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Use of Silkworm Larvae to Study Pathogenic Bacterial Toxins

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Injection of stationary phase culture-supernatants of *Staphylococcus aureus* and *Pseudomonas aeruginosa* into the hemolymph of silkworm larvae caused their death, whereas a culture-supernatant of a non-pathogenic strain of *Escherichia coli* did not. A culture-supernatant of a mutant of *agr*, a global virulence regulator of *S. aureus* that is required for exotoxin production, was much less toxic to silkworm larvae. A culture-supernatant of a disruption mutant of the *S. aureus* beta-toxin gene did not kill larvae, whereas one of a deletion mutant of alpha-toxin, gamma-toxin, or aureolysin killed larvae, indicating that the beta-toxin gene is required for staphylococcal supernatant–mediated killing of silkworm larvae. The 50% lethal doses (LD_{50}) of staphylococcal alpha-toxin and beta-toxin, *Pseudomonas* exotoxin A and diphtheria toxin were 12 μg/g, 9 μg/g, 0.14 μg/g and 1.1 μg/g, respectively. As the purified toxins killed the larvae, silkworm larvae could be used as a model to study the actions of pathogenic bacterial toxins in animal bodies.

Key words: animal model, infection, pathogenic bacterial toxins, silkworm larva, *Staphylococcus aureus*.

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Toxins produced by microorganisms, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, provide a selective growth advantage in certain environments. These toxins not only help pathogenic bacteria combat the immune systems of their hosts, but also secure nutrients required for growth. *S. aureus* can produce a number of different components that might contribute to virulence, including surface-associated adhesions, capsular polysaccharides, exoenzymes and exotoxins. The expression of these virulence factors is regulated by a number of genetic loci, including *agr* (accessory gene regulator) and *sar* (staphylococcal accessory regulator) (*1*). Mutants of these global regulators exhibit attenuated virulence in both experimental animal models (*2–5*) and cell lines (*6*). Mice and rabbits are used as animal models for research on purified toxins of pathogenic bacteria, although the use of large numbers of mammals is difficult for logistical, ethical, and financial reasons. Invertebrate animals offer alternative, less expensive animal models. Although the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* have been used as less expensive, alternative animal models to study host-microbe interactions (*7–9*), these invertebrate animal models are difficult to use for experiments in which large-volume injections are required for quantitative analysis; for example, evaluation of the effects of purified toxins. Furthermore, to our knowledge, invertebrate animals have not been used to study pathogenic bacterial toxins that are lethal for mammals.

We reported that the larvae of silkworm *Bombyx mori* are useful not only as an animal model to study infections by bacteria or fungi that are pathogenic to humans, but also for quantitative evaluation of the therapeutic effects of antibiotics (*10*, *11*). We previously reported that injection of live *S. aureus* or *P. aeruginosa* into silkworm larvae killed the animals, whereas injection of a non-virulent strain of *Escherichia coli* did not (*10*). Using silkworm larvae as an animal model, we recently identified novel conserved virulence factors, *cvfA*, *cvfB*, and *cvfC*, which are required for the maximum virulence of *S. aureus* (*5*). In the present study, we examined the effects of pathogenic bacterial toxins, and found that injection of culture-filtrates of pathogenic bacteria or purified bacterial toxins killed silkworm larvae. Based on the findings in the present study, we propose that silkworm larvae can be used as an animal model for studying both host-toxin interactions and the mechanisms of action of pathogenic bacterial toxins *in vivo*.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions—A list of the bacterial strains used in this study is given in Table 1. *S. aureus* strains were cultured in either Luria-Bertani (LB) medium (1% tryptone (Becton-Dickinson, Cockeysville, MD), 0.5% yeast extract (Becton-Dickinson), 1% NaCl (Sigma-Aldrich Chemical Co., St. Louis, MO), or tryptic soy broth (TSB; Becton-Dickinson) at 37°C. *P. aeruginosa* strain PAO1 and *E. coli* strain W3110 were cultured in LB medium at 37°C. When required, a culture was supplemented with antibiotics. Phage transduction was performed using phage 80α, as described previously (*12*). An *agr* mutant of 8325-4, YM0113, was prepared by phage-transduction using Δ*agr* strain

