The Bacterial Spore

Volume 2

Edited by

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Preface

It is now some fourteen years since the first volume of "The Bacterial Spore" was published. The editors first considered revising and bringing up-to-date this volume. However, much of the material has withstood the test of time and clearly required little or no revision. Consequently, we decided to produce a completely new book on the same subject, retaining the old title, but aiming to cover the recent advances that have been made, rather than recapitulating and reporting data already dealt with in the previous volume. In Volume 2 of "The Bacterial Spore" we have therefore included some chapters which provide new insights on topics previously dealt with. They are on spore germination and outgrowth, mechanisms of resistance and dormancy and the pathogenic spore-forming bacteria. We also have completely new topics. One of these is a comprehensive and scholarly account of the history of spore research, another concerns the role of spores in nature, and another, the injury of spores by sublethal treatments. We hope that the description and taxonomy of endospore-forming bacteria will be useful practically. This deals not only with the genera *Bacillus* and *Clostridium*, but also with the less studied endospore-forming genera. The chapter on genetic and metabolic control of sporulation reflects the great advances which have recently taken place in this field; and illustrates the wealth of information which can be gathered by using the elegant techniques of molecular biology.

Modern molecular biology has emerged from the study of several systems, mainly microbiological, which have been accepted as models for general biological phenomena. Initially, molecular biologists were a relatively homogeneous group, but now as the fruits of their fundamental studies are approaching application, this group has become more heterogeneous. In contrast, sporologists have been a diverse group from the start. They include molecular biologists and geneticists interested in cellular differentiation, control mechanisms, dormancy and its significance in nature. Other sporologists come from the food industry, interested in reducing the severity of heat treatments so that foods may be more safely preserved close to their natural state in appearance, texture, flavour and nutritive properties. Other sporologists are interested in obtaining enzymes, solvents and antibiotics and other products of interest or of value within the fermentation industry. There is a small but devoted group interested in using insect pathogenic organisms for biological control, motivated by the belief that biological control is specific and non-polluting and thus preferable to chemical insecticides. We hope that this diverse group of scientists, united only by their interest in spore biology, will benefit from Volume 2 of "The Bacterial Spore".

The research structures and institutions concerned with spore research have remained essentially unchanged in the fourteen years between the first volume in 1969 and the publication of the present volume. In the USA, International Spore Conferences continue to be held every two or so years. The proceedings, published, by the American Society for Microbiology, have now reached "Spores" Volume VIII. During the years in between the British Spore Group has met regularly and most recently has held meetings sponsored by the relatively new Federation of European Microbiological Societies, along with the UK Society for Applied Bacteriology. The *Spore Newsletter* continues to be produced in Australia by its devoted editors W. G. Murrell and J. A. Lindsay, and abstracts usually appear a few months before papers are published.

Although activity in spore research has at least doubled since 1969, recently it appears to have reached a plateau. This is partly because of increased costs and world-wide recession and partly because eukaryotic research on differentiation and control mechanisms has progressed so far that some biologists feel that the value of bacterial model systems has been superseded. However, it would be tragic if, at this point, we could not press home our advantage. We are not far from gaining a full understanding of several facets of sporology such as the basic mechanism of heat resistance and dormancy, regulation of sporulation and the processes involved in spore outgrowth. As with bacterial genetics, fundamental knowledge may soon lead to practical application. Will Volume 3, to be published a decade hence, contain all this information? Sporologists will then have discharged their obligation to the communities that have supported them. Meanwhile, this book is offered to present day sporologists, researchers and teachers in this area. We hope that the broad picture which emerges and the information it contains will cement existing knowledge and stimulate further research activity.

June 1983

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CHAPTER 1

Spore Research in Historical Perspective

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THE BACTERIAL SPORE, VOLUME 2
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I. The Discovery of the Bacterial Spore

Today, over a century after the discovery of the bacterial endospore by Ferdinand Cohn in 1876 and, independently, by Robert Koch shortly afterwards in the same year, it is worth recalling the circumstances in which that momentous discovery occurred. Those were the heroic days of classic bacteriology. Pasteur had made microbes famous. The possible role of bacteria in disease had been suggested and was discussed widely. It so happened that the same studies which led to the discovery of spores also led to the final proof that an infectious disease was caused by micro-organisms.

