JOURNAL OF BACTERIOLOGY, Apr. 2006, p. 2711–2714 Vol. 188, No. 7
0021-9193/06/$08.00+0 doi:10.1128/JB.188.7.2711–2714.2006

PlyPH, a Bacteriolytic Enzyme with a Broad pH Range of Activity and Lytic Action against *Bacillus anthracis*

Pauline Yoong, Raymond Schuch, Daniel Nelson, and Vincent A. Fischetti*

Laboratory of Bacterial Pathogenesis and Immunology, The Rockefeller University, New York, New York 10021

Received 11 August 2005/Accepted 13 January 2006

We have cloned a lytic enzyme, PlyPH, with a specific lytic effect on *Bacillus anthracis* strains. PlyPH remains active between pH 4 and 10.5, and a single dose rescued a significant percentage of mice infected intraperitoneally with an attenuated *B. anthracis* strain. We propose PlyPH as a novel therapeutic agent.

Bacillus anthracis is classified as a category A biological weapon (24) with the capacity of developing multidrug resistance (1–3, 5, 11, 29). Thus, new and improved ways of treating anthrax infections are needed. In this regard, lytic enzymes (lysins) expressed by bacteriophages (34) have been applied successfully in the control of a number of bacterial pathogens (4, 13, 15, 16, 22, 26). Structurally, lysins are commonly found as modular proteins with an N-terminal domain that confers the enzymatic activity for a peptidoglycan bond and a C-terminal domain that confers binding specificity to primarily carbohydrate epitopes in the bacterial cell wall (17, 19, 20, 27). Lysins have an advantage over antibiotics in that lysins generally affect only targeted bacterial strains, while having minimal effects on other bacteria, including commensals (6, 7), making them suitable as alternative therapeutic agents. In addition, their efficient bactericidal effects may be useful in the decontamination of bacteria on inanimate surfaces and objects.

Our laboratory reported the development of a lysin named PlyG, isolated from the γ phage, demonstrating both in vitro and in vivo lytic activity against *B. anthracis* and *B. anthracis*-like *B. cereus* (26). In this work, PlyPH, a bacteriolytic enzyme of putative bacteriophage origin which could also be applied in the treatment and decontamination of *B. anthracis*, was characterized. *B. cereus* strain RSVF1, a strain representative of *B. anthracis* cured of its virulence plasmids (9, 14, 24, 26, 30–32), and the ΔSterne strain of *B. anthracis* (12) were used as safe alternatives to fully virulent *B. anthracis* strains.

PlyPH was identified through a BLAST search of four *B. anthracis* strains with the amino acid sequence of PlyG as the query sequence (26). Using the *B. anthracis* Ames strain nomenclature, the open reading frame (ORF) BA2805 was identified (accession number NC_003997). Subsequently, BA2805 was renamed *plyPH* and its corresponding gene product was called PlyPH. The *plyPH* ORF was amplified by PCR using DNA from the attenuated *B. anthracis* strain ΔSterne with primers G**GAATTC**ATGGGTTATATTGTAGATATTTCG (EcoRI restriction site boldfaced) and GC**TCTAGA**TTATTTAACTTCATACCACCAAC (XbaI restriction site boldfaced). The PCR product was directionally cloned into the EcoRI and XbaI restriction sites within pBAD24 (8), and protein was induced as described elsewhere (26). PlyPH was purified to >90% homogeneity (data not shown) and used in all experiments.

The amino acid sequences of PlyPH and PlyG were aligned (Fig. 1A) and revealed little sequence identity in the N-terminal half of the proteins, suggesting that the catalytic domains differ. Since for most phage lysins, the C-terminal half confers the specificity of the molecule, usually by binding to a polysaccharide epitope in the cell wall, the high degree of sequence identity between the putative binding domains of PlyPH and PlyG suggests that both enzymes may recognize and bind the same bacterial cell wall epitope.

A BLAST search using the PlyPH sequence identified a number of *Bacillus* phage lysins with a high degree of sequence identity. An alignment of PlyPH with these lysins (Fig. 1B) revealed that the greatest identity was located within the N-terminal half of the molecules comprising the catalytic domains, suggesting similarities in catalytic activity.

