THE INDUCTION OF MOTILITY IN BACILLUS ANTHRACIS BY MEANS OF BACTERIOPHAGE LYSATES

SIGNIFICANCE FOR THE RELATIONSHIP OF *Bacillus anthracis* TO *Bacillus cereus*

ERIC R. BROWN, WILLIAM B. CHERRY, MAX D. MOODY, AND MORRIS A. GORDON[1]

Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare, Atlanta, Georgia

Received for publication November 18, 1954

Manninger and Nográdi (1948) first reported the induction of encapsulation and motility in a nonmotile, unencapsulated, avirulent strain of *Bacillus anthracis*. Following Griffith's (1928) method they plated *B. anthracis* together with cell-free filtrates of a motile, encapsulated culture of *Bacillus mesentericus* var. *vulgatus*. After incubation either in CO_2 or in air, typical colonies of *B. anthracis* were found, but, in addition, there appeared a number of slimy colonies corresponding neither to *B. anthracis* nor to *B. mesentericus*. The cells of these atypical colonies were characterized by a loose capsule and the presence of flagella. The authors concluded that such organisms were variants of *B. anthracis* in which encapsulation and motility had been induced by a substance found in the capsular material of the "mesentericus" organism.

Tomcsik (1949), although unable to confirm all of the results reported by Manninger and Nográdi, was successful in producing encapsulated mutants from unencapsulated strains of *B. anthracis*. He showed that the mutant isolated by Manninger and Nográdi (1948) possessed capsular antigens common to both *B. anthracis* and *B. mesentericus* as well as other antigens not found in either of these species, and drew comparisons between this system and that involved in pneumococcus capsule type transformation reactions (McCarty, 1946). Later Tomcsik (1950) reported the observation of motility in some of the cultures of encapsulated mutants and showed that the motile mutants were related, both serologically and morphologically, more closely to *B. anthracis* than to any of the saprophytes tested. However, the motile mutants had lost their pathogenicity for test animals.

[1] Present address: Doctrine Division, Chemical Corps Training Command, Fort McClellan, Alabama.

A general review of the literature on the subject of transformation reactions of bacteria has been published by Austrian (1952). Subsequently, Zinder and Lederberg (1952) described, in salmonellae, a type of genetic exchange which they designated "transduction". Evidence that specific desoxyribonucleic acid can direct the inheritance of certain traits in *Neisseria meningitidis* was presented by Alexander and Redman (1953). The production of toxigenic mutants of *Corynebacterium diphtheriae* as a result of treatment with bacteriophage was reported by Freeman (1951).

It is the purpose of this paper to describe the isolation, from a naturally occurring lysogenic culture of *B. anthracis*, of an agent capable of conferring motility upon certain strains of *B. anthracis* and to discuss the possible role of desoxyribonucleic acid (DNA) in this phenomenon. The characteristics of the motile *B. anthracis* organisms are described, and their relationship to *B. cereus* and *B. anthracis* are discussed.

MATERIALS AND METHODS

A search first was undertaken for a phage capable of lysing both motile and nonmotile forms of various species of the genus *Bacillus*, since it was felt that transduction might be mediated best through such an agent. For this purpose 93 strains of *B. anthracis* and 46 strains of other species of the genus *Bacillus* were examined for lysogenicity. The 139 cultures yielded 32 phages, obtained as follows: 50 ml of heart infusion broth (HIB, Difco) were inoculated with the host organism, incubated at 37 C on a rotary shaker for 2 to 4 days, and passed through a Seitz E K G filter. After the filtrates

were tested for bacterial sterility,[2] they were screened for phage by testing their ability to lyse a series of indicator strains. For titration, 0.2 ml of a 6 hour broth culture of each indicator strain was spread over the surface of a nutrient agar plate and allowed to dry for 15 minutes at 37 C, following which 0.1 ml of each given phage dilution (tenfold dilution series) was spread over the surface of the plate, which then was incubated at 37 C. All titrations were done in duplicate and read at intervals up to 24 hours. Titers were calculated in terms of the number of plaques produced per ml of the undiluted phage.

In the course of these experiments, 103 strains of *B. anthracis*, 47 of *B. cereus*, 30 of *B. cereus* var. *mycoides*, 30 of *B. megaterium*, and 10 of *B. subtilis* were investigated. All strains were tested periodically for purity. The histories of 7 of the *B. anthracis* strains (Brown and Cherry, 1955) which played a major role in this investigation are given below:

Strain Ohio—received in 1952 from Dr. E. H. Bohl of the Ohio State Board of Health. Isolated from an outbreak of animal anthrax caused by bone-meal imports.

Strain North Carolina (N.C.)—isolated in 1953 by Gordon *et al.* (1954) from an outbreak of human anthrax in Monroe, N. C.

Strain 1014—received in 1953 from Dr. Ruth E. Gordon from the Nathan R. Smith collection at Rutgers University.

Strains UK 19NV and UK 11—received as *B. anthracis* from a U. S. Government agency during 1953.

Strains Ax 16 and Ax 189—received in 1953 from Mrs. Mildred Galton of the Communicable Disease Center, assigned to the Florida State Board of Health. Isolated from an outbreak of animal anthrax in Florida in 1952.

Cultures were tested repeatedly in transmigration and semisolid media to exclude the possibility that motile mutants had occurred spontaneously. Furthermore, each time that a culture was exposed to phage in an attempt to induce motility, a comparable inoculum of unexposed culture was plated in semisolid or in transmigration medium. Motility was never observed in these control cultures. In induction experiments, sterile broth was used regularly as a phage control, and phage inactivated by boiling for 30 minutes was used several times. The symbiotic phage (omega) which was used successfully to induce motility was reisolated from its host strain (Ohio) from time to time to eliminate the possibility of latent contamination of any one batch of phage.

The range of activity of each symbiotic phage was determined by testing it on a number of cultures of *B. anthracis* and *B. cereus* as described previously.

