

## Bases for Applications of Bacterial and Fungal Spores

HILLEL S. LEVINSON, FLORENCE E. FEEHERRY, AND GABRIEL R. MANDELS

*Food Sciences Laboratory, U.S. Army Natick Research and Development Command,  
Natick, Massachusetts 01760*

INTRODUCTION .....	3
APPLICATIONS BASED ON RESISTANCE .....	3
APPLICATIONS BASED ON PRODUCTS OF BACTERIAL SPORES OR WITH SPORULATION .....	5
Bacterial insecticides .....	5
Antibiotics .....	7
Enzymes .....	11
Toxins .....	12
APPLICATIONS OF FUNGAL SPORES .....	13
EPILOGUE .....	14
LITERATURE CITED .....	14
APPENDIX .....	15
1. Aspects of Inactivation and Injury of Spores at Ultrahigh Temperatures .....	15
2. Toxins of Entomocidal Sporeforming Bacilli .....	16
3. Relationship Between <i>Clostridium perfringens</i> Enterotoxin and Sporulation .....	16
4. Speculations on the Role(s) of Antibiotics in Sporeforming Microorganisms .....	16

*All applied research is founded upon basic research. Before the basic facts are known, it is a sheer waste of money to support application on nonexistent basic knowledge.*

Hugo Theorell

*Whoever, in the pursuit of science, seeks after immediate practical utility may rest assured that he seeks in vain.*

H. L. F. von Helmholtz

### INTRODUCTION

No attempt will be made, in this brief paper, to review all aspects of the applied microbiology of spores. To review every past, present, and potential application would, in effect, amount to a review of all of spore science. We shall refer mainly to those published reviews of aspects of spore science which seem most closely related to practical applications. For more details and for reference to reports of original research, the reader should consult these reviews. Application is often dependent on basic science, and, while the following discussion is broadly organized around application, it will often refer to fundamental aspects of spore science. For example, a logical approach to food preservation must be based on an understanding of heat resistance, heat inactivation, heat activation, thermal injury and repair, germination, outgrowth, etc. It is apparent, therefore, that we shall be able, in the space available, to do little more than to skim the surface.

The Seventh International Spore Conference included a number of symposia, one of which was entitled "Applied Microbiology of Spore-forming Bacteria." This symposium was organized around two major aspects of spores: applications based on resistance and applications based on products of spores or associated with the sporulation process. The first aspect was covered in papers by G. W. Gould, by D. M. Adams, and by P. Gerhardt and W. G. Murrell. The second aspect was represented in the symposium by R. G. Labbe, by L. A. Bulla, Jr., and by A. L. Demain and J. Piret. The comments of Gould and of Gerhardt and Murrell appear as separate papers in this volume. Abstracts of the remarks of the other speakers are included as an appendix to this paper.

### APPLICATIONS BASED ON RESISTANCE

If there is any single practical implication of the physiology of bacterial spores which has made them the object of continuing investigations for many years, it is their extraordinary resistance to adverse environmental influences. Methods for preservation of foods and sterilization of such diverse industrial products as metal-cutting fluids, spacecraft, pharmaceutical preparations, and medical and surgical materials must all be geared to the resistance of bacterial spores to such agents as heat (wet and dry), ultraviolet and ionizing radiations, chemicals, desiccation, etc.

Useful reviews appear in the book edited by Block (2), and some aspects of resistance to and inactivation by heat are discussed in the present volume in Appendix 1 to this paper and in the papers by Gerhardt and Murrell and by Gould.

The resistance of the bacterial spore makes it the microbial form of choice in tests for determining the sterilizing efficiency of various devices, chemicals, and processes (2), whether for food preservation or other applications. In selection of a spore indicator of sterilizing efficiency, there are questions as to the choice of species, since spores of one strain may be the most resistant under one set of circumstances but less resistant under another; e.g., dried spores of *Clostridium sporogenes* may be more resistant to ethylene oxide and less resistant to phenol than those of *Bacillus subtilis* var. *niger*. The strain, medium, and temperature, as well as age and concentration of the culture, should be selected to give the most resistant spores for each application. In this regard, spores of particular strains of *B. stearothermophilus* have been produced commercially for use as indicators of steam sterilization; it has been recommended by the Association of Official Analytical Chemists that spores of specific strains of *B. subtilis* and *C. sporogenes* be used in the evaluation of gaseous and liquid chemicals for sporicidal activity; and spores of *C. botulinum* and of *B. pumilus* have been recommended as indicators of radiation sterilization.

Commercially available biological indicators may not necessarily be the most resistant types, but they are orders of magnitude more resistant than many harmful microbes of public health concern and have the advantage of ease and convenience of handling and stability over long periods. In some cases, e.g., food preservation, where one may be concerned over effects of the heating menstruum (the food) on microbial resistance, it is often considered more appropriate to inoculate samples of the foods with bacterial spores (inoculated pack), simulating the real conditions of food processing.

The use of spore indicators has become increasingly important as a result of the demand for sterile products, some of which cannot tolerate sterilization by conventional procedures. In the sterilization of spacecraft, for example, delicate electrical work might be damaged by sterilizing doses of either heat or radiation, but both heat and radiation doses can be reduced if both forms of energy are applied. Reliable indicators of sterility are essential, since the entire spacecraft will not be available for testing.

In essence, the problem in spoilage of foods by such sporeforming anaerobes as *C. botulinum*, *C. perfringens*, *C. sporogenes*, *C. bifermentans*,

*C. butyricum*, and *C. pasteurianum* and by the sporeforming aerobes *B. thermoacidurans*, *B. stearothermophilus*, *B. subtilis*, *B. licheniformis*, *B. macerans*, *B. polymyxa*, and *B. cereus* is in treating the food so that spores are destroyed and in maintaining the heated food under conditions which do not permit the adventitious entry and subsequent growth of spores. In heat treatment aimed at destruction of spores, it is also important to consider that rare spores may have an unusually high heat resistance (65).

The sporicidal efficacy of a treatment is usually judged by the ability of the treated spore to multiply. One must consider whether the "sterilizing" treatment has affected mechanisms of germination, outgrowth, or division (see Appendix 1) and whether optimal conditions for recovery of treated spores differ from those for recovery of untreated spores. These distinctions become increasingly important when the spores have been heated with, or their viability has been estimated in the presence of, chemical additives, such as curing salts. A major question is whether the colony count from treated spores has been affected by "injury" (with the possibility of repair) or whether these spores have been irreversibly inactivated. The existence of such injury, its basis, and its repair or circumvention become exceedingly important in judging the sterility of foods (see Appendix 1). "Knowledge of sublethal injury is indispensable in evaluating laboratory data, in developing or modifying food processes, and in preserving culture activity" (8). For example (9), radiation-injured spores of *C. botulinum* formed colonies at 40°C, but not at 30°C. It was suggested that the temperature of postirradiation incubation be considered in determining the survival of radiation-damaged spores of *C. botulinum* or in determining the sterility of irradiated foods.

Sporeforming organisms, both aerobic and anaerobic, are involved in human and animal disease (1, 59). Transmission of the pathogenic organisms is closely related to the resistance of their spores. Diseases caused by these organisms are usually initiated by spores which have survived desiccation, lack of nutrient, and other conditions unsuitable for vegetative cell survival and growth, as shown by the following examples. (i) *C. botulinum* survives food preservation treatments because of the heat resistance of the spores. There are so many variables influencing heat resistance (59) that generalizations are often inaccurate. Since spores of *C. botulinum* are more resistant to radiation than those of other spoilage organisms, they have been extensively used in inoculated pack studies concerned with radiation preservation of foods. Spores surviving thermal or radiation treatments

