THE ACTION OF BACILLUS CEREUS AND RELATED SPECIES ON THE LECITHIN COMPLEX OF EGG YOLK

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A bacterial spoilage of some fertile eggs used in the cultivation of mouse mammary tumor produced an unusual reaction in the eggs' constituents. The yolks of these eggs were hardened, had the appearance of ping-pong balls, were creamy in color, and upon standing in a container would crack open indicating their marked change in consistency. Their albumen was tan in color, and it and the yolk had a sour, cheesy odor. The organism causing these changes was found to be a member of the aerobic mesophilic sporeforming group, and was identified as *Bacillus cereus*.

Since the changes brought about in the eggs by the bacterium were so unusual and since the inoculated eggs did not possess those odors commonly associated with many bacterial spoilages, it was thought to be of interest to determine what substance or substances in the egg were involved in the chemical and physical alterations and also to determine whether other members of this group of organisms possessed similar powers.

EXPERIMENTAL RESULTS

Preliminary tests with the sporeformer on the albumen and yolk of aseptically opened fresh eggs indicated that the action that brought about the changes in the whole egg was centered in the yolk. Since Winton and Winton (1937) indicate that water (48.8 per cent), glycerides (18 per cent), protein (16 per cent), and phospholipids (10 per cent) are the major ingredients in egg yolk, tests were made for the detection of a lipase from the organism that might be capable of acting on the yolk fat. The tests were negative. Tests made on yolk protein extracted from fresh eggs showed a rapid proteolysis of it by the organism, but there was no indication that the physical alterations of the yolk in the inoculated whole egg could be due to this type of action.

MacFarlane, Oakley, and Anderson (1941), van Heyningen (1941), and Weed, Minton, and Carter (1942) worked with a lecitho-vitellin suspension in their study of the α-toxin of *Clostridium welchii*, and found that this toxin altered the suspension and produced a characteristic flocculation. McClung, Heidenrich, and Toabe (1946) in their work with members of the genus *Clostridium* suggested a substitute egg yolk agar medium for that used by Hayward (1943) and Nagler (1945). In this medium a precipitate formed in the agar following the growth of some of the organisms. These findings suggested that the phospholipid fraction of the yolk might be concerned in the alterations noted in the spoiled eggs.

To determine whether the isolated organism had any action upon the phospholipid component, the McClung, Heidenrich, and Toabe (1946) egg yolk medium

was used as the solid substrate with the substitution of 10 grams of tryptone for the 40 grams of proteose peptone no. 2. The lecitho-vitellin suspension of van Heyningen (1941), dispensed in sterile 12-by-100-mm test tubes, was used as the liquid medium. Cells from an agar slant were used for inoculation. Incubation was at 37 C for 48 to 72 hours.

The isolated organism acted upon the lecitho-vitellin suspension to produce, at first, an opalescence that upon continued incubation became flocculent and then later appeared as a curd floating upon a clear liquid. On the egg yolk medium there was a rapid formation of a creamy precipitate extending below and out from the spot of inoculation. These two types of action indicated that the isolate in its growth in the whole egg was probably decomposing the phospholipid component of the yolk. Since lecithin has been reported (Winton and Winton, 1937) to be in a larger amount than cephalin in the phospholipid fraction of egg yolk, and since MacFarlane (1942) concluded that the α-toxin of *C. welchii* does not attack either the amino-ethanol or the serine form of cephalin, a test of the action of the organism on lecithin was thought to be advisable.

Lecithin was prepared according to the methods of MacFarlane and Knight (1941) and Levene and Rolf (1927). The former method does not free the lecithin from cephalin and sphingomyelin, whereas the latter does. The method for putting the lecithins in suspension was that of King (1931). One volume of a 2.5 per cent alcoholic solution of a lecithin was run into 10 volumes of hot borate buffer at pH 7.5 with vigorous shaking. This gave a stable emulsion.

The isolate produced an opalescence in both lecithin suspensions similar to the reaction in the van Heyningen (1941) medium; however, there was less reaction in the lecithin suspensions than that normally found in the lecitho-vitellin preparation. It was noted that a cell-free filtrate, secured by Seitz-filtering the liquid portions of eggs inoculated with the isolate, acted readily upon either the egg yolk medium or upon the lecithin-borate suspension. Figure 4 shows the action of this filtrate upon the lecithin-borate suspension.

