SYMPOSIUM ON INITIATION OF BACTERIAL GROWTH[1]

IV. Dormancy, Germination, and Outgrowth

H. ORIN HALVORSON

Department of Bacteriology, University of Illinois, Urbana, Illinois

The bacterial spore is the most nearly inert living cell known, and yet it responds rapidly to unique trigger mechanisms that initiate growth. It is intended to discuss here some of the characteristics of these cells as well as some factors involved in the trigger mechanism and the initial growth that results.

The resistance of these unique cells to heat and injurious chemicals is well known and needs no comment. However, the fact that the mechanism of resistance is so little understood reflects our lack of knowledge of the composition and organic structure of the cells. Some progress in this connection is evident from recent researches which have revealed a few substances that are abundantly present in spores but absent in the corresponding vegetative cells. Notable among these are dipicolonic acid (DPA) revealed by Powell (1), a special spore peptide revealed by Strange and Powell (2), and several heat-resistant enzymes reported by the Illinois group, Stewart and Halvorson (3), Lawrence and Halvorson (4), Lawrence (5), and Nakata (6).

DPA has been demonstrated in all spores, both aerobic and anaerobic, that have been studied to date. It has never been found in vegetative cells, but is present in spores to the extent of from 6 to 12 per cent of the dry weight of the cells. Although DPA itself is readily water soluble, it can be extracted from spores only by means of digestion with strong mineral acid. It is synthesized late in the process of sporulation. In the case of anaerobic sporeformers, Collier (7) has shown that it is produced after the sporelike structure is formed. When the spore germinates, the DPA is released into the medium. It is apparent, therefore, that the DPA must be tied up in some insoluble structure. When germination takes place, a number of other chemicals are also released to the surrounding medium, notably the spore peptide, a number of amino acids, and other substances as yet unidentified. The DPA is therefore probably complexed with the peptide and other substances in the spore.

Church and Halvorson (8) and unpublished results of the writer and G. G. K. Murty have been able to produce sporelike structures that do not contain DPA, or that contain less than the normal amount. Cells having no DPA are as heat sensitive as vegetative cells, and those having reduced amounts have a reduced heat resistance. In fact, over a certain range of concentration, there appears to be a direct correlation between heat resistance and the DPA content. These facts indicate that this acid, as it is complexed in the cell, is somehow responsible for the resistance of the spores. To date little progress has been made in elucidating the nature of this complex because mechanical rupture of the cell produces the same result as germination, and no one has yet found a way of preserving the complex while mechanically rupturing the cell.

Cytological studies on the spore have revealed a structure quite different from that of the vegetative cell. This has been studied extensively by Robinow and Fitz-James (9) but time does not permit a review of their work here. Suffice it to say that marked changes take place in the spore structure during germination and during the release of the DPA and spore peptide, indicating a relationship between the structure as seen in cytological studies and the biochemical changes occurring during germination.

Although the spores are in most respects inert, several active enzymes have been clearly demonstrated in the intact heat-resistant cell. Stewart and Halvorson (3) have found racemase that converts L- or D-alanine to a racemic mixture; Lawrence (5) and Nakata (6) have found ribosidase that breaks adenosine to adenine and ribose; and Lawrence and Halvorson (4) have

[1] This symposium was held at the 59th Annual Meeting of the Society of American Bacteriologists in St. Louis, Missouri, on May 13, 1959, under the sponsorship of the Agricultural and Industrial Division.

demonstrated a heat-resistant catalase. All of these enzymes are active in the intact spore and are remarkably heat resistant, even retaining this heat resistance during and after germination or following mechanical rupture. Church *et al.* (10), found racemase in many but not all spores of aerobes. It has not been found in the spores of anaerobes. The other two enzymes have so far been demonstrated only in strains of *Bacillus cereus*. Spores of other species have not yet been studied in this respect.