may then germinate and grow in the treated food, producing the toxin responsible for botulism. (ii) In animals, the major mode of transmission of anthrax is through ingestion of *B. anthracis* spores (in soil or in contaminated feeds), with involvement of abraded pharyngeal or intestinal mucosa. Humans are usually infected through the handling of animals or animal products, and the main routes of infection are by inhalation of spores or by spore contamination of an abrasion or wound. Disease transmission in humans depends largely on the resistance of spores to desiccation on animal carcasses or hides. (iii) Humans and horses are among the species most susceptible to infection with *C. tetani*. There is a high correlation between infection and the incidence of spores in the soil. Spores may germinate aerobically, but vegetative growth and the potent neurotoxin are produced anaerobically in the necrotized tissue. (iv) Although *C. sporogenes* is probably not pathogenic, it often accompanies such pathogens as *C. perfringens* and *C. novyi* in wounds, perhaps playing a synergistic role by increasing invasiveness. Spores are readily produced and survive in the soil to accompany other, more pathogenic, organisms in contamination of wounds or abrasions. (v) *C. novyi* produces a lethal toxin and, in humans, is important as an agent of wound infection. It is a highly fatal wound infection, particularly when accompanied by *C. sporogenes* (59). *C. novyi* and the closely related *C. haemolyticum* are also significant causes of disease in animals (59). (vi) *C. septicum*, a producer of toxins, some of which are enzymatically active, is a cause of gas gangrene, either alone or when accompanied by other members of the genus. Cattle and other domesticated animals are also susceptible to this organism (59). (vii) *C. chauvoei* sporulates readily, produces toxins, and is economically important as the cause of blackleg of cattle and sheep. It is probably transmitted through spores surviving in the soil. (viii) *C. histolyticum* produces several active extracellular toxins, four of which have known enzymatic function. Like *C. perfringens*, it is often transmitted in soil and is a minor cause of gas gangrene. It is one of the few *Clostridium* species for which there are definite data associating lethal toxin production with sporulation (see Toxins, below). (ix) In *C. perfringens* infections, the spore is probably involved mainly in initiation of infection of wounds, leading to gas gangrene. Some *C. perfringens* toxins have been characterized by their enzymatic activity, but data relating to their association with sporulation are not firmly established (1, 2, 59). However, the organism also produces an enterotoxin, synthesis of which has been associated with

sporulation (see Toxins, below). Although *B. cereus* is commonly considered to be a harmless saprophyte, it does produce at least three important extracellular toxins. Certain strains are also associated with food-poisoning incidents, these strains presumably producing an enterotoxin (24). It is uncertain whether growth or sporulation must occur in the intestine to produce a food-poisoning syndrome.

#### APPLICATIONS BASED ON PRODUCTS ASSOCIATED WITH SPORES OR WITH SPORULATION

##### Bacterial Insecticides

Demands for increased production of food and other natural products have been partially met through the use of a wide range of chemical insecticides. These synthetic chemicals, while often quite effective in control of destructive or disease-carrying insects, may pollute the environment, may disturb the natural ecology and predator-prey relationships, may leave a persistent toxic residue on foods, may poison agricultural workers, or may become progressively ineffective as insects develop resistance.

For control of a number of insect species, insecticidal preparations based on microorganisms which cause insect epizootics may be successful alternatives to synthetic chemicals. Although viruses, fungi, and bacteria have been intensively investigated, we will discuss here only the sporeforming bacteria. Sporeforming bacteria are associated with, or kill, a wide variety of insects, including moths, butterflies, beetles, houseflies, and cockroaches. These bacteria are safe and are specific for the target insects. They show no toxicity for humans, mammals, birds, bees, earthworms, or plants. Insects do not appear to develop resistance to these insecticides as they do to chemical insecticides (43).

Much research is going on to improve production and toxicity of the microbes. Of great importance also are parallel studies of the mechanism of action, the nature of the susceptibility and resistance of insect species, and the environmental impact of insect control, including hazards for nontarget insects. Biological insecticides can help to form the basis for control of certain insects, but they are often too specific to suppress the total pest population. The future of microbial insecticides probably lies not in total replacement of chemical agents, but in a rational, integrated program with both types of agents (4). This concept of "integrated control," taking into account the normal mortality, the ecosystem, and the effects of insecticides, is now

recognized as the practical way to deal with insect pest problems.

The following brief discussion of pathogenic bacteria and of bacterial toxins for insects has been derived from several reviews and compendia of papers (4-6, 15, 28, 43, 48, 49, 52; see also Appendix 2).

Bacteria other than sporeformers (*Pseudomonodaceae*, *Enterobacteriaceae*, *Lactobacillaceae*, and *Micrococcaceae*) have been isolated and demonstrated to be pathogenic for various insects. Among the *Bacillaceae*, species of *Clostridium* have only rarely been isolated from diseased insects, perhaps because of the necessity for anaerobic techniques. An exception was the experimental demonstration of the susceptibility of tent caterpillars (*Malacosoma* spp.) to the disease brachyosis induced by spores of *C. brevifaciens* (52). The genus *Bacillus* includes those species which make a reality of bacterial control of insects.

*B. alvei-circulans* and *B. brevis* produce a material toxic to mosquito larvae early in sporulation, and the insecticidal activity declines during sporulation. In contrast, insecticidal activity of *B. sphaericus* (58) first appears during the initial stages of sporulation, but peaks midway through the sporulation cycle and eventually declines. Overall, however, *B. sphaericus* toxicity may be unrelated to spore formation (Appendix 2). However, the commercially useful *Bacillus* species are *B. popilliae* and the closely related *B. lentimorbus*, which cause "milky disease" of the larvae of the Japanese beetle, *Popillia japonica*, and the "crystalliferous" sporeforming pathogens closely related to *B. cereus* and characterized by a large toxic inclusion body which is formed during sporulation ("parasporal body"). Since the *B. cereus* relatives, collectively grouped as *B. thuringiensis*, readily grow and sporulate on artificial media, they have been much more thoroughly studied than the obligate pathogens, *B. popilliae* (also forming a crystal) and *B. lentimorbus*, which have only recently been grown and sporulated on artificial media (4). *B. thuringiensis* produces not only the commercially important crystalline toxic protein (delta-toxin), accounting for as much as 30% of the cellular dry weight, but several others as well, including: the thermostable beta-exotoxin, which is probably not sporulation associated; a thermolabile soluble exotoxin, toxic to larch sawfly larvae, but which, because of its lability, is not commercially useful; and extracellular enzymes, including phospholipase and hyaluronidase, which may attack insect gut wall epithelium (28, 43, 49).

The toxic crystal forms outside the exosporium. Turnover of vegetative cell protein prob-

ably contributes to its synthesis, which may be associated with protease produced early in sporulation (41). To be effective as an insecticide, *B. thuringiensis* preparations (spores plus delta-toxin) must be ingested by the susceptible lepidopterous insect. Susceptible species in other orders (*Hymenoptera*, *Coleoptera*, *Diptera*, and *Orthoptera*) have been reported. The chemistry, activation, and mode of action of the toxin are discussed by Bulla (Appendix 2). Crops which have been protected by use of the spore plus delta-toxin mixture include broccoli, cabbage, cauliflower, celery, lettuce, potato, melon, tobacco, tomato, alfalfa, forest trees, fruit trees, etc.

*B. popilliae* and *B. lentimorbus* are effective in controlling a number of scarabaeid larvae. Pathogenesis in Japanese beetle larvae, initiated by spores of *B. popilliae*, has been discussed by Dutky (15) and by Bulla and his collaborators (4, 5, 52). In vivo, as many as  $2 \times 10^{10}$  *B. popilliae* spores/ml may be produced in 1 to 3 weeks of growth in hemolymph. The spores, which are resistant to desiccation, remain in the soil when the insect dies and become a potent means for perpetuating the disease (Appendix 2). The use of *B. popilliae* spore preparations represents one of the outstandingly successful programs of biological control because they are not environmental pollutants and because of their resistance to adverse environmental conditions, their safety, the lack of development of insect resistance, their specificity, and their relatively low cost.

"Although preparations of *B. thuringiensis* have reached the market without the nature and the mode of action of the toxin being clearly understood, it is probable that in the future a more detailed understanding will be required for development of any new microbial toxins. This will require a multidisciplinary research effort. . . ." (60). Such efforts might well include genetic analysis of the relation between toxin production and sporulation. We agree with Norris (43) that "the search for chemicals which are highly toxic for insects but which have no effect on other forms of life . . . implies the discovery and exploitation of features in the structure, biochemistry, or behavior of insects which differentiate them from other living things. The microorganisms which infect insects are usually highly specific for their hosts—they have found and exploited just those features for which man is looking in his search for new insecticides. When we understand fully the mechanisms by which microorganisms and their toxins kill insects we shall have a much deeper understanding of the differences between insects and other life forms, and the knowledge may . . . open up

entirely new approaches to the development and use of insecticidal chemicals."