Not only were the two co-discoverers of the bacterial spore, Cohn and Koch, very different in background and personality, but the motivations behind the scientific work of each man were quite dissimilar. Cohn conducted his experiments in an attempt to understand the reasons for conflicting observations on heat sterilization of organic material. Such observations remained from the debates on spontaneous generation of micro-organisms. Thus, while Cohn engaged in fundamental research, Koch sought to understand the aetiology of a specific infectious disease, anthrax, which caused both human suffering and great economic loss. From its very beginnings, up to and including the present, the investigation of bacterial spores has always had this duality of aspect, a mixture of basic and applied research.

In his classic paper published in 1876, Cohn addressed himself to those experiments in which it was shown that hay infusions could not be sterilized by boiling. He wondered "why is it that 100°C is not sufficient to kill bacteria when much lower temperatures are enough to kill most living organisms?" In order to resolve this paradox, he first repeated the earlier experiments which had demonstrated that hay could not be sterilized by boiling. He then compared the micro-organisms that grew in the heated hay infusions with those that grew in unheated hay infusions, observing them under the microscope to determine "whether the nature of their unbelievable resistance to boiling water could reside in some characteristic specific (morphological) properties".

Remarking on the organisms that survived heating versus those that developed in the unheated controls, he stated "even the first glance indicated that a completely different sequence of developments occurred in the boiled hay infusion than in the unboiled one". In the former he described "swarms of countless fine, straight, active, motile rods" which "all belong to a single species – *Bacillus subtilis*".

Between October 1875 and July 1876, Cohn studied the life history of these rods in detail, and noticed that the sequence was indeed different from that of other bacteria. He reported that in the "homogeneous content" of the filaments formed by this bacteria, "strongly refracting bodies appear. Each of these bodies develops into an oblong or short, cylindrical, strongly refracting, dark-rimmed spore. When these spores have completely separated from their mother cell, they show a delicate, jelly-like enclosure (spore membrane) and a strongly refractile interior."

Although their appearance was unusual, Cohn stated that "the spores are viable. It appears that they do not germinate in the same liquid in which they were formed, for when I placed a small number of spores formed a few months ago into a freshly boiled hay infusion, I was fortunate in being able to observe their germination directly. The strong, separated body of the spore soon disappeared, and the germ tube immediately developed into a short bacillus which then became mobile." Trying to learn whether spores could explain some of the unusual properties of hay, he investigated the heat resistance of spores and observed "they can be heated to 100°C without losing their viability, in the same way that has been shown for mould spores".

Cohn now returned to the issue of "spontaneous generation" addressing himself to the various observations in the literature which claimed that bacteria grew in heated, organic solutions. He noted "in all cases in which organisms have developed in boiled organic substances, I have found only spore-producing bacilli". In the conclusion to this paper, he recalled that in 1875 he had noted that "since bacilli might reproduce by endospores, it could also be expected that the rods found in animals infected by anthrax would also form spores", and that these "could be the germs of the infection in apparently bacteria-free food" (Cohn, 1876).

While Cohn was working on this classical paper, he was contacted by Koch. Cohn had never met Koch, who was then simply a country doctor. But Koch knew about Cohn, who was famous for his studies on bacteria and their classification. Cohn referred to this correspondence in his 1876 publication: "To my great pleasure, I received a letter from Dr Koch of Wollstein on April 22 (1876). He has been occupied with studies on the anthrax contagium for a long time, and has finally been able to discover its complete life history. He was willing to demonstrate this to me at my Institute, in order to obtain my opinion on these discoveries."

Indeed, Koch, the District Physician of the County of Wollstein came to Breslau and performed his critical public experiments at the Plant Physiological Institute between 30 April and 3 May of that year. He grew anthrax bacilli on a microscope slide in such a way that he could observe the burgeoning "micro-culture". As a growth medium, he used sterile beef serum which had been inoculated with spleen from an infected animal. Cohn invited no fewer than six learned professors (whose names are carefully cited in Cohn's publication) to join him as witnesses to these experiments:

"The closer to the edge of the cover glass you looked, the longer the filaments were", Koch observed. "They finally reached a size a hundred or more times the length of the original bacilli." The filaments that lay right at the edge of the cover glass "contained completely formed spores which were embedded in the substance of the filaments at regular distances, and were somewhat oval, strongly light-refracting bodies. In form the filaments were of a remarkable appearance which can best be compared to a string of pearls." By means of a series of microscopic examinations at 10- to 20-min intervals over a period of many hours, Koch was able therefore to watch the actual process of sporulation in individual bacilli. This culture was then subcultured and, after several further passages, injected into animals which then died of typical anthrax infections (Koch, 1876).

