Optical Screening for Rapid Antimicrobial Susceptibility Testing and for Observation of Phenotypic Diversity among Strains of the Genetically Clonal Species *Bacillus anthracis*

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ABSTRACT During high-impact events involving *Bacillus anthracis*, such as the Amerithrax incident of 2001 or the anthrax outbreaks in Russia and Sweden in 2016, critical decisions to reduce morbidity and mortality include rapid selection and distribution of effective antimicrobial agents for treatment and postexposure prophylaxis. Detection of antimicrobial resistance currently relies on a conventional broth microdilution method that requires a 16- to 20-h incubation time for *B. anthracis*. Advances in high-resolution optical screening offer a new technology to more rapidly evaluate antimicrobial susceptibility and to simultaneously assess the growth characteristics of an isolate. Herein, we describe a new method developed and evaluated as a rapid antimicrobial susceptibility test for *B. anthracis*. This method is based on automated digital time-lapse microscopy to observe the growth and morphological effects of relevant antibiotics with an optical screening instrument, the oCelloScope. *B. anthracis* strains were monitored over time in the presence or absence of penicillin, ciprofloxacin, or doxycycline. Susceptibility to each antibiotic was determined in ≤ 4 h, 75 to 80% less than the time required for conventional methods. Time-lapse video imaging compiled from the optical screening images revealed unexpected differences in growth characteristics among strains of *B. anthracis*, which is considered to be a clonal organism. This technology provides a new approach for rapidly detecting phenotypic antimicrobial resistance and for documenting growth attributes that may be beneficial in the further characterization of individual strains.

KEYWORDS *Bacillus anthracis*, morphological differentiation, multidrug resistance, optical screening technology

Bacillus anthracis is the etiological agent of anthrax and has been designated a tier 1 select agent by the United States Federal Select Agent Program, as it presents a high risk of deliberate misuse as a biological threat agent. In 2001, spores of *B. anthracis* were maliciously distributed in letters sent through the U.S. postal system. The epidemiologic investigation identified 22 cases of anthrax (cutaneous and inhalation) in seven states (New York, New Jersey, Florida, Pennsylvania, Virginia, Maryland, and Connecticut) that ultimately resulted in five deaths (1). Globally, there are also naturally occurring outbreaks of anthrax in animals, and these may include infection of humans associated with their care.

B. anthracis is a member of the *Bacillus cereus* group, which is composed of six species, including *B. cereus*, *B. thuringiensis*, *B. anthracis*, *B. weihenstephanensis*, *B. mycoides*, and *B. pseudomycoides*. *B. anthracis* has remarkably low genetic diversity across strains compared to other species of the *B. cereus* group (2). This species is one

Received 10 November 2016 Returned for modification 12 December 2016 Accepted 30 December 2016

Accepted manuscript posted online 4 January 2017

Citation McLaughlin HP, Gargis AS, Michel P, Sue D, Weigel LM. 2017. Optical screening for rapid antimicrobial susceptibility testing and for observation of phenotypic diversity among strains of the genetically clonal species *Bacillus anthracis*. J Clin Microbiol 55:959–970. <https://doi.org/10.1128/JCM.02209-16>.

Editor Robin Patel, Mayo Clinic

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of the most genetically monomorphic bacteria known (3), and isolates may differ by only a limited number of single nucleotide polymorphisms (SNPs) (4). Variable number tandem repeats (VNTRs) or canonical SNPs in the *B. anthracis* genome have been used to differentiate and subdivide isolates into three major clades (A, B, and C) (5–7), with the A lineage adapted to more diverse environmental conditions than strains of the B lineage (8). Extrachromosomal elements account for most of the differences in phenotypic properties observed in the *B. cereus* group. In *B. anthracis*, two large plasmids encode the major virulence factors; toxins composed of lethal factor or edema factor and protective antigen are encoded on pXO1, and the characteristic poly- γ -D-glutamic acid capsule genes are located on pXO2 (9). However, little information is available regarding the general microbiological characterization of strains of *B. anthracis* in terms of diversity of growth patterns and colony morphologies. Recognition of any characteristic differences could prove valuable for rapid identification during an anthrax incident or outbreak.

The consideration of antimicrobial resistance phenotypes is an essential part of the ongoing effort to prepare for and respond to an intentional release or an outbreak of anthrax because without timely, effective therapy, this disease is associated with high rates of mortality (10). *B. anthracis* is usually susceptible to the antimicrobial agents approved for treatment and postexposure prophylaxis, although naturally occurring resistance to penicillin (PEN) has been documented, and a single isolate with resistance to erythromycin has been reported (11–15). The introduction of antimicrobial resistance genes and the use of selective pressure for mutations associated with resistance have been described in the literature (16, 17). Such studies were specifically for research purposes and usually limited to the use of attenuated or avirulent strains. Given the potential consequences of antimicrobial resistance when distributing medical countermeasures to large populations in the aftermath of a deliberate release of *B. anthracis* spores, it is essential to detect and characterize resistant strains of *B. anthracis* as quickly as possible (18).

The gold standard for antimicrobial susceptibility testing is conventional broth microdilution (BMD), and this method requires an incubation period of 16 to 20 h for *B. anthracis* according to guidelines published by the Clinical and Laboratory Standards Institute (CLSI) (19). A number of rapid molecular methods designed to reduce the incubation time have been reported, including real-time PCR, microarrays, and flow cytometry (20–22). However, if the method targets specific resistance genes or mutations, prior knowledge of these is required to include them in the assay. Additionally, approaches that depend on genotypic analysis of resistance determinants, such as targeted genome sequencing, have the key limitation of being unable to detect novel mechanisms of resistance. While these approaches would provide information on the presence or absence of a specific DNA target, the presence of a target does not necessarily correlate with the resistance phenotype. This is the case for *B. anthracis*, which has two β -lactamase genes on the chromosome. These genes are not expressed in most strains, and the organisms remain susceptible to β -lactam antibiotics. Another potential issue with the genomics-based approach occurs when the whole genome sequence of a novel resistant strain is compared with that of a susceptible reference strain. Any resistance genes or small resistance plasmids in the isolate to be characterized will not be present in the reference; consequently, those sequences may be discarded as irrelevant when they do not align with the reference sequence.

