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MINIREVIEW

# Utilization of a silkworm model for understanding host-pathogen interactions

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**Abstract**

Studies of the interactions between humans and pathogenic microorganisms require adequate representative animal infection models. Further, the availability of invertebrate models overcomes the ethical and financial issues of studying vertebrate materials. Insects have an innate immune system that is conserved in mammals. The recent utilization of silkworms as an animal infection model led to the identification of novel virulence genes of human pathogenic microorganisms and novel innate immune factors in the silkworm. The silkworm infection model is effective for identifying and evaluating novel factors involved in host-pathogen interactions.

**Key Words**: insect model; innate immune factor; bacteria; fungi; virulence factor

## Advantages of the silkworm as an animal infection model

Invertebrate animals possess an innate immune system, but lack an acquired immune system. Many aspects of the innate immune system of invertebrate animals are conserved in mammals. For example, cationic antimicrobial peptides and Toll receptors recognizing pathogens are found in both invertebrate animals and mammals (Okada and Natori, 1983; Hoffmann, 1995; Natori, 2010). Therefore, studies using invertebrate animals can be performed to develop a better understanding of the host-pathogen interactions in mammals without the ethical and financial issues (Seabra and Bhogal, 2009).

Silkworms are larvae of the moth *Bombyx mori*, a lepidopteran species (Fig. 1). Silkworms form cocoons where they develop into pupae. Humans have used these cocoons as raw materials for silk for over 5000 years (Goldsmith *et al.*, 2005). *Bombyx mori* is the only domesticated insect species, and the silkworm cannot survive in the natural world, probably due to their ineffective locomotion. In contrast to wild insects, silkworms can barely bite human fingers or escape from a breeding cage. Silkworms typically consume mulberry leaves, but an artificial diet for silkworms has also been established and is commercially available. Thus, rearing silkworms in the laboratory is easy.

Studies of host-pathogen interactions require quantitative evaluation of the virulence properties of pathogenic microorganisms. To evaluate pathogenic virulence quantitatively, injection of a precise amount of the pathogen solution into model animals is essential. The large body size of the fifth instar silkworm (~ 5 cm) allows for the injection of a very precise amount of the pathogen solution into the silkworm hemolymph using a tuberculin syringe equipped with a 27-gauge needle (Kaito and Sekimizu, 2007), whereas injection of a precise sample amount is more difficult in small body-sized invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans*. Injection of human pathogenic bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* into the silkworm hemolymph kills the silkworm (Kaito *et al.*, 2002). *S. aureus* injected into silkworms proliferates in the hemolymph. The lethal effects of *S. aureus* injection in silkworms are blocked by the injection of antibiotics. These observations suggest that the lethal effects of *S. aureus* in silkworms require bacterial proliferation (Kaito *et al.*, 2002). The silkworm-*S. aureus* infection model allows for the identification of biologic molecules involved in the ability of *S. aureus* to escape various innate immune factors of the silkworm and to proliferate in the silkworm hemolymph. Importantly, infection experiments using silkworms can be performed at 37 ˚C, the temperature at which most human pathogenic microorganisms exhibit high virulence properties (Kaito *et al.*, 2011).

Genetic and biochemical analyses of silkworms are essential for identifying biologic molecules of silkworms that are involved in host-pathogen interactions. The *Bombyx mori* genome project was recently completed and genome data are now

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**Fig. 1** 5th instar larvae of *Bombyx mori.* Tuberculin syringe equipped with a 27-gauge needle is shown above the silkworm.

available on line (Shimomura *et al.*, 2009). In addition, construction of transgenic silkworms is established (Tomita*, et al.*, 2003). For biochemical analysis, biologic molecules from crude silkworm biomaterials must first be purified and identified. A fifth instar silkworm weighs around 2 grams, and thus an adequate amount of silkworm biomaterial can easily be prepared for purifying biologic molecules.

