Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity

Nichole A. Broderick*†‡, Kenneth F. Raffa*, and Jo Handelsman†§

Departments of *Entomology and †Plant Pathology and ‡Microbiology Doctoral Training Program, University of Wisconsin, 1630 Linden Drive, Madison, WI 53706

Edited by Jeffrey I. Gordon, Washington University School of Medicine, St. Louis, MO, and approved August 17, 2006 (received for review June 12, 2006)

Bacillus thuringiensis **is the most widely applied biological insecticide and is used to manage insects that affect forestry and agriculture and transmit human and animal pathogens. This ubiquitous spore-forming bacterium kills insect larvae largely through the action of insecticidal crystal proteins and is commonly deployed as a direct bacterial spray. Moreover, plants engineered with the *cry* genes encoding the *B. thuringiensis* crystal proteins are the most widely cultivated transgenic crops. For decades, the mechanism of insect killing has been assumed to be toxin-mediated lysis of the gut epithelial cells, which leads to starvation, or *B. thuringiensis* septicemia. Here, we report that *B. thuringiensis* does not kill larvae of the gypsy moth in the absence of indigenous midgut bacteria. Elimination of the gut microbial community by oral administration of antibiotics abolished *B. thuringiensis* insecticidal activity, and reestablishment of an *Enterobacter* sp. that normally resides in the midgut microbial community restored *B. thuringiensis*-mediated killing. *Escherichia coli* engineered to produce the *B. thuringiensis* insecticidal toxin killed gypsy moth larvae irrespective of the presence of other bacteria in the midgut. However, when the engineered *E. coli* was heat-killed and then fed to the larvae, the larvae did not die in the absence of the indigenous midgut bacteria. *E. coli* and the *Enterobacter* sp. achieved high populations in hemolymph, in contrast to *B. thuringiensis*, which appeared to die in hemolymph. Our results demonstrate that *B. thuringiensis*-induced mortality depends on enteric bacteria.**

insect–microbe interactions | microbe–microbe interactions | synergy | Lepidoptera | pore-forming toxin

Bacillus thuringiensis is an opportunistic insect pathogen that was discovered almost a century ago (1–7). The salient feature of this species is accumulation of crystalline parasporal inclusions during sporulation. These inclusions are composed of one or more protoxins, known as δ-endotoxins, each of which is specific primarily at the level of insect orders, particularly Lepidoptera, Diptera, and Coleoptera (6). In Lepidoptera, specificity is due in part to the extremely alkaline midgut environment that is required to solubilize the protoxin into the active form. Solubilized protoxins are activated by midgut proteases and bind to receptors on the epithelial surface. Then, by a process that remains unclear, the toxins appear to insert into the membranes of gut cells, where they form pores that lead to cell lysis (2). It has been proposed that disruption of the midgut epithelium results in a prolonged cessation of feeding and eventual death by starvation. An alternative proposed mechanism of killing is that extensive cell lysis provides spores access to the more favorable environment of the hemocoel, where they germinate and reproduce, leading to septicemia and death (2, 3). Although these two models have been cited many times in the literature, neither is entirely consistent with experimental observations. For example, the starvation model is not supported by the fact that *B. thuringiensis* kills insects much more rapidly than does starvation: *B. thuringiensis* toxin-induced mortality typically takes 2–5 days, whereas starvation-induced mortality often takes 7–10 days. In addition, rapid turnover of gut epithelial cells permits larvae to recover from toxin-induced feeding inhibition (8–10). The septicemia model is challenged by the observation that the toxin causes mortality when it is separated from the bacterial cells, which has been accomplished with purified toxin and transgenic plants that produce the toxin (1–4, 6).

