BACTERIAL SPORES

I. A STUDY IN HEAT RESISTANCE AND DORMANCY[1]

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Our knowledge regarding the properties of bacterial spores, *per se*, has been impeded by the more or less universal impression that spores are metabolically inactive or inert. Except for occasional theories concerning spore-cycles and the like, the usual conception of spore function has been limited to *ipso facto* sporulation under conditions of unfavorable environment, and germination when conditions again become favorable. Some exceptions are noted in the cases of certain organisms having peculiar tendencies toward dormancy or latent germination.

Spore properties, such as minimal metabolism, respiration, enzyme production and the possibility of varying degrees of pre-germination stimulation have received little attention or credence. There have been isolated attempts to gain information concerning these still hypothetical properties; some of these will be discussed later.

Just as studies of the intimate details of metabolism have aided greatly in other fields of biological research, so a finer comprehension of spore properties and spore function should result in material improvement and greater precision in studies of the heat resistance and dormancy of bacterial spores, a subject which so vitally affects various routine laboratory procedures and their industrial applications.

The two strains of the organism with which all of the preliminary work reported here was concerned were isolated in pure cul-

[1] This communication is based on a portion of a thesis submitted to the Graduate School of Yale University by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

ture from spoiled cans of evaporated milk from two widely separated sections of the country. These strains were considered as identical or of extremely close relationship, for certainly in all of their major characteristics, particularly resistance to heat, and in the type of spoilage produced in milk, they were alike. These organisms are non-thermophilic and produce a non-acid, non-gaseous coagulation or solid curd formation in the sealed cans or tubes of evaporated milk, totally different from the so-called flat-sour spoilage caused by thermophiles, which is so commonly encountered in various canned foods, including milk.

Although the type of spoilage produced is unusual, the occurrence of this organism in cans of milk from divergent sources suggested that this invading type is a common one, or as will be intimated later, perhaps a common dissociant of one of the ordinary aerobic, spore-forming species. In many of their characteristics these strains conformed to the descriptions of *B. vulgatus*, but, on account of dissociative tendencies, could not be positively identified as such.

LABORATORY DETERMINATION OF HEAT RESISTANCE

The heat resistance properties of the spores of the spoilage organisms were studied at the outset in order to obtain, if possible, data of immediate practical value in prescribing a safe sterilization temperature and time for factory use, in view of the fact that the spoilage obviously has been due to under-sterilization. For this reason also evaporated milk was used as the heating menstruum in the preliminary experiments.

SPORE SUSPENSION PREPARATION

Uniform, viable spore suspensions free from clumps and cellular débris are basically essential to many, if not all, forms of spore study, and more particularly experiments on resistance to heat. After investigation of several methods for the preparation of suspensions, the following procedure was devised and adopted because of its simplicity and reliability.

Plain nutrient agar slants, pH 6.8 to 7.0, are used in the cultivation of the organism. Incubation is continued until maximum sporulation

occurs. With the organisms under discussion, holding for two weeks at room temperature results in the presence of abundant free spores. The growth is washed from the agar surface with distilled water, and the crude suspension filtered through a cotton plunger tube to free it from débris and clumps. One filtration usually results in a surprisingly uniform, clump-free suspension. The cotton plunger tube consists of an ordinary test tube having a compact, closely fitting plug of non-absorbent cotton about one-third of the way down from the usual plug. The suspension is introduced below the plunger by means of a capillary-tipped pipette which is passed through or along the side of the cotton plug. The plunger is forced down through the suspension evenly with the pipette which is used for withdrawing the spore material. The spores are washed two or three times in distilled water by centrifugation, and finally suspended in phosphate-buffered distilled water, heated at 80°C. for ten minutes and stored in the ice box as the stock suspension.

Phosphate-buffered distilled water

NaH_2PO_4	4.0 grams
K_2HPO_4	6.0 grams
Distilled water	1000 cc.

This double phosphate solution has a pH value of 6.8 to 7.0 which does not change on heating in ordinary soft glass tubes unless the temperature is extremely high or the heating prolonged.

Except for certain modifications, the more or less standard method for heat resistance determination suggested by Bigelow and Esty (1920) was used in this work.

