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Defining the natural habitat of *Bacillus* spore-formers

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Abstract

Our understanding of the genetics and physiology of the spore-forming genus *Bacillus* is remarkable. On the other hand, though, where these Gram-positive bacteria live and grow is far from clear. The soil, once considered their habitat, may simply serve as a reservoir. A growing number of studies show that *Bacillus* spores can be found in the intestinal tracts of animals, raising the question of whether this could be where they live and grow. In this study, we have conducted the first evaluation of *Bacillus* spore formers in soil and in human faeces. Our aim is simply to determine the abundance of aerobic spore-formers. Our results show that soil carries approximately $\sim 10^6$ spores/g while human faeces an average of up to 10^4 spores/g. The numbers of spores found in faeces, we reason, is too high to be accounted for principally by ingestion of food contaminated with spores from soil. This provides further evidence that *Bacillus* spore formers may have adapted to survival within the intestinal tract of insects and other animals that ingest them; if so they may well be hitherto undiscovered gut commensals.

Keywords: *Bacillus* species; Endospores; Spore-formers; Soil microorganisms; Gut commensals

1. Introduction

Bacterial endospore-formers typically fall under two major groupings of Gram-positives, the *Bacilli* and *Clostridia*. Many other spore-forming genera exist, including Gram-negatives, but for the most part, these remain poorly understood [12]. In the case of *Bacillus,* most members have long been considered soil organisms [25]. This assumption is based upon culture-dependant methods of isolation that enrich for the presence of endospores, implying abundance [11]. In recent years, it has become apparent that this may be an oversimplification and *Bacillus* endospores have been found in diverse environments including rocks, dust, aquatic environments and the gut of various insects and animals [25]. Endospores are uniquely robust life forms enabling them to be dispersed easily and, as a result, found everywhere [24,25]. From a purely academic viewpoint, it is ironic that, for an organism that is genetically so well defined, on a par with *Escherichia coli*, its true habitat and life cycle is so poorly understood. One question that has been raised is whether there is a symbiotic relationship between *Bacillus* spp. and insects and animals [25]. For insects, in particular, there is stronger evidence for commensalism where the host benefits. For example, cockroaches fed with *Bacillus cereus* exhibited direct and positive weight gains [10]. An obligate, endosymbiotic, relationship with the Gram-positive endospore former, *Metabacterium polyspora* has been shown in the gut of the guinea pig [1] demonstrating that for some endospore formers, at least, there must also be direct benefits to the bacterium. *Bacillus* spp. are being used as probiotics for livestock, aquaculture as well as functional foods for human consumption [16]. For animal use, there are direct and substantiated benefits to animal health including weight gains and prevention of disease. In rabbits for example, *Bacillus subtilis* has been shown to have a direct effect on the development of the gut-associated lymphoid tissue (GALT)

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[28]. Interestingly, these studies showed that sporulation, per se, was required for this phenomenon. This has been supported by murine studies that have shown, in vivo, that ingested endospores are able to germinate, proliferate and then re-sporulate in the small intestine [33]. *Bacillus* endospores then must have a much more intimate relationship with insects and animals than might be expected if their primary habitat were only the soil. We have raised the possibility that *Bacillus* species are able to exploit the intestinal tract as a habitat [33]. This has been supported by a number of studies that have recovered *Bacillus* spp. from the small intestine and from faecal samples [9,17]. In this work, we have examined the abundance of aerobic endospore formers in samples of human faeces as well as soil samples. Our results suggest that the human gastrointestinal (GI) tract is populated with *Bacillus* spore-forming species but these counts are at least 100-fold lower than the counts found in soil.

2. Materials and methods

2.1. Collection of faecal samples

Freshly voided faeces was collected from healthy volunteers and weighed by difference. Volunteers had not taken antibiotics or probiotics within the preceding 12 months and were recruited from the vicinity of either London or Naples. Faeces was stored at 4 °C and processed within 2–3 h of collection.

2.2. Soil samples

Approximately 10–20 g of soil (weighed by difference) was collected from selected locations in the London region. In each case, soil at a depth of 5–10 cm below the surface was collected. Samples were stored at 4 °C prior to analysis.