**Identification of bacterial and fungal virulence factors using silkworms**

*S. aureus* is a pathogenic Gram-positive bacterium present in the noses of 30 % of healthy individuals. To identify novel virulence factors of *S. aureus*, 100 hypothetical genes that are conserved among bacteria were disrupted and examined for lethal activity against silkworms. Gene-disrupted mutants of three novel genes, named *cvfA, cvfB,* and *cvfC* (conserved virulence factor A, B, and C), exhibited attenuated lethality in silkworms (Kaito *et al.*, 2005) (Table 1). These gene-disrupted mutants also showed attenuated virulence in mice, indicating that these genes contribute to the virulence of *S. aureus* not only in insects but also in mammals (Kaito *et al.*, 2005; Matsumoto *et al.*, 2007; Marincola *et al.*, 2012). *Streptococcus pyogenes* is a human pathogenic Gram-positive bacterium that causes various diseases, including adenoiditis and necrotizing fasciitis. The *cvfA* gene is also required for the lethality of *S. pyogenes* in silkworms and mice, and it is involved in the expression of various genes in *S. pyogenes* (Kaito *et al.*, 2005; Kang *et al.*, 2010; Kang *et al.*, 2012) (Table 1).

The *cvfA* gene is required for hemolysin production in both *S. aureus* and *S. pyogenes*. CvfA protein is a cyclic phosphodiesterase that cleaves a 2',3'-cyclic phosphodiester linkage at the 3'-terminal nucleotide of RNA (Kaito *et al.*, 2005; Nagata *et al.*, 2008). The *cvfB* gene contributes to *S. aureus* hemolysin production *via* a virulence regulatory gene, *agr* (Matsumoto *et al.*, 2007). Crystal structure analysis revealed that CvfB has a novel L-shaped structure comprising three S1 RNA binding domains and a winged-helix domain (Matsumoto *et al.*, 2010). The *cvfC* gene contributes to *S. aureus* resistance to detergents *via the* expression of thymidylate synthetase (Ikuo *et al.*, 2010). These novel virulence factors are conserved in many human pathogenic bacteria and their molecular functions are different from those of other well-known virulence factors.

To determine whether *S. aureus* virulence factors against mammals contribute to *S. aureus* lethality in silkworms, *S. aureus* gene-disrupted mutants of hemolysins, cell wall proteins, and virulence regulators were examined for their attenuated lethality against silkworms (Miyazaki *et al.*, 2012) (Table 1). The results demonstrated that *S. aureus* hemolysins are not required for virulence in silkworms. In contrast, several cell wall proteins and virulence regulators are required for *S. aureus* lethality in silkworms. Thus, although not all *S. aureus* virulence factors against mammals can be evaluated in silkworms, silkworms are useful for evaluating the effects of *S. aureus* cell wall proteins and virulence regulators. That is, interactions between the host animal and *S. aureus* cell wall proteins or between the host animal and *S. aureus* virulence regulators are conserved among invertebrates and vertebrates.

The silkworm model is also applicable for evaluating virulence factors of Gram-negative human pathogenic bacteria. Enterohemorrhagic *Escherichia coli* (EHEC) is a human pathogen that causes encephalopathy and nephropathy. EHEC O157:H7 produces Shiga toxins that are toxic to mammalian cells. The EHEC gene-deleted mutant of Shiga toxin exhibits attenuated virulence in a mouse infection model (Eaton *et al.*, 2008), but not in a silkworm model (Miyashita *et al.*, 2012). In contrast, the EHEC gene-deleted mutant of lipopolysaccharide (LPS) O-antigen synthase showed attenuated lethality in both silkworms and mice (Miyashita *et al.*, 2012) (Table 1). The LPS O-antigen mutant of EHEC is sensitive to both silkworm and porcine antimicrobial factors (Miyashita *et al.*, 2012). Therefore, LPS O-antigen is required for the lethal effects of EHEC in silkworms and mice *via* conferring resistance against innate immune factors of insects and mammals. A transposon mutant library of *Serratia marcescens*, a

