Review

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Understanding of bacterial virulence using the silkworm infection model

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Summary **We have used silkworms, larva of *Bombyx mori*, to investigate host-pathogen interactions. Silkworms have several advantages, such as high availability of a large number of animals and ease of injection of quantitative amounts of samples. Human pathogenic bacteria such as *Staphylococcus aureus*, *Streptococcus pyogenes*, or *Pseudomonas aeruginosa* kill silkworms. In this review, I would like to summarize our approach identifying *S. aureus* virulence factors by using the silkworm infection model.**

Keywords: *Bombyx mori*, *Staphylococcus aureus*, virulence factors, infection model

1. Introduction

Staphylococcus aureus is a human pathogenic bacteria causing various diseases. Especially, methicillin-resistant *S. aureus*, MRSA, has afflicted humans from its emergence in the 1960's. In USA, death by MRSA is over 18,000/year, which is more than those by AIDS (*1*).

S. aureus produces various factors in the human body causing diseases, including defensive factors against the host immune system, adhesive factors to host tissues, and toxins that destroy host tissues. Expression of these various virulence factors is regulated by several virulence regulatory factors. *S. aureus* has 16 species of two-component systems that are composed of the sensor protein detecting environmental stimuli and the response regulator acting as a transcription factor (*2*). In these two-component systems, association with *S. aureus* virulence has been reported in the *agr* system (*3*), which acts in quorum-sensing, the *arlRS* (*4*), and *saeRS* (*5*), both of which recognize unidentified signals, and the *graSR* (*6,7*), which is involved in resistance against antimicrobial peptides. In addition, there are many transcription factors including SarA family proteins that regulate the expression of *S. aureus* virulence factors (*8,9*). Identification of novel virulence factors other than these known virulence factors is important for understanding the whole picture of the regulatory network for *S. aureus* virulence factors.

In the past, *S. aureus* virulence factors have been identified by transposon mutagenesis (*10,11*). Most transposons have a tendency to be integrated into some specific DNA sequence, resulting in a biased mutant library (*11*). Using a mutant library constructed with a popular transposon is assumed not to be effective to identify novel virulence factors, since it contains many mutants of known virulence factors. In the recent two decades since the completion of many genome projects, targeting of all genes in monocellular model organisms such as *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, and *Saccharomyces pombe* has been accomplished to reveal the functions of all genes (*12-15*). Such reverse genetic approaches are effective to identify novel gene functions, because there is no opportunity to spend efforts to handle mutants of known genes. We have tried to identify novel virulence factors based on the *S. aureus* genome information (*16*).

2. Establishment of silkworm infection model for human pathogenic bacteria

To identify bacterial virulence factors, it is essential to evaluate the virulence of gene-knockout mutants in animal infection models. Since early times, mammals have mainly been used as infection models for *S. aureus* (*10,17,18*). It is difficult, however, to use large numbers of mammals for infection experiment because of cost and ethical problems. Especially, for the purpose of screening virulence-attenuated mutants from gene-

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knockout mutant libraries, it is needed to use animals other than mammals so that we can use large numbers. We focused our attention on silkworms, moth larva, *Bombyx mori*, a lepidopteran species. Silkworms have been utilized by humans to produce silk for more than 4000 years and thus a great amount of technological information.about the insect has been accumulated. In addition, the silkworm is a solitary insect that has been capitalized on by humans, and has various handy characteristics such as, no biting and no escaping. Before we utilized the silkworm as an infection model for human pathogenic bacteria, *Caenohabditis elegans*, an invertebrate animal, has been used as an infection model for *P. aeruginosa*, a human pathogenic bacteria (*19*). Since the genetic analysis method has been established in *C. elegans*, it is useful to investigate host factors using *C. elegans*. *C. elegans* is, however, a small size animal and is not suitable for injection of quantitative amounts of bacterial solution and for quantitative evaluation of bacterial virulence. In contrast, the 5th-instar silkworm is around 5 cm long, has 700 μL of hemolymph (*20*), and we can easily inject 50 μL solution into the hemolymph by using a tuberculin syringe equipped with a 27 gauge needle (*6,21*). This enabled a quantitative evaluation of bacterial virulence as lethal dose 50% (LD_{50}) (*22*). Furthermore, silkworms are resistant to 37˚C, the human body temperature, and can be used for infection experiments at 37˚C (*23*). Insects, including silkworms, have innate immune systems conserved with mammals. In addition, silkworms have a primed immune system that has several characteristics that resemble acquired immune systems in vertebrates (*24,25*).

Injection of human pathogenic bacteria, such as *S. aureus*, *S. pyogenes*, *P. aeruginosa*, *Vibrio cholerae*, and enteroharnollogic *Escherichia coli*, killed silkworms (*26,27*). *S. aureus* cells injected into the silkworm hemolymph have proliferated in the hemolymph. Injection of antibiotics suppressed silkworm death caused by *S. aureus*, indicating that bacterial proliferation in hemolymph is required for the killing of silkworms. On the other hand, non-pathogenic bacteria against humans such as *Bacillus subtilis* or laboratory strains of *Escherichia coli* did not kill silkworms. These results indicate that virulence of bacteria against humans is reflected in the silkworm infection model.