In the *B. anthracis* genomes, it was not evident that *plyPH* originated from a phage, since no obvious phage genes were found in the ORFs surrounding it (results not shown). Furthermore, the *plyPH* ORF was not among the loci attributed to prophage functions (25). However, a BLAST search with PlyPH revealed a high percentage of identity with lysins from various *Bacillus* phages including the *B. anthracis* prophage

TABLE 1. Bacterial strains on which the lytic action of PlyPH was tested

Bacterial strain[a]	Lytic activity
Bacillus anthracis ΔSterne	2+
Bacillus cereus RSVF1	4+
Bacillus cereus ATCC 10987	±
Bacillus cereus 14579	−
Bacillus cereus 13472	−
Bacillus cereus T	−
Bacillus thuringiensis HD1	−
Bacillus thuringiensis HD73	−
Bacillus subtilis SL4	−
Bacillus pumilus SL4680	−
Bacillus megaterium RS77	−
Brevibacillus laterosporus ATCC 9141	−

[a] All strains were from The Rockefeller University Bacteria Collection, New York, NY.

* Corresponding author. Mailing address: Laboratory of Bacterial Pathogenesis and Immunology, The Rockefeller University, 1230 York Avenue, New York, NY 10021. Phone: (212) 327-8166. Fax: (212) 327-7584. E-mail: vaf@rockefeller.edu.