Results

Effect of Antibiotics on Larval Susceptibility to *B. thuringiensis*. In a previous study, we found that a number of treatments that altered the susceptibility of the gypsy moth to *B. thuringiensis* also affected the bacterial community in the larvae's midgut (11, 12). To determine whether there was a causal relationship between changes in this community and susceptibility to *B. thuringiensis* toxin, we explored the impact of antibiotics. We reared larvae from hatching on either sterile artificial diet or diet amended with a range of concentrations (8–500 μg/ml of diet) of an antibiotic mixture that consisted of penicillin, gentamicin, rifampicin, and streptomycin. Upon molting to the third instar, larvae from each treatment were fed with 0, 1, or 10 units of *B. thuringiensis*, and mortality was monitored for 7 days. *B. thuringiensis*-induced larval mortality was reduced by amendment of the larval diet with antibiotics in a dose-dependent fashion (Fig. 1). Mortality of insects fed *B. thuringiensis* was inversely proportional to the antibiotic concentration, with no mortality caused by *B. thuringiensis* among larvae fed 10 units and reared on antibiotic mixtures containing 125 μg/ml or more of each antibiotic. This reduction in mortality was accompanied by reduced populations of culturable *Enterococcus* and *Enterobacter* spp. from the midguts of larvae (Table 1). These species were previously identified as the dominant culturable members of the gypsy moth midgut (11). This result suggested that the microbial community in the midgut contributed to larval death due to *B. thuringiensis* treatment.

Impact of *Enterobacter* sp., a Midgut Inhabitant, on Larval Susceptibility to *B. thuringiensis*. To test the possibility that midgut bacteria were contributing to *B. thuringiensis*-induced mortality directly, a resident member of the midgut microbial community, *Enterobacter* sp. NAB3 (11), was reestablished in larvae reared on antibiotic-amended medium. After feeding of the *Enterobacter* sp. to newly molted third-instar gypsy moth larvae, this bacterium established populations of 9.0×10^2 to 2.1×10^4 per midgut and restored the insecticidal activity of *B. thuringiensis* to nearly that of larvae not treated with antibiotics (Fig. 2). Other members of the gut community, *Enterococcus casseliflavus* and *Staphylococcus xylosus*, did not restore insecticidal activity to *B.*

Author contributions: K.F.R. and J.H. contributed equally to this work; N.A.B., K.F.R., and J.H. designed research; N.A.B. performed research; N.A.B., K.F.R., and J.H. analyzed data; and N.A.B., K.F.R., and J.H. wrote the paper.

The authors declare no conflict of interest.

This paper was submitted directly (Track II) to the PNAS office.

§To whom correspondence should be addressed at: Department of Plant Pathology, University of Wisconsin, 1630 Linden Drive, Room 589, Madison, WI 53706. E-mail: joh@plantpath.wisc.edu.

www.pnas.org/cgi/doi/10.1073/pnas.0604865103

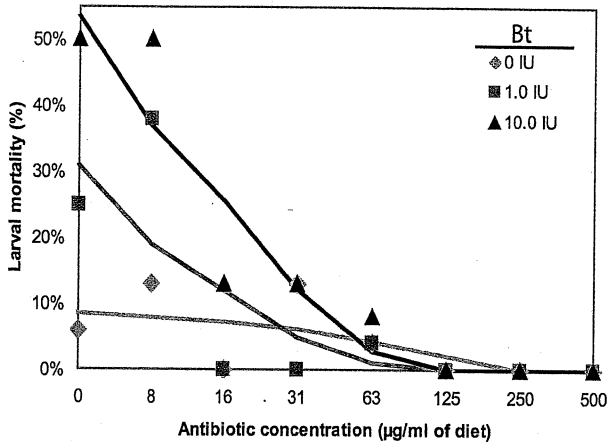

Fig. 1. Effect of antibiotic concentration on toxicity of *B. thuringiensis* to third-instar *Lymantria dispar*. Larvae were reared on sterile artificial diet that was unamended or amended with an antibiotic mixture containing up to 500 μg each of penicillin, gentamicin, rifampicin, and streptomycin per ml. Curves were fit by using PROC NLIN in SAS 9.1.3 (28). All are of the general form $y = ae^{-bx}$. Dose 0 IU, $a = 0.0862$, $b = 0.0108$, $F_{2,6} = 5.47$, $P > 0.05$; dose 1.0 IU, $a = 0.3125$, $b = 0.0608$, $F_{2,6} = 8.12$, $P > 0.02$; dose 10.0 IU, $a = 0.5351$, $b = 0.0474$, $F_{2,6} = 41.12$, $P > 0.0003$.