The heat resistance of racemase and ribosidase has been shown to be associated with the complexing of the enzyme into a particulate structure, sedimented at a gravity of less than 40,000. When this fraction, freed from any soluble portion, is subjected to sonic treatment, two fractions can be demonstrated in the resulting suspension, one particulate that still contains the heat-resistant enzyme and the other soluble that is enzymatically active but heat sensitive. This soluble portion remains in the supernatant after centrifuging at 140,000 times gravity. These findings are the basis for our conclusion that heat resistance is associated with the complexing of the enzymes into larger structures. The nature of the particles that protect these two enzymes is not known. If the same type of process is involved in the protection of the other enzymes of the spore, those that are dormant in the intact cell, the structure must be somewhat different because the dormant enzymes are not enzymatically active while they are thus protected and the protection is lost when the spore germinates. At any rate, it would be interesting to know whether the DPA is involved in the complex that protects the racemase and the ribosidase.

Further studies may reveal other enzymes in the spore that are similar to the racemase. So far no one has demonstrated heat-resistant active enzymes in the spores of anaerobes, but in view of the findings with the aerobes, there seems to be no reason why such enzymes should not be found.

A number of enzymes in the intact spore in a dormant state are resistant to heat, but become heat sensitive upon germination or mechanical rupture of the spore. It is reasonable to assume that these also owe their heat stability to a colloidal particle that complexes them into stable structures. In this case, however, the prosthetic groups have been rendered inactive.

The so-called inertness of the bacterial spore is only relative. A spore population will undergo marked changes on storage even at low temperatures and under relatively dry conditions. The rate of change is naturally hastened at higher temperatures and at higher moisture levels. If one makes a quantitative study of the viability and ease of germination of a spore population, one will find a decrease in viability and a simplification of the germination requirements with time of storage. Wolfe and Mahmoud (11) have suggested that the loss of viability is due to a slow rate of germination and death of the germinated cells. Our studies indicate that this is not the case, because we find that these so-called "dead" spores still react normally so far as staining, morphology, and response to the germination nutrients are concerned. When the "dead" cells, in the presence of the germination nutrients, lose their refractility, they also lose their DPA, as do normal spores. We believe, therefore, that these "dead" spores have not undergone previous germination.

Aging simplifies the germination requirements, and this, we believe, is due to slow autolytic changes within the spore resulting in a release of germination nutrients (Murty and Halvorson, (12)).

The trigger mechanism for germination is one of the most interesting and unique mechanisms found in nature for the conversion of an inactive cell into a rapidly growing one. Detailed studies of this mechanism have been made in only a few species. Hills (13) was the first to demonstrate that spores of aerobes could be made to germinate in a few minutes with a mixture of amino acids and adenosine.

Before continuing this discussion we need to define our terms. In the past, "germination" has designated all the changes taking place during conversion of the spore to the new vegetative cell. With the discovery of the complex trigger mechanism, it became apparent that this process consists of at least two independent steps. The initial changes, occurring within minutes, involve a loss in heat resistance and refractility, and a gain in stainability. These changes are followed by the second stage, involving the initiation of growth, which is relatively slow, taking an hour or more. Bacteriolo-

gists working with spores have restricted the term germination to mean the initial changes only, and refer to second stage as "outgrowth." We shall follow this usage here.

Although spores from different species may require different mixtures of amino acids to trigger germination, most of them require L-alanine either alone or in combination with other substances. A typical example is *B. cereus* that requires L-alanine and adenosine. With the proper amounts of these substances, germination will occur in from 2 to 5 min. It can be followed by observing the change in the amount of light transmitted through a suspension. As germination proceeds, the spores lose their refractility and more of the incident light is transmitted through the suspension. The heat resistance diminishes and the cells become more easily stainable. Simultaneously also the DPA and other organic materials are released to the outside medium.

The trigger mechanism is not understood. Harrell and Halvorson (14) found that such a small amount of alanine was used during germination that it could hardly be detected with radioactive tracer techniques. This fact indicates that the alanine served as a catalyst. The spore extracts, however, would enzymatically convert alanine to pyruvic acid and ammonia; perhaps, therefore, pyruvic acid is the active substance. In this connection it is of interest to note that Murty and Halvorson (15) have found ethyl pyruvate to be an inhibitor of germination.