Previous work has demonstrated that the use of digital time-lapse microscopy provides rapid automated antimicrobial susceptibility results for some common clinical isolates in both monoculture experiments and complex samples such as urine (23, 24). In this report, we describe the development and evaluation of a rapid susceptibility test for *B. anthracis* based on optical screening to detect growth or inhibition of growth in the presence of antimicrobial agents with a digital time-lapse microscopy instrument. While *B. anthracis* is considered to be a genetically monomorphic pathogen, this technology also revealed unexpected, intriguing differences in cell morphology and growth characteristics between strains.

FIG 1 Growth kinetics of attenuated *B. anthracis* control strains. Sterne (PEN^s CIP^s DOX^s) and JB34 (PEN^{ns} CIP^{ns} DOX^{ns}) over 8 h of incubation at 35°C evaluated in the presence or absence of PEN (A), CIP (B), or DOX (C). Growth was measured by the SESA algorithm. Graphs represent the average growth value $\pm$ the standard deviation from three replicate wells. Optical screening images of bacterial growth were taken from a 90- μ l cell suspension after 6 h (top), and stereomicroscope images ($\times 8$) of single colonies are displayed at the bottom (D).

RESULTS

Rapid antimicrobial susceptibility testing of attenuated *B. anthracis* control strains by optical screening.

Four attenuated *B. anthracis* control strains (Table 1), including one with multidrug resistance, were tested against the three antimicrobial agents currently approved for treatment or postexposure prophylaxis of anthrax. Each strain had been previously classified as susceptible or resistant to PEN or as susceptible or nonsusceptible to ciprofloxacin (CIP) and doxycycline (DOX) on the basis of the MIC of each antibiotic and the established breakpoint for susceptibility as designated by CLSI guidelines. Initial evaluation of the optical screening method for two attenuated *B. anthracis* strains was performed by growth kinetic analysis (Fig. 1). Growth values were recorded every 10 min for the susceptible Sterne strain and the nonsusceptible JB34 strain over 8 h of incubation at 35°C in the presence or absence of PEN (Fig. 1A), CIP (Fig. 1B), or DOX (Fig. 1C). The optical screening method confirmed that the nonsusceptible control strain was not inhibited by the presence of the antibiotics at the concentrations tested and the susceptible strain was inhibited by the same concentrations. Divergence of the growth curves between the susceptible and resistant or nonsusceptible strains was recorded in < 2 h. However, unexpectedly, in the absence of antibiotics, the growth curves had distinctively different characteristics. The Sterne strain displayed an oscillating pattern instead of the typical smooth curve encompassing lag, exponential, and stationary phases of growth (Fig. 1). Video imaging supported these kinetic data and revealed that these strains differ in the way reproducing cells developed into chains and the way the chains aggregated in broth culture (Fig. 1D). Images taken after 6 h show the Sterne strain multiplying as long chains of cells entwined together to form rope-like structures that aggregated in a clumped phenotype, whereas the cell chains of JB34 were relatively short and homogeneously dis-

FIG 2 Single time point experiments with attenuated *B. anthracis* control strains after incubation for 4 h at 35°C in the presence or absence of PEN (A), DOX (B), or CIP (C). CLSI susceptibility interpretations for these strains are denoted as resistant (R), nonsusceptible (NS), or susceptible (S). Growth was measured by using the BCA algorithm. Growth of strains in the absence of antibiotics was normalized to 1, and growth in the presence of antibiotics was calculated as a fraction of 1. Bar graphs represent the average of quadruplicate samples $\pm$ the standard deviation, and a superscript lowercase letter a indicates the CLSI breakpoint for susceptibility. MICs determined by conventional BMD for bacterial strains are listed in the tables on the right along with the average percent growth reduction for each susceptible strain at the antibiotic concentration 1 doubling dilution above the CLSI susceptibility breakpoint (superscript lowercase letter b).

persed through the medium. The clumped chains of Sterne cells moved in and out of the focal plane of the instrument, which resulted in a graph composed of peaks and troughs. A similar clumping phenotype was also observed in *B. anthracis* 411A2, a CIP-nonsusceptible Sterne strain derivative, during growth in broth culture (data not shown).

Having discovered that *B. anthracis* strains could potentially reproduce with the cell chains aggregated into clumps presented a challenge for interpretation of susceptibility testing based on real-time growth kinetics. Therefore, a modification of the rapid antimicrobial susceptibility method was developed to address the issue. As an alternative to monitoring bacterial growth over time, *B. anthracis* strains were incubated for 4 h at 35°C in the presence or absence of antibiotics. A mixing step was incorporated to disperse potential clumps, and a single growth value was recorded by the instrument using the background corrected absorption (BCA) algorithm. An incubation time of 4 h was selected on the basis of the minimum time required to establish antimicrobial susceptibility from growth kinetic experiments and from results obtained from initial single-read experiments using various incubation times (data not shown). Subsequently, the growth of four attenuated *B. anthracis* control strains, two of which, Sterne and 411A2, exhibited clumping phenotypes, was evaluated in single time point experiments at 4 h of incubation with or without PEN (Fig. 2A), DOX (Fig. 2B), or CIP (Fig. 2C). The growth of each strain in the absence of antibiotics was normalized to 1, and their

FIG 3 Growth kinetics of *B. anthracis* strains SK57 (A), UT223 (B), Ames (C), Vollum (D), A0264 (E), A0102 (F), and 240 (G) evaluated over 18 h at 35°C in the absence of antibiotics. Growth was measured by the SESA algorithm. With the exception of *B. anthracis* A0264 and A0102, in which graphs display a representative single replicate because of their clumping phenotype, graphs represent the average growth value $\pm$ the standard deviation from three replicate wells. Optical screening images of cell aggregation taken between 6 and 8 h and stereomicroscope images of single colonies are displayed to the right.

