Effects of Long-Term Storage on Plasmid Stability in Bacillus anthracis

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The plasmid profiles of 619 cultures of Bacillus anthracis which had been isolated and stored between 1954 and 1989 were analyzed using the Laboratory Response Network real-time PCR assay targeting a chromosomal marker and both virulence plasmids (pXO1 and pXO2). The cultures were stored at ambient temperature on tryptic soy agar slants overlaid with mineral oil. When data were stratified by decade, there was a decreasing linear trend in the proportion of strains containing both plasmids with increased storage time (P < 0.001). There was no significant difference in the proportion of strains containing only pXO1 or strains containing only pXO2 (P = 0.25), but there was a statistical interdependence between the two plasmids (P = 0.004). Loss of viability of B. anthracis cultures stored on agar slants is also discussed.

Preservation of microorganisms is an important component of microbiological research. Maintenance of a well-defined collection of isolates is crucial for providing reproducible results and continuity in research, as well as for validating newly developed diagnostic and detection methods and treatment and postexposure prophylaxis protocols. While the ultimate objectives for preservation are maximum viability, genetic stability, and prevention of contamination, the preservation method is often chosen primarily based on ease of recovery and cost. Historically, the preservation methods that have been used include storage on agar slants, lyophilization, cryopreservation, and desiccation. There have been several reports regarding the effect that a chosen preservation method can have on bacteria (1, 3, 9, 12), but no study has focused specifically on regarding the effect that a chosen preservation method can have on bacteria (1, 3, 9, 12), but no study has focused specifically on

**MATERIALS AND METHODS**

**Isolates.** Of 1,150 storage slants, 769 were cultured successfully; the isolates on the remaining slants were no longer viable. All isolates had been stored at room temperature on tryptic soy agar slants overlaid with mineral oil in glass screw-cap tubes. The collection was stored in several different physical locations at the Centers for Disease Control and Prevention. For each slant, the mineral oil was removed from the tube, and 5 ml of heat infusion broth (Remel, Lenexa, KS) was added. The surface of the agar was scraped with a loop to suspend spores on the slant in the broth. The tube was then incubated at 37°C for 18 to 48 h. After incubation, the broth was subcultured on a blood agar plate (Trypticase soy agar with 5% defibrinated sheep blood [SBA]; BD Diagnostic Systems, Sparks, MD). The SBA plate was incubated at 37°C for 18 to 48 h. Colonies were analyzed using standard microbiological procedures (11), such as lack of hemolysis, ground-glass appearance, flat, opaque, tenacious, and grayish-white, and gamma phage susceptibility, and were processed for PCR analysis.

**Real-time PCR for B. anthracis.** A DNA template for each recovered isolate was obtained by heat lysis of a single colony after overnight growth on SBA. Using a 1-μl loop, one colony was suspended in 10 μl of 10 mM Tris-HCl (pH 8.0) in a 1.5-ml tube containing a 0.22-μm filter unit (Millipore, Bedford, MA). The suspension was heated at 95°C for 20 min and centrifuged in a microcentrifuge at 6,000 × g for 2 min. The filter unit was discarded, and the cell lysate was held at −20°C until testing. Five microliters of lysate was used in each PCR. Isolates were assayed using the Laboratory Response Network real-time PCR assay as described by Hoffmaster et al. (7). The assays were performed with Lightcycler (Roche Diagnostics GmbH, Mannheim, Germany) or Smart Cycler (Cepheid, Sunnyvale, CA) instruments. All three primer-probe sets (targeting both B. anthracis virulence plasmids and a chromosomal marker) were used.

**Statistical analysis.** The degree of association between plasmid profiles and decade of storage was measured using a chi-square statistic. The trend in proportions of recovered B. anthracis isolates containing both plasmids by decade of storage was examined using the chi-square for linear trend statistic.

**RESULTS**

The recovery rates were significantly different for isolates from the first two decades (1950s and 1960s) (423/731 isolates, 58%) than for isolates from the last two decades (1970s and 1980s) (349/419 isolates, 83%) (P < 0.001). The identities of 619 recovered isolates were confirmed to be B. anthracis by gamma phage lysis and the Laboratory Response Network real-time PCR; 127 slants yielded nonanthrax Bacillus species, while 23 isolates gave inconsistent PCR and gamma phage susceptibility results requiring further study at a later date. Only the 619 confirmed B. anthracis cultures were evaluated in this study.