Koch's paper describing the life cycle of *Bacillus anthracis* followed Cohn's report in *Beitrage zur Biologie der Pflanzen* (1876), a journal founded and edited by Cohn. In this manner, the first proof of a specific micro-organism causing a specific disease in an animal was demonstrated for the spore-former *B. anthracis*, and the first report on this discovery was published in a journal of plant physiology as an appendix to a paper announcing the discovery of bacterial spores (Koch, 1876). Koch's appendix immediately overshadowed Cohn's original paper and Cohn himself remarked "I consider this the greatest discovery in the field of bacteriology". While Koch's contribution to the aetiology of anthrax was recognized at once as a milestone in the history of science, the discovery of the bacterial spore did not create an impact.

The existence of heat-resistant "spores" of micro-organisms had already been demonstrated for *Penicillium* some years before by Pasteur, who consequently introduced, based on empirical trials, the routine sterilization temperature of 121°C still in use today. Therefore, the discovery that such heat-resistant stages also existed in other, subsequently discovered organisms did not surprise scientists and even came as a relief to those who still had to deal with certain unexplained experiments in which "spontaneous generation" had been proposed.

Moreover, the concept of "dormant life" was also not new; it played an important role in the debates of the European Academies of Science in the latter half of the nineteenth century. Curiously, the discovery of bacterial spores did not enter into these debates (Keilin, 1959), for the extreme character of their dormancy and resistance was fully appreciated only much later. So greatly were the basic biological aspects of the work of Cohn and

Koch on sporulation underestimated at the time, that Pasteur, who developed an anthrax vaccine in 1881 (using an asporogenous strain of anthrax), never credited Koch with the discovery of spore formation in this organism (Pasteur, 1881) (see also Pasteur's letter to Koch as cited in Lechevalier and Solotorowsky, 1974).

II. The Discovery of Many Kinds of Spore-forming Bacteria and the Problem of their Classification

The decades following the discoveries of Cohn and Koch might be characterized as a period in which spore-forming bacteria, rather than the spores of bacteria as such, were investigated. Because the first micro-organism proven to be pathogenic to men and animals happened to be a spore-former, many scientists looked for spore formation in each new organism that was shown to produce disease. The literature of those days is thus replete with unverified reports of "spore-like" morphological structures in pathogenic organisms. Nonetheless, a variety of new and interesting spore-forming bacteria were found.

Ever since Pasteur's studies on lactic acid bacteria, yeast and especially on butyric acid fermentation, anaerobic life was known to exist. Pasteur demonstrated that the strictly anaerobic fermentation of butyric acid could be interrupted by aeration. Therefore, it is understandable that some of the first spore-forming bacteria studied were anaerobes (Pasteur, 1861).

Butyric acid fermentation, a peculiar process in which butyric acid, butanol and, sometimes, acetone were produced, was shown to be caused by a group of anaerobic spore-formers. The first of those to be studied was *Clostridium butyricum* (Adamson, 1919). Based on such early studies, anaerobic spore-formers were used subsequently for the production of butanol, acetone and other solvents.

Some of the anaerobic spore-formers turned out to be of medical importance. Pasteur and Koch, independently studying septicaemia in rabbits and guinea-pigs by inoculating them with "putrid blood", both discovered "malignant oedema". This was the first experimental induction of gas gangrene in animals (Lechevalier and Solotorowsky, 1974).

The early studies of these anaerobic, wound-infecting spore-formers were a source of great confusion in the bacteriological literature of the time. Because it was technically difficult to isolate anaerobes in pure cultures, much of what was known about them was based either on direct microscopic observation or on observation of cultures whose purity was doubtful. The observed changes in the forms of the bacteria intensified the great debate between the "monomorphists" and the "pleomorphists".

The monomorphists, including Cohn and Koch, believed in the stability of the morphological character of bacteria. Pleomorphists, such as Naegli (1877) and Zop (1885), held that the natural variability of bacteria was great and that the bacteria actually changed many of their properties; hence, it was virtually impossible to arrange them in any systematic classification, making bacteria different from all other known organisms (Winogradsky 1895, reprinted in 1949; Penn and Dworkin, 1976). The plethora of references to "polymorphism" in pathogenic *Clostridia* kept this controversy alive.

However, in 1889 Kitasato succeeded in isolating the tetanus bacillus in pure culture and Van Ermengem (1896) isolated *C. botulinum*. Both of these organisms were described in great detail and exhibited the stable property of producing some of the most potent toxic substances known to man.

