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Roles of *Bacillus* endospores in the environment

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Abstract. The occurrence and diverse roles of *Bacillus* spp. and their endospores in the environment is reviewed, with particular emphasis on soil ecology, host-symbiont and host-parasite interactions, and human exploitation of spores as biological control agents and probiotics.

Key words. *Bacillus* spp.; ecology; endospore; environment; pathogenesis; symbiosis.

Introduction

The independent discoveries of the bacterial spore by Tyndall, Koch and Cohn in the last quarter of the 19th century [1–3] marked the beginning of the movement of spore research from the environment into the laboratory. With few exceptions, the laboratory is still where the bulk of spore research has been performed during the past 120 years, and within the past 40 years spore research has focused progressively more narrowly upon the descendents of a particular strain of one bacterial species, *Bacillus subtilis* strain 168 [4]. Slepecky and Leadbetter [5] best described the prevailing situation in spore research as follows: 'As a result of such attention we are by now aware of many exciting details regarding the physiology, biochemistry, and, now, molecular biology of endospore formation and germination. However, we are far less cognizant of the roles that endospore-forming organisms play in natural habitats' [5]. Sporeforming bacteria indeed exhibit a bewildering array of environmental niches and habits, few of which have been explored in detail (reviewed in [4, 5]). In this brief review I will concentrate upon spores of *Bacillus* spp., the bacteria about which we possess the most information. I will make an attempt to consider the environmental roles of *Bacillus* spores in the context of a few broad basic ecological themes such as niche occupancy, competition, symbiosis, parasitism and human exploitation.

Roles of spores in natural settings

Spores as time capsules

In considering the question, What is the role of *Bacillus* spores in the environment? a simple and obvious answer immediately presents itself – to preserve and to propagate the genetic information contained within the bacterium. Based on the well-known practice of inducing sporulation in the laboratory by nutrient limitation, it is generally accepted that spore formation evolved as a mechanism for both spatial and temporal escape from local conditions unfavorable to rapid growth [4]. (The elaborate molecular mechanisms underlying spore longevity and survival in the environment have recently been reviewed extensively [4 and references therein], and will not be reconsidered here.) As a device for preserving and dispersing genetic information in the environment, the spore is an incredible success. Spores can be found in environmental samples obtained from virtually all parts of both the Earth's surface and subsurface [4, 6]. Reports of the recovery of spores from environmental samples ranging in age from decades to hundreds of thousands of years are common [4, 7], and the scientific world has recently been treated to more controversial reports of spore longevity spanning geologic time scales. In 1994 and 1995 there appeared reports that viable *Bacillus* spp. spores had been isolated from the gut of a bee fossilized in Dominican amber for an astounding 25–40 million years [8, 9]. Even this incredible age for a spore has been dwarfed by the report in 2000 of the discovery of *Bacillus* species 2-9-3, which was recovered from a brine inclusion within a 250 million-year-old salt crystal from the Permian Salado

Formation near Carlsbad, New Mexico [10]. Such claims are always met with a high degree of skepticism, and indeed, it is an exceedingly difficult task to ensure that bacteria isolated from ancient environmental samples are truly ancient and not recently acquired contaminants. The subject of the criteria and controversy surrounding claims of isolation of ancient microorganisms has been reviewed [7].

Ecology of *Bacillus* spores in soil

In the laboratory, *Bacillus* spores are usually prepared by cultivating the bacterium at 37 °C in a liquid nutrient broth-based sporulation medium at a high growth rate and to high cell density until some essential nutrient, such as the carbon source, is exhausted from the medium [11]. Although very little is known about the growth or sporulation of *Bacillus* spp. in their natural habitats, it is not difficult to envision that the process probably differs significantly from the manner in which spores are prepared in the laboratory. Growth of sporeforming bacteria in their natural environments (e.g. soil, decaying organic matter, plant surfaces, insect and mammalian guts, and so on) (i) is almost certainly slower; (ii) probably takes place as microcolonies on and within a solid substrate (e.g., large soil particle aggregates [12, 13]); (iii) is subject to wide variations in temperature, humidity, nutrient and oxygen availability; and (iv) probably occurs within consortia or in direct competition with other micro- and macroorganisms [14].