### Antibiotics

Near the end of logarithmic growth and early in (or during) sporulation, sporeforming bacteria produce a large variety of antibiotics. Most of these are oligopeptides, often containing unusual amino acids, generally refractory to hydrolysis by proteolytic enzymes, and synthesized by a mechanism which is independent of ribosomes. In addition to the peptide antibiotics, some chemically different antibiotics are also produced by *Bacillus* species, e.g., butirosin, an aminoglycoside produced by *B. circulans*, and proticin, a phosphorus-containing triene from *B. licheniformis* var. *mesentericus*. Katz and Demain (32) listed 45 bacillary antibiotics (Table 1). Most of these are active against gram-positive bacteria; some are active almost exclusively against gram-negative bacteria (polymyxin, colistin, circulin); and some are effective agents against molds and yeasts (bacillomycin, mycobacillin, fungistatin). Some of the peptide antibiotics have medical application (44; Table 2). Some, e.g., subtilin, bacitracin, and tyrothricin, have enjoyed nonmedical use (25, 45) in plant disease control and in food preservation, and others may be or may become useful as research tools because of their spectra (3).

The following discussion deals almost exclusively with the peptide antibiotics. Literature references will be limited principally to review articles on bacillary antibiotics (13, 17, 32, 50) and to those pertinent to the biological role of antibiotics (27, 41, 55; see also Appendix 4).

One of the most intriguing problems relating to antibiotic production concerns the relation of antibiotics to sporulation, a problem which arises because the production of bacillary antibiotics coincides with the early stages of sporulation. A number of factors support the hypothesis that the peptide antibiotics of bacilli function in sporulation (13, 32). Whether or not antibiotic synthesis is an obligatory prelude to spore formation is unknown, but most investigators (13, 32, 50) are convinced that antibiotic synthesis is not a gratuitous event and that antibiotics perform some function necessary to the organism. Demain and co-workers (13, 32; Appendix 4) believe that, while sporulation and antibiotic formation may be regulated by a common mechanism, the antibiotic is not an obligatory part of sporulation. It may function at a later stage in the life cycle of the producer, perhaps by inhibiting competitors after germination or by inhibiting germination until favorable growth conditions arise. Sadoff and his collaborator (50, 67), on the other

TABLE 1. Some antibiotics elaborated by species of the genus *Bacillus*<sup>a</sup>

Species	Antibiotics
<i>B. brevis</i>	Gramicidin S Tyrocidine Linear gramicidin Brevin Edeine Eseine Bresseine Brevistin
<i>B. subtilis</i>	Mycobacillin Subtilin Bacilysin Bacillomycin Fungistatin Bulbiformin Bacillin Subsporin Bacillocin Mycosubtilin Fungocin Iturin Neocidin Eumycin
<i>B. pumilus</i>	Micrococцин P Pumilin Tetain
<i>B. mesentericus</i>	Esperin
<i>B. thiaminolyticus</i>	Octopytin (Thianosine) Baciphelacin
<i>B. licheniformis</i>	Bacitracin Licheniformin Proticin
<i>B. polymyxa</i>	Polymyxin Colistin Gatavalin Jolipeptin
<i>B. circulans</i>	Butirosin Circulin Polypeptin EM-49 Xylostatin
<i>B. laterosporus</i>	Laterosporamine Laterosporin
<i>B. cereus</i>	Biocerin Cerexin Thiocillin

<sup>a</sup> Reproduced, with permission, from reference 32.

hand, believe that the regular production of antibiotics by cells after exponential growth and during sporulation argues for a specific function in sporulation. For example, bacitracin, which may be produced from a constituent of vegeta-

TABLE 2. Bacillary peptide antibiotics with medical application<sup>a</sup>

Antibiotic	Producer	Stage of production	Structure	Target	Biochemical action
Bacitracins (10 known bacitracins) A <sup>b</sup>	<i>B. licheniformis</i> <sup>b</sup> <i>B. subtilis</i> <i>B. cereus</i> (baci- tracin-like compound)	Throughout sporulation	Dodecapeptide, contain- ing a cyclic hexapep- tide and a thiazoline ring structure	Bactericidal: gram-posi- tive bacteria	Inhibits cell wall synthe- sis by interrupting the transport of cell wall components; inhibits protein synthesis; anti- membrane agent
Colistins <sup>b</sup>	<i>B. colistinus</i>		Branched, cyclic deca- peptides linked to a fatty acid residue. Re- lated to polymyxins	Bactericidal: gram- negative bacteria Bacteriostatic: some yeasts and fungi	Anti-spasmodic; more active than polymyxin B vs coli-aerogenes group
Linear gramicidins (A, B, C, and D) <sup>b</sup>	<i>B. brevis</i>	Formation occurs after exponential growth has stopped and prior to the appearance of free spores-engulfment	Linear pentadecapep- tides consisting of hydrophobic amino acids in alternating D and L configurations. Gramicidins A, B, and C are produced by the same strain of <i>B.</i> <i>brevis</i> that produces tyrocidines	Bacteriostatic: gram- positive bacteria	Affect integrity of cell membrane leading to cell lysis or leakage of nuclear components
Polymyxins (9 known polymyxins) B <sup>b</sup> E <sup>b,c</sup>	<i>B. polymyxa</i> <sup>b</sup> <i>B. circulans</i>	Stage V Maximum concn in me- dium at time of spor- angial lysis	Branched cyclic deca- peptides linked to a fatty acid residue	Bactericidal: gram- negative bacteria	Bind to subsurface layers of sensitive organisms and pro- mote leakage of cell constituents; modify or destroy membranes
Tyrocidines Tyrocidines (A-D) <sup>b</sup>	<i>B. brevis</i>	Stages II to III Time course for forma- tion of tyrocidines parallels engulfment process of sporulation	Cyclic decapeptides composed principally of hydrophobic amino acids, Tyrocidines A, B, and C, produced by same strains of <i>B.</i> <i>brevis</i> that produce linear gramicidins, but not gramicidin S	Bactericidal: gram- positive and some gram-negative bac- teria	See gramicidins. Topical application

Gramicidin S <sup>a</sup>	<i>B. brevis</i>	Enzymes for synthesis appear in late logarithmic phase. Synthesis: t <sub>1</sub> (max rate) to t <sub>3</sub> (complete)	Cyclic decapeptide, a dimer of a pentapeptide occurring in tyrocidine A	Bactericidal: gram-positive and some gram-negative bacteria	See gramicidins
Tyrothrycins <sup>b</sup>	<i>B. brevis</i>	Throughout sporulation; See gramicidins and tyrocidines	Complex: 20% gramicidin (linear); 80% tyrocidine	Gram-positive and some gram-negative bacteria Bacteriostatic at low concn; bacteriocidal at high concn Active against some fungi	See gramicidins. Topical application

<sup>a</sup> The following references were used in construction of the table: 3, 17, 26, 32, 44, 50.

<sup>b</sup> Principal component (or producing organism) of commercial product.

<sup>c</sup> Identical to colistin.

tive cells by a specific sporulation-related seryl protease (67), functions (Table 2) by inhibition of cell wall synthesis in sensitive organisms (61). In sporulation, bacitracin may function in inhibition of cell wall synthesis in the cell division preceding spore protoplast formation (stage II) and engulfment (stage III). Linear gramicidins, tyrocidines (including gramicidin S), and polymyxin all affect cell membranes (18) and may function: in the transport of hydrogen, ammonium, and potassium ions; by generation of sites of membrane synthesis in the engulfment process during the stage II-III transition; or by transport of dipicolinic acid from the sporangial cell into the spore.