Germinated spores also contain enzymes that convert alanine to pyruvic acid and ammonia, and metabolize the resulting pyruvic acid. It is difficult to demonstrate this activty in the intact spore, possibly because it is too slight to be measured. The activity may still be enough, however, to initiate germination. As this enzyme acts on the alanine, and germination is started, enough enzyme may be released so that the catalytic effect can be observed. The germination process would also release bound alanine, to increase this activity still more.

Some heat shock is usually necessary to start the trigger mechanism. Since heating itself can activate some of the dormant enzymes, as demonstrated by Church and Halvorson (16) and Murty and Halvorson (12), such activation may be the real function of heat shock, for whereas fairly long heat treatment at 65 C (several hours) is required to activate enough enzyme to permit a demonstration of its activity by conventional methods, a much shorter heat treatment may suffice to initiate the trigger mechanism.

There are some other facets of the trigger mechanism that are not readily explainable. It will function well at room temperature but not at all at refrigerator temperatures or at 65 C. However, cells that have been kept at 65 C for some time will germinate rapidly when returned to room temperature. If, however, the spores of *B. cereus* are kept at 65 C for a considerable length of time in the presence of the minimum amount of alanine required for germination but with adequate amounts of adenosine, the spores will fail to germinate on being brought back to room temperature unless more alanine is added. This shows that the alanine can be decomposed at 65 C but in such a way that the products do not participate in the germination process. Similar results can be obtained with extended heating at 65 C in the presence of the minimal amount of adenosine required for germination, but with adequate amounts of alanine. Such spores will not germinate when restored to room temperature unless more adenosine is added. Thus, the products of the decomposition of adenosine cannot serve as trigger activators.

A number of studies have been made on the effect of various types of inhibitors on germination. Arsenate, arsenite, azide, fluoride, cyanide, fluoroacetic acid, and dinitrophenol have no effect. This could mean either that the reactions these chemicals inhibit are not involved in germination or that none of these substances can penetrate the spore wall.

The following substances, however, have been found to inhibit germination of some species: D-alanine, 8-hydroxyquinoline, heavy metals such as Cu, Zn, Ni, Cr, and Co, organic mercurials, *bis*-1,3-β-ethylhexyl-5-methyl-5-aminohexahydropyrimidine (W-1435), octyl alcohol, and ethyl pyruvate.

As first observed by Hills (13), D-alanine interferes with the L-alanine-induced germination of aerobic spores, but only when the D-alanine is added along with the L-alanine to spores that are not subsequently heat shocked. If they are added prior to a prolonged heat shock, there is no evidence of inhibition. This suggests that the

D-alanine merely prevents penetration of the spore by L-alanine.

Inhibition with 8-hydroxyquinoline was reported by Powell (17) and Murty and Halvorson (18), who found also that the inhibition could be reversed by certain metallic ions. One may reasonably assume that the hydroxyquinoline chelates essential metallic ions. This is consistent with the findings of Levinson and Sevag (19) that Mg and Mn ions enhance germination by L-alanine in some species of spores. The inhibition of germination by heavy metals and organic mercurials reported by Murty and Halvorson (18) is probably due to the binding of essential sulfhydryl groups. This inhibition can be overcome by glutathione and thioglycolate. Of interest in this connection is the finding of Brown (20) that Versene (ethylenediaminetetraacetic acid) will initiate the germination of spores of a putrefactive anaerobe. It would seem that some adsorbed metallic ion prevented these spores from undergoing spontaneous germination.