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Table 1. **Bacterial strains used in this study.**

Strain	Genotype	Characteristics	Source/Ref.
S. aureus strain			
8325-4	*rsbU*	NCTC8325 cured of prophages; defective RsbU and SigB	T.J. Foster (*26*)
YM0113	*agr*	Agr mutant of 8325-4	Present study
DU1090	*hla*	Alpha-toxin mutant of 8325-4	T.J. Foster (*26*)
DU5719	*hlb*	Beta-toxin mutant of 8325-4	T.J. Foster (*26*)
DU5942	*hlg*	Gamma-toxin mutant of 8325-4	T.J. Foster (*26*)
DU5996	*aur*	Aureolysin-deficient mutant of 8325-4	T.J. Foster (*26*)
8325-4/pCU1hlb	hlb^{+}/hlb^{+}	8325-4 with plasmid harboring wild-type *hlb* gene	T.J. Foster (*26*)
DU5719/pCU1hlb	hlb/hlb^{+}	Beta-toxin mutant of 8325-4 with plasmid harboring wild-type *hlb* gene	Present study
RN4220	*rsbU*	Derivative of 8325-4, restriction-mutant, partially *agr* suppressed	R.P. Novick (*27*)
CK501	agr^{+}	*agr* knock-in strain of RN4220	Kaito *et al.* (5)
CK3	*agr*	Agr-deficient mutant of RN4220	Kaito *et al.* (*5*)
RN6390	*rsbU*	Derivative of 8325-4	R.P. Novick (*28*)
RN6911	*agr*	Agr mutant of RN6390	R.P. Novick (*28*)
P. aeruginosa strain			
PAO1	$algR^{+}$	Wild-type prototroph, non-mucoid	ATCC 15692

RN6911 (Table 1). Plasmid pCU1hlb was phage-transduced from strain 8325-4 to DU5719.

Toxins—Alpha- and beta-toxin, exotoxin A, and diphtheria toxin were purchased from Sigma-Aldrich Chemical Co. Before injection into larvae, the toxins were each solubilized in sterilized 0.9% NaCl to a final concentration of 1.0 mg/ml.

Preparation of Culture-Filtrates—For preparation of culture-supernatants, overnight bacterial cultures were diluted with 5.0 ml LB medium to A_{600} = 0.01, followed by incubation in a 50 ml tube (Becton Dickinson Labware, Franklin Lakes, NJ) at 37°C, being grown to the early-stationary phase, A_{600} = 6.0 for *S. aureus* 8325-4, 5.0 for *P. aeruginosa* PAO1, and 3.0 for *E. coli* W3110. Log phase culture-supernatants were prepared in a similar way from bacterial cultures of A_{600} = 0.5. In TSB medium, *S. aureus* strains were grown until $A_{600} \sim 10.0$ (early-stationary phase). Cell cultures were centrifuged at 8,000 × *g* at 4°C for 5 min and the supernatants were filter-sterilized through 0.22 μm Millex-GV (Millipore, Millipore Corp., Bedford, Mass).

Preparation of Concentrated Culture-Supernatants of S. aureus—Exoproteins from *S. aureus* strains were precipitated from 100 ml of bacterial culture grown as described previously (*13*). The precipitate was resuspended in 10 ml distilled water and the suspension was centrifuged at 10,000 × *g* for 20 min at 4°C to remove cell debris, followed by overnight dialysis (MW cut-off 14,000) of the supernatant against 2 liters of distilled deionized water at 4°C. The protein concentrations in the preparations were 1.23 mg/ml (strain 8325-4) and 0.58 mg/ml (strain DU5719; *hlb* mutant), as determined with a Bradford Assay kit (Pierce Chemicals Co., Rockford, Ill).

