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Occurrence, recognition, and reversion of spontaneous, sporulation-deficient *Bacillus anthracis* mutants that arise during laboratory culture

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Abstract

Bacillus anthracis is a spore-forming, soil-dwelling bacterium. This review describes the occurrence of spontaneous mutations leading to loss of sporulation and the selective pressures that can lead to their enrichment. We also discuss recognition of the associated phenotypes on solid medium, thereby allowing researchers to employ measures that either prevent or favor selection of sporulation-deficient mutants.

Keywords

Bacillus anthracis; sporulation; spo0A; mutation; Amerithrax

1. Introduction

The ability to form endospores sets the *Bacillus* genera apart from many others. This trait allows the bacteria to survive for long periods in harsh and unfavorable conditions, such as heat, desiccation, and low nutrient availability. In nature, *Bacillus anthracis* spores can reside in the soil for decades before they are inhaled by grazing animals, which initiates germination and vegetative growth, followed by production of capsule and anthrax toxins. The anthrax letter attacks in 2001, which became the subject of the FBI's "Amerithrax" investigation, involved mailing of *B. anthracis* spore preparations to several news organizations and to two U.S. Senate offices, leading to the death of five people and infection of many others. In nature, the formation of spores, usually induced by starvation signals, is an essential part of the developmental life cycle of *B. anthracis*. However, in the rich media used in laboratory cultures, where nutrient availability is not obviously limited and the pressure to sporulate is low, spontaneous, and initially silent, mutations in genes involved in sporulation pathways can accumulate, leading to loss of the ability of *Bacillus* to initiate or complete the sporulation cycle.

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2. Sporulation-deficient mutants spontaneously formed by *Bacillus* have a distinct phenotype on solid medium

The frequency of spontaneous mutations occurring in *B. anthracis* was estimated to be between 10^{-7} to 10^{-9} per base pair per generation [1]. Mutations can have many origins, including replication errors, DNA damage caused by mutagens, and ineffective repair mechanisms [2]. Because of the low frequency with which these errors occur, they are generally lost during propagation of a population. However, it was shown that in certain liquid growth media, asporogenic bacteria can quickly accumulate and become a large fraction of a population, as was observed for several different *Bacillus* species [3,4,5]. It appears that in these cases the growth or passage conditions imposed an unrecognized selective pressure that favored the growth or survival of sporulation-defective mutants. As long as growth was continued in a liquid culture, the identification and elimination of the spontaneous, asporogenic members of the population was not possible.

In contrast to the situation in liquid medium, *B. anthracis* mutants grown on solid media that fail to initiate sporulation are easily recognizable by their unique colony morphologies. In comparison to their sporogenic counterparts, colonies consisting of asporogenic bacteria are more translucent and larger [6], or can have a yellow or yellow-gray color [7]. The addition of certain indicator dyes such as Congo Red to solid media can enhance the differences in appearance between sporulating and non-sporulating colonies [8] (Fig. 1). We have also noticed this atypical colony phenotype of *B. anthracis* in less than 1% of colonies after 2 days of growth on some solid media such as Luria-Bertani, while it was less obvious on other media [9]. Additionally, factors such as the age of colonies, the number of passages performed for a single colony, and the type of solid medium on which bacteria are maintained can greatly influence the frequency of mutants altered in sporulation pathways. In particular, we found frequent mutations of various kinds in the global regulator Spo0A, which is involved in the initiation of sporulation [6]. Loss of Spo0A function leads to a complete block of sporulation.

