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Bacteria in the gut of Japanese honeybee, *Apis cerana japonica*, and their antagonistic effect against *Paenibacillus larvae*, the causal agent of American foulbrood

Mikio Yoshiyama *, Kiyoshi Kimura

Honeybee Research Group, Animal Breeding and Reproduction Research Team, National Institute of Livestock and Grassland Science, 2 Ikenodai, Tsukuba, Ibaraki 305-0901, Japan

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ABSTRACT

We assessed the complexity of bacterial communities occurring in the digestive tract of the Japanese honeybee, *Apis cerana japonica*, using histological and 16S rRNA gene sequence analyzes. Both Gram-positive and -negative bacteria were observed, and the number of gut bacteria was higher in old larvae compared with young larvae. A total of 35 clones were obtained by a culture-dependent method, and 16S rRNA gene sequence analysis revealed that the bacterial population in the gut of Japanese honeybee was diverse, including the phyla firmicutes, actinobacteria, and alpha-, beta-, and gammaproteobacteria. Further investigation by *in vitro* inhibition assays was carried out to determine the ability of an isolate to inhibit *Paenibacillus larvae*, the causal agent of American foulbrood. Out of 35 isolates, seven showed strong inhibitory activity against *P. larvae*. Most of the antagonistic bacteria belonged to *Bacillus* species, suggesting that the bacterial isolates obtained in this study appear to be potential candidates for the biological control of *P. larvae*.

1. Introduction

Honeybees, *Apis mellifera*, play an essential role as pollinators of crop and fruit species, and pollination by honeybees in agricultural production is estimated to be worth 15 billion dollars per year in the USA (Morse and Calderone, 2000). Honeybee colonies are threatened by numerous pathogens, including viruses, fungi, bacteria, and protozoa. One of the most serious honeybee diseases is American foulbrood (AFB), caused by the Gram-positive spore-forming bacterium *Paenibacillus larvae* (Genersch et al., 2006). AFB is a contagious and destructive disease affecting the larval and pupal stages of honeybees. It is difficult to prevent this disease because of the resilience and long life of the pathogen spores (Matheson and Reid, 1992). Two antibiotics, oxytetracycline and tylosin, are currently approved for their use in the control of AFB; however, the emergence of oxytetracycline-resistant *P. larvae* has been reported in USA, Canada, and Argentina (Alippi, 2000; Evans, 2003; Miyagi et al., 2000).

There is increased interest in investigations on new effective AFB control methods. According to pioneering work by Evans and Armstrong (2005, 2006), certain bacteria that show antagonistic activity against *P. larvae* were found in the gut of *A. mellifera*, and these provide a novel way of controlling AFB.

The Japanese honeybee, *Apis cerana japonica*, is a native honeybee that is found in various parts of Japan. Compared with *A. mellifera*, which is commonly used in beekeeping, *A. cerana* subspecies possess greater antipathogenic potential, including resistance to *Varroa* mites and AFB (Peng et al., 1987; Chen et al., 2000). These useful traits might contribute to the control of bee pathogens in apiculture. However, little is known about the interactions between gut bacteria and pathogens in *A. c. japonica*. In particular, the actions of gut bacteria against AFB remain elusive. To improve understanding of gut microbial communities with a view to the future use of antagonistic bacteria as biological agents, we identified the bacterial species present in the gut of *A. c. japonica* by 16S rRNA gene sequence analysis and evaluated their activity against *P. larvae*.

2. Materials and methods

2.1. Bees

Two *A. c. japonica* colonies were collected independently in the Tsukuba area of Japan in March and May 2008, respectively, and reared in wooden hives in an apiary at the Honeybee Research Group, National Institute of Livestock and Grassland Science, Tsukuba.

2.2. Histology

Adults ($n = 8$), 1-day-old larvae ($n = 8$), and 4-day-old larvae ($n = 8$) (age estimated by body size; Evans, 2004) were randomly

* Corresponding author. Fax: +81 29 838 8606.
E-mail address: yoshiyam@affrc.go.jp (M. Yoshiyama).