The B. anthracis isolates in the current study were collected and stored from 1954 to 1989; 275 isolates were collected from...
TABLE 1. Plasmid profiles of 619\textit{B. anthracis} isolates collected from 1954 to 1989

<table>
<thead>
<tr>
<th>Years</th>
<th>Total no. of isolates</th>
<th>pXO1(^{-}), pXO2(^{-})</th>
<th>pXO1(^{+}), pXO2(^{-})</th>
<th>pXO1(^{-}), pXO2(^{+})</th>
<th>pXO1(^{+}), pXO2(^{+})</th>
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<tbody>
<tr>
<td>1954–1966</td>
<td>275</td>
<td>118 (43%)</td>
<td>49 (18)</td>
<td>16 (16)</td>
<td>64 (23)</td>
</tr>
<tr>
<td>1974–1979</td>
<td>319</td>
<td>84 (26)</td>
<td>60 (19)</td>
<td>39 (12)</td>
<td>136 (43)</td>
</tr>
</tbody>
</table>

1954 through 1966, 319 isolates were collected from 1974 through 1979, and 25 isolates were collected from 1981 through 1989. No isolates were collected from 1967 to 1973 and in 1980. Information regarding the sources and/or geographic origins of the isolates was available for 446 of the 619 \textit{B. anthracis} isolates. There were 37 human, 45 animal, and 364 environmental isolates from 23 of the states in the United States and nine other countries.

PCR results for 619 \textit{B. anthracis} isolates indicated that lengthy storage may have affected the plasmid stability of these isolates. For isolates collected in the 1950s and 1960s there was a much higher percentage of strains that were cured of both plasmids (43\%) than there was for strains collected and stored in the later decades (26\% for 1970s isolates) (Table 1). In addition, the proportion of isolates which were stored for a shorter period that harbored both virulence plasmids was higher (43\% for 1970s versus 23\% for 1950s and 1960s).

Although plasmid profiling was not done at the time when the \textit{B. anthracis} cultures were collected, it is reasonable to assume that all human and animal isolates (\(n = 82\); collected from 1955 to 1979) were fully virulent, as both plasmids are needed to cause disease, and therefore contained both plasmids at the time of collection. In addition, detection of a capsule (genes located on \(pXO2\)) was part of the phenotypic identification procedure for \textit{B. anthracis} for isolates collected after 1972. Although there are a limited number of isolates, Table 2 gives an indication of the plasmid loss with increased time in storage for isolates that were fully virulent when they were first collected and stored. While 45\% of the human and animal isolates stored in the 1950s and 1960s were cured of both plasmids, only 29\% of the human and animal isolates from the 1970s were cured of both plasmids. Conversely, 52\% of the 1970s isolates retained both virulence plasmids, while only 22\% of the 1950s and 1960s isolates had both plasmids.

For environmental isolates collected in the same time period (\(n = 364\)), it cannot be assumed that they were all fully virulent at the time of collection. However, the changes in the proportions of the four plasmid categories paralleled the changes in the human and animal strains (Table 2), indicating that the majority of environmental strains were most likely also fully virulent when they were collected. Altogether, when data were analyzed by decade, as storage time increased, the proportion of recovered \textit{B. anthracis} isolates containing both plasmids decreased linearly (\(P < 0.001\)). In addition, there was no significant difference in the proportion of strains cured of only one plasmid (pXO1 versus pXO2) over time (\(P = 0.25\)). However, a chi-square analysis aimed at determining whether loss of one of the plasmids affected the loss of the other plasmid indicated that the two plasmids were interdependent (\(P = 0.004\)).

**DISCUSSION**

The loss of viability with increased duration of storage provides valuable insight into the longevity of anthrax spores. Much has been written about the ability of anthrax spores to remain viable for decades, but there are few reliable data. Wilson and Russell reported that anthrax spores survived in dry soil for 60 years (16). Jacquot and Virat found that anthrax spores prepared by Pasteur in 1888 were viable 68 years later (8). In 1992, Bowen and Turnbull found \textit{B. anthracis} in the plaster and lagging of London’s Kings Cross railway station roof space and attributed this to the use of contaminated horse hair to bind the plaster when the building was constructed a century earlier (P. C. B. Turnbull, unpublished data and personal communication). The longest claim is that of De Vos, who recovered anthrax spores from bones recovered during an archaeological excavation at a site in Kruger National Park in South Africa, which were estimated by carbon dating to be 200 ± 50 years old (4). Conversely, Turnbull reported the loss of viability of \textit{B. anthracis} and other Bacillus species stored on agar slants in his collection at the Centre for Applied Microbiology and Research, Porton Down, England, which occurred particularly when the lids of the bottles containing the storage slants had become loose, allowing the agar to dry (Turnbull, personal communication), a situation similar to the situation described in this study. While it is not known why this difference in viability exists for long-term storage of \textit{B. anthracis}, some factors that may play a role include the original storage conditions (dry storage versus overlay in mineral oil), drastic changes in storage (e.g., drying out of agar), or the sporulation conditions.