"A correlation does not establish a causal relationship. . ." (41). In spite of the obviously intimate relationship between antibiotic synthesis and sporulation, there is no proof that they are causally related (32). Resolution of the question of the role of antibiotics in the life of the producing organism remains equivocal. Peptide antibiotics (tyrothricin complex, gramicidin) may regulate the vegetative cell-to-spore transition by selectively terminating the expression of vegetative genes by alterations in RNA polymerase. This regulation of sporulation may involve a complex interaction between groups of antibiotics (53, 54). However, Demain and co-workers (32; Appendix 4) believe that most of the published data are consistent with the hypothesis that sporulation and antibiotic synthesis are independent phenomena, regulated by a common or similar mechanism. They believe that the crucial experiment—isolation of an asporogenous mutant which can be induced to sporulate by the addition of antibiotic—is lacking.

Not all antibiotics necessarily function in sporulation (13), no more than do all enzymes produced during sporulation (e.g., amylase). If an antibiotic does indeed function in sporulation, it might be excreted only in "loosely regulated" strains; those that are "tightly regulated" would appear to produce none (55). With refinement of techniques for the detection of antibiotics, application of Schaeffer's (55) rules will be vital for determining whether synthesis of sporulation-associated products (enzymes, toxins, and antibiotics) is an integral part of the sporulation process.

Although this discussion has been devoted exclusively to *Bacillus* antibiotics, *Streptomyces* species are much more important sources of industrially important antibiotics (44). Ensign (16) proposed that *Streptomyces* antibiotics are formed as a strategy for developing and maintaining the dormant state and extended this hypothesis to suggest a function in the timing of *Streptomyces* spore germination.

TABLE 3. *Bacillus* extracellular enzymes with current or potential application<sup>a</sup>

Enzyme	Application	Producer organism	Comments
Amylases	Hydrolysis of cereal mashes prior to alcoholic fermentation in brewing Syrup production in food industry Desizing of textiles Starch modification for sizing of paper	$\alpha$ -Amylase: <i>B. amylolyticus</i> , <i>B. caldolyticus</i> , <i>B. coagulans</i> , <i>B. licheniformis</i> , <i>B. macerans</i> , <i>B. stearothermophilus</i> , <i>B. subtilis</i> , alkalophilic <i>Bacillus</i> spp. $\beta$ -Amylase: <i>B. cereus</i> , <i>B. megaterium</i> , <i>B. polymyxa</i> , alkalophilic <i>Bacillus</i> spp.	Produced stages I to II $\alpha$ -Amylase not sporulation specific
Proteases	Detergents; spot removal in dry cleaning Mashing and "chill proofing" in brewing; flour treatment lowering protein content, increasing workability, enhancing flavor; processing inedible waste in fish industry for meal, oil solubles; meat tenderizing Modification of penicillin molecule in drug design; in mouthwash, with amylase, for dental plaque removal; debridement of wounds; relief of inflammation, bruises, blood clots; as digestive aid Soaking, dehairing, bating in leather industry Recovery of silver from photographic film	Alkalophilic protease: alkalophilic <i>Bacillus</i> spp. Aminopeptidase: <i>B. licheniformis</i> , <i>B. subtilis</i> Esterase: <i>B. subtilis</i> Metal protease: <i>B. amyloliquefaciens</i> , <i>B. cereus</i> , <i>B. licheniformis</i> , <i>B. megaterium</i> , <i>B. polymyxa</i> , <i>B. subtilis</i> , <i>B. thuringiensis</i> Serine protease (subtilisins): <i>B. amyloliquefaciens</i> , <i>B. licheniformis</i> , <i>B. pumilus</i> , <i>B. subtilis</i> Serine-metal protease: <i>B. licheniformis</i> , <i>B. pumilus</i>	Produced stages 0 to I Metal protease not in main sequence leading to sporulation
Penicillinases	Destruction and identification of penicillin in pharmaceutical industry Treatment of penicillin allergic reactions Modification of penicillin molecule in drug design Production of cheese from milk of cows fed antibiotics	$\beta$ -Lactamases: <i>B. anthracis</i> , <i>B. cereus</i> , <i>B. licheniformis</i> , <i>B. megaterium</i> , <i>B. subtilis</i> Penicillin amidase: <i>B. megaterium</i>	
Pectinases	Retting of hemp and flax	Pectic lyase: <i>B. circulans</i> , <i>B. polymyxa</i> , <i>B. pumilus</i> , <i>B. sphaericus</i> , <i>B. stearothermophilus</i> , <i>B. subtilis</i> , alkalophilic <i>Bacillus</i> spp.	
$\beta$ -Glucanases	Aid in the utilization of unmalted cereals in brewing industry	$\beta$ -1,3-Glucanase: <i>B. circulans</i> , <i>B. polymyxa</i> , <i>B. subtilis</i> , alkalophilic <i>Bacillus</i> spp. $\beta$ -1,6-Glucanase: <i>B. circulans</i>	
Cellulase and xylanase	Transformation of plant waste products into fermentable substrates for single-cell protein, ethanol, etc.; however, some fungi, e.g., <i>Trichoderma reesei</i> , may have greater industrial potential	Cellulase: <i>B. brevis</i> , <i>B. firmus</i> , <i>B. polymyxa</i> , <i>B. pumilus</i> , <i>B. subtilis</i> Xylanase: <i>B. amyloliquefaciens</i> , <i>B. firmus</i> , <i>B. polymyxa</i> , <i>B. subtilis</i>	Some members of genus <i>Clostridium</i> are superior to <i>Bacillus</i> as cellulase producers
Ribonuclease	Production of nucleoside-5'-phosphates for the flavor industry Lowering RNA levels in microbial feedstuffs Large-scale purification of intracellular enzymes	<i>B. amyloliquefaciens</i> , <i>B. cereus</i> , <i>B. pumilus</i> , <i>B. subtilis</i>	Produced stages I to II; involvement in sporulation is questionable

TABLE 3. (Continued)

Enzyme	Application	Producer organism	Comments
Deoxy-ribonuclease	Research tool in biochemistry	<i>B. amyloliquefaciens</i> , <i>B. cereus</i> , <i>B. subtilis</i> , <i>B. pumilus</i>	
Alkaline phosphatase	Production of IMP, GMP, and XMP for flavor enhancement	<i>B. amyloliquefaciens</i> , <i>B. cereus</i> , <i>B. subtilis</i> , alkalophilic <i>Bacillus</i> spp.	Produced stage III

<sup>a</sup> The table is reproduced, in large measure, from those of Erickson (17) and Priest (47), with permission. Other information was from Fogarty et al. (19, 20), Hanson et al. (27), and Mandelstam (41). Some cell-associated *Bacillus* enzymes also have practical potential, e.g., lytic enzymes (cell disruption for industrial and research applications), glucose isomerase (conversion of glucose to fructose for medical and food industries), polynucleotide phosphorylases (synthesis of polynucleotides for medical and research use), restriction endonuclease (gene modification for research), and dextranases (prevention of dental caries, and in juice and sugar industries). There is no clear indication that synthesis of these enzymes is associated with sporulation (except, perhaps, for the lytic enzymes, which are produced at stage VI).

### Enzymes

Microbial enzymes are becoming increasingly important in such diverse fields as foods, medicine, and brewing. The genus *Bacillus* has played an important role in this development.

There is such extensive protein turnover that ca. 75 to 90% of the soluble proteins of *B. subtilis* spores are synthesized during sporulation (33). Indeed, quite a few enzymes first appear or increase significantly during sporulation and either are not detectable or are present at very low levels during exponential growth. Kornberg et al. (33) listed at least 60 enzymes found in the spore. Priest (47) has published an extensive list of extracellular enzymes of the genus *Bacillus*, and he stated that their maximal synthesis normally occurs just before or during sporulation. The time of appearance of many of the bacillary enzymes suggested the possibility of a causal relationship, to which we shall allude in later paragraphs.

Whatever their function in the producing organism, a substantial portion of the *Bacillus* enzymes have found, or have potential for, a wide range (Table 3) of commercial or industrial applications (17, 19, 20, 30, 64; H. Dellweg [ed.], Fifth International Fermentation Symposium Abstracts, Berlin, 1976). Yields of many of these enzymes have been increased to commercially useful levels through the use of sporulation mutants (17).