When the whole egg was used as the test medium, it was thoroughly washed, the air sac end was treated with 1:100 $HgCl_2$, a small pore was drilled into the treated area with the heated end of a metal file, and then 1 ml of a saline suspension of cells from a 24-hour-old agar slant was injected into the yolk by means of a 5-ml syringe equipped with an 18-gauge needle $1\frac{1}{2}$ inches long. Immediately after the inoculation was made, the pore was closed with sterile paraffin. A control egg was used that had received 1 ml of sterile saline. Incubation was at 37 C for 48 to 72 hours.

To determine how widespread the possession of lecithinase might be within the genus *Bacillus*, other species were tested for their ability to form a precipitate on the egg yolk agar medium, to form an opalescence in the lecitho-vitellin suspension, and to harden the yolk in a whole egg. Since Smith, Gordon, and Clark (1946) have proposed certain modifications in the grouping of the aerobic mesophilic sporeforming bacteria, and particularly for the relation of *Bacillus mycoides*, *Bacillus anthracis*, *Bacillus praussnitzii*, and *Bacillus albolactis* to *Bacillus cereus*, cultures of these were especially noted. The results of these tests are given in table 1.

TABLE 1

Reactions of some bacilli on lecithin-containing media

CULTURES USED	HARDENING OF YOLK IN WHOLE EGG	PRECIPITATE IN EGG YOLK AGAR	FLOCCULATION IN LECITHO-VITELLIN SUSPENSION
Departmental cultures			
Isolate	++++	+++	+++
B. cereus	+++	++	+++
B. mycoides	++	++	+
B. subtilis	−	−	−
B. mesentericus	−	−	−
B. circulans	−	−	−
B. megatherium	−	−	−
3 unidentified bacilli	−	−	−
N. R. Smith cultures			
B. cereus	+++	++	++
B. cereus var. *mycoides*	++	++	++
B. megatherium	−	−	−
B. pumilus	−	−	−
B. subtilis	−	−	−
B. alvei	−	−	−
B. circulans	−	−	−
B. brevis	−	−	−
B. macerans	−	−	−
B. polymyxa	−	−	−
Kenneth Burdon cultures			
B. cereus	+++	++	++
B. mycoides	+++	++	+
B. anthracis	+	+	±
B. sphaericus	−	−	−
B. brevis	−	−	−
B. subtilis (Marburg)	−	−	−
B. subtilis (Ford)	−	−	−
B. mesentericus	−	−	−
B. megatherium	−	−	−
O. B. Williams cultures			
B. cereus	+++	++	++
B. mycoides	++	++	+
B. mycoides variant	+++	++	+
W. B. Sarles cultures			
B. cereus	+++	++	++
B. mycoides	+++	+	+
B. albolactis	+++	+++	++
B. anthracis	+	+	±
ATCC cultures			
B. albolactis	+++	++	++
B. praussnitzii	++	+	+

DISCUSSION

Possible explanation for alterations in egg yolk. The early work of Osborne and Campbell (1900) on the protein of egg yolk showed that it is largely, if not wholly, a lecithin compound that dissolves in salt solution and behaves like a globulin. Sommer (1935) suggested a hypothesis that depended upon this lecithin-protein