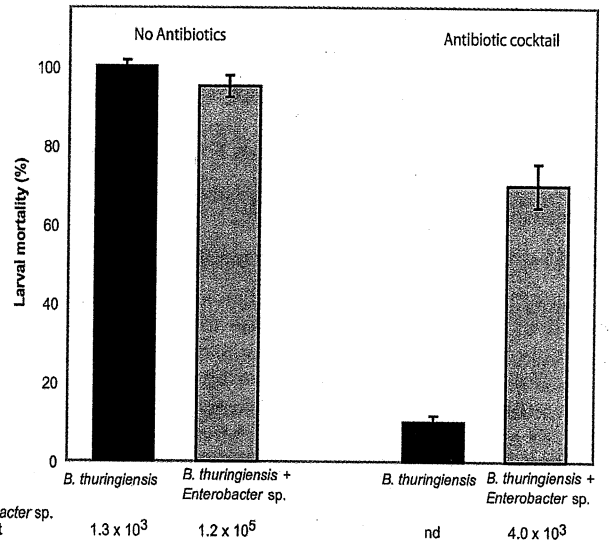

Fig. 2. Restoration of *B. thuringiensis* toxicity by an *Enterobacter* sp. after elimination of detectable gut flora and *B. thuringiensis* activity by antibiotics. "Antibiotic cocktail" *L. dispar* larvae were reared until the third instar on sterile artificial diet amended with 500 μg each of penicillin, gentamicin, rifampicin, and streptomycin per ml. Each bar represents the mean mortality ± SEM of 48 larvae (four replications of 12 larvae each). Values at the bottom represent the sizes of the populations of the *Enterobacter* sp. as detected by culturing. nd, not detected.

thuringiensis when fed to larvae in the same manner. These results suggest that the *Enterobacter* sp. is largely responsible for the septicemia associated with *B. thuringiensis*-induced mortality in gypsy moth larvae.

***In Vitro* Growth of *Enterobacter* sp. NAB3 and *B. thuringiensis* in Larval Hemolymph.** To cause septicemia, bacteria must be able to enter and multiply in the hemolymph, so we compared the growth of *B. thuringiensis* and an *Enterobacter* sp. *in vitro* in larval hemolymph. We introduced $\approx 10^5$ bacterial cells per ml of hemolymph withdrawn from fifth-instar gypsy moth larvae. The *Enterobacter* sp. reached a population density of 1.3×10^9 cfu/ml after 6 h, which was equivalent to its growth in tryptic soy broth. In contrast, *B. thuringiensis* populations dropped to below detectable limits (200 cfu/ml) in hemolymph after 6 h and remained undetectable throughout the 18.5-h assay, even though *B. thuringiensis* grew as quickly as the *Enterobacter* sp. in tryptic soy broth (Fig. 3).

