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Title	Studies on the risk of emergence of tylosin-resistant Paenibacillus larvae in Japan
Author(s)	岡本, 真理子
Degree Grantor	北海道大学
Degree Name	博士(獣医学)
Dissertation Number	乙第7216号
Issue Date	2024-09-25
DOI	https://doi.org/10.14943/doctoral.r7216
Doc URL	https://hdl.handle.net/2115/93842
Type	doctoral thesis
File Information	Mariko_Okamoto.pdf

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Studies on the risk of emergence of tylosin-resistant *Paenibacillus larvae* in Japan

（日本におけるタイロシン耐性アメリカ腐蛆病菌の出現リスクの研究）

Mariko Okamoto

Contents

Abbreviations

ABC	ATP-binding cassette
ABC-F	ATP binding cassette F
AFB	American foulbrood
CARD	the Comprehensive Antibiotic Resistance Database
CC	clonal complex
CFU	colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CP	chloramphenicol
CSA	Columbia blood agar containing 5% sheep blood
DDBJ	the DNA Data Bank of Japan
EFB	European foulbrood
EM	erythromycin
EMBL	European Molecular Biology Laboratory
ERIC	enterobacterial repetitive intergenic consensus
GAMSA	modified GAM agar containing 5% sheep blood
ICE	integrative conjugative element
kbp	kilobase pair
LCM	lincomycin
MGE	mobile genetic element
MH	Mueller-Hinton
MIC	minimum inhibitory concentration
MLSB	macrolide-lincosamide-streptogramin B
MLST	multilocus sequence typing

MRM	mirosamicin
NPET	nascent peptide exit tunnel
OIE	Office International des Epizooties
oriT	the origin of transfer
OTC	oxytetracycline
PCI	phenol, phenol-chloroform-isoamyl alcohol (25:24:1)
PCR	polymerase chain reaction
PTC	peptidyl-transferase center
Rep	rolling circle replication initiator protein
RGI	Resistance Gene Identifier
RT-PCR	reverse transcription PCR
ST	sequence type
T4CP	type IV coupling protein
T4SS	type 4 secretion system
TS	tylosin
USA	United States of America
WOAH	World Organisation for Animal Health

Notes

Contents of the present thesis were published in the following articles.

1. **Okamoto M, Kumagai M, Kanamori H, Takamatsu D.** Antimicrobial resistance genes in bacteria isolated from Japanese honey, and their potential for conferring macrolide and lincosamide resistance in the American foulbrood pathogen *Paenibacillus larvae*. Front. Microbiol. 12:667096, 2021.
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2. **Okamoto M, Furuya H, Sugimoto I, Kusumoto M, Takamatsu D.** A novel multiplex PCR assay to detect and distinguish between different types of *Paenibacillus larvae* and *Melissococcus plutonius*, and a survey of foulbrood pathogen contamination in Japanese honey. J. Vet. Med. Sci. 84, 390–399, 2022.
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3. **Okamoto M, Furuya H, Sugimoto I, Takamatsu D.** Detection of macrolide resistance genes, *ermC* and *ermB*, in Japanese honey using real-time PCR assays. J. Vet. Med. Sci. 84, 1453–1456, 2022.
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General Introduction

The long-standing relationship between people and honey bees dates back to BC. The evidence of honey hunting to obtain wild honey is archived in Spanish cave murals from 6000 BC, and in 3000 BC, practical beekeeping techniques were established in Egypt (1). In Japan, although beekeeping and honey production were first archived in the "Nihon Shoki" written in the Nara period, practical beekeeping started in the Edo period (2). At that time, honey had been consumed as a luxury food. Honey bees had mainly been kept for honey and royal jelly in modern times. In recent years, however, they have also been used as an agricultural resource to pollinate a variety of crops, and these are the reasons why honey bees are treated as one of domestic animals. In agricultural industries, more than 90% of the leading global crop types are visited by wild and/or managed bees, including the western honey bee (*Apis mellifera*), eastern honey bee (*Apis cerana*), some bumble bees and stingless bees. The annual market value of the production with animal pollination services is estimated at $235 billion-$577 billion (in 2015) worldwide (3). In Japan, the economic contribution amount by managed *A. mellifera* is estimated at approximately ¥180 billion (4). In addition, approximately 87.5% of the world's flowering wild plants depend on animal pollination for sexual reproduction (3), and managed honey bees also contribute to the maintenance of natural ecosystems outside of agricultural lands. Therefore, appropriate managements of honey bees is extremely important for the long and healthy existence of humankind.

Honey bees are social insects that live in colonies of many individuals. One colony of honey bees consists of one queen bee, thousands to tens of thousands of worker bees, and drones which only appear in numbers of about 10% of the number of worker bees in the breeding season. The queen bee is devoted solely to laying eggs, and the drones are only responsible for mating the queens of other colonies; therefore, the worker bees undertake all tasks to maintain the colony, including the cleaning and construction of hives, rearing larvae, colony defense, foraging, and honey production and storage. Since most larvae that hatch from eggs eventually develop into worker

bees, the healthy development of larvae is of critical importance for the maintenance of healthy colonies, and thus, diseases that affect bee larvae seriously threaten the maintenance of healthy colonies.

American foulbrood (AFB) and European foulbrood (EFB) are bacterial diseases of honey bee broods that can cause colony collapses. Both diseases occur in most areas of the world where beekeeping is practiced, causing economic losses in the beekeeping and agricultural industries (5). From their importance, both diseases are designated as listed diseases in the World Organisation for Animal Health (WOAH [OIE, Office International des Epizooties]) and are also designated as monitored infectious diseases under the Act on Domestic Animal Infectious Disease Control in Japan. According to the annual statistics of foulbrood diseases compiled by the Ministry of Agriculture, Forestry and Fisheries in Japan, approximately 30–60 AFB/EFB cases have been officially reported annually in recent years.

AFB is caused by a spore-forming and Gram-positive bacterium, *Paenibacillus larvae*. Larvae are most susceptible to *P. larvae* infection during the early larval stages, i.e., 12–36 h after egg hatching, and become infected through the ingestion of brood foods contaminated with *P. larvae* spores (6). Ingested spores germinate and massively proliferate in the larval midgut. *P. larvae* vegetative cells then penetrate the peritrophic membrane that lines the midgut epithelium, breach the midgut epithelium via the paracellular route, enter the haemocoel, and kill the larvae (6, 7, 8). Dead brood becomes ropy, dries out, and then forms hard dark scales. The scales contain millions of *P. larvae* spores and drive disease transmission within and between colonies (6, 9, 10). *P. larvae* spores are highly resistant to heat and chemical agents and can survive for many years in dried larval scales, hive products and equipment (11); therefore, burning of diseased colonies and contaminated hive materials is considered the most effective control measure for AFB.

The pathogen of EFB is a Gram-positive lanceolate coccus, *Melissococcus plutonius*. Larvae are also infected via oral ingestion of brood foods contaminated with *M. plutonius*, and the ingested bacteria colonize and proliferate in the larval midgut.

The peritrophic membrane is degraded during the course of infection, and *M. plutonius* directly interacts with the midgut epithelium. Although the bacteria do not invade or proliferate in the body cavity actively, midgut epithelial cells degenerate with time in infected larvae, and most infected larvae eventually die. Dead larvae change from pearly white to yellow and then brown, decompose, and finally become grayish black. Furthermore, larvae often give off a foul or sour smell due to the decomposition of larval remains by secondary invaders, such as *Enterococcus faecalis* and *Paenibacillus alvei* (12, 13, 14, 15).

In Japan, when AFB or EFB occur, diseased colonies and contaminated hive materials have to be burned to control the diseases. Furthermore, movement restrictions will be imposed on items that could spread the pathogens (e.g., hives, hive frames, combs, honey, and bee wax) in the outbreak area and surrounding areas based on the administrative standards of each prefectural government. As these control measures lead to significant economic losses in apicultural and agricultural industries, the prevention of AFB and EFB development is crucial to keep these industries healthy.

In several countries, antimicrobials are used to prevent and treat foulbroods in beekeeping. Oxytetracycline (OTC) is the most commonly used antimicrobial in the control of AFB (16). However, the intensive use of antimicrobials could induce the development and selection of antimicrobial-resistant bacteria. Indeed, OTC-resistant *P. larvae* strains have been detected in several countries, including the USA, Canada and Argentina (17, 18, 19, 20). In previous studies, approximately 40% of Argentinian and 16% of North American *P. larvae* isolates tested were OTC-resistant (17, 20). The United States Food and Drug Administration additionally approved a macrolide antibiotic (tylosin tartrate [TS]) in 2005 and a lincosamide antibiotic (lincomycin hydrochloride [LCM]) in 2012 for the control of AFB; however, in the USA, TS- and/or LCM-resistant *P. larvae* were isolated from AFB cases in 2007, 2010 and 2013 (20).

OTC-resistant phenotypes observed in *P. larvae* develop, at least in part, due to the acquisition of the tetracycline resistance gene, *tet*(L) (21, 22, 23, 24).

Intriguingly, López et al. (25) isolated *Bacillus* strains carrying a variety of tetracycline resistance genes, including *tet*(L), from honey, and suggested that bacteria in honey are a reservoir for tetracycline resistance genes. Since honey is a brood food for worker larvae, *P. larvae* may have contacted such honey-derived tetracycline-resistant bacteria in the larval gut and acquired tetracycline resistance genes. As a wide variety of bacteria are known to exist in honey (26, 27, 28, 29), bacteria other than *Bacillus* spp. may also harbor antibiotic resistance genes. Therefore, *P. larvae* may have many opportunities to come into contact with and acquire resistance genes from other bacteria in honey.