The enzymes which hydrolyze starch ( $\alpha$ -amylase,  $\beta$ -amylase, and amyloglucosidases) or protein have found the widest application. Different industries require enzyme preparations with different properties (17, 19, 20), and the microbiologist plays an important part in the selection of strains desirable for each application. For example (17), the  $\alpha$ -amylase used in the brewing industry to supplement natural malt

in the mashing process must not detract from the organoleptic qualities of the finished product. Some protease is helpful in this application. In other applications, the presence of protease and glucosidase would be undesirable (17). With present-day emphasis on the abatement of pollution, extracellular enzymes, like cellulase and xylanase, may be economically useful in the transformation of waste products into single-cell protein, chemical feedstocks, fuel, etc.

Industry has achieved high levels of enzyme productivity (particularly of amylases and proteases) because the enzyme-producing *Bacillus* strains have high growth rates and secrete enzymes into the medium and because of the large fund of basic information available on the conditions for growth and sporulation of these species (30). Areas of study required for further exploitation of the *Bacillus* species as protein factories (30) include investigations of genetic control of hyperproductivity, more precise definition of mechanisms and factors controlling enzyme synthesis and secretion (35), and genetic manipulation for practical applications. On the basis of their experience in the production of bacillary enzymes, Ingle and Boyer (30) suggested that membrane changes, transformability, and hyperproductivity may be related, and that catabolite repression is a major controlling factor in  $\alpha$ -amylase biosynthesis in *B. amyloliquefaciens*. They predicted that *B. subtilis* will be the species of choice for producing a wide variety of new and improved fermentation products through strain selection and genetic manipulation. It is expected (17) that, as basic knowledge concerning the mechanisms of control of exocellular protein synthesis and genetic modification expands, rational design of bacillary strains will supplant random screening processes.

Not only are amylases and proteases the enzymes with the widest industrial application, but

they are also the enzymes produced by a great many members of the genus *Bacillus*—at least 32 species of *Bacillus* degrade starch (47). In addition to the starch-degrading enzymes, a variety of other carbohydrases and other extracellular enzymes are also produced by *Bacillus* species (47; Table 3). Alkaline phosphatase from *B. megaterium* and from some strains of *B. subtilis* may be membrane bound rather than extracellular (47). Lytic enzymes produced by a number of species may be sporulation associated, being produced at stage VI of sporulation (27, 41).

The proteases of *Bacillus* species are outstanding in the extent of their application, both industrially and in basic studies. Much of the latter effort has been devoted to assessment of the role of this group of enzymes in sporulation. The bacillary proteases are, in common with most extracellular bacillary enzymes, produced early in sporulation. Several major proteases have been described (11, 42, 46, 47): (i) metal protease, most active near neutrality and inactivated by EDTA; (ii) alkaline serine protease, resembling subtilisin, inactivated by phenylmethylsulfonyl fluoride (PMSF) but unaffected by EDTA; and (iii) an esterase with high esterolytic and low proteolytic activity. About 80% of the proteolytic activity excreted by *B. subtilis* (42) was attributable to the serine protease. The serine proteases hydrolyze simple terminal esters and resemble trypsin and chymotrypsin. They have a molecular weight of 25,000 to 30,000. The metal proteases require  $\text{Ca}^{2+}$  for stability and  $\text{Zn}^{2+}$  for activity and are produced by several species, some of which (e.g., *B. megaterium*) secrete only this one protease. Many investigators (11, 12, 21, 27, 33, 41, 47, 55), struck by the temporal relationship between appearance of enzymes and initiation of sporulation and by the importance of proteases in other differentiating systems (41), have attacked the question of a cause-effect relation. Freese (21) pointed out that, since the change in culture conditions near the end of exponential growth can depress the synthesis of many enzymes, it is futile to argue whether an enzyme is sporulation specific (27). He suggested that the issue is whether the enzyme is necessary, useful, or of no value for sporulation or for subsequent germination (22). In many instances, the use of mutants has clarified this situation; e.g.,  $\alpha$ -amylase production in *B. subtilis* has been dissociated from sporulation in this way (47). The great danger in studies of this kind, using asporogenous mutants lacking enzyme-synthesizing ability, is misinterpretation of pleiotropic mutations as suggesting necessity for an enzyme in sporulation (55).

In an attempt to resolve these difficulties, Mandelstam and collaborators (12, 41) proposed

three main categories of biochemical events associated with sporulation: "(i) the primary sequence of dependent events specifically concerned in spore formation; (ii) a class of side effects triggered by some events in (i), but not themselves part of the primary sequence; and (iii) changes in vegetative function that occur because of changes in cultural conditions" (e.g., release from catabolic repression). The PMSF inhibition and thymidine starvation studies of Dancer and Mandelstam (11, 12) suggested that serine protease is part of the main sequence of events leading to spore formation (category i, above). The metallo-protease was categorized as a side effect (ii), ribonuclease could be either in the main sequence or a side effect associated with it (i or ii), and amylase was neither directly nor indirectly connected with the sporulation sequence. Most studies have suggested that, of the major proteases, the serine protease is likely to be functional in sporulation, i.e., to be required for the initiation and subsequent stages of sporulation (41, 46), although the precise function of serine protease is open to question. Serine protease functions, such as modification of vegetative RNA polymerases (37) and the conversion of vegetative cell aldolase to the spore form of this enzyme (51), have been postulated. An intracellular serine protease, immunologically distinct from extracellular protease (63), has also been investigated, and it has been demonstrated that a mutant, temperature sensitive for the cytoplasmic serine protease, is asporogenous and deficient in protein turnover at the nonpermissive temperature.

Proteases, synthesized and accumulated during sporulation, may become functional at a later developmental stage. For example, Setlow (57) has described a protease, inactivated by both PMSF and EDTA, which functions dramatically and specifically in the early stages of germination.

As with antibiotics, pleiotropic mutations involving protease synthesis and sporulation may be quite common. Cross-feeding experiments have, thus far, been unsuccessful, and no causal relationship can be implied by the existence of asporogenous mutants, defective in synthesis of a particular enzyme (47). To us, however, the sheer multitude of papers dealing with enzymes and sporulation (which we have not attempted to cite individually) suggests that resolution of some enzyme-sporulation relationships may not be too distant.

#### Toxins

It has been reported that the lethal toxins produced by several species of *Clostridium*, including *C. botulinum*, *C. histolyticum*, *C. tetani*,

etc., first appear when the phase of active growth is over (55), but of these lethal toxins only that of *C. histolyticum* (56) has been directly related to spore formation. In that species, asporogenic strains blocked at stage 0 did not produce lethal toxin, whereas those blocked at stage II or later did produce toxin. The ability of *C. perfringens* type A to produce an enterotoxin has been directly related to the ability of the organism to sporulate (14). The organism and its food-poisoning ability have been described (10, 69). Foods, usually various types of meats, perhaps fecally contaminated with *C. perfringens*, are cooked, but not thoroughly enough to inactivate all spores. These heat-activated spores germinate readily and grow in the meat rendered anaerobic by having had dissolved oxygen driven off. The "culture" is then ingested, multiplies, and sporulates in the gut, synthesizing the enterotoxin, which is liberated during sporangial lysis. Enterotoxin was produced only in a sporulation medium and not in a growth medium (14). Studies with mutants have led to the conclusion that *C. perfringens* enterotoxin protein is a sporulation-specific gene product, perhaps related to spore coat protein (14; Appendix 3). No function in sporulation has, as yet, been attributed to the enterotoxin. Although a direct relationship exists between sporulation and enterotoxin synthesis (14), complete sporulation is not necessary for enterotoxin synthesis because the toxin is synthesized during the early stage of sporulation; asporogenic mutants blocked at stage III or later may still be enterotoxigenic (14).

#### APPLICATIONS OF FUNGAL SPORES

In *Spores VI*, Weber and Hess (70) summarized the status of the physiology, biochemistry, and structure of fungal spores (see also 39, 62, 71). The objectives of the present comments will be to point out the significance of fungal spores to life and to indicate the importance of applying knowledge of fungus spores to the welfare of mankind.

Lamanna et al. (34), after speculating at some length, concluded that the only clear function of the bacterial spore is to tide the organism over an unfavorable environmental situation. The necessity for such a survival mechanism must be rather limited since relatively few bacterial species sporulate. Fungal spores, on the other hand, have clear and essential functions in heredity, survival, and distribution. Practically all fungi reproduce by means of spores, sporulation constituting one or more sexual and/or asexual links in the life cycle. Vegetative forms of fungi (mycelium) have low survival values; spores are more resistant to environmental

stresses. Many fungal spores are well adapted for aerial distribution of the species. Further, the relative stability of such morphological characteristics as size, shape, ornamentation, and number of cells has made spores invaluable (to the mycologist, if not to the fungus) for taxonomic use.