2.3. Determination of counts of spore-forming bacteria

Bacterial endospore formers were counted using two methods, heat or ethanol treatment. Faecal or soil samples (approx. 1–10 g) were suspended in the minimal volume of sterile phosphate-buffered saline (PBS) that allowed adequate suspension of solid matter by vigorous vortexing. In some cases, sterile glass beads (2 mm) were used to aid homogenisation of solid matter. For heat treatment, homogenised samples were heated in an oven at 65 °C for 45 min. For ethanol treatment 1 volume of homogenised sample was mixed with an equal volume of absolute alcohol and incubated for 1 h at room temperature (RT). After both heat or ethanol treatment, samples were immediately serially diluted and plated out on three solid media, Difco-Sporulation medium (DSM; [26]), MRS agar (Oxoid) and MacConkey's agar (Oxoid). Plates were incubated for 2–3 days at 30 °C and in the case of MRS agar plates were incubated anaerobically using a Don Whitley anaerobic cabinet.

2.4. Other methods

Catalase activity was confirmed by emulsification of single colonies in a 3% solution of hydrogen peroxide (Sigma). The one-way ANOVA test was used to compare the significance between groups.

3. Results and discussion

Faecal and soil samples were examined for quantification of bacterial endospores using a culture-dependent approach. Spore counts from faecal samples were taken from volunteers in an Italian study (Fig. 1A and Supplementary Table 1) and 20 from the UK (Fig. 1B and Supp. Table 2). As controls, the number of enterobacteria and lactobacilli were enumerated using growth on MacConkey's and MRS agar [14] respectively. Faecal counts for the enterobacteria and lactobacilli ranged between 10^6–10^7 for the former and 10^7–10^9 for the latter. Counts of lactobacilli using MRS medium were in agreement with other work [13] as were counts for the enterobacteria, demonstrating that our methodology was sound. In addition, 50 soil samples were examined (Fig. 2 and Supp. Table 3). Our analysis was designed to identify aerobic endospore formers and therefore excluded species of the strict anaerobes, i.e., *Clostridia*. Colonies were randomly tested for their catalase reaction and always tested positive, further excluding the possibility of *Clostridia*. The Gram stain was also performed on some isolates confirming in all cases the presence of Gram-positive rods. Thus, the vast majority of recovered strains were most probably species of *Bacillus*.

3.1. Spore formers are recovered from human faecal samples

Heat treatment (65 °C, 45 min.) should kill all vegetative cells, but it is worth mentioning that endospores of some species are resistant to only 60 °C. For example, endospores of some strains of *B. cereus* showed a reduction in viability after heat treatment at 65 °C [15]. We used 65 °C since, at 60 °C, residual fungal contamination was sometimes observed, and therefore our counts are probably a slight underestimate of the true numbers of aerobic endospore formers. While a surprisingly high number of bacterial species produce endospores [12], the most ubiquitous is *Bacillus* and it is likely that these represent the majority of the recovered counts. Our counts averaged at about 10^4 CFU/g and were therefore similar to those from a study of heat-treated human samples in Vietnam [33], although counts for adults were somewhat higher in the Vietnamese study. Using ethanol produced somewhat different results with lower endospore counts ($\sim 10^3$) compared to heat treatment. There are a number of explanations: first, the study was performed in two different laboratories and two different countries and we cannot rule out the possibility of a slight variation in the methodology or reagents. A second possibility is that this reveals a real difference perhaps caused, for example, by diet. We believe, though, that the lower counts

