Original Article

DOI: 10.5582/ddt.2013.v7.4.153

Use of silkworms to evaluate the pathogenicity of bacteria attached to cedar pollen

Yuan Hu, Hiroshi Hamamoto, Kazuhisa Sekimizu*

Laboratory of Microbiology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan.

ABSTRACT: Injection of a Japanese cedar pollen suspension into silkworm hemolymph kills the silkworms. A certain species of bacteria proliferated in the hemolymph of the dead silkworms. A 16S rDNA analysis demonstrated that the proliferating bacteria were *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis*, and *Bacillus amyloliquefaciens*. Among them, *B. cereus*, *B. thuringiensis*, and *B. weihenstephanensis* exhibited hemolysis against sheep red blood cells and were lethal to mice. A culture filtrate of *B. amyloliquefaciens* showed enzyme activity toward the pectic membrane of cedar pollen. These results suggest that silkworms as an animal model are useful for evaluating the pathogenicity of bacteria attached to cedar pollen.

Keywords: Silkworm, cedar pollen, bacillus, pathogenicity, cedar pollinosis

1. Introduction

Cedar pollinosis is one of the most prevalent causes of allergic rhinitis in Japan, causing many patients to suffer from runny nose, sneezing, and fatigue. Antigenic proteins inside cedar pollen, Cry j 1 and Cry j 2, are considered to cause pollinosis by promoting antigen-antibody reactions in the human nasal cavity (*1-3*). Immunoglobulin E antibody titers in the blood of patients with pollinosis are not related to the severity of clinical symptoms (*4*), suggesting that unknown factors determine the severity of pollinosis. These factors and their functions remain to be elucidated. We previously reported that cedar pollen undergoes morphologic changes in weak alkaline solution. Breaking down of the external walls is followed by expansion of the pectic membrane (*5*). Because the pH in the nasal cavity of patients with allergic rhinitis is higher than 8 (*6,7*), cedar pollen can be expected to undergo morphologic changes in the nasal cavity. The mechanism of destruction of the pectic membrane of cedar pollen and the release of antigenic proteins in the human nasal cavity, however, remain unclear. Bacteria and fungi are attached to pollen of various plants (*8,9*). Thus, we hypothesized the presence of bacteria on cedar pollen that may have a role in pollinosis.

We previously reported that silkworms were killed when injected with bacteria pathogenic to mammals such as *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Vibrio cholerae* (*10*). We also reported that pathogenicities of bacteria from soil and fish could be evaluated in silkworms (*11,12*). Silkworms as experimental model animals have several advantages: low rearing costs, fewer ethical issues regarding experimentation than vertebrate animals, and culturally established rearing method. For these reasons, we considered that silkworms were highly suitable for evaluating pathogenic microbes from the environment. In the present study, we attempted to isolate pathogenic bacteria from silkworms killed by the injection of cedar pollen into the hemolymph. This is the first report that pathogenic bacteria attached to pollen could be evaluated using silkworms.

2. Materials and Methods

2.1. *Animals*

Silkworms eggs (Hu•Yo × Tsukuba•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 for 1 day and then used for infection experiments. ICR mice (4 weeks old, female) were purchased from CLEA (Tokyo, 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 (p24-49).

**Address correspondence to:*
Dr. Kazuhisa Sekimizu, Laboratory of Microbiology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.
E-mail: sekimizu@mol.f.u-tokyo.ac.jp

2.2. *Cedar pollen injection into silkworms*

Japanese cedar pollen was collected from four different regions in Japan (Oita, Ibaraki, Chiba, and Tottori) in March in 2012 and 2013, dried, and preserved at 4˚C (ITEA, Tokyo, Japan). Cedar pollen (0.1 g) was suspended in 2 mL of 50 mM Tris/HCl (pH 8) buffer to promote the release of the external shell followed by expansion of the pectic membrane (*5*). Immediately or after 4 days of incubation of the cedar samples at 30˚C, aliquots of 0.05 mL were injected into silkworms, and the injected silkworms were reared at 27°C.

2.3. *Isolation of bacteria from silkworm*

Silkworms killed by the injection of bacteria were cut with dissecting scissors. The body fluid was spread onto Brain Heart Infusion (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) agar plates with an inoculating loop, and the plates were incubated at 30°C to isolate colonies.

