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Opinion

Anthrax, but Not *Bacillus anthracis*?

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Bacillus anthracis, the etiologic agent of anthrax, is a close relative of *B. cereus*, a soil organism and known opportunistic pathogen that causes a variety of human infections [1]. *B. anthracis* is very similar to *B. cereus* and *B. thuringiensis* except that all confirmed samples of *B. anthracis* suggest that it is a monophyletic clone derived from the *B. cereus* and *B. thuringiensis* clade. The major distinguishing feature of *B. anthracis* is the presence of two large virulence plasmids, pXO1 and pXO2, that harbor the tripartite toxin complex [2] and the genes responsible for the synthesis of a poly-γ-D-glutamic acid capsule [3,4], respectively. Although virulence factors associated with pathogenic *B. cereus* isolates are not understood, large plasmids are known to be associated with many of the soil and pathogenic isolates [5] and are likely to impart advantageous phenotypes that promote opportunistic pathogenic properties and/or growth in soil. Recent studies now demonstrate that the "genetic backbones" for both the pXO1 and pXO2 plasmids are not restricted to *B. anthracis* but rather can be found in related *B. cereus* and *B. thuringiensis* isolates as well [6–9].

Importantly, several close relatives of *B. anthracis* were recently identified because they were associated with diseases that resembled anthrax [10–14]. Whole-genome sequencing of one of these isolates, *B. cereus* G9241, revealed a homolog of pXO1 that includes an expressed *pagA* gene and a complete pathogenicity island. This isolate did not harbor pXO2, but it did express a capsule under experimental conditions that is not poly-γ-D-glutamic acid. Two other *B. cereus* isolates (03BB102 and 03BB108) with clinical properties similar to *B. cereus* G9241 have recently been characterized and shown by PCR to be positive for the pXO1 toxin complex and, in one case (03BB102), positive for the pXO2 *cap* genes [13]. Unlike *B. anthracis,* neither of these isolates was sensitive to the γ-phage, and both were penicillin-resistant. A third series of isolates was obtained from chimpanzees that died in Tai National Park, Côte d'Ivoire (CI), and from chimpanzees and a gorilla that died in Dja Reserve, Cameroon (CA), reportedly from an anthrax-like disease [12]. Isolates from CI were genetically indistinguishable, but different from those obtained from CA. The CI and CA isolates contained the *pagA* gene and *capC* genes as measured by real-time PCR assays, suggesting the presence of pXO1- and pXO2-like sequences [11].

What, then, is *B. anthracis*? Should these new isolates be categorized as *B. anthracis*? Should the definition be based purely upon clinical disease definitions or based upon other phenotypes? Historically, conventional bacteriology has suggested that motility, hemolysis, and the production of capsule were the only useful markers that could distinguish *B. anthracis* from *B. cereus* [15]. Hoffmaster et al. have taken a similar tack in maintaining the *B. cereus* nomenclature for the *B. cereus* G9241 isolate by expanding the strict definition of *B. anthracis* at the United States Centers for Disease Control and Prevention Special Bacteriology Reference Laboratory to include the following phenotypic and biochemical properties: (a) capsule-producing, (b) nonmotile, (c) susceptible to γ-phage, (d) nonhemolytic, (e) susceptible to penicillin, and (f) having other cell-wall, capsule, and 16S RNA features [10]. More recently, a definitive molecular genotypic marker has been found in the *B. anthracis plcR* gene in the form of a nonsense mutation [16]. This mutation was present in all *B. anthracis* isolates (89) tested but not in any of an array of close and distant *B. cereus* relatives [17]. While this paper was in review, an additional study of the CI and CA isolates showed that they are motile bacilli and that their primary cultures are not susceptible to the γ-phage [14]. These results, combined with several other features—including the lack of the nonsense mutation in the *plcR* gene and the absence of large *B. anthracis*–specific prophage regions in their chromosomes—indicate that the CI and CA isolates are not *B. anthracis*.

All currently accepted isolates and strains of *B. anthracis* fall into a monophyletic clade, and only the combined use of rapidly evolving variable number tandem repeat markers and single nucleotide polymorphism analysis using whole-genome comparisons allowed for discrimination between individual isolates and construction of a highly accurate phylogeny with precise rooting for this species [18–20]. These results led to the conclusion that *B. anthracis* was derived from the clonal expansion of a single ancestral *B. cereus* that acquired the two virulence plasmids and the nonsense *plcR* mutation. Strains that diverged close to this species boundary are being discovered principally because they share many *B. anthracis*–like traits, but correct nomenclature is dependent on determining where isolates fall in relation to this boundary.

