STUDIES ON AN AUTOLYTIC SUBSTANCE PRODUCED BY AN AEROBIC SPOREFORMING BACTERIUM[1]

RICHARD A. GREENBERG[2] AND H. ORIN HALVORSON

Department of Bacteriology, University of Illinois, Urbana, Illinois

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The capacity of many "surface-active" materials to cause lysis of bacterial cultures is a well documented fact. In addition to lytic activity by nonbiologic detergents, one finds that many biologically produced substances have the ability to destroy the structural integrity of living microorganisms. Lysozyme lyses susceptible bacteria by hydrolyzing certain mucopolysaccharides necessary to the cell's gross structure (Thompson, 1940).

Filtrates of cultures of *Bacillus mycoides* and *Bacillus megaterium* are listed by Waksman (1941) as being actively lytic against a wide spectrum of organisms. *Bacillus brevis* produces the actively lytic polypeptide tyrocidine, which has been studied intensively (Hotchkiss, 1944). These and other antibacterial substances have at least two common characteristics; they are active against a fairly wide spectrum of microorganisms and they do no injury to the microbial strains which produce them.

Observations made during an investigation of factors involved in sporulation established the presence of a factor, produced during the sporulation of an aerobic bacillus, which can lyse vegetative cells of the same species.

MATERIALS AND METHODS

Organisms. The bulk of this investigation was carried out with the aerobic sporeforming bacterium, *Bacillus terminalis*, taken from the culture collection of the Department of Bacteriology, University of Illinois (Stewart and Halvorson, 1953). This organism produces acid, but no gas, from glucose, xylose, salicin, and lactose and does not ferment mannitol and inositol. It reduces nitrate to nitrite, liquefies gelatin, hydrolyzes starch, and produces acetylmethylcarbinol. No growth occurs at 55 C. Growth is observed in alkaline nutrient broth plus nitrate under anaerobiosis. The pH of cultures of *B. terminalis* after growth in glucose broth drops to 5.0. These characteristics, in addition to its morphology, suggest that *B. terminalis* is a strain of *Bacillus cereus* (Smith *et al.*, 1952).

Cultures of *B. megaterium*, *B. subtilis*, *B. polymyxa*, *B. cereus*, strain 300, and *Escherichia coli* were also taken from the culture collection of the University of Illinois. Cultures of *B. cereus*, strain 9139, and *B. subtilis* (9943, Ford strain) were obtained from the American Type Culture Collection.

Medium. A medium consisting of yeast extract, 0.2 per cent; glucose, 0.8 per cent; ammonium sulfate, 1.4 per cent; K_2HPO_4, 0.2 per cent; magnesium sulfate heptahydrate, 0.8 per cent; and trace amounts of manganese, calcium, zinc, copper, and ferrous iron was employed for the growth of *B. terminalis*. The "G" medium mentioned henceforth refers to the manganese-supplemented medium (Stewart and Halvorson, 1953).

Lytic factor preparation. Solutions containing the lytic material were prepared by inoculating flasks of "G" medium with a loop of *B. terminalis* spores and incubating on a Gump type shaker at 30 C until growth had terminated and sporulation and subsequent lysis of vegetative residues had gone to completion (48–60 hours). The cultures were then centrifuged in a model G Sorvall centrifuge to remove the spores and intact cells. The resulting supernatant contained the lytic material.

Assay of lytic factor. Manganese-deficient "G" medium was used for the production of vegetative cultures of *B. terminalis* for assay of the lytic factor since it was found that in the presence of only small amounts of manganese (0.0002 per cent instead of 0.02 per cent) it allowed more uniform germination and growth of vegetative cells. One hundred milliliters of this medium in a

