

A silkworm model of pathogenic bacterial infection

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ABSTRACT: Silkworms are invertebrate animals that are killed by bacteria pathogenic against humans, such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and *Vibrio cholerae*. Injection into the hemolymph of antibiotics that are clinically used for human patients abolishes the killing effects. There are several advantages to using silkworms as an infection model, such as low cost, the absence of ethical problems that are associated with the use of mammals, and a body size large enough to handle while injecting sample solution into the hemolymph. We screened *S. aureus* mutants with attenuated virulence against silkworms and found three novel virulence regulatory genes, *cvfA*, *cvfB*, and *cvfC*. These genes contribute to virulence against mice and are required for exotoxin production. The *cvfA* gene is required for expression of the *agr* locus, which regulates most exotoxin genes, and a novel DNA binding protein SarZ. Silkworms are susceptible to *S. aureus* beta toxin, *P. aeruginosa* exotoxin A, and diphtheria toxin. Therefore, silkworms are a promising infection model animal for the identification and evaluation of virulence-associated genes.

Key Words: Silkworms, virulence, *Staphylococcus aureus*, exotoxin

Introduction

Animal models are essential for identifying and evaluating virulence factors of pathogenic microorganisms. The purpose of this review is to provide evidence that silkworms are very useful for evaluating virulence factors of pathogenic bacteria. The

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review is divided into two parts. First, we introduce the advantages of the silkworm infection model for studying bacterial pathogenicity and evaluating the therapeutic effects of antibiotics. Then, we report the results of our recent studies in which we identified bacterial virulence genes using the silkworm infection model.

Results and Discussion

1. Advantages of the silkworm infection model

To better understand bacterial pathogenicity, the use of animal models is essential. There are problems inherent in the use of animal models, however, such as the high cost housing animals, as well as ethical issues surrounding the use of mammals, which has become more serious in recent years. Especially in European countries, laws strictly regulate the use of mammals for the development of medicine. To overcome these problems, the use of invertebrates, such as the nematode *C. elegans*, the insect *D. melanogaster*, and the amoeba *Dictyostelium discoideum*, has been suggested (1-6). In particular, *C. elegans* and *D. melanogaster* are powerful tools for identifying host proteins involved in immune systems because they are genetically tractable and many mutant lines have been constructed (7-9). Because these animals are too small to handle, however, they are not suitable for injecting precise volumes of samples into the body fluid, a technique that is essential for quantitative evaluation of bacterial pathogenicity and the therapeutic effects of antimicrobial compounds.

Silkworms have several advantages as model animals for studying bacterial pathogenicity and the therapeutic effects of antibiotics (Figure 1). Silkworm, Lepidoptera *Bombyx mori*, was domesticated over

Advantages of silkworms as animal models

- (1) Low cost
- (2) No ethical issues
- (3) Available to accurate injection into hemolymph and gut
- (4) Available to pharmacological test with isolated organs

Figure 1. Silkworm as a model animal. Silkworms have favorable features as a non-mammalian hosts. In particular, No. 3 and 4 are difficult to perform in small-sized non-mammalian hosts, such as flies and nematodes.

the past 5,000 years from the wild species *Bombyx mandarina* for obtaining silk fibers from its cocoons. The long history of the silk industry has provided methods to produce thousands of silkworms at any time of the year. The fertilized eggs can be stored in a refrigerator for a year and can be obtained commercially. Silkworms can be raised from fertilized eggs to 5th instar larvae for three weeks by feeding artificial diet. There are no ethical problems associated with the use of a large number of silkworms. In addition, silkworms have great advantages over other invertebrate animals. Because the body size of silkworm 5th instar larvae (5 cm) is large enough to handle, sample solutions of pathogens and drug samples can be injected into the hemolymph or gut of the larvae using syringes equipped with needles. In *Drosophila* or *C. elegans*, manipulations must be performed under a microscope. When red ink is injected into the hemolymph of silkworms, the whole body color immediately changes to red, because the insect has an open circulatory system. If the needle is introduced deep enough to reach into the midgut, the red ink disperses throughout the midgut without changing the body color (10). These characteristics allow for the evaluation of bacterial pathogenicity and the therapeutic effects of antibiotics.

Furthermore, various tissues responsible for multiplying bacteria and drug metabolism can be isolated from silkworm larva, thus allowing for tracing of bacterial infectious process and pharmacologic experiments to study the pharmacodynamics of compounds. The hemolymph of the larvae that was injected with pathogenic bacteria can be easily collected and thus the bacterial cell number in the hemolymph counted. The proliferation process of the pathogenic bacteria in silkworms can be closely monitored (11). Drug transport assays can be performed using the isolated midgut. For this, the test sample is injected into the midgut and incubated in an appropriate buffer. If the compound passes through the midgut membrane, it will be detected in the buffer (12,13).