B. cereus G9241 and the CI/CA chimpanzee isolates diverged from the *B. anthracis* ancestor before the species boundary and are not included in the *B. anthracis* clade. Amplified fragment length polymorphism analysis (AFLP) indicates that *B. cereus* G9241 falls into a large cluster that includes *B. anthracis* and a number of clinical isolates known as AFLP group F [21,22]. Included in this AFLP cluster are two of the closest confirmed relatives of *B. anthracis: B. cereus* E33L and *B. thuringiensis* 97–27 [21]. Neither of these genomes contains a

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Abbreviations: AFLP, amplified fragment length polymorphism analysis; CA, Cameroon; CI, Côte d'Ivoire

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pXO1 plasmid, but *B. thuringiensis* 97–27 has a pXO2-like plasmid that lacks the pathogenicity island that contains the synthetic machinery for the polysaccharide capsule [9]. Both of these genomes contain what appear to be active *plcR* genes. Multilocus sequence typing of the CI and CA isolates indicates that they are close relatives of *B. thuringiensis* 97–27 [14], which would therefore place them in close proximity to the *B. anthracis* species boundary, albeit clearly on the side of *B. cereus*.

The *B. cereus* G9241 isolate and the CI and CA chimpanzee/gorilla isolates have another common feature. These three have *pagA* gene sequences that contain two mutations at positions 1,999 and 2,672 that result in serine-to-proline and isoleucine-to-serine amino acid changes that appear exclusively in these isolates [10,12]. These unique and shared mutations suggest that the pXO1 plasmids from the CI, CA, and *B. cereus* G9241 strains are closely related. Two models can be suggested for the existence of these *B. anthracis*–like plasmids in these non–*B. anthracis* isolates: (a) these sister taxa may have acquired the virulence plasmids or genes by lateral gene transfer of a promiscuous pXO1 from an ancestral *B. anthracis* into at least two divergent *B. cereus* ancestors from the AFLP F group, or (b) an ancestor outside the *B. anthracis* species boundary may have acquired the pXO1 and pXO2 plasmids, which were subsequently lost by some descendents. In both cases, the presence or absence of the virulence plasmids is not diagnostic and still begs the question, what is anthrax?

A strict phylogenetic definition for a clonally derived *B. anthracis* lineage has been documented over a number of decades and includes a battery of phenotypic and biochemical properties unique to this species. A newer group of *B. cereus* isolates has now been identified because they caused anthrax-like illnesses. Members of this group possess pXO1- and pXO2-like plasmids, and at least one has been shown to express the *pagA* gene. But these isolates differ from *B. anthracis* because the plasmids and the chromosomal background are distinct from those of the monophyletic *B. anthracis* clade. Despite anthrax-like disease manifestations, there are many unknowns left to be deciphered. Is the presence of pXO1 and pXO2 in a different genetic background sufficient to cause anthrax? Hoffmaster et al., for example, point to the presence of fully functional *atxA* and *plcR* regulatory genes as possibly being incompatible in *B. anthracis* [10,16]. Would this potential conflict affect the overall phenotype of a fully functioning pXO1? These questions should be considered in the context of a large body of information regarding history, etiology, epidemiology, pathology, evolution, vaccines, structure/function of toxins, host interactions, genetics, and regulation that was used to define the nomenclature for a classic *B. anthracis* (*B. anthracis* sensu stricto). The recently discovered strains that cause an anthrax-like disease should be defined as "*B. cereus/B. anthracis* sensu lato" until phylogenetic relationships and phenotypic characteristics can be firmly established. The benefits of such nomenclature are two-fold. First, confusion and potentially misplaced public concern regarding this widely recognized biological agent could be avoided. Secondly, since this loose designation would eventually be updated with more information, erroneous initial designations would not be perpetuated through scientific databases and publications.

Despite the presence of multiple, well-established phenotypic and molecular markers to define *B. cereus, B. thuringiensis,* and *B. anthracis,* there are often isolates that are misdesignated because of unusual properties (for instance, *B. thuringiensis* 97–27 [23]) or because of a lack of sufficient information [11,12]. For example, subsequent and more thorough analyses of the CI and CA chimpanzee isolates have demonstrated that they are not in fact in the monophyletic clade that defines *B. anthracis* [14]. An initial designation of the CI and CA isolates as *B. cereus/B. anthracis* sensu lato could have avoided the initial misdiagnosis and erroneous conclusions presented in Leendertz et al. [12] and in their subsequent opinion [11]. In the United States, select agents are highly regulated and "dual-use" research could become highly regulated as well (http://www.biosecurityboard.gov). The designation *B. anthracis* in publications requires responsible practices in such a politically charged environment.

At present there does not appear to be a single molecular trait that defines the sensu lato class. Pneumonia-causing *B. cereus* isolates can harbor either one or both of the *B. anthracis* plasmids, and they may or may not harbor functional anthrax toxin genes [10,14,24]. In addition, while most of the isolates that reside in the *B. cereus/B. anthracis* sensu lato class appear to be part of a single AFLP phylogenetic cluster, not all the residents of this cluster would cause anthrax-like disease [22]. The investigation of several cases of fatal respiratory illness apparently caused by *B. cereus* isolates harboring *B. anthracis*–like *pagA* sequences has created a new clinical awareness for anthrax-like manifestations. These cases have been largely ignored and treated as *B. cereus* contaminants in the past [10,24]. New *B. cereus/B. anthracis* sensu lato strains that cause anthrax-like illness in humans, gorillas, and chimpanzees appear to reside at the boundary between *B. cereus* and *B. anthracis,* and these new isolates may shed light on the evolutionary acquisition of the diagnostic characters that define *B. anthracis* sensu stricto. ■

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