After determining the approximate range of the resistance of the spores in evaporated milk at the temperature employed (115.5°C.) a number of series of sealed tube vials were exposed to heat in the oil bath and tubes were removed at intervals of one minute within this range. Subcultures were made in standard nutrient broth and on standard agar plates. Agar plates were found to be most reliable in the detection of surviving spores.

Both of the spoilage strains, when heated in evaporated milk at 115.5°C., showed a survival time of fifteen minutes, and both were killed in sixteen minutes in numerous tests. All of the tubes in these series which were heated for shorter periods than the maximum survival time of fifteen minutes gave prompt growth in

the subcultures. Likewise, the killing was 100 per cent in tubes heated for longer periods than the survival time of fifteen minutes.

Immediately after completing these tests in the laboratory, experimental sterilization studies were undertaken in one of the milk canning factories. The technic used in this work was briefly as follows:

Cans of evaporated milk were taken from the track conveyor at the filling machine before being soldered. In the plant laboratory 0.5 cc. quantities of the stock spore suspension that had been used in the preliminary tests were introduced into the cans through the small vent hole, immediately before complete soldering. Extensive series of such inoculated cans were subjected to various sterilization temperatures and times

TABLE 1

Showing survival of spores as determined by visible spoilage and cultural tests

STERILIZATION TEMPERATURE	STERILIZATION TIME*	TIME OF INCUBATION AT 37°C.	PHYSICAL APPEARANCE	CULTURAL EXAMINATION
°C.	*minutes*			
110.0	30	24–48 hours	Curd; spoiled	Positive
115.0	15	24–48 hours	Curd; spoiled	Positive
115.5	15	24–48 hours	Curd; spoiled	Positive
116.0	15	48–96 hours	Curd; spoiled	Positive
116.5	15	3 months	Normal	Negative

* Exclusive of calculated time for heat penetration, as in the case of times reported for laboratory determination.

in the regular and in the sample sterilizers. After the required time of exposure the cans were cooled by turning cold water into the sterilizer. They were then labelled and stacked in the 37°C. incubator room.

The results of these sterilization experiments are presented in table 1.

It is interesting to note the close correlation between the results of the preliminary laboratory determinations of heat resistance and those obtained in the experimental sterilization work at the plant, the maximum survival temperatures being 115.5° and 116.0°C. for fifteen minutes respectively.

DISSOCIATIVE TENDENCIES OF THE SPOILAGE ORGANISM

When first isolated, both strains of the organism appeared on agar slant cultures as flat, even, dull, powdery gray growths

which were characteristic and constant in repeated transplants. Within three months, however, particularly in cultures over three weeks old, the appearance on agar had changed. Erosion or semi-lysis was apparent. Agar slant growths disappeared almost completely, leaving clear areas or a filmy secondary growth. A constant observation was that of papillate, secondary colonies superimposed on the agar surface growth in the older cultures. These colonies were smooth, white, glistening, opaque and raised.

The morphology of the bacterial cells in these colonies was at decided variance with that of the usual dull, powdery gray growth on the same medium. Filamentous forms, varying in size and shape and characterized by profuse irregularity in staining, were seen. Some of these stained deeply, some lightly, while others presented pictures of mottled, granular or banded light and dark staining. Also, depending upon the time of examination, regular rods and sporangia could be seen, varying from normal to forms approaching the filamentous stage.

This apparent dissociation was not of a stable or permanent nature, however, since transplants made from these raised, papillate colonies to fresh agar invariably resulted in immediate reversion to the rough, dull gray type of growth.

Limited attempts were made to obtain stabilized dissociants through the usual procedures of plating old broth or peptone water cultures, phenol-broth, or "R"-exhausted-broth cultures. Although these attempts failed to induce permanent dissociation, the possibility of obtaining stabilized forms by more intensive methods was recognized. However, a comprehensive study of dissociation is not within the scope of this work, although the tendencies of these organisms toward dissociation suggest that this phenomenon, particularly the possibility of deriving super-resistant "roughs" from common strains of the aerobic spore-forming bacilli, may play an important rôle in the bacteriology of food preservation.