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TABLE 1

Correlation of drop in optical density with decrease in viable cell count

Time	Viable Cells per Ml	Per Cent Cells Killed	Optical Density
	Supernatant A		
0	9.00×10^7	—	0.3325
30	1.11×10^7	87.7	0.2050
60	5.41×10^6	94.1	0.1675
	Supernatant B		
0	9.30×10^7	—	0.337
30	1.46×10^7	84.3	0.2334
60	1.24×10^7	86.7	0.2334
	Buffer		
0	2.7×10^7	—	0.2577
30	1.9×10^7	—	0.2717
60	3.5×10^7	—	0.2717

one liter Erlenmeyer flask were inoculated with a loop of *B. terminalis* spores and shaken 12 hours at 30 C. One ml of this culture was then transferred to a fresh flask of the medium. The assay cells were harvested from the second culture about 4½ hours after inoculation, washed once with 30th molar pH 5.0 buffer, and suspended in the test material. When organisms are added to solutions containing the lytic material, there occurs a steady decrease in the turbidity of the suspension. This was followed by measuring the light transmission in a Bausch and Lomb monochromatic colorimeter equipped with a 6300 A filter. This change in the turbidity may be due only to actual lysis of cells or to a change in the refractive index of the cell wall followed by lysis. This exact sequence was not studied. In the report, these changes in turbidity will be referred to as lysis.

EXPERIMENTAL RESULTS

Young vegetative cells of *B. terminalis* showed definite signs of lysis after being suspended in supernatants taken from completely sporulated cultures of the same organism grown in "G" medium. No entire cells were visible 5 hours after suspension. On the other hand, identical cells suspended in phosphate buffer persisted until sporulation ensued and exhibited no evidence of lysis. It was also observed that supernatants taken from unsporulated cultures of *B. terminalis* contained no lytic activity.

Turbidimetric measurement of lysis was investigated as a possible means of more accurate analysis. Turbidimetric assay of lytic activity was compared with plate counts of surviving organisms. In this experiment, 4½ hour cells of *B. terminalis*, washed once in pH 5.0 30th M phosphate buffer, were suspended in equal volumes of two different batches of active lytic supernatant and also in buffer. Optical density readings at 630 mμ and platings on nutrient agar were made at 0, 30, and 60 minutes. The data listed in table 1 indicate that the change in light transmission correlates with the change in viable count. The data, however, do not show that there is a direct correlation of these two at precisely the same time because while the measurements of light transmission can be made shortly after the cells have been added to the active solutions, considerable time must elapse before colonies can be formed on a plate. The killing of the cells may, therefore, occur later than the observed change in turbidity.

Determination of optimal age of cells used in assay of lytic material. In order to establish the age at which growing cultures of *B. terminalis* were the most sensitive to the lytic factor, cells were harvested at various times from 2½ hours until 8 hours after inoculation, washed in buffer, and suspended in aliquots of active lytic supernatant. Cells harvested during experimental growth were susceptible to the lytic agent while those harvested during the lag period and after experimental growth had stopped were not. The maximum susceptibility occurred in cells harvested after 4–5 hours.

Some properties of the lytic material. The active material was precipitated at 0.45–0.70 per cent ammonium sulfate saturation at 4 C. It was not dialyzable. An aliquot of active lytic material was dialyzed in cellophane tubing against distilled water at 8 C for twenty hours. Washed 4½ hour *B. terminalis* cells were suspended in dialyzed material, undialyzed material, and buffer and checked at 20 minute intervals turbidimetrically. The results show that dialysis under the conditions cited did not remove any lytic activity from the active material tested. This suggests that the agent may be a fairly large molecule. Centrifugation at about 12,000 × G for one hour left all of the activity in the supernatant.