3. Key role of sporulation-impaired *B. anthracis* mutants in the Amerithrax investigation

The accumulation of mutant bacteria within a laboratory culture as described above played a key role in solving the Amerithrax investigation [7]. The microbial forensics studies performed in support of the investigation produced a unique fingerprint of the culture used in the anthrax letter attacks and allowed it to be traced back to the presumed perpetrator's laboratory. Rare colonies within the population were noted to have aberrant morphologies ("morphotypes"). The genomes of these variants were sequenced and found to be highly similar to the Ames Ancestor strain, which is studied in laboratories worldwide [10]. Furthermore, these analyses showed that four chromosomal loci harbored mutations unique to the variants present in the spore preparations of the anthrax letters. Of these four loci, three could be linked to Spo0F, a bacterial response regulator that, like Spo0A, is activated at the onset of sporulation [11,12,13]. Unlike the totally sporulation-deficient Spo0A mutants that we obtained [9], the mutants identified in the anthrax letter cultures retained a limited ability to form spores, as expected considering their isolation from spore preparations. Interestingly, some of the mutations occurred through mechanisms like those that caused the Spo0A mutations we found. For example, both groups of mutants included mutations caused by illegitimate recombination events [14,15] that occurred between short direct repeats, resulting in deletion of a larger region of the genome. Thus, it appears that the culture that was the origin of the spores used in the letter attacks had been repeatedly

passaged and/or expanded in a way that enriched for variants that sporulated poorly. We discuss below mechanisms that may have led to this enrichment.

4. Possible reasons for the selection of asporogenic mutants during laboratory culture

Laboratory manipulations as simple as growth in a specific medium unintentionally impose selective pressures that can lead to the enrichment of bacteria harboring particular mutations. Thus, cultures passed repeatedly can quickly acquire a genetic fingerprint unique to a bacterial culture that is kept in a particular laboratory. However, the mechanisms that drive induction, selective growth, and enrichment of asporogenic mutants are not well understood. While a shortened lag phase could be an explanation for the larger colony size observed in asporogenic colonies of *B. anthracis* [9], in *B. subtilis* it was found that asporogenic mutants with mutations in particular sporulation-dependent genes have an improved overall growth rate and higher biomass yield [16]. The higher biomass yield in those *B. subtilis* mutants could result from a loss of the cannibalistic behavior described for *B. subtilis*, whereby sporulating bacteria eliminate their non-sporulating siblings to acquire nutrients [17,18]. Asporogenic bacteria with a mutated Spo0A would not be able to kill their neighbors, resulting in a higher overall biomass.

An alternative phenomenon that might select for sporulation-defective mutants also depends on competition for nutrients. When bacteria grow on agar plates, it is likely that the very dense population of bacteria in a colony ($>10^7$ organisms in a 2–3 mm colony) will lead to severe competition for the nutrients that slowly diffuse into the colony from the surrounding agar. The resulting nutrient deficiency will be recognized as a signal to sporulate, especially by bacteria at the center of the colony, so that the wildtype members of the population will stop dividing and sporulate. Any rare spontaneous mutant bacteria that fail to recognize or successfully act on the signal to sporulate will continue to grow, perhaps making use of nutrients released by lysis of mother cells of nearby spore-forming bacteria. In this way, the sporulation mutants can become enriched as the colony ages.

5. Advantages and disadvantages of sporulation-deficient mutants for research

Bacillus mutants defective at various stages of sporulation have proven invaluable for the analysis of biochemical markers characteristic of sporulation, the identification of genes involved in sporulation, and for the elucidation of complex sporulation pathways. For example, early studies comparing sporogenic and asporogenic colonies of *B. subtilis* showed that certain proteases and antibiotics are only secreted by sporulating bacteria [19,20]. Similarly, the many genomic loci required for sporulation were identified by transduction analyses (for reviews see [6,21]) and later by cloning of genes involved in these pathways [22,23,24,25,26]. The rate with which *Bacillus* loses the ability to sporulate in liquid culture has further been useful for the analyses of mutation frequencies as a model for calculating the rate with which trait losses occur during evolution [27,5].

Bacillus species are valuable as expression hosts for production of industrial enzymes (e.g., proteases), as recently reviewed [28], and are offered commercially as expression hosts to the research market (e.g., *Bacillus megaterium* by MoBiTec, Inc.). The currently licensed (non-recombinant) anthrax vaccines are partially purified supernatants of attenuated *B. anthracis* strains [29], and some candidate second generation, recombinant anthrax vaccines are produced in improved *B. anthracis* host strains. An improvement relevant to this discussion is to render the expression host unable to sporulate. Strains that are sporulation deficient can be isolated as spontaneous mutants using the simple morphological screens