growth in the presence of antibiotics was calculated as a fraction of 1. With the mixing step, growth values of nonsusceptible strains in the presence of antibiotics, including *B. anthracis* 411A2, which displayed a clumping phenotype, were comparable to those of the no-drug growth control, with normalized fractions observed between 0.95 and 1.13. In the presence of 1 μ g/ml PEN, susceptible *B. anthracis* strains Sterne and 411A2 had at least a 60% reduction in their average growth values (Fig. 2A). This concentration of PEN is 1 doubling dilution above the current CLSI breakpoint for susceptibility. Growth at this concentration would be interpreted as resistance, and growth inhibition at this concentration would indicate susceptibility. These antibiotic concentrations (1 μ g/ml PEN, 2 μ g/ml DOX, and 0.5 μ g/ml CIP) and the average percent growth reduction values for susceptible strains in the presence of these concentrations are listed in Fig. 2. At 2 μ g/ml DOX and 0.5 μ g/ml CIP, the average percent growth of the susceptible strains was reduced by $\geq 40\%$ (Fig. 2B and C). On the basis of the percent growth reductions of all replicates of the susceptible *B. anthracis* control strains at the drug concentrations above the CLSI breakpoints for susceptibility, minimum growth reduction cutoffs of 60%, 35%, and 30% were established for PEN, DOX, and CIP, respectively.

Strain-specific growth characteristics of *B. anthracis*. This optical screening technology also revealed different growth characteristics among a diverse set of *B. anthracis* strains (Fig. 3). Seven strains that included representatives from *B. anthracis* clades A, B, and C were evaluated in broth culture over a period of 18 h. The kinetic graphs of *B. anthracis* strains SK57, UT223, Ames, Vollum, and 240 revealed classical growth curves consisting of an exponential growth phase followed by a stationary phase (Fig. 3A to D and G). Images from four of these strains, SK57, UT223, Ames, and Vollum, revealed

similar long chains of cells dispersed homogeneously throughout the medium during growth. A representative video (see Movie S1 in the supplemental material) captured the growth of *B. anthracis* Ames as the length and density of the chains of cells increased over time until the surface area was completely occupied by reproducing cells. The colony morphologies of these four strains (Fig. 3) were opaque and rough with undulate margins. The average colony diameter varied by strain, ranging from 4.2 mm for Vollum to 6.7 mm for Ames after 18 h of growth on sheep blood agar (SBA). Interestingly, although the growth curves of *B. anthracis* strain 240 cells were similar, images of their growth were different. The reproducing cells of this strain were initially arranged in long chains in broth culture (Fig. 3G). However, this changed over the incubation time. In a video recording of strain 240 during a 20-h period, long chains of cells appeared during the first 5 h of growth, followed by the chains breaking up, and individual cells were then dispersed throughout the medium (see Movie S2). Strain 240 also had a unique colony morphology among the strains analyzed, with very small, smooth, transparent colonies that had well-defined margins. This was one of the smallest average colony diameter sizes, only 3.22 mm after 18 h at 35°C on SBA plates.

Two of the pXO2⁺ strains, A0264 and A0102, also had growth curves that oscillated over time (Fig. 3E and F), suggesting that these strains may have a clumping growth phenotype similar to that previously observed in the Sterne strain. The associated optical screening images confirmed the presence of clumps formed from aggregated long chains of cells of both strains. For the growth curve and video imaging of strain A0102 with rope-like structures aggregated into clumps, see Movie S3. The colony morphologies of these two strains on solid medium differed, with A0102 appearing more opaque, rougher in texture, and approximately 1.6 times the diameter of A0264 colonies.

Rapid antimicrobial susceptibility testing of diverse *B. anthracis* strains by optical screening. The accuracy of the optical screening technology as an antimicrobial susceptibility test was evaluated with a diverse set of *B. anthracis* strains and testing panels that included PEN, CIP, and DOX. Two strains with naturally occurring PEN resistance, UT223 and SK57, and two PEN-susceptible strains, Vollum and Ames, were used. Each strain was incubated with or without PEN over a period of 12 h (Fig. 4A and B). Inhibition of the growth of both susceptible strains was observed within 2 h of exposure to 0.5 and 1 $\mu\text{g/ml}$ PEN. The growth values and the complementary video images of *B. anthracis* Ames assembled over a period of 20 h in the presence of 0.5 $\mu\text{g/ml}$ PEN revealed growth continuing for the first 1.5 h, followed by cell lysis due to inhibition of cell wall biosynthesis (see Movie S4). Differences between the growth curves of PEN-susceptible and PEN-resistant strains in the presence of this antimicrobial agent were observed within 4 h in these kinetic experiments. For PEN-susceptible strains Vollum and Ames, the times (in hours) required to determine susceptibility upon exposure to increasing doubling dilutions of PEN ranging from 0.12 to 1 $\mu\text{g/ml}$ was calculated by using a two-tailed *t* test, with confidence levels of 90, 95, and 99% (Fig. 4C). We included confidence levels of 90% to demonstrate the rapidity of this assay and 99% to minimize very major errors and increase the accuracy for patient treatment. These times were inversely proportional to the concentrations of the antimicrobial agent and were directly proportional to the percent confidence levels. The time required to determine the PEN susceptibility of *B. anthracis* Vollum was slightly less than that required for Ames across all concentrations and confidence levels. At 0.5 $\mu\text{g/ml}$ PEN and with a confidence level of 95% ($P < 0.05$), the times required to determine susceptibility were 1.67 ± 0.24 h and 1.84 ± 0.47 h for Vollum and Ames, respectively. As expected, for both PEN-resistant strains UT223 and SK57, a statistically significant difference between growth with and without the drug could not be established.

