C., or 37° C. Two days later a little bacteria material was taken from each tube with the exception of that containing 335, which in this case too had not survived the pasteurization, and inoculated in an ERLENMEYER bulb containing 1 % peptone water. These cultures were incubated for two days at 30° C., and then filtrated. The filtrates which were therefore derived from strains which had been treated with 899 ⊙ phage before pasteurization, led to the following results on the mutilate 338*b'* :

Filtrate 333 : With a dilution of 10^{-4} hundreds of plaques with a diameter averaging 0.5 m.m., belonging to the O and the ⊙ types ; plaques with 2 m.m. were not found.

Filtrate 334 : *With a dilution of 10^{-5} hundreds of plaques with a diameter averaging 2 m.m. all of the* ⊙ *type.*

Filtrate 336 : *With a dilution of 10^{-4} tens of plaques with a diameter averaging 2 m.m., the greater number belonging to the* ⊙*type, and a few to the* O *type.*

Filtrate 337 : *With a dilution of 10^{-5} hundreds of* ⊙ *plaques with a diameter of 2 m.m. and a few* O *plaques with a diameter of 2.5 m.m.*

Filtrate 338 : With a dilution of 10^{-1} no plaques.

Hence from these experiments with the two pure 899 phages described under *A* and *B*, it seemed that the "inclusion" of these phages in the forming spores led to the presence of the lytic principle in the pasteurized spores. It is remarkable, however, that in a few cases (working with strain 336 and 337) the spores seemed able to transform the ⊙ phage into the O

phage, although only to a slight extent, a circumstance which, at any rate in the case of the 899 phage, has never been observed under normal conditions (without the intervention of living spores).

C. Experiments with the 333 O phage.

A 333 filtrate was then made in exactly the same way as has been described under *A*, and then put on 338*b'* spreadings in various degrees of dilution. The O plaques were taken from the mixture and pure cultures were made from them. The 333 O filtrate thus obtained formed at 37° C. per c.c. 36×10^6 plaques of 0.5 m.m. diameter on 338*b'* spreadings. And again in the same way as has been described above, 0.3 c.c. of this filtrate was poured on young still vegetative cultures of the *megatherium* strains 334, 336, 337, and 338, and a week later pasteurized for 10' at 90° C., and then inoculated in 1 % peptone water in an ERLENMEYER bulb. After being incubated for two days at 30° C. the cultures were filtrated and the filtrates obtained were tested on the mutilate 338*b'*. The following result was obtained:

Filtrate 334: With a dilution of 10^{-1} no plaques.
Filtrate 336: With a dilution of 10^{-1} no plaques.
Filtrate 337: *With a dilution of 10^{-4} hundreds of plaques of the O type, of which by far the majority were 0.2 m.m. in diameter, and the rest 1 m.m. in diameter.*
Filtrate 338: *With a dilution of 10^{-4} hundreds of plaques of the O type and 1 m.m. in diameter.*

D. Experiments with the 333 ⊙ phage.

Then young vegetative cultures of the *megatherium* strains 334, 336, 337, and 338 were covered with 0.3 c.c. of a 333 ⊙ filtrate, resulting in the formation of 48×10^4 plaques of 0.5 m.m. in diameter per c.c. on spreadings of 338*b'*. A week later they were pasteurized at 90° C. for 10' and then inoculated in 1 % peptone water in ERLENMEYER bulbs. After two days incubation at 30° C. the fluids were filtered and the filtrates tested on the mutilate 338*b'*. The result was as follows:

Filtrate 334: With a dilution of 10^{-1} no plaques.
Filtrate 336: With a dilution of 10^{-1} no plaques.
Filtrate 337: *With a dilution of 10^{-3} hundreds of plaques with a diameter of 0.5 m.m., 70 % belonging to the ⊙ type, and 30 % to the O type.*
Filtrate 338: *With a dilution of 10^{-3} ten plaques with an average diameter of 0.5 m.m., belonging to the ⊙ type and also to the O type.*

Hence we see in the experiments with the two 333 phages described under C and *D* that the absorption of both these lytical principles by the

forming spores leads to the presence of the phage in the pasteurized spores. It is very interesting to note that the *megatherium* strains 334 and 336 which could be activated by the 899 phages repeatedly proved to be unsusceptible to the action of 333 phages, whereas strain 338 which repeatedly was found to be unsusceptible to the 899 phages, could be affected by the 333 phages. Strain 337 was susceptible both to the 899 phages and to the 333 phages.

It is worthy of note, moreover, that in the spores the transformation of 333 ☉ phage into 333 O phage took place to a still greater degree than the transformation of 899 ☉ phage into 899 O phage. This led me to examine the properties of the 333 phage *in vitro* more carefully than had been done up till now. To my surprise I found that dissemination of 333 ☉ phage led to the formation of a majority of ☉ phage plaques, but also to a small but varying number of O plaques, this being contrary to what was found in the case of the 899 ☉ phage. Hence it is not necessary in this case to call in the help of the spore to effect the transformation, and although it has not yet been confirmed in vitro, it is most probable that the transformation of the 899 ☉ phage into the 899 O phage may also take place apart from the spore.