2.4. *Colony count and determination of LD_{50} against silkworms*

Single colonies of bacteria isolated from silkworms were incubated in 1 mL of Brain Heart Infusion broth at 30°C. One hundred microliters of $10^{-4} \sim 10^{-7}$ diluted full growth samples were spread onto Brain Heart Infusion agar plates and the plates were incubated at 30°C for 12 h. The bacterial numbers in full growth were determined based on the number of colonies appearing on the plates. To determine the number of bacteria needed to kill 50% of the silkworms (LD_{50}), 0.05 mL of diluted sample was injected into the silkworm hemolymph, and silkworms were reared for 2 days.

2.5. *Determination of bacterial species*

Gram staining of the bacteria isolated from silkworms was performed using a Gram staining kit (Merck, Darmstadt, Germany) and the stained bacteria were observed under a microscope. The 16S rDNA regions were amplified by colony polymerase chain reaction (30 cycles; 94°C 15 sec, 50°C 30 sec, 68°C 1 min) using DNA polymerase KOD Fx Neo (Toyobo, Tokyo, Japan) and primer pairs U1(5'-CCAGCAGCCGCGGTAATACG-3'), U2(5'-ATCGGCTACCTTGTTACGACTTC-3'), and 9F(5'-GAGTTTGATCCTGGCTCAG-3'), 1541R(5'-AAGGAGGTGATCCAGCC-3'). The amplified DNA was separated by 1% agar gel electrophoresis, and extracted from gel using a DNA gel extraction kit (QIAGEN, The Netherlands). A cycle sequence reaction (25 cycles; 96°C 10 sec, 50°C 5 sec, 60°C 4 min) was conducted using a Big Dye Terminator (Applied Biosystems by Life Technologies, Carlsbad, CA, USA) and primers as mentioned above. DNA sequences were analyzed using a 3130xl Genetic Analyser (Applied Biosystems by Life Technologies). A BLAST search against 16S rDNA sequences of more than 400 bp was performed to identify the bacterial species. The bacterial species were identified based on sequences that matched with over 99% identity.

2.6. *Assay of digestion enzymes on pectic membrane*

Cedar pollen was suspended in 50 mM Tris/HCl (pH 8) buffer to promote the expansion of the pectic membrane. Bacteria were cultured in Brain Heart Infusion broth overnight, centrifuged to obtain supernatants, which were then filtered through a 0.22-µm Filter Unit (Millipore, Billerica, MA, USA). The filtrate (200 µL) was added to cedar pollen samples (200 µL). After incubation at 30°C for 12 h, the pectic membrane of the cedar pollen was observed under a microscope. Pectinase (11 units) from *Aspergillus niger* (Sigma-Aldrich, Steinheim, Germany) was used as a control.

2.7. *Hemolysin production assay*

Bacterial full growth was spread onto EHT agar plates (Kyokuto, Tokyo, Japan) and was incubated at 30°C for 12 h. When a transparent region appeared around colonies, the bacteria were judged to produce hemolysin.

2.8. *Pathogenicity against mice of bacteria obtained from cedar pollen*

Overnight cultures of the bacteria in Brain Heart Infusion broth at 30°C were washed with saline. Aliquots (500 µL) were injected into the mouse peritoneal cavity (n = 3). The number of mice alive after 5 days was monitored.

3. Results

3.1. *Killing of silkworms by cedar pollen injection*

Cedar pollen collected from four prefectures in Japan (Oita, Ibaraki, Chiba, Tottori) was suspended in a weak alkaline solution and injected into silkworms. Of 20 silkworms, 5 were killed in 3 days when injected with cedar pollen preserved at 4°C for 1 year after collection. Of 47 silkworms, 15 were killed when injected with cedar pollen stored for 2 months. In both cases, almost 30% of the silkworms were killed, suggesting that storage of the cedar pollen did not affect the silkworm killing effects. Furthermore, 35 of 36 silkworms were killed when injected with cedar pollen suspension incubated at 30°C for 4 days. These findings indicate that incubation of cedar pollen samples increases their lethality to silkworms.

Next, we attempted to isolate germs from the

silkworms killed by cedar pollen injection. We collected the body fluid from dead silkworms and spread it onto Brain Heart Infusion agar plates. After incubation at 30°C for 12 h, one or two species of numerous colonies appeared. Colonies were not observed when using body fluid from silkworms injected with control buffer without cedar pollen. All germ colonies reached full growth in Brain Heart Infusion broth after overnight incubation. All silkworms (n = 5) were killed within 20 h when the full growth (0.05 mL) was injected, suggesting that the microorganisms isolated on agar plates caused the killing effects on silkworms.