Injection of Culture-Supernatants or Purified Toxins into Silkworm Larvae—Silkworm eggs (Hu·Yo × Tukuba·Ne) were reared to the fourth molt stage as described previously (*11*, *14*). On the first day of the fifth-instar stage, the larvae were fed for 1 d with an artificial antibiotic-free diet, Silkmate (Katakura Industries Co., Ltd., Tokyo, Japan). The average weight of the fifth-instar, day 2 larvae was 2 g. A culture-supernatant, protein-precipitate, or purified toxin was injected into the hemolymph through the dorsal surface of the silkworm. After injection, the silkworms were incubated in a safety cabinet (BHC-1303IIA; AirTech Japan Ltd., Tokyo, Japan) at 27°C with 50% humidity. Silkworm larvae were not fed after the injection. Survival was monitored at various times after the injection for 24 h. Larvae were considered to be dead when they showed no movement in response to touch. Five or 10 larvae were used for each sample. Statistical analysis was performed using the Student *t*-test.

RESULTS

Culture-Filtrates of S. aureus and P. aeruginosa Kill Silkworm Larvae—To determine whether or not silkworm larvae can be used as an animal model for studying pathogenic bacterial toxins, we first tested the effect of injection of an extracellular culture-filtrate (supernatant) derived from stationary phase cells into the hemolymph of larvae. Injection of a culture-supernatant obtained at the early-stationary phase of *S. aureus* strain 8325-4 or *P. aeruginosa* strain PAO1 grown in LB medium at 37°C killed silkworm larvae (Fig. 1), whereas injection of up to 200 μl of a supernatant obtained from a non-virulent strain of *E. coli*, W3110, did not kill the larvae (Fig. 1). The *P. aeruginosa* supernatant was less-toxic than the *S. aureus* one (Fig. 1). Injection of the log phase culture-supernatant of either *S. aureus* or *P. aeruginosa* did not kill the larvae (data not shown). The results indicate that the stationary phase culture-supernatants of *S. aureus* and *P. aeruginosa* are lethal for silkworm larvae.

agr, a Global Virulence Regulator of S. aureus, Is Required for the Lethal Effect of Staphylococcal Culture-Supernatants on Silkworm Larvae—We then examined whether or not *agr*, a global virulence regulator that is required for exotoxin production by *S. aureus*, is required for supernatant-mediated killing of silkworm larvae. In this experiment, we used TSB medium, because it is widely used for virulence studies on *S. aureus*. *agr* mutant YM0113 exhibited reduced hemolytic activity in the supernatant when grown in TSB (data not shown).

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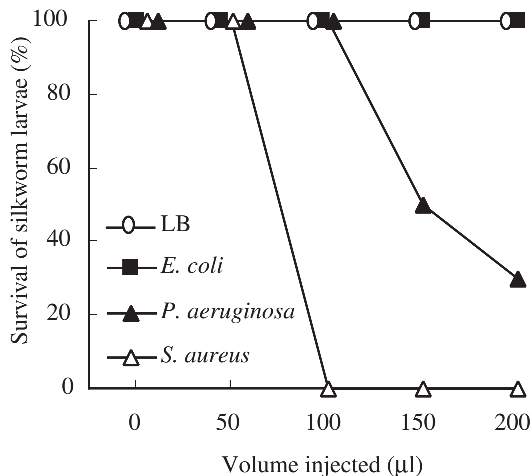

Fig. 1. **Stationary phase culture-supernatants of *S. aureus* and *P. aeruginosa* kill silkworm larvae.** Each supernatant was injected into the hemolymph of larvae ($n = 10$). Survival was examined after 24 h. The data shown are representative of three independent experiments.