Fungi, and consequently fungus spores, are important to mankind (36). As pathogens, they have a major destructive impact in agriculture and a lesser role in medicine. On the positive side, fungi are used in fermentations for the production of foods, enzymes, antibiotics, chemicals, and pharmaceuticals. In a less obvious, but more important, way, fungi are essential for the decomposition of vegetative material (7) in the biological cycling of carbon, nitrogen, etc. Without such cycles, these elements would accumulate in the soil, and life, as we know it, could not have developed.

The primary method of dispersal of fungus diseases of plants and animals is by spores (31). Fungus spores can be carried thousands of miles by air currents. The continental spread of wheat rust, for example, is effected by air dispersal of spores. The high losses in agricultural crops—fruits, vegetables, ornamental plants, cereal grains—due to fungal disease plus the costs of control represent a significant portion of the value of crops. Efforts to control plant diseases are directed mainly against spores of the pathogen by spraying with an appropriate fungicide to kill the spores before or after germination.

Although fungal infections of animals, including humans, do not approximate the frequency or significance of plant diseases, most are also acquired by exposure to spores of the pathogen. Allergic responses can also result from inhalation of spores.

Pathogenic fungi may also be utilized in a beneficial application, e.g., in the control of certain weeds and of destructive insects. In such cases, spores of the pathogen are used as inoculum. Further, the spores of pathogens of certain crops, particularly cereals and grains, have potential application as agents in biological warfare.

An interesting development in the ability to forecast epidemics of early blight of potato and tomato (caused by *Alternaria solani*) has been described (68). Environmental factors such as humidity, wind speed, sunlight, and temperature, coupled with knowledge of the effects of these factors on various phases of sporulation, dissemination, germination, etc., are written into a computer program. Success of the method in predicting epidemics would permit more timely, effective, and economical control.

Fungi are most useful because of the chemical transformations they can effect. Spores are normally the source of inoculum in such in-

stances, e.g., cheese manufacture, oriental fermented foods (29), fermentations producing antibiotics, vitamins, organic acids, and enzymes, and in steroid transformations. Additionally, in the development of improved strains for industrial usage, mutants with desired characteristics are produced by exposure of spores to mutagenic agents.

An interesting, potentially useful attribute of certain fungal spores arises from the presence of active enzymes on the external surface of the spore, from which they cannot be easily washed off or eluted (a natural immobilized enzyme system). The surface-located enzymes include carbohydrases, ascorbic acid oxidase, and sulfhydryl oxidase (38, 40). Fungus spores have been used to carry out transformations of steroids, fatty acids, triglycerides, and penicillin (66). In contrast to the situation with bacterial spores, which do not appear to be endowed with externally located active enzymes, these transformations occur in the absence of germination or vegetative growth.

In another vein, investigations with *Neurospora* and *Saccharomyces* have made fundamental contributions to modern genetics and to our knowledge of basic life processes. Spores have played a key role in these studies.

### EPILOGUE

The joining of the paths of basic and applied scientists (at best, a vague distinction) has been characteristic of spore science since its earliest days (23). The two major reasons for the continuing importance of bacterial spores as objects of scientific investigation are (i) their usefulness as models for mechanisms regulating cellular differentiation and (ii) the practical consequences of their resistance to heat and other deleterious agents in food preservation and disease control (23). Gerhardt (23) noted that, at the Sixth International Spore Conference, reports on resistance and dormancy and on applied sporology were underrepresented. The Seventh Conference has remedied these deficiencies to some extent. As the two quotations with which we began this essay imply, application and basic studies are inevitably and inextricably intertwined. We hope that, at the least, both of these aspects will continue to be represented at future Spore Conferences.

### LITERATURE CITED

1. Baird-Parker, A. C. 1969. In G. W. Gould and A. Hurst (ed.), *The bacterial spore*, p. 517-548. Academic Press, London.
2. Block, S. S. (ed.). 1976. *Disinfection, sterilization, and*

- preservation, p. 481-638. Lea and Febiger, Philadelphia.
3. Bodanszky, M., and D. Perlman. 1964. *Nature (London)* 204:840-844.
4. Bulla, L. A., Jr. (ed.). 1973. Regulation of insect populations by microorganisms. *Ann. N.Y. Acad. Sci.* 217: 1-243.
5. Bulla, L. A., Jr., R. A. Rhodes, and G. St. Julian. 1975. *Annu. Rev. Microbiol.* 29:163-190.
6. Burges, H. D., and N. W. Hussey (ed.). 1971. *Microbial control of insects and mites*. Academic Press, London.
7. Burges, N. A., and F. Raw. 1967. *Soil biology*. Academic Press Inc., New York.
8. Busta, F. F. 1976. *J. Milk Food Technol.* 39:138-145.
9. Chowdhury, M. S. U., D. B. Rowley, A. Anellis, and H. S. Levinson. 1976. *Appl. Environ. Microbiol.* 32:172-178.
10. Collee, J. G. 1974. In F. A. Skinner and J. G. Carr (ed.), *The normal microbial flora of man*, p. 205-219. Academic Press Inc., New York.
11. Dancer, B. N., and J. Mandelstam. 1975. *J. Bacteriol.* 121: 406-410.
12. Dancer, B. N., and J. Mandelstam. 1975. *J. Bacteriol.* 121:411-415.
13. Demain, A. L. 1974. *Ann. N.Y. Acad. Sci.* 235:601-612.
14. Duncan, C. L., D. H. Strong, and M. Sebold. 1972. *J. Bacteriol.* 110:378-391.
15. Dutky, S. R. 1963. In E. A. Steinhaus (ed.), *Insect pathology*, vol. 2, p. 75-114. Academic Press Inc., New York.
16. Ensign, J. C. 1976. In D. Schlessinger (ed.), *Microbiology—1976*, p. 531-533. American Society for Microbiology, Washington, D.C.
17. Erickson, R. J. 1976. In D. Schlessinger (ed.), *Microbiology—1976*, p. 406-419. American Society for Microbiology, Washington, D.C.
18. Feingold, D. S., C. C. HsuChen, and I. J. Sud. 1974. *Ann. N.Y. Acad. Sci.* 235:480-492.
19. Fogarty, W. M., P. J. Griffin, and A. M. Joyce. 1974. *Process Biochem.* 9:11-18, 24.
20. Fogarty, W. M., P. J. Griffin, and A. M. Joyce. 1974. *Process Biochem.* 9:27-35.
21. Freese, E. 1972. *Curr. Top. Dev. Biol.* 7:85-124.
22. Freese, E., and Y. Fujita. 1976. In D. Schlessinger (ed.), *Microbiology—1976*, p. 164-184. American Society for Microbiology, Washington, D.C.
23. Gerhardt, P. 1975. In P. Gerhardt, R. N. Costilow, and H. L. Sadoff (ed.), *Spores VI*, p. v-viii. American Society for Microbiology, Washington, D.C.
24. Goepfert, J. M., W. M. Spira, and H. U. Kim. 1972. *J. Milk Food Technol.* 35:213-227.
25. Goldberg, H. S. 1959. *Antibiotics, their chemistry and non-medical uses*. D. van Nostrand, New York.
26. Gottlieb, D., and P. D. Shaw (ed.). 1967. *Antibiotics*, vol. 1. Springer-Verlag, New York.
27. Hanson, R. S., J. A. Peterson, and A. A. Yousten. 1970. *Annu. Rev. Microbiol.* 24:53-90.
28. Heimpel, A. M. 1967. *Annu. Rev. Entomol.* 12:287-322.
29. Hesseltine, C. W., E. W. Swain, and H. L. Wang. 1976. *Dev. Ind. Microbiol.* 17:101-115.
30. Ingle, M. B., and E. W. Boyer. 1976. In D. Schlessinger (ed.), *Microbiology—1976*, p. 420-426. American Society for Microbiology, Washington, D.C.
31. Ingold, C. T. 1953. *Dispersal in fungi*. Oxford University Press, London.
32. Katz, E., and A. L. Demain. 1977. *Bacteriol. Rev.* 41: 449-474.
33. Kornberg, A., J. A. Spudich, D. L. Nelson, and M. P. Deutscher. 1968. *Annu. Rev. Biochem.* 37:51-78.
34. Lamanna, C., M. F. Maffette, and L. N. Zimmerman. 1973. *Basic bacteriology*. The Williams & Wilkins Co., Baltimore.
35. Lampen, J. O. 1976. In D. Schlessinger (ed.), *Microbiology—1976*, p. 540-542. American Society for Microbiology, Washington, D.C.