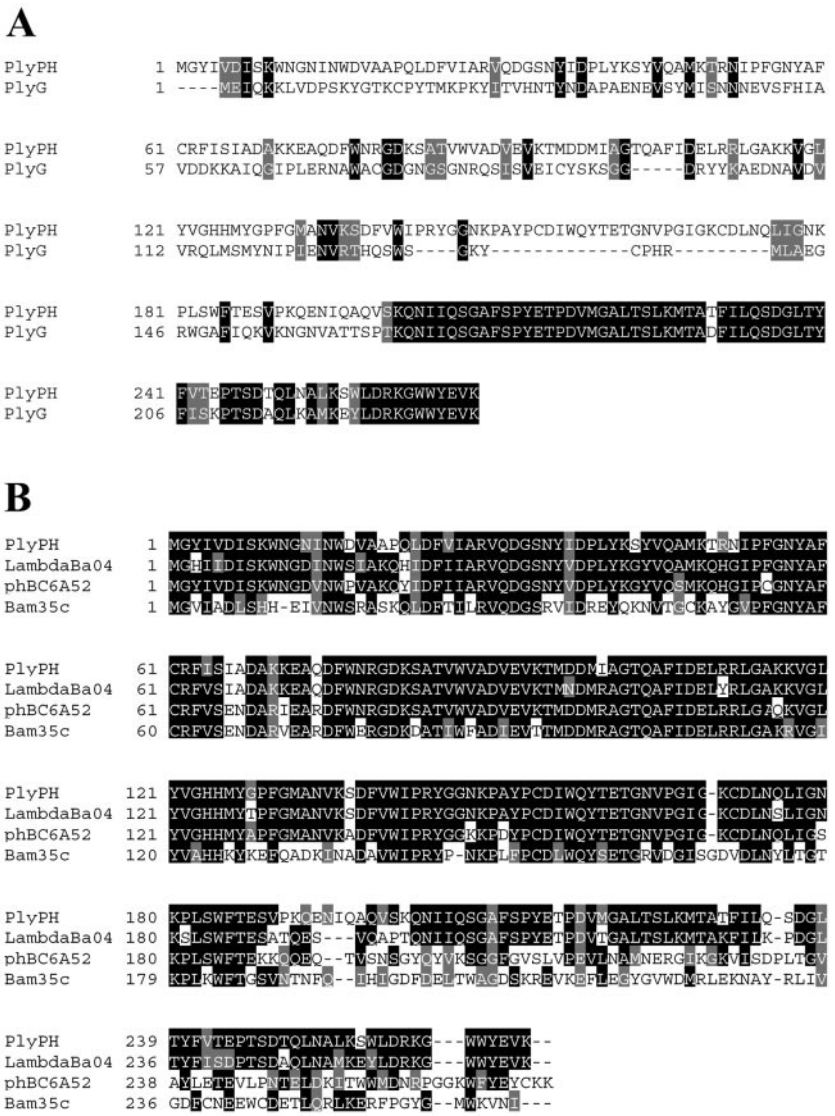

FIG. 1. Alignment of the amino acid sequence of PlyPH with those of bacteriophage lysins. (A) Alignment of amino acid sequences of PlyPH and PlyG, the *B. anthracis* γ phage lysin. (B) Alignment of the amino acid sequence of PlyPH with those of lysins from three *Bacillus* bacteriophages. Those phages include LambdaBa04, a prophage within *B. anthracis* (NC_003997.3); phBC6A52a, *B. cereus* prophage (NP_852605); and Bam35c, a *B. thuringiensis* phage (NP_943776). Identical residues are highlighted by black boxes, while conserved residues are highlighted with gray boxes.

LambdaBa04 (84%), the *B. cereus* prophage phBC6A52 (66%), and the *B. thuringiensis* phage Bam35c (49%), suggesting that PlyPH is closely related to phage lysins, if not a phage lysin itself (Fig. 1B). Therefore, we propose that PlyPH was acquired by the *B. anthracis* genome from a phage source. This is not the first incidence of an apparent phage lysin located on a bacterial chromosome away from known prophages. There are at least four such examples in *B. subtilis* (10, 18, 23, 28).

Bacillus strains to be assayed were grown as previously described (26). The lytic activity of PlyPH and how it was measured are described below. A universal buffer was prepared with equal parts of 40 mM boric acid and 40 mM phosphoric acid, followed by titration of the buffer from pH 3 to 11 with sodium hydroxide using a pH meter. To test the activity of PlyPH at different pH values, purified PlyPH was dialyzed against 5 mM acetate buffer at pH 5.5. The *B. cereus* suspension (180 μl) was added to 20 μl of purified PlyPH at 300 μg · ml^{-1}. In controls, phosphate-buffered saline (PBS) replaced PlyPH. Reaction mixtures were incubated at 21°C for 15 min, with the final pH of each reaction checked by pH paper and recorded. Viability counts were determined by serial dilutions and plating. The killing efficiency of PlyPH at a particular pH was represented as the ratio of *B. cereus* RSVF1 viability at that pH to *B. cereus* RSVF1 viability after exposure to the PlyPH enzyme at the same pH. The optical density of each reaction was noted at the beginning and end of each assay and calculated as percent decrease.

We found that PlyPH remained active over a wide range of pH values. Its lytic effect was found to be greatest between the pH values of 4.5 and 8, while it maintained partial activity at pH 4, 9, 9.5, and 10.5 (Fig. 2). The addition of 50 mM and

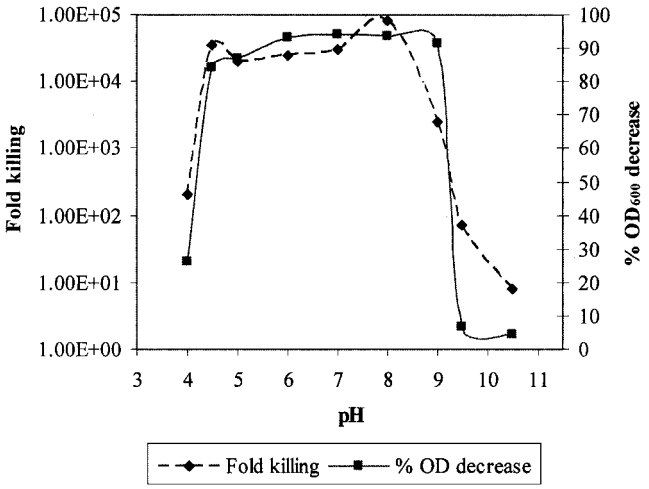

FIG. 2. pH profile of PlyPH activity. PlyPH activity against *B. cereus* strain RSVF1 in buffers with pH values between 3 and 11 was tested in viability and optical density assays that proceeded for 15 min. Killing (*n*-fold) in the viability assay was calculated based on the ratio of *B. cereus* RSVF1 viability at a particular pH to *B. cereus* RSVF1 viability after exposure to the PlyPH enzyme at the same pH and was plotted as a dashed black line. The percent decrease in optical density at 600 nm of the same reaction mixtures is plotted as a solid gray line. Final pH readings for each reaction are recorded on the *x* axis.