Killing Ability of *Escherichia coli* ECE52 and *in Vitro* Growth in Hemolymph. Population densities of *B. thuringiensis* were similar in midguts of larvae that had been reared on antibiotic-amended and unamended sterile artificial diet, suggesting that the reduction in insect mortality was not due to direct elimination of *B. thuringiensis*. To test this possibility more directly, we assayed the effect of *E. coli* strain ECE52, which produces the *B. thuringiensis* toxin (13), and compared its toxicity to larvae reared on antibiotic-amended versus unamended diet. *E. coli* ECE52 killed larvae that had been reared on antibiotics as effectively as larvae containing the native gut community (Fig. 4). When the *E. coli* cells were heat-killed before feeding, mortality was equivalent to treatment with live *E. coli* in larvae reared on an unamended diet. However, in larvae reared on antibiotics, there was no mortality due to toxin alone in the absence of live *E. coli* cells (Fig. 4). *E. coli* lacking the crystal toxin genes (JM103) did not induce significant mortality in either group of larvae (0–4%). In hemolymph, *E. coli* ECE52 reached a population density of $>10^9$ cfu/ml after 14.5 h (Fig. 3). These results suggest that the *B. thuringiensis* toxin alone is not sufficient to cause larval mortality, because an enteric bacterium (a member of the Enterobacteriaceae family) such as *E. coli* or *Enterobacter* must also be present to induce septicemic death.

Table 1. Effect of antibiotic mixture concentration on the culturable midgut bacteria of third-instar *L. dispar*

Antibiotic concentration, μg/ml of diet*	*Enterococcus* sp., cfu per gut	*Enterobacter* sp., cfu per gut
0	4.80×10^6	5.21×10^5
8	1.17×10^5	6.08×10^7
16	nd	1.13×10^8
31	1.00×10^1	1.67×10^1
63	1.30×10^5	2.50×10^4
125	5.33×10^1	nd
250	nd	nd
500	nd	nd

nd, not detected.

*Larvae were reared on sterile artificial diet amended with an antibiotic mixture composed of 500 μg each of penicillin, gentamicin, rifampicin, and streptomycin per ml.

Discussion

We present three lines of evidence that indicate that the resident midgut bacteria of gypsy moth larvae contribute to mortality associated with consumption of *B. thuringiensis*. First, insecticidal activity was abolished by eliminating the detectable midgut bacterial community. Second, insecticidal activity was restored by reestablishing an *Enterobacter* sp., a member of the normal midgut community. Third, a live *E. coli* strain that produces the *B. thuringiensis* toxin killed larvae whether or not they contained other bacteria in their midguts, but if the *E. coli* was heat-killed before feeding, it killed only those larvae that contained the normal gut community. These results, coupled with the observation that the *Enterobacter* sp. and *E. coli* grew rapidly in larval hemolymph, whereas *B. thuringiensis* did not, indicate that the enteric bacteria are responsible for septicemia associated with *B. thuringiensis* toxicity. The enteric bacteria alone do not induce

MICROBIOLOGY

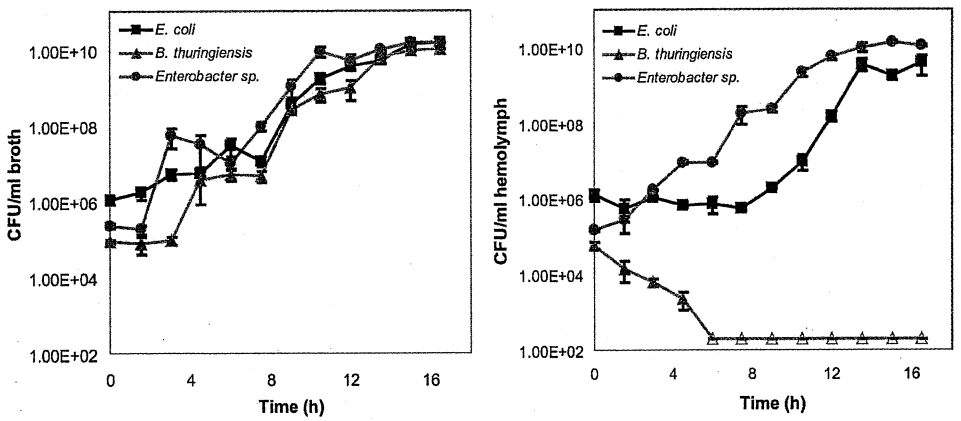

Fig. 3. Growth of *B. thuringiensis*, *Enterobacter* sp. NAB3, and *E. coli* ECE52 in tryptic soy broth (*Left*) and *L. dispar* hemolymph (*Right*). The detection limit was 200 cfu/ml; samples in which no colonies were detected were assigned this value and are indicated by open symbols. Each point represents the mean cfu/ml ± SEM of three replicate cultures.

mortality, suggesting that *B. thuringiensis* enables them to reach the hemocoel by permeabilizing the gut epithelium.