Induction of motility was tested for as follows: 1.0 ml of bacteriophage was inoculated into a 50 ml HIB culture of the test organism (usually a 1 to 2 hour culture grown on the shaker as described above), this mixture was placed on a rotary shaker, incubated at 37 C for 24 hours, and the culture examined microscopically for motility. Aliquots (0.05 ml) of each phage-cell mixture then were added to each of 8 to 10 tubes containing 10 ml of transmigration medium (Nordberg, 1953), were incubated at 37 C, and observed periodically for motility. An alternative procedure was that of centrifuging the phage-bacteria cultures and placing 0.05 ml of the sediment on the surface of the transmigration medium. It was found later that more rapid results could be obtained by using plates containing 10 ml each of the semisolid agar of Edwards and Bruner (1942). Freshly prepared plates of medium were inoculated by spreading 0.01 ml of phage-cell mixture in a line across the center of the plate. A control plate, utilizing sterile broth instead of phage, was made for each culture tested in order to exclude the possibility that spontaneous motility had occurred.

The morphological and biochemical characterization of all strains was studied by means of the procedures described by Smith *et al.* (1952). Colonial characteristics were studied on potato glucose agar, 10 per cent rabbit blood agar, and nutrient agar and heart infusion agar; the type of growth in nutrient broth was noted also. The susceptibility of all cultures to both W and Gamma phages (Brown and Cherry, 1955) was

[2] Controls for bacterial sterility on all phages were carried out as follows: 1 to 10 ml of each phage were inoculated into 100 ml of HIB and checked for bacterial growth for periods up to 60 days. At the same time plates of nutrient and heart infusion agar (HIA) were streaked with 1.0 ml aliquots of the phages and, after incubation both at 37 C and at room temperature, were examined for evidences of bacterial growth.

determined. Following the technique of Nordberg (1953), bacilli were observed over a 4 day period for growth and encapsulation in pure, inactivated mule serum. Controls consisting of *B. megaterium*, *B. subtilis*, *B. cereus*, and *B. cereus* var. *mycoides* were included.

In order to test the persistence of motility in the motile mutants and of nonmotility in the parent strains, the organisms were transferred daily on transmigration medium for 32 consecutive generations. At intervals streak plates were prepared and single, typical colonies picked for further studies. Ultimately, semisolid agar (Edwards and Bruner, 1942) plates were employed for the routine demonstration of motility.

Pathogenicity for mice, guinea pigs, and rabbits was determined in all cases by injection, subcutaneously or intraperitoneally, of 0.02 ml of 18 hour HIB cultures (shaken at 37 C) into duplicate test animals. Controls for the experiments in mice and guinea pigs consisted of sterile broth and 7 strains each of *B. cereus*, *B. cereus* var. *mycoides*, *B. megaterium*, and *B. subtilis*. All cultures were stained and examined for purity prior to inoculation. Those cultures of *B. cereus* found pathogenic for mice were used as controls in the experiments with rabbits. Autopsies were performed on all animals, and cultures made of the spleen and heart's blood.

EXPERIMENTAL RESULTS

Experiment 1

Induction of motility in B. anthracis. From the 93 strains of *B. anthracis* and 46 strains of other members of the genus *Bacillus* which were examined for lysogenicity, a total of 32 phages was isolated. These phages were low in titer, only eight of them acting upon both the motile bacilli tested and upon *B. anthracis*. One phage lysed 9 of the 103 strains of *B. anthracis*, 3 of 47 strains of *B. cereus*, and 4 of 22 strains of *B. cereus* var. *mycoides*. This phage was naturally and constantly associated with strain Ohio of *B. anthracis* and could be isolated easily as a broth culture filtrate. In this paper it will be referred to subsequently as "omega" followed by the designation of the strain on which it was propagated, i.e., omega/Ohio phage. Omega/Ohio phage was assayed by plaque count either on a strain of *B. cereus* or on its own host strain (Ohio). The latter was possible since the nature of the relationship of omega phage to the Ohio strain resulted in the presence in the host of a sufficient number of phage susceptible cells to permit an homologous assay. Thus, omega/Ohio phage could be assayed on Ohio by the difference in the number of plaques on plates receiving added omega as compared to those receiving Ohio culture alone. As reported in an earlier paper (Brown and Cherry, 1955) the Ohio strain of *B. anthracis* was found to be fully virulent for rabbits in doses of 0.01 ml of an 18 hour HIB culture. Biochemically and morphologically it showed no variation from the classical description in *Bergey's Manual* (Breed *et al.*, 1948) of *B. anthracis* as demonstrated by repeated tests during the past two years. The initial titer of omega/Ohio phage, when assayed on the Ohio culture, was approximately 1.5×10^4 plaques per ml. It was found that an appreciable rise in titer (5.9×10^7) could be obtained by propagation of the phage in a shake culture of the same host organism.

Since it was felt that the lack of motility in *B. anthracis* might be due to a deficiency of one or more genetic factors (Stocker *et al.*, 1953), it seemed possible that a phage propagated on a motile organism (*B. cereus*) might convey such a factor(s) to a related nonmotile organism (*B. anthracis*). To test this point, each of the 32 phages was propagated upon a suitable culture of *B. cereus* and then tested for ability to induce motility in each of 89 strains of *B. anthracis*. The omega/cereus lysate, which had a titer of 6×10^8 plaques per ml on *B. cereus*, was able to confer motility upon the following 6 strains of *B. anthracis*: Ax 16, Ax 189, UK 11, UK 19NV N.C., and 1014. All other phages were negative.

Since the source of the inducing agent in these experiments was a motile sporeforming organism (*B. cereus*), the possibility of contamination was difficult to eliminate. However, it was felt that this problem could be resolved if the same phage propagated only on nonmotile cultures of *B. anthracis* could be shown to possess this activity. The hypothesis, that more than one genetic unit might be involved in the expression of motility and that the factor(s) missing in nonmotile cultures might vary from one strain to another, suggested that the omega/Ohio phage might induce motility in other strains of the anthrax bacillus. Accordingly it was found that

TABLE 1

Induction of motility in strains of Bacillus anthracis by means of lysates produced by the action of omega phage

Nonmotile Recipient Strains†	Motility Resulting from the Application of Lysates of Donor Strains						
	Omega/Ohio (nonmotile)	Omega/189 (motile)	Omega/Ax 16 (motile)	Omega/1014 (motile)	Omega/N.C. (motile)	Omega/UK 11 (motile)	Omega/UK 19NV (motile)
Ohio	−	−	−	−	no growth	−	−
Ax 16	+	−	−	−	no growth	−	−
Ax 189	+	−	−	−	−	−	−
UK 11	+	−	−	−	no growth	−	−
UK 19NV	+	−	−	−	−	−	+
N.C.	+	−	−	−	−	−	−
1014	+	−	−	−	no growth	−	−
920*	−	−	+	−	no growth	−	−
936*	−	−	+	−	−	−	−
965*	−	−	+	−	−	−	−

* Cultures originating from the same outbreak of anthrax in Louisiana.