In Japan, as foulbrood disease colonies have to be burned, antimicrobials cannot be used for the treatment of foulbroods. However, the use of antibiotics for the prevention of AFB is allowed, and 16-membered ring macrolides (mirosamicin [MRM] and TS) have been used in honey bee colonies for more than 20 years (30). MRM was approved in 1999 by the Ministry of Agriculture, Forestry and Fisheries of Japan, and TS was additionally approved in 2017. As the sale of the MRM-containing prophylactic drug (Apiten for Honey bees; FEED ONE, Yokohama, Japan) was discontinued, TS is the only approved prophylactic available for controlling AFB at present. Fortunately, TS-resistant *P. larvae* was not found among Japanese strains isolated between 1993 to 2017 in the previous study (30). In addition, the isolation of TS-resistant *P. larvae* has not been reported yet from AFB cases after the TS approval in 2017. Therefore, Japan is probably free from TS-resistant *P. larvae* at present. However, as mentioned above, TS-resistant *P. larvae* strains have already been detected in North America (20). Although the TS-resistant mechanisms in the North American *P. larvae* strains are unknown, TS-resistant *P. larvae* may emerge in Japan in the future through the acquisition of macrolide resistance genes. However, bacteria in honey that may confer TS resistance to *P. larvae* have not been investigated in Japan. If macrolide resistance genes and *P. larvae* coexist in one colony, the use of prophylactics in that colony increases the risk of TS-resistant *P. larvae* being generated and selected for, and if TS-resistant *P. larvae* emerges through the acquisition of macrolide resistance genes, the effectiveness of the only prophylactic may be lost.

However, there were no appropriate assays for the presence of foulbrood pathogens and macrolide resistance genes in honey bee colonies.

Therefore, in the present thesis, in order to elucidate the risk of the emergence of TS-resistant *P. larvae* in Japan in the future, the author identified and analyzed honey-derived bacteria, which had the potential for conferring TS resistance to *P. larvae*. Furthermore, for the prudent use of TS, an evaluation system that determines the contamination status of foulbrood pathogens and macrolide resistance genes in honey bee colonies was developed to assess the risk of TS-resistant *P. larvae* emergence.

The present thesis consists of four chapters; in Chapter I, TS-resistant bacteria were isolated from Japanese honey, and putative macrolide resistance genes with the potential for horizontal transmission to *P. larvae* were investigated by analyzing the genomes of the honey-derived bacteria. In Chapter II, gene transfer studies to *P. larvae* were conducted using an expression vector to determine whether the detected putative macrolide resistance genes could confer TS resistance to *P. larvae*. Furthermore, the mating tests for conjugative transmission of a macrolide-resistant plasmid between *P. larvae* and honey-derived bacteria were performed, and the stability of the plasmid in *P. larvae* was evaluated. In Chapter III, the detection methods for foulbrood pathogens and macrolide resistance genes in honey were developed in order to understand the risk of TS-resistant *P. larvae* emergence in apiaries. In addition, the most efficient kit for bacterial DNA extraction from honey was selected from commercially available kits. With these assays, in Chapter IV, the author investigated the contamination rate of foulbrood pathogens and macrolide resistance genes in Japanese commercial honey. The assays provide important information to promote the prudent use of the prophylactic in apiaries, and this study will contribute to the further improvement of honey bee management techniques.

Chapter I

Tylosin-resistant bacteria in Japanese honey and search for their macrolide resistance genes and mobile genetic elements by genome analysis

Introduction

Honey bee larvae cannot get food by themselves and thus are fed by young worker bees called nurse bees. In a hive, worker larvae are firstly fed worker jelly. This is a mixture of the hypopharyngeal and mandibular gland secretions of nurse bees (31, 32). In addition, worker larvae are fed some pollen and honey on or after the third day of larval life (33, 34). When foulbrood pathogens contaminate such larval foods, larvae become infected with those pathogens by taking them in orally.

Honey is produced by worker bees through several steps (33). First, floral nectar, which is an aqueous plant secretion, is collected by the worker bees in the honey stomach. During the transmission of the nectar to the nest, various enzymes from the hypopharyngeal gland are added to the nectar. These enzymes break down the sugars, resulting in the most easily digestible forms by bees. The collected nectar is stored in the hive, and the worker bees responsible for processing and storing honey evaporate the water in in-process honey to less than 18% to protect the honey from yeasts. The ripened honey is sealed beneath a wax capping and stored until fed to worker bees or larvae. Because honey production is affected by many worker bees and environments such as flowers, plants, soils, waters, and other field factors, a wide range of bacteria distributed in such an environment can contaminate the honey.

In beekeeping, the unique food processing and larval feeding systems of honey bees are used to administer the AFB prophylactic, TS, to larvae. TS is currently used by dusting methods in Japan, and 150 mg of TS is used for 10,000 adult bees; i.e., the mixture consisting of 5 g of confectioner's sugar and 50 mg of TS is sprinkled over the hive's top bars three times at one-week intervals, and the dose can be scaled up depending on the number of adult bees. In the hive, TS-containing sugar is ingested by worker bees and processed into honey as described above, and TS is administered orally to larvae by worker bees via TS-containing honey. The ingested TS is transported to the larval midgut and inhibits *P. larvae* proliferation in the midgut, resulting in preventing the development of AFB.

In the country where TS is used for the treatment of AFB, TS-resistant *P.*

larvae has already been found (20). Although TS resistance mechanisms in *P. larvae* strains has not been elucidated in the previous study, those of OTC, the most commonly used antimicrobial in beekeeping, have already been reported to be due to the acquisition of the *tet*(L)-harboring plasmids (21, 22). The tetracycline-resistant plasmids are suggested to be small mobilizable plasmids with a rolling-circle replication system (21, 22, 23, 24). The mobilizable plasmid is one of the mobile genetic elements (MGEs). MGE is a type of genetic material that can move around within a genome and/or that can be transferred between bacteria. In addition to plasmids, integrative conjugative elements (ICEs) and phage are also known as MGEs. Various genes that contribute to the adaptation of bacteria to their environment can be carried on MGEs, and their transmission to other bacteria via MGEs can allow bacteria to acquire new traits including antimicrobial resistance and increase their survival in the environment. Interestingly, *tet*(L) was also detected from a *Bacillus* sp. isolated from honey (25). Although it is not yet clear whether this *tet*(L) gene is located on MGEs, given that honey is one of the larval foods and that the larval midgut is the only place in nature where *P. larvae* becomes the vegetative form and proliferates, it is thought that *P. larvae* has received tetracycline-resistant genes from the bacteria in honey via MGEs in the larval midgut and has become OTC-resistant.

If this hypothesis regarding the OTC-resistant *P. larvae* is correct, TS-resistant *P. larvae* may also emerge through the same mechanism. That is, if honey is a reservoir for bacteria harboring macrolide resistance genes and those resistance genes are on MGEs, *P. larvae* may become TS-resistant by receiving macrolide resistance genes from honey-derived bacteria via MGEs in the midgut. However, it has not yet been investigated whether bacteria possessing macrolide resistance genes exist in honey. Therefore, in the present study, the author searched Japanese honey samples for TS-resistant bacteria and macrolide resistance genes on MGEs.

Materials and Methods

Japanese honey used in this study

Fifty-three Japanese honey samples (lot nos. J1−J53) were used in the study of this chapter (Table 1). All of them were commercially available honey from different origins (i.e., different apiaries, different producers and/or different floral origins) and purchased between 2017 and 2018. Four honey samples (J12, J15, J24 and J42) were produced by Japanese honey bees, *A. cerana japonica*, and the others were produced by western honey bees, *A. mellifera*. All samples were stored at room temperature until use.

Isolation of bacteria from honey

Twenty milliliters of each honey sample was diluted to 50% (v/v) with sterile water and centrifuged at 11,000 rpm for 45 min. The supernatant was removed and the sediment was suspended with 1 ml of sterile water. In order to isolate as many bacterial species as possible, one hundred microliters of each suspension was plated on following media: Columbia blood agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing 5% sheep blood (CSA) (for various bacteria including *P. larvae*), KSBHI agar (12) (for *M. plutonius*), Lactobacilli MRS agar (Becton, Dickinson and Company) (for lactic acid bacteria) and modified GAM agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 5% sheep blood (GAMSA) (for anaerobic bacteria including *Clostridium*). For selective isolation of TS-resistant bacteria, all agar media used for the isolation were supplemented with 1 μg/ml of TS. CSA was incubated at 35℃ under air plus 5% CO_2 conditions for two days, and the other agar media were incubated at 35℃ under anaerobic conditions for four days. Colonies grown on the agar media were subcultured for purification. Pure-cultured bacterial isolates were suspended in LB broth (Becton, Dickinson and Company) containing 30% glycerol and stored at –80℃ until use.

Identification of honey-derived bacteria

Genomic DNA of honey derived bacteria was extracted using InstaGene Matrix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions and 16S rRNA gene sequences of the isolates were determined as described previously (12). Briefly, an approximately 1.5-kbp region of the 16S rRNA gene was amplified from the genomic DNA by TaKaRa ExTaq DNA polymerase (Takara Bio, Kusatsu, Japan) using primers F1 (5'-GAGTTTGATCCTGGCTCAG-3') and R13 (5'-AGAAAGGAGGTGATCCAGCC-3') (35). The amplified fragments were purified using QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and sequenced by a BigDye terminator v3.1 cycle sequencing kit using a 3130xl Genetic Analyzer (Applied Biosystems, Tokyo, Japan). Sequencher ver. 5.4.6 (Gene Codes Corp., Ann Arbor, MI, USA) was used to assemble the sequences. The species or genus was identified by analyzing the 16S rRNA gene sequences using the EzBioCloud server (https://www.ezbiocloud.net) (36). The 16S rRNA gene sequences determined in this study were deposited in the DNA Data Bank of Japan (DDBJ)/GenBank/European Molecular Biology Laboratory (EMBL) database under the accession numbers LC588427–LC588635. The presence of the cereulide synthetase gene was investigated in some *Bacillus* isolates using the *Bacillus cereus* (CRS gene) PCR Detection Kit (Takara Bio) according to the manufacturer's instructions.