Since the appearance of these dissociative tendencies persisted in all subsequent transplants, some attention was given in the further experimental work to the possibility of alteration or reversion of the organisms employed with respect to heat resistance.

To obtain direct evidence on this point, spores of the two strains were heated in sealed tube vials of evaporated milk, as in the original determinations, six months after the first tests. The resistance was found to be the same as it was at the outset.

DORMANCY OF THE HEATED SPORES

In further work on the heat resistance of the spores of the spoilage organisms, observations were made pertaining to the conditions under which the spores exhibited properties of dormancy or delayed germination after heating. In this work spore suspensions prepared in phosphate-buffered distilled water, as

TABLE 2

Showing heat resistance of spores in water, and dormancy of the heated spores incubated at 37°C. in nutrient broth

TEMPERATURE HEATED	TIME HEATED	TIME OF GROWTH IN DAYS																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	30	45	60
°C.	*minutes*																		
115.5	1	−	−	−	−	−	−	+											
115.5	2	−	−	−	−	−	−	−	−	−	−	−	−	+					
115.5	3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+		
115.5	4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+		
115.5	5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
115.5	etc. to 10	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
115.5	15	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

described previously, containing approximately 500,000 spores per cubic centimeter, were sealed in thin-walled tube vials in 1 cc. quantities and subjected to heat in the oil bath at 115.5°C. Tubes were removed from the bath at one minute intervals, subcultured in standard nutrient broth (Difco-Bacto meat extract and peptone) and incubated at 37°C.

The data given in table 2 were based on the average duration of dormancy in a number of tests, since in the various individual trials irregular results were obtained. "Skips" occurred, as described by Esty and Williams (1924), and by other authors, and variable periods of dormancy were noted in the subcultures of different tests. In some instances the results of heat resistance

tests in water, with sub-cultivation in plain broth, indicated that the resistance of the spores was lower than that shown in tests made only a short time before. These results seemed offhand to refute the earlier observations on the properties of the same spores heated in milk, in which no "skips" occurred and in which the thermal death time was sharply defined and apparently constant. Such would have been the conclusion were it not for the fact that parallel or confirmatory tests in milk revealed that the spores

TABLE 3

Showing the variable response and dormancy of the spores in plain nutrient broth following heating in buffered distilled water at 100°C. Incubation at 37°C.

TEMPERATURE HEATED	TIME HEATED	TIME OF GROWTH IN DAYS																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
°C.	*minutes*																				
100.0	0	−	−	+																	
100.0	1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+			
100.0	5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+					
100.0	10	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+					
100.0	15	−	−	−	−	−	−	−	−	−	−	−	−	+							
100.0	20	−	−	−	−	+															
100.0	25	−	−	−	−	+															
100.0	30	−	−	−	−	−	−	+													
100.0	40	−	−	−	−	−	−	−	−	+											
100.0	50	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+					
100.0	60	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+			

Note: Critical point not determined at 100°C.; see page 305.

possessed their original resistance and showed no "skips" or dormancy when the heated milk suspensions were subcultured by plating out on plain nutrient agar.

Since the range of resistance of the spores heated in water at 115.5°C. was limited (maximum four minutes) a series of tests was run at a lower temperature to determine whether or not these idiosyncrasies were retained by the spores when the heating was not so severe.

It will be seen from table 3 that the same sort of irregular response is obtained at 100° as at 115.5°C., even though the maximum or critical time for the spores at 100° was not approached.

The occurrence of irregular results and variable periods of dormancy in all heat resistance tests on this organism in which the spores were heated in water and subcultured in plain nutrient broth, while regular results and no dormancy were obtained in tests in which milk was used as the heating menstruum and standard agar in plates as the subculture medium, suggested that the dormancy in the first instance was due to unfavorable environment, that is, that some factor, nutritional or otherwise, is lacking in the standard nutrient broth medium.

TABLE 4

Showing the action of the spoilage organisms as compared with that of other aerobic spore-forming bacteria

ORGANISM	DEGREE OF CLEAR ZONE FORMATION	TIME OF COLONY APPEARANCE	TIME OF CLEAR ZONE APPEARANCE	COLONY SIZE	MAXIMUM WIDTH OF CLEAR ZONE
		hours	*hours*	*mm.*	*mm.*
B. cereus	+	7	9	1–2	1
				4*	None*
B. subtilis	0	10			
B. vulgatus	+	9	12–16	3	1
				5*	None*
Spoilage str. I	++++	10	10	0.5	2.5
				1.5*	4.5*
Spoilage str. II	++++	10	10	0.5	2.5
				1.5*	4.0*

* Colony size and clear zone width at twenty-four hours.