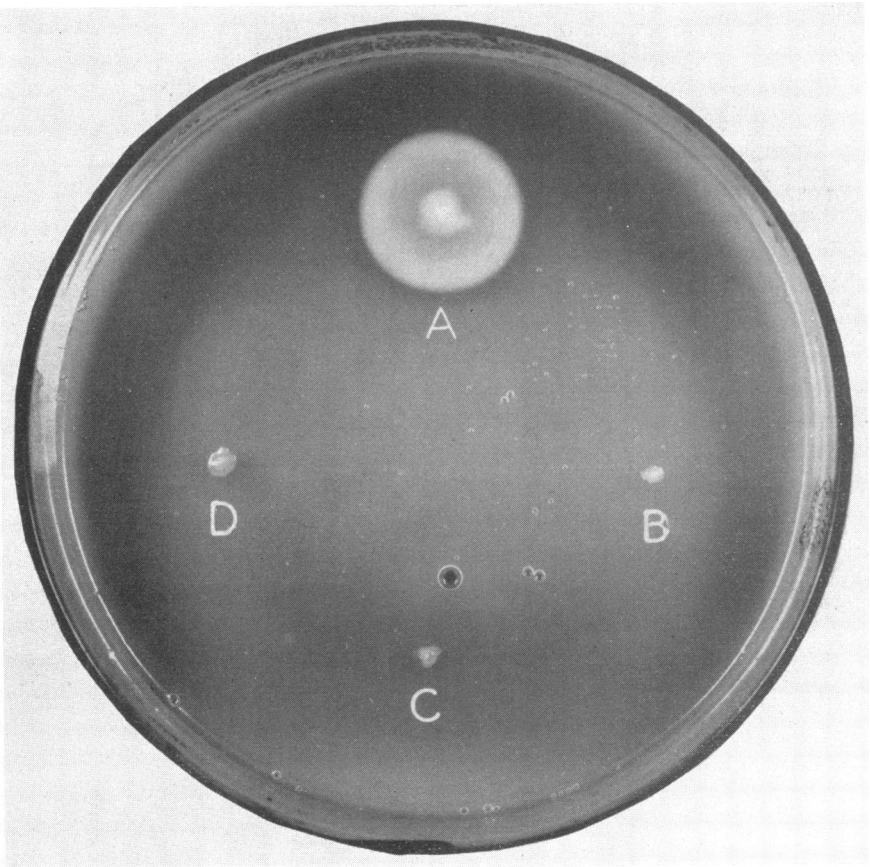

FIG. 1. COLONIES ON EGG YOLK AGAR

A, *B. cereus* (isolate); B, *B. subtilis*; C, *B. mesentericus*; D, *B. megatherium* (Burdon's culture).

complex to explain the action of egg products in increasing the "whip" of ice cream mixes. He stated:

> Lecithin with its glycerol and two fatty acid radicals in the molecule will dissolve in fat, but because of the other groups in the molecule, will be held at the surface of the fat globules. If the lecithin is in combination with proteins, the lecithin-protein combination will, therefore, cover the surface of the fat globules. The affinity of water for proteins is greater than for fat; such a covered fat globule will, then, adhere to the serum more tenaciously; the fat globule represents a less serious point of weakness in the lamellae and, therefore, better whipping results.

Probably the yolk of the egg with its balanced system of water, protein, glycerides, phospholipids, etc., is not too far removed from the system studied by Sommer. There is the probability that the lecithinase, acting upon the lecithin of the lecithin-protein fraction of the yolk, breaks down this binder and thus brings about the changes in the colloidal state of the components of the yolk. With the loss of the binder action of the lecithin, the fat and protein change from their dispersed state to that found after the isolate has grown in the egg.