† Inocula consisted of 0.5 ml of 18 hour HIB cultures.

such a phage preparation induced motility in the same 6 strains of *B. anthracis*. Two additional phages, W and Gamma, previously reported (Brown and Cherry, 1955) to be specific for *B. anthracis*, were devoid of such activity.

Host adaptation studies showed that three strains of *B. anthracis* (Ax 22, Ax 150, and V 864), in addition to the Ohio strain, were not rendered motile by the omega/Ohio lysate although they were lysed by it. Omega phage propagated on strains Ax 16, Ax 189, UK 11, N.C., or 1014 of *B. anthracis* was unable to induce motility in any of the cultures which were previously susceptible, and the lysate of Ax 16 was no longer able to lyse Ax 22, Ax 150, and V 864. The data in table 1 show the results obtained in induction tests using lysates of motile cultures previously treated with omega/Ohio phage. They will be analyzed in some detail in the discussion section of this paper. It should be noted that although hundreds of tests have been performed, it has not yet been possible to induce motility in the Ohio strain with any of the lysates listed in table 1. The induction of motility in anthrax cultures 920, 936, and 965 by a lysate of omega/Ax 16 (motile) suggests that the latter strain (Ax 16) is the source of the active material. The similarity of behavior of this lysate towards the above three cultures is of interest since they originated from a single outbreak of anthrax. With a single exception the motility inducing activity of omega phage was lost by propagating it upon any of the six motile cultures. Also worthy of note is the highly lytic action shown by lysates of the motile N.C. strain for several of the nonmotile strains of *B. anthracis*.

In other experiments the lytic activity of various generations of motile mutants was compared with that of their parent nonmotile strains. The results are summarized in table 2 and show that lysogenization, as measured by lytic ability for indicator strains, followed treatment with omega/Ohio phage (compare control filtrate with that from generation 5 and 89). The majority of the 9 cultures of *B. anthracis* which were susceptible to the action of filtrates of motile mutants maintained this relationship through as many as 89 consecutive transfers, alternate transfers consisting of plating and reisolation from a single colony. However, it has not been proved that the phage associated with these motile cultures of *B. anthracis* is that originally added (omega) since most normal strains are carriers of phage. Of the 6 cultures of *B. anthracis* in which motility could be induced by omega/Ohio phage, 5 were found to be naturally lysogenic for one or more of 12 indicator strains. No suitable indicator strains are available for assaying omega phage in mixtures. Another difficulty is that of making accurate plaque counts of weakly lytic phages on an organism which characteristically is very rough and nonuniform in its growth.

All experiments concerned with attempts to

TABLE 2

The lytic ability of filtrates of motile mutants of Bacillus anthracis

Filtrate from Motile Strains	Generation Serving as Source of Filtrate	No. Nonmotile Cultures Lysed by Filtrates
Ax 16	5	6/9†
	89	6/9
	Control*	3/9
Ax 189	5	9/9
	89	9/9
	Control	0/9
UK 11	5	9/9
	89	9/9
	Control	2/9
UK 19NV	5	7/9
	89	9/9
	Control	1/9
N.C.	5	8/9
	89	8/9
	Control	1/9
1014	5	8/9
	89	8/9
	Control	1/9
Ohio—nonmotile		10/10

* Filtrate of nonmotile, untreated, parent culture.

† Numerator denotes number of positive results; denominator number of cultures studied. The 9 nonmotile strains consisted of Ax 22, Ax 150, and V 864 in addition to the first 6 listed above.

induce motility in *B. anthracis* were controlled by careful examination of both phage and cultures.

Experiment 2

Characteristics of B. anthracis and motile mutants. All strains of *B. anthracis* were plated, and single typical colonies were selected for study. The results of the biochemical and morphological investigations, summarized in tables 3 and 4, represent replicate tests. No significant variation was found in single colony isolates of mutant or parent strains or in the various generations of the motile forms. However, as indicated in table 4, the motile cultures grew in broth with uniform turbidity and, upon longer incubation, formed heavy surface rings of growth which tended to adhere to the glass walls of the tube. These cultures were more aerobic in growth characteristics than were their nonmotile precursors. The growth of motile mutants on infusion agar tended to be thinner, more gummy, and more tenacious than that of their progenitors. These observations suggest a change in the nature of the cell surface, and such changes may be related to a possible increase in the pathogenicity of the motile strains.

The manifestation by the motile mutants of motility on semisolid agar or in transmigration

TABLE 3

A comparison of the biochemical characteristics of motile mutants of Bacillus anthracis with those of the nonmotile parent strains and with Bacillus cereus

	Typical Mutant	Parent *B. anthracis*	*B. cereus*
Nitrate reduction	+1*	+1	+1
Starch broth	A 1†	A 1	A 1
H_2S	−4	−4	−4
V.P.	+2	+2	+2
Motility (Difco)	+1	−	+1
Glucose	A 1	A 1	A 1
Sucrose	A 1–8	A 1–4	A 1
Lactose	−‡	−	−
Dulcitol	−	−	−
Rhamnose	−	−	−
Salicin	A 1–8	A 1–8	A 1–4
Xylose	−	−	−
Maltose	A 1	A 1	A 1
Mannitol	−	−	−
Arabinose	−	−	−
Trehalose	A 1	A 1	A 1
Sorbitol	−	−	−
Inositol	−	−	−
Adonitol	−	−	−
Glycerol	A 1	A 1	A 1
Raffinose	A 5–7	A 5–7	A 1–5
Methylene blue reduction	+7–9	+7–9	+1–8
Gelatin liquefaction	24–72 hours	24–72 hours	24–72 hours

* Numerals refer to day on which reaction was recorded.