200 mM NaCl resulted in slightly enhanced killing of *B. cereus* RSVF1 compared with a reaction mixture with no added salt (results not shown). PlyPH also retains 100% of its lytic activity when incubated at temperatures between 4°C and 60°C for 1 h prior to testing with *B. cereus* RSVF1 (results not shown). These results indicate that PlyPH remains stable to relatively high salt concentrations, including the physiological salt concentration of 150 mM, and is highly thermostable.

Like that of PlyG, the lytic activity of PlyPH is very specific toward *B. anthracis* ΔSterne and *B. cereus* RSVF1 compared to other *Bacillus* strains tested, including other strains of *B. cereus* and *B. thuringiensis* (Table 1). PlyPH was also able to selectively lyse *B. cereus* RSVF1 in a mixture of bacteria, as measured by a luminescent assay based on the release of bacterial ATP (results not shown). This property sets it apart from other bactericidal agents, which generally have a broad spectrum of activity. The identical range of PlyPH and PlyG (26) activities reinforces the possibility that both enzymes may recognize and bind the same cell wall epitope.

Recently, a phage lysin was characterized within the genome of *B. anthracis* (21). This lysin, PlyL, exhibited 93% and 60% identity to the catalytic and binding domains of PlyG, respectively. In contrast to the selective lytic activities of PlyG and PlyPH, full-length PlyL was demonstrated to have lytic activity against a number of diverse *Bacillus* species. However, it still remains to be determined if either PlyL or PlyPH is expressed during the life cycle of *B. anthracis* and how they may function in the host bacterium.

A previously described *B. cereus* RSVF1 model of peritonitis was used to test the in vivo protective capacity of PlyPH (26). An RSVF1 suspension containing approximately 2.5×10^6 CFU/100 μl was injected into the peritoneal cavity of each mouse. Ten minutes postinfection, mice were injected either with 400 μl of purified PlyPH enzyme containing an estimated 3 mg · ml^{-1} of protein or with 400 μl of sterile 50 mM acetate buffer at pH 5.5. Three independent mouse experiments were carried out with five, four, and four mice in each group. Results from all three experiments were combined, with a total of 26 mice, 13 in the control buffer-treated group and 13 in the PlyPH-treated group. Mice were followed for 5 days and clinical signs recorded. As a control, two mice were injected with

