

Research Note

Growth Characteristics of Virulent *Bacillus anthracis* and Potential Surrogate Strains

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ABSTRACT

The objectives of this study were to compare generation and lag times of virulent *Bacillus anthracis* strains with those of other *Bacillus* strains, to identify possible surrogates for growth studies, and to determine if the *B. cereus* module of the U.S. Department of Agriculture Pathogen Modeling Program (PMP) had predictive value for *B. anthracis*. Growth characteristics of *B. anthracis*, *B. cereus*, *B. mycoides*, and *B. subtilis* strains in brain heart infusion broth at pH 6.5, 6.0, and 5.5 were determined by absorbance measurements. Growth curves of *B. anthracis* Sterne and *B. cereus* strains appeared similar, and the generation times for strain Sterne fell within the PMP's 95% confidence interval for *B. cereus*. However, the virulent *B. anthracis* strains Vollum and Pasteur had shorter generation times than the avirulent Sterne strain and most other surrogates and were lower than the PMP's 95% confidence interval for *B. cereus*. Growth curves of *B. cereus* ATCC 9818 and *B. subtilis* ATCC 6633 were more similar to those of virulent *B. anthracis* strains, but all potential surrogates had significantly different generation times and lag times under some conditions.

Bacillus anthracis is a nonmotile, gram-positive, spore-forming rod that is pathogenic to animals and humans (7). This bacterial species can cause anthrax disease when spores enter the body through inhalation, ingestion, or skin abrasions (4). Human anthrax is often associated with work-related infections in the animal hide and wool industries. However, in October 2001, inhalation anthrax was linked to bioterrorism when *B. anthracis* spores were intentionally released through the U.S. postal system (3). Despite the use of *B. anthracis* for bioterrorism, a significant amount of information about it is still unknown. One aspect about which we have little information is the growth behavior, including generation time and lag time, of *B. anthracis*. Because this pathogen could be used for bioterrorism again, it is critical that we understand the basic growth characteristics of *B. anthracis*.

Working with pathogens, particularly select agents, can be challenging. Ideally, researchers would prefer to gather information about pathogenic organisms while avoiding the risk inherent in their direct manipulation. For this reason, surrogate organisms are frequently used as models for pathogens. A surrogate is defined as a nonpathogenic organism that behaves similarly to the pathogenic organism when exposed to the same conditions or treatment (6). The surrogate concept can be applied to *B. anthracis*.

Genetically, *B. anthracis*, *B. cereus*, and *B. mycoides* are very closely related (5, 11), although detailed comparisons of the species' growth behaviors have not been made.

In this study, we measured and statistically analyzed the growth characteristics of three *B. anthracis* strains at three pH values and compared them to the growth characteristics of the putative surrogates *Bacillus subtilis*, *B. mycoides*, and *B. cereus*. The results from this study will help identify suitable surrogates for *B. anthracis* for use in future studies.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. cereus* ATCC 4342, ATCC 7004, and ATCC 9818, *B. subtilis* ATCC 6633, and *B. mycoides* ATCC 21929 were purchased from the American Type Culture Collection (ATCC; Manassas, Va.). *B. cereus* F4810, B4AC, and T1 were obtained from the U.S. Department of Agriculture (USDA; Eastern Regional Research Center, Wyndmoor, Pa.). A *B. cereus* mixture containing equal populations of *B. cereus* F4810, B4AC, and T1 was also prepared so that the data from our experiments could be compared with those of Benedict et al. (2) and with predictions generated by the *B. cereus* module of the USDA Pathogen Modeling Program (PMP version 6.0, USDA). *B. anthracis* Sterne, the avirulent vaccine strain, was received from Dr. Darcy Haynes (Center for Food Safety and Applied Nutrition, College Park, Md.). The virulent strains of *B. anthracis* Vollum and Pasteur were obtained from the Centers for Disease Control and Prevention (Atlanta, Ga.). Cells were cultured in brain heart infusion broth (Becton Dickinson, Franklin Lakes, N.J.), maintained at 4°C on tryptic soy agar slants (Difco, Becton Dickinson, Sparks, Md.), and renewed monthly.