PEN-resistant *B. anthracis* strains UT223 and SK57 differed in their cell chain morphologies in the presence of the drug (Fig. 4D). While both strains have a PEN MIC of >32 $\mu\text{g/ml}$, cell chains of UT223 appeared to take on a crinkled type of morphology when exposed to 0.5 and 1 $\mu\text{g/ml}$ PEN while chains of SK57 cells remained straight. In

FIG 4 Growth kinetics of *B. anthracis* Vollum (PEN^s) and UT223 (PEN^r) (A) and Ames (PEN^s) and SK57 (PEN^r) (B) evaluated over 12 h at 35°C in the presence or absence of PEN by optical screening. Growth was measured by the SESA algorithm. Graphs represent the average growth in three replicate wells. The table in panel C lists the times (in hours) required to determine the susceptibilities of *B. anthracis* Ames and Vollum in the presence of PEN. The times are reported as the mean ± the standard deviation of duplicate biological experiments and include confidence levels (CL) of 90, 95, and 100% with *P* values of <0.10, <0.05, and <0.01, respectively. (D) Optical screening images of resistant *B. anthracis* strains taken after 8 h at 35°C in the presence or absence of PEN.

the presence of 1 µg/ml PEN, the chains of UT223 cells begin to crinkle within 2 h of exposure to the antibiotic and remained entangled over time (see Movie S5). Attenuated strains UT308 and JB34, which, like UT223, are derivatives of parent strain *B. anthracis* 32, also displayed crinkled cell chain morphologies in the presence of PEN (data not shown). However, cell chains of SK57 remained straight and smooth and did not develop the crinkled morphology when exposed to PEN.

Rapid antimicrobial susceptibility testing of *B. anthracis* strains was also evaluated by using the modified method to permit a single time point analysis. Single time point growth values were recorded for six strains in the presence of PEN (Fig. 5A), DOX (Fig. 5B), or CIP (Fig. 5C). Growth values after 4 h of incubation with each antibiotic were calculated as a fraction of the growth values achieved without the antibiotic. Statistical analysis from previously performed growth kinetic experiments indicated that the optimal incubation time for unambiguous separation of susceptible and resistant strains was 4 h. In the presence of 1 µg/ml DOX, the CLSI breakpoint for susceptibility, and in the two doubling dilutions above the breakpoint, a minimum average growth reduction of 50% was observed for all of the susceptible *B. anthracis* strains tested compared to their no-drug controls (Fig. 5B). On the basis of the minimum growth reduction cutoff of 35% established by the control strains at 1 doubling dilution concentration above the CLSI breakpoint for susceptibility, no major errors occurred for any DOX-susceptible strain tested in this study by this rapid method. Similarly, a minimum average growth reduction of 50% was recorded for the four PEN-susceptible strains at the newly designated CLSI susceptibility breakpoint of 0.5 µg/ml, as well as 1 µg/ml (Fig. 5A). The growth of resistant strains SK57 and UT223 in the presence of each PEN concentration tested was comparable to the growth of their respective

<i>B. anthracis</i> strain	PEN MIC by BMD ($\mu\text{g/ml}$)	Avg. % Growth Reduction ^b
SK57 ^R	> 32	-
UT223 ^R	> 32	-
Ames ^S	0.06	73.10
Vollum ^S	0.03	80.27
A0264 ^S	0.06	65.71
A0102 ^S	0.12	78.87

<i>B. anthracis</i> strain	DOX MIC by BMD ($\mu\text{g/ml}$)	Avg. % Growth Reduction ^b
SK57 ^S	≤ 0.06	57.42
UT223 ^S	≤ 0.06	56.72
Ames ^S	≤ 0.06	68.58
Vollum ^S	≤ 0.06	67.04
A0264 ^S	≤ 0.06	77.57
A0102 ^S	≤ 0.06	56.28

<i>B. anthracis</i> strain	CIP MIC by BMD ($\mu\text{g/ml}$)	Avg. % Growth Reduction ^b
SK57 ^S	0.06	60.82
UT223 ^S	0.06	67.74
Ames ^S	0.12	50.41
Vollum ^S	0.06	72.04
A0264 ^S	0.06	59.45
A0102 ^S	0.06	70.79

FIG 5 Single time point experiments with *B. anthracis* strains after incubation for 4 h at 35°C in the presence or absence of PEN (A), DOX (B), or CIP (C). CLSI susceptibility interpretations for these strains are denoted as resistant (R) or susceptible (S). Growth was measured by the BCA algorithm. Growth of strains in the absence of antibiotics was normalized to 1, and growth in the presence of antibiotics was calculated as a fraction of 1. Bar graphs represent the average of triplicate samples $\pm$ the standard deviation, and a superscript lowercase letter a indicates the CLSI susceptibility breakpoint. MICs determined by conventional BMD for bacterial strains are listed in the tables at the right along with the average percent reduction in the growth of each susceptible strain at the antibiotic concentration 1 doubling dilution above the CLSI susceptibility breakpoint (superscript lowercase letter b).

no-drug controls, with fractions observed between 0.98 and 1.12. As naturally occurring PEN-resistant strains were included in this study, we can report that no major errors or very major errors occurred when the minimum growth reduction cutoff of 60% was used. While *B. anthracis* A0264 had only a 24% average growth reduction in the presence of 0.25 $\mu\text{g/ml}$ PEN, a concentration above its MIC but below the CLSI breakpoint for susceptibility, the associated video imaging revealed that cell lysis had already begun, with more cell debris remaining for A0264 than the other susceptible strains in the same concentration of PEN (data not shown). These real-time video images provide the auxiliary aspect of being able to visualize the effect an antibiotic has on a susceptible strain. In the presence of 0.5 and 1 $\mu\text{g/ml}$ CIP, relevant concentrations that would classify a growing *B. anthracis* strain as nonsusceptible, a minimum average growth reduction of 50% was observed for all of the susceptible strains evaluated compared to growth without CIP (Fig. 5C). No major errors were observed for CIP-susceptible strains with the established cutoff of 30%, and very major errors could not be determined since there are no isolates with naturally occurring resistance to CIP or DOX.

DISCUSSION

B. anthracis has the potential to be a bioterrorism risk to the public because of the stability of its spores, its ability to be widely disseminated by aerosolization, and its potential for a high rate of lethality (25). Antimicrobial resistance is an essential factor to be considered in efforts to prepare for and respond to an intentional release, as *in vitro* selection of CIP-resistant mutants (16), genetically engineered strains for tetracycline resistance (17), and naturally occurring beta-lactam resistance (26–28) have all been reported for *B. anthracis*. Decreasing the time required to detect antimicrobial

resistance is essential to prevent delays in the distribution of effective treatment or postexposure prophylaxis in response to an anthrax outbreak or a detected biothreat situation. Susceptibility testing based on genetic analysis alone has limited utility, as knowledge of specific gene targets is required, a gene sequence that predicts a resistance phenotype may not be expressed, and new mechanisms of resistance are still being discovered. It is essential to explore novel approaches and evaluate new technologies for phenotypic antimicrobial susceptibility testing.