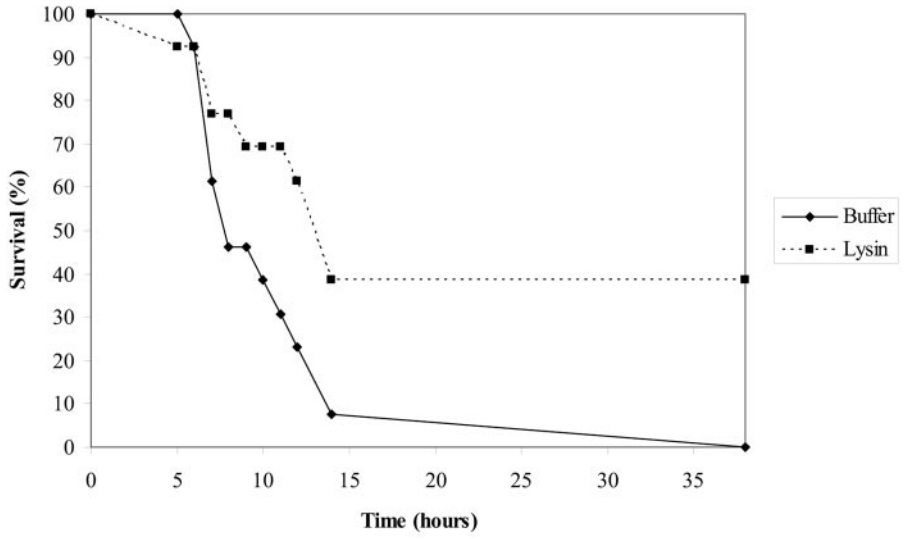

FIG. 3. Survival of BALB/c mice infected with *B. cereus* RSVF1 through the intraperitoneal route, followed by treatment with buffer or PlyPH. Each mouse was injected intraperitoneally with 100 μl of RSVF1 in PBS, followed by injection 10 min later with 400 μl of either sterile buffer or PlyPH. Three independent mouse experiments were carried out with five, four, and four mice in each experimental group. The results represent the combination of the three experiments, with a total of 26 mice, 13 in the control buffer-treated group and 13 in the PlyPH-treated group. This graph charts the survival of buffer- and PlyPH-treated mice over the initial 40-h period posttreatment.

100 μl of sterile brain heart infusion broth (instead of *B. cereus* RSVF1), followed by 400 μl of purified PlyPH 10 min later.

The results of these experiments show that PlyPH was able to rescue ~40% of mice completely, while 100% of buffer-treated mice died within 38 h of infection (Fig. 3). The survival curves were significantly different ($P < 0.02$). Purified PlyPH alone had no adverse effects on control mice.

Results from this study revealed PlyPH to be a resilient enzyme that retains its catalytic activity under various conditions that would inactivate phage lysins. PlyPH's most impressive property is its pH range (hence its name): it retains lytic activity between pH 4 and 10.5, with maximal activity between pH 4.5 and 8 (Fig. 2). Because lysins are most active between pH 5 and 7, with activity diminishing rapidly beyond those values (16, 33), PlyPH may be the first putative lysin described to be active over such a wide pH range. Significantly, PlyPH seems to be highly specific for *B. anthracis* ΔSterne and *B. cereus* strain RSVF1, which possesses *B. anthracis*-like properties (results not shown). Considering the ability of PlyPH to retain lytic activity under such a broad range of conditions, it may be possible for PlyPH to function under environmental conditions that render PlyG inactive.

While the majority of natural *B. anthracis* isolates are sensitive to most antibiotics, the window of treatment opportunity for spore-exposed individuals is only 48 h. Bacteriolytic enzymes, used in combination with antibiotics, may extend this narrow treatment window by controlling the growth of bacilli in the blood. In addition, *B. anthracis* has been shown to be able to acquire resistance to certain antibiotics with relative ease (1, 3, 5, 29). Should infections occur with a resistant strain of *B. anthracis* that cannot be treated with conventional antibiotics, cell wall-cleaving enzymes such as PlyPH may be considered as an alternative form of therapy.

This work was supported by grants from the Defense Advanced Research Project Agency (DARPA) and by U.S. Public Health Service grant AI057472 to V.A.F.

We thank Alexander Tomasz, Joshua Lederberg, and Fischetti lab members for insightful discussions of this work. We also acknowledge Jutta Loeffler's expertise and guidance in the animal experiments.