Recently, the silkworm genome project was completed by Japanese and Chinese groups (14,15). The reverse genetic method, RNA interference, was established in silkworms (16-18). The information obtained based on the genome and RNA interference method will facilitate the study of host factors involved in infectious processes.

When *S. aureus* was injected into the hemolymph of silkworm larvae, all of the larvae were killed by *S. aureus* within 2 d (Figure 2). All of the larvae in the control group, which were injected with saline, lived. Chloramphenicol has therapeutic effects. All of the *S. aureus*-injected silkworms injected with 100 µg chloramphenicol lived. Thus, this is a basic system for the study of "infection and therapy". Many pathogenic bacteria, such as *S. pyogenes*, *P. aeruginosa*, *Listeria*

(A) Control (B) *S. aureus* (C) *S. aureus* + Chloramphenicol



Figure 2. Infection and therapy using the silkworm model. Ten silkworms were injected with saline (A), 1×10^7 cells of *S. aureus* (B), or 1×10^7 cells of *S. aureus* with 100 µg chloramphenicol (C). Photograph was taken 2 d after the injection.

monocytogenes, *Serratia marcescens*, *Salmonella typhimurim*, and *Vibrio cholerae* kill silkworms (11). Antibiotics used clinically in humans also have therapeutic effects against silkworms injected with *S. aureus* and *P. aeruginosa* (11,12). Bacterial exotoxins kill silkworms (19). The 50% lethal dose (LD_{50}) of staphylococcal alpha-toxin is 12 µg/g; and that of staphylococcal beta-toxin is 9 µg/g; that of *Pseudomonas* exotoxin A is 0.14 µg/g; that of diphtheria toxin is 1.1 µg/g. Most of the LD_{50} values obtained were similar to the reported values in mice, suggesting that silkworms can be used as a model to study the general effects of bacterial exotoxins on multicellular organisms, including humans.

2. Identification of bacterial virulence genes

In this chapter, we describe our recent studies for the identification of bacterial virulence genes.

S. aureus causes opportunistic diseases in patients whose ability to protect themselves from bacterial infection is compromised. An understanding of the molecular mechanisms underlying the expression of *S. aureus* pathogenicity in animal bodies will help in establishing therapeutic methods against this pathogen. Based on the genome of this bacterium, there are 589 genes that are conserved among pathogenic bacteria, although their functions remain to be elucidated (20).

We constructed 100 gene mutants by disrupting these genes by targeting using a homologous recombination technique (Figure 3). Targeting vectors with the erythromycin-resistant gene and the internal region of the open reading frame were constructed, and introduced into the RN4220 strain. Gene disruption was confirmed by Southern blotting analysis. We injected 100 strains of disruption mutants into the hemolymph of silkworms, and found three mutants with decreased pathogenicity. The parent strain killed half the population of silkworms within 36 h, whereas these three strains required more than 80 h (Table 1). We named these genes *cvfA*, *cvfB*, and *cvfC* (conserved

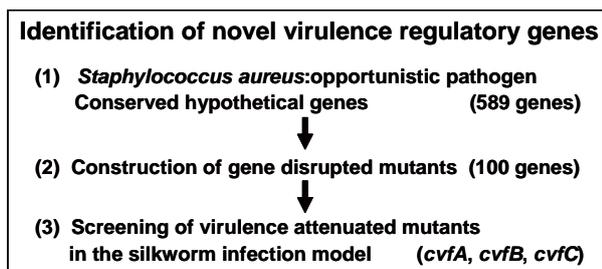


Figure 3. Strategy for identifying of novel virulence genes. The strategy for identifying novel virulence regulatory genes is illustrated. Gene deletions were performed by integrating the targeting vector into the desired hypothetical genes. Silkworms were injected with 50 µL of 20-fold diluted overnight cultures (1×10^7 cells) and larval survival was monitored. When the LT_{50} value of the mutant was 2 times greater than that of the parent strain, the mutant was selected as a virulence-attenuated mutant.

Table 1. Decreased killing ability in silkworms of the *cvfA*, *cvfB*, *cvfC* mutants of *S. aureus* and the *cvfA* mutant of *S. pyogenes*

Strain	Genotype	LT_{50} (h)	Fold change
<i>S. aureus</i>			
RN4220	Parent	36	1
M1129	$\Delta cvfA$	84	2.3
M1223	$\Delta cvfB$	94	2.6
M1262	$\Delta cvfC$	98	2.7
<i>S. pyogenes</i>			
SSI-9	Parent	21	1
M1633	$\Delta cvfA$	102	4.9

Ten silkworms were injected with *S. aureus* (1.3×10^7 CFU) and *S. pyogenes* (9×10^8 CFU) and larval survival was monitored. The time point when half of the larvae were killed is represented as LT_{50} . Fold change indicates the ratio of LT_{50} for the mutant to that of the parent.

