Strain stability in a research culture collection is critical for valid and consistent experimental results. The University of South Florida Center for Biological Defense (CBD) stocks well-characterized methicillin-resistant *Staphylococcus aureus* (MRSA) strains for use in validating molecular typing methods such as multilocus sequence typing, staphylococcal protein A typing, multilocus variable-number tandem repeat analysis, pulsed-field gel electrophoresis (PFGE), and staphylococcal cassette chromosome mec (SCCmec) typing. The CBD collection largely consists of community-acquired strains received from hospitals and clinical laboratories in Florida and Washington state as well as strains obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) and from H. de Lencastre (13). Based on the known association of pulsed-field type with SCCmec type (15), the majority of the strains typed in our collection were USA300 (159 presumed SCCmec type IV), followed by USA100 (103 presumed SCCmec type II), and 84 sporadic pulsed-field types were SCCmec types II, IIA, III, IIIA, and IV and undetermined. The integrity and stability of mecA and SCCmec within our MRSA strains are critical to CB research.

In 2005, van Griethuysen et al. reported the loss of mecA in a MRSA strain collection after two years of cryostorage at −80°C in a Microbank bead-based preservation system (MBPS) (Pro-Lab Diagnostics, Austin, TX) (16). Prior to storage in the MBPS, the strains were previously held as long-term stocks at room temperature (RT). Two years later, approximately 14% of the collection stored in the MBPS no longer carried mecA.

This report prompted an evaluation of the stability of 360 MRSA strains from the CBD collection. After confirmation of methicillin resistance and phenotypic characterization from a single, representative colony, each MRSA strain was cryostocked using a standardized cryostorage protocol as follows. The progenitor colony was inoculated to 100 ml of tryptic soy broth (TSB) and incubated at 35°C for approximately 15 h in a shaking water bath at 125 rpm. Thirty milliliters of overnight culture was pelleted at 3,571 × g at RT for 10 min, resuspended in 25 ml of TSB with 10% glycerol, and aliquoted as 1 ml into cryovials. After 30 min of equilibration at RT with the cryoprotectant, cryovials were placed in Nunc controlled freezing units (Nalgene, Rochester, NY) following the manufacturer’s instructions and held at −85°C for 75 min to foster cooling of approximately −1°C/min to near −30°C. Immediately thereafter, cryovials were removed from the units and stored at −85°C.

One week after cryostorage, during which most cell death occurs (1), a single cryovial of each CBD MRSA strain was quick-thawed at 35°C and plate counts were obtained from a 1:10 dilution series to review viability and purity. To confirm that methicillin resistance had been maintained in the population after cryopreservation, disk diffusion susceptibility (DDS) was performed using 30-μg cefoxitin and 1-μg oxacillin antibiotic disks (Oxoid, Hants, United Kingdom) on Mueller Hinton (MH) plates. Zones of inhibition were measured after incubation at 35°C for 24 h, followed by incubation at 30°C for another 24 h. The Clinical and Laboratory Standards Institute (CLSI) standards for these antibiotics were used to interpret the resistance of the strains (3, 4). Control strains for this method and all others used in this study were a methicillin-susceptible *Staphylococcus aureus* strain, ATCC 25923 (American Type Culture Collection, Manassas, VA) and a confirmed SCCmec type IV MRSA strain, CBD 804 (HDE288). CB strains were also tested for the presumptive presence of PBP2, the mecA gene product, from cultures grown at 35°C for 24 h on blood agar following the manufacturers’ instructions (Oxoid, Hants, United Kingdom; Denka Seiken, Ltd., Derbyshire, United Kingdom).

At one- and two-year intervals, plate counts were performed on the 360 CBD MRSA strains to ensure that a robust heteroresistant population of MRSA had persisted after prolonged cryostorage at −85°C. CBD MRSA stocks were quick-thawed, and a 1:10 dilution series was spread in 100-μl volumes on tryptic soy agar (TSA) and MH agar containing 4% NaCl and 6 μg/ml oxacillin (MH-OXA) (Remel, Lenexa, KS). By comparing the CFU/ml of the entire population on TSA to that of the MRSA subpopulation on MH-OXA at each yearly time

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**Two-Year Study Evaluating the Potential Loss of Methicillin Resistance in a Methicillin-Resistant *Staphylococcus aureus* Culture Collection**

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Received 7 April 2008/Returned for modification 26 June 2008/Accepted 7 August 2008

A reported loss of mecA prompted us to monitor 360 cryostocked methicillin-resistant *Staphylococcus aureus* strains for stability. Concurrently, 14 well-characterized strains were stored in a Microbank preservation system and subjected to multiple freeze-thaw events. There were no significant declines in the methicillin-resistant populations with either method over a two-year period.