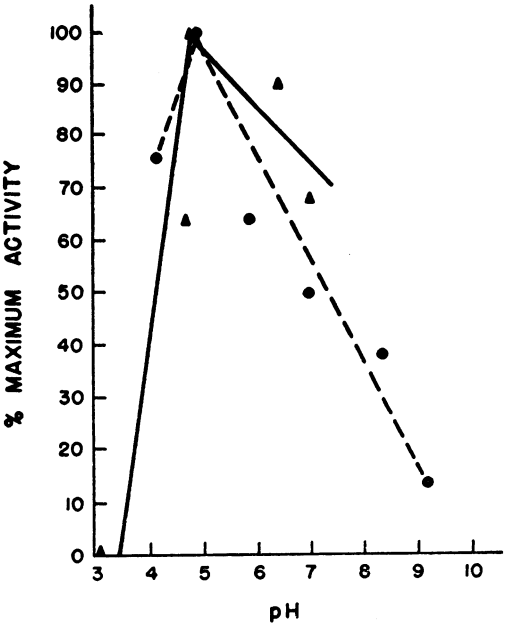

Figure 1. pH activity curves

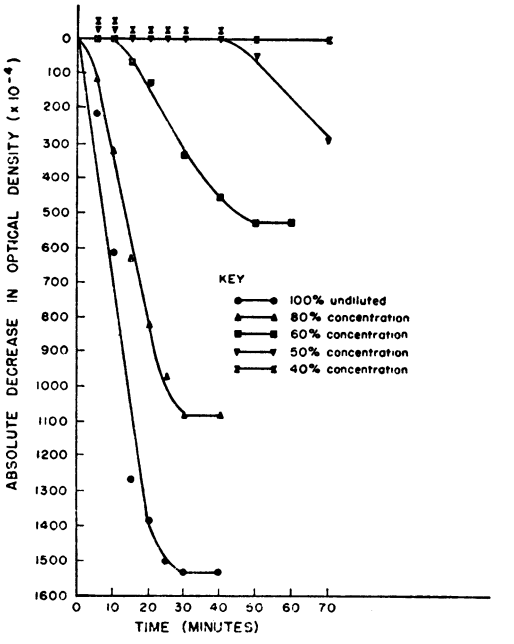

Figure 2. Effect of dilution upon activity of lytic supernatant.

The agent has a pH optimum between 5.0 and 5.5. This was shown by testing a series of supernatants in which the pH was adjusted to values between 3.3 and 9.2. Buffer washed 4½ hour *B. terminalis* cells were suspended therein and the rate of lysis of each suspension determined turbidimetrically. Optimal activity, based upon net drop in optical density per unit time, occurred between pH 5.0–5.5. Figure 1 shows the comparative rates of lytic activity at different pH values in two separate experiments.

Batches of active *B. terminalis* supernatant were diluted with buffer so as to prepare a series of solutions containing known concentrations of the lytic supernatant. The results of a typical experiment in which washed 4½ hour *B. terminalis* cells were suspended in a series of different supernatant concentrations are depicted in figure 2. Lysis of the suspended cells is linear in respect to duration of contact with active supernatant. Decrease in optical density begins almost immediately after suspension in concentrated supernatant and is linear in respect to time. Decreasing the supernatant concentrations delays the onset of these changes, the length of curve shoulder being characteristic of the particular dilution used. Lysis, when it begins in diluted supernatant, proceeds at a rate which also seems to be relatively characteristic of a given dilution.

Studies were made to determine whether or not susceptible cells can adsorb lytic material; assay cells were suspended in active supernatant for one, three, and five minutes at room temperature. After the term of exposure, the cells were separated from the supernatants by centrifugation in a clinical centrifuge. Figure 3 shows the rate of lysis of the adsorbing cells resuspended in pH 5.0 30th M phosphate buffer. The optical density of the resuspended adsorbing cells decreased from an initial value of 0.2007 to 0.1707 in 80 minutes. Control cells which had not been suspended in supernatant before resuspension in buffer showed no lysis. Since all three adsorbed suspensions lysed at approximately the same rate, adsorption apparently takes place during the initial minute of contact. Prolonging the cell-supernatant contact period to five minutes resulted in no additional loss in supernatant lytic activity.