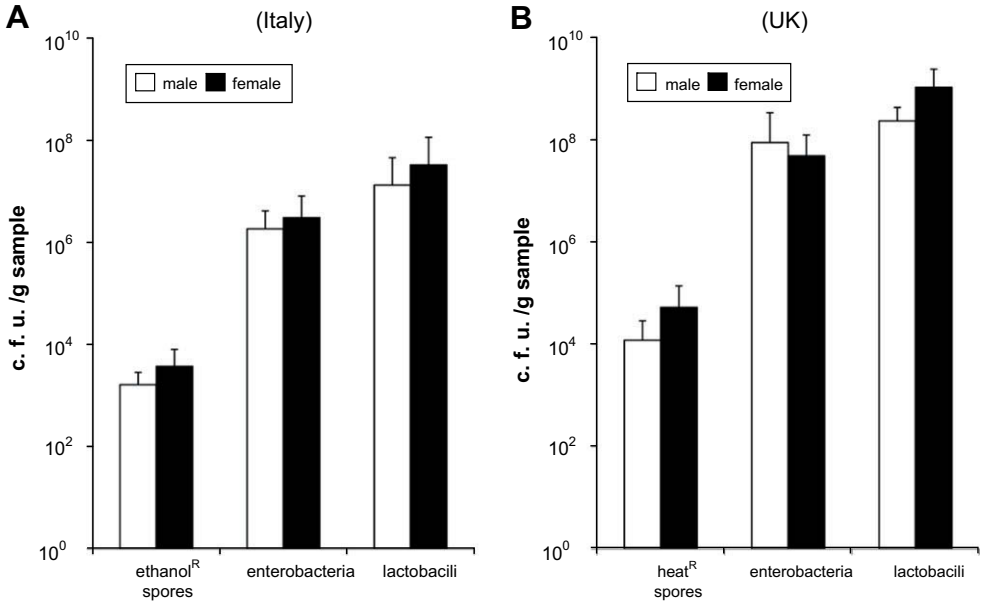

Fig. 1. Aerobic spore formers isolated from human faeces. Panel A shows counts of spores (CFU/g) obtained by ethanol treatment of freshly voided samples. Panel B shows counts obtained by heat-treatment. Plates were grown aerobically. Levels of enterobacteria and lactobacilli were determined by plating untreated samples on MacConkey's and MRS agar respectively. Raw data is given in Supp. Tables 1 and 2.

reflect the choice of ethanol for detection of endospore counts. The premise for using ethanol as a selective treatment was a previous study suggesting better enumeration of endospore counts compared to heat treatment [19]. In this work though, endospores evaluated were, at most, 5 days old. It is well understood that 'aged' spores are less capable of germinating synchronously and they must be heat-activated prior to culture on rich media [22]. If endospores found in the faeces (and also the soil samples; see below) are in this state of heightened dormancy, then this might explain the slight difference in endospore counts between the two studies. Although some differences existed between sexes, endospore counts were always higher in females, although the differences were not significant ($P > 0.05$).

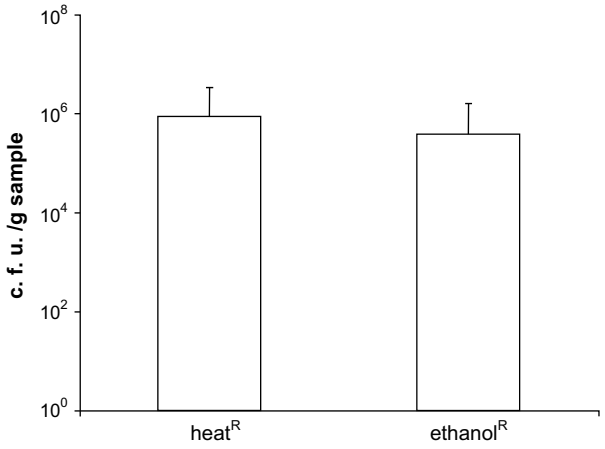

Fig. 2. Aerobic spore formers isolated from soil samples. Fifty soil samples were examined for the presence of aerobic spore formers (CFU/g) using ethanol or heat treatment. Raw data is given in Supp. Table 3.

