Original Article

DOI: 10.5582/ddt.2011.v5.2.66

Isolation of mammalian pathogenic bacteria using silkworms

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ABSTRACT: We developed a method to predict bacterial pathogenicity against mammals by measuring bacterial virulence in silkworms at 37°C, human body temperature. One hundred and twenty-two strains of bacteria were isolated from the intestines of fish and shellfish and tested for their virulence against silkworms. Overnight cultures of 50 strains killed at least 50% of the silkworms when injected into the hemolymph. Of 10 strains that showed the most potent pathogenicity against silkworms, 8 also killed mice within 4 days after injection, including *Staphylococcus simiae* and *Staphylococcus pasteuri*, neither of which was previously reported to be pathogenic against mammals. These findings suggest that bacterial pathogenicity against mammals can be predicted based on measurements of silkworm-killing activity.

Keywords: Bacterial pathogenicity, mammals, silkworm infection model, *S. simiae*, *S. pasteuri*

1. Introduction

Infectious disease can be life-threatening in humans (*1*), and are an important public health challenge. Many pathogenic bacteria are present in the environment and in foods. Bacteria indigenous to the environment are potential emerging sources of human infectious diseases. Efficient methods to detect environmental pathogens are needed to prepare against the threat of emerging infectious diseases. In general, bacteria isolated from the environment are usually analyzed by comparing the morphologic aspects, biochemical characteristics, and 16S rRNA sequence to those of previously reported pathogens. Little attention has been paid to the potential pathogenicity of environmental bacteria. Therefore, potential pathogens in samples may escape identification. A cost-effective and efficient method for evaluating bacterial pathogenicity in an animal infection model is therefore crucial.

Infection experiments are generally performed using mammals. The use of a large number of mammals for infection experiments, however, is associated with ethical problems and is costly. The development of invertebrate infection models, therefore, is highly desirable. We recently established a silkworm infection model as an alternative to a mammalian infection model. Silkworms are sensitive to human pathogens and the silkworm infection model is useful for studying the virulence mechanisms of pathogens (*2-7*). The silkworm model is also useful for identifying exotoxins secreted from pathogens (*8*). We recently reported the purification of an exotoxin secreted from the soil bacterium *Bacillus sp*. by monitoring its toxicity in silkworms (*9*). It remains uncertain, however, whether most bacteria that are pathogenic to silkworms are also pathogenic in mammals. In the present study, we demonstrated that pathogens can be easily isolated by monitoring their pathogenicity in silkworms and the results can be used to predict pathogenicity in mammals.

2. Materials and Methods

2.1. *Animals*

Silkworms eggs (Hu·Yo × Tukuba·Ne) were purchased from Ehime Sansyu (Ehime, Japan). The hatched larvae were raised to fourth-instar larvae with artificial diets and the fifth-instar larvae were fed antibiotic-free food (Katakura Industries, Japan) for 1 day and then used for infection experiments. ICR mice (4 weeks old, female) were purchased from CLEA Japan. All mouse protocols followed the Regulations for Animal Care and Use of the University of Tokyo and were approved by the Animal Use Committee at the Graduate School of Pharmaceutical Science at the University of Tokyo (approval number: 19-28).

2.2. *Fish and shellfish*

Japanese horse mackerel, sea eel, oyster, marbled rock fish, barracuda, splendid alfonsino, flying fish, red sea bream, marbled flounder, and yellow tail were purchased from a fish market in Tokyo, Japan.

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2.3. *Isolation of bacteria from intestines of fish and shellfish and determination of species*

Intestinal contents of fish and shellfish were spread on brain-heart infusion (BHI) agar plates. Colonies were isolated after overnight incubation at 37°C. The 16S rRNA regions of bacteria were amplified by Colony PCR (35 cycles: 95°C 30 s, 55°C 15 s, 72°C 1 min) using the following primer pairs: forward (5'-GAGTTTGATCCTG GCTCAG-3' and 5'-CCAGCAGCCGCGGTAATACG-3') and reverse (5'-AAGGAGGTGATCCAGCC-3' and 5'-A TCGGCTACCTTGTTACGACTTC-3'), and sequenced by the dye-terminator method. A BLAST search against more than 1,000 bp of 16S rRNA was performed. Bacterial species were identified when more than 99.5% identity was obtained.