These results suggest that *B. thuringiensis* toxicity depends on an interaction with microorganisms of the normal gut community. This finding contrasts sharply with established models that assume that *B. thuringiensis* itself induces mortality through starvation or direct septicemia. Thus, much of the research on *B. thuringiensis* since its discovery in 1918 and its development for commercial use in the 1950s has been predicated on a mechanism that is quite different from the one we present here. Studies of the mode of action of *B. thuringiensis* in the last 25 years have clarified the molecular specificity of toxin binding and pore formation but have not addressed the subsequent events that lead to insect death (1–3). The pervasive acceptance of the models of starvation and *B. thuringiensis*-induced septicemia over the last decade appears to be associated with two review articles that have been cited >800 times since 1992; each article postulates the models, but neither provides empirical evidence to support either one.

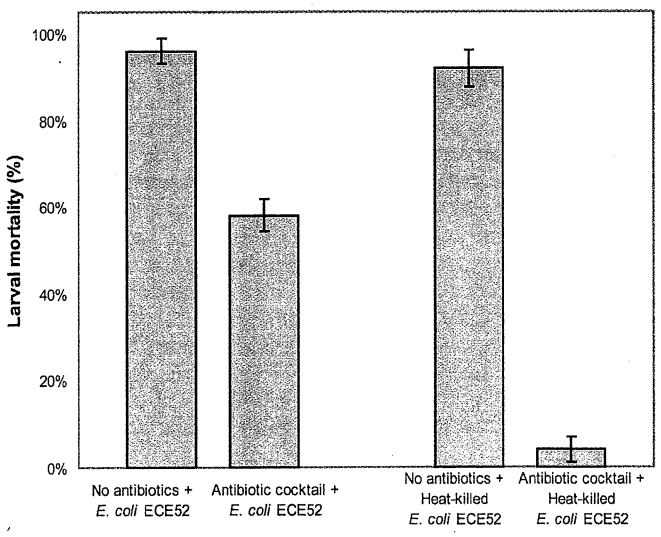

Fig. 4. Insecticidal activity of a *B. thuringiensis* toxin-producing *E. coli* strain (ECE52) is abolished by heat-killing in the absence of normal gut microbiota. Larvae were fed live ECE52 with or without antibiotics, or they were fed heat-killed ECE52 with or without antibiotics, as indicated. Each bar represents the mean mortality ± SEM of 36 third-instar *L. dispar* (three replications of 12 larvae each).

A number of published results that are inconsistent with the starvation and *B. thuringiensis*-induced septicemia models are easily reconciled with the gut microbial community model that is presented here. *B. thuringiensis* spores, for example, are typically absent from the hemocoel until very late in the infection process and frequently do not appear until well after the death of the insect (14–16). This finding challenges the idea that *B. thuringiensis* induces death by septicemia but is consistent with the model that proposes that septicemia is initiated by the enteric bacteria in the insect gut. In addition, some studies indicate that *B. thuringiensis* is unable to grow in the hemocoel, which is consistent with our evidence that it does not grow in hemolymph (17, 18) and also makes it difficult to ascribe septicemia to *B. thuringiensis* alone. Most studies have not included an examination of dead larvae to verify the septicemic agent, thereby obscuring the contribution of the gut bacteria. Despite the prevailing models, there have been hints of the involvement of enteric bacteria in *B. thuringiensis* killing. For example, Dubois and Dean (19) demonstrated that some species of forest epiphytic bacteria enhance the toxicity of *B. thuringiensis* proteins, although these researchers and others fall short of concluding that the gut bacteria are required for *B. thuringiensis* killing activity, as we suggest here. Furthermore, a number of studies use a procedure known as "immunizing" larvae and hemolymph with enteric bacteria to enable *B. thuringiensis* to grow, casting doubt on the ability of *B. thuringiensis* to proliferate in this environment independently (20). This latter result suggests that the enteric bacteria might remove an immunological barrier, such as defensive enzymes or antimicrobial peptides that prevent growth of *B. thuringiensis*.