## APPENDIX

36. Large, E. C. 1940. The advance of the fungi. H. Holt and Co., New York.
37. Leighton, T. J., P. K. Freese, R. H. Doi, R. A. J. Warren, and R. A. Kelln. 1972. In H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V, p. 238-246. American Society for Microbiology, Washington, D.C.
38. Loewenberg, J., and E. T. Reese. 1957. Can. J. Microbiol. 3:643-650.
39. Madelin, M. F. (ed.). 1966. The fungus spore. Proceedings of the Colston Research Society, vol. 18. Butterworths, London.
40. Mandels, G. R., R. Vitols, and F. W. Parrish. 1965. J. Bacteriol. 90:1589-1598.
41. Mandelstam, J. 1969. Symp. Soc. Gen. Microbiol. 19:377-402.
42. Millet, J. 1970. J. Appl. Bacteriol. 33:207-219.
43. Norris, J. R. 1969. In G. W. Gould and A. Hurst (ed.), The bacterial spore, p. 485-516. Academic Press, London.
44. Perlman, D. 1973. ASM News 39:648-655.
45. Prescott, S. C., and C. G. Dunn. 1959. Industrial microbiology. McGraw-Hill Book Co., Inc., New York.
46. Prestidge, L., V. Gage, and J. Spizizen. 1971. J. Bacteriol. 107:815-823.
47. Priest, F. G. 1977. Bacteriol. Rev. 41:711-753.
48. Rogoff, M. H. 1966. Adv. Appl. Microbiol. 8:291-313.
49. Rogoff, M. H., and A. A. Yousten. 1969. Annu. Rev. Microbiol. 23:357-386.
50. Sadoff, H. L. 1972. In H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V, p. 157-166. American Society for Microbiology, Washington, D.C.
51. Sadoff, H. L., E. Celikkol, and H. L. Engelbrecht. 1970. Proc. Natl. Acad. Sci. U.S.A. 66:844-849.
52. St. Julian, G., L. A. Bulla, Jr., E. S. Sharpe, and G. L. Adams. 1973. Ann. N.Y. Acad. Sci. 217:65-76.
53. Sarkar, N., D. Langley, and H. Paulus. 1977. Proc. Natl. Acad. Sci. U.S.A. 74:1478-1482.
54. Sarkar, N., and H. Paulus. 1972. Nature (London) New Biol. 239:228-230.
55. Schaeffer, P. 1969. Bacteriol. Rev. 33:48-71.
56. Sebal, M., and P. Schaeffer. 1965. C. R. Acad. Sci. 260:5398-5400.
57. Setlow, P. 1976. J. Biol. Chem. 251:7853-7862.
58. Singer, S. 1974. Dev. Ind. Microbiol. 15:187-194.
59. Smith, L. D.S., and L. V. Holdeman. 1968. The pathogenic anaerobic bacteria. Charles C Thomas, Publisher, Springfield, Ill.
60. Somerville, H. J. 1973. Ann. N.Y. Acad. Sci. 217:93-108.
61. Storm, D. R. 1974. Ann. N.Y. Acad. Sci. 235:387-398.
62. Sussman, A. S., and H. O. Halvorson. 1966. Spores, their dormancy and germination. Harper and Row, New York.
63. Szulmajster, J., and E. Keryer. 1975. In P. Gerhardt, R. N. Costilow, and H. L. Sadoff (ed.), Spores VI, p. 271-278. American Society for Microbiology, Washington, D.C.
64. Terui, G. (ed.). 1972. Fermentation technology today. Society of Fermentation Technology, Osaka, Japan.
65. Vas, K., and G. Prosz. 1957. J. Appl. Bacteriol. 20:431-441.
66. Vézina, C., S. N. Sehgal, and K. Singh. 1968. Adv. Appl. Microbiol. 10:221-268.
67. Vitković, L., and H. L. Sadoff. 1977. J. Bacteriol. 131:897-905.
68. Waggoner, P. E., and V. G. Horsfall. 1969. Conn. Agric. Exp. Stn., New Haven, Bull. 698.
69. Walker, H. W. 1975. Crit. Rev. Food Sci. Nutr. 7:71-104.
70. Weber, D. J., and W. M. Hess. 1975. In P. Gerhardt, R. N. Costilow, and H. L. Sadoff (ed.), Spores VI, p. 97-111. American Society for Microbiology, Washington, D.C.
71. Weber, D. J., and W. M. Hess (ed.). 1976. The fungal spore—form and function. Wiley-Interscience, New York.

### I. Aspects of Inactivation and Injury of Spores at Ultrahigh Temperatures by D. M. Adams (Department of Food Science, North Carolina State University, Raleigh, North Carolina 27607)

Heating foods at ultrahigh temperatures (UHT) takes advantage of differences in the temperature coefficients of reactions leading to bacterial spore death and product damage. Processes of 121 to 149°C for 0.5 to 60 s (depending on the product) should achieve the desired spore inactivation but with reduced loss of product quality. However, the behavior of spores at UHT may present serious problems. The influence of temperature on inactivation rates may be less at UHT than at lower temperatures. Spores of *Bacillus subtilis*, *B. stearothermophilus*, and putrefactive anaerobe 3679 are much more heat resistant above 118 to 132°C than would be expected on the basis of heat treatments at lower temperatures. Although difficult, it is essential that the heat resistance of spores at UHT be determined directly rather than estimated by extrapolation of heat resistance data obtained for lower temperatures, which could result in underestimates of spore heat resistance.

Also, bacterial spore injury can be much more extensive at UHT than at sub-UHT and can lead to serious overestimates of process effectiveness. One manifestation of injury is the requirement for non-nutritive germination agents by surviving spores. UHT-treated *B. subtilis* spores required calcium dipicolinate for maximal recovery of survivors; *Clostridium perfringens* and *C. botulinum* spores required lysozyme for colony formation. Secondly, after severe heat treatment, survivors may be recovered best at temperatures below the optimum for unheated or mildly heated spores, or in a narrower range of temperatures. This has been reported for spores of *B. stearothermophilus*, *B. subtilis*, *C. botulinum*, *C. sporogenes*, and *C. perfringens*, and both germination and outgrowth systems have been implicated. A third type of spore injury involves the increased sensitivity of survivors to inhibitors. This has been reported for *B. subtilis*, *B. stearothermophilus*, *C. botulinum*, *C. perfringens*, putrefactive anaerobe 3679, and others. The classical studies of Olsen, Scott, and Murrell revealed a heat-induced requirement for starch in enumeration media to achieve maximal recovery of survivors. Heat-injured *C. perfringens* spores are increasingly sensitive to surface-active agents, suggesting membrane damage, and heat-induced membrane alterations in *C. botulinum* spores have been observed by electron microscopy. The fourth type of spore injury is a heat-induced requirement for blood, starch, yeast or liver extracts, certain sugars, amino acids, or vitamins. These requirements probably reflect the need to adsorb inhibitors, stimulate secondary germination systems after inactivation of the primary system, or fulfill the requirements of heat-induced mutations to auxotrophy, all of which have been reported for bacterial spores.

UHT technology involves new processes and frequently new products or new formulations. These must be developed with adequate testing to avoid poten-

tially serious economic or public health problems resulting from the survival of spores more heat resistant at UHT than expected or from the activation of germination or outgrowth systems capable of repairing or bypassing heat injury damage.