Although it is generally accepted that the primary reservoir of sporeforming microbes and their spores is the soil, almost nothing is known of the physiology and population dynamics of germination, growth and sporulation in the soil environment [14]. Indeed, his observation that sporeforming bacteria are found in soil mostly as inert dormant spores prompted Conn in 1916 to question their ecological significance [15]. Later studies from the 1930s through the 1960s, which more closely examined the distribution of soil microflora within different soil horizons, suggested a much more dynamic picture of sporeforming *Bacillus* spp. in soils than Conn's static dormant spores, including seasonal variations in the numbers, distribution and predominance of various *Bacillus* spp. [16, 17 and references therein]. Using fluorescent antibodies directed against either vegetative cells or spores of *B. subtilis*, Siala et al. [17] performed a thorough examination of the distribution of vegetative cells and spores within the upper 20 cm of a pine forest soil and found that (i) cells and spores were associated mostly with particles of decaying organic matter, even though 85% of the total soil particles were of mineral (quartz sand) origin; (ii) mostly vegetative cells were found in the near-surface organic (A1) acidic horizon associated with particles of decaying pine leaves and stems; (iii) mostly spores were found in the alkaline mostly mineral (C) horizon located immediately below the A1 horizon, and were associated with particles of decaying pine roots [17]. Siala and Gray [18] performed experiments to monitor the fate of *B. subtilis* cells or spores grown in laboratory media and applied to glass slides which were then placed into contact with soil. They showed that germination of spores and growth of vegetative cells of *B. subtilis* in soil exhibited completely different properties when experiments were performed in sterilized soil vs. soil containing its normal microflora. Growth and germination of *B. subtilis* cells or spores inoculated into the A1 soil horizon were inhibited by contact with pine roots. In sharp contrast, spore germination and vegetative cell growth were specifically associated with the growth and development of fungal hyphae in the soil; indeed, vegetative cells were observed growing in direct proximity to the fungal mycelia [18]. However, it was uncertain in this study whether the bacteria were simply using nutrients excreted by the living hyphae, or were causing death of the fungus and living off the products of hyphal lysis [18]. More recent reports of *B. subtilis* biocontrol strains which produce fungicidal compounds [19–21] suggest the latter possibility, however. More recently, numerous reports have appeared documenting the occurrence of *Bacillus* spp. as members of the rhizoplane and rhizosphere of various wild and cultivated plants [22–26]. These studies have begun to offer the beginnings of a more complex and detailed portrait of the life of sporeforming *Bacillus* spp. in soil, and have led to the use of *Bacillus* spores as biocontrol and probiotic agents (see below).

Spores in rocks

An extensive recent literature indicates that a wide variety of microbial life (called *endolithic* microbes) reside within rock extending from the surface regolith (the layer of broken rock and soil which covers consolidated bedrock on the majority of Earth's land masses) well into the deep subsurface rock itself [4, 27]. Several *Bacillus* spp. have been isolated from the interiors of such locations as manganese rock varnish from Sonoran, Mohave and Negev desert rocks [28–30], deep subsurface boreholes [31, 32], and near-surface granite formations [W. L. Nicholson, unpublished data]. The question naturally arises; Have spores found within rocks or ancient soils arisen from growth and sporulation of bacterial cells in situ, or have they simply become trapped by sedimentary deposition or groundwater percolation from overlying soil? Both scenarios are possible, and indeed not mutually exclusive. Two observations favor of the in situ growth hypothesis. First, the newly described species *Bacillus infernus* was discovered growing vegetatively nearly 3 km below the Earth's surface [32]. Second, *Bacillus* spp. have been found associated with the bio-

degradative activities which are slowly destroying certain granite buildings and monuments [33, 34].