Table 2. Conservation of the *cvfA* gene among bacteria

Phylum	Section	Genus	Identities
Proteobacteria	Epsilonproteobacteria	<i>Campylobacter</i>	47%
		<i>Helicobacter</i>	36%
Firmicutes	Bacillales	<i>Bacillus</i>	69%
		<i>Oceanobacillus</i>	58%
		<i>Listeria</i>	63%
		<i>Staphylococcus</i>	100%
	Clostridia	<i>Clostridium</i>	55%
		<i>Thermoanaerobacter</i>	60%
	Lactobacillales	<i>Enterococcus</i>	65%
		<i>Lactobacillus</i>	53%
		<i>Lactococcus</i>	59%
<i>Streptococcus</i>		53%	
Mollicutes	<i>Mycoplasma</i>	36%	
	<i>Ureaplasma</i>	27%	
Fusobacteria	Fusobacteriales	<i>Fusobacterium</i>	49%
Thermotogae	Thermotogales	<i>Thermotoga</i>	64%
<i>Thermus/Deinococcus</i>	Deinococci	<i>Deinococcus</i>	46%
Spirochaetes	Spirochaetales	<i>Borrelia</i>	42%
		<i>Treponema</i>	50%
Bacteroidetes	Bacteroides	<i>Bacteroides</i>	44%
Aquificae	Aquificales	<i>Aquifex</i>	60%
Chlorobi	Chlorobia	<i>Chlorobium</i>	39%

Bacterial species that have proteins homologous to *S. aureus* CvfA with more than 20% amino acid identity and E-values lower than e^{-10} .

virulence factor A, B, and C) (21).

The *cvfA*, *cvfB*, and *cvfC* genes are conserved among various bacteria. The *cvfA* gene is ubiquitously present in the most bacterial genomes, although its function is unknown (Table 2). To test the ubiquitous function of the *cvfA* gene in other pathogenic bacteria, we constructed a deletion mutant of the *cvfA* homologue in *S. pyogenes* and tested its virulence in the silkworm infection model. The result demonstrated that the deletion mutant also had a delayed killing effect in silkworms (Table 1). When the mutant was complemented with the wild-type *cvfA* gene, the transformant had pathogenicity indistinguishable from

the wild-type strain, confirming that deletion of the *cvfA* gene was responsible for the phenotype.

We then tested whether the *cvfA*, *cvfB*, and *cvfC* gene deletion mutants were less pathogenic in mice. The result showed that the number of *S. aureus* and *S. pyogenes* mutant bacterial cells required to kill 50% of the mice was greater than that of the corresponding wild-type bacteria, suggesting that these genes are necessary for pathogenicity in mammals.

The *S. aureus* *cvfA*, *cvfB*, and *cvfC* gene mutants produce less exotoxin than wild-type strains. In an agar plate assay containing sheep blood erythrocytes, a substrate for beta-toxin, the mutants produced a

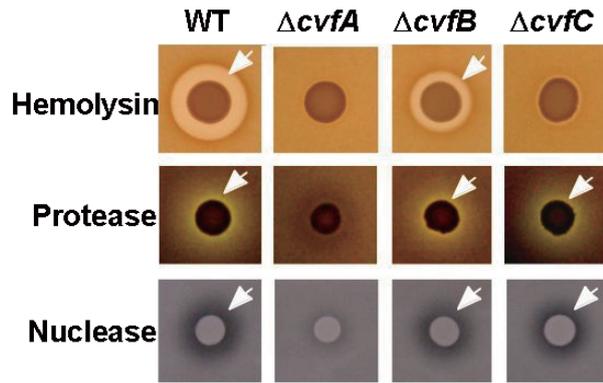


Figure 4. Decreased exotoxin production in the *cvfA*, *cvfB*, and *cvfC* mutants. Overnight cultures of the *cvfA*, *cvfB*, and *cvfC* mutants were spotted onto sheep blood agar plates, skim milk agar plates, and DNA agar plates, and incubated at 37°C. The cleared zone indicated by white arrows reflects the activity of each exotoxin.

smaller lysis zone than did the wild-type strain (Figure 4). The phenotype was complemented by introduction of the wild-type genes. For the *cvfA* mutant, the amounts of protease and nuclease secreted from the cells were lower than those secreted by wild-type cells, and the phenotype was complemented by the wild-type *cvfA* gene. We hypothesized that proteins encoded by the *cvfA*, *cvfB*, and *cvfC* genes act to regulate exotoxin genes.