To address these issues, we have developed and evaluated a rapid, functional antimicrobial susceptibility test for *B. anthracis* by using an optical screening system, the oCelloScope. Real-time, automated detection of bacterial growth with or without the antimicrobial agents PEN, CIP, and DOX provided phenotypic susceptibility data. All optical screening results were consistent with results from conventional susceptibility tests and were available in ≤ 4 h. This decreased the time required to determine antimicrobial susceptibility profiles by 75 to 80%, as conventional testing by BMD requires sufficient visible growth for interpretation of results and has been standardized by CLSI as a 16- to 20-h incubation time for *B. anthracis*. An ideal antimicrobial susceptibility test should also facilitate the high-throughput processing of samples. In an outbreak situation, there will likely be a need to process multiple samples, both environmental and clinical. The optical screening method is capable of detecting the real-time growth of up to 96 bacterium-antibiotic combinations in a single run. Each 96-well plate format could test multiple bacterial isolates and multiple antibiotics, and each antibiotic can be tested at various concentrations.

The ability to visualize the growth of *B. anthracis* in real time directed the method development for our rapid antimicrobial susceptibility test. Although *B. anthracis* is considered to be genetically monomorphic and clonal in nature (2, 6, 29, 30), this technology revealed unexpected variations in growth characteristics between strains. Data were acquired from growth kinetics and complementary video imaging by optical screening, as well as observations of colony morphologies. These data did not reveal any clade-specific or VNTR-related characteristics but rather highlighted the variability in the growth characteristics of *B. anthracis* strains. These results also highlight the importance of evaluating an assay with a diverse set of isolates.

Although numerous reports on the antimicrobial resistance or susceptibility of *B. anthracis* have been published, there is little information available regarding the manner in which replicating *B. anthracis* cell chains develop and organize during growth in broth culture, how these growth patterns are affected by the presence of antibiotics, or the variability of growth characteristics between strains. Three distinct morphotypes of *B. anthracis* cells replicating in broth culture without antibiotics were recorded. Additionally, the time required to visualize cell lysis of the susceptible Ames strain in the presence of PEN is consistent with the mechanism of action of this antibiotic and is in line with findings that demonstrate significantly increased membrane permeability after 1 h (31). There were also morphological differences between two PEN-resistant strains when they were exposed to various concentrations of the drug. Work is ongoing to investigate whether these unique growth characteristics support any fitness advantage in certain environments. Whole-genome sequence comparisons may provide clues to the genes responsible for the observed variations. We are currently evaluating this optical screening method for the characterization of other biothreat agents, including *Yersinia pestis*, *Burkholderia pseudomallei*, and *Francisella tularensis*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *B. anthracis* strains included in this study are listed in Table 1. Four attenuated control strains with distinctive antimicrobial susceptibility profiles were evaluated. Nonsusceptible attenuated control strains were generated by our laboratory with the approval and oversight of the CDC Institutional Biosafety Committee (32). A diverse set of seven *B. anthracis* strains representing clades A, B, and C were also evaluated, including two naturally occurring, PEN^r isolates. All *B. anthracis* strains were cultured at 35°C on tryptic soy agar II with 5% sheep's blood agar (SBA) (Fisher Scientific, Pittsburgh, PA) from glycerol stocks maintained at -70°C . Growth in broth culture was

TABLE 1 Bacterial strains used in this study

<i>B. anthracis</i> strain	Clade/cluster	Plasmids	Susceptibility interpretation ^a	Origin	Reference
Sterne (34F2)	A/3.b	pXO1 ⁺ , pXO2 [−]	PEN ^s , CIP ^s , DOX ^s	South Africa	35
411A2	A/3.b	pXO1 ⁺ , pXO2 [−]	PEN ^s , CIP ^{ns} , DOX ^s	Derived from <i>B. anthracis</i> Sterne	20
UT308	A/1.b	pXO1 [−] , pXO2 [−]	PEN ^r , CIP ^s , DOX ^s	Derived from <i>B. anthracis</i> 32	11
JB34	A/1.b	pXO1 [−] , pXO2 [−]	PEN ^r , CIP ^{ns} , DOX ^{ns}	Derived from <i>B. anthracis</i> UT308	32
Ames	A/3.b	pXO1 ⁺ , pXO2 ⁺	PEN ^s , CIP ^s , DOX ^s	Texas	6
Vollum	A/4	pXO1 ⁺ , pXO2 ⁺	PEN ^s , CIP ^s , DOX ^s	United Kingdom	6
A0264	A/1.b	pXO1 ⁺ , pXO2 ⁺	PEN ^s , CIP ^s , DOX ^s	Sivas, Turkey	6
A0102	B/1	pXO1 ⁺ , pXO2 ⁺	PEN ^s , CIP ^s , DOX ^s	Maputo, Mozambique	6
240	C	pXO1 ⁺ , pXO2 ⁺	PEN ^s , CIP ^s , DOX ^s	Unknown	36
SK57	Unknown	pXO1 ⁺ , pXO2 ⁺	PEN ^r , CIP ^s , DOX ^s	Unknown	37
UT223	A/1.b	pXO1 [−] , pXO2 ⁺	PEN ^r , CIP ^s , DOX ^s	Derived from <i>B. anthracis</i> 32	38

^aCLSI designates *B. anthracis* strains susceptible (superscript s) or resistant (superscript r) to PEN and susceptible or nonsusceptible (superscript ns) to CIP and DOX.

assessed in cation-adjusted Mueller-Hinton broth with *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (CAMHBT; Remel Inc., Lenexa, KS).