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Published ahead of print on 20 August 2008.
point, we were able to track the stability of the heteroresistant population. All the strains tested produced methicillin-resistant colony counts on MH-OXA equal to or within a log of the heteroresistant CFU/ml on TSA at one- and two-year time points. The means of the counts from year 1 on TSA and MH-OXA were $1.66 \times 10^5 \pm 4.28 \times 10^4$ and $1.26 \times 10^5 \pm 3.14 \times 10^4$, respectively, and in year 2, $1.89 \times 10^5 \pm 5.98 \times 10^4$ and $1.46 \times 10^5 \pm 3.87 \times 10^4$ (data not shown). Thus, none of the 360 cryostocked MRSA strains tested from the CBD culture collection demonstrated a significant decline in stability of their heteroresistant populations of MRSA after the two-year review (nonparametric Kruskal-Wallis test; $P = 0.7175$) (Table 1).

During this evaluation of our CBD MRSA collection stability, we also sought to recreate the loss of mecA reported by van Griethuysen et al. for strains stored in the MBPS (16). We subjected six previously described (13) MRSA strains comprised of SCCmec types I, IA, II, III, IIIA, and IV and eight PFGE types (USA100 through -800) obtained from NARSA to two years of cryostorage in the MBPS with periodic freeze-thaw events (Table 1). In contrast to our CBD cryostorage protocol, the MBPS technical instructions directed the inoculation of a cell suspension of young growth for each strain into a cryostorage vial containing 25 porous beads and a proprietary cryoprotectant. The vials were inverted five times and then immediately well aspirated of most liquid. This action gave the cells that had adhered to the beads very little equilibration with the cryoprotectant. The vials were frozen in an uncontrolled manner to $-85^\circ C$; the manufacturer recommends storage at $-70^\circ C$.

To monitor the 14 strains for a stable methicillin-resistant subpopulation, a single MBPS bead for each strain was tested at intervals spanning 1 to 6 weeks during the two-year period. At each time point, the MBPS vials were removed from $-85^\circ C$ and held on ice for approximately 10 to 15 min while a bead for each strain was aseptically transferred to 1 ml TSB with 10% glycerol at RT. The MBPS vials were immediately returned to $-85^\circ C$. Following 5 s of vigorous vortexing of each sacrificed bead per strain, plate counts comparing the heteroresistant population on TSA to the MRSA subpopulation on MH-OXA were performed as described above. The vials containing individual beads suspended in TSB with glycerol were placed directly in $-85^\circ C$ to freeze and were saved for future testing. Within 10 min on ice, the MBPS vials transitioned from approximately $-85^\circ C$ to near 0°C and rose to 5°C during handling to remove a bead, as determined by inserting a temperature probe among the beads. This procedure allowed us to replicate freeze-thaw events.

During cryostorage in the MBPS for 104 weeks, the 14 MRSA strains were subjected to 24 such freeze-thaw events. Thirteen strains did not have a significant decline in the methicillin-resistant population from initial storage in the MBPS to final evaluation at 104 weeks (nonparametric Kruskal-Wallis test; $P = 0.1528$) (Table 1). The means of the counts from week 0 on TSA and MH-OXA were $5.17 \times 10^5 \pm 1.59 \times 10^5$ and $2.62 \times 10^5 \pm 6.39 \times 10^4$, respectively, and in week 104, $8.10 \times 10^6 \pm 2.56 \times 10^6$ and $4.73 \times 10^6 \pm 1.23 \times 10^6$ (Table 1). Two strains struggled to tolerate a relatively high concentration of oxacillin from the beginning of the study. CBD 801 (N315), a SCCmec type II strain, repeatedly failed to grow on MH-OXA, and therefore, the methicillin-resistant cells in its population were not enumerated at each time point. CBD 804 (HDE288), a SCCmec type IV strain, also had difficulty thriving on MH-OXA, as it required extended incubation time to produce countable colonies. DDS and PBP2* latex agglutination testing performed at each freeze-thaw event on both strains demonstrated that they were resistant to 1 μg oxacillin and that the mecA gene product was presumptively present.

To investigate whether a decrease in resistance to methicillin occurred among the 14 strains over two years, we examined the stored beads from the first freeze-thaw event, which were col-
lected 1 week after initial storage in the MBPS, and from week 104, in which the beads had undergone the stress of 24 freeze-thaw events. Susceptibility tests using the microbroth dilution method were performed with a Sensititre system (TREK Diagnostics Systems, Cleveland, OH) following the manufacturer’s instructions and with CLSI standards (3). A methicillin-susceptible S. aureus ATCC 29213 strain was the control in all MIC testing. The microtiter plates containing ampicillin, penicillin, oxacillin, and vancomycin were incubated at 35°C for 24 h. The susceptibility tests gave identical or near-identical MIC testing. The microtiter plates containing ampicillin, penicillin, oxacillin, and vancomycin were incubated at 35°C for 24 h. The susceptibility tests gave identical or near-identical MICs (a <2-fold dilution difference) on all the antimicrobials for the sacrificed beads stored at week 1 and week 104 for all 14 cryostocked strains in the MBPS.