The inhibition of germination of spores of *B. cereus* by W-1435, as reported by Halvorson and Church (21), is attributed to the blocking of pyruvate oxidation, in accordance with their theory that L-alanine is converted to pyruvate during germination. If this be true, a different mechanism must be involved here than in the spores of *Bacillus megaterium*. According to Powell (*personal communication*), W-1435 has no effect on the germination of *B. megaterium* spores. Inhibition by octyl alcohol (G. G. K. Murty and H. Orin Halvorson, *unpublished data*) may be related to its ability to interfere with the oxidation of L-alanine as well as with L-amino acid oxidases in general. This inhibition has been tried on the spores of *B. cereus* only and may not hold for other species. The inhibition by ethyl pyruvate (15), also tried only on *B. cereus* spores, again points to the importance of pyruvate metabolism during germination. We have found that this ester prevents the decarboxylation of pyruvic acid.

Stedman *et al.* (22) have reported that some heat-labile inhibitors of germination are secreted into the outside medium during germination and claim that these inhibitors prevent the germination of some spores in a suspension that contains suitable stimulants. It has been the experience of most workers that from 5 to 10 per cent of the spores in any population always remain ungerminated. These dormant cells, though normal, may be inhibited by the secretions of the other 90 to 95 per cent that do germinate, as postulated by Stedman, or they may be abnormal cells that somehow resist germination. To find out which is the correct hypothesis, it will be necessary to remove the ungerminated cells and investigate them separately.

Another phenomenon closely allied to the above is the influence of the density of the spore population on germination. Powell and Hunter (23), working with spores of *B. megaterium*, found that closely packed cells had a higher rate of germination than did cells in thin suspension. We noted an opposite effect with *B. cereus* spores, obtaining a higher rate of germination in dilute suspensions. For example, when 10^8 spores per ml were suspended in a 10 per cent skim milk solution, only 10 per cent of the spores germinated; with 10^6 spores per ml in the same type of solution, 90 per cent did so. This indicates some quantitative relationship between the cell density and the required concentration of the germination nutrients.

It is the writer's opinion that germination, as defined above to mean only the early stage of the process, involves (a) the breakdown of the complexing structure which protects the vital functions and (b) the activation of the enzymes that remain dormant in the intact spore. When these enzymes are activated, particularly the enzymes that yield energy, the synthesis of new enzymes can take place and when these become fully functional, normal growth can occur.

Although many studies have been made of the early changes, comparatively little work has been reported on the later and slower changes. The work of Hyatt and Levinson (24) is of special interest in this connection. These workers prefer to consider four separate steps in the over-all process: germination, swelling, elongation, and cell division. The first is characterized by a rapid rise in respiratory activity, together with the other changes mentioned above. This respiratory rate reaches a plateau and remains at this level during the next stage (swelling), during which the cells enlarge by imbibing water, salts, and nutrients from solution. During this period also, lasting 1 hr or more, the cell is adjusting itself to the synthetic activity that is to occur later.

In the third stage (elongation), a new burst of metabolic activity takes place and this has to be supported by minerals and nutrients that are not necessarily required for germination. Levinson and his group found that when the spores were suspended in a solution containing only the minimal requirements for germination, the normal increase in activity at this stage did not occur. One of the important requirements for this new burst of activity was sulfate in the form of any one of a number of sulfate salts. The period of elongation takes 1 hr or more and is followed by the first cell division. By this time the metabolism of the cell has reached the level of a mature vegetative cell and presumably the full complement of enzymes has been synthesized and normal growth follows.