THE ENZYMIC MECHANISM OF THE ACTION OF THE SPOILAGE ORGANISM ON MILK

The apparent predilection of the spoilage organism for milk, as evidenced by its prompt development in milk, even after severe heating, led to the following experiment, which was designed to gain some information regarding the nature of the action on milk.

A modification of the auxanographic method used by Eijkman (1901) in the study of bacterial enzymes was employed. Milk agar was pre-

pared by adding whole or evaporated milk to standard plating agar. Dilutions of the washed spore suspensions of the spoilage organisms, as well as of other forms, calculated to result in well-isolated colonies, were plated out on the milk agar and incubated at 37°C.

Around the colonies of the spoilage organisms clear zones appeared in the opaque milk agar, indicating that some agent capable of producing changes in the milk had diffused out from the colonies. Frequently these zones extended for a distance many times greater than the diameter of the colony itself. Table 4 indicates the action of the spoilage organisms as compared with that of other aerobic, spore-forming organisms.

It is noteworthy that with the milk spoilage strains the appearance of the clear zone is simultaneous with the appearance of the colony, while with *B. cereus* and *B. vulgatus* (laboratory strains) the colonies reach considerable size (two to seven hours growth after their first visible appearance) before the slightest clear zone can be detected. Also, the clear zones formed by the milk spoilage organisms not only appear earlier but are much more extensive and persist longer than do those produced by the other forms.

It is quite evident from these data that the spoilage strains produce secretions of an active substance, probably a caseolytic enzyme, that is at least partly accountable for the avidity of these strains for milk.

GENERAL DISCUSSION

Some of the observations made in the work reported here are at variance with previous findings and generally accepted theories concerning spore behavior.

Contrary to the present findings, variability in spore resistance to heat has been reported by numerous investigators, including Weiss (1921), Dickson et al. (1922), Esty and Williams (1924), Magoon (1926) and others. In general the view of most of these authors is that resistance is not a fixed property in any case, but an extremely variable one, being influenced by a host of conditions. Some are inclined to the view that there are wide variations in the resistance of individual spores in a single suspension.

In view of the complexity and multiplicity of factors that have been reported from time to time as influencing resistance, it would be difficult indeed to offer an explanation of the apparent constancy in resistance observed by us in the spores of the spoilage organisms when uniform suspensions were heated in evaporated milk and incubated, with or without subcultivation on agar. The marked variability shown by spores from the same suspensions when heated in water and subcultured in standard nutrient broth, suggests that evaporated milk in this instance supplies a consistently favorable, protective and uniform environment, which, together with the maintenance of other desirable factors, results in reducing any tendency toward variation to an absolute minimum

The writers' views are in accord with those of the above mentioned authors in so far as recognition of the possibility of variation in spore resistance is concerned, since it occurred in this work under certain conditions, and because variability is a law of living things. On the other hand, the opinion is offered that the extreme variability so often reported for the spores of various organisms is associated with conditions that are unfavorable or extreme, either during storage of the stock spore suspensions, or in the course of testing, or in the subculture environment. Just as there are conditions which tend to favor or induce variability in the resistance of spores, so there are also conditions that influence or bring about constancy of spore resistance.

Similarly, the absence of "skips" in single tube series of heat resistance tests on spores of the spoilage organisms, when the heating menstruum is evaporated milk and the subculture medium is agar, must be attributed to the favorable nature of nutritional and other factors in the environment, for the reason that with changes in the heating and subculture mediums to water and standard nutrient broth, respectively, duplicate sets of spore suspensions from identical stocks exhibited "skips" in the majority of instances.

In any discussion of "skips" and "killing range" in heat resistance determinations the uniformity of the spore suspensions of course cannot be ignored. In this work emphasis has been

placed upon the importance of this factor and on the heating of sufficiently large numbers of spores in each tube of a test series to further obviate unequal distribution of the spores.