Antimicrobial susceptibility tests

Antimicrobial susceptibility tests were performed on 209 Japanese honey-derived bacterial isolates cultured under the appropriate culture conditions (Table 2). TS (Combi- Blocks Inc., San Diego, CA, USA), OTC (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) and LCM (Sigma-Aldrich, St. Louis, MO, USA) were used for the tests. Except for the *Clostridium beijerinckii* isolate, minimum inhibitory concentrations (MICs) of these antimicrobials were determined by broth microdilution methods according to standard methods of Clinical and Laboratory Standards Institute

(CLSI, M07-A10). For the quality control of each test, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 cultured on Mueller Hinton (MH) agar (Becton, Dickinson and Company) for 20 h at 37℃ under aerobic conditions were employed. MICs for *C. beijerinckii* J33TS5 were measured by agar dilution methods according to CLSI, M07-A10, except that MICs were measured on GAMSA after a 24-h incubation at 35℃ under anaerobic conditions. The antimicrobial concentration range employed for the tests was 0.25–256 μg/ml.

DNA extraction for genome sequence analysis

Bacterial cells cultured on agar media under appropriate culture conditions (Table 2) were harvested and suspended in 550 μl of TE (10 mM Tris–HCl [pH 8.0] and 1 mM EDTA [pH 8.0]). The bacterial suspensions were then mixed with 60–80 μl of a mixed solution of two enzymes (100 mg/ml of lysozyme [Sigma-Aldrich] and 500 U/ml of mutanolysin [Sigma-Aldrich]) and incubated for 1h at 37℃. After incubation, bacterial cells were lysed by adding 60–70 μl of 10% sodium dodecyl sulfate, and the lysates were extracted with an equal volume of phenol, phenol-chloroform-isoamyl alcohol (25:24:1) (PCI), and chloroform at least once, three times, and once, respectively. Nucleic acids were then precipitated by ethanol, rinsed with 70% ethanol, and dissolved in sterile H_2O. DNA was further treated with 100 μg/ml of RNase (NIPPON GENE Co., Ltd., Tokyo, Japan) at 37℃ for 2 h, extracted with PCI and chloroform twice and once, respectively, and precipitated by ethanol. Extracted DNA was rinsed with 70% ethanol, dissolved in 10 mM Tris–HCl (pH 8.5), and stored at –20℃ until use.

Whole genome shotgun sequencing

Genome sequencing data were obtained using the Miseq and/or Novaseq 6000 platforms (Illumina, Inc., San Diego, CA, USA). Sequencing by the Miseq platform (300-bp paired-end reads) was performed at the Institute of Crop Science,

National Agriculture and Food Research Organization as recommended by the manufacturer. Genomic libraries for the Miseq sequencing were prepared by the TruSeq Nano DNA low-throughput library preparation kit (Illumina). Sequencing by the Novaseq 6000 platform (151-bp paired-end reads) was performed by Macrogen Japan Corp (Tokyo, Japan). Genomic libraries for the Novaseq 6000 sequencing were prepared by the TruSeq DNA PCR Free kit (Illumina). Adapter sequences and low-quality bases in Illumina paired end reads were removed using Trimmomatic v.0.36 (37) with options of 'ILLUMINACLIP:TruSeq3-PE.fa:3:40:15 LEADING:20 TRAILING:20 MINLEN:20'. Whole genome *de novo* assembly was conducted using SPAdes v.3.13.2 with options of –careful and –cov-cutoff auto (38). Genomes were annotated using Prokka v.1.14.5 (39). The genome sequences were deposited in the DDBJ/GenBank/EMBL database under the DRA accession number DRA011480, BioProject accession number PRJDB11087, and BioSample accession numbers SAMD00277588–SAMD00277637.

Analysis of genome sequencing data

Putative antimicrobial resistance genes in the genome sequences were searched using Resistance Gene Identifier (RGI) in the Comprehensive Antibiotic Resistance Database (CARD, https://card.mcmaster.ca/analyze/rgi) (40). Genes that exhibited > 50% best identity (percent identity of match to top hit in CARD) and percentage length of 60–140% with the reference sequence were selected as antimicrobial resistance genes. ICEs and prophages were searched using ICEberg 2.0 (http://db-mml.sjtu.edu.cn/ICEberg/) (41) and PHAST (42), respectively. Scaffolds including plasmid replicons were selected by PlasmidFinder 2.1 (43), and complete sequences of the plasmids were determined by PCR and primer walking using primers listed in Table 3. The plasmid sequences were deposited in the DDBJ/GenBank/EMBL database under the accession numbers LC586958 (pJ18TS1mac), LC596398 (pJ18TS1tet) and LC597664 (pJ45TS6).

Results

Bacteria isolated from Japanese honey

Two hundreds and nine bacterial strains were isolated from 48 of the 53 Japanese honey samples under 1 μg/ml TS conditions (Table 2). As of January 2024, the 209 isolates were classified into 15 genera by 16S rRNA gene sequencing, and at least 57 species existed in the isolates (Figure 1 and Table 2). *Paenibacillus* (84 isolates, 40.2%) and *Bacillus* (58 isolates, 27.8%) were the two most common genera found in the isolates, followed by *Shouchella* (21 isolates, 10.1%), *Heyndrickxia* (13 isolates, 6.2%), *Lederbergia* (10 isolates, 4.8%), *Cytobacillus* (5 isolates, 2.4%), *Siminovitchia* (4 isolates, 1.9%), *Oceanobacillus* (3 isolates, 1.4%), *Robertmurraya* (3 isolates, 1.4%), *Brevibacillus* (2 isolates, 1.0%), and *Cohnella* (2 isolates, 1.0%) (Figure 1). *Clostridium*, *Niallia*, *Ornithinibacillus*, and *Virgibacillus* were also found, but only in one isolate each (Figure 1). All isolates were Gram-positive spore-forming bacteria. Except for the anaerobic bacterium *Clostridium beijerinckii* J33TS5, all isolates were aerobic or facultatively anaerobic. At the species level, *Bacillus licheniformis*, *Paenibacillus cineris*, *Paenibacillus dendritiformis*, and *Shouchella tritolerans* were frequently isolated, and found in 45.3, 35.8, 26.4, and 22.6% of honey samples, respectively. Under the 1 μg/ml TS conditions, neither *P. larvae* nor *M. plutonius* was isolated from the Japanese honey. *Clostridium botulinum*, cereulide synthetase gene-positive *Bacillus cereus*, and *Bacillus anthracis*, which may cause public health problems, were also not isolated.

Antimicrobial susceptibility tests

Results of the antimicrobial susceptibility tests are shown in Figures 2 and Table 2. MICs of the antimicrobials for the honey-derived isolates were widely distributed, and the MIC range of TS, LCM, and OTC was ≤ 0.25– ≥ 256, 1– ≥ 256, and ≤ 0.25–128 μg/ml, respectively. Although bacteria in honey were screened under 1 μg/ml TS conditions, MICs of TS in some isolates were 1 μg/ml or lower. However, most of the isolates exhibited lower susceptibility to TS than Japanese *P. larvae* strains

(MIC of TS, 0.125–0.25 μg/ml) (30), and 12 isolates had a MIC of 256 μg/ml or higher (Figure 2A and Table 2). Most of the isolates also demonstrated low susceptibility (MIC50, ≥256 μg/ml) to LCM, which is structurally different but functionally similar to macrolides (Figure 2B and Table 2). Moreover, in 122 (58.4%) isolates, MICs of OTC were higher than those for Japanese *P. larvae* strains (MIC, ≤ 1 μg/ml) (30) (Figure 2C and Table 2). This suggests a wide distribution of antimicrobial resistance genes in honey-derived bacteria.

Antimicrobial resistance genes in honey-derived bacteria detected by genome sequence analysis

In order to identify genes conferring antimicrobial resistant phenotypes of the honey-derived isolates, the author selected 50 isolates for genome sequencing (Table 4). The isolates were selected from various species. When there were multiple isolates in one species, 1–3 isolate(s), which demonstrated low susceptibility to TS and/or LCM, were selected as representatives. The sizes of the assembled genome of the isolates ranged from 3.48 to 7.44 Mbp, and there were 3,463– 6,685 predicted coding sequences (Table 4). RGI detected 379 macrolide, lincosamide, and/or tetracycline resistance genes, and 59 met the perfect or strict criteria of the analysis (Table 5). Among the resistance genes, 156, 166, and 207 genes, including 28, 46, and 11 perfect or strict hit genes, were predicted to confer macrolide, lincosamide, and tetracycline resistance, respectively (Table 4 and Table 5). There were from one to seven macrolide resistance genes per strain.

PlasmidFinder and additional sequence analysis identified three plasmids carrying antimicrobial resistance genes. Two of the three plasmids, which carry the macrolide resistance gene *ermC* (pJ18TS1mac) (Table 4 and Figure 3) and tetracycline resistance gene *tet*(L) (pJ18TS1tet) (Figure 3), were found in *Oceanobacillus oncorhynchi* subsp. *incaldanensis* isolate J18TS1. Both plasmids were relatively small in size (less than 4.7 kbp) and possessed the *mob* gene (Figure 3) (accession numbers LC586958 [pJ18TS1mac] and LC596398 [pJ18TS1tet]), suggesting that they are

mobilizable plasmids. Another plasmid pJ45TS6 found in *Paenibacillus* sp. isolate J45TS6 (Table 4) was 51.4 kbp in size and carried the macrolide resistance gene *ermB* and tetracycline resistance gene *tet*(L). Although not detected by RGI, *ermL*, which was predicted to control the expression of *ermB*, was identified in the upstream region of the *ermB* gene by Blast analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table 4). In addition, two putative macrolide resistance genes, *lsaB* and *oleC*, were identified in an ICE region of *Paenibacillus cineris* isolate J43TS9 (Table 4). No intact prophages with antimicrobial resistance genes were identified.