REFERENCES

1. Powell, J. F. 1953 Isolation of dipicolinic acid (pyridine-2:6-dicarboxylic acid) from spores of *Bacillus megaterium*. Biochem. J., **54**, 210–211.
2. Strange, R. E. and Powell, J. F. 1954 Hexosamine-containing peptides in spores of *Bacillus subtilis*, *B. megaterium*, and *B. cereus*. Biochem. J., **58**, 80–85.
3. Stewart, B. T. and Halvorson, H. Orin. 1954 Studies on the spores of aerobic bacteria. II. The properties of an extracted heat-stable enzyme. Arch. Biochem et Biophys., **49**, 168–178.
4. Lawrence, N. L. and Halvorson, H. Orin. 1954 Studies on the spores of aerobic bacteria. IV. A heat resistant catalase from spores of *Bacillus terminalis*. J. Bacteriol., **68**, 334–337.
5. Lawrence, N. L. 1955 The cleavage of adenosine by spores of *B. cereus* var. *terminalis*. J. Bacteriol., **70**, 577–582.
6. Nakata, H. M. 1956 Studies on the enzymatic cleavage of adenosine by the spores of *Bacillus cereus*. M.S. thesis, University of Illinois.
7. Collier, R. E. 1958 A study of sporogenesis in *Clostridium roseum*. Ph.D. thesis, University of Illinois.
8. Church, B. D. and Halvorson, H. 1959 Dependence of the heat resistance of bacterial endospores on their dipicolinic acid content. Nature, **183**, 124–125.
9. Robinow, C. F. and Fitz-James, P. C. 1957 Cytological changes during germination. In *Spores*, Publ. No. 5, pp. 83–93. American Institute of Biological Sciences, Washington, D. C.
10. Church, B. D., Halvorson, H., and Halvorson, H. Orin. 1954 Studies on spore germination: its independence from alanine racemase activity. J. Bacteriol., **68**, 393–399.
11. Wolf, J. and Mahmoud, S. A. Z. 1957 The germination and enzymic activities of *Bacillus* spores at low temperatures. J. Appl. Bacteriol., **20**, 124–136.
12. Murty, G. G. K. and Halvorson, H. Orin. 1957 Effect of duration of heating, L-alanine and spore concentration on the oxidation of glucose by spores of *Bacillus cereus* var. *terminalis*. J. Bacteriol., **73**, 235–240.
13. Hills, G. M. 1949 Chemical factors in the germination of spore-bearing aerobes. The effect of yeast extract on the germination of *Bacillus anthracis* and its replacement by adenosine. Biochem. J., **45**, 353–362.
14. Harrell, W. K. and Halvorson, H. 1955 Studies on the role of L-alanine in the germination of spores of *Bacillus terminalis*. J. Bacteriol., **69**, 275–279.
15. Murty, G. G. K. and Halvorson, H. Orin. 1959 The role of the glyoxylic acid cycle in the sporulation of *Bacillus cereus* T. Bacteriol. Proc., **1959**, 39.
16. Church, B. D. and Halvorson, H. 1957 Intermediate metabolism of aerobic spores. I. Activation of glucose oxidation in spores of *Bacillus cereus* var. *terminalis*. J. Bacteriol., **73**, 470–476.
17. Powell, J. F. 1951 The sporulation and germination of a strain of *Bacillus megaterium*. J. Gen. Microbiol., **5**, 993–1000.
18. Murty, G. G. K. and Halvorson, H. Orin. 1957 Effect of enzyme inhibitors on the germination and respiration of and growth from *Bacillus cereus* var. *terminalis* spores. J. Bacteriol., **73**, 230–234.
19. Levinson, H. S. and Sevag, M. G. 1953 Stimulation of germination and respiration of the spores of *Bacillus megaterium* by manganese and monovalent anions. J. Gen. Physiol., **36**, 617–629.
20. Brown, W. L. 1956 The production and germination requirements of putrefactive anaerobe 3697 spores. Ph.D. thesis, University of Illinois.
21. Halvorson, H. and Church, B. D. 1957 Biochemistry of spores of aerobic bacilli with special reference to germination. Bacteriol. Revs., **21**, 112–131.
22. Stedman, R. L., Kravitz, E., Anmuth, M.,

AND HARDING, J. 1956 Autoinhibition of bacterial endospore germination. Science, **124**, 403–405.

23. POWELL, J. F. AND HUNTER, J. R. 1955 Spore germination in the genus *Bacillus*: The modification of germination requirements as a result of preheating. J. Gen. Microbiol., **13**, 59–67.

24. HYATT, M. T. AND LEVINSON, H. S. 1957 Sulfur requirement for postgerminative development of *Bacillus megaterium* spores. J. Bacteriol., **74**, 87–93.