**2. Toxins of Entomocidal Sporeforming Bacilli** by Lee A. Bulla, Jr. (U.S. Grain Marketing Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Manhattan, Kansas 66502)

A number of bacteria are pathogenic to insects, but relatively few have been successfully used as bacterial insecticides. The foremost candidates are *Bacillus thuringiensis*, *B. popilliae*, *B. lentimorbus*, and *B. sphaericus*. The first two are "crystalliferous," and the last two are acrySTALLIFEROUS. *B. popilliae* and *B. lentimorbus* are obligate pathogens, whereas *B. thuringiensis* and *B. sphaericus* are not true pathogens but facilitate various disease symptoms as a result of elaboration of toxic metabolites and end products.

*B. thuringiensis* is one of the best-known crystalliferous bacteria. The parasporal crystal formed during sporulation is toxic to lepidopteran insects. The intracellular parasporal crystal is a protoxin that is formed outside the exosporium during stages III to V of sporulation. It contains a single glycoprotein subunit with a molecular weight of ca.  $1.2 \times 10^5$ . The carbohydrate consists of glucose (3.8%) and mannose (1.8%). At alkaline pH, characteristic of most lepidopteran insect guts, the protoxin is apparently solubilized and activated by an autolytic mechanism involving an inherent sulfhydryl protease that renders the protoxin insecticidal. In vivo activation of the protoxin and proteolytic activity of the toxin could cause any one of the pathological disorders that have been ascribed to the crystal: (i) separation of gut cells and detachment from the basement membrane, (ii) enhancement of secretory activity of gut epithelial cells, (iii) increase in permeability of the gut wall to sodium ions with a slower rate of glucose uptake into the hemolymph, (iv) elevated level of potassium ion concentration in the hemolymph, and (v) gut paralysis and sometimes general paralysis of the body.

*B. popilliae* is a pathogen of various scarabaeid beetles. The bacterium, when ingested by beetle larvae, invades the hemocoel, where it grows and sporulates, causing death of the larvae. The spores that accumulate are ultimately released to the surrounding soil, and consequently the pathogen can survive for an extended period. These spores are eaten by newly hatched beetle larvae and, upon germination and outgrowth in the alimentary tract, begin the infectious process again. The name given to this infection is "milky disease" because of the milky appearance of the hemolymph containing spores.

*B. popilliae* and *B. lentimorbus*, a closely related milky disease organism, represent a unique category among bacteria because they are gram variable, facultatively anaerobic, and catalase negative, in addition to being insecticidal. Whether the parasporal crystal of *B. popilliae* is toxic to larvae is not known. The predominantly protein crystal can be separated into three cathodic components by high-voltage electrophoresis. It contains no lipid or nucleic acids but does contain carbohydrate.

*B. sphaericus* is a gram-variable facultative anaerobe that is ubiquitous to terrestrial and aquatic habitats. Many strains exhibit extreme specificity to a number of medically important mosquitoes, are inert to nontarget vertebrates and invertebrates, and can be cultured and produced commercially at prices competitive with those of chemical insecticides. The toxicity of *B. sphaericus* apparently is not associated with sporulation since vegetative cells kill mosquitoes quite effectively. Also, nonsporulating cells and oligosporogenic mutants are as toxic as the parental strain despite little or no sporulation.

**3. Relationship Between *Clostridium perfringens* Enterotoxin and Sporulation** by Ronald G. Labbe (Department of Food Science and Nutrition, University of Massachusetts, Amherst, Massachusetts 01002)

The production of *Clostridium perfringens* enterotoxin is a sporulation-specific event. Toxin, measured by overt diarrhea, by fluid accumulation in the ligated rabbit ileum following injection of cells, or by erythema in guinea pig or rabbit skin, was produced only when cells were grown in a sporulation medium (Duncan and Strong medium). In this medium, maximum numbers of heat-resistant spores formed by 7 h. Intracellular accumulation of enterotoxin followed shortly afterward, reaching a maximum between 10 and 12 h. Coincident with a decrease in toxin in cell extracts was the appearance of free spores. Detection of enterotoxin in culture supernatant fluid accompanied sporangial lysis. Mutants of NCTC 8798 blocked at various stages of sporulation were obtained either as spontaneous mutants or by treatment with acridine orange or nitrosoguanidine. All  $sp^-$  mutants that were blocked at stage 0 lost the ability to produce enterotoxin. Mutants blocked at stage III or beyond retained the ability to produce enterotoxin. Revertants isolated from an  $sp^- ent^-$  mutant regained not only the ability to sporulate but also the ability to produce enterotoxin. Spores of enterotoxin-positive and enterotoxin-negative strains were extracted with urea-mercaptoethanol or dithiothreitol, and the immunological homology between extracted spore protein fractions and enterotoxin protein was compared by means of double diffusion in agar gels. Protein extracted from all strains was precipitated by anti-enterotoxin serum, suggesting that spore protein and enterotoxin were structurally similar. Protein extracted from spores by urea-mercaptoethanol or dithiothreitol produced erythema in the guinea pig skin test. This activity was neutralized by anti-enterotoxin serum. In addition, antisera produced against urea-mercaptoethanol-solubilized spore protein neutralized the erythematous activity of purified enterotoxin. A relatively minor amount of enterotoxin was associated with the core fraction. A stable messenger RNA which coded for production of enterotoxin also coded for a protein species which corresponded to spore coat protein. These results suggest that enterotoxin protein of *C. perfringens* is sporulation specific and apparently is a component of spore coat protein.

**4. Speculations on the Role(s) of Antibiotics in Spore-forming Microorganisms** by A. L. Demain and J. M.

Piret (Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139).

A large body of circumstantial evidence suggests that the antibiotics made by sporeformers may be necessary for the differentiation (sporulation) of those organisms. The necessity of antibiotics in sporulation has been analyzed genetically, but, unfortunately, most of the mutants have been extremely pleiotropic. As suggested by Schmitt and Freese, the crucial experiment would be to isolate an asporogenous mutant that could sporulate *only* upon addition of pure antibiotic. This experiment has not yet been reported. The most damaging evidence to the hypothesis involving antibiotics in sporulation is the existence of mutants which are antibiotic negative yet can still sporulate. Kambe et al. isolated gramicidin S-negative mutants of *Bacillus brevis*, and we have found the mutants to sporulate well. Also, Haavik and Thomassen's mutant of *B. licheniformis* with a defective bacitracin synthetase complex sporulates normally, as do mutants of *Penicillium urticae* unable to make patulin (Sekiguchi and Gaucher).

However, Sarkar and Paulus noted a specific inhibition of RNA synthesis by the tyrothricin complex during growth of *B. brevis* ATCC 8185, and purified *B. brevis* RNA polymerase was inhibited by the complex. Both components of the tyrothricin complex (linear gramicidin and tyrocidine) inhibited transcription to a similar degree, suggesting that peptide antibiotics may regulate transcription. Kleinkauf and co-workers found opposing effects of the two peptides and argued that both peptides are produced to

ensure that not all vegetative genes are turned off during sporulation. However, linear gramicidin reversed the effect of tyrocidine on growth and RNA synthesis, but sporulation was fully inhibited. Thus, a causal relationship between the inhibition of RNA synthesis and sporulation remains in question.

Mukherjee and Paulus found mutants of *B. brevis* ATCC 8185 that made normal levels of tyrocidine and spores but did not produce linear gramicidin. The spores, however, were less heat resistant and contained 20% of the normal level of dipicolinic acid. Revertants regained heat resistance, dipicolinic acid content, and the ability to make linear gramicidin. Of particular note was the fact that addition of linear gramicidin to the mutant culture at the end of log phase restored dipicolinic acid content, heat resistance, and serine protease activity. Whether linear gramicidin acts by turning off transcription of vegetative genes which are nonessential for sporulation remains to be proven.

The close connection between sporulation and antibiotic production could be a result of independent phenomena controlled by a common regulatory mechanism. Certain antibiotics in bacilli and actinomycetes are found in spores, indicating a possible function in the survival of the dormant or germinating spores. One possibility is that the antibiotic is packaged in the spore and functions to inhibit or activate germination. Alternatively, the antibiotic may function to protect dormant spores from bacteria-consuming amoebae. Finally, antibiotics may function by eliminating competitors in the environment during germination of the spore. (Our work on gramicidin S was supported by National Science Foundation grant GI-34284.)