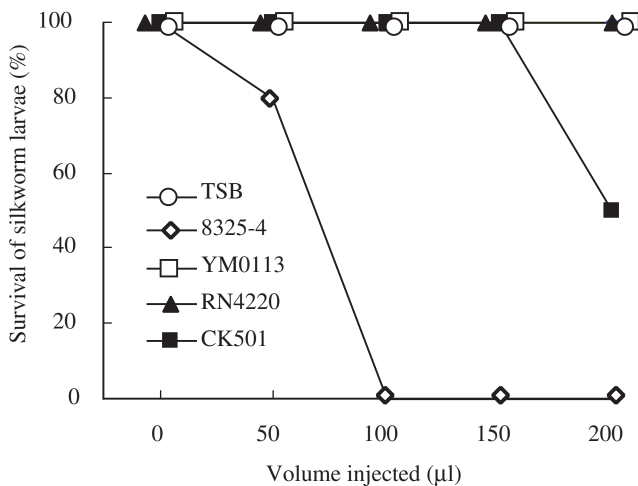

Fig. 2. ***agr*, a global virulence regulator of *S. aureus*, is required for the supernatant-mediated killing of silkworm larvae.** Stationary phase culture-supernatants were collected from 8325-4 and isogenic *agr* mutants YM0113 and RN4220, and isogenic *agr* knock-in strain CK501. Each supernatant was injected into the hemolymph of larvae ($n = 10$). Survival was examined after 24 h. The data shown are representative of three independent experiments.

When silkworm larvae were injected with the stationary phase culture-supernatant of YM0113, all the larvae survived, whereas the wild-type supernatant killed all the larvae (Fig. 2). Similar results were obtained with an *agr* mutant (RN6911) of another laboratory strain RN6390 (data not shown). A supernatant obtained from strain RN4220, in which *agr* expression is partially suppressed, did not kill the larvae, whereas one obtained from a wild-type *agr* knock-in strain with the RN4220 background, CK501, killed some of the larvae (Fig. 2). These results indicate that *agr*, a global virulence regulator of *S. aureus*, is required for culture-supernatant-mediated killing of silkworm larvae.

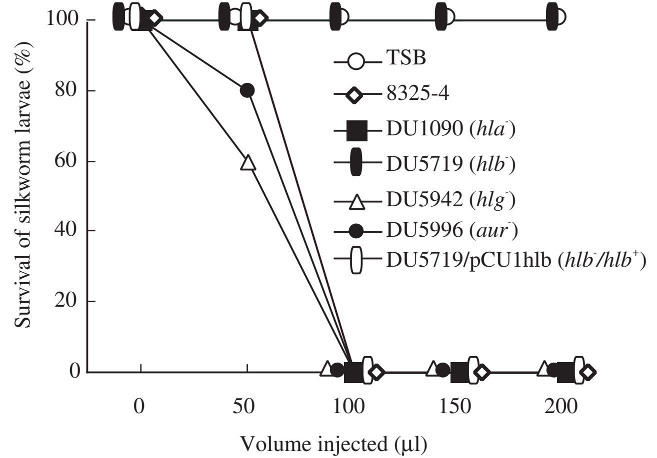

Fig. 3. **Beta-toxin gene *hlb* is required for the supernatant-mediated killing of silkworm larvae by *S. aureus* strain 8325-4.** Stationary phase culture-supernatants of 8325-4 and isogenic mutants as to various toxin genes (Table 1) were collected. Each supernatant was injected into the hemolymph of larvae ($n = 10$). Survival was examined after 24 h. The data shown are representative of four independent experiments.

Table 2. **The LD_{50} values of staphylococcal culture-supernatants.**

S. aureus strain	LD_{50} (µl/g larva)
8325-4	37 ± 0
DU1090 (*hla*$^-$)	37 ± 0
DU5719 (*hlb*$^-$)	>100*
DU5942 (*hlg*$^-$)	32 ± 8
DU5996 (*aur*$^-$)	36 ± 3
DU5719/pCU1hlb (*hlb*$^-$/*hlb*$^+$)	37 ± 0

The culture-supernatants were prepared as described under "MATERIAL AND METHODS." LD_{50} was determined as the volume of supernatant per gram of silkworm larvae required to kill 50% of the larvae at 24 h post-injection. Data are the averages ± S.D. of four independent experiments. *$p < 0.001$ compared to other strains.