Discussion

In addition to TS, LCM and OTC are used for controlling AFB in other countries (16, 20). Although LCM and OTC are not drugs approved in Japan, the use of these drugs may be considered in the future if TS-resistant *P. larvae* will spread in Japan. Therefore, we also employed LCM and OTC for the tests.

OTC and tetracycline-resistant *P. larvae* strains have been isolated in several countries. The resistant strains were suggested to develop, at least in part, by horizontal gene transfer of tetracycline resistance genes via MGEs (19, 20, 21, 22, 23, 24), and bacteria in honey may be a source of the antimicrobial resistance genes (25). Therefore, elucidation of the resistome in honey is important to predict the future evolution of antimicrobial resistance in foulbrood pathogens. In this study, we isolated bacteria from Japanese honey for the resistome analysis and found macrolide resistance genes on MGEs, which may confer TS resistance if transmitted to *P. larvae*.

In the recent 16S rRNA gene amplicon sequencing for investigating microbiota in honey, *Lactobacillus kunkeei* (*Apilactobacillus kunkeei*), *Parasacharribacter apium* (*Bombella apis*), *Fructobacillus fructosus*, *Gilliamella apicola*, Xanthomonadales and Actinomycetales were detected as major bacterial species/groups at the DNA level (29). Other previous studies reported that aerobic spore-forming bacteria of the genera *Paenibacillus* and *Bacillus* were commonly found in honey (25, 26, 27, 44). In this study, to isolate TS-resistant bacteria in honey, 1 μg/ml TS-containing media were used. Although the use of TS and the antimicrobial characteristics of honey itself may have affected the repertoire of live bacteria recovered by the culture methods, all isolates from the Japanese honey were spore-forming bacteria, and *Paenibacillus* and *Bacillus* were the two major genera found in the isolates. On the other hand, major bacterial species/groups detected by 16S rRNA gene amplicon sequencing (29) were not isolated in this study. Some honey samples used in this study may have been heated after harvesting, and the heating step may also have affected non-spore-forming bacteria in the honey such as *A. kunkeei*, *B. apis*, and *F. fructosus*.

P. larvae and *M. plutonius* can also exist in honey in beehives (14, 45, 46). In this study, however, neither pathogen was isolated from Japanese honey under the 1 μg/ml-TS conditions. This suggests that Japan is still free from TS-resistant foulbrood pathogens. However, we cannot rule out the possibility that we have missed TS-resistant foulbrood pathogens in honey samples. For isolation of foulbrood pathogens from samples with other bacteria, it is necessary to use selective media supplemented with appropriate antimicrobials such as nalidixic acid and pipemidic acid (14, 47). In this study, we did not use such selective media, and this may have caused overgrowth of other honey-derived bacteria, making the recovery of the pathogens difficult. In addition, culture methods are known to not be effective for detecting foulbrood pathogens from honey (14, 48, 49, 50). Therefore, further investigation using selective media and/or molecular techniques is needed to clarify the contamination status of *P. larvae* and *M. plutonius* in Japanese honey.

Although TS-containing media was used, some honey-derived isolates were highly susceptible to TS. In particular, the TS susceptibility of 12 isolates was at a similar level to that of Japanese *P. larvae* strains (i.e., MIC of TS, 0.25 μg/ml). As suggested by previous studies (25, 26, 27, 44), the presence of spore-forming bacteria in honey was expected. Therefore, in order to germinate as many spores as possible, we cultured honey sediment-inoculated agar media for 2–4 days at the first isolation step. During the relatively long cultivation step, the potency of TS may have decreased gradually, and TS-susceptible bacteria may have grown on the media. Alternatively, because of efficient TS inactivation by macrolide-resistant bacteria coexisting in the same honey, the antibiotic may not be able to inhibit the growth of these TS-susceptible bacteria. Indeed, our genome analysis suggested the presence of bacteria in honey, which produce enzymes involved in macrolide inactivation (macrolide phosphotransferases) (Table 5). However, such highly TS-susceptible bacteria were the minority among the honey-derived isolates retrieved in this study. MICs of TS for the honey-derived isolates varied (≤ 0.25– ≥ 256 μg/ml), and the majority of the isolates exhibited lower TS susceptibility than Japanese *P. larvae* strains (30). Although breakpoints for antimicrobials tested have not yet been established for the

bacterial species identified in this study, some isolates were highly resistant to TS (Figure 2 and Table 2). These suggested the presence of multiple macrolide resistance mechanisms in honey-derived bacteria. Indeed, a variety of putative macrolide resistance genes involved in macrolide efflux, inactivation, target alteration, and target protection were found in the representative isolates analyzed by genome sequencing (Table 5).

In addition to macrolide resistance genes, a variety of lincosamide and tetracycline resistance genes were also detected in the genomes of the representative isolates by RGI (Table 5). Consistent with this, many isolates from honey exhibited low susceptibility to LCM and OTC. In particular, 60.8% of the isolates were highly resistant to LCM (MICs, ≥ 256 mg/ml). Although the details were not mentioned in this study, RGI also detected many putative resistance genes for other drug classes, including aminoglycoside, phenicol, fluoroquinolone, glycopeptide and β-lactam antibiotics, in the genome of the honey-derived isolates. This suggests the presence of many (and probably multidrug) resistant bacteria in honey and support the previous hypothesis that bacteria in honey are a reservoir of resistance genes (25).

Summary

A macrolide antibiotic, TS, is the only approved prophylactic against AFB in Japan. Although TS-resistant *P. larvae* has not so far been isolated in Japan, we will lose the important countermeasure for AFB if TS-resistant *P. larvae* widely spread in Japan. Therefore, in order to use the only prophylactic effectively for a long time, it is important to assess the risk of the emergence of TS-resistant *P. larvae* and seek more appropriate use of the prophylactic. Honey, a brood food for worker larvae, is known to be contaminated with various bacteria. If bacteria possessing macrolide resistance genes exist in honey, *P. larvae* and such bacteria may encounter in the larval gut, and *P. larvae* may acquire TS resistance genes from the honey-derived bacteria. Therefore, in Chapter I, to investigate the hypothesis that honey is a reservoir of macrolide-resistant bacteria that may confer TS resistance to *P. larvae*, the author searched 53 commercially available Japanese honey samples for TS-resistant bacteria and macrolide resistance genes on MGEs. In total, 209 bacterial isolates were obtained from the Japanese honey samples under 1 μg/ml TS conditions, and all of them were Gram-positive and spore-forming bacteria. Genome sequencing analysis of 50 representative isolates revealed that all of them possessed putative macrolide resistance genes, and four of the resistance genes (*ermC*, *ermB*, *lsaB*, and *oleC*) were located on MGEs (i.e., plasmids and an ICE), which may be transmitted to *P. larvae*. These results suggested that bacteria latent in honey are a reservoir for macrolide resistance genes and that they may cause the emergence of TS-resistant *P. larvae* via transfer of macrolide resistance genes.

Table 1. Japanese honey used for isolation of tylosin-resistant bacteria

Lot number	Species of honey bee	Floral origins	Isolation of bacteria under 1 μg/ml-tylosin conditions (Number of isolates used for further analyses)[a]
J1	*Apis mellifera*	Multiflower	+ (5)
J2	*Apis mellifera*	Multiflower	+ (6)
J3	*Apis mellifera*	Multiflower	+ (5)
J4	*Apis mellifera*	Acacia	+ (3)
J5	*Apis mellifera*	Multiflower	+ (3)
J6	*Apis mellifera*	Japanese horse chestnut	+ (7)
J7	*Apis mellifera*	Acacia	+ (5)
J8	*Apis mellifera*	Apple	+ (4)
J9	*Apis mellifera*	Multiflower	−
J10	*Apis mellifera*	Japanese wax tree	+ (2)
J11	*Apis mellifera*	Mandarine orange	+ (2)
J12	*Apis cerana japonica*	Multiflower	+ (1)
J13	*Apis mellifera*	Multiflower	+ (4)
J14	*Apis mellifera*	Lotus	+ (5)
J15	*Apis cerana japonica*	Multiflower	+ (8)
J16	*Apis mellifera*	Loquat	−
J17	*Apis mellifera*	Multiflower	+ (2)
J18	*Apis mellifera*	Japanese pagoda tree	+ (2)
J19	*Apis mellifera*	Acacia	+ (4)
J20	*Apis mellifera*	Japanese wax tree	+ (1)
J21	*Apis mellifera*	Cherry blossoms	+ (6)
J22	*Apis mellifera*	Japanese raisin tree	+ (4)
J23	*Apis mellifera*	Acacia	+ (6)
J24	*Apis cerana japonica*	Multiflower	+ (1)
J25	*Apis mellifera*	Multiflower	+ (4)
J26	*Apis mellifera*	Multiflower	+ (4)
J27	*Apis mellifera*	Japanese linden	+ (6)
J28	*Apis mellifera*	Lotus	+ (7)
J29	*Apis mellifera*	Lotus	−
J30	*Apis mellifera*	Multiflower	+ (3)
J31	*Apis mellifera*	Multiflower	+ (7)

Table 1. (Continued)

Lot number	Species of honey bee	Floral origins	Isolation of bacteria under 1 μg/ml-tylosin conditions (Number of isolates used for further analyses)[a]
J32	*Apis mellifera*	Acacia	+ (6)
J33	*Apis mellifera*	Acacia	+ (4)
J34	*Apis mellifera*	Multiflower	+ (1)
J35	*Apis mellifera*	Multiflower	+ (4)
J36	*Apis mellifera*	Multiflower	+ (3)
J37	*Apis mellifera*	Japanese horse chestnut	–
J38	*Apis mellifera*	Burr cucumber	+ (5)
J39	*Apis mellifera*	Sunflower	+ (5)
J40	*Apis mellifera*	Acacia	+ (2)
J41	*Apis mellifera*	Multiflower	+ (9)
J42	*Apis cerana japonica*	Multiflower	+ (1)
J43	*Apis mellifera*	Multiflower	+ (6)
J44	*Apis mellifera*	Acacia	+ (4)
J45	*Apis mellifera*	Multiflower	+ (6)
J46	*Apis mellifera*	Multiflower	+ (7)
J47	*Apis mellifera*	Apple	+ (9)
J48	*Apis mellifera*	Japanese horse chestnut	+ (3)
J49	*Apis mellifera*	Multiflower	+ (8)
J50	*Apis mellifera*	Lotus	+ (4)
J51	*Apis mellifera*	Japanese horse chestnut	+ (3)
J52	*Apis mellifera*	Multiflower	–
J53	*Apis mellifera*	Multiflower	+ (2)

[a] +, isolation positive; -, isolation negative.