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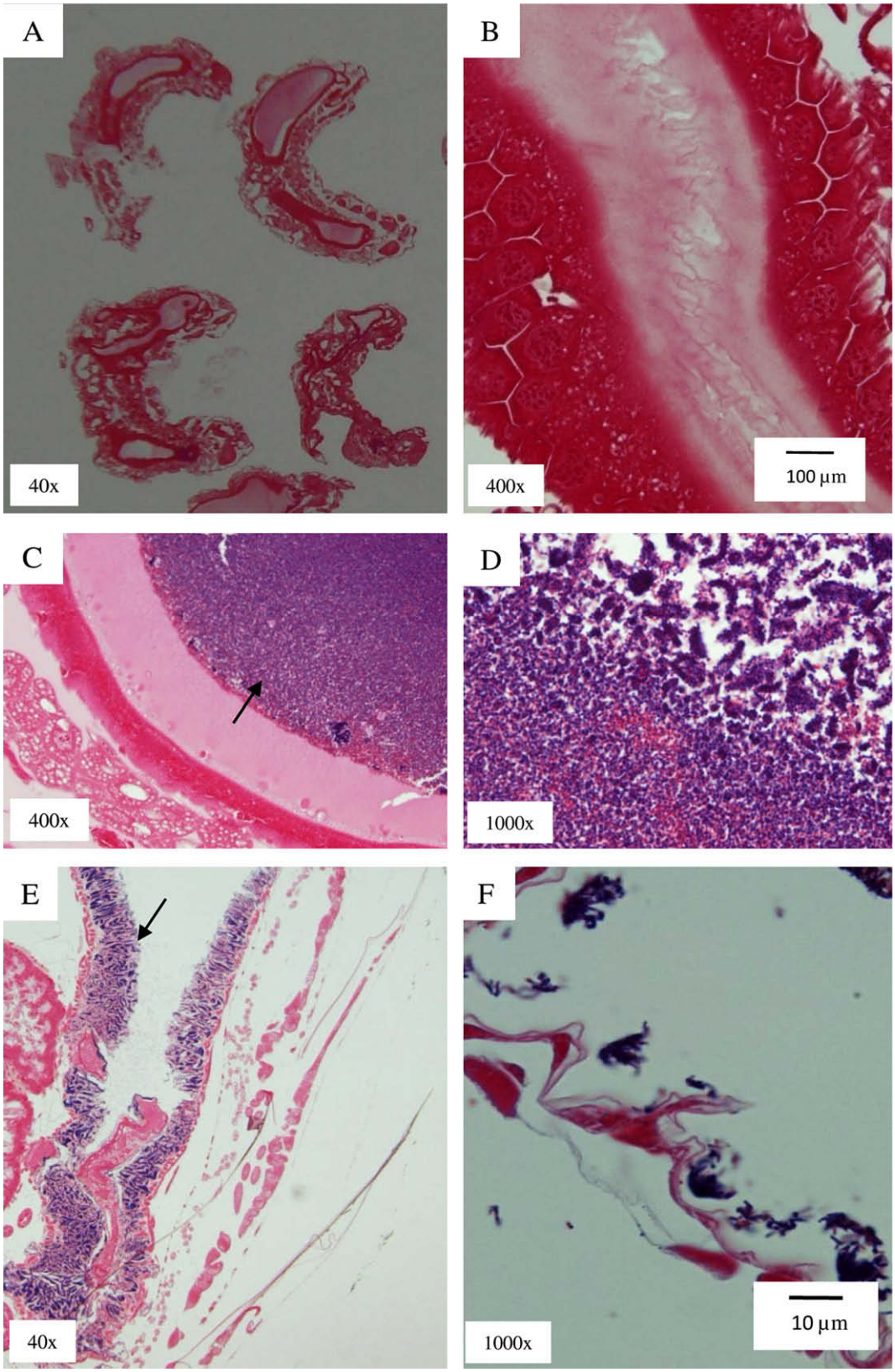

Fig. 1. Histological sections of *Apis cerana japonica*. Light micrographs of the midgut of the first instar larvae (A, B) showing no obvious bacteria. The midguts of fourth instar larvae (C, D) were filled with bacteria. Forager (E, F) honeybees possessed a number of bacteria. Gram-positive and -negative bacteria are stained purple and red, respectively. Arrows indicate bacteria. (For interpretation of the reference to color in this figure legend, the reader is referred to the web version of the article.)

collected from the two colonies, fixed in 10% phosphate-buffered formaldehyde for 4 h, and dehydrated in a series of ethanol concentrations. They were embedded in paraffin wax, and sections of approximately 3 µm thickness were stained using hematoxy-