REFERENCES

1. **Athamna, A., M. Athamna, N. Abu-Rashed, B. Medlej, D. J. Bast, and E. Rubinstein.** 2004. Selection of *Bacillus anthracis* isolates resistant to antibiotics. J. Antimicrob. Chemother. **54:**424–428.
2. **Brook, I.** 2002. The prophylaxis and treatment of anthrax. Int. J. Antimicrob. Agents **20:**320–325.
3. **Bryskier, A.** 2002. *Bacillus anthracis* and antibacterial agents. Clin. Microbiol. Infect. **8:**467–478.
4. **Cheng, Q., D. Nelson, S. Zhu, and V. A. Fischetti.** 2005. Removal of group B streptococci colonizing the vagina and oropharynx of mice with a bacteriophage lytic enzyme. Antimicrob. Agents Chemother. **49:**111–117.
5. **Choe, C. H., S. S. Bouhaouala, I. Brook, T. B. Elliott, and G. B. Knudson.** 2000. In vitro development of resistance to ofloxacin and doxycycline in *Bacillus anthracis* Sterne. Antimicrob. Agents Chemother. **44:**1766.
6. **Fischetti, V. A.** 2003. Novel method to control pathogenic bacteria on human mucous membranes. Ann. N. Y. Acad. Sci. **987:**207–214.
7. **Fischetti, V. A.** 2001. Phage antibacterials make a comeback. Nat. Biotechnol. **19:**734–735.
8. **Guzman, L.-M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose pBAD promoter. J. Bacteriol. **177:**4121–4130.
9. **Helgason, E., O. A. Okstad, D. A. Caugant, H. A. Johansen, A. Fouet, M. Mock, I. Hegna, and A.-B. Kolsto.** 2000. *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*—one species on the basis of genetic evidence. Appl. Environ. Microbiol. **66:**2627–2630.
10. **Howell, A., S. Dubrac, K. K. Anderson, D. Noone, J. Fert, T. Msadek, and K. Devine.** 2003. Genes controlled by the essential YycG/YycF two-component system of *Bacillus subtilis* revealed through a novel hybrid regulator approach. Mol. Microbiol. **49:**1639–1655.
11. **Inglesby, T. V., T. O'Toole, D. A. Henderson, J. G. Bartlett, M. S. Ascher, E. Eitzen, A. M. Friedlander, J. Gerberding, J. Hauer, J. Hughes, J. McDade, M. T. Osterholm, G. Parker, T. M. Perl, P. K. Russell, and K. Tonat.** 2002. Anthrax as a biological weapon, 2002. JAMA **287:**2236–2252.
12. **Ivins, B. E., J. W. Ezzell, Jr., J. Jemski, K. W. Hedlund, J. D. Ristoph, and S. H. Leppla.** 1986. Immunization studies with attenuated strains of *Bacillus anthracis*. Infect. Immun. **52:**454–458.
13. **Jado, I., R. Lopez, E. Garcia, A. Fenoll, J. Casal, and P. Garcia.** 2003. Phage lytic enzymes as therapy for antibiotic-resistant *Streptococcus pneumoniae* infection in a murine sepsis model. J. Antimicrob. Chemother. **52:**967–973.
14. **Koehler, T. M.** 2000. *Bacillus anthracis*, p. 519–528. *In* V. A. Fischetti, R. P. Novick, J. J. Ferretti, D. A. Portnoy, and J. I. Rood (ed.), Gram-positive pathogens. American Society for Microbiology, Washington, D.C.
15. **Loeffler, J. M., D. Nelson, and V. A. Fischetti.** 2001. Rapid killing of *Streptococcus pneumoniae* with a bacteriophage cell wall hydrolase. Science **294:**2170–2172.
16. **Loeffler, J. M., S. Djurkovic, and V. A. Fischetti.** 2003. Phage lytic enzyme Cpl-1 as a novel antimicrobial for pneumococcal bacteremia. Infect. Immun. **71:**6199–6204.
17. **Loessner, M., K. Kramer, F. Ebel, and S. Scherer.** 2002. C-terminal domains of *Listeria monocytogenes* bacteriophage murein hydrolases determine specific recognition and high-affinity binding to bacterial cell wall carbohydrates. Mol. Microbiol. **44:**335–349.
18. **Longchamp, P. F., C. Mauel, and D. Karamata.** 1994. Lytic enzymes associated with defective prophages of *Bacillus subtilis*: sequencing and characterization of the region comprising the *N*-acetylmuramoyl-L-alanine amidase gene of prophage PBSX. Microbiology **140:**1855–1867.