mentioned above and then validated by sequencing [9,8], or can be engineered by intentional deletion of specific sequences [30]. Thus, strains derived by these methods and specifically deleted in the *spo0A* gene are being used to produce candidate anthrax vaccine proteins [31,8,32,33], although whether mutations in *spo0A* or other sporulation-related genes enhance toxin production has not yet been determined. Nevertheless, in this and several other laboratories, Spo0A-deleted strains are routinely used to produce recombinant anthrax toxin proteins [34,35]. Sporulation-deficient mutants are advantageous as expression hosts because they eliminate the risk that a laboratory, production facility, or expensive fermentation equipment may become permanently contaminated with viable spores. Asporogenic *B. anthracis* strains can die rapidly at the end of a batch culture process; within the first 48 h after inoculation in rich medium, a reduction in viable cells of least 100-fold can be observed (Sastalla and Leppla, unpublished data). Apparently, once a signal to sporulate is produced by nutrient depletion, the bacteria enter a “do or die” program. If the bacteria initiate the sporulation process but cannot execute it successfully, they undergo an alternative death process. We have demonstrated this for the *spo0A* mutant strain BH460, a protease-deletion strain that was developed and is used in our laboratory [31,8,32,33].

Conversely, for studies of *B. anthracis* pathogenesis, sporulation-deficient strains are of little value and in fact constitute a problem. Since nearly all animal models of *B. anthracis* virulence involve administration of spores, a strain that has lost the ability to sporulate cannot easily be compared to other strains. Thus, the accidental generation and selection of spontaneous asporogenic mutants during genetic manipulation of other, defined loci, is undesirable, and if not promptly recognized, may later require that the experiments accidently performed with genetically-altered strains be repeated, once the strain has been “repaired” (see below) or constructed again.

6. A method to reverse accidentally generated asporogenic mutants

We found that accidentally generated asporogenic mutants can be “repaired” and that complex genetic strain construction projects can be rescued by applying phage transduction to restore sporulation [9]. The bacteriophage CP51 [36] has been found useful for generalized transduction in *B. anthracis*, where it has enabled transfer of the virulence plasmid pXO2 [37] and marked mutations from one strain to another [38]. The thermo-sensitive version of CP51 (CP51ts) is a lytic phage that occasionally packages random fragments of up to 60 kb of host DNA instead of the viral genome. These large fragments of DNA are homologous to the DNA of the recipient strain and can replace portions of the genome by homologous recombination. These rare events will often not be evident or retrievable, except when the incoming DNA contains a selective marker. In the present case, the restoration to an asporogenic population of the ability to produce heat-resistant spores provides such a selection. By infecting asporogenic *B. anthracis* with CP51ts propagated on sporulation-sufficient bacteria, we successfully reverted asporogenic derivatives [9], thereby allowing the reversion and preservation of desirable strains.

7. Conclusions

The observation that bacteria exhibit spontaneous mutations that can result in distinct phenotypes is not new. For example, in *Salmonella typhimurium*, spontaneously occurring mutations in the gene encoding the response regulator PmrA result in an increased resistance of these mutants to the antibiotic polymyxin B [39]. In other bacteria such as *Yersinia pestis* and *Mycoplasma pneumonia*, spontaneous mutations can lead to loss of virulence [40,41], while it was observed for *Streptococcus pyogenes* that during animal infection, *in vivo* selection pressures enrich for strains containing spontaneous mutations in a regulator responsible for repression of capsule expression, thereby resulting in increased virulence of

the mutants [42]. Thus, it is important that researchers not only pay attention to obvious bacterial morphotypes observed on agar plates, but also ensure that observed phenotypes of genetically engineered deletion strains are not caused by accidental secondary mutations introduced during laboratory passage.