The implications of this work encompass both applications and fundamental knowledge. The role of enteric bacteria in insect mortality by *B. thuringiensis* presents opportunities for designing approaches to manage insect pests by harnessing their indigenous communities or combining *B. thuringiensis* with bacteria that can induce septicemia. This work also raises the possibility that the genes encoding the *B. thuringiensis* toxins could be deployed more effectively in transgenic crops by exploiting the role of insect-borne bacteria that enhance insecticidal activity. Additionally, research on both the efficacy and nontarget impacts of *B. thuringiensis* has yielded a perplexingly wide range of results with regard to the environmental persistence of activity (21). Recent studies show that the composition and populations of gut communities can vary widely within insect species (11, 12); hence, a more thorough understanding of gut communities could help clarify these issues. Recent interest in the role of intestinal bacteria in sepsis invites a parallel between human disease and

insect killing by *B. thuringiensis* (22). A number of human pathogens are similar to *B. thuringiensis* in that they deploy toxins that produce pores in the wall of the host gut. The possibility that normal gut microbiota contribute to human diseases that involve toxins such as colicin A, diphtheria toxin, and *Pseudomonas aeruginosa* exotoxin (23–25) bears exploration. Thus, the cooperative relationship between *B. thuringiensis* and the indigenous gut microbiota might provide a model for studying polymicrobial diseases in animals that are not as experimentally accessible as the gypsy moth.

Methods

Bacterial Strains. The *B. thuringiensis* used was a commercial formulation of *B. thuringiensis* subspecies *kurstaki* (DiPel DF, Valent Biosciences, Libertyville, IL) consisting of proteins and spores. *Enterobacter* sp. NAB3 (11) was originally cultured from the midguts of gypsy moth larvae feeding on sterile artificial diet. *E. coli* strain ECE52 was provided by the Bacillus Genetic Stock Center (Columbus, OH). For assays with heat-killed *E. coli* ECE52, a washed overnight culture was heated to 70°C for 15 min before assay use.

Insects. Gypsy moth egg masses were obtained from colony NJSS at the U.S. Department of Agriculture–Animal and Plant Health Inspection Service laboratory at Otis Air National Guard Base (Cape Cod, MA) and reared as described in ref. 26, except that larvae reared on the antibiotic mixture were fed sterile artificial diet amended with 500 μg each of rifampicin, gentamicin, penicillin, and streptomycin per ml.

Mortality Assays. *B. thuringiensis* mortality assays were performed as described in ref. 26. All assays were performed on newly molted third-instar larvae. Within a treatment, the same dose of 1, 10, or 100 units of *B. thuringiensis* was applied each day. All treatments were applied in a volume of 1 μl to a standard diet disk (4-mm diameter and 1-mm height) and fed to the larvae on 2 consecutive days. Mortality was recorded every 24 h for 7 days.

Bacterial Growth Curves in Hemolymph. Hemolymph was collected from ≈250 larvae by the method of Orchard and Goodrich-Blair (27), and bacterial growth in hemolymph was measured by dilution plating on tryptic soy agar. Approximately 10^5 cfu from an overnight culture grown in 1/10-strength tryptic soy broth was inoculated into 1 ml of hemolymph and grown at 25°C with shaking for 18.5 h. Subsamples (10 μl) were taken every 1.5 h for dilution plating.