In this study, 85 isolates were found to harbor only pXO2. Environmental isolates of \textit{B. anthracis} lacking pXO2 have been recovered from sites with a history of anthrax spore contamination in the distant past (15). Less frequently, isolates lacking both plasmids have been found, but, in interesting contrast to the results presented in this paper, naturally occurring isolates harboring only pXO2 have never been found in the environment. For the isolates in this study, there was no significant difference in the proportion of strains containing only pXO1 or only pXO2 over time (\(P = 0.25\)). However, the chi-square statistic indicated that the loss of the two plasmids was interdependent (\(P = 0.004\)). Due to the nature of the study presented here, in which all isolates were recovered and analyzed for plasmid loss at one time, it is difficult to determine the
dependence of the two plasmids on plasmid stability. Ideally, in
order to determine the interdependence of plasmid stability,
isolates should be monitored for plasmid loss at periodic in-
tervals over time. Thus, any conclusions based on the data
provided in this study regarding interdependence of plasmid
stability or loss would be speculative at best. However, Bowen
and Quinn observed that some control elements governing the
maintenance of both plasmids were at least partly located on
pXO2 (2).

Under appropriate environmental conditions, such as within
sewage or in the harsh conditions of Etosha National Park in
Africa, B. anthracis could be spontaneously cured of one or
both virulence plasmids (15). As mentioned above, the collect-
ion of isolates included in this study was stored in various
locations, and, although the exact history of the collection’s
storage is unknown, anecdotal reports indicate that the cul-
tures were transported to various rooms spacious enough to
hold a collection of this size. The inconsistent conditions of the
various storage rooms in which the cultures were held may
have contributed to the plasmid instability.

To our knowledge, there have been no other studies regard-
ing the effects that different storage methods have on B. an-
thracis vegetative cells or spores. However, various studies
have evaluated different preservation methods. While not nec-
ecessarily applicable to B. anthracis, these studies, which mostly
compared the effects of lyophilization to the effects of freezing
or cryopreservation, provide some insight into the effects of
preservations methods on certain bacteria. Ashwood-Smith (1)
and Sinskey and Silverman (14) reported that lyophilization
may cause mutations in DNA and/or damage to the cell mem-
brane, resulting in increased permeability during the preser-
vation process. On the other hand, Breese and Sharp (3) and
Sidjakina et al. (12) concluded that while lyophilization re-
duced viability, it did not affect the genetic stability of Esche-
richia coli. Niermans and Feldblyum reported that preservation
of Bacillus subtilis vegetative cells by lyophilization resulted in
reduced viability and retention of the pTL12 plasmid in viable
cells after 10 months of storage at 4°C, although lyophilization
of B. subtilis spores had no effect on viability or plasmid reten-
tion (10). However, none of the studies covered storage peri-
ods of more than 2 years or evaluated the effect of long-term
preservation on a large number of strains of any one species. In
general, most reports indicated that cryopreservation is likely
the best method for preserving the viability and genetic sta-
lility of strains (13). Currently, the permanent storage method
for the isolates in this study is to prepare spore suspensions
from fresh cultures and to store these suspensions at −70°C in
water containing 25% glycerol.

The mechanisms of plasmid loss from the B. anthracis iso-
lates used in this study are not known. However, it is possible
that there may be some genetic damage over time that may
affect plasmid replication or partitioning and result in plasmid
loss upon germination and vegetative growth. It is also possible
that spores stored on media (such as slants or stabs) exhibit low
levels of sporulation and germination resulting from leftover
nutrients from the media and amino acids and sugars released
during mother cell lysis. Under these stressful conditions, non-
essential elements, such as plasmids, could be cured. Stresses
such as elevated temperatures (42°C) and the addition of no-
lovobioin to laboratory media have been shown to result in the
loss of pXO1 and pXO2, respectively (5). Green et al. observed
spontaneous loss of pXO2 from colonies of the Pasteur strain
during growth on agar for several days under conditions con-
ductive to capsule formation (6). In addition, as mentioned
above, Sinskey and Silverman reported that lyophilization may
damage the cell membrane, causing increased permeability
during the preservation process (14). While this may not occur
in B. anthracis plasmid loss, it is another mechanism by which
plasmids may be lost from the cell.

This is the first documentation of the effects of long-term
storage on the plasmid stability of B. anthracis. The length of
time and the storage method may have had an effect on the
plasmid retention in the recovered isolates, as the majority of
isolates were cured of at least one plasmid. Further studies to
evaluate other media that could be used for long-term storage,
as well as studies to determine the exact nature of the plasmid
curing, are needed.

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