† "A" indicates acid production.

‡ All negative fermentation tests were held for a minimum of three weeks.

TABLE 4

Description of mutants and parent strains of Bacillus anthracis

Property Investigated	Typical Parent *B. anthracis* Strains	Typical Mutants
Motility	Consistently nonmotile	Motile
Gamma and W phage results	Strong lysis	Moderate lysis
Vegetative rod	1.0 by 3.0 to 5.0 μ. Usually in short to long tangled chains; ends square, stained protoplasm granular or foamy, no shadow forms, moderately encapsulated. Gram positive.	Same
Sporangia	Not appreciably swollen	Same
Spores	1.0 by 1.5 μ, oval, central, or paracentral, thin walled, not many appearing before 48 hours.	Same
Colonies (nutrient agar)	Moderately large, cuneiform appearance under oblique lighting; shows typical "Medusa Shape".	Same
Width of zone of hemolysis* (rabbit blood agar)	0.1 to 0.2 mm	0.1 to 0.3 mm
Growth in nutrient broth	Light turbidity with soft easily dispersed sediment or flocculent sediment with clear supernatant without ring or pellicle.	Heavy, uniform turbidity; soft, easily dispersed sediment; rings after 48 hours' growth.
Inactivated mule serum	Good growth—appeared encapsulated	Same

* Zones of hemolysis given by *B. cereus* controls averaged 0.5 to 1.0 mm.

media appeared to be a two-step process. The first stage was the formation of "microcolonies", which sometimes remained static but characteristically were precursors of a second stage in which the organisms migrated throughout the medium. The "microcolonies" took the form of filaments just beneath the surface, from which the motile organisms broke loose and swarmed throughout the transmigration medium. On semisolid plates the first stage appeared as a slight transparent bulb which formed along the margin of growth and from which cells subsequently swarmed across the plate. Figure 1 shows the two stages of motility in tubes of transmigration media; figure 2 shows the first stage on semisolid media after 10 hours' incubation; and figure 3 shows the same plate after 72 hours' incubation. The two stages occurred almost simultaneously in strains N.C. and Ax 189, while in other strains (Ax 16, UK 19NV, 1014, and UK 11) several days might elapse from the appearance of the "microcolony" until the appearance of actively motile organisms. When these "microcolonies" were removed and examined microscopically, they were found to consist of sluggishly motile cells.

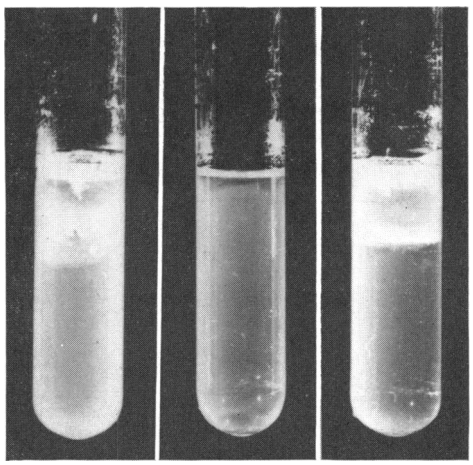

Figure 1. Stages of motility in transmigration medium of *Bacillus anthracis* treated with Ohio phage.

Left—Motility after 72 hours' incubation in transmigration medium.

Center—Negative control showing growth of untreated organisms on the surface (10 hours' incubation at 37 C).

Right—First stage of motility: "microcolonies" shown extending below ring of growth (10 hours' incubation at 37 C).

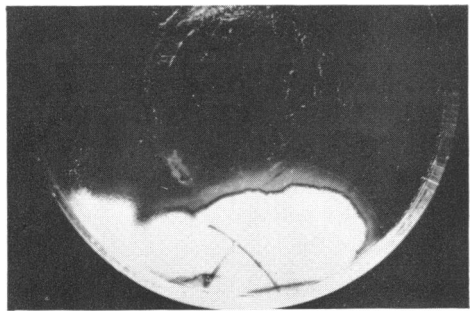

Figure 2. Semisolid agar plate, inoculated at point "X" with phage treated *Bacillus anthracis*, showing fringe of motile organisms after 10 hours' incubation at 37 C.

The results of the pathogenicity tests suggested that motile mutants may be more pathogenic than the parent cultures. The 6 motile strains of *B. anthracis*, the 6 nonmotile parent strains from which these were derived, and 3 of 7 strains of *B. cereus* were pathogenic for mice and guinea pigs. None of the *B. cereus* strains produced fatal infections in rabbits when 0.02 ml was injected subcutaneously. While the number of animals in this experiment was too small for a quantitative analysis of pathogenicity, the motile mutants of N.C., UK 11, 1014, and Ax 16 were found to be lethal for rabbits at this dosage within 48 hours and Ax 189 was lethal in 5 days. Strain UK 19NV, motile or nonmotile, proved to be nonpathogenic when 0.02 ml was injected. The parent nonmotile strains (UK 11, 1014, and Ax 16) required 10 to 21 days to produce death while strain N.C. was lethal in 4 days and strain Ax 189 failed to kill during a period of 21 days. All parent strains of *B. anthracis* except UK 19NV produced fatal infections in rabbits in doses of 1.0 ml; all motile mutants produced fatal infections within 9 days. The gross lesions produced by the parent *B. anthracis* organisms and by the motile mutants were identical and were consistent with those of classical anthrax as described in the literature. Impression smears from the spleens of all animals revealed the presence of typical encapsulated organisms. Cultures obtained from the spleen or heart blood were found to be pure, and no changes in motility were observed.

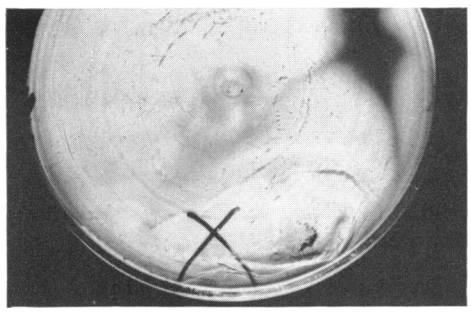

Figure 3. The same semisolid agar plate as figure 2 after 72 hours' incubation. Complete motility.