**Table 1** Summary of biologic molecules identified in the silkworm infection model

| Pathogenic microorganism | Gene | Category | Function | References |
|---|---|---|---|---|
| **Gram-positive bacteria** | | | | |
| *Staphylococcus aureus* | *cvfA* | regulator | 2', 3'-cyclic phosphodiesterase | (Kaito *et al.*, 2005) |
| | *cvfB* | regulator | RNA binding protein | (Matsumoto*, et al.*, 2010) |
| | *cvfC* | regulator | conributing to detergent resistance | (Ikuo *et al.*, 2010) |
| | *sarZ* | regulator | transcription factor | (Kaito *et al.*, 2006) |
| | *agr* | regulator | transcription factor and regulatory RNA | (Miyazaki *et al.*, 2012) |
| | *saeRS* | regulator | a two-component regulatory system | (Miyazaki *et al.*, 2012) |
| | *arlRS* | regulator | a two-component regulatory system | (Miyazaki *et al.*, 2012) |
| | *srtA* | cell wall protein | anchoring proteins to cell wall | (Miyazaki *et al.*, 2012) |
| | *clfB* | cell wall protein | binding mammalian cytokeratins | (Miyazaki *et al.*, 2012) |
| | *fnbB* | cell wall protein | binding mammalian fibronectin | (Miyazaki *et al.*, 2012) |
| | *sdrC* | cell wall protein | adherence to mammalian epithelial cells | (Miyazaki *et al.*, 2012) |
| *Streptococcus pyogenes* | *cvfA* | regulator | 2', 3'-cyclic phosphodiesterase | (Kaito *et al.*, 2005) |
| **Gram-negative bacteria** | | | | |
| Enterohemorrhagic *Escherichia coli* | *rfbE* | lipopolysaccharide | lipopolysaccharide O-antigen synthesis | (Miyashita *et al.*, 2012) |
| | *waaL* | lipopolysaccharide | lipopolysaccharide O-antigen ligation | (Miyashita *et al.*, 2012) |
| *Serratia marcescens* | *wecA* | lipopolysaccharide | lipopolysaccharide O-antigen synthesis | (Ishii *et al.*, 2012) |
| | *flhD* | flagella | flagella synthesis | (Ishii *et al.*, 2012) |
| | *fliR* | flagella | flagella synthesis | (Ishii *et al.*, 2012) |
| *Pseudomonas aeruginosa* | *toxA* | toxin | exotoxin A | (Chieda *et al.*, 2011) |
| | *exoS* | toxin | type III effector protein | (Okuda *et al.*, 2010) |
| | *sodM* | stress response | manganese-superoxide dismutase | (Iiyama *et al.*, 2007) |
| | *sodB* | stress response | iron-superoxide dismutase | (Iiyama *et al.*, 2007) |
| **Fungi** | | | | |
| *Cryptococcus neoformans* | *gpa1* | regulator | G-protein alpha subunit | (Matsumoto *et al.*, 2012) |
| | *pka1* | regulator | catalytic subunit of protein kinase A | (Matsumoto *et al.*, 2012) |
| | *cna1* | regulator | catalytic subunit of calcineurin | (Matsumoto *et al.*, 2012) |
| *Candida albicans* | *cmp1* | regulator | protein phosphatase | (Hanaoka *et al.*, 2008) |
| | *yvh1* | regulator | protein phosphatase | (Hanaoka *et al.*, 2008) |
| | *sit4* | regulator | protein phosphatase | (Hanaoka *et al.*, 2008) |
| | *PTC1* | regulator | protein phosphatase | (Hanaoka *et al.*, 2008) |
| *Candida glabrata* | *cyb2p* | metabolism | lactate dehydrogenase | (Ueno *et al.*, 2011) |
| Host animal | | | | |
| **Silkworms** | *apoLp-II/I* | virulence inhibitor | suppressing *S. aureus* hemolysin production | (Hanada *et al.*, 2011) |
| | *PP* | cytokine | inducing innate immune responses | (Ishii*, et al.*, 2010) |

Silkworm hybrid (Kinshu × Showa) was used in studies of *P. aeruginosa* (Iiyama *et al*., 2007; Chieda *et al*., 2011). Silkworm hybrid (Hu • Yo × Tukuba • Ne) was used in other studies.

human pathogenic Gram-negative bacterium, was screened for its attenuated lethality in silkworms, leading to the identification of LPS O-antigen synthase as the factor required for silkworm lethality (Ishii *et al.*, 2012). Exotoxin A, a type III effector protein ExoS, and superoxide dismutase of *P. aeruginosa*, which are virulence factors in mammals, are also required for killing silkworms (Iiyama *et al.*, 2007; Okuda *et al.*, 2010; Chieda *et al.*, 2011) (Table 1). In contrast, *P. aeruginosa* pyocyanin, which is a virulence factor in mammals, is not required for killing silkworms (Chieda *et al.*, 2008). Many factors in Gram-negative bacteria are required for virulence in both silkworms and mammals, although some factors are specifically required for virulence in mammals.