2.4. *Pathogenicity of bacteria from intestines of fish and shellfish against silkworms*

A 10-fold serial dilution of overnight culture was injected into the silkworm hemolymph (50 µL, n = 2). After injection, silkworms were raised at 37°C in a fasting state. The number of silkworms alive after 18-22 h was counted.

2.5. *Pathogenicity of bacteria obtained from intestines of fish and shellfish against mice*

Overnight cultures of 10 potent pathogens against silkworms were prepared in Brain Heart Infusion broth at 37°C. These cultures (500 µL) were injected into the mouse peritoneal cavity. After injection, the number of live mice was counted each day.

3. Results

3.1. *Isolation of pathogens against silkworms from the intestinal contents of fish and shellfish*

We previously reported that some human pathogens kill silkworms (*2,3,10*). These experiments were performed at 27°C, a standard temperature for raising silkworms. Temperature affects pathogen exotoxin production (*11-13*), however, and therefore we performed the silkworm infection experiments at 37°C, human body temperature. Silkworms can be kept alive for 3 days at 37°C. We isolated 122 bacteria from the intestinal contents of fish and shellfish and evaluated their pathogenicity by injecting overnight cultures into the silkworm hemolymph. Of the 122 bacteria obtained, 50 killed silkworms. These 50 bacteria were classified as 22 individual bacteria based on the morphologic aspects of the colonies on BHI agar plates. We further performed quantitative evaluations of the pathogenicity of these pathogens against silkworms by injecting serial dilutions of full-growth cultures. Fourteen pathogens (sample No. 1-14) showed LD_{50} values less than 1×10^6 (Table 1). The bacterial species were determined by analyzing the 16S rRNA sequences. Seven species, *Bacillus thuringiensis*, *Staphylococcus pasteuri*, *Staphylococcus simiae*, *Proteus vulgaris*, *Morganella morganii*, *Bacillus amyloliquefaciens*, and *Proteus mirabilis* were high virulence against silkworms (Table 1).

3.2. *Bacteria with potent pathogenicity in silkworms also killed mice*

From the 14 most potent pathogens against silkworms,

Table 1. Pathogenicity in silkworms of bacteria isolated from the intestine of fish and shellfish

Sample No.	Species	Identity (%)	Materials used for isolating bacteria	LD_{50} in silkworms ($\times 10^4$ CFU)
1	*Bacillus thuringiensis*	99.9	Oyster	3.3
2	*Staphylococcus pasteuri*	100	Japanese horse mackerel	8.5
3	*Staphylococcus pasteuri*	100	Japanese horse mackerel	8.5
4	*Staphylococcus simiae*	100	Japanese horse mackerel	10
5	*Staphylococcus simiae*	100	Yellow tail	14
6	*Staphylococcus simiae*	100	Flying fish	15
7	*Staphylococcus simiae*	100	Yellow tail	16
8	*Staphylococcus simiae*	100	Japanese horse mackerel	19
9	*Proteus vulgaris*	99.8	Marbled flounder	65
10	*Morganella morganii*	99.8	Marbled flounder	75
11	*Bacillus amyloliquefaciens*	99.5	Marbled rockfish	75
12	*Staphylococcus pasteuri*	100	Japanese horse mackerel	75
13	*Staphylococcus pasteuri*	100	Splendid alfonsino	90
14	*Proteus mirabillis*	99.7	Oyster	100
15	*Bacillus licheniformis*	99.9	Marbled rockfish	115
16	*Staphylococcus pasteuri*	100	Marbled flounder	175
17	*Staphylococcus pasteuri*	99.5	Marbled rockfish	550
18	*Pectobacterium carotovorum*	99.9	Sea eel	900
19	*Macrococcus caseolyticus*	99.9	Barracuda	3,400
20	*Hafnia alvei*	99.5	Japanese horse mackerel	5,500
21	*Edwardsiella tarda*	99.9	Red sea bream	18,000
22	*Staphylococcus epidermidis*	100	Japanese horse mackerel	22,500

Bacterial species were determined by 16S rRNA sequencing. "Identity" indicates identity between the sequenced and registered sequences. Pathogenicity was evaluated by injecting an overnight culture of bacteria into the silkworm hemolymph.