Experiment 3

The effect of purified desoxyribonuclease and of extracellular products of Group A streptococci on the motility inducing agent and on the phage itself. Since the work of Tomcsik (1950) indicated that desoxyribonucleic acid was concerned in the transformation of capsular material and perhaps in the production of motility in *B. anthracis*, it seemed logical to investigate the role of DNA in the induction of motility by means of phage. DNA can be identified best in a complex mixture by determining its susceptibility to the action of desoxyribonuclease (DNase). Therefore a stock solution containing 1 mg per ml of purified enzyme (Worthington Biochemical Corp.) was prepared and, for use in the experiments, diluted to contain 1 μg of enzyme per ml. The diluent consisted of a solution of 0.035 per cent of gelatin in distilled water which was made 0.025 M

TABLE 5

Effect of desoxyribonuclease on the agent inducing motility in Bacillus anthracis and on phage activity

Time of Exposure of Phage to Enzyme at 30 C Before Plating	Motility of Organisms† Exposed to Phage Treated with		Motility of Organisms Unexposed to Phage	Plaque Count of Phage Treated with	
	DNase	Broth		Broth	Enzyme
min					
0	0/5*	4/4	0/5	5.0×10^7	8.6×10^7
15				4.2×10^7	10.1×10^7
30	0/5	4/4	0/5	7.7×10^7	6.1×10^7
60	0/5	4/4	0/5		
90	0/5	4/4	0/5		
120	0/5	4/4	0/5	2.6×10^7	7.6×10^7

* Numerator indicates number of plates showing swarms of motile organisms; denominator indicates number of plates used.

† Strain N.C. of *B. anthracis* was used.

in respect to $MgSO_4$ and adjusted to a pH of 7.5. In one experiment, phage and diluted DNase were mixed in equal proportions, and 2.0 ml of this were added immediately to 50 ml of a culture prepared by growing *B. anthracis* (N.C.) in HIB on the shaker for 2 hours at 37 C. The undiluted omega/Ohio phage suspension had a titer of 5.9×10^7 per ml. The final concentration of DNase in the mixture of cells, enzyme, and phage was 2×10^{-8} g per ml. This mixture was incubated at 30 C, and, at intervals over a period of 2 hours, aliquots of 0.25 ml were removed and plated in duplicate on semisolid agar. Sterility controls were prepared on all reagents. Experiments with omega phage lysate which had not been exposed to the action of DNase were carried out in the same way, using broth as the phage diluent in one control and the gelatin-$MgSO_4$ mixture in the other. The data presented in table 5 show that the small amount of enzyme used was sufficient to inactivate completely the motility inducing agent. All aliquots of cells treated with phage in the absence of DNase showed motility in semisolid plates. Cultures which had not been subjected to phage were never observed to be motile. Every experiment was repeated at least once with similar results.

The effect of DNase on the lytic activity of the omega phage was tested at the same time. Plates of HIA were spread with approximately 0.1 ml of a 2 hour broth culture of *B. anthracis* (Ohio strain). Enzyme and phage were mixed in equal amounts and immediately diluted 10^{-1} through 10^{-5} in broth, the first and last dilutions being plated in duplicate for phage assay after 0, 15, 30, and 120 minutes at 30 C. A control consisting of a mixture of equal amounts of broth and phage was diluted and titered in the same manner. The plates were incubated at 37 C for 12 hours, and plaques were counted. Phage subjected to different concentrations of enzyme failed to reveal any significant decrease in lytic activity as compared with untreated phage (table 5).

Previously, similar experiments had been carried out using as a source of DNase the broth in which certain types of Group A streptococci had been cultured. Tillett *et al.* (1948) reported the presence of this enzyme in such material. The failure of omega/Ohio phage lysates to induce motility in *B. anthracis* after treatment with streptococcal enzymes suggested the possible role of DNA in the process. In agreement with the data given above, the lytic ability of omega/Ohio phage was not impaired by this treatment. Thus, both purified DNase and a streptococcal preparation presumably containing this enzyme destroyed the ability of lysates of the Ohio strain of *B. anthracis* to induce motility in other strains of this organism. The same material had no significant effect on the titer of the phage as measured by the plaque count. It therefore is apparent that the motility inducing agent and the lytic activity of the phage are separable. It is postulated that the failure of enzyme treated lysates to induce motility resulted from inactivation of DNA.

The rapidity of the inactivation of the active principle by DNase was indicated by failure to obtain motile swarms on any of 5 plates inoculated immediately following the mixing of phage and enzymes with the cells, while motility was evident on all four plates receiving phage and

TABLE 6

The relationship of desoxyribonuclease to the induction of motility in Bacillus anthracis

Tube Number	Experimental Conditions*	Motility Results†
1	1.0 ml phage + 1.0 ml broth; plate after 1 hour	3/4
2	1.0 ml broth; after 1 hour add 1.0 ml phage and plate	4/4
3	1.0 ml strep. enzyme; after 1 hour add 1.0 ml phage and plate	0/4
4	1.0 ml phage; after 1 hour add 1.0 ml strep. enzyme and plate	4/4
5	1.0 ml DNase; after 1 hour add 1.0 ml phage and plate	0/4
6	1.0 ml phage; after 1 hour add 1.0 ml DNase and plate	4/4
7	1.0 ml strep. enzyme + 1.0 ml phage; plate after 1 hour	1/4‡
8	1.0 ml DNase + 1.0 ml phage; plate after 1 hour	0/4
9	2.0 ml broth; plate after 1 hour	0/4
10	1.0 ml $MgSO_4$-gelatin diluent + 1.0 ml broth; plate after 1 hour	0/4

* Each tube contained 5.0 ml of a 6 hour broth culture of *B. anthracis* strain N.C. All tests were incubated at 30 C.

† Numerator indicates number of plates showing swarms; denominator indicates number of plates prepared.