The *agr* locus is responsible for the expression of exotoxins in *S. aureus* (22,23). We examined whether deletion of the *cvfA* gene affects the expression of the *agr* locus. The results of Northern blot analysis demonstrated that the expression levels of RNAII, a product of the *agr* locus, and RNAIII, whose expression is stimulated by RNAII, were very low in the *cvfA* gene deletion mutants. The results strongly suggest that the *cvfA* gene regulates expression of the *agr* locus, which is required for the expression of exotoxin genes encoding hemolysins, proteases, and nucleases.

The primary amino acid sequence of the CvfA protein indicates that the CvfA protein contains a transmembrane domain, an RNA binding domain named KH (24) and a metal-dependent phosphohydrolase domain named HD (25,26). The KH and HD domains are needed for the pathogenicity of *S. aureus*, because the point mutations of these domains attenuated virulence in the silkworm infection model (21). CvfA protein is the first example of a protein harboring both the KH and HD domains having a physiologic role. A database search analysis (27) indicates that these proteins might function as ribonucleases, although this has not been verified experimentally.

The isolation and characterization of suppressor mutations is a basic technique in genetics. To understand the molecular network between pathogenic genes of *S. aureus*, including the *cvfA* gene, we screened multicopy suppressors of the *cvfA* gene deletion mutant from an *S. aureus* genomic library. We identified SA2174, which was previously designated as *sarZ*, which suppresses the

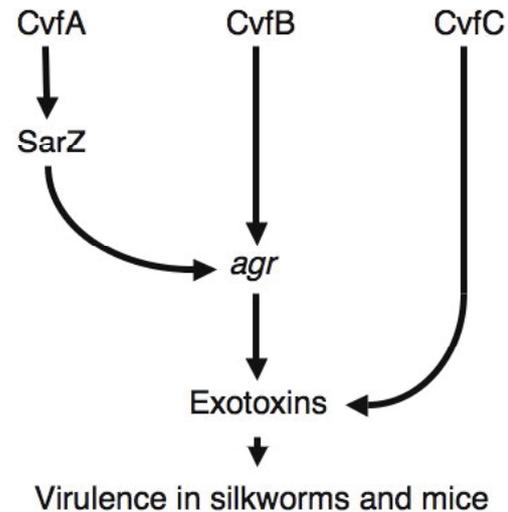


Figure 5. A model of virulence gene regulation. CvfA, CvfB, CvfC, and SarZ contribute to the expression of exotoxins and virulence in both silkworms and mice. CvfA, CvfB, and SarZ are required for maximum expression of the *agr* locus (30). CvfA and CvfB also exert their functions via an *agr*-independent pathway, because their deletion in the *agr* null background also attenuated virulence (21,30).

decreased hemolysin production of the *cvfA* mutant (28). SarZ has sequence similarity with the MarR family (29), which are known as transcription factors of various genes. Because the *sarZ* gene was initially characterized as a multicopy suppressor of the *cvfA* gene deletion mutant, we considered the possibility that the *cvfA* gene activates the *sarZ* gene. This would explain how overproduction of the SarZ protein restores hemolysin production in the absence of the CvfA protein. Northern blot analysis demonstrated that the amount of *sarZ* transcript was much lower in the *cvfA* deletion mutant than in the parent. Introduction of the wild type *cvfA* gene into the *cvfA* deletion mutant restored the level of the *sarZ* transcript. Therefore, the *cvfA* gene is required for expression of the *sarZ* gene. Because the deletion of the *sarZ* gene induces a loss of pathogenicity in silkworms and mice, the *sarZ* gene itself is a virulence gene that is activated by the *cvfA* gene.

Concluding remarks

We discovered three pathogenic genes in *Staphylococcus aureus* using the silkworm infection model. We suggest that silkworms are useful for identifying and evaluating virulence regulatory genes. The *cvfA*, *cvfB*, and *sarZ* genes locate upstream of the *agr* locus, a well-studied virulence regulatory gene of this bacterium (Figure 5). Together with the CvfB and CvfC proteins, CvfA protein activates the expression of genes encoding hemolysin and other pathogenic genes. We propose that these genes act as regulators of pathogenic gene expression. Their conservation among pathogenic bacteria might indicate their contribution to the central

pathway of the virulence gene expression. Although uncovering the molecular function of novel genes is quite difficult, the effort will benefit our understanding of how pathogenic bacteria exert virulence against humans.

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