3. Identification of novel virulence factors of *S. aureus*

S. aureus genome contains 589 gene products that are conserved among bacteria but the functions have not been revealed, which are called "conserved hypothetical proteins" (*16*). We hypothesized that these genes contain novel virulence genes responsible for virulence mechanisms conserved among bacteria. According to the gene-disruption method used in *B. subtilis* by single homologous recombination of a suicide vector into the chromosome (*28*), we tried to construct *S. aureus* knockout strains of these conserved hypothetical proteins (*29*). In *B. subtilis*, it was reported that 200 bp of DNA fragments targeting plasmids homologous to the target DNA sequence in the chromosome causes homologous recombination. In *S. aureus*, we did not obtain gene-disrupted strains by using a 200 bp homologous region in the targeting vector, but obtained gene-disrupted strains by using a 600 bp homologous region. Since the transformation frequency of the *S. aureus* RN4220 strain by plasmids is not much different than *B. subtilis* (*30,31*), there may be a different DNA recombination system between the two bacteria. We constructed gene-disrupted strains of around 100 genes by using a targeting vector harboring around a 600 bp homologous DNA fragment to the target gene. We evaluated the virulence of these gene-disrupted mutants in the silkworm infection model and screened virulence-attenuated mutants. We identified three genes necessary to kill silkworms and named them, *cvfA*, *cvfB*, and *cvfC* (conserved virulence factor) (*32*). These genes also contribute to *S. aureus* virulence in mice and have roles in producing several toxins. To know whether *cvfA* is required for virulence in other bacteria, we examined the *cvfA* function in *S. pyogenes*. We found that *cvfA* is required for *S. pyogenes* virulence in silkworms and mice, and is necessary for *S. pyogenes* production of several toxins including hemolysin (*32*). These results suggest that utilization of the silkworm infection model to evaluate bacterial virulence is a powerful tool to identify novel virulence factors.

4. Functions of novel virulence factors

To reveal the functions of novel virulence factors, we performed biochemical studies based on *in silico* information for protein domains and genetic studies utilizing transcriptome analysis or isolating a genetic suppressor. In the *cvfA*-disrupted mutant, 20% of all gene transcripts were differentially expressed compared with the parent strain (*33*). Downregulated genes in the *cvfA* mutant include *hla* encoding alpha hemolysin, *sarZ* encoding SarA family transcription factor, and *agr* encoding virulence regulator (*34*). Based on the information that CvfA protein has an RNA binding domain and metal-dependent phosphohydrolase domain, we found that CvfA protein has hydrolytic activity against 2',3'-cyclic phosphodiester bond at 3'-terminus of RNA and produces RNA with a 3'-phosphate (*35*). The structural alteration of 3'-terminus of RNA by CvfA conferred resistance against degradation by an exonuclease PNPase (*33*). The phenotype of the *cvfA*-disrupted mutant with decreased hemolysin production was suppressed by disruption of the PNPase gene (*33*). These results suggest that modification of RNA 3'-terminus by CvfA is important for the stability of RNA to regulate the expression of *S. aureus* virulence

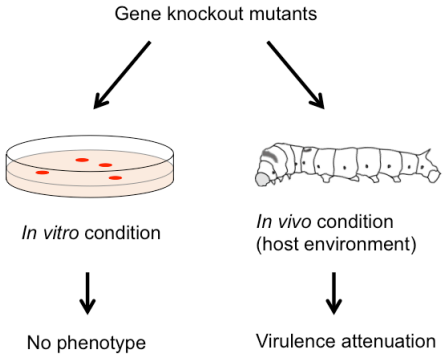

Figure 1. Scheme of phenotypic characterization of gene-knockout mutants. When a gene knockout mutant shows no phenotype under *in vitro* culture condition, more physiological conditions for the bacteria to be exposed should be tested. Silkworm infection model can be used as a host environment to evaluate bacterial virulence properties.

genes. CvfA homolog in *B. subtilis* named RNase Y has an endonuclease activity (*36*). Recently, RNA sequence analysis of the *S. aureus cvfA*-deleted mutant revealed that CvfA acts as an endonuclease against many transcripts (*37*). 2',3'-Cyclic nucleotide at 3'-terminus of RNA is observed at the endonuclease cleavage site of RNA (*38*). The information suggests that CvfA cleaves RNA, produces 3'-phosphorylated RNA, and controls RNA stability.

Biochemical and structural analyses revealed that CvfB protein is an RNA binding protein (*39,40*). The *cvfC* gene regulates expression of a nucleotide synthetase and is required for resistance against detergent (*41*). Recently, we found that ribosomal RNA methyltransferases are required for *S. aureus* virulence *via* conferring resistance against oxidative stress (*42,43*). The novel virulence factors identified from conserved hypothetical genes by using the silkworm-infection model have functions in RNA binding, RNA modification, or nucleotide metabolism, which are different from those of the known virulence regulatory factors such as the two-component systems or transcription factors. Because the gene-disrupted mutants of the novel virulence factors show almost indistinguishable growth from the parent strain, the modification of RNA or alteration of nucleotide metabolism has an important role in the *S. aureus* infectious process in host animals. Molecular mechanisms underlying the requirement of these factors in the host environment should be further clarified in the future.

5. Concluding remarks

We have utilized silkworms as an infection model animal for human pathogenic bacteria and used the model to identify *S. aureus* virulence factors from the conserved hypothetical proteins. Novel virulence factors have been identified and revealed to have functions in RNA modification or nucleotide metabolism. The factors had little report in all organisms, possibly because the phenotype other than virulence is difficult to find. In fact, there are many factors where the gene knockout does not show any phenotypes in model organisms such as *E. coli* (*44-46*). To reveal functions of such factors, it is important to evaluate gene-knockout phenotypes in more physiological conditions than the laboratory culture condition (Figure 1). The method to evaluate bacterial virulence using silkworm infection model is expected to be effective to identify gene functions and contribute to our understanding of bacterial virulence.

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