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UNEARTHING ANTHRAX — APPLICATION OF GENOTYPING FOR EXPLORING A CRYPTIC LIFE CYCLE OF *BACILLUS ANTHRACIS* IN SOIL

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Summary. *Bacillus anthracis*, the etiological agent of the zoonotic disease anthrax is a very monomorphic species. Typically, the epidemiology of anthrax outbreaks is investigated employing progressive hierarchical resolving assays using nucleic acids (PHRANA). For resolution of relationships of *B. anthracis* originating from a single animal this approach is not suited. In order to close this gap PHRANA can be amended with whole genome sequencing data and subsequent analysis of Single Nucleotide Polymorphisms (SNPs). Doing so, it was possible to resolve the genetic diversity of isolates from anthrax outbreaks in Sweden, Italy as well as of drug use-related infections in Europe. The Swedish outbreak was confined to a short time-period whereas the Italian anthrax foci were revisited for analysis ten years after the host animals have died. Data from the study in Italy contrast with the established view concerning a strict resting stage of *B. anthracis* in soil. This review discusses the plausibility that *B. anthracis* multiplies in a limited soil-borne life cycle after the spores have diffused to near the surface where the bacteria encounter favorable conditions in non-animal hosts or rhizosphere.

Keywords: *Bacillus anthracis*, anthrax, soil, genotyping, life cycle

Evidence that *B. anthracis* is not fully inert while residing as a spore in soil. Pathogenesis of *B. anthracis* infections in animals and man is well understood and the pathogen's virulence factors are studied in great detail (Turnbull, 2008). Much less is known about the genetic diversity of the bacterium within a single infected animal or within different sick animals of the same herd. All but nothing is known about the period after the bacterium has killed its host and sporulation has been completed. What happens after *B. anthracis* has entered the part of its life cycle known as endospore in the environment, e.g., soil, must be considered a black box. What is known is that endospores can endure for decades in soil constituting the natural reservoir of *B. anthracis* (Hugh-Jones and Blackburn, 2009). These sites of contamination pose a high risk of reinfection for grazing animals presumably either via inhalation or ingestion of endospores. However, outbreaks occur only sporadically with time intervals ranging from years to decades and typically these outbreaks are seasonal following a period of hot-dry weather and rain fall (Turnbull, 2008).

Despite the prevailing theory that in nature *B. anthracis* is an obligate pathogen restricted to a metabolically inactive endospore state outside the host, an early hypothesis postulated multiplication of *B. anthracis* in 'incubator areas' (soils rich in organic matter and calcium with a pH above 6.0 and an ambient temperature above 15.5 °C) (Van Ness, 1971). The sporadic occurrence of anthrax outbreaks under certain climatic and environmental circumstances could then be explained by local accumulations of *B. anthracis* that reach the required dose for infection of grazing animals due to multiplication in near-surface soil. A competing hypothesis suggests that these local accumulations emerge from the physical pooling of endospores in rainwater depressions because of the endospores' hydrophobic surface character. Furthermore, vegetative cells of *B. anthracis* were suggested to be unable to successfully compete with resident soil microbiota (Sterne, 1959) and have never been found in the environment. Also, the clonal genetic character of the organism argues against frequent episodes of soil proliferation. Thus, the lifestyle of *B. anthracis* in the environment has jokingly been summarized as 'sporulate or die' (Turnbull, 2008).

An increasing amount of laboratory findings, however, contrast with this established view of an obligate pathogen. Since other members of the genetically homogenous *B. cereus* sensu lato group was found to multiply in guts of soil associated invertebrates, as a saprophyte in the rhizosphere of plants and also in sterilized soil, it would be surprising if multiplication of *B. anthracis* is strictly limited to an animal host body. Indeed, repeated probing of a natural focus in Namibia at which an animal had died from anthrax, showed that *B. anthracis* spore counts increased around grass roots over the years (Saile and Koehler, 2006) indicating multiplication in the rhizosphere which could already be observed under laboratory conditions. Later work strongly suggested the proliferation of *B. anthracis* within soil-dwelling amoebae (Dey, Hoffman, and Glomski, 2012). Additional experiments showed that lysogeny of *B. anthracis* with bacteriophages may enable the bacterium the colonization of the intestinal tract of earthworms (Schuch and Fischetti, 2009). These findings suggest that *B. anthracis* is principally