Sporeformers as symbionts

An area which has been well documented among entomologists, but virtually ignored by spore researchers is the symbiotic (literally, 'living together') relationship which exists between sporeforming *Bacillus* spp. and a number of insects, including honeybees, solitary bees and stingless bees [35–37]; termites [38, 39]; moths [40, 41]; and cockroaches, millipedes and sow bugs [41]. Without a doubt this list will grow as new insects are tested. What do these close insect-*Bacillus* associations imply? Are they examples of mutualism (in which both partners benefit) or commensalism (in which one partner benefits and the other neither benefits nor is harmed)? As is the case for obligate endosymbionts such as *Buchnera* spp., which colonize the guts of aphids [42], a nutritional advantage to the insect has been postulated for the existence of *Bacillus* spp. in insect guts. For example, in cockroaches fed different diets, a positive correlation was observed between *B. cereus* population densities in the gut and the rate of insect weight gain [41].

Other roles for insect-symbiotic *Bacillus* spp. have been postulated as well. A wide variety of *Bacillus* spp. have been found associated with brood provisions, pollen, larval feces and in the alimentary canals of insects, including *B. licheniformis. B. cereus, B. subtilis, B. sphaericus, B. circulans, B. megaterium, B. alvei* and *B. pumilus* [35–37], among others. Due to the metabolic capabilities and antibiotic production of these various *Bacillus* isolates, their role in the microecology of the insect nest has been postulated to be involved in conversion of various substrates to forms more nutritionally suitable for the insects, and prevention of overgrowth of pathogenic- or food-spoilage microorganisms [35–37]. Thus it seems clear that the insect benefits from the association – but what about the *Bacillus* spp.? Unlike *Buchnera* spp. in the guts of aphids, the above-mentioned *Bacillus* species are obviously not obligate inhabitants of the insect gut, as they readily grow in laboratory culture, but their long-term residence in the insect gut likely implies some advantage to the bacterium, rather than passive accidental ingestion and passage through the alimentary tract.

Spores as delivery vehicles for animal pathogens

Several diseases of animals, including humans, are initiated by environmental contact of a susceptible host with spores of *Bacillus* spp. Below are summarized some of the best studied. *B. thuringiensis* will be considered separately below as a biological control agent.

***Bacillus* (*Paenibacillus*) larvae**

P. larvae (formerly *B. larvae*) is the causative agent of American foulbrood disease (AFB) of domestic honeybees. Within the brood chamber, vegetative *P. larvae* are rapidly killed by royal jelly, but spores survive [43]. Spores ingested by very young larvae (but not older larvae [44]) germinate, grow to a high density and sporulate within the larval host, killing it. Once established, *P. larvae* spores are spread throughout the brood by contaminated nurse bees. The disease is highly contagious and can quickly destroy an entire hive. However, the mere presence of *P. larvae* in a hive does not indicate active disease, and no strict correlation has been found between the concentration of *P. larvae* spores in honey and presence or absence of AFB in a hive [45]. In a recent experiment to assess interhive spread of *P. larvae* and disease, AFB was experimentally induced in 5 hives of a 20-hive apiary [46]. Although adult bees contaminated with *P. larvae* were subsequently detected in 12 hives, no additional hives became infected with AFB, suggesting that AFB is not easily spread between hives by drifting bees [46].

B. popilliae* and *B. lentimorbus

These two closely-related species are natural soil inhabitants which cause milky disease in the larvae (grubs) of various Coleopteran (beetle) species. Rather than killing the host outright as in *B. thuringiensis* infection, ingested spores germinate and release a parasporal crystal which apparently aids in passage of the vegetative cell from the gut into the hemolymph of the grub, where the bacterium establishes parasitic vegetative growth [47]. The complex ecology of these organisms with their hosts in soil is largely unexplored, despite the fact that *B. popilliae* has been used as a biological control agent against Japanese beetles for the past 50 years [48].