In order to determine whether any lytic activity is lost from active lytic supernatant during the lysis of susceptible cells, washed 4½ hour cells of *B. terminalis* were suspended in active supernatants for 60 minutes. The optical density

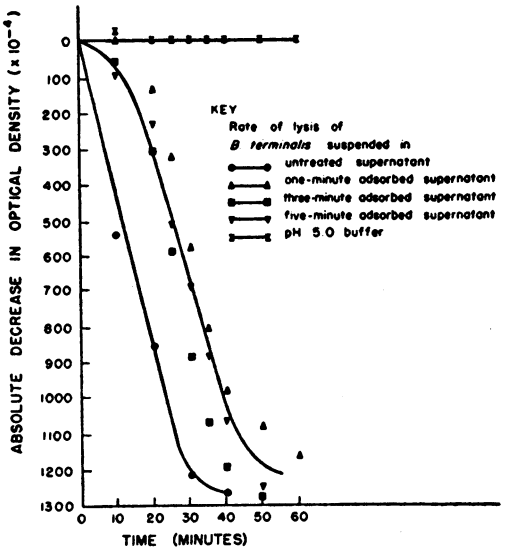

Figure 3. Removal of lytic activity by susceptible cell adsorption.

of this suspension dropped from its original value of 0.2779 to 0.1805. Particulate matter was removed by centrifugation and the supernatant decanted and labeled "lysed supernatant". Fresh assay cells were suspended in untreated supernatant, lysed supernatant, and pH 5.0 30th M phosphate buffer. The lysis of each suspension was determined turbidimetrically. Lysis rate of both the untreated supernatant suspension and the lysed supernatant suspension was almost identical, suggesting that the lytic material is not destroyed during the course of lysis and that the lytic material adsorbed by the cells is released when they lyse.

Attempts to demonstrate the presence of any active lytic material within intact cells of *B. terminalis* of various ages were without success. Mechanical disruption of such cells released no detectable lytic material.

Washed 4½ hour cells of the following organisms grown in aerated flasks of "G" medium were suspended in equal amounts of a batch of active *B. terminalis* supernatant: *B. subtilis*, *B. megaterium*, *B. polymyxa*, *E. coli*, *B. terminalis*, *B. cereus*, strain 300, *B. cereus*, strain 9139, and *B. subtilis*, strain 9943. Equal quantities of each culture were also suspended in pH 5.0 30th M phosphate buffer in order to detect any spurious lysis. Lysis was determined turbidimetrically. Table 2 lists the percentage of net drop in optical

TABLE 2

Resistance of a series of organisms to lysis by terminalis supernatant

Organism	Per Cent Change in Optical Density per 30 min in:	
	B. terminalis supernatant	pH 5.0 buffer
B. terminalis	100	00
B. cereus, strain 300	112	00
B. cereus, strain 9139	105	3.2
B. polymyxa	17.5	00
B. subtilis, strain 9943	13.5	8.0
B. subtilis	00	2.9
B. megaterium	00	00
E. coli	00	00

density of each culture in 30 minutes, using *B. terminalis* as the standard. *B. terminalis* supernatant produced lysis of both strains of *B. cereus* at least as rapidly as with *B. terminalis*. *B. polymyxa*, *B. subtilis*, strain 9943, and *E. coli* were affected very slightly, if at all.

The two strains of *B. cereus* tested in the above experiment exhibited striking similarity to *B. terminalis* in respect to their growth and sporulation in "G" medium. All three of these organisms germinate, multiply, and sporulate at the same rate after inoculation of the medium. A group of eight other aerobic sporeforming organisms grew well under these conditions but sporulated poorly during seven days of incubation. The *B. cereus* cultures sporulated completely and lysed almost all of their extra-spore vegetative residue within 2–3 days after inoculation into "G" medium.

Filtrates were prepared from completely sporulated cultures of *B. cereus*, strain 300, and *B. cereus*, strain 9139, grown in "G" medium. The capacity of these supernatants to lyse *B. terminalis* cells was then compared with actively lytic terminalis supernatant. Figure 4 shows the rate of change in optical density of washed 4½ hour *B. terminalis* cells suspended in these supernatants and in pH 5.0 30th M phosphate buffer.