Antibiotics. CLSI breakpoints for interpretation of *B. anthracis* antimicrobial susceptibility testing results are available for CIP, DOX, and PEN. Sensititre drug panels (Trek Diagnostics, ThermoFisher Scientific) were custom manufactured in a 96-well plate format with desiccated antibiotics at the following concentrations: PEN, 0, 0.12, 0.25, and 0.5 $\mu\text{g/ml}$; CIP, 0, 0.25, 0.5, and 1 $\mu\text{g/ml}$; DOX, 0, 1, 2, and 4 $\mu\text{g/ml}$. The lowest concentrations of CIP and DOX were equivalent to the CLSI breakpoints for susceptibility, followed by two doubling dilutions. Because of the recent adjustment of the CLSI breakpoint for *B. anthracis* susceptibility to PEN, published as an update of M45-Ed3 (August 2016), the new CLSI breakpoint (0.5 $\mu\text{g/ml}$), plus and minus one doubling dilution (1 and 0.25 $\mu\text{g/ml}$, respectively), was evaluated (33).

Biosafety procedures. All procedures using attenuated strains were performed in a biosafety level 2 (BSL2) laboratory. All procedures involving pXO2⁺ strains (Table 1) were performed in a class II type A2 biological safety cabinet located in a BSL3 laboratory registered with the U.S. Federal Select Agent Program by trained personnel wearing personal protective equipment, including a powered air-purifying respirator and protective laboratory clothing (34).

Microscopy of single colony isolates. From glycerol stocks maintained at -70°C , *B. anthracis* strains were cultured at 35°C on SBA. Each strain was subcultured at 35°C for 18 h on SBA, and images of individual colonies were acquired with a Leica EZ4 HD digital stereomicroscope (Leica Microsystems, Wetzlar, Germany).

Imaging of bacterial growth in broth culture by optical screening. As described by Fredborg et al. (23), optical screening images are generated from scans through a fluid sample using digital time-lapse microscopy with the oCelloScope instrument (Philips BioCell A/S, Allerød, Denmark). Briefly, a z-stack image was produced by combining 10 images taken from a tilted imaging plane in each well of a 96-well flat-bottom plate containing 90 μl of the culture sample. Videos were composed of z-stack images taken over time from each well. Preceding image acquisition, each well was automatically and individually focused through the UniExplorer software v. 5.0.3 program. Subsequent two-dimensional images obtained with this technology have a magnitude comparable to that of a $\times 200$ light microscope.

Susceptibility testing by optical screening. *B. anthracis* strains were inoculated into drug panels by preparing cell suspensions in CAMHBT. Briefly, a cell suspension equivalent to a 0.5 McFarland density standard was prepared from colonies grown overnight on SBA. The cell suspension was diluted 1:100 in CAMHBT. A 100- μl aliquot of each diluted cell suspension was transferred into the wells of a Sensititre drug panel. For growth kinetic experiments, the inoculated panels were first incubated at room temperature for 10 min to rehydrate desiccated antibiotics. The cell suspensions and the reconstituted antibiotics were then mixed by pipetting and 90 μl was transferred to a 96-well flat-bottom plate. The inoculated plates were sealed with a breathable film cover (Breathe-Easy Sealing Membranes; Sigma-Aldrich, St. Louis, MO) and placed in the instrument, and growth values were recorded every 10 min over a period of 8, 12, or 18 h of incubation at 35°C . Alternatively, for single time point experiments, the inoculated Sensititre panels were incubated for 4 h, after which the cell suspensions were mixed by pipetting and transferred to a 96-well flat-bottom plate. The breathable seal was applied, and growth values were immediately recorded by the instrument. The accuracy of the optical screening rapid susceptibility test was determined by comparison of results with the MIC. The MIC of each drug for each strain tested was previously determined by conventional BMD performed in accordance with CLSI guidelines.

Analysis of optical screening instrument data. Data processing was performed with UniExplorer software v. 5.0.3, and graphical figures were compiled with Microsoft Excel Professional Plus 2013. For both growth kinetic and single time point experiments, the instrument-derived growth values were obtained with Segmentation and Extraction Surface Area (SESA) normalized and BCA algorithms, respectively. The SESA normalized algorithm determines microbial growth based on segmentation and contrast-based identification where growth curves are generated by summarizing the surface area covered by all of the identified objects in a scan area. The BCA algorithm determines growth based on changes in object pixels. All of the experiments in this report were performed in duplicate, and growth results from duplicate experiments were comparable. The growth kinetic data represent the average of

triplicate values $\pm$ standard deviations from one representative experiment. Data from single time point experiments represent average values $\pm$ standard deviations from one representative experiment, where growth in the absence of a drug is normalized to 1 and growth in the presence of a drug is presented as a fraction of 1. Statistical analysis of growth data was used to define the minimum time required to determine the susceptibility or resistance of a particular strain. The statistical significance of the difference between a susceptible strain incubated with or without each antibiotic over time was achieved with a two-tailed *t* test, where *n* = 3. The minimum times required to determine susceptibility were reported as the mean $\pm$ standard deviation of duplicate biological experiments and confidence levels of 90, 95, and 100% with *P* values of <0.10, <0.05, and <0.01, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JCM.02209-16>.

VIDEO S1, MOV file, 0.5 MB.

VIDEO S2, MOV file, 1.4 MB.

VIDEO S3, MOV file, 1.7 MB.

VIDEO S4, MOV file, 1.5 MB.

VIDEO S5, MOV file, 1.4 MB.

ACKNOWLEDGMENTS

We thank Theresa Koehler for providing *B. anthracis* strains UT223 and UT308. We acknowledge Klaus R. Andersen, Stine Kroghsbo, and other members of the oCelloScope team, part of Philips BioCell A/S in Allerød, Denmark, for their technical support. We also acknowledge Thomas Taylor, in the Division of Laboratory Systems at the U.S. Centers for Disease Control and Prevention, for support in statistical analysis.

This work was funded by interagency agreement HDTRA1213740 with the Department of Defense (DoD) Defense Threat Reduction Agency (DTRA) and Joint Science and Technology Office (JSTO). The funding agency had no role in the study design or in the data collection and interpretation.