B. anthracis

The disease anthrax in mammals can be contracted by ingestion, inhalation or cutaneous inoculation with spores of the soil bacterium *B. anthracis*. While the disease usually strikes grazing animals whose lifestyle places them in close contact with soil and dust, anthrax is a zoonotic infection of humans; human infection is generally through accidental contact with hides, wool or hair of infected animals. Studies on the ecology of anthrax have mostly been limited to bison herds in northwestern Canada [49] and grazing animals in Etosha National Park, Namibia [50]. Both studies have noted seasonal variations in anthrax incidence and differences in long-term survival of *B. anthracis* spores dependent upon soil type. Interestingly, in both studies it was found that anthrax preferentially struck adult male animals [49, 50]. In the case of Canadian bison it has been postulated that during dry periods following wet spring seasons, *B. an-*

thracis spores become concentrated into low-lying wallows preferentially utilized by sexually mature bulls [49]. In the Etosha study, presence of high concentration of *B. anthracis* spores detected in scavenger feces collected from the vicinity of infected carcasses implicated scavengers as a route of anthrax spread during epizootics [50].

Human exploitation of spores

Spores as biodosimeters

Due to their extreme resistance properties, bacterial spores have long been used as biodosimeters for verification of various disinfection and sterilization regimes. A common example is the use of *B. stearothermophilus* spore strips to test the efficiency of autoclaves. The subjects of using spores of *B. subtilis* as biodosimeters for performance monitoring of ultraviolet (UV) disinfection reactors for drinking water purification systems [51, 52] and solar UV radiation exposure [4, 53, 54] have been reviewed extensively recently and will not be reiterated here.

Spores as biological control agents

The use of spores of various *Bacillus* spp. as natural pesticides is not new, and 'biopesticides' have long been touted as safe and environmentally friendly alternatives to traditional pesticides. However, natural biopesticides also exert environmental impacts which often are not appreciated until long after their use has become institutionalized. For example, as mentioned above, *B. popilliae* has been in use for several decades as an agent to control Japanese beetle infestations, but large-scale spore production is hampered by inability of the bacterium to sporulate outside the host grub [48]. Furthermore, widespread distribution of *B. popilliae* in the environment by humans has recently been brought into question, due to the fact that the bacterium encodes natural resistance to vancomycin, an important 'last line of defense' antibiotic for human use [55, 56]. For control of lepidopteran larvae (caterpillars), *B. thuringiensis* (Bt) spores and parasporal crystal preparations have been a resounding commercial success: Bt accounts for greater than 90% of all marketed bioinsecticides, with a worldwide market of over $100 million [20, 57]. Despite the fact that Bt generally has performed better than traditional chemical pesticides in terms of target selectivity [58–61], concerns persist about Bt insecticides also killing potentially beneficial nontarget predator insects. Such concerns have become especially acute coincident with the introduction and expression of the Bt crystal toxin directly within crop plants [62, 63]. Similarly, *Bacillus sphaericu*s spore preparations are widely used for control of dipteran species, particularly mosquito larvae, and studies to date also tend to indicate few noticeable ill effects on nontarget organisms [64–67].

The recent successes of *B. cereus* [20] and *B. subtilis* [20, 21, 68, 69] strains as both natural fungicides and plant-growth-promoting bacteria when inoculated directly into soils as seed pretreatments has spawned a new generation of studies concerning the establishment and survival of sporeforming bacterial populations in soils [70–74]. Van Elsas et al. [75] studied parameters affecting the establishment and survival of engineered bacterial populations in soils, and showed that inoculation of vegetative cells of *B. subtilis* into two different soils at ~10^6 cfu/g of soil led to a rapid decline within 3 days of 2–3 logs in cell number. The population then stabilized at $3–4 \times 10^3$ cfu/g (as nearly 100% spores) for at least the next 120 days. The population density of the inoculant spores was roughly the same as the indigenous spore population, indicating both (i) the existence of factors antagonistic to survival of the vegetative inoculum and (ii) a finite carrying capacity of the soil [75]. Tokuda et al. [19] demonstrated that this limitation could be overcome, and high-level populations of *B. subtilis* spores (10^7 spores/g soil) could be established in soil by a combination of nutritional amendment of soil with a readily metabolized carbon source such as glucose and raising the soil incubation temperature from 15 to 25 °C. Additional success in enhancement of the establishment and survival of bacterial inoculant populations in soil has been achieved by encapsulation of microbes within a variety of polymeric materials, which can provide a source both of nutrition for the inoculant and protection of the inoculant from harmful soil factors [74].