Aliquots of 100 µL of 0.05 g/mL cedar pollen suspension yielded 0 to 6 colonies when spread onto Brain Heart Infusion agar plates. In our protocol, the amount of cedar pollen injected to silkworms was 0.0025 g, thus the number of germs administered was less than 5, suggesting that a very small number of germs was sufficient to kill the silkworms. Therefore, we attempted to determine the LD_{50} of separated germs against silkworms. The LD_{50} of all separated germs against silkworms was less than 170 (cfu/larvae) (Table 1).

3.2. *Identification of isolated bacterium*

We attempted to identify the species of microorganisms that killed the silkworms. First, we performed ampicillin and ketoconazol sensitivity test to determine whether the germs were bacteria or fungi. All germs were sensitive to 12.5 µg/mL ampicillin and tolerant to 25 µg/mL ketoconazol. Therefore, all separated germs were considered to be bacteria. Furthermore, the results of the Gram staining demonstrated that all germs were Gram-positive, rod-shaped bacteria.

Next, we determined the 16S rDNA sequence to identify the species of the separated bacteria. These bacteria were identified as *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis*, and *Bacillus amyloliquefaciens* (Table 1). Therefore, all bacteria that attached to cedar pollen and that showed high pathogenicity against silkworms belonged to *Bacillus* sp.

3.3. *Production of hemolysin and pathogenicity of separated bacteria against mice*

We previously reported that bacteria such as *Staphylococcus* sp., *Proteus* sp., and *Morganella* sp., which were isolated from soil and fish, exhibited pathogenicity against both silkworms and mice (*11,12*). Thus, we considered that the bacteria isolated from cedar pollen that showed high pathogenicity against silkworms would also exhibit pathogenicity against mice as well. To test this, we first examined whether the separated bacteria showed hemolytic activity on sheep red blood cells. *B. cereus*, *B. thuringiensis*, and *B. weihenstephanensis* separated from cedar pollen all formed colonies on EHT agar plates with a transparent region around them. These findings suggest that these *Bacillus* sp. bacteria produce hemolysin against sheep red blood cells (Table 1).

Next, these germs were injected into the mouse peritoneal cavity to evaluate their pathogenicity against mammals. Three mice per group were injected with *B. cereus*, *B. thuringiensis*, or *B. weihenstephanensis*, and at least two mice died within 7 h in each group, whereas none of the mice injected with saline was killed. These results suggest that *B. cereus*, *B. thuringiensis*, and *B. weihenstephanensis* isolated from cedar pollen were all pathogenic against mice (Table 1).

Table 1. *Bacillus* sp. bacteria isolated from cedar pollen

Colony Number	Bacterial specieces	Locality	Date of collection	LD_{50} against silkworms (cells/larva)	Hemolysis against sheep red blood cells	Lethality in mice	Destructive activity against the pectic membrane
1	*Bacillus amyloliquefaciens*	Oita	March 2012	170	–	0/3	+
2	*Bacillus thuringiensis*	Ibaraki	March 2012	12	+	2/3	–
3	*Bacillus thuringiensis*	Chiba	March 2012	1.9	+	3/3	–
4	*Bacillus weihenstephanensis*	Totoori	March 2012	1.3	+	2/3	–
5	*Bacillus weihenstephanensis*	Totoori	March 2012	2.3	+	2/3	–
6	*Bacillus weihenstephanensis*	Totoori	March 2012	0.6	+	3/3	–
7	*Bacillus weihenstephanensis*	Totoori	March 2013	8.0	+	3/3	–
8	*Bacillus cereus*	Chiba	March 2012	26	+	3/3	–
9	*Bacillus cereus*	Oita	March 2012	0.9	+	2/3	–
10	*Bacillus cereus*	Oita	March 2013	0.5	+	3/3	–
11	*Bacillus cereus*	Ibaraki	March 2013	7.7	+	3/3	–
12	*Bacillus cereus*	Ibaraki	March 2013	2.5	+	3/3	–
13	*Bacillus cereus or Bacillus thuringiensis*	Chiba	March 2013	3.9	+	3/3	–
14	*Bacillus cereus or Bacillus thuringiensis*	Tottori	March 2013	2.6	+	3/3	–

Properties of *Bacillus* bacteria isolated from cedar pollen are presented. The species of bacteria were determined by 16S rDNA sequencing. The LD_{50} against silkworms, hemolysis against sheep red blood cells, lethality in mice, and destructive activity against the pectic membrane in culture filtrate are shown.