The Beta-Toxin Gene hlb Is Required for the Lethal Effect of the Culture-Supernatant of the S. aureus Strain 8325-4 on Silkworm Larvae—To determine which toxins are required for the lethal effect of staphylococcal supernatant-mediated killing of silkworm larvae, we used disruption mutants of various toxin genes of *S. aureus* (Table 1). There was no significant difference with respect to the doubling time among the parent and mutants when grown in TSB medium at 37°C (data not shown). Injection of the stationary phase supernatant obtained from the mutant as to the *hlb* gene, whose expression is regulated by the *agr* locus (*1*), produced no dead larvae, whereas that of a mutant as to *hla*, *hlg*, or *aur* killed silkworm larvae to a similar extent to observed with the wild-type (Fig. 3). The LD_{50} values for the culture-supernatants of the strains are shown in Table 2. Complementation of the *hlb* mutant strain with plasmid pCU1hlb, harboring the wild-type *hlb* gene, resulted in complete restoration of the supernatant killing effect (Fig. 3). Measurement of beta-hemolysin activity in supernatants using sheep blood confirmed that the *hlb* mutant exhibited decreased activity compared to the parent strain, but the activity in the mutant was restored upon complementation with plasmid pCU1hlb

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Table 3. **The LD_{50} values of purified bacterial toxins for silkworm larvae.**

Toxin	LD_{50}		
	Silkworm (/g·larva)	Mouse (29) (/g·mouse)	Other mammals (/g·animal)
Staphylococcal alpha-toxin	12 μg	40–60 pg	1.3 μg (rat) (*29*)
Staphylococcal beta-toxin	9 μg	>7 μg	0.01–0.06 μg (rabbit) (*30*)
Pseudomonas exotoxin A	140 ng	3 ng	
Diphtheria toxin	1.1 μg	1.6 μg	0.16 μg (pigs) (*29*)

LD_{50} was determined as the amount of protein per gram of silkworm larvae required to kill 50% of the larvae at 24 h post-injection for alpha-toxin, beta-toxin and exotoxin A, or at 72 h post-injection for Diphtheria toxin. Ten larvae were used for each experiment. The data are the averages of at least two independent experiments.

(data not shown). These results indicate that beta-toxin gene *hlb* is required for the lethal effect of staphylococcal culture-supernatants on silkworm larvae.

Purified Bacterial Toxins Kill Silkworm Larvae—To determine whether or not staphylococcal toxins other than beta-toxin kill silkworm larvae, we prepared a concentrated culture-supernatant of the *hlb* mutant by means of ethanol precipitation followed by dialysis. Injection of the concentrated culture-supernatant of the *hlb* mutant resulted in the killing of silkworm larvae with an LD_{50} of 44 μg/g larva, whereas the LD_{50} for the concentrated supernatant of the wild-type was 13 μg/g larva. This result indicates that exotoxin(s) other than beta-toxin produced by strain 8325-4 can kill silkworm larvae.

Next, we injected the purified toxins into the hemolymph of silkworm larvae. Injection of staphylococcal alpha-toxin, beta-toxin, *Pseudomonas* exotoxin A, and diphtheria toxin into the hemolymph of larvae killed the larvae (LD_{50} values are shown in Table 3). These results indicate that purified staphylococcal alpha-toxin, beta-toxin, *Pseudomonas* exotoxin A, and diphtheria toxin can kill silkworm larvae.

The concentrations of alpha-toxin and beta-toxin in the culture-supernatant of *S. aureus* 8325-4 determined by Western blot analysis and bioassaying (*15*) were 2.1 μg/ml and 57 μg/ml, respectively. We injected 0.05 ml to 0.2 ml of culture-supernatant into silkworm larvae (2 g/larva). These results suggest that beta-toxin might be responsible for the killing of silkworms by the culture-supernatant.

DISCUSSION

In the present study, the injection of culture-supernatants of pathogenic bacteria or purified bacterial toxins killed silkworm larvae. These findings suggest that silkworm larvae can be used as an animal model for studying pathogenic bacterial toxins.