Table 2. Bacterial isolates from Japanese honey and their antimicrobial susceptibility

Isolate[a]	Species[b]	Accession number of 16S rRNA gene sequence	EzBioCloud results[c]		Culture conditions[d]	MIC (μg/ml)[e]		
			Species of the closest type strain(s)	16S rRNA gene sequence similarity (%)		TS	LCM	OTC
J1TS1	*Shouchella tritolerans*	LC588427	*Shouchella tritolerans*	**100**	CSA, aerobically, 24h	64	>256	4
J1TS2	*Bacillus licheniformis*	LC588428	*Bacillus licheniformis*	**99.86**	CSA, aerobically, 24h	1	>256	≤0.25
J1TS3	*Siminovitchia fordii*	LC588429	*Siminovitchia fordii*	**99.66**	CSA, aerobically, 24h	8	128	8
J1TS5	*Paenibacillus macerans*	LC588430	*Paenibacillus macerans*	**99.72**	CSA, aerobically, 24h	16	>256	4
J1TS6	*Paenibacillus dendritiformis*	LC588431	*Paenibacillus dendritiformis*	**99.79**	CSA, aerobically, 24h	≤0.25	2	0.5
J2TS1	*Heyndrickxia sporothermodurans*	LC588432	*Heyndrickxia sporothermodurans*	**98.7**	CSA, aerobically, 24h	128	>256	4
J2TS2[f]	*Bacillus* sp.	LC588433	*Bacillus norwichensis*	**100**	CSA, aerobically, 24h	2	64	≤0.25
J2TS3	*Siminovitchia terrae*	LC588434	*Siminovitchia terrae*	**100**	CSA, aerobically, 24h	8	128	8
J2TS4[g]	*Paenibacillus* sp.	LC588435	*Paenibacillus residui*	95.82	CSA, aerobically, 24h	8	>256	8
J2TS5	*Bacillus licheniformis*	LC588436	*Bacillus licheniformis*	**99.93**	CSA, aerobically, 24h	2	>256	0.5
J2TS6[f]	*Paenibacillus* sp.	LC588437	*Paenibacillus albilobatus*	**99.64**	CSA, aerobically, 24h	4	256	32
J3TS1	*Cytobacillus kochii*	LC588438	*Cytobacillus kochii*	**100**	CSA, aerobically, 24h	2	32	≤0.25
J3TS2	*Paenibacillus cineris*	LC588439	*Paenibacillus cineris*	**100**	CSA, aerobically, 24h	32	256	64
J3TS3	*Shouchella tritolerans*	LC588440	*Shouchella tritolerans*	**100**	CSA, aerobically, 24h	64	256	4
J3TS4[g]	*Cohnella* sp.	LC588441	*Cohnella lubricantis*	97.98	CSA, 5% CO_2, 24h	2	32	2
J3TS5	*Paenibacillus dendritiformis*	LC588442	*Paenibacillus dendritiformis*	**99.79**	CSA, aerobically, 24h	≤0.25	2	1
J4TS1	*Bacillus licheniformis*	LC588443	*Bacillus licheniformis*	**99.93**	CSA, aerobically, 24h	2	>256	0.5

Table 2. (Continued)

Isolate[a]	Species[b]	Accession number of 16S rRNA gene sequence	EzBioCloud results[c]		Culture conditions[d]	MIC (μg/ml)[e]		
			Species of the closest type strain(s)	16S rRNA gene sequence similarity (%)		TS	LCM	OTC
J4TS2	*Lederbergia ruris*	LC588444	*Lederbergia ruris*	**100**	CSA, aerobically, 24h	8	32	0.5
J4TS5	*Paenibacillus dendritiformis*	LC588445	*Paenibacillus dendritiformis*	**99.79**	CSA, aerobically, 24h	≤0.25	1	0.5
J5TS1	*Bacillus licheniformis*	LC588446	*Bacillus licheniformis*	**99.93**	CSA, aerobically, 24h	2	>256	0.5
J5TS2	*Brevibacillus halotolerans*	LC588447	*Brevibacillus halotolerans*	**99.44**	CSA, aerobically, 24h	8	32	8
J5TS4	*Bacillus tequilensis*	LC588448	*Bacillus tequilensis*	**100**	CSA, aerobically, 24h	1	128	≤0.25
J6TS1	*Siminovitchia terrae*	LC588449	*Siminovitchia terrae*	**99.93**	CSA, aerobically, 24h	>256	128	8
J6TS2	*Heyndrickxia sporothermodurans*	LC588450	*Heyndrickxia sporothermodurans*	**98.77**	CSA, aerobically, 24h	256	>256	8
J6TS3	*Bacillus licheniformis*	LC588451	*Bacillus licheniformis*	**99.93**	CSA, aerobically, 24h	1	256	≤0.25
J6TS4	*Heyndrickxia oleronia*	LC588452	*Heyndrickxia oleronia*	**99.73**	CSA, aerobically, 24h	8	256	8
J6TS5[f]	*Bacillus* sp.	LC588453	*Bacillus norwichensis*	**100**	CSA, aerobically, 24h	2	64	≤0.25
J6TS6[g]	*Paenibacillus* sp.	LC588454	*Paenibacillus telluris*	98.05	CSA, aerobically, 24h	4	>256	16
J6TS7	*Paenibacillus dendritiformis*	LC588455	*Paenibacillus dendritiformis*	**99.79**	CSA, aerobically, 24h	0.5	32	1
J7TS1	*Lederbergia galactosidilytica*	LC588456	*Lederbergia galactosidilytica*	**100**	CSA, aerobically, 24h	4	16	1
J7TS2	*Paenibacillus cineris*	LC588457	*Paenibacillus cineris*	**100**	CSA, aerobically, 24h	4	256	1
J7TS3	*Bacillus paralicheniformis*	LC588458	*Bacillus paralicheniformis*	**100**	CSA, aerobically, 24h	2	>256	8
J7TS4[h]	*Bacillus* sp.	LC588459	*Bacillus cereus* *Bacillus paranthracis* *Bacillus nitratireducens*	**100**	GAMSA, 5% CO_2, 24h	2	8	≤0.25
J7TS5	*Paenibacillus dendritiformis*	LC588460	*Paenibacillus dendritiformis*	**99.79**	CSA, aerobically, 24h	≤0.25	8	0.5

Table 2. (Continued)

Isolate[a]	Species[b]	Accession number of 16S rRNA gene sequence	EzBioCloud results[c]		Culture conditions[d]	MIC (μg/ml)[e]		
			Species of the closest type strain(s)	16S rRNA gene sequence similarity (%)		TS	LCM	OTC
J8TS1	*Bacillus licheniformis*	LC588461	*Bacillus licheniformis*	**99.86**	CSA, aerobically, 24h	2	>256	≤0.25
J8TS2	*Lederbergia ruris*	LC588462	*Lederbergia ruris*	**100**	CSA, aerobically, 24h	>256	>256	≤0.25
J8TS3	*Paenibacillus dendritiformis*	LC588463	*Paenibacillus dendritiformis*	**99.79**	CSA, aerobically, 24h	≤0.25	8	0.5
J8TS7	*Paenibacillus timonensis*	LC588464	*Paenibacillus timonensis*	**99.46**	CSA, aerobically, 24h	2	2	4
J10TS1	*Shouchella tritolerans*	LC588465	*Shouchella tritolerans*	**100**	CSA, aerobically, 24h	>256	>256	2
J10TS2	*Paenibacillus dendritiformis*	LC588466	*Paenibacillus dendritiformis*	**99.8**	CSA, aerobically, 24h	≤0.25	16	0.5
J11TS1[g]	*Oceanobacillus* sp.	LC588467	*Oceanobacillus oncorhynchi* subsp. *incaldanensis*	98.45	CSA, aerobically, 24h	4	16	0.5
J11TS3	*Paenibacillus dendritiformis*	LC588468	*Paenibacillus dendritiformis*	**99.73**	CSA, aerobically, 24h	≤0.25	8	0.5
J12TS1[g]	*Lederbergia* sp.	LC588469	*Lederbergia galactosidilytica*	98.57	CSA, aerobically, 24h	8	16	2
J13TS1	*Bacillus licheniformis*	LC588470	*Bacillus licheniformis*	**99.86**	CSA, aerobically, 24h	1	>256	≤0.25
J13TS2	*Bacillus licheniformis*	LC588471	*Bacillus licheniformis*	**99.93**	CSA, aerobically, 24h	4	>256	0.5
J13TS4	*Bacillus paralicheniformis*	LC588472	*Bacillus paralicheniformis*	**100**	CSA, aerobically, 24h	2	8	16
J13TS5	*Paenibacillus dendritiformis*	LC588473	*Paenibacillus dendritiformis*	**99.73**	CSA, aerobically, 24h	≤0.25	8	1
J14TS2[g]	*Lederbergia* sp.	LC588474	*Lederbergia galactosidilytica*	98.63	CSA, aerobically, 24h	8	16	2
J14TS3	*Bacillus licheniformis*	LC588475	*Bacillus licheniformis*	**100**	CSA, aerobically, 24h	4	>256	1
J14TS4[h]	*Bacillus* sp.	LC588476	*Bacillus paranthracis* *Bacillus nitratireducens* *Bacillus anthracis*	**99.86**	CSA, aerobically, 24h	2	16	1
J14TS5	*Paenibacillus lautus*	LC588477	*Paenibacillus lautus*	**99.73**	CSA, aerobically, 24h	8	256	16