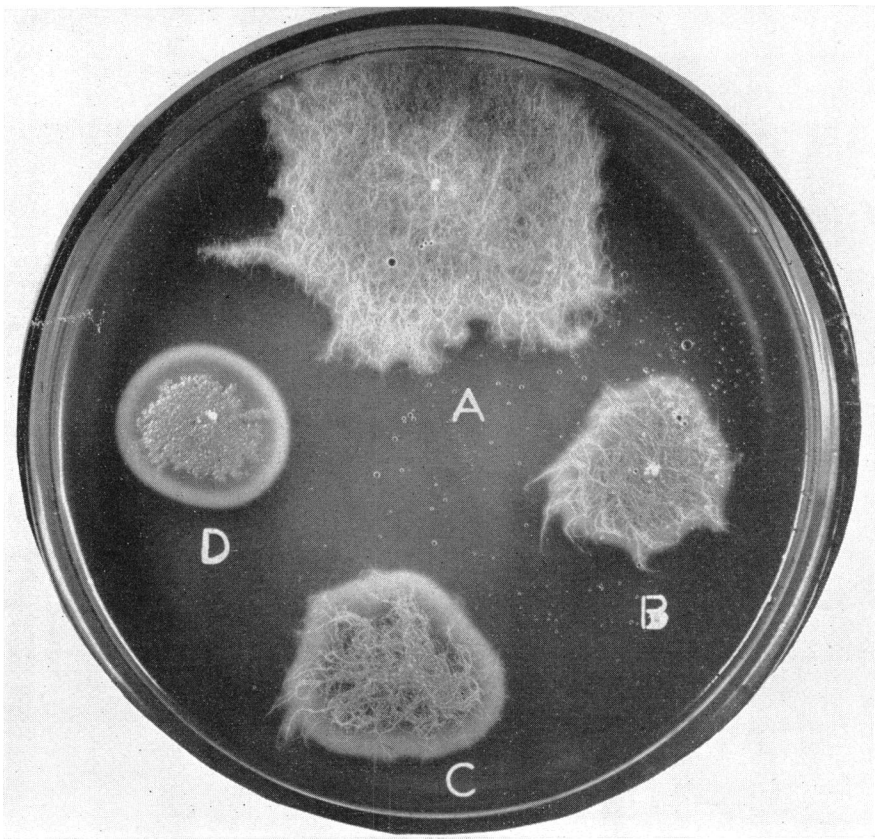

FIG. 2. COLONIES ON EGG YOLK AGAR

A, *B. mycoides* (Sarles's culture); B. *B. praussnitzii* (ATCC culture); C, *B. mycoides* (Williams' culture); D, *B. mycoides* variant (Williams' culture).

In inoculated eggs 10 to 15 days old there appear to be areas of the yolk where they are not as hardened as in other areas. This response of the yolk to the action of lecithinase is in conformity with the findings of Jukes and Kay (1932), who state that the white yolk, in intimate contact with the egg nucleus, is less rich in phospholipids, neutral fat, and protein than the yellow yolk.

Further work is under way to investigate the relation of this lecithinase to those reported by Belfanti, Contardi, and Ercoli (1936).

Lecithinase activity of some members of the genus Bacillus. A number of workers have noted that some bacteria other than the clostridia can act upon the egg yolk agar and can change the lecitho-vitellin suspension. Hayward (1941) said that in routine tests 3 hemolytic aerobic sporeformers were Nagler-treated and 2 were feebly positive. The same worker (1943) found an aerobic sporeformer that gave to Nagler plates an unneutralized opacity. Weed, Minton, and Carter (1942) found some common sporeformers to give a positive reaction in the lecitho-vitellin