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

REFERENCES

- Jernigan DB, Raghunathan PL, Bell BP, Brechner R, Bresnitz EA, Butler JC, Cetron M, Cohen M, Doyle T, Fischer M, Greene C, Griffith KS, Guarner J, Hadler JL, Hayslett JA, Meyer R, Petersen LR, Phillips M, Pinner R, Popovic T, Quinn CP, Reefhuis J, Reissman D, Rosenstein N, Schuchat A, Shieh WJ, Siegal L, Swerdlow DL, Tenover FC, Traeger M, Ward JW, Weisfuse I, Wiersma S, Yeskey K, Zaki S, Ashford DA, Perkins BA, Ostroff S, Hughes J, Fleming D, Koplan JP, Gerberding JL, National Anthrax Epidemiologic Investigation Team. 2002. Investigation of bioterrorism-related anthrax, United States, 2001: epidemiologic findings. *Emerg Infect Dis* 8:1019–1028. <https://doi.org/10.3201/eid0810.020353>.
- Harrell LJ, Andersen GL, Wilson KH. 1995. Genetic variability of *Bacillus anthracis* and related species. *J Clin Microbiol* 33:1847–1850.
- Keim P, Kalif A, Schupp J, Hill K, Travis SE, Richmond K, Adair DM, Hugh-Jones M, Kuske CR, Jackson P. 1997. Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. *J Bacteriol* 179:818–824. <https://doi.org/10.1128/jb.179.3.818-824.1997>.
- Pearson T, Busch JD, Ravel J, Read TD, Rhoton SD, U'Ren JM, Simonson TS, Kachur SM, Leadem RR, Cardon ML, Van Ert MN, Huynh LY, Fraser CM, Keim P. 2004. Phylogenetic discovery bias in *Bacillus anthracis* using single-nucleotide polymorphisms from whole-genome sequencing. *Proc Natl Acad Sci U S A* 101:13536–13541. <https://doi.org/10.1073/pnas.0403844101>.
- Van Ert MN, Easterday WR, Huynh LY, Okinaka RT, Hugh-Jones ME, Ravel J, Zanecki SR, Pearson T, Simonson TS, U'Ren JM, Kachur SM, Leadem-Dougherty RR, Rhoton SD, Zinser G, Farlow J, Coker PR, Smith KL, Wang B, Kenefic LJ, Fraser-Liggett CM, Wagner DM, Keim P. 2007. Global genetic population structure of *Bacillus anthracis*. *PLoS One* 2:e461. <https://doi.org/10.1371/journal.pone.0000461>.
- Keim P, Price LB, Klevytska AM, Smith KL, Schupp JM, Okinaka R, Jackson PJ, Hugh-Jones ME. 2000. Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. *J Bacteriol* 182:2928–2936. <https://doi.org/10.1128/JB.182.10.2928-2936.2000>.
- Girault G, Blouin Y, Vergnaud G, Derzelle S. 2014. High-throughput sequencing of *Bacillus anthracis* in France: investigating genome diversity and population structure using whole-genome SNP discovery. *BMC Genomics* 15:288. <https://doi.org/10.1186/1471-2164-15-288>.
- Smith KL, DeVos V, Bryden H, Price LB, Hugh-Jones ME, Keim P. 2000. *Bacillus anthracis* diversity in Kruger National Park. *J Clin Microbiol* 38:3780–3784.
- Moayeri M, Leppla SH, Vrentas C, Pomerantsev AP, Liu S. 2015. Anthrax pathogenesis. *Annu Rev Microbiol* 69:185–208. <https://doi.org/10.1146/annurev-micro-091014-104523>.
- Pillai SK, Huang E, Guarnizo JT, Hoyle JD, Katharios-Lanwermyer S, Turski TK, Bower WA, Hendricks KA, Meaney-Delman D. 2015. Antimicrobial treatment for systemic anthrax: analysis of cases from 1945 to 2014 identified through a systematic literature review. *Health Secur* 13:355–364. <https://doi.org/10.1089/hs.2015.0033>.
- Ross CL, Thomason KS, Koehler TM. 2009. An extracytoplasmic function sigma factor controls beta-lactamase gene expression in *Bacillus anthracis* and other *Bacillus cereus* group species. *J Bacteriol* 191:6683–6693. <https://doi.org/10.1128/JB.00691-09>.
- Coker PR, Smith KL, Hugh-Jones ME. 2002. Antimicrobial susceptibilities of diverse *Bacillus anthracis* isolates. *Antimicrob Agents Chemother* 46:3843–3845. <https://doi.org/10.1128/AAC.46.12.3843-3845.2002>.
- Brook I, Elliott TB, Pryor HI, II, Sautter TE, Gnade BT, Thakar JH, Knudson GB. 2001. In vitro resistance of *Bacillus anthracis* Sterne to doxycycline, macrolides and quinolones. *Int J Antimicrob Agents* 18:559–562. [https://doi.org/10.1016/S0924-8579\(01\)00464-2](https://doi.org/10.1016/S0924-8579(01)00464-2).