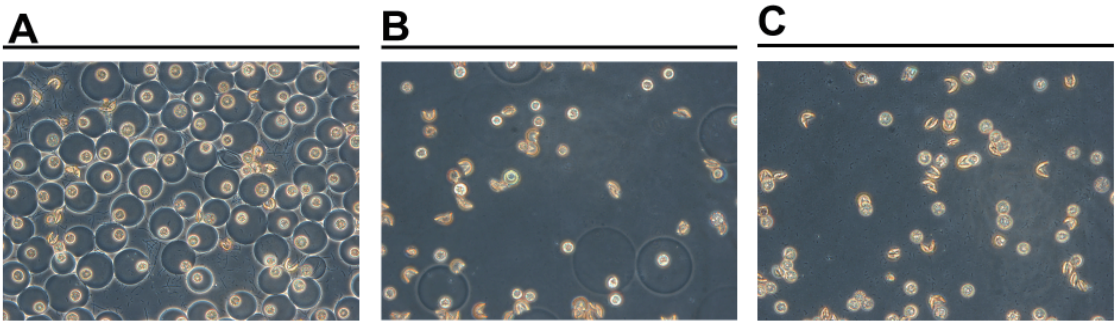

Figure 1. Phase-contrast microscope pictures of cedar pollen suspended in weak alkaline solution. Cedar pollen was incubated with BHI broth **(A)**, culture filtrate of *Bacillus amyloliquefaciens* **(B)**, or pectinase from *Aspergillus niger* **(C)** in a weak alkaline solution at 30°C for 12 h, and was observed under a microscope.

3.4. *Digestion activity in a bacterial culture against the pectic membrane of cedar pollen*

We examined whether bacteria isolated from cedar pollen secrete digestion enzymes against the pectic membrane of cedar pollen. Bacterial cultures were centrifuged and filtered, and each sample was incubated with cedar pollen suspended in a weak alkaline solution for 12 h at 30°C. The pectic membrane of cedar pollen disappeared after incubation with the sample from *B. amyloliquefaciens*, indicating that the cultural supernatant of *B. amiloliquefaciens* has enzymatic activity that digests the pectic membrane of cedar pollen (Figure 1).

4. Discussion

4.1. *Isolation of Bacillus sp. bacteria from cedar pollen*

Silkworms injected with cedar pollen were killed and four species of *Bacillus* sp. bacteria were isolated from the body fluid of the dead silkworms. Bacteria in genus *Bacillus* form spores, thus it is considered that *Bacillus* spores were attached to cedar pollen. The lack of difference in the mortality of cedar pollen preserved for 1 year or 2 months supports the notion that bacterial spores attached to cedar pollen killed the silkworms. Among many species of bacteria attached to cedar pollen, bacteria with the strongest pathogenicity might proliferate in the silkworm body fluid. Therefore, we conclude that *Bacillus* spores attached to cedar pollen proliferated in the body fluid of silkworms, resulting in the death of the silkworms.

It is uncertain when and how the *Bacillus* spores attach to the cedar pollen. Fungi proliferate in the stamens of cedar (*9*), thus it is highly conceivable that bacteria had already attached to the pollen before collection. The finding that all cedar pollen collected from four different prefectures in Japan contained the attached *Bacillus* spores supports the notion that the attachment of *Bacillus* spores to cedar pollen is a universal phenomenon. It is therefore likely that *Bacillus* spores are attached to cedar pollen flying from forest to urban areas.

4.2. *Evaluation of Bacillus pathogenicity using silkworms*

We previously reported that the LD_{50} against silkworms of bacteria separated from soil and fish was $10^4 \sim 10^8$ cfu (*11,12*). In contrast, the LD_{50} of bacteria separated from cedar pollen was much lower, suggesting high pathogenicities among environmental bacteria. Invertebrate animals such as silkworms possess no antibodies, and therefore, have no acquired immunity system. Invertebrate animals combat against invading pathogens by relying solely on their natural immunity systems (*13*). Innate immunity systems in invertebrates are highly conserved in mammals. Therefore, we speculate that pathogenic bacteria against silkworms isolated from cedar pollen can tolerate attacks by innate immune systems of mammalian hosts.

B. cereus, *B. thuringiensis*, and *B. weihenstephanensis* isolated from cedar pollen produce hemolycin on agar plates. Also, these *Bacillus* sp. bacteria are lethal in mice when injected into the mouse peritoneal cavities. The findings suggest that these bacteria are pathogenic to mammals as well as to silkworms. By monitoring pathogenicity against silkworms, we demonstrated that 3 of 4 bacterial species isolated from cedar pollen were pathogenic in mice. Therefore, silkworms can be considered a useful animal model for easy and efficient evaluation of the pathogenicity of environmental bacteria against mammals.