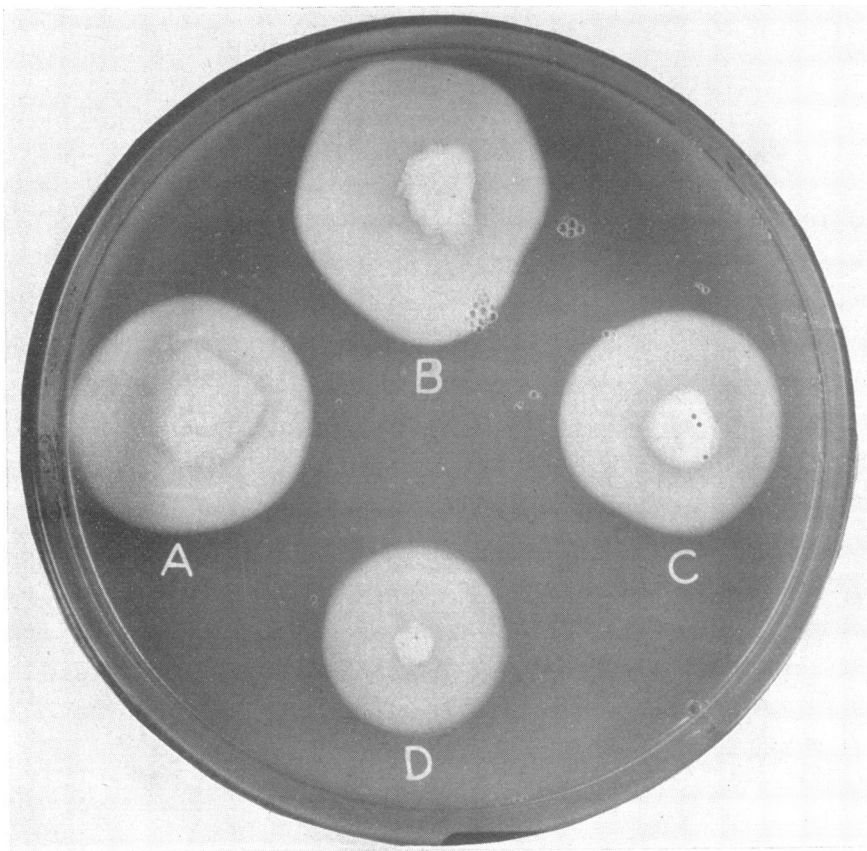

FIG. 3. COLONIES ON EGG YOLK AGAR

A, *B. cereus* (isolate); B, *B. cereus* (Smith's culture); C, *B. albolactis* (ATCC culture); D, *B. albolactis* (Sarles's culture).

suspension. Nagler (1945) stated that a number of species of aerobic sporeformers of the genus *Bacillus* had been tested, but none of them gave the pearly film. McClung and Toabe (1947) obtained positive results with their medium (precipitation but no luster) with strains of *Bacillus* designated as *B. lacticola*, *B. tumefaciens*, *B. ellenbachensis*, *B. megatherium*, *B. cereus*, *B. mycoides*, and several unidentified cultures that appeared as contaminants. They found *B. anthracis* to be among their cultures giving negative results.

It thus appears that the action of aerobic mesophilic sporeforming bacteria on egg yolk components has not gone unnoted. Although the species tested in this work were limited, there is an indication that the possession of the enzyme lecithinase is not widespread among them. Probably wider sampling of authentic cultures might reconcile the reported differences in the possession of lecithinase by some of the members of this genus. Smith, Gordon, and Clark (1946) have