Determination of growth parameters. Overnight cultures (~24 h at 28°C) of each strain were adjusted to ~0.5 A₅₉₅ (SmartSpec 3000, Bio-Rad, Hercules, Calif.) with additional brain heart infusion broth, if necessary, to give a concentration of ~10⁷ CFU/ml. One milliliter of each adjusted culture was diluted 1,000-

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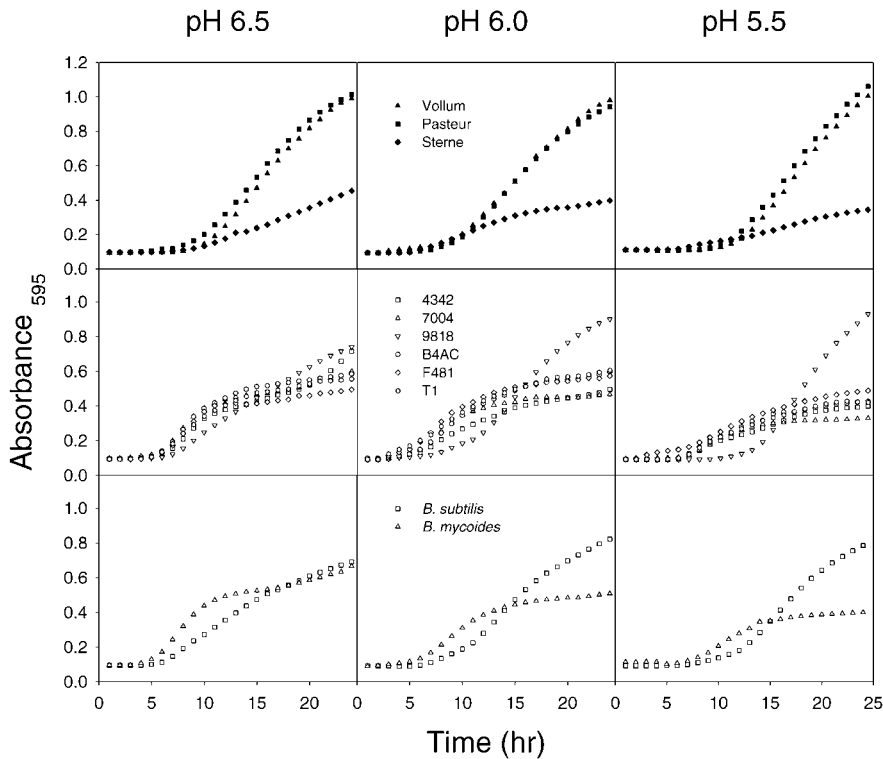


FIGURE 1. Growth behavior of *Bacillus anthracis* and potential surrogates in brain heart infusion broth at pH 5.5, 6.0, and 6.0 as measured by changes in absorbance at 595 nm. The top three panels contain results for the three *B. anthracis* strains using closed symbols as noted. The middle three panels contain results for the six *B. cereus* strains using open symbols as noted. The bottom three panels contain results for *B. subtilis* and *B. mycooides* using gray symbols as noted.

fold in 0.1% peptone water (Difco, Becton Dickinson) for use as the inoculum. Wells of a 96-well microtiter plate (Corning Inc., Corning, N.Y.) were filled with 135 μ l of brain heart infusion broth adjusted to pH 6.5, 6.0, or 5.5, and 15 μ l of inoculum was added to give an initial inoculum of $\sim 10^3$ CFU/ml. The study was conducted in brain heart infusion broth, since that medium was used to generate the data in the PMP *B. cereus* module (2). At least four wells containing the same strain were on each plate, and at least two separate plates were used to generate the data for each strain-pH combination. Control wells containing uninoculated media were included on each plate. The inside of the plate cover was wiped with an anti-fog cloth wipe (RainX, SOPUS Products, Houston, Tex.) to prevent condensation formation inside the cover. The sides of the microtiter plate were secured with tape to prevent spillage, and the plate was placed in a plate reader (model 550, Bio-Rad) in a 25°C incubator. The plate reader was programmed to record the A_{595} every 30 min over a 24-h period with 5 s of shaking before each reading.

Data analysis. The optical density for each well was recorded in Excel (Microsoft, Redmond, Wash.) and analyzed by DMFit ((1); available at <http://www.ifr.bbsrc.ac.uk/Safety/DMFit/>; accessed 29 August 2005), an Excel add-in that fits growth curve data. DMFit calculated a growth rate and lag time for each curve. Averages and standard deviations of these parameters were calculated with Excel. Experiments were replicated at least eight times per pH value per strain, and control experiments verified that the relationship between A_{595} and concentration (CFU per milliliter) was linear and was similar among the strains (data not shown). The final growth curves (Fig. 1) were produced using an average of absorbance readings for all wells of each strain at each time point. Data were analyzed by the General Linear Models procedure of SAS, version 8.2 (SAS Institute, Raleigh, N.C.). Lag time and generation time of each strain were compared and grouped by Duncan's multiple range test. Data falling into the same group at the same pH were not statistically different ($P > 0.05$).