Several virulence factors of human pathogenic fungi, including *Cryptococcus neoformans*, *Candida glabrata*, and *Candida albicans,* were identified by infecting silkworms with gene-deletion mutants (Hanaoka *et al.*, 2008; Ueno *et al.*, 2011; Matsumoto *et al.*, 2012). Gene-deletion mutants of the virulence factors of *C. neoformans* and *C. albicans* in mammals showed attenuated virulence in silkworms (Hanaoka *et al.*, 2008; Matsumoto *et al.*, 2012) (Table 1). Cyb2p of *C. glabrata* and PTC2 of *C. albicans* have been identified as virulence factors in silkworms and these genes are also required for virulence in mice (Hanaoka *et al.*, 2008; Ueno *et al.*, 2011) (Table 1).

These results suggest that human pathogen virulence factors of Gram-positive bacteria, Gram-negative bacteria, and fungi can be identified and evaluated in a silkworm model by infecting silkworms with gene-disrupted mutants.

**Identification of innate immune factors in silkworms**

Injection of *S. aureus* hemolysins into silkworms kills silkworms (Hossain *et al.*, 2006). In contrast, *S. aureus* hemolysin gene-deleted mutants did not exhibit attenuated killing ability against silkworms (Miyazaki *et al.*, 2012). These findings suggest that silkworm hemolymph contains a factor that inhibits *S. aureus* hemolysin production. A lipid carrier protein, apolipophorin (ApoLp), purified from silkworm hemolymph shows inhibitory activity against *S. aureus* hemolysin production (Hanada *et al.*, 2011) (Table 1). The addition of ApoLp to *S. aureus* culture decreases the expression of *saeRS*, which is a positive regulator of *S. aureus* hemolysin genes. Injection of anti-ApoLp antibodies into silkworms sensitizes silkworms against *S. aureus*. These findings suggest that ApoLp inactivates *S. aureus saeRS* and decreases hemolysin expression, leading to silkworm resistance against *S. aureus*. Mammalian mucin also inhibits *S. aureus* hemolysin production, indicating that resistance to infection by the inhibition of hemolysin production is conserved among insects and mammals. Most innate immune factors contribute to infection resistance by killing pathogenic microorganisms. Novel innate immune factors that do not inhibit bacterial proliferation and inhibit bacterial virulence are not well understood. In addition to ApoLp, apolipoprotein B in mammalian blood and hydrogen peroxide produced by macrophages inhibit *S. aureus* virulence (Rothfork *et al.*, 2004; Peterson *et al.*, 2008). ApoLp is the first invertebrate biologic molecule found to inhibit bacterial virulence.

Silkworm hemolymph contains a cytokine-like peptide named paralytic peptide (PP) (Ishii *et al.*, 2008) (Table 1). PP is synthesized as an inactive precursor and constitutively exists in silkworm hemolymph. Bacterial peptidoglycans and fungal glucans induce reactive oxygen species (ROS) from silkworm hemocytes and ROS activate serine protease. The activated serine protease digests the PP precursor to produce matured PP. The matured PP activates humoral and cellular immune responses, including phagocytosis by silkworm hemocytes, phosphorylation of p38 mitogen-activated protein kinase, and production of antimicrobial peptides (Ishii *et al.*, 2010). Because injection of the anti-PP antibody into silkworms sensitizes silkworms against *S. aureus* (Ishii *et al.*, 2008), PP contributes to silkworm resistance against *S. aureus*. PP was originally identified as a biologic molecule that induces muscle contraction in silkworms (Ha *et al.*, 1999). The biologic significance of the muscle-contracting activity of PP in the innate immune system is unknown.

**Concluding remarks**

This minireview describes biologic molecules identified in the silkworm infection model. In most cases, the biologic molecules identified in the silkworm infection model are involved in mammalian host-pathogen interactions. Utilization of a multitude of silkworms allows for quantitative evaluation of the virulence of many gene-disrupted mutants of pathogenic microorganisms. The silkworm infection model will be a powerful tool to further our understanding of host-pathogen interactions.

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