19. **Lopez, R., E. Garcia, P. Garcia, and J. L. Garcia.** 1997. The pneumococcal cell wall degrading enzymes: a modular design to create new lysins? Microb. Drug Resist. **3:**199–211.
20. **Lopez, R., M. P. Gonzalez, E. Garcia, J. L. Garcia, and P. Garcia.** 2000. Biological roles of two new murein hydrolases of *Streptococcus pneumoniae* representing examples of module shuffling. Res. Microbiol. **151:**437–443.
21. **Low, Y. L., C. Yang, M. Perego, A. Osterman, and R. C. Liddington.** 2005. Structure and lytic activity of a *Bacillus anthracis* prophage endolysin. J. Biol. Chem. **280:**35433–35439.
22. **Nelson, D., L. Loomis, and V. A. Fischetti.** 2001. Prevention and elimination of upper respiratory colonization of mice by group A streptococci by using a bacteriophage lytic enzyme. Prot. Natl. Acad. Sci. USA **98:**4107–4112.
23. **Nugroho, F. A., H. Yamamoto, Y. Kobayashi, and J. Sekiguchi.** 1999. Characterization of a new sigma-K-dependent peptidoglycan hydrolase gene that plays a role in *Bacillus subtilis* mother cell lysis. J. Bacteriol. **181:**6230–6237.
24. **Oncu, S., S. Oncu, and S. Sakarya.** 2003. Anthrax—an overview. Med. Sci. Monit. **9:**RA276–RA283.
25. **Read, T. D., S. N. Peterson, N. Tourasse, L. W. Baillie, I. T. Paulsen, K. E. Nelson, H. Tettelin, D. E. Fouts, J. A. Eisen, S. R. Gill, E. K. Holtzapple, O. A. Okstad, E. Helgason, J. Rilstone, M. Wu, J. F. Kolonay, M. J. Beanan, R. J. Dodson, L. M. Brinkac, M. Gwinn, R. T. DeBoy, R. Madpu, S. C. Daugherty, A. S. Durkin, D. H. Haft, W. C. Nelson, J. D. Peterson, M. Pop, H. M. Khouri, D. Radune, J. L. Benton, Y. Mahamoud, L. Jiang, I. R. Hance, J. F. Weidman, K. J. Berry, R. D. Plaut, A. M. Wolf, K. L. Watkins, W. C. Nierman, A. Hazen, R. Cline, C. Redmond, J. E. Thwaite, O. White, S. L. Salzberg, B. Thomason, A. M. Friedlander, T. M. Koehler, P. C. Hanna, A.-B. Kolste, and C. M. Fraser.** 2003. The genome of *Bacillus anthracis* Ames and comparison to closely related bacteria. Nature **423:**81–86.
26. **Schuch, R., D. Nelson, and V. A. Fischetti.** 2002. A bacteriolytic agent that detects and kills *Bacillus anthracis*. Nature **418:**884–888.
27. **Sheehan, M. M., J. L. Garcia, R. Lopez, and P. Garcia.** 1997. The lytic enzyme of the pnemococcal phage Dp-1: a chimeric enzyme of intergeneric origin. Mol. Microbiol. **25:**717–725.
28. **Smith, T. J., S. A. Blackman, and S. J. Foster.** 2000. Autolysins of *Bacillus subtilis*: multiple enzymes with multiple functions. Microbiology **146:**249–262.
29. **Stepanov, A. V., L. I. Marinin, A. P. Pomerantsev, and N. A. Staritsin.** 1996. Development of novel vaccines against anthrax in man. J. Biotechnol. **44:**155–160.
30. **Swartz, M. N.** 2001. Recognition and management of anthrax—an update. N. Engl. J. Med. **345:**1621–1626.
31. **Ticknor, L. O., A.-B. Kolsto, K. K. Hill, P. Keim, M. T. Laker, M. Tonks, and P. J. Jackson.** 2001. Fluorescent amplified fragment length polymorphism analysis of Norwegian *Bacillus cereus* and *Bacillus thuringiensis* soil isolates. Appl. Environ. Microbiol. **67:**4863–4873.
32. **Turnbull, P. C. B.** 2002. Introduction: anthrax history, disease and ecology. Curr. Top. Microbiol. Immunol. **271:**1–19.
33. **Yoong, P., R. Schuch, D. Nelson, and V. A. Fischetti.** 2004. Identification of a broadly active phage lytic enzyme with lethal activity against antibiotic-resistant *Enterococcus faecalis* and *Enterococcus faecium*. J. Bacteriol. **186:**4808–4812.
34. **Young, R.** 1992. Bacteriophage lysis: mechanism and regulation. Microbiol. Rev. **56:**430–481.