3.2. Aerobic spores present in soil

Counts of aerobic endospore formers were, on average, 10- to 100-fold higher than faecal counts with an average of 10^5 CFU/g. As with faeces, heat-treatment generated somewhat higher numbers. Colonies identified were, in general, more pleiomorphic in appearance with rhizoid-type colonies, pinpoint as well as crenated forms. We also found that up to 15% of colonies identified from soil were pigmented. Pigmentation in endospores provides them with an extra level of protection against UV irradiation and an attribute of high-value if they are to remain dormant in the soil for long periods of time [21]. A variety of pigments have been found in *Bacillus* species including reds (*Bacillus atrophaeus* [21] and *Bacillus megaterium* [20]), dark-grey (*B. atrophaeus* [21]), yellow and orange (*Bacillus indicus* [32] and *Bacillus cibi* [35]). In many cases, the pigments are carotenoids that provide natural antioxidative properties [6,21]. Intriguingly, we found that at low dilutions, pigmentation was difficult to detect even when colonies were well isolated. On the other hand, at higher dilutions, for example, with 20–100 colonies on a plate, as many as 15% of colonies derived from heat-or ethanol-treated soil were pigmented (data not shown). It is possible that in a nutrient-rich environment (i.e., at high dilution), pigmentation is enhanced implying some form of catabolite repression. In any event, the main discovery, we surmise, is that a large number of endospore-formers are able to form pigments and the use of classical culture-dependant methods of identifying soil endospore formers may have

failed to identify these. We believe then that the soil potentially offers a large reservoir of yet undiscovered pigmented endospore formers.

3.3. Endospores found in food products

A potential source of the spores found in the human GI tract is through food. Endospores are commonly found in food products where their presence can be linked to the soil. For example, populations of endospores (typically, *B. cereus*, *B. licheniformis* and *B. subtilis*) in pasteurised milk can reach 10^3 CFU/ml and they have been shown to contaminate milk from silage, bedding as well as faeces [34]. During prolonged storage, germination, outgrowth and proliferation of endospores can substantially increase counts of live bacteria to as high as 10^6 CFU/ml [27]. Other food sources that carry *Bacillus* endospores at levels reaching 10^2 CFU/g are rice, grain and vegetables. In all cases, the origin of these endospores can be attributed to soil but as with milk storage, if endospores can germinate and proliferate the numbers of bacteria can increase substantially [2,29].

3.4. The true habitat of Bacillus species

If we assume that soil is the true habitat of *Bacillus,* then their presence in faeces is a direct consequence of the host having consumed food contaminated with soil. Our data reveals a basal level of endospores in the human GI tract of about 10^4 spores/g of faeces. For a healthy adult living on a Western diet they would be expected to have a mean daily stool weight of about 200 g [5] which, using our findings here, would contain in total approximately 2×10^6 endospores. To produce this a person would need, for example, to consume 2 litres of milk a day, or 20 kg of rice and cereals. While these are generalizations we doubt that the counts found in human faeces can be accounted for based solely on intake with food. A more reasonable explanation is that intake with food introduces endospores into the GI tract which then germinate and proliferate as part of their life cycle. Germination is a process designed to occur in the presence of nutrients and nowhere else is this more apparent than in the small intestine. If endospores are designed to survive within the GI tract we might ask what attributes they possess that facilitates this. One important finding is that *Bacillus* can grow and sporulate under anaerobic conditions [23,33], as well as molecular studies showing endospore germination, proliferation and re-sporulation [4,33]. The endospore itself, is encased in a protective coat of protein, the spore coat, whose natural protective role has surprisingly, until recently, been poorly understood. Work has now shown that the spore coat enables protection from immersion in gastric juices [7,31]. Interestingly, a role for the spore coat in avoiding phagocytic predation by the protozoan *Tetrahymena theromophila* has also been demonstrated [18]. Perhaps then, the endospore is designed to survive predation whether by simple microbes or large animals. Intriguingly, few studies have been made on the analysis of live bacillus in the soil environment and, as with faecal analysis, there are a number of logistic and technical reasons why this approach is problematic. Still, those studies that examined soil using fluorescent antibodies failed to convincingly prove the existence of vegetative *B. subtilis* in the soil other than an association with decaying plant matter [30]. Other studies have demonstrated that sporulation of *B. subtilis* cannot occur at temperatures below 15 °C [3]. For an organism purported to live in the soil, this result is difficult to explain, but rather supports the hypothesis that endospores, while found in the soil, have adapted to survive within the GI-tract of animals that ingest them. How these Gram-positive endospore formers have adapted to life within a host remains to be seen, yet *B. subtilis* is now being subject to microarray-based comparative genomics, revealing a remarkable diversity within this single species [8].

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Appendix. Supplementary information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.resmic.2009.06.006.

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