Table 2. Pathogenicity in mice of bacteria isolated from the intestine of fish and shellfish

Sample No.	Species	Injected CFU	Survival of mice (n = 3)				
			0 day	1 day	2 days	3 days	4 days
2	*Staphylococcus pasteuri*	8.5×10^8	3/3	3/3	2/3	2/3	2/3
3	*Staphylococcus pasteuri*	8.5×10^8	3/3	3/3	3/3	1/3	0/3
4	*Staphylococcus simiae*	1.0×10^9	3/3	3/3	0/3		
5	*Staphylococcus simiae*	1.4×10^9	3/3	3/3	0/3		
6	*Staphylococcus simiae*	1.5×10^9	3/3	3/3	0/3		
7	*Staphylococcus simiae*	1.6×10^9	3/3	3/3	2/3	2/3	2/3
8	*Staphylococcus simiae*	1.9×10^9	3/3	3/3	0/3		
9	*Proteus vulgaris*	6.5×10^9	3/3	0/3			
10	*Morganella morganii*	7.5×10^9	3/3	0/3			
14	*Proteus mirabillis*	1.0×10^{10}	3/3	0/3			
	Saline		3/3	3/3	3/3	3/3	3/3

we tested virulence in mice of 10 strains other than *B. thuringiensis* that was an insect pathogen, *B. amyloliquefaciens* that was an plant root-colonizing bacterium and did not produce toxins (*14,15*), and *S. pasteuri* strains (No. 12 and 13) that were less virulence than *S. pasteuri* strains (No. 2 and 3). Overnight culture of each bacterium was injected into the mouse peritoneal cavity. Of these 10 bacteria, 8 killed all the mice within 4 days (Table 2). *Staphylococcus pasteuri* (No. 3), *Staphylococcus simiae* (No. 4, 5, 6, 8), *Proteus vulgaris*, *Morganella morganii*, and *Proteus mirabilis* killed mice within 4 days after injection. Mice injected with *Staphylococcus pasteuri* (No. 2) or *Staphylococcus simiae* (No. 7) were not all killed within 4 days after injection. *S. simiae* has been isolated from the gastrointestinal tracts of South American squirrel monkeys in 2005 (*16*). *S. pasteuri* has been isolated from foods, animals, and humans in 1993 (*17*), and the pathogenicity against humans are controversial (*18*). There are no previous reports of the pathogenicity of *S. simiae* and *S. pasteuri* in mammals. Therefore, these data are the first to demonstrate the virulence of those bacteria in mammals.

3.3. *Effect of temperature on the pathogenicity of Staphylococcus simiae in silkworms*

Temperature affects the exotoxin production by pathogens (*11-13*). Therefore, we examined the effects of temperature on the pathogenicity of *S. simae* in silkworms. After injection of a serial dilution culture of *S. simiae* into the silkworm hemolymph, silkworms were raised at 27 or 37°C. The number of silkworms alive after 24 h was counted. Injection of bacteria killed silkworms raised at 37°C, with an LD_{50} of 9×10^4 CFU *S. simiae*. On the other hand, at 27°C, injection of 2.8×10^8 CFU *S. simiae* did not kill silkworms (Figure 1). These results indicate that the pathogenicity of *S. simiae* in silkworms is dramatically affected by temperature.