‡ After 4 days' incubation, 1 swarm appeared.

cells only (table 5). From the data shown in table 6 it appears that if the enzymes are added to the recipient cells either before or at the time that the phage is added, induction of motility will not occur. However, when phage was added first, followed by enzyme one hour later, the process was unaffected, showing that the reaction was irreversible.

Experiment 4

Temporal relationships and the frequency of the induction phenomenon. No precise measurements have been made of the time required for the induction of motility to become irreversible, but several experiments have suggested that, in the presence of fairly high concentrations of the inducing agent, the sequence of events resulting in motility is very rapid. However, plating immediately following the mixing of the cells with small quantities of phage resulted in failure, whereas plating of the same mixture after 10 or 15 minutes resulted in motile swarms. This suggests that the active agent produces its effect on the cells very rapidly but that when the concentration of this agent is near a minimum, rapid plating with the attendant decrease in adsorption rate results in failure. Unless the inducing agent is inactivated before plating, continued adsorption on the plate would be expected to increase the number of motile swarms. In summary, results of all experiments showed that motility usually was obtained in replicate aliquots plated immediately after mixing of phage and cells and always was apparent after 30 or more minutes of mixture incubation prior to plating.

TABLE 7

Proportionality of phage concentration to frequency of appearance of motile mutants of Bacillus anthracis

Flask	Phage per Aliquot Plated	Phage/Cell* Ratio	Motility Result†
1	9.4×10^3	1.0×10^{-2}	4/4
2	4.7×10^3	5.0×10^{-3}	4/4
3	2.3×10^3	2.5×10^{-3}	4/4
4	1.2×10^3	1.25×10^{-3}	3/4
5	5.8×10^2	6.24×10^{-4}	4/4
6	2.9×10^2	3.12×10^{-4}	4/4
7	1.5×10^2	1.56×10^{-4}	3/4
8	7.3×10^1	7.8×10^{-5}	1/4
9	3.7×10^1	3.9×10^{-5}	1/4
10	1.8×10^1	1.95×10^{-5}	0/4

* Average number of cells inoculated on each plate = 9.4×10^5. Strain N.C. of *B. anthracis* was used.

† Numerator indicates number of positive plates; denominator indicates number of plates used.

A rough measurement of the proportionality of omega phage concentration to the frequency of induction of motility was obtained by exposing a constant volume of cells to a series of twofold dilutions of the phage. The titer of the undiluted phage used in this experiment was 5.9×10^7 plaque-forming particles per ml. The cell suspension contained approximately 4.7×10^6 bacteria per ml as determined from a standard plate count corrected for average chain length of the bacterial filaments appearing in stained preparations. The results of this experiment are presented in table 7 and emphasize that the frequency of appearance of motile swarms is related to the concentration of phage added. Since each plate of semisolid agar received an inoculum of 0.2 ml of the cell-phage mixture, the estimated number of phage particles contained in this amount was recorded. The data suggest that an amount of lysate containing approximately 2.9×10^2 phage particles is needed for consistent induction of motility to a single organism of a suspension containing 9.4×10^5 cells.

Experiment 5

Behavior of the motility inducing principle in the high speed centrifuge. Further information relating to the degree of association between the omega phage and the motility inducing principle was obtained by studying the behavior of an active lysate when subjected to high speed centrifugation. Two tubes each containing 27 ml of cell-free lysate (omega/Ohio phage) were centrifuged for one hour at approximately 30,000 rpm in the Spinco Model L preparative ultracentrifuge. Under these conditions the average centrifugal force of about 78,000 × G would be expected to sediment rapidly particles having diameters in the range of 100 mμ. Following centrifugation 15 ml of supernatant were removed from one tube, care being taken not to disturb the pellet, while the second tube was shaken vigorously to resuspend the sedimented material. The contents of the latter tube served

TABLE 8

The effect of high speed centrifugation on the agent inducing motility in Bacillus anthracis

Nonmotile Recipient Cultures	Motility Induced by Various Materials Tested			
	Negative control (broth only)	Positive control (omega/Ohio lysate)	Supernatant of centrifuged omega/Ohio lysate	Centrifuged and resuspended omega/Ohio lysate
Ax 16	0/2	2/2	0/2	1/2
Ax 189	0/2	2/2	0/2	1/2
UK 11	0/4	1/2	0/6	2/2
UK 19NV	0/2	2/2	0/2	1/1
N.C.	0/4*	3/4	0/4	4/4
1014	0/2	2/2	0/2	1/2
Total.....	0/16	12/14	0/18	10/13

* Numerator indicates number of positive tests; denominator indicates number of tests performed.

as a control on possible deleterious effects on the active material produced by manipulation and aggregate formation during centrifugation. Fifteen ml aliquots from each of the above tubes and from a third tube consisting of the uncentrifuged lysate were filtered through separate millipore filters and tested for sterility by the inoculation of broth and semisolid agar.

The above preparations were tested, by the procedures previously described, for ability to induce motility in susceptible strains of *B. anthracis*. An inspection of table 8 reveals that all of the activity was removed from the supernatant by centrifugation but that it reappeared upon resuspension of the sediment. These results suggest that the active DNA is physically associated either with the omega phage particles themselves or with other cellular fragments which were sedimented along with the phage.

DISCUSSION

The work of Tomcsik and his colleagues, as well as that of Manninger and Nográdi, indicates that some "specific substance" found in extracts of encapsulated motile organisms can be transferred to nonmotile, unencapsulated strains of *B. anthracis* in such a way that the latter become motile and encapsulated. The role of phage in the transfer of genetic characteristics among the salmonellae and in *C. diphtheriae* has been established firmly. The participation of DNA in the transformations of types of pneumococci and *Hemophilus influenzae* also is well known. Presumably the latter transformations are not concerned with phage although it is difficult to see how this possibility could be eliminated, particularly if a phage were acting as a carrier of the genetic material. On first examination, the work of Manninger and Nográdi might seem to involve a mechanism of transformation which differs from that reported in this paper. However, neither they nor Tomcsik reported having examined their material for phage. On the other hand, we cannot rule out the possibility that the active agent of our preparations is associated with phage only fortuitously even though it sediments with the phage during high speed centrifugation. No further attempts have been made to separate the phage from the lysates and to study the effect. It is possible that we and the workers mentioned above have observed transformations brought about by the same mechanisms.