competent to grow in suitable soil environments, but this has not been observed in the environment thus far. However, frequent probing of contaminated soils over several years revealed isolates of *B. anthracis* lacking one or both virulence plasmids (Antwerpen et al., 2011; Aikembayev et al., 2010). This loss, on the background of the aforementioned laboratory findings, can be explained most simply by a soil-borne life cycle of *B. anthracis*. Recent work conducted as part of a collaborative project between partners from Germany and Italy has explored using genetic methodologies the possibility of a cryptic life cycle in soil. The results and conclusions are outlined in the next section.

***B. anthracis* retrieved from aged anthrax foci appears to have undergone some degree of microevolution.** Although occurring only sporadically, anthrax is endemic in southern Italy. The most severe epidemic during the last decade started in 2004 in the region of Basilicata with a total of 124 animals, mostly cattle but also sheep, goats, horses and red deer (Fasanella et al., 2010). In 2014, sites were revisited where in 2004 cows diseased from anthrax had been buried. From two of these aged foci samples were drawn (Fig. 1) to test the hypothesis of a soil-borne life cycle (Braun et al., 2015). The rationale of this work was if there was *B. anthracis*-proliferation then genotypes of strains isolated from near the surface (5 cm) of contaminated soil should be on a different evolutionary trajectory from those residing at 100 cm depth near the bovine carcass. It was the expectation that the surface population would yield a higher genetic diversity and such possible microevolution was evaluated using genomic tools.