Table 2. (Continued)

Isolate[a]	Species[b]	Accession number of 16S rRNA gene sequence	EzBioCloud results[c]		Culture conditions[d]	MIC (μg/ml)[e]		
			Species of the closest type strain(s)	16S rRNA gene sequence similarity (%)		TS	LCM	OTC
J14TS10	*Bacillus licheniformis*	LC588478	*Bacillus licheniformis*	**100**	CSA, aerobically, 24h	2	>256	0.5
J15TS1	*Bacillus licheniformis*	LC588479	*Bacillus licheniformis*	**100**	CSA, aerobically, 24h	1	128	≤0.25
J15TS2	*Heyndrickxia oleronia*	LC588480	*Heyndrickxia oleronia*	**99.59**	CSA, aerobically, 24h	16	>256	8
J15TS3	*Paenibacillus cineris*	LC588481	*Paenibacillus cineris*	**100**	CSA, aerobically, 24h	8	256	64
J15TS4	*Bacillus paralicheniformis*	LC588482	*Bacillus paralicheniformis*	**100**	CSA, aerobically, 24h	1	>256	8
J15TS5	*Bacillus licheniformis*	LC588483	*Bacillus licheniformis*	**99.86**	CSA, aerobically, 24h	1	256	≤0.25
J15TS6	*Heyndrickxia oleronia*	LC588484	*Heyndrickxia oleronia*	**99.66**	CSA, aerobically, 24h	8	32	8
J15TS7	*Paenibacillus lactis*	LC588485	*Paenibacillus lactis*	**99.86**	CSA, aerobically, 24h	16	256	16
J15TS10	*Paenibacillus woosongensis*	LC588486	*Paenibacillus woosongensis*	**99.86**	CSA, aerobically, 24h	8	128	≤0.25
J17TS1	*Heyndrickxia oleronia*	LC588487	*Heyndrickxia oleronia*	**99.59**	CSA, aerobically, 24h	16	128	8
J17TS2	*Paenibacillus cineris*	LC588488	*Paenibacillus cineris*	**100**	CSA, aerobically, 24h	16	>256	64
J18TS1	*Oceanobacillus oncorhynchi* subsp. *incaldanensis*	LC588489	*Oceanobacillus oncorhynchi* subsp. *incaldanensis*	**100**	CSA, aerobically, 24h	>256	>256	64
J18TS3	*Paenibacillus cineris*	LC588490	*Paenibacillus cineris*	**100**	CSA, aerobically, 24h	16	256	32
J19TS1	*Heyndrickxia oleronia*	LC588491	*Heyndrickxia oleronia*	**99.59**	CSA, aerobically, 24h	16	128	8
J19TS2	*Cohnella xylanilytica*	LC588492	*Cohnella xylanilytica*	**99.93**	CSA, 5% CO_2, 24h	8	>256	1
J19TS3	*Heyndrickxia oleronia*	LC588493	*Heyndrickxia oleronia*	**99.64**	CSA, aerobically, 24h	16	128	32
J19TS4	*Paenibacillus cineris*	LC588494	*Paenibacillus cineris*	**100**	CSA, aerobically, 24h	8	256	32

Table 2. (Continued)

Isolate[a]	Species[b]	Accession number of 16S rRNA gene sequence	EzBioCloud results[c]		Culture conditions[d]	MIC (μg/ml)[e]		
			Species of the closest type strain(s)	16S rRNA gene sequence similarity (%)		TS	LCM	OTC
J20TS1	*Paenibacillus cineris*	LC588495	*Paenibacillus cineris*	**100**	CSA, aerobically, 24h	16	>256	64
J21TS1	*Bacillus licheniformis*	LC588496	*Bacillus licheniformis*	**99.93**	CSA, aerobically, 24h	1	>256	≤0.25
J21TS2	*Heyndrickxia sporothermodurans*	LC588497	*Heyndrickxia sporothermodurans*	**98.7**	CSA, aerobically, 24h	8	>256	4
J21TS3	*Paenibacillus cookii*	LC588498	*Paenibacillus cookii*	**99.93**	CSA, aerobically, 24h	64	>256	8
J21TS5	*Bacillus licheniformis*	LC588499	*Bacillus licheniformis*	**99.93**	CSA, aerobically, 24h	1	128	≤0.25
J21TS7	*Paenibacillus cineris*	LC588500	*Paenibacillus cineris*	**100**	CSA, aerobically, 24h	32	>256	32
J21TS8	*Paenibacillus vini*	LC588501	*Paenibacillus vini*	**99.23**	CSA, aerobically, 24h	4	64	0.5
J22TS1	*Siminovitchia terrae*	LC588502	*Siminovitchia terrae*	**100**	CSA, aerobically, 24h	8	64	16
J22TS2[g]	*Paenibacillus* sp.	LC588503	*Paenibacillus residui*	95.89	CSA, aerobically, 24h	4	>256	8
J22TS3[g]	*Paenibacillus* sp.	LC588504	*Paenibacillus telluris*	98.04	CSA, aerobically, 24h	8	>256	32
J22TS4	*Paenibacillus dendritiformis*	LC588505	*Paenibacillus dendritiformis*	**99.73**	CSA, aerobically, 24h	≤0.25	4	1
J23TS1	*Bacillus licheniformis*	LC588506	*Bacillus licheniformis*	**99.93**	CSA, aerobically, 24h	2	>256	≤0.25
J23TS2	*Shouchella tritolerans*	LC588507	*Shouchella tritolerans*	**100**	CSA, aerobically, 24h	64	>256	4
J23TS3	*Oceanobacillus sojae*	LC588508	*Oceanobacillus sojae*	**100**	CSA, aerobically, 24h	1	128	16
J23TS4	*Paenibacillus cookii*	LC588509	*Paenibacillus cookii*	**100**	CSA, aerobically, 24h	16	256	4
J23TS8	*Bacillus paralicheniformis*	LC588510	*Bacillus paralicheniformis*	**100**	CSA, aerobically, 24h	4	>256	16
J23TS9	*Paenibacillus dokdonensis*	LC588511	*Paenibacillus dokdonensis*	**99.58**	CSA, aerobically, 24h	4	128	4

Table 2. (Continued)

Isolate[a]	Species[b]	Accession number of 16S rRNA gene sequence	EzBioCloud results[c]		Culture conditions[d]	MIC (μg/ml)[e]		
			Species of the closest type strain(s)	16S rRNA gene sequence similarity (%)		TS	LCM	OTC
J24TS1	*Paenibacillus lactis*	LC588512	*Paenibacillus lactis*	**100**	CSA, aerobically, 24h	16	256	4
J25TS1	*Bacillus paralicheniformis*	LC588513	*Bacillus paralicheniformis*	**100**	CSA, aerobically, 24h	2	>256	16
J25TS3	*Lederbergia galactosidilytica*	LC588514	*Lederbergia galactosidilytica*	**100**	CSA, aerobically, 24h	2	32	32
J25TS5	*Paenibacillus faecis*	LC588515	*Paenibacillus faecis*	**99.86**	CSA, aerobically, 24h	16	64	8
J25TS6	*Paenibacillus cineris*	LC588516	*Paenibacillus cineris*	**100**	CSA, aerobically, 24h	16	256	64
J26TS1	*Bacillus licheniformis*	LC588517	*Bacillus licheniformis*	**99.93**	CSA, aerobically, 24h	2	>256	≤0.25
J26TS2	*Shouchella tritolerans*	LC588518	*Shouchella tritolerans*	**100**	CSA, aerobically, 24h	128	>256	8
J26TS3	*Bacillus licheniformis*	LC588519	*Bacillus licheniformis*	**99.93**	CSA, aerobically, 24h	1	256	≤0.25
J26TS4	*Bacillus licheniformis*	LC588520	*Bacillus licheniformis*	**99.86**	CSA, aerobically, 24h	1	>256	0.5
J27TS1	*Bacillus paralicheniformis*	LC588521	*Bacillus paralicheniformis*	**100**	CSA, aerobically, 24h	2	>256	16
J27TS2	*Cytobacillus kochii*	LC588522	*Cytobacillus kochii*	**100**	CSA, aerobically, 24h	2	32	≤0.25
J27TS4[h]	*Shouchella* sp.	LC588523	*Shouchella clausii* *Shouchella tritolerans*	**99.93**	CSA, aerobically, 24h	>256	>256	4
J27TS5	*Bacillus licheniformis*	LC588524	*Bacillus licheniformis*	**100**	CSA, aerobically, 24h	2	256	0.5
J27TS7	*Paenibacillus dendritiformis*	LC588525	*Paenibacillus dendritiformis*	**99.66**	CSA, aerobically, 24h	4	64	64
J27TS8	*Robertmurraya siralis*	LC588526	*Robertmurraya siralis*	**99.93**	CSA, aerobically, 24h	32	>256	≤0.25
J28TS1[h]	*Bacillus* sp.	LC588527	*Bacillus paralicheniformis* *Bacillus haynesii*	**100**	CSA, aerobically, 24h	1	>256	16
J28TS2	*Shouchella tritolerans*	LC588528	*Shouchella tritolerans*	**99.93**	CSA, aerobically, 24h	>256	256	4