The data shown in figure 5 are similar to those obtained from supernatant dilution experiments. Washed 4½ hour cells of *B. cereus*, strain 300, and *B. cereus*, strain 9139, were suspended in supernatants from both organisms as well as in terminalis supernatant to determine whether the data could be explained in terms of lytic agent concentration differences in the supernatant or as evidence of cell resistance due to strain specific-

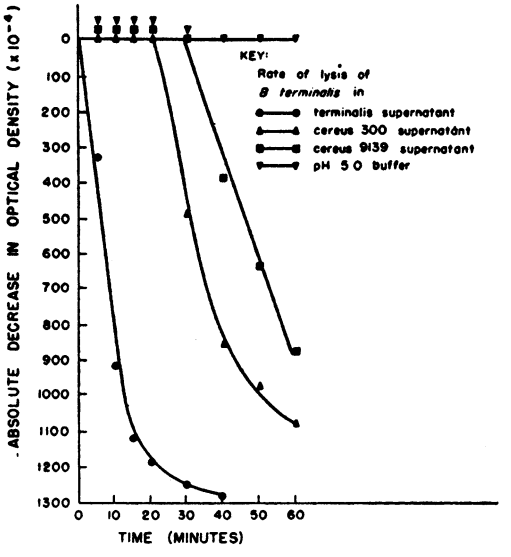

Figure 4. Lysis of *Bacillus terminalis.*

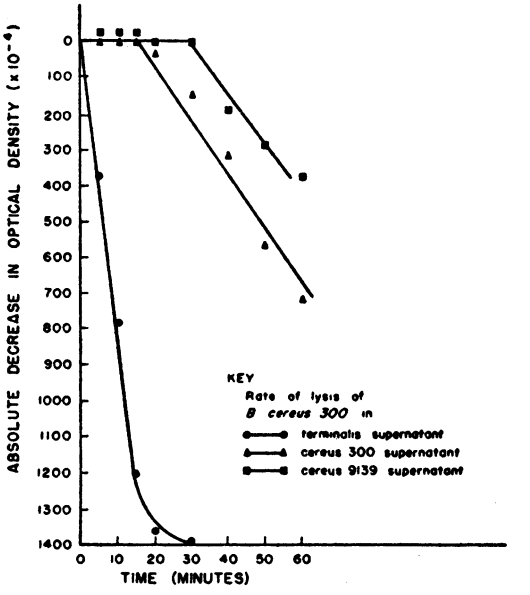

Figure 5. Lysis of *Bacillus cereus*, strain 300.

ity. The results of these experiments indicated that the differences are due to a variation of the concentration of lytic material rather than to a variation in susceptibility of the three microbial strains. *B. terminalis*, having exhibited marked morphological and cultural similarity to *B. cereus*, strain 300, and *B. cereus*, strain 9139, as well as having been shown to be closely related biochemically to *B. cereus*, is thought to be a strain of *B. cereus*.

The observations made on lytic terminalis supernatants suggested that other sporeforming aerobes might also produce an autolytic substance which could be found in the supernatant of completely sporulated cultures. *B. polymyxa*, which grows well and sporulated completely within 48 hours in brain heart infusion broth fortified with 0.01 per cent manganese sulfate, was selected for study.

Washed 4½ hour *B. polymyxa* cells grown in brain heart infusion-manganese broth were found to be rapidly lysed by supernatant taken from completely sporulated cultures of the organism grown in the same medium. As had previously been observed in the case of *B. terminalis*, older cells were not lysed by active polymyxa supernatant. Polymyxa supernatant, in contrast to terminalis supernatant, was more active at alkaline pH than on the acid side of the pH scale. It would appear, therefore, that these lytic materials are species specific.

The observations reported here have many features in common with lysis produced by bacteriophages. We, therefore, attempted in a number of ways to show that a bacteriophage was involved in our observations. We have not been able to demonstrate plaque formation nor an increase of lytic agent during lysis. While we cannot from our evidence to date rule out the possibility of a bacteriophage, we are inclined to believe that it is not. Further investigations are being made which we hope will reveal the true nature of the substance.