At first, little work was done to clarify the habitat of spore-forming bacteria. But Winogradsky, with marvellous insight into soil microbes, realized very early that these are primarily organisms that degrade organic material whenever present in the soil. They survive in spore form when their nutrient is exhausted (Winogradsky, 1902). It was also Winogradsky who reported that an anaerobic spore-forming bacterium, which he named *C. pasteurianum*, could grow in a nitrogen-free medium. By measuring the amount of nitrogen before and after growth, he discovered that these bacteria were actually fixing free nitrogen. For the first time, a free-living soil organism was shown to use and fix free nitrogen (Winogradsky, 1895).

In 1895, Omeliansky demonstrated that anaerobic cellulose decomposition could be caused by spore-forming bacilli. Schardinger isolated *B. macerans* as the organism responsible for the retting of flax (Bergey *et al.*, 1923).

In the same year, while investigating a disease of honeybees, White (1904) pin-pointed a spore-forming bacterium, which he named *B. larvae*, as the agent causing this disease. This opened a line of research which led to the isolation of many spore-formers responsible for insect disease. The most studied of these insect pathogens is *B. thuringiensis*, first isolated by Berliner in 1915. The bacteria of this group are potential instruments of biological insect control, and *B. thuringiensis* is used currently as such.

Another group of organisms with unusual qualities are the thermophilic spore-formers. *B. stearothermophilus* was the first of these to be isolated (Donk, 1920). It has an optimal temperature between 50°C and 65°C, will not grow below 28°C and grows only slightly at 70°C.

During the First World War, when anaerobic wound infection was a frequent occurrence, techniques for isolating anaerobic bacteria in pure cultures were improved vastly. As a result, it became possible to establish conclusively that the variability among anaerobic spore-forming bacteria was not greater than that of other groups; thus, their classification should be similar to all other bacteria (Wilson and Miles, 1945; Reports, 1917, 1919).

In the original classification, which is still used today (Bergey *et al.*, 1923), all rod-shaped spore-forming bacteria were classified as belonging to one family, Bacillaceae (Fisher, 1895), in the order Eubacteriales. They are divided into two genera, the aerobic *Bacillus* and the anaerobic *Clostridium*. By any criterion, all species in this family seem closely related.

The restriction of the class of spore-formers to rod-shaped bacteria ran into difficulties when round, rather than rod-like, spore-forming bacteria were discovered. The first of these, the spore-forming *Sporosarcina ureae*, was described as early as 1901 (Beijerinck, 1901). "Bergey's Manual of Determinative Bacteriology" (Bergey *et al.*, 1923) claimed that this species had "heat-resistant spore-like bodies", and included in his description was the comment, "typical endospores absent". This implied a difference between the "spore-like bodies" of this sarcina and "real spores".

The word "spore" has been applied in the literature to very different biological entities. Here it is used only to describe those structures produced intracellularly by Eubacteriales which differ from their mother cell both in structure and chemical composition, contain dipicolinic acid, have little or no metabolism and are more resistant than vegetative cells to extreme adverse conditions. Defined in this way, the spores of *S. ureae* are no different from the spores of rod-shaped bacilli in either fine structure or chemical composition. When this was shown to be true, it was suggested that this group should also be included in the family Bacillaceae (Slepecky and Leadbetter, 1976).

On the other hand, several groups of organisms have been discovered which form spores that contain dipicolinic acid and have the same general fine structure as the spores of Bacillaceae, but clearly cannot belong to this family because, as DNA hybridization experiments have now demonstrated, their DNA has little in common with that of Bacillaceae. These groups also differ from one another in various respects. Among such organisms known today are a group of thermophilic actinomycetes, a group which resembles lactic acid bacteria (*Sporolactobacillus*), and a group of sulphate-reducing vibrios. Obviously, such findings create problems for those interested in classification and nomenclature.

The use of spore formation as a criterion for classifying bacteria is complicated by the fact that it depends very much on the conditions under which the bacteria is tested. Several species have been shown to sporulate only under certain narrowly defined conditions. For example, a Gram-negative rod, which grows anaerobically, sporulates only under semi-aerobic conditions. Others, such as the giant spore-formers in the guts of amphibia and cockroaches, have been observed under the microscope, but have never been cultured.

Two conclusions emerge. The first is that endospore formation is certainly not limited to one bacterial family and might be discovered in additional, hitherto unknown organisms. Given the fact that similar spores are formed by very different organisms, the second conclusion is that sporulation may have evolved more than once during prokaryotic evolution. Comparative sporology in prokaryotes is a field worth pursuing (Slepecky, 1972; Slepecky and Leadbetter, 1976).