There is a synergistic increase in the efficacy of bioinsecticides which contain both spores and endotoxin of *B. thuringiensis* [76, 77]. However, when commercial preparations containing mixtures of *B. thuringiensis* spores and crystal inclusions are used for insect control, the spore component disappears rapidly from the field, attributable at least in part to solar UV sensitivity of the spores [78–80]. Empirical attempts at circumventing this problem have concentrated on encapsulation of the biocontrol agent [81–83] or isolation of UV-resistant strains by UV irradiation of spores in the lab [84] or exposing spores to sunlight [85]. Recently, however, Du and Nickerson [86] presented evidence indicating that during *B. thuringiensis* sporulation some of the insecticidal crystal toxin molecules produced actually insert into the spore coat where they are displayed for binding to specific receptors in the insect midgut, thereby facilitating spore attachment and germination in the insect host. The authors postulated that disruption of spore coat integrity by toxin insertion greatly enhances insect pathogenicity at the expense of a loss of some of the resistance properties of the spore [86]. In support of this hypothesis, it was recently demonstrated that mutational disruption of the spore coat layers in *B. subtilis* resulted in decreased spore resistance to solar UV [87]. The above

sampling of studies underscores the importance of understanding the basic ecology of sporeformers in their natural habitats for the continued rational design of safe and effective biopesticides.

Spores as 'probiotics' in plant, animal and human health

There has been a recent upsurge in interest of the use of various species of *Bacillus* as probiotics, loosely defined as live microbial feed supplements which beneficially affect the growth or nutrition of the host. Growth-promotion effects of various *Bacillus* spp. have been documented both for plants [20, 21, 68, 69, 87a, 88, 89] and animals [90, 91]. The exact mechanism(s) by which probiotic microorganisms exert their beneficial effect are currently the subject of much investigation and debate; however, some recurring themes appear to be that addition of probiotic *Bacillus* spp. to a plant or animal system alters the microbial community structure in the rhizosphere or gut, respectively, to (i) suppress the growth of antagonistic microorganisms either by production of antimicrobial compounds [91] or competitive exclusion [92]; (ii) boost the immune or defense response of the host; or (iii) increase the local availability of nutrients to the host. However, extreme care must be taken by scientists in evaluating the claims and components in commercial probiotic preparations; it was recently shown that when two commercial probiotic products, both of which claim to contain *B. subtilis*, were evaluated (trade names Enterogermina and Biosubtyl), neither product were found to contain detectable *B. subtilis* [93].

In humans, the use of probiotics to enhance intestinal health and to treat intestinal diseases has been proposed for many years. Indeed, unsubstantiated claims exist that *Bacillus subtilis* itself was originally isolated by the German Medical Corps in 1941 as an effective probiotic treatment for dysentery which was decimating Nazi troops during the North African campaign. However, despite much anecdotal information and many products on the poorly regulated nutritional and food supplements market (with trade names such as Bacti-Subtil, Biosporin, Subalin and EarthFlora), the scientific basis of *Bacillus* spp. use as human probiotic has been established only recently, and sound clinical studies have only begun to be published [94]. While probiotics represent an exciting and potentially useful prophylactic and therapeutic advance, intensive systematic investigations must be undertaken before their role in intestinal health can be delineated clearly [94]. In light of the current crisis in the rise of pathogenic microorganisms resistant to traditional antibiotics, it is anticipated that the research and use of *Bacillus* spp. and other microbes as probiotic replacements for antibiotics will be an area of considerable growth in the upcoming decades.

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