We thank Peter M. Crump for assistance with statistical analysis, Matthew McMahon and Michelle Preston for maintenance of the gypsy moth colony, the U.S. Department of Agriculture for providing gypsy moth eggs, and the Bacillus Genetic Stock Center for providing *E. coli* ECE52. This work was supported by the University of Wisconsin College of Agricultural and Life Sciences Hatch Project, Valent Biosciences, and the Howard Hughes Medical Institute.

1. Gill SS, Cowles EA, Pietrantonio PV (1992) *Annu Rev Entomol* 37:615–636.
2. Knowles BH (1994) *Adv Insect Physiol* 24:275–308.
3. Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998) *Microbiol Mol Biol Rev* 62:775–806.
4. Entwistle PF, Cory JS, Bailey MJ, Higgs S (1993) in *Bacillus thuringiensis, an Environmental Biopesticide: Theory and Practice* (Wiley Interscience, New York).
5. Bulla LA, Jr, Rhodes RA, St Julian G (1975) *Annu Rev Microbiol* 29:163–190.
6. Höfte H, Whiteley HR (1989) *Microbiol Rev* 53:242–255.
7. Heimpel AM, Angus TA (1960) *Bacteriol Rev* 24:266–288.
8. Heimpel AM (1967) *Annu Rev Entomol* 12:287–322.
9. Lüthy P, Ebersold HR (1981) in *Pathogenesis of Invertebrate Microbial Diseases*, ed Davidson EW (Allanheld Osmun, Totowa, NJ), pp 235–267.
10. Retnakaran A, Lauzon H, Fast P (1993) *Entomol Exp Appl* 34:233–239.
11. Broderick NA, Raffa KF, Goodman RM, Handelsman J (2004) *Appl Environ Microbiol* 70:293–300.
12. Broderick NA, Goodman RM, Handelsman J, Raffa KF (2003) *Environ Entomol* 32:387–391.
13. Ge AZ, Shivarova NI, Dean DH (1989) *Proc Natl Acad Sci USA* 86:4037–4041.
14. Heimpel AM (1955) *Can J Zool* 33:311–326.
15. Nishiitsutsuji-Uwo J, Endo Y (1980) *Appl Entomol Zool* 15:416–424.
16. Toumanoff C, Vago C (1953) *Ann Inst Pasteur (Paris)* 84:376–385.
17. Zhang M-Y, Lövgren A, Low MG, Landén R (1993) *Infect Immun* 61:4947–4954.
18. Suzuki MT, Lereclus D, Nagy Arantes OM (2004) *Can J Microbiol* 50:973–975.
19. Dubois NR, Dean DH (1995) *Environ Entomol* 24:1741–1747.
20. Fedhila S, Nel P, Lereclus D (2002) *J Bacteriol* 184:3296–3304.
21. Johnson KS, Scriber JM, Nitao JK, Smitley DR (1995) *Environ Entomol* 24:288–297.
22. Wells CL, Hess DJ, Erlandsen SL (2004) *Shock* 22:562–568.
23. Grochulski P, Masson L, Borisova S, Pusztai-Carey M, Schwartz JL, Brousseau R, Cygler M (1995) *J Mol Biol* 254:447–464.
24. Allured VS, Collier RJ, Carroll SF, McKay DB (1986) *Proc Natl Acad Sci USA* 83:1320–1324.
25. Tilley SJ, Saibil HR (2006) *Curr Opin Struct Biol* 16:230–236.
26. Broderick NA, Goodman RM, Raffa KF, Handelsman J (2000) *Environ Entomol* 29:101–107.
27. Orchard SS, Goodrich-Blair H (2004) *Appl Environ Microbiol* 70:5621–5627.
28. SAS Institute (2006) SAS 9.1.3 (SAS Institute, Cary, NC).