Hence since it appeared to be possible that cultures of *megatherium* strains could be so influenced by *megatherium* phages that these strains appeared to possess the power of phage production even after pasteurization, it seemed to me very important to find out whether this power could be communicated to other strains not belonging to the species *megatherium*. To do this young asporogenous cultures of *Bac. undulatus* [1]), *Bac. mycoides* and *Bac. mesentericus* were treated with a very active *megatherium* lysate in exactly the same way as was done with the *Bac. megatherium*. A week after they had formed spores these cultures were subjected to pasteurization, which would have been destructive to the phage in vitro. The filtrate of the culture fluids of these strains was examined in the usual way.

E. Experiments with Bac. undulatus.

Young vegetative cultures of the *undulatus* strains 1101, 1102, 1106, 1108*, 1109*, 1110* [2]) were treated with 0.3 c.c. of a 899 ☉ phage, which formed 8×10^7 per c.c. plaques in 338*b'* spreadings. A week later these cultures were pasteurized at 100° C. for 5', and then inoculated in 1 % peptone water in ERLENMEYER bulbs, to which a little of the mutilate 338*b'* had been added. This was done in case only a very few 899 phage particles should be set free from the above mentioned strains; they would then have the opportunity of multiplying and so be demonstrable. After incubating for two days at 30° C. the contents of the ERLENMEYER bottles were filtered and the filtrates obtained tested on 338*b'* mutilates.

[1]) I have described this bacillus in Zbl. Bakter. I, Orig. Bd. **122** p. 277 (1931).

[2]) The numbers marked with an asterisk produce an undulatus phage.

The result was as follows:

Filtrate 1101: With a dilution of 10^{-1} no plaques.
Filtrate 1102: „ „ „ „ „ „ „ .
Filtrate 1106: „ „ „ „ „ „ „ .
Filtrate 1108: „ „ „ „ „ „ „ .
Filtrate 1109: „ „ „ „ „ „ „ .
Filtrate 1110: „ „ „ „ „ „ „ .

The same negative result was obtained after 0.3 c.c. of an 899 O phage had been added.

F. Experiments with Bac. mycoides.

Young still vegetative cultures of the *mycoides* strains 384, 386, 1161, 1167, 1169 and 1171 were treated with 0.3 cc of a 899 ⊙ phage, which led to the formation of 25×10^6 plaques per c.c. in *338b'* spreadings. A week later these cultures were pasteurized for 10' at 90° C. and then inoculated in 1 % peptone water in ERLENMEYER bottles to which some mutilate *338b'* had been added. After two days incubation at 30° C. the contents were filtered and the filtrates tested on the mutilate *338b'*. The results were as follows:

Filtrate 384: With a dilution of 10^{-1} no plaques.
Filtrate 386: „ „ „ „ „ „ „ .
Filtrate 1161: „ „ „ „ „ „ „ .
Filtrate 1167: „ „ „ „ „ „ „ .
Filtrate 1169: „ „ „ „ „ „ „ .
Filtrate 1171: „ „ „ „ „ „ „ .

The same negative result was obtained after adding 0.3 c.c. of an 899 O phage.

G. Experiments with Bac. mesentericus.

Young still vegetative cultures of the *mesentericus* strains 999, 1076, 1082, and 1083 were treated with 0.3 c.c. of a 899 ⊙ phage, which led to the formation of 26×10^8 plaques per c.c. in *338b'* spreading. A week later these cultures were pasteurized of 5' at 90° C. and then inoculated in 1 % peptone water in ERLENMEYER bottles to which some of the mutilate *338b'* had been added. After 2 days incubation at 37° C. they were filtered and the filtrates obtained tested on the mutilate *338b'*.

The result was as follows:

Filtrate 999: With a dilution of 10^{-1} no plaques.
Filtrate 1076: „ „ „ „ „ „ „ .
Filtrate 1082: „ „ „ „ „ „ „ .
Filtrate 1083: „ „ „ „ „ „ „ .

The same negative result was obtained after 0.3 c.c. of a 899 O phage had been added.

The results of the experiments described under *E, F* and *G* show that the spores of *Bac. undulatus, Bac. mycoides* and *Bac. mesentericus* when treated with a *megatherium* phage, contrary to the spores of *Bac. megatherium,* never lead to the formation of cultures containing this phage. We may point out that this result is not contrary to what COWLES found seeing he worked with the homologous phages of these bacilli.

Considering too the fact that even a *megatherium* phage is not able to "infect" all *megatherium* spores, it is highly probable that the influence of a certain bacteriophage on certain spores which we have found by experiment to be present, cannot be explained simply by an inclusion during which there is protection against heat of an ultra microbe by the spores, as formely might be thought possible. The influence found to exist can more reasonably be attributed to an induction phenomenon, i.e. the stimulation to perceptible activity of a latent power of phage production in the spores, by the presence of a specific bacteriophage, i.e. one adapted to act upon the same species of spore forming bacillus.

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