The majority of exotoxins in pathogenic bacteria are produced during the post-exponential or early-stationary phase *in vitro* (*16*), which is consistent with our results (Fig. 1A). We cannot rule out the possibility that differences in cell number in cultures might cause the avirulence of the log phase culture-supernatants.

The production of exoproteins with virulence properties is controlled by cell density, energy availability, and environmental signals. In *S. aureus*, most of the accessory genes involved in pathogenesis encode proteins that either bind to the bacterial surface or secreted into the surroundings. A key global regulator of *S. aureus* virulence is *agr*, which controls the expression of most of the exoprotein genes (*1*). Attenuated virulence of *agr* mutant bacteria was observed in several vertebrate animal models (*17–19*). Recently, we reported that the expression of *agr* is regulated by a novel gene, *cvfA*, because inactivation of *cvfA* in *S. aureus* decreased *agr* expression (*5*). There is attenuated virulence of *agr* mutants in both silkworm larvae and mice (*5*). In the present study, supernatants of *agr* mutants of *S. aureus* failed to kill silkworm larvae (Fig. 2), indicating that *agr*-dependent exoprotein production is critical for culture-supernatant-mediated killing of silkworm larvae.

The results of injection of culture-supernatants or concentrated culture-supernatants derived from disruption mutants as to various toxin genes of *S. aureus* suggested that (i) beta-toxin is the major factor in the supernatant-mediated killing of silkworm larvae; and (ii) staphylococcal toxin(s) other than beta-toxin can also kill silkworm larvae. Because beta-toxin is a sphingomyelinase, it is possible that there is rapid degradation of one or more sphingomyelin-rich organs, if any, in silkworm larvae when the toxin is present in the hemolymph. Insects have an open circulatory system in which all the tissues are exposed to the hemolymph (*20*). It is also possible that the sphingomyelinase-independent activity of beta-toxin is responsible for the killing of silkworm larvae, because human monocytes and leukocytes with low sphingomyelin contents in membranes are sensitive to purified beta-toxin *in vitro* (*21*, *22*).

We do not know what lethal factor is required for the *P. aeruginosa* strain PAO1 supernatant-mediated killing of silkworm larvae. Although a sphingomyelinase C (similar to staphylococcal beta-toxin) has been identified in the aquatic bacterium *Pseudomonas* sp. strain TK4 (*23*), the genome project on *P. aeruginosa* strain PAO1 indicates that the strain does not have a gene for sphingomyelinase C (*24*). From this result (Table 3), it is possible that *P. aeruginosa* supernatant-mediated killing depends, at least in part, on exotoxin A.

The LD_{50} values of bacterial toxins for silkworms were almost the same as those for mammalian animals. The extremely low LD_{50} value of staphylococcal alpha-toxin for mouse might be explained by the presence of highly specific receptors for the toxin in the animal. It is possible that the receptors, if any, for *Pseudomonas* exotoxin A and diphtheria toxin in the silkworm do not exhibit a high affinity for the toxins. Regarding staphylococcal alpha-toxin and beta-toxin, the toxins might cause membrane damage and induce cytolysis in silkworm larvae via non-specific adsorption rather than interaction with a specific receptor (*25*). It is known that human red blood cells require a 1,000-fold higher amount of alpha-toxin to be

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affected than rabbit red blood cells due to the high affinity receptors for the toxin in rabbit (*25*).

In summary, we established a convenient system for studying the effects of staphylococcal toxins in animals using silkworm larvae. The system can also be used to analyze the structure–function relationships of purified toxins. To our knowledge, the present study is the first to demonstrate that purified, pathogenic bacterial toxins can be lethal to invertebrate animals. Knowledge of the precise mechanisms underlying the death of silkworm larvae caused by purified toxins of pathogenic bacteria will be useful for extending the findings to vertebrate animals. Studies to determine whether or not toxins of other microorganisms have lethal effects on silkworm larvae are ongoing.

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