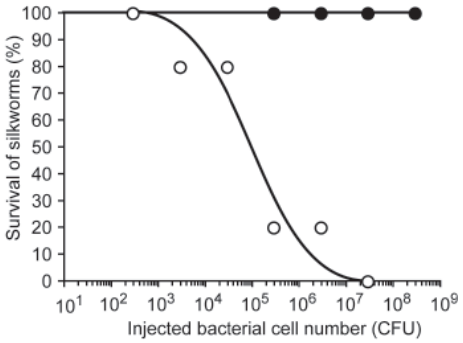

Figure 1. Effect of temperature on the pathogenicity of *Staphylococcus simiae* in silkworms. Overnight culture of *S. simiae* was diluted with saline. A total of 0.05 mL of diluted bacterial culture was injected into silkworm (n = 5). After injection, silkworms were maintained at 27°C (closed circle) or 37°C (open circle). The number of surviving silkworms was determined after 24 h.

3.4. *Silkworm-killing activity of culture supernatant or cell wall components of S. simiae*

Pathogenicity of bacteria is generally due to the exotoxin or cell wall components of the bacteria. We examined silkworm-killing activity of the culture supernatant and cell wall components of *S. simiae* at 37°C. Both the culture supernatant and cell wall components killed silkworms with an LD_{50} of 14 μg protein and 1.7×10^9 equivalent cells, respectively (Figures 2A and 2B). These results suggest that pathogenicity of *S. simiae* depends on both exotoxin and cell wall components.

3.5. *Therapeutic effects of erythromycin in silkworms infected with S. simiae*

The silkworm infection model may be helpful to prepare methods for medical treatment against predictable emerging infectious diseases. We first screened antibiotics that inhibit the growth of *S. simiae in vitro*. The minimum inhibitory concentrations for the following antibiotics are listed in Table 3: chloramphenicol, erythromycin, kanamycin, oxacillin, tetracycline, and vancomycin. We further demonstrated that injection of

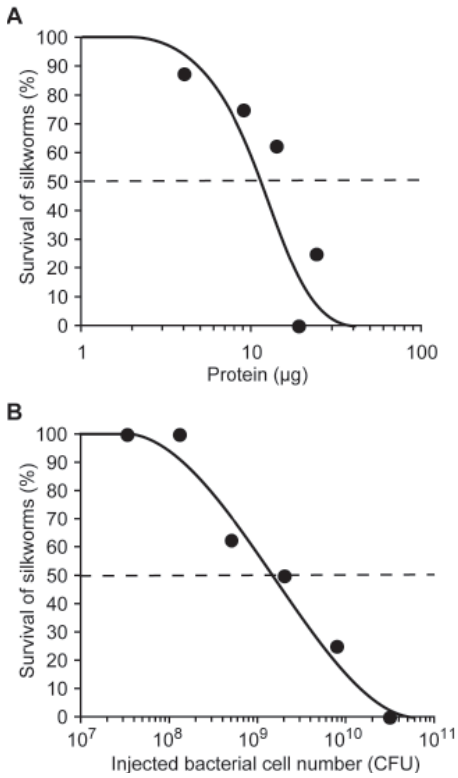

Figure 2. Culture supernatant and heat-killed bacteria of *S. simiae* killed silkworms. (A) The silkworm-killing activity of the supernatant of a bacterial culture of *S. simiae* was evaluated. Silkworms ($n = 8$) were injected with serially diluted solutions of bacterial culture supernatants. Concentration of proteins was determined using the Bradford assay. The silkworms were maintained at 37°C. The number of surviving silkworms was determined after 48 h. **(B)** The silkworm-killing activity of heat-killed *S. simiae* was evaluated. Silkworms ($n = 8$) were injected with heat-killed bacteria. The silkworms were maintained at 37°C. The number of surviving silkworms was determined after 48 h. LD_{50} was 1.7×10^9 CFU.

Table 3. Minimum inhibitory concentrations (MIC) of antibiotics for *S. simiae*

Antibiotics	MIC (μg/mL)
Chloramphenicol	2.5
Erythromycin	0.33
Kanamycin	2.5
Oxacillin	0.17
Tetracycline	0.65
Vancomycin	1.3

S. simiae was cultured in the presence of antibiotics at 37°C for 1 day. The concentrations of antibiotics that inhibited bacterial growth were determined.

erythromycin (400 μg/silkworm) showed therapeutic effects in silkworms infected with *S. simiae* (Figure 3).