We have shown that a lysate containing a phage constantly associated with a nonmotile strain (Ohio) of *B. anthracis* is capable of inducing motility in cells of 6 other typical strains of the anthrax bacillus. The possible genetic relationships which were deduced from the data of table 1 were as shown in the accompanying table. Both donor and recipient strains were grouped according to the pattern of motility which was found, and similar groups were represented by the same capital letters (A through E). For the purpose of discussion the group designation or

	Donor	Recipient					Apparent Genotype of Donor
		A	B	C	D (920, 936, 965)	E	
	B. cereus (motile)	−	+	+		+	M_n^+
A	*B. anthracis* (Ohio, nonmotile)	−	+	+	−	+	M_2^-, M_3^-, M_1^+
B	*B. anthracis* (189, 1014, N.C., UK 11, motile)	−	−	−	−	−	M_1^-
C	*B. anthracis* (UK 19NV, motile)	−	−	+	−	−	?
E	*B. anthracis* (Ax 16, motile)	−	−	−	+	−	M_1^-

postulated genetic factors of the donor organisms are written first followed by that of the recipient, i.e., A/B. M_n denotes any number of motility factors in the genotype of the donor strain. *B. cereus*, a naturally motile organism, is assumed to have a genetically complete series of motility factors. If these are designated M_1, M_2, M_3, etc., it appears that the organisms of series B, C, and E are complete except for the M_1 factor, which can be supplied by A. However, the results of C/C and E/D suggest complexities which are not found in B donor strains. From the results of A/B, B carries at least one negative factor (M_1^-) which is supplied by A; from B/A, C/A, and E/A one concludes that A carries more than one negative factor, i.e., M_2^-, M_3^- but also M_1^+ (from A/B). Although it is tempting to speculate on the nature of the induction process, insufficient data are at hand to warrant this. Some results in the table above, for example those of series A and B, are not inconsistent with "transduction" in the sense of Zinder and Lederberg (1952). However, certain discrepancies, i.e., C/C, cannot be explained by "transduction", and an understanding of the nature of the genetic mechanism awaits further study. Apparently, factor(s) in the omega/Ohio lysate can be transferred to the susceptible strains in such a fashion as to cause them to become motile, and this motility is a reproducible, stable characteristic in subsequent generations. The active principle, unlike that of the salmonella system (Zinder and Lederberg, 1952), was inactivated by purified DNase. In this respect it resembles the DNA responsible for the transformation of capsular antigens of the pneumococcus (McCarty, 1946) and of *H. influenzae* (Alexander and Leidy, 1951). The degree of association existing between the genetically active fragments and the phage itself has not been determined although the removal of both by centrifugation of the lysate suggests either that the active material is physically a part of the phage or that it is associated with other particles of somewhat similar size. The properties of the omega phage are similar to those of phages used by other investigators for the induction of genetic changes: namely, specificity for a limited number of strains, high titer, and poor lytic ability. It appears that the genetic component(s) necessary for motility in the 6 susceptible strains can be released from the Ohio strain of *B. anthracis* by its symbiotic phage and that none of the other 32 phages studied has such characteristics.

The ability of low concentrations (10^2) of omega/Ohio phage to induce motility in some strains of *B. anthracis* suggests that motility may be a direct result of lysogenization as suggested by Groman (1953) in studies on *C. diphtheriae*. That something more is required for transformation of cells of *B. anthracis* is indicated by the failure to observe motility in the Ohio strain from which the active lysate was prepared and in a lack of correlation between the occurrence of lysogenization and motility when host adaptation of omega phage was permitted (tables 1 and 2). Three other strains of *B. anthracis* were lysed directly by this phage but remained nonmotile. Furthermore, the lytic activity of the active lysates remained after transforming ability had been destroyed by DNase. Hewitt (1954) has called attention to the complexities of the interactions of symbiotic phages and their hosts. These may result in genetic recombination between the infecting and symbiotic viruses. Since most cultures of *B. anthracis* in which motility was induced were known to be lysogenic, the factors discussed by Hewitt may play a part in the experiments reported here.

A careful study of phage (omega) excluded the possibility that it was contaminated with a motile sporeformer in that each batch of phage was tested repeatedly for bacterial sterility by inoculating into either semisolid or transmigration agar. The phage lysates were always sterile. Furthermore, it is unlikely that, in repeated tests for purity of over 100 strains of nonmotile anthrax organisms subjected to the omega phage, only the same 6 strains would be contaminated consistently.

It was observed that motility test medium (Difco) was unsatisfactory for the demonstration of motility of *B. anthracis*. The motility of sluggish cultures was stimulated by growth on the surface of plates as compared to cultivation as stab cultures, apparently because of increased exposure to air.

On the basis of their morphological characteristics it appears that the mutants described by Tomcsik and those produced by phage induction take an intermediate taxonomic position between *B. cereus* and *B. anthracis*. However, changes in

the surface characteristics of the cells as well as the orientation of growth to more aerobic conditions suggest that basic changes other than the development of motility have occurred. The definition of the relationship of these changes to pathogenicity may be a problem of primary importance for the study of the pathogenesis of anthrax. No significant biochemical differences could be found between the motile and the nonmotile cultures of *B. anthracis* or between these and *B. cereus*. Our motile organisms appeared slightly more pathogenic than the parent strains of *B. anthracis*.

At the present time *B. anthracis* and *B. cereus* are differentiated primarily on the basis of pathogenicity and motility. The experiments reported in this paper do not affect the validity of these characteristics for diagnosis even though spontaneously motile mutants of *B. anthracis* do occur occasionally. However, Nordberg showed conclusively that over 40 per cent of *B. cereus* strains were pathogenic for mice in doses commonly used in diagnosing anthrax. About 14 per cent of these produced fatal infections in guinea pigs while none was pathogenic for rabbits. Our experience has been similar. The artificial induction of motility in *B. anthracis*, as reported by Manninger and Nográdi, and by Tomcsik, and as described here, lends strong support to the classification of this organism as a variety of *B. cereus*, as suggested by Smith *et al.* (1952). These results suggest an analogy with those reported by Stocker *et al.* (1953), who were able to transduce motility to certain nonmotile mutants of some *Salmonella* serotypes which are characteristically motile. However, they could obtain only indirect evidence for the existence of genotypic flagellar determinants in characteristically nonmotile serotypes such as *Salmonella pullorum* and *Salmonella gallinarum*. The successful induction of motility in *B. anthracis* strengthens the belief that this organism is simply a nonmotile mutant of *B. cereus* or of a common antecedent.