Safety and security considerations. All procedures involving *B. anthracis* were conducted by immunized personnel working in a Select Agent Laboratory registered with the Centers for Disease Control and Prevention in compliance with the U.S. Patriot Act. All culture manipulations were performed inside a class Iia biosafety cabinet using protocols approved by the University Biosafety Committee.

RESULTS

All strains tested followed two general trends: as the pH decreased, the lag time increased, and the final cell density generally decreased (Fig. 1). Surprisingly, pH did not have a large effect on the growth rates of most strains. It is clear from the top three panels of Figure 1 that the virulent strains, *B. anthracis* Vollum and Pasteur, grew much faster than the nonpathogenic strain, *B. anthracis* Sterne, and had higher final cell densities that were not greatly affected by pH. The generation times of Sterne compared with the generation times of Vollum and Pasteur were clearly statistically significantly different (Table 1).

Most *B. cereus* strains (Fig. 1, middle row panels) behaved similarly to each other and to *B. anthracis* Sterne. Generally, the *B. cereus* strains had significantly longer generation times and significantly shorter lag times than the virulent *B. anthracis* strains Vollum and Pasteur, with several notable exceptions. *B. subtilis* 6633, *B. cereus* F4810, and *B. cereus* B4AC had generation times similar to the virulent strains at pH 6.5, while no strains except for Sterne had similar lag times (Table 1). At pH 6.0 and 5.5, neither generation times nor lag times of *B. cereus* ATCC 9818 were significantly different from Vollum, and generation times were not significantly different from Pasteur (Table 1). *B. cereus* ATCC 9818 lag time was not significantly different from Pasteur at pH 6.0 and was only slightly different from Pasteur at pH 5.5.

TABLE 1. Effect of pH on generation time and lag time of *Bacillus anthracis* and potential surrogates

pH	Bacterial species and strain	Mean generation time (min)	<i>n</i> ^a	Significant difference group ^b			Mean lag time (h)	<i>n</i>	Significant difference group		
6.5	<i>B. anthracis</i> Sterne	31.2	19	A			8.9	19	A		
	<i>B. anthracis</i> Pasteur	10.0	14				8.2	14	A		
	<i>B. anthracis</i> Vollum	10.0	14				9.1	14	A		
	<i>B. subtilis</i> 6633	14.4	12				5.6	12	B C		
	<i>B. cereus</i> 9818	15.6	16				5.9	16	B		
	<i>B. cereus</i> 4342	24.9	12	B			2.2	6			
	<i>B. cereus</i> 7004	17.0	12				3.2	10	E F		
	<i>B. cereus</i> B4AC	14.2	10				3.9	10	D E		
	<i>B. cereus</i> F4810	12.9	10				4.6	10	C D		
	<i>B. cereus</i> T1	17.1	10				3.5	10	D E		
	<i>B. cereus</i> mixture	17.6	16				5.4	16	B C		
<i>B. mycooides</i> 21929	14.4	12				3.4	9	D E F			
6.0	<i>B. anthracis</i> Sterne	27.1	14	A			4.6	14	C		
	<i>B. anthracis</i> Pasteur	10.8	14				8.1	14	A B		
	<i>B. anthracis</i> Vollum	10.4	14				8.3	14	A B		
	<i>B. subtilis</i> 6633	12.6	12				7.3	12	B		
	<i>B. cereus</i> 9818	9.7	16				8.8	16	A		
	<i>B. cereus</i> 4342	23.6	18	B			4.1	16	C		
	<i>B. cereus</i> 7004	16.7	12				3.9	11	C		
	<i>B. cereus</i> B4AC	17.7	12				3.5	12	C		
	<i>B. cereus</i> F4810	15.6	12				4.0	10	C		
	<i>B. cereus</i> T1	18.5	20				4.1	16	C		
	<i>B. cereus</i> mixture	20.2	14				4.6	13	C		
<i>B. mycooides</i> 21929	15.8	14				4.7	14	C			
5.5	<i>B. anthracis</i> Sterne	40.5	18	A			8.5	18	D		
	<i>B. anthracis</i> Pasteur	10.5	9				10.4	9	B C		
	<i>B. anthracis</i> Vollum	10.6	13				11.2	13	A B		
	<i>B. subtilis</i> 6633	16.0	12	E			8.9	12	C D		
	<i>B. cereus</i> 9818	8.2	8				12.5	8	A		
	<i>B. cereus</i> 4342	31.6	13	B			4.7	13	E F		
	<i>B. cereus</i> 7004	27.0	16	B C			5.0	15	E F		
	<i>B. cereus</i> B4AC	27.7	9	B C			4.9	9	E F		
	<i>B. cereus</i> F4810	24.5	16				5.7	13	E F		
	<i>B. cereus</i> T1	27.3	9	B C			4.4	9	F		
	<i>B. cereus</i> mixture	26.5	12	B C			5.9	12	E F		
<i>B. mycooides</i> 21929	20.0	13				6.6	13	E			

^a *n*, number of observations used to determine the mean.