Bacterial strain stocks are usually kept in laboratory freezers for years, and one can draw from these stocks with confidence that their genotypes have not changed. In contrast, strains that are serially and frequently passaged may contain unrecognized mutations. The definitive identification of the source of the particular culture used in the anthrax letter attacks, through analysis of the phenotypic and sequence heterogeneities of the rare mutants in the culture [7], provides a good example of how mutation can impact genetic uniformity. Most researchers soon become aware through their own experience that genetic variation is common, and design their experiments to prevent this, or to detect it when it occurs. The ever-decreasing cost of DNA sequencing offers the prospect that before long it will become routine to sequence a bacterial isolate at both the beginning and end of an experiment (e.g., after re-isolation from an experimentally infected animal). This approach is likely to produce frequent instances where cultures considered to be pure will be found to contain a variety of distinct mutations. More importantly, resequencing of isolates is likely to decrease the instances where significant phenotypic changes that are attributed to particular genetic changes are in fact due to unrecognized spontaneous mutations at other loci. For *B. anthracis*, spontaneous mutations often result in the alteration of sporulation efficacy. Because some genes involved in sporulation also impact functions in vegetative cells, these spontaneous mutations may produce phenotypes that will be incorrectly attributed to other, known genetic alterations. Awareness of this hazard may decrease the frequency of such events.

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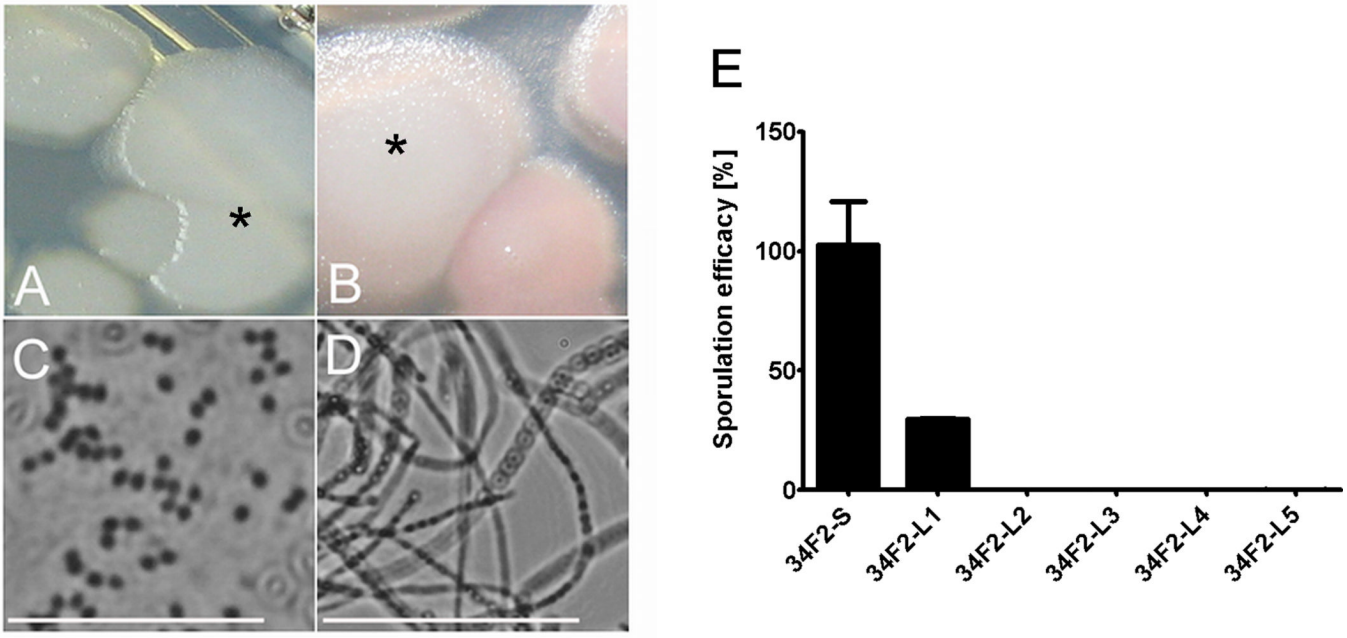

Figure 1.

Colony phenotype of *B. anthracis* on (A) LB and (B) LB containing Congo Red, an indicator that allows phenotypic identification of asporogenic *B. anthracis* by lack of salmon color. Asterisks indicate large (asporogenic) colony morphologies. Microscopy of spores and bacteria derived from (C) small and (D) large colony phenotype. Bar represents 25 μm. (E) Sporulation efficacies of 5 large, asporogenic (34F2-L1 through L5) and 1 small, sporogenic (34F2-S) colony phenotypes, measured by colony counts retrieved before and after heat-treatment.