DISCUSSION

It has been shown that the autolytic substance produced by *B. terminalis* is not present in culture supernatants until the onset of sporulation. Although it is believed that this substance is responsible for the lysis of the vegetative cells which contain spores, rapid lysis was observed only in the case of cells obtained from the exponential portion of the growth curve.

Examination of the lytic substance has revealed some interesting properties. It is precipitated at 0.45–0.70 ammonium sulfate saturation at 4 C. Dialysis against distilled water through cellophane removes no activity in 20 hours. Centrifugation at 12,000 × G for one hour leaves all of the activity in the supernatant. The lytic

agent has an activity peak in the range pH 5.0–5.5.

Data obtained from the experiments in which vegetative cells were suspended in a series of various dilutions of active supernatant imply a rather complex kinetic. It is, very probably, a multimolecular phenomenon since the family of curves obtained from such an experiment shows that dilution results in delay in the onset of lysis. The greater the dilution, the longer the curve shoulder. Lysis, when it begins in diluted supernatant, proceeds at a rate which also seems to be relatively characteristic of a given dilution.

It has been demonstrated that susceptible cells adsorb lytic material. *B. terminalis* cells suspended in active lytic supernatant for one minute at room temperature adsorbed about 30 per cent of the activity initially present in the supernatant. Some of these cells proceeded to lyse in buffer following their removal from the active supernatant in which they had been suspended. The fact that adsorbed lytic material is not destroyed during the course of lysis has been shown by testing active supernatant in which suspended vegetative cells had been lysed. These showed lytic ability almost identical to that in active supernatant controls, suggesting also that the lytic material is released by the adsorbing cells when they lyse.

A very interesting fact revealed in the course of the work is the specificity of action displayed. Of a series of bacterial species tested, only *B. terminalis* and *B. cereus* proved to be highly susceptible to lysis by terminalis supernatant. The two strains of *B. cereus* studied also produced supernatants lytic to themselves as well as to *B. terminalis*. Previous investigation of the taxonomic characteristics of *B. terminalis* had suggested that this organism is a strain of *B. cereus*. The results obtained with the lytic material tend to confirm the conclusion that this is, in fact, the case.

Subsequent studies of supernatants taken from sporulated cultures of *B. polymyxa* showed that it also produces an autolytic material, but that it differs from the substance produced by *B. terminalis*. The optimum pH value differs; for *B. polymyxa* supernatant it is approximately 8.8, while for *B. terminalis* supernatant it is 5.3. Polymyxa supernatant will lyse *B. polymyxa* cells, but it is not active against *B. terminalis*. The reverse is true of terminalis supernatant. Thus, these two lytic materials appear to be lytic only against those species which produce them. If further work demonstrates the presence of similar specific lytic materials in cultures of other members of the genus *Bacillus*, a new method of species identification may be possible.

SUMMARY

A specific autolytic substance has been observed in the supernatants of sporulating cultures of *Bacillus terminalis*. This material is relatively heat stable, nondialyzable, and has a pH optimum in the range 5.0–5.5. It is precipitated at 0.45–0.70 per cent saturation with ammonium sulfate.

Cells of *B. terminalis* suspended in lytic supernatant for one minute at room temperature adsorb about 30 per cent of the activity initially present in the supernatant. Adsorbed lytic material is not destroyed during the course of lysis and is released by the adsorbing cells when they lyse.

Of a series of bacterial species tested, only *B. terminalis* and *Bacillus cereus* proved to be highly susceptible to lysis by active terminalis supernatant. These organisms are thought to be strains of the same species.

Bacillus polymyxa produces an autolytic material that is different from the substance produced by *B. terminalis*. The polymyxa supernatants have a different pH optimum than do terminalis supernatants, and they lyse cells of *B. polymyxa* but not cells of *B. terminalis*.

The possibility of a new method of species identification in the genus *Bacillus* is discussed.

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