III. Early Work on Spore Biology

In 1911, commenting on the chaotic state of bacterial cytology in general and of spore research in particular, Clifford Dobell wryly opined that the reason was that "the bacteria have been entrusted to the bacteriologists, yet to the bacteriologist, the bacteria are but a means to an end – they study them to cure a cold or to make cheese". A perusal of the early literature on spore research validates this assessment. Nevertheless, although their work was motivated primarily by practical considerations, the early bacteriologists did investigate spore biology and spore formation. The roots of some of the problems engaging the modern sporologist can thus be found in the "old" literature, as the following examples illustrate.

Pasteur was the first to note the existence of asporogenous strains of normal spore-forming bacilli. He tried to develop a vaccine from attenuated cultures of *B. anthracis* by growing them at 43–47°C. In the process, he found that they not only lost their pathogenic properties, but many of the strains also permanently lost their ability to form spores. This discovery of the sensitivity of spore formation to environmental conditions was surprising. The great resistance of the spores to heat, desiccation, radiation and chemicals was already well documented, mostly as a result of the practical investigations of sterilization techniques by medical microbiologists (Koch, 1881; Koch and Wolffhügel, 1881; Geppert, 1889, 1891; Krönig and Paul, 1897).

Factors responsible for the onset of sporulation were first investigated by Buchner in 1880 (published in detail in 1890). He concluded that "it is the decreasing concentration of nutrients which induces spore formation" provided that the mother cell came from a rich medium. Buchner also was the first to notice sporulation in distilled water. His conclusion that starvation induced sporulation led to a great controversy. Many investigators claimed that some of his experiments were not sufficiently rigorous to preclude the possibility that other factors such as change of reaction, accumulation of metabolites or unsuitable salt balance might be responsible for spore formation.

As late as 1932, Cook wrote "it is impossible to state definitely that spore formation is brought about by reason of any one physical or chemical effect". It was only in 1951 that Grelet confirmed Buchner's conclusion that sporulation was indeed induced by starvation, and in the meantime the controversy about the factors inducing sporulation generated some interesting information about other aspects of the sporulation process (Grelet, 1957).

For instance, Schreiber (1886) found that glucose inhibited or delayed spore formation. Weil (1890) discovered that certain bacilli needed oxygen for sporulation. The importance of salts, temperature and pH also was well documented. Henrici (1926) showed that spore formation started only when the logarithmic growth phase was over.

Microscopic observations of sporulation were largely limited to morphological descriptions useful for systematic studies. However, two scientific debates that lasted for several decades also contributed to the early descriptions of the sporulation process. One of these discussions was about the existence of a nucleus in bacteria, while the other concerned the occurrence of sexual processes in bacteria. These issues merged in 1902 when Schaudin suggested that a sexual process of "autogamy" preceded spore formation.

In order to observe nuclear material in bacteria, Schaudin searched for very large bacteria, and finally found some in the intestinal tract of cockroaches. Fortuitously, these large bacteria happened to be spore-formers. Schaudin described what he considered to be a diffuse nucleus existing during most of the life cycle in the form of scattered granules of chromatic substances. During spore formation, these granules fused and organized themselves into a spiral. He also observed what he thought to be incomplete cell division before sporulation, albeit after the formation of the chromatin filament. This was interpreted as a kind of cell fusion "of the two incomplete daughter cells". Since conjugation between single cells had been shown to occur in yeast, in diatoms and in some protozoa, Schaudin announced that he had discovered sexuality in bacteria as well (Schaudin, 1902).

This investigation aroused the curiosity of Dobell, whose main interest was the occurrence of nuclear material in bacteria. He adapted the Giemsa stain for demonstrating chromatic material, and was able to see chromatin bodies in bacteria. Dobell not only documented the existence of a "chromatic filament" in bacterial cells prior to sporulation, but also reported that the "chromatic material was surrounded by a membrane at the onset of sporulation". Repeating Schaudin's experiments, Dobell confirmed the observation on the fusion of chromatic material before sporulation and noticed that cell division usually preceded sporulation. However, he could find no evidence that a process of conjugation had taken place (Dobell, 1909).

Most of these early observations on sporulation were forgotten, mainly because they were isolated observations by individual scientists rather than the results of systematic investigation. It was only much later that sporulation, germination and the biology of spore-forming bacteria became objects of continuous study by several groups of scientists. In fact, solid knowledge of