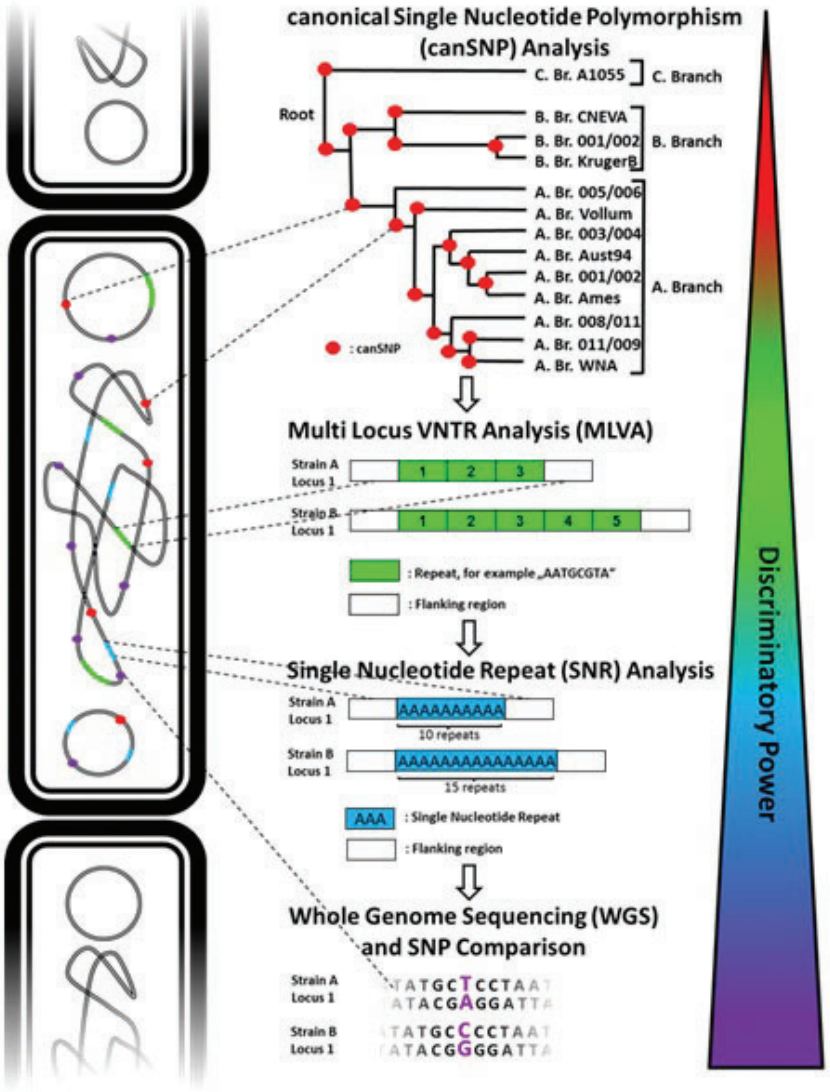

Figure 1. Unearthing *Bacillus anthracis*. Left Panel: Drilling core of a hand-held ground auger with a soil-sample from an anthrax burial site at Pollino National Park (Italy). Right panel: Agar plate after processing such a soil sample using the GABRI method (Fasanella et al., 2013). Non-hemolytic colonies of *B. anthracis*, which appeared whitish and medusa-head shaped, can be easily identified and distinguished from contaminants

B. anthracis can be a challenging organism for genetic analysis in terms of differentiating the species from its close relatives of the *B. cereus* sensu lato group. Conversely, on the genomic level, *B. anthracis* itself is a very clonal organism with little if at all horizontal gene transfer. Evolution is considered to be restricted to the limited reproduction phases of 20–40 generations during host infection while the ensuing endospores may lay dormant for years away from any host (Keim et al., 2004). Since its rather recent emergence as a pathogen about 3,000–6,000 years ago (Van Ert et al., 2007) only a small degree of genetic variations is observed when comparing strains from different parts of the world. Analysis of whole genome data revealed variations mainly in Variable Number of Tandem Repeats (VNTR), Single Nucleotide Repeats (SNR) and Single Nucleotide Polymorphisms (SNP). A subset of the SNPs is representative for the three major lineages A, B and C of *B. anthracis* which can be further divided into thirteen different original canonical SNP (canSNP) branches that reflect the global phylogenetic relationships among *B. anthracis* strains (Van Ert et al., 2007). Within a single distinct canSNP group, Multi Locus VNTR Analysis (MLVA) can be used to further differentiate strains, e.g., in outbreak investigations because of the greater diversity values within these markers due to higher mutation rates in VNTRs compared to point mutations (Keim et al., 2004; Van Ert et al., 2007). Finally, analysis of highly mutable SNR markers serves as a tool to discriminate between very closely related isolates even within a single MLVA genotype during enzootics (Keim et al., 2004). This hierarchical fingerprinting system is called PHRANA (Progressive Hierarchical Resolving Assays using Nucleic Acids) and is commonly used for epidemiological investigation of outbreaks as well as for trace back analyzes in bioforensics and was for instance used in the 2001 Amerithrax event (Keim et al., 2004). PHRANA can be amended with whole genome sequencing data for elucidating close relationships of strains responsible for injectional anthrax among heroin consumers injecting the anthrax-contaminated drug (Keim et al., 2015).

The recent study on *B. anthracis* microevolution at aged anthrax foci (Pollino National Park, Italy) (Braun et al., 2015) amended PHRANA with whole genome sequencing in order to increase resolution power of genetic analysis (Fig. 2). For this, the genetic diversity of randomly picked *B. anthracis*-isolates was compared by 31-Loci MLVA, 4-loci SNR analysis and whole genome sequencing. MLVA-31 analysis of 114 isolates only revealed three differences, none of which in near-surface isolates. Similarly, SNR 4-loci-screening of 174 isolates yielded 18 differences with very similar percentage of occurrences of SNR-variants in near-surface (9.9%) and near-carcass (10.9%) isolates.

Figure 2. Application of molecular bioforensic tools used for investigating the genetic diversity of B. anthracis

Finally, genome sequencing of nine 5 cm- and 100 cm-isolates, respectively, revealed five isolate-specific SNPs, four of which only found in different isolates from 5 cm. Notably, one of these randomly-picked surface-isolates harbored two different SNPs and another one lacked plasmid pXO1. Possibly, the observed loss of pXO1 could have been an artifact of laboratory growth, however, such a loss in the laboratory was determined to be rare (Okinaka et al., 1999), and since DNA of the bacteria analyzed in (Braun et al., 2015) was extracted after a single passage, this idea can likely be discarded. While isolation of *B. anthracis* lacking pXO2 or both virulence plasmids from contaminated soils over the years is well known, natural pXO1-negative isolates are

rather rare but emerged exclusively from soil (Okinaka et al., 1999). Such strains represent an evolutionary dead end as they are likely no longer able to successfully infect new hosts due to the lack of anthrax toxins. Since pXO1-negative isolates were neither detected in infected animals during the outbreak in 2004, it is likely that this loss occurred in the soil environment. The most obvious assumption concerning the absence of pXO1 is that it reflects a soil-borne life cycle in which the plasmid was spontaneously lost during replication.