Table 2. (Continued)

Isolate[a]	Species[b]	Accession number of 16S rRNA gene sequence	EzBioCloud results[c]		Culture conditions[d]	MIC (μg/ml)[e]		
			Species of the closest type strain(s)	16S rRNA gene sequence similarity (%)		TS	LCM	OTC
J28TS3	*Shouchella tritolerans*	LC588529	*Shouchella tritolerans*	**99.93**	CSA, aerobically, 24h	256	128	4
J28TS4	*Paenibacillus lautus*	LC588530	*Paenibacillus lautus*	**99.59**	CSA, aerobically, 24h	16	256	4
J28TS5	*Bacillus licheniformis*	LC588531	*Bacillus licheniformis*	**99.8**	CSA, aerobically, 24h	2	>256	≤0.25
J28TS7	*Bacillus paralicheniformis*	LC588532	*Bacillus paralicheniformis*	**100**	CSA, aerobically, 24h	2	>256	16
J28TS8	*Robertmurraya siralis*	LC588533	*Robertmurraya siralis*	**99.93**	CSA, aerobically, 24h	16	>256	≤0.25
J30TS1	*Shouchella tritolerans*	LC588534	*Shouchella tritolerans*	**100**	CSA, aerobically, 24h	64	>256	4
J30TS2[h]	*Bacillus* sp.	LC588535	*Bacillus albus* *Bacillus luti* *Bacillus sanguinis* *Bacillus basilensis*	**100**	CSA, aerobically, 24h	2	16	16
J30TS3[f]	*Niallia* sp.	LC588536	*Niallia alba*	**99.84**	CSA, aerobically, 24h	1	128	≤0.25
J31TS1	*Bacillus paralicheniformis*	LC588537	*Bacillus paralicheniformis*	**100**	CSA, aerobically, 24h	2	>256	16
J31TS2	*Bacillus licheniformis*	LC588538	*Bacillus licheniformis*	**100**	CSA, aerobically, 24h	4	>256	0.5
J31TS3	*Paenibacillus lactis*	LC588539	*Paenibacillus lactis*	**100**	CSA, aerobically, 24h	32	256	1
J31TS4[g]	*Paenibacillus* sp.	LC588540	*Paenibacillus chitinolyticus*	95.24	CSA, aerobically, 24h	8	256	≤0.25
J31TS5	*Shouchella tritolerans*	LC588541	*Shouchella tritolerans*	**99.93**	CSA, aerobically, 24h	128	>256	1
J31TS6	*Brevibacillus reuszeri*	LC588542	*Brevibacillus reuszeri*	**100**	CSA, aerobically, 24h	8	>256	8
J31TS7	*Paenibacillus lautus*	LC588543	*Paenibacillus lautus*	**99.52**	CSA, aerobically, 24h	16	128	16
J32TS1	*Bacillus paralicheniformis*	LC588544	*Bacillus paralicheniformis*	**100**	CSA, aerobically, 24h	2	>256	8

Table 2. (Continued)

Isolate[a]	Species[b]	Accession number of 16S rRNA gene sequence	EzBioCloud results[c]		Culture conditions[d]	MIC (μg/ml)[e]		
			Species of the closest type strain(s)	16S rRNA gene sequence similarity (%)		TS	LCM	OTC
J32TS2	*Shouchella tritolerans*	LC588545	*Shouchella tritolerans*	**99.56**	CSA, aerobically, 24h	>256	>256	4
J32TS3	*Bacillus licheniformis*	LC588546	*Bacillus licheniformis*	**99.93**	CSA, aerobically, 24h	2	>256	0.5
J32TS5[h]	*Bacillus* sp.	LC588547	*Bacillus paranthracis* *Bacillus nitratireducens*	**100**	CSA, aerobically, 24h	1	16	≤0.25
J32TS6	*Virgibacillus pantothenticus*	LC588548	*Virgibacillus pantothenticus*	**100**	CSA, aerobically, 24h	256	>256	1
J32TS7	*Paenibacillus cineris*	LC588549	*Paenibacillus cineris*	**100**	CSA, aerobically, 24h	32	>256	64
J33TS2	*Lederbergia ruris*	LC588550	*Lederbergia ruris*	**100**	CSA, aerobically, 24h	8	32	0.5
J33TS3	*Paenibacillus cineris*	LC588551	*Paenibacillus cineris*	**100**	CSA, aerobically, 24h	32	>256	128
J33TS4	*Paenibacillus timonensis*	LC588552	*Paenibacillus timonensis*	**99.86**	CSA, aerobically, 24h	4	>256	2
J33TS5	*Clostridium beijerinckii*	LC588553	*Clostridium beijerinckii*	**99.86**	GAMSA, anaerobically, 24h	0.5	8	2
J34TS1	*Paenibacillus azoreducens*	LC588554	*Paenibacillus azoreducens*	**100**	CSA, aerobically, 24h	2	32	64
J35TS1	*Robertmurraya siralis*	LC588555	*Robertmurraya siralis*	**99.93**	CSA, aerobically, 24h	8	>256	≤0.25
J35TS2[h]	*Shouchella* sp.	LC588556	*Shouchella clausii* *Shouchella tritolerans*	**99.93**	CSA, aerobically, 24h	>256	256	8
J35TS3	*Bacillus licheniformis*	LC588557	*Bacillus licheniformis*	**99.86**	CSA, aerobically, 24h	1	256	≤0.25
J35TS4	*Paenibacillus vini*	LC588558	*Paenibacillus vini*	**99.68**	CSA, aerobically, 24h	8	64	1
J36TS1	*Paenibacillus cineris*	LC588559	*Paenibacillus cineris*	**100**	CSA, aerobically, 24h	16	256	64
J36TS2	*Bacillus paralicheniformis*	LC588560	*Bacillus paralicheniformis*	**100**	CSA, aerobically, 24h	2	256	8
J36TS3	*Bacillus licheniformis*	LC588561	*Bacillus licheniformis*	**99.86**	CSA, aerobically, 24h	2	256	≤0.25

Table 2. (Continued)

Isolate[a]	Species[b]	Accession number of 16S rRNA gene sequence	EzBioCloud results[c]		Culture conditions[d]	MIC (μg/ml)[e]		
			Species of the closest type strain(s)	16S rRNA gene sequence similarity (%)		TS	LCM	OTC
J38TS1	*Paenibacillus cineris*	LC588562	*Paenibacillus cineris*	**99.8**	CSA, aerobically, 24h	32	256	64
J38TS2	*Cytobacillus kochii*	LC588563	*Cytobacillus kochii*	**100**	CSA, aerobically, 24h	2	32	≤0.25
J38TS3	*Bacillus glycinifermentans*	LC588564	*Bacillus glycinifermentans*	**100**	CSA, aerobically, 24h	2	256	16
J38TS4	*Paenibacillus cineris*	LC588565	*Paenibacillus cineris*	**100**	CSA, aerobically, 24h	16	>256	128
J38TS5	*Shouchella tritolerans*	LC588566	*Shouchella tritolerans*	**100**	CSA, aerobically, 24h	32	256	4
J39TS1	*Paenibacillus cineris*	LC588567	*Paenibacillus cineris*	**100**	CSA, aerobically, 24h	16	>256	32
J39TS2	*Paenibacillus cookii*	LC588568	*Paenibacillus cookii*	**99.93**	CSA, aerobically, 24h	8	64	32
J39TS3	*Paenibacillus ginsengihumi*	LC588569	*Paenibacillus ginsengihumi*	**100**	CSA, aerobically, 24h	1	8	≤0.25
J39TS6	*Bacillus licheniformis*	LC588570	*Bacillus licheniformis*	**99.93**	CSA, aerobically, 24h	2	256	≤0.25
J39TS10	*Paenibacillus lautus*	LC588571	*Paenibacillus lautus*	**99.46**	CSA, aerobically, 24h	8	128	4
J40TS1	*Paenibacillus montaniterrae*	LC588572	*Paenibacillus montaniterrae*	**99.78**	CSA, aerobically, 24h	4	>256	8
J40TS2	*Shouchella clausii*	LC588573	*Shouchella clausii*	**99.86**	CSA, aerobically, 24h	>256	256	4
J41TS2	*Bacillus sonorensis*	LC588574	*Bacillus sonorensis*	**99.93**	CSA, aerobically, 24h	2	128	8
J41TS3	*Paenibacillus faecis*	LC588575	*Paenibacillus faecis*	**99.79**	CSA, aerobically, 24h	16	256	8
J41TS4	*Paenibacillus apis*	LC588576	*Paenibacillus apis*	**99.86**	CSA, aerobically, 24h	4	64	1
J41TS5[h]	*Shouchella* sp.	LC588577	*Shouchella clausii* *Shouchella tritolerans*	**99.93**	CSA, aerobically, 24h	64	>256	4
J41TS6[h]	*Shouchella* sp.	LC588578	*Shouchella clausii* *Shouchella tritolerans*	**99.93**	CSA, aerobically, 24h	64	>256	0.5

Table 2. (Continued)