The phenomenon of dormancy or delayed germination of aerobic spores after heating has been frequently reported as occurring in environments entirely favorable for the germination of the spores in question. Magoon (1926), for example, reported that spores of *B. mycoides* remained dormant in subcultures (heat resistance tests) for periods varying from one to sixty-four days. He attributed this dormancy to normal, inherent properties within the spores, rather than to "heat inhibition" or injury due to heat, which has been suggested by Burke, Weiss and others as the cause of dormancy after heating. Neither of these views offers an explanation for the dormancy observed in the plain standard nutrient broth subcultures in the heat resistance tests on the spoilage organisms under consideration here. Both theories are untenable in this case, for the reason that with a change in pabulum from plain broth to milk the dormancy is eliminated. This fact limits the cause of the dormancy in this instance to environmental factors.

There is considerable evidence, direct and indirect, in support of the view that dormancy of heated or unheated spores is a function of the environment; in other words, that it is dependent on stimuli supplied by the environment. Further experimental work bearing on various environmental factors, particularly nutrition, in relation to dormancy, and discussion of this subject, will appear in a later communication.

A brief discussion concerning the function of nutrition in the germination of spores of the spoilage organisms under consideration is included here, since a certain amount of experimental evidence has been presented to show the mechanism of the action of these organisms on milk. It has been indicated that the peculiar adaptability of these organisms to milk may be due in part to the predominance of enzymes elaborated by them which are capable of attacking certain materials present in milk. It is not a far cry, then, to presume that the effectiveness of milk in stimulating

germination of the spores after heating is due to the presence of like enzymes in the spores, themselves, and that the secretion of these enzymes takes place prior to the actual germination.

The objection to such an hypothesis will be raised that enzymes are thermolabile and therefore can have no part in the rapid development of the spores in milk after heating to the extremely high temperatures mentioned. Granting this objection, the fact remains that the vital, life forces of the spores have been in no way impaired by the heat, as evidenced by their prompt germination. It is conceivable, then, that the enzymes may be regenerated by the spores, perhaps at an unusually rapid rate due to the stimulation of the temperature shock. The view, in this instance, that enzyme production and secretion precedes and facilitates germination by rendering the substrate utilizable by the germinating spores, is in direct confirmation of the work of Ruehle (1923) and that of Effront (1917) concerning the enzymic content of bacterial spores. The following statement of Effront is particularly apropos in this case: "Bacterial spores attenuated either by heat or antiseptics show themselves the more productive of enzymes the more difficult their germination. Under certain conditions and in the presence of antiseptics the spores may produce an intense secretion of enzymes in a liquid without, however, arriving at germination."

The suggestion has been advanced that the spoilage organisms are possibly super-resistant varieties of some common aerobic, spore-forming organism because of the tendencies toward dissociation. In this connection particular interest attaches to the work of Kelly (1926) on bacteria causing spoilage of evaporated milk. He has observed three types of spoilage, and it is significant that he designated the causative agents as atypical strains of *B. cereus*, *B. simplex* and *B. megatherium*, respectively. His results are highly suggestive as a possible confirmation of the contention that spoilage may be due frequently to super-resistant variants of common forms. In the writers' experience, ordinary strains of *B. cereus* and *B. megatherium* are notably lacking in resistance to heat.

SUMMARY

Observations made in a study of the properties of a heat-resistant, aerobic spore-forming organism suggest that the response or behavior of bacterial spores after heating is dependent upon environmental conditions, and that what appears to be extreme variability in spore resistance to heat is due to the lack of specific favorable conditions in the culture medium rather than to inherent qualities of variability.

Spores of the milk spoilage organism in question gave regular results, without dormancy or delayed germination, in heat resistance determinations when evaporated milk was employed both as the heating and subculture medium, but extremely irregular results were obtained when nutrient broth was used as the subculture medium. It is significant that this organism is peculiarly adapted in its enzymic function to the environment supplied by milk.

Because of certain dissociative tendencies, this spoilage organism was thought to be a relatively stable variant of a common aerobic spore-forming species, *B. vulgatus*, which it resembles in many ways.

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