ACKNOWLEDGMENTS

We wish to thank Dr. Ruth E. Gordon of Rutgers University for cultures and for valuable advice and criticism. We are indebted to Dr. J. Lederberg, Department of Genetics, University of Wisconsin, for reading the manuscript and offering many valuable suggestions. Dr. Francis Binkley of Emory University supplied us with a solution of desoxyribonuclease. We also are grateful for the assistance received from Dr. Ralph B. Hogan, Chief, Laboratory Branch, Communicable Disease Center, under whose general supervision this work was done. Mr. John P. Gust, Jr., Photographer, Training Branch, Communicable Disease Center, supplied the photographs.

SUMMARY

Induction of motility in 6 of 89 strains of *Bacillus anthracis* was effected by means of a symbiotic phage (omega). Only one of 32 such phages isolated from bacilli had such activity. Omega phage propagated on nonmotile cultures of *B. anthracis* was as effective in engendering motility as was the same phage propagated on motile cultures of *Bacillus cereus*. The motile mutants did not differ significantly in virulence or with respect to cultural, morphological, and biochemical characteristics from the cultures of *B. anthracis* from which they were derived.

The induction of motility appears to be mediated by specific desoxyribonucleic acid carried by phage particles or fortuitously associated with them. Such a supposition is based on the evidence of inactivation of the inducing substance by extracellular products (enzymes) of Group A streptococci and by highly purified desoxyribonuclease. Although the frequency of successful transformations is high, little support can be given to the thesis of direct induction of motility solely as a result of lysogenization. Although the data suggest that genetically active fragments are carried from donor cell to recipient by means of phage lysates, certain discrepancies exist which are not consistent with the process of "transduction" as understood in the salmonellae.

The implications of these observations for the classification of *Bacillus anthracis* and *Bacillus cereus* and for the diagnosis of anthrax are discussed.

REFERENCES

ALEXANDER, H. E., AND LEIDY, G. 1951 Determination of inherited traits of *H. influenzae* by desoxyribonucleic acid fractions isolated from type-specific cells. J. Exptl. Med., **93**, 345–359.

ALEXANDER, H. E., AND REDMAN, W. 1953 Transformation of type specificity of meningococci. J. Exptl. Med., **97**, 797–806.

AUSTRIAN, R. 1952 Bacterial transformation reactions. Bacteriol. Revs., **16,** 31–50.

BREED, R. S., MURRAY, E. G. D., AND HITCHENS, A. P. 1948 *Bergey's manual of determinative bacteriology.* 6th Edition. The Williams and Wilkins Co., Baltimore, Md.

BROWN, E. R., AND CHERRY, W. B. 1955 Specific identification of *Bacillus anthracis* by means of a variant bacteriophage. J. Infectious Diseases, **96,** 34–39.

EDWARDS, P. R., AND BRUNER, D. W. 1942 Serological identification of *Salmonella* cultures. Univ. Kentucky Agr. Expt. Sta. Cir. 54, Lexington, Ky.

FREEMAN, V. J. 1951 Studies on the virulence of bacteriophage-infected strains of *Corynebacterium diphtheriae.* J. Bacteriol., **61,** 675–688.

GORDON, M. A., MOODY, M. D., BARTON, A. M., AND BOYD, F. M. 1954 Industrial air sampling for anthrax bacteria. Arch. Ind. Hyg. and Occupational Med., **10,** 16–22.

GRIFFITH, F. 1928 The significance of pneumococcal types. J. Hyg., **27,** 113–159.

GROMAN, N. B. 1953 Evidence for the induced nature of the change from nontoxigenicity to toxigenicity in *Corynebacterium diphtheriae* as a result of exposure to specific bacteriophage. J. Bacteriol., **66,** 184–191.

HEWITT, L. F. 1954 Autoadaptation of bacterial viruses and its effect on bacterial variation and evolution. J. Gen. Microbiol., **11,** 261–271.

MANNINGER, R., AND NOGRÁDI, A. 1948 Induced mutation on *Bacillus anthracis* and *Bacillus mesentericus.* Hung. Acta. Physiol., **1,** 248–252.

MCCARTY, M. 1946 Chemical nature and biological specificity of the substance inducing transformation of pneumococcal types. Bacteriol. Revs., **10,** 63–71.

NORDBERG, B. K. 1953 Continued investigations of some important characteristics in anthrax-like microorganisms as viewed from a point of view of differential diagnosis. Nord. Veterinaermed., **5,** 915–924.

SMITH, N. R., GORDON, R. E., AND CLARK, F. E. 1952 Aerobic sporeforming bacteria. U. S. Dept. Agr., Agr. Monograph no. 16.

STOCKER, B. A. D., ZINDER, N. D., AND LEDERBERG, J. 1953 Transduction of flagellar characters in *Salmonella.* J. Gen. Microbiol., **9,** 410–433.

TILLETT, W. S., SHERRY, S., AND CHRISTENSEN, L. R. 1948 Streptococcal desoxyribonuclease: Significance in lysis of purulent exudates and production by strains of hemolytic streptococci. Proc. Soc. Exptl. Biol. Med., **68,** 184–188.

TOMCSIK, J. 1949 Zur Frage der induzierten Mutation der Milzbrandbazillen. Schweiz. Z. Pathol. u. Bakteriol., **12,** 489–499.

TOMCSIK, J. 1950 Über eine bewegliche Mutante des *B. anthracis.* Schweiz. Z. Pathol. u. Bakteriol., **13,** 616–624.

ZINDER, N. D., AND LEDERBERG, J. 1952 Genetic exchange in *Salmonella.* J. Bacteriol., **64,** 679–699.