The presence of mecA was also tested in sacrificed beads stored at week 1 and week 104. Genomic DNA was extracted from overnight bacterial growth in TSB using a MagNa Pure LC instrument (Roche Diagnostics Corp., Indianapolis, IN) with the supplied DNA isolation kit III following the manufacturer’s instructions. PCR for mecA was performed as described previously (13). The results confirmed that the mecA gene was present in all 14 strains from their initial storage in the MBPS to the endpoint of our MBPS evaluation.

There are various insults to cells that occur during a freeze-thaw event (11, 12) that may explain the previously reported loss of mecA-positive cells (16). However, a distinction should also be made between the deletion or disruption of mecA and the overgrowth and eventual replacement of MRSA by a co-existing mecA-negative population. MRSA strains are heterogeneous, consisting of two subpopulations wherein the resistant population grows slower than the more-robust susceptible population (2). Stressful environments such as storage at RT and prolonged freezing with repeated freeze-thaw events can play a significant role in evolving lineages that are better able to survive these stresses (14). Therefore, the CBD protocol began with selection and enrichment of a predominantly MRSA population prior to preservation. Confirmation of a robust MRSA population was also done shortly after cryostorage in TSB with 10% glycerol.

The cryostocking process itself could have decreased the mecA-positive subpopulation, since it is known to inflict detrimental changes to bacterial cells, such as ice crystal formation, dehydration, decreased and/or increased activity of enzymes, accumulation of metabolites, increased molecular contacts, disruption of weak hydrogen bonds, distortion of molecules, and breakdown of the cell membrane (12). The CBD cryostocking protocol incorporates osmotic equilibration with a 10% glycerol cryoprotectant. Equilibration with a cryoprotectant minimizes damage during freezing by penetrating and stabilizing the cell membrane and delaying freezing. While we allow 30 min or more for cells to equilibrate with the glycerol cryoprotectant, the MBPS protocol allows <1 min for cells to equilibrate with the proprietary cryoprotectant. It is possible that the removal of the cryoprotectant leaves the mecA-positive cells in the MBPS more vulnerable to injury during freeze-thaw events. Also, the MBPS technical instructions do not specify how to handle the vials while retrieving beads. The injurious complexities of a freeze-thaw event stem largely from uncontrolled rates of cooling and warming during which ice in the cells (re)crystallizes (8, 11). The MBPS vials in our experiment were held on ice during bead retrieval, in keeping with a common practice that results in relatively rapid thawing and reduced cell injury (8, 11). Our standardized protocol includes a program of controlled, slow freezing to minimize intracellular ice formation, followed by uncontrolled, rapid freezing to forestall further dehydration.

We did not detect a significant decline in 13 MRSA populations in the MBPS after 24 freeze-thaw cycles in two years; the MRSA population for CBD 801 (N315) could not be enumerated on MH-OXA after initial evaluation, but methicillin resistance was confirmed at each time point. However, it is possible that low-temperature storage or multiple uncontrolled freeze-thaw events give “freeze-hardy” methicillin-susceptible cells in the heterogeneous population a “cryopreservation selection” advantage over the methicillin-resistant subpopulation during thawing and reculturing (11). To avoid these potential problems, we stock multiple single-use cryovials for every strain in our collection and perform quality control checks with each use.

A previous study reported that genetic background affects the stability of mecA in MRSA (10). The distribution of SCCmec contributes to the efficiency and stability of PBP2’ expression, which propagates methicillin resistance. The mecA gene is well maintained on an engineered plasmid in methicillin-susceptible Staphylococcus aureus strains from major clonal complexes, including the complexes containing USA100 and USA300 (10). Conversely, strains from other lineages are more likely defective in mecA production (10). A further analysis of the lineages of the strains that lose mecA could address any inherited instability, since apparent genetic insertion and deletion events may serve as indicators for strain stability (16).

The reported loss of methicillin resistance in MRSA is rare, and a confirmed explanation as to why it occurs is still elusive (6, 7, 9, 16). The suggestion of genetic instability of MRSA strains in cryostorage prompted us to review our MRSA strain collection for the potential loss of a robust heteroresistant population. We have seen no decline in the methicillin-resistant population among the 360 MRSA strains cryostocked using a standardized protocol for more than two years in our collection, possibly due to the major presence of inherently stable strains. The genetic background of MRSA strains as well as the complexities of the cryostocking process can play a significant role in long-term preservation of these strains. Therefore, it is important to enrich and confirm a robust mecA-positive subpopulation before, during, and after cryostorage of MRSA strains critical to research.

We are grateful to Lindsey Shaw for reading and commenting on the manuscript.

Isolates NRS 382 to 387, NRS 22, and NRS 123 were obtained through the NARSAM supported under NIAID/NH contract no. HHSN2722007006055C. This work was supported by U.S. Army Research, Development and Engineering Command contract no. W911SR-06-C-0020.

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5. Reference deleted.