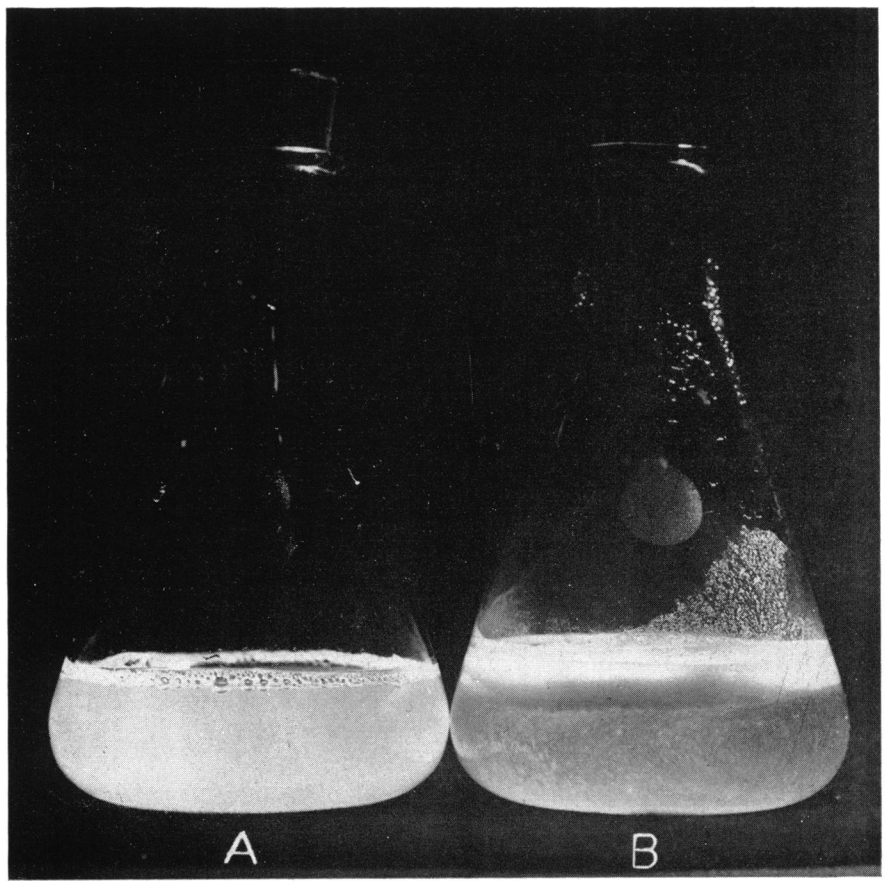

FIG. 4. ACTION OF ENZYME LECITHINASE ON LECITHIN SUSPENSION

A, control flask, lecithin-borate suspension with heat-inactivated cell-free filtrate; B, lecithin-borate suspension after 24 hours' incubation with cell-free filtrate.

found that some of their cultures labeled *B. lacticola* when received were later identified as *B. cereus*. A similar happening occurred with cultures labeled *B. ellenbachensis*; they, too, were *B. cereus*.

It is of interest to note that the production of lecithinase by two cultures aided in detecting a mislabeling. One culture designated as *Bacillus subtilis* and one designated as *B. megatherium* of the departmental culture collection gave a positive test for lecithinase activity. Later morphological, cultural, and physiologi-

cal tests showed that both organisms were *B. cereus*, confirming the placement originally indicated by their possession of this enzyme. No record of the labeled *B. subtilis* culture was available to show whether it was a *B. subtilis* of the Michigan strain, an organism claimed to be *B. cereus* (Smith, Gordon, and Clark, 1946).

The lecithinase activity of the two cultures of *B. anthracis* was feeble. Whereas the zone of precipitation was rather wide about the *B. cereus* colony, with *B. anthracis* the zone extended but little beyond the edge of the colony.

Smith, Gordon, and Clark (1946) have treated in detail certain relationships among the aerobic mesophilic sporeforming bacteria. They state:

> *B. cereus* possesses quite a wide range of characters and some of its variants and biotypes have been given names as species. For instance, certain strains ferment lactose and have been called *B. albolactis*, *B. lactis*, and *B. lacticola;* others produce a yellowish-green fluorescent pigment and have been called *B. cereus-fluorescens* or *B. fluorescens* (not *Pseudomonas fluorescens*); others produce a rhizoid growth on agar, and if they do not ferment lactose they are *B. mycoides*, if they do ferment it they are *B. praussnitzii*. Each one of these characters may be easily lost during studies on dissociation, and the resulting cultures cannot be distinguished from the typical *B. cereus*. The writers consider, therefore, that *B. cereus* is a "parent" or "basic species."

Figure 1 shows the typical reaction on egg yolk agar of a lecithinase producer contrasted with three common nonlecithinase organisms, *B. subtilis*, *Bacillus mesentericus* (*pumilus*), and *B. megatherium*. Figure 2 shows that the enzyme was produced by the rhizoid lactose-fermenting *B. praussnitzii* and the rhizoid non-lactose-fermenting *B. mycoides*. This figure also shows that the nonrhizoid dissociant of *B. mycoides* possessed the enzyme. These last two cultures were from the culture collection of the late I. M. Lewis. Figure 3 indicates that the lactose-fermenting *B. albolactis* and the non-lactose-fermenting *B. cereus* had the same relation to each other in regard to lecithinase as did the rhizoid pair shown in figure 2.

It is felt that the conclusions drawn by Smith, Gordon, and Clark (1946) regarding these organisms and their over-all relation to *B. cereus* can be augmented by the finding that there is a common possession of lecithinase by all of them, a property which does not seem to be possessed outside this group in the genus *Bacillus*.

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SUMMARY

Cultures of *Bacillus cereus*, *B. cereus* var. *mycoides*, *B. cereus* var. *anthracis*, and cultures labeled *Bacillus albolactis* and *Bacillus praussnitzii* possessed lecithinase.

The possession of this extracellular enzyme by this group of sporeformers is another fact emphasizing their inherent relationship. Other members of the genus that were tested did not produce lecithinase.

It is suggested that the action of lecithinase upon the lecithin fraction of the egg yolk is to destroy its binder action, and the loss of this property causes a marked modification in the colloidal state of the constituents of the egg.

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