14. Luna VA, King DS, Gullledge J, Cannons AC, Amuso PT, Cattani J. 2007. Susceptibility of *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus pseudomycoides* and *Bacillus thuringiensis* to 24 antimicrobials using Sensititre automated microbroth dilution and Etest agar gradient diffusion methods. *J Antimicrob Chemother* 60:555–567. <https://doi.org/10.1093/jac/dkm213>.
15. Kim HS, Choi EC, Kim BK. 1993. A macrolide-lincosamide-streptogramin B resistance determinant from *Bacillus anthracis* 590: cloning and expression of *ermJ*. *J Gen Microbiol* 139:601–607. <https://doi.org/10.1099/00221287-139-3-601>.
16. Price LB, Vogler A, Pearson T, Busch JD, Schupp JM, Keim P. 2003. In vitro selection and characterization of *Bacillus anthracis* mutants with high-level resistance to ciprofloxacin. *Antimicrob Agents Chemother* 47:2362–2365. <https://doi.org/10.1128/AAC.47.7.2362-2365.2003>.
17. Pomerantsev AP, Shishkova NA, Marinin LI. 1992. Comparison of therapeutic effects of antibiotics of the tetracycline group in the treatment of anthrax caused by a strain inheriting *tet*-gene of plasmid pBC16. *Antibiot Khimioter* 37:31–34. (In Russian.)
18. Jones ME, Goguen J, Critchley IA, Draghi DC, Karlowsky JA, Sahm DF, Porschen R, Patra G, DelVecchio VG. 2003. Antibiotic susceptibility of isolates of *Bacillus anthracis*, a bacterial pathogen with the potential to be used in biowarfare. *Clin Microbiol Infect* 9:984–986. <https://doi.org/10.1046/j.1469-0691.2003.00775.x>.
19. CLSI. 2014. Performance standards for antimicrobial susceptibility testing; twenty-fourth informational supplement. M100-S24, vol. 34 no. 1. Clinical and Laboratory Standards Institute, Wayne, PA.
20. Weigel LM, Sue D, Michel PA, Kitchel B, Pillai SP. 2010. A rapid antimicrobial susceptibility test for *Bacillus anthracis*. *Antimicrob Agents Chemother* 54:2793–2800. <https://doi.org/10.1128/AAC.00247-10>.
21. Antwerpen MH, Schellhase M, Ehrentreich-Forster E, Bier F, Witte W, Nubel U. 2007. DNA microarray for detection of antibiotic resistance determinants in *Bacillus anthracis* and closely related *Bacillus cereus*. *Mol Cell Probes* 21:152–160. <https://doi.org/10.1016/j.mcp.2006.10.002>.
22. Broeren MA, Maas Y, Retera E, Arents NL. 2013. Antimicrobial susceptibility testing in 90 min by bacterial cell count monitoring. *Clin Microbiol Infect* 19:286–291. <https://doi.org/10.1111/j.1469-0691.2012.03800.x>.
23. Fredborg M, Andersen KR, Jorgensen E, Droce A, Olesen T, Jensen BB, Rosenvinge FS, Sondergaard TE. 2013. Real-time optical antimicrobial susceptibility testing. *J Clin Microbiol* 51:2047–2053. <https://doi.org/10.1128/JCM.00440-13>.
24. Fredborg M, Rosenvinge FS, Spillum E, Kroghsbo S, Wang M, Sondergaard TE. 2015. Rapid antimicrobial susceptibility testing of clinical isolates by digital time-lapse microscopy. *Eur J Clin Microbiol Infect Dis* 34:2385–2394. <https://doi.org/10.1007/s10096-015-2492-9>.
25. Weigel LM, Morse SA. 2009. Implications of antibiotic resistance in potential agents of bioterrorism, p 1293–1312. In Mayers DL (ed), *Antimicrobial drug resistance*. Humana Press, Totowa, NJ.
26. Turnbull PC, Sirianni NM, LeBron CI, Samaan MN, Sutton FN, Reyes AE, Peruski LF, Jr. 2004. MICs of selected antibiotics for *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus mycoides* from a range of clinical and environmental sources as determined by the Etest. *J Clin Microbiol* 42:3626–3634. <https://doi.org/10.1128/JCM.42.8.3626-3634.2004>.
27. Athamna A, Athamna M, Abu-Rashed N, Medlej B, Bast DJ, Rubinstein E. 2004. Selection of *Bacillus anthracis* isolates resistant to antibiotics. *J Antimicrob Chemother* 54:424–428. <https://doi.org/10.1093/jac/dkh258>.
28. Ågren J, Finn M, Bengtsson B, Segerman B. 2014. Microevolution during an anthrax outbreak leading to clonal heterogeneity and penicillin resistance. *PLoS One* 9:e89112. <https://doi.org/10.1371/journal.pone.0089112>.
29. Henderson I, Duggleby CJ, Turnbull PC. 1994. Differentiation of *Bacillus anthracis* from other *Bacillus cereus* group bacteria with the PCR. *Int J Syst Bacteriol* 44:99–105. <https://doi.org/10.1099/00207713-44-1-99>.
30. Perreten V, Vorlet-Fawer L, Slickers P, Ehrlich R, Kuhnert P, Frey J. 2005. Microarray-based detection of 90 antibiotic resistance genes of Gram-positive bacteria. *J Clin Microbiol* 43:2291–2302. <https://doi.org/10.1128/JCM.43.5.2291-2302.2005>.
31. Xing YH, Wang W, Dai SQ, Liu TY, Tan JJ, Qu GL, Li YX, Ling Y, Liu G, Fu XQ, Chen HP. 2014. Daptomycin exerts rapid bactericidal activity against *Bacillus anthracis* without disrupting membrane integrity. *Acta Pharmacol Sin* 35:211–218. <https://doi.org/10.1038/aps.2013.159>.
32. Bugrysheva JV, Lascols C, Sue D, Weigel LM. 2016. Rapid antimicrobial susceptibility testing of *Bacillus anthracis*, *Yersinia pestis*, and *Burkholderia pseudomallei* using laser light scattering technology. *J Clin Microbiol* 54:1462–1471. <https://doi.org/10.1128/JCM.03251-15>.
33. CLSI. 2016. Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria M45, 3rd ed. Clinical and Laboratory Standards Institute, Wayne, PA.
34. Centers for Disease Control and Prevention. 2009. Biosafety in microbiological and biomedical laboratories, 5th ed. Centers for Disease Control and Prevention, Atlanta, GA.
35. Sterne M. 1939. The use of anthrax vaccines prepared from avirulent (unencapsulated) variants of *Bacillus anthracis*. *Onderstepoort J Vet Sci Anim Ind* 13:307–312.
36. Sue D, Marston CK, Hoffmaster AR, Wilkins PP. 2007. Genetic diversity in a *Bacillus anthracis* historical collection (1954 to 1988). *J Clin Microbiol* 45:1777–1782. <https://doi.org/10.1128/JCM.02488-06>.
37. Gill SC, Rubino CM, Bassett J, Miller L, Ambrose PG, Bhavnani SM, Beaudry A, Li J, Stone KC, Critchley I, Janjic N, Heine HS. 2010. Pharmacokinetic-pharmacodynamic assessment of faropenem in a lethal murine *Bacillus anthracis* inhalation postexposure prophylaxis model. *Antimicrob Agents Chemother* 54:1678–1683. <https://doi.org/10.1128/AAC.00737-08>.
38. Chen Y, Tenover FC, Koehler TM. 2004. Beta-lactamase gene expression in a penicillin-resistant *Bacillus anthracis* strain. *Antimicrob Agents Chemother* 48:4873–4877. <https://doi.org/10.1128/AAC.48.12.4873-4877.2004>.