The genotyping approach called PHRANA (Progressive Hierarchical Resolving Assays using Nucleic Acids) is commonly used for outbreak and epidemiological investigations or for bioforensics of *B. anthracis* : The method comprises the consecutive application of canSNP-, MLVA and SNR analysis (Keim et al., 2004). Recently, the PHRANA fingerprinting system has been amended by whole genome sequencing (WGS) and multiple-isolate SNP comparison (Ågren et al., 2014; Keim et al., 2015; Braun et al., 2015). The left part of panel depicts simplified *B. anthracis* cells growing in chains. Plasmids pXO1 and pXO2 (open circles) and chromosome (twisted circle) are indicated with arbitrary canSNP positions (red dots), VNTRs (green bars) as well as SNRs (blue bars) and WGS SNPs (purple dots). Discriminatory power increases in every step from canSNPs to WGS SNPs and genetic resolution is dependent on the markers' intrinsic mutations rates.

The power of bioforensic tools for investigating genetic diversity in pathogens. Because of high homoplasy in MLVA and SNR markers (Keim et al., 2004), a phylogenetic explanation on the origin of length variations can hardly be given and therefore the different alleles might have originated during the course of infection as well. This would agree with the findings of Stratilo and Bader (2012), who compared SNR profiles of *B. anthracis* soil isolates distributed around carcass sites of bisons diseased from anthrax. The authors found similar distributions of SNR variations within soil samples of single carcass sites (Stratilo and Bader, 2012) which were proposed to be acquired during host passaging.

Whole genome sequencing more and more becomes the tool of choice in molecular epidemiology of infectious diseases and was already used for investigation of anthrax outbreaks (Braun et al., 2015; Keim et al., 2015; Ågren et al., 2014). Similar to variations obtained by MLVA and SNR, the SNPs might be unintentionally introduced during laboratory growth after sampling. However, the *in vitro* mutation rate of *B. anthracis* single nucleotide variations, which is due to DNA-polymerase errors, was observed to be 8.3×10^{-10} mutations/bp/generation, about 10–20 times lower than the estimated rate for *in vivo* mutations (Ågren et al., 2014). The authors did not find any mutation in three different colonies after five passages. Therefore, the possibility of *in vitro* mutation being the source of the SNPs should be negligible. Instead, given the higher mutation rate *in vivo* than *in vitro,* SNPs might be incorporated during host passaging. Regarding the whole population in a respective burial site, the occurrence of a unique SNP in any specific isolate is very rare because it must have originated from an error during the last rounds of DNA-replication before sporulation in one or only a few bacteria of the population within the dying animal. Otherwise, this SNP would be more common. It is noteworthy, that Ågren et al. (2014) found specific SNVs (Single Nucleotide Variants) for every isolate even between those from a single host animal or soil samples taken from nearby. Notably, in this Swedish outbreak, animals were treated with the antibiotic penicillin, whereas this had not been the case at Pollino National Park. The phenomenon of a higher mutation rate compared to the Pollino National Park sites can thus most easily be explained by the selective pressure applied during antibiotic treatment of the infected Swedish bovines (Ågren et al., 2014).

Conclusions. Recent experimental data support the idea that a soil-borne life cycle of *B. anthracis* exists at anthrax burial sites, which leads to microevolution increasing the genetic diversity from the depth of the carcass to the near-surface. It can be expected that improvements in technology will make possible to provide a clear-cut higher answer to this question if genotype variations are more likely due to mutation events during host infection or are a result of a soil-borne life cycle of *B. anthracis*. Currently, a model on the events at aging anthrax foci is favored which states physical diffusion to be the cause of local accumulations of endospores at or near the surface where only sporadic soil replication takes place under favorable conditions, possibly within the rhizosphere or in soil-dwelling protists as proposed earlier (Saile and Koehler, 2006; Dey, Hoffman, and Glomski, 2012).

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