4.3. *Digestion of the pollen pectic membrane by pectinase produced by bacteria*

Here we demonstrated that the culture supernatant of *B. amyloliquefaciens* isolated from cedar pollen digested the pectic membrane of cedar pollen. This finding suggests that this bacterium secretes pectinase. We previously reported that cedar pollen undergoes morphologic changes, release of external walls, and expansion of pectic membrane when suspended in a weak alkaline solution (*5*). Nasal cavities of human patients with allergic rhinitis are reported to have an alkaline pH (*6,7*). Therefore, cedar pollen that invade the nasal cavity undergoes morphologic changes, as

mentioned above. Digestion of the pectic membrane by pectinase secreted from bacteria may cause the release of antigenic proteins Cry j 1 and Cry j 2, which cause allergic reactions. Thus, the removal of pectinase-producing bacteria or the administration of pectinase inhibitors can contribute to preventing cedar pollinosis.

Acknowledgements

This study was supported by the Genome Pharmaceutical Institute, and supported in part by a Grant-in-Aid for Young Scientists (A) (24689008) and a Grant-in-Aid for Challenging Exploratory Research (24659041).

References

1. Yasueda H, Yui Y, Shimizu T, Shida T. Isolation and partial characterization of the major allergen from Japanese cedar (*Cryptomeria japonica*) pollen. J Allergy Clin Immunol. 1983; 71:77-86.
2. Taniai M, Ando S, Usui M, Kurimoto M, Sakaguchi M, Inouye S, Matuhasi T. N-terminal amino acid sequence of a major allergen of Japanese cedar pollen (Cry j I). FEBS Lett. 1988; 239:329-332.
3. Sakaguchi M, Inouye S, Taniai M, Ando S, Usui M, Matuhasi T. Identification of the second major allergen of Japanese cedar pollen. Allergy. 1990; 45:309-312.
4. Somville MA, Machiels J, Gilles JG, Saint-Remy JM. Seasonal variation in specific IgE antibodies of grass-pollen hypersensitive patients depends on the steady state IgE concentration and is not related to clinical symptoms. J Allergy Clin Immunol. 1989; 83:486-494.
5. Ishii K, Hamamoto H, Sekimizu K. A novel method to suppress the dispersal of Japanese cedar pollen by inducing morphologic changes with weak alkaline solutions. Drug Discov Ther. 2007; 1:124-129.
6. Washington N, Steele RJ, Jackson SJ, Bush D, Mason J, Gill DA, Pitt K, Rawlins DA. Determination of baseline human nasal pH and the effect of intranasally administered buffers. Int J Pharm. 2000; 198:139-146.
7. England RJ, Homer JJ, Knight LC, Ell SR. Nasal pH measurement: A reliable and repeatable parameter. Clin Otolaryngol Allied Sci. 1999; 24:67-68.
8. Heydenreich B, Bellinghausen I, Konig B, Becker WM, Grabbe S, Petersen A, Saloga J. Gram-positive bacteria on grass pollen exhibit adjuvant activity inducing inflammatory T cell responses. Clin Exp Allergy. 2012; 42:76-84.
9. Hirooka Y, Akiba M, Ichihara Y, Masuya H, Takahata Y, Suda T, Yada Y, Yamamoto S, Kubono T. A novel approach of preventing Japanese cedar pollen dispersal that is the cause of Japanese cedar pollinosis (JCP) using pollen-specific fungal infection. PLoS One. 2013; 8:e62875.
10. Kaito C, Akimitsu N, Watanabe H, Sekimizu K. Silkworm larvae as an animal model of bacterial infection pathogenic to humans. Microb Pathog. 2002; 32:183-190.
11. Kaito C, Usui K, Kyuma T, Sekimizu K. Isolation of mammalian pathogenic bacteria using silkworms. Drug Discov Ther. 2011; 5:66-70.
12. Usui K, Miyazaki S, Kaito C, Sekimizu K. Purification of a soil bacteria exotoxin using silkworm toxicity to measure specific activity. Microb Pathog. 2009; 46:59-62.
13. Ishii K, Hamamoto H, Imamura K, Adachi T, Shoji M, Nakayama K, Sekimizu K. *Porphyromonas gingivalis* peptidoglycans induce excessive activation of the innate immune system in silkworm larvae. J Biol Chem. 2010; 285:33338-33347.

(Received July 29, 2013; Accepted August 5, 2013)