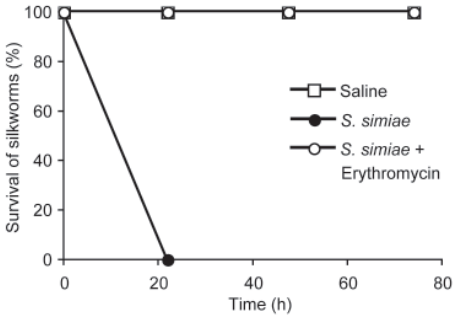

Figure 3. Therapeutic effect of erythromycin in silkworms infected with *S. simiae*. Silkworms ($n = 8$) were injected with 2×10^5 CFU *S. simiae*, followed by injection with 400 μg of erythromycin (final concentration, 0.6 mg/mL hemolymph). The silkworms were maintained at 37°C. The number of surviving silkworms was monitored.

4. Discussion

4.1. *Prediction of pathogenicity of bacteria in mammals based on measurements of silkworm-killing activity*

To examine the pathogenicity of bacteria, it is very important to determine whether the bacteria fulfill the criteria of Koch's postulates (*19*). In many cases, it is difficult to judge the pathogenicity because of the lack of an animal infection model. In general, mammals, such as mice or rats, are used to evaluate bacterial pathogenicity. The use of many mammals for infection experiments is associated with high costs and ethical concerns. Silkworms as model animals have a number of advantages for investigating pathogenicity: *i*) The methods of breeding and growing genetically homogeneous silkworms are well established because of the long history of the silk industry, *ii*) The silkworm body size is large enough to handle and inject specific volumes of bacterial samples using syringes, and *iii*) There are generally no ethical problems associated with the use of invertebrates, such as silkworms. We previously reported that silkworm larvae are killed by injection into the hemolymph of bacteria and true fungi pathogenic for humans, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Vibrio cholerae*, *Stenotrophomonas maltophilia*, *Candida albicans*, and *Candida tropicalis* (*2*,*3*,*10*). In this report, we examined whether bacteria that killed silkworms are also pathogenic to mice. Most of the bacteria with potent pathogenicity in silkworms also killed mice (Tables 1 and 2). Among them, the pathogenicity of *S. simiae* or *S. pasteuri* in mammals has not been previously reported. These findings suggest that silkworms are highly valuable as an infection model to predict the pathogenicity of bacteria in mammals.

Infection experiments with invertebrate animals are usually performed at room temperature. The expression of some virulence factors in pathogenic bacteria is greatly affected by temperature (*11-13*). Silkworms can be kept alive for at least 3 days at 37°C, although further incubation at 37°C kills silkworms probably due to various heat-induced damages. We found that *S. aureus* and other bacteria had more potent pathogenicity at 37°C than at 27°C (K. Sekimizu *et al*., unpublished results). In the present report, *S. simiae* killed silkworm larva more potently at 37°C than at 27°C (Figure 1). Thus, evaluation of pathogenicity of bacteria in silkworms at 37°C is important to predict pathogenicity in mammals.

4.2. *Availability of silkworm infection model for study of virulence mechanism of pathogenic bacteria*

We previously identified *cvfA*, *cvfB*, and *cvfC* as new virulence genes of *S. aureus* by using silkworm model. We further examined the functions of the protein products of these genes (*3,6,7,20,21*). We also reported purification of exotoxin secreted from environmental pathogens using the silkworm infection model (*9*). In the present report, we demonstrated that the supernatant of overnight culture of *S. simiae* and the heat-killed *S. simiae* have silkworm-killing activity. These findings suggest that the exotoxin and the cell wall component of this bacterium are virulence factors. Cell wall component of this bacterium supposed to stimulate excessively innate immune response to cause death of worms (*22,23*). Purification and characterization of the exotoxin is important toward understanding the pathogenicity of this bacterium. We therefore propose that the silkworm infection model is useful for studying the virulence mechanisms of pathogenic bacteria.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research. This study was supported in part by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) and Genome Pharmaceuticals Institute.

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(Received March 04, 2011; Accepted April 12, 2011)