^b Means followed by the same letter, within the same pH value, are not statistically significantly different.

B. mycooides ATCC 21929 (Fig. 1, bottom panels) followed the same growth pattern as most *B. cereus* strains and the Sterne strain. The growth behaviors of *B. mycooides* ATCC 21929 were all significantly different from Pasteur and Vollum, except for the generation time at pH 6.5 (Table 1). *B. subtilis* ATCC 6633 (Fig. 1, bottom panels) had the fast growth rate characteristic of the virulent *B. anthracis* strains and *B. cereus* 9818. At pH 6.5 and 6.0, the generation times of *B. subtilis* ATCC 6633 were not significantly different from Pasteur and Vollum (Table 1).

DISCUSSION

The results presented indicate that the growth characteristics of the virulent strains of *B. anthracis* Pasteur and Vollum were generally different from *B. anthracis* Sterne, most *B. cereus* strains, and *B. mycooides* ATCC 21929.

These strains would be inappropriate surrogates in experiments concerning the growth of pathogenic *B. anthracis*. The difference between strain Sterne, which lacks the virulence plasmid pOX2, and the fully virulent strains was unexpected. To the best of our knowledge, pOX2, which contains the gene for the capsular material required for full virulence, has not been sequenced. This large (96,231-bp) megaplasmid (12) may contain other genes related to growth in rich media. Sequencing of plasmid pOX1, which contains the three toxin genes, has revealed regulatory elements, three germination response genes, and 143 predicted open reading frames (10). So it would be naïve to think that, except for their ability to produce toxins or capsules, strains lacking pOX1 or pOX2 (respectively) are identical to virulent strains.

B. cereus ATCC 9818 had growth behavior similar to

TABLE 2. Comparison of generation times from this study with those predicted by the Pathogen Modeling Program

pH	95% confidence interval for generation time (min)			
	<i>B. cereus</i> (predicted PMP)	<i>B. anthracis</i> Sterne	<i>B. anthracis</i> Pasteur	<i>B. anthracis</i> Vollum
6.5	24–48	7–43	8–12	9–11
6.0	24–54	18–36	8–12	9–12
5.5	30–60	18–61	9–12	10–12

B. anthracis at pH 6.0 and 5.5, and its spores were previously validated as a conservative heat resistance surrogate for *B. anthracis* spores (8). *B. subtilis* ATCC 6633, which had growth characteristics similar to those of the virulent *B. anthracis* strains at pH 6.5 and 6.0, has been validated as a surrogate for *B. anthracis* spores for both heat resistance (8) and UV resistance (9) and has the added benefit of being nonpathogenic. None of the strains examined were appropriate surrogates for virulent *B. anthracis* strains over the whole pH range examined. The validity of specific strains as surrogates could be expected to vary with temperature, growth media, and other factors, underscoring the importance of validating surrogates under conditions of actual use.

The generation time results for the nonpathogenic Sterne strain of *B. anthracis* fell within the 95% confidence intervals of the *B. cereus* growth section of the USDA PMP (Table 2). This model was generated from data using the mixture of *B. cereus* strains at various temperatures, pH values, and concentrations of sodium chloride and nitrite (2). The generation time results for the *B. cereus* mixture reported here were shorter but were generally within the lower limits of the PMP prediction, indicating the robustness of that model. The virulent *B. anthracis* strains had generation times significantly shorter than those predicted by the PMP *B. cereus* growth module under all conditions examined. All of the lag times from the *B. anthracis* strains and *B. cereus* mixture in this study were longer than the outer limits of the PMP 95% confidence limit. This is not surprising given that the PMP lag times were generated from plate count data, which can determine the actual point at which exponential growth starts, while our lag times are calculated from the time turbidity starts to change. In this method, the actual lag time and the subsequent time required to produce a measurable change in absorbance are summed as a perceived lag time. It should also be noted that both PMP and this study estimated the lag time from inocula of vegetative cells; lag times from spore inocula would be longer and more variable. However, regardless of whether the inocula were spores or vegetative cells, they would not be expected to affect growth rates.

B. cereus ATCC 9818 and *B. subtilis* ATCC 6633 would be good surrogates for virulent *B. anthracis* given the similarities in generation times and previously published spore thermal resistance. If combined in a cocktail, they would be valid over the pH range 5.5 to 6.5. The other *Bacillus* strains tested, including *B. anthracis* Sterne, have different growth characteristics and would be inappropriate surrogates for studies in which growth of the organism is an important factor.

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