Isolate[a]	Species[b]	Accession number of 16S rRNA gene sequence	EzBioCloud results[c]		Culture conditions[d]	MIC (μg/ml)[e]		
			Species of the closest type strain(s)	16S rRNA gene sequence similarity (%)		TS	LCM	OTC
J41TS7	*Bacillus licheniformis*	LC588579	*Bacillus licheniformis*	**99.8**	CSA, aerobically, 24h	1	>256	0.5
J41TS8[h]	*Bacillus* sp.	LC588580	*Bacillus paralicheniformis* *Bacillus haynesii*	**100**	CSA, aerobically, 24h	2	>256	8
J41TS12	*Paenibacillus antibioticophila*	LC588581	*Paenibacillus antibioticophila*	**99.79**	CSA, aerobically, 24h	8	64	0.5
J41TS13	*Paenibacillus lactis*	LC588582	*Paenibacillus lactis*	**100**	CSA, aerobically, 24h	32	>256	8
J42TS3	*Paenibacillus vini*	LC588583	*Paenibacillus vini*	**99.44**	CSA, aerobically, 24h	32	32	1
J43TS1[h]	*Bacillus* sp.	LC588584	*Bacillus licheniformis* *Bacillus haynesii*	**99.52**	CSA, aerobically, 24h	2	256	0.5
J43TS2	*Paenibacillus cookii*	LC588585	*Paenibacillus cookii*	**100**	CSA, aerobically, 24h	32	128	16
J43TS3	*Ornithinibacillus bavariensis*	LC588586	*Ornithinibacillus bavariensis*	**99.86**	CSA, aerobically, 24h	1	128	1
J43TS4	*Paenibacillus lautus*	LC588587	*Paenibacillus lautus*	**99.8**	CSA, aerobically, 24h	8	128	32
J43TS5	*Paenibacillus yonginensis*	LC588588	*Paenibacillus yonginensis*	**99.69**	CSA, aerobically, 24h	1	32	2
J43TS9	*Paenibacillus cineris*	LC588589	*Paenibacillus cineris*	**100**	CSA, aerobically, 24h	64	>256	64
J44TS2	*Paenibacillus albilobatus*	LC588590	*Paenibacillus albilobatus*	**99.52**	CSA, aerobically, 24h	8	>256	32
J44TS3	*Paenibacillus macerans*	LC588591	*Paenibacillus macerans*	**99.38**	CSA, aerobically, 24h	16	128	16
J44TS4	*Paenibacillus dendritiformis*	LC588592	*Paenibacillus dendritiformis*	**99.73**	CSA, aerobically, 24h	≤0.25	8	0.5
J44TS5	*Bacillus licheniformis*	LC588593	*Bacillus licheniformis*	**99.86**	CSA, aerobically, 24h	2	>256	≤0.25
J45TS1	*Paenibacillus lautus*	LC588594	*Paenibacillus lautus*	**99.38**	CSA, aerobically, 24h	8	128	16
J45TS2	*Paenibacillus lactis*	LC588595	*Paenibacillus lactis*	**100**	CSA, aerobically, 24h	32	256	8

Table 2. (Continued)

Isolate[a]	Species[b]	Accession number of 16S rRNA gene sequence	EzBioCloud results[c]		Culture conditions[d]	MIC (μg/ml)[e]		
			Species of the closest type strain(s)	16S rRNA gene sequence similarity (%)		TS	LCM	OTC
J45TS3	*Paenibacillus cineris*	LC588596	*Paenibacillus cineris*	**99.93**	CSA, aerobically, 24h	4	>256	32
J45TS4	*Lederbergia galactosidilytica*	LC588597	*Lederbergia galactosidilytica*	**98.7**	CSA, aerobically, 24h	4	128	2
J45TS5[g]	*Lederbergia* sp.	LC588598	*Lederbergia galactosidilytica*	98.57	CSA, aerobically, 24h	4	8	1
J45TS6[f]	*Paenibacillus* sp.	LC588599	*Paenibacillus gallinarum*	**99.66**	CSA, aerobically, 24h	32	>256	64
J46TS1	*Bacillus licheniformis*	LC588600	*Bacillus licheniformis*	**99.86**	CSA, aerobically, 24h	1	256	0.5
J46TS2	*Bacillus haynesii*	LC588601	*Bacillus haynesii*	**99.93**	CSA, aerobically, 24h	2	>256	16
J46TS4[f]	*Cytobacillus* sp.	LC588602	*Cytobacillus stercorigallinarum*	**100**	CSA, aerobically, 24h	2	64	≤0.25
J46TS5	*Shouchella tritolerans*	LC588603	*Shouchella tritolerans*	**100**	CSA, aerobically, 24h	16	>256	4
J46TS6	*Shouchella clausii*	LC588604	*Shouchella clausii*	**99.86**	CSA, aerobically, 24h	8	256	4
J46TS7	*Paenibacillus dendritiformis*	LC588605	*Paenibacillus dendritiformis*	**99.73**	CSA, aerobically, 24h	≤0.25	8	1
J46TS8	*Heyndrickxia oleronia*	LC588606	*Heyndrickxia oleronia*	**99.64**	CSA, aerobically, 24h	4	64	32
J47TS1	*Bacillus licheniformis*	LC588607	*Bacillus licheniformis*	**99.86**	CSA, aerobically, 24h	2	>256	0.5
J47TS3	*Heyndrickxia oleronia*	LC588608	*Heyndrickxia oleronia*	**99.59**	CSA, aerobically, 24h	8	128	4
J47TS4	*Paenibacillus cineris*	LC588609	*Paenibacillus cineris*	**100**	CSA, aerobically, 24h	8	256	8
J47TS5	*Paenibacillus dendritiformis*	LC588610	*Paenibacillus dendritiformis*	**99.73**	CSA, aerobically, 24h	≤0.25	8	1
J47TS7[g]	*Heyndrickxia* sp.	LC588611	*Heyndrickxia sporothermodurans*	98.44	CSA, aerobically, 24h	2	4	≤0.25
J47TS8	*Bacillus paralicheniformis*	LC588612	*Bacillus paralicheniformis*	**100**	CSA, aerobically, 24h	2	>256	8

Table 2. (Continued)

Isolate[a]	Species[b]	Accession number of 16S rRNA gene sequence	EzBioCloud results[c]		Culture conditions[d]	MIC (μg/ml)[e]		
			Species of the closest type strain(s)	16S rRNA gene sequence similarity (%)		TS	LCM	OTC
J47TS9	*Bacillus licheniformis*	LC588613	*Bacillus licheniformis*	**99.86**	CSA, aerobically, 24h	2	>256	0.5
J47TS10	*Paenibacillus lactis*	LC588614	*Paenibacillus lactis*	**100**	CSA, aerobically, 24h	32	256	8
J47TS12[h]	*Bacillus* sp.	LC588615	*Bacillus cereus* *Bacillus albus* *Bacillus luti* *Bacillus sanguinis* *Bacillus basilensis*	**100**	CSA, aerobically, 24h	1	16	2
J48TS1	*Cytobacillus horneckiae*	LC588616	*Cytobacillus horneckiae*	**100**	CSA, aerobically, 24h	2	>256	≤0.25
J48TS2	*Paenibacillus lautus*	LC588617	*Paenibacillus lautus*	**99.52**	CSA, aerobically, 24h	16	128	8
J48TS3[h]	*Bacillus* sp.	LC588618	*Bacillus cereus* *Bacillus albus* *Bacillus luti* *Bacillus sanguinis* *Bacillus basilensis*	**100**	CSA, aerobically, 24h	2	8	1
J49TS1	*Bacillus licheniformis*	LC588619	*Bacillus licheniformis*	**99.93**	CSA, aerobically, 24h	1	>256	0.5
J49TS2	*Shouchella tritolerans*	LC588620	*Shouchella tritolerans*	**100**	CSA, aerobically, 24h	64	>256	4
J49TS3	*Paenibacillus cineris*	LC588621	*Paenibacillus cineris*	**100**	CSA, aerobically, 24h	16	>256	32
J49TS4[f]	*Paenibacillus* sp.	LC588622	*Paenibacillus oralis*	**99.57**	CSA, aerobically, 24h	16	256	16
J49TS5	*Shouchella tritolerans*	LC588623	*Shouchella tritolerans*	**99.93**	CSA, aerobically, 24h	64	256	4
J49TS6	*Shouchella tritolerans*	LC588624	*Shouchella tritolerans*	**99.93**	CSA, aerobically, 24h	64	>256	1
J49TS7	*Bacillus licheniformis*	LC588625	*Bacillus licheniformis*	**99.93**	CSA, aerobically, 24h	2	256	0.5
J49TS8	*Paenibacillus lautus*	LC588626	*Paenibacillus lautus*	**99.73**	CSA, aerobically, 24h	8	128	4

Table 2. (Continued)

Isolate[a]	Species[b]	Accession number of 16S rRNA gene sequence	EzBioCloud results[c]		Culture conditions[d]	MIC (μg/ml)[e]		
			Species of the closest type strain(s)	16S rRNA gene sequence similarity (%)		TS	LCM	OTC
J50TS1	*Heyndrickxia oleronia*	LC588627	*Heyndrickxia oleronia*	**99.45**	CSA, aerobically, 24h	8	256	8
J50TS3[g]	*Lederbergia* sp.	LC588628	*Lederbergia galactosidilytica*	98.57	CSA, aerobically, 24h	2	16	0.5
J50TS4	*Paenibacillus lautus*	LC588629	*Paenibacillus lautus*	**99.46**	CSA, aerobically, 24h	8	256	32
J50TS5	*Paenibacillus lautus*	LC588630	*Paenibacillus lautus*	**99.53**	CSA, aerobically, 24h	4	256	32
J51TS1	*Paenibacillus cineris*	LC588631	*Paenibacillus cineris*	**100**	CSA, aerobically, 24h	16	>256	32
J51TS2	*Paenibacillus cineris*	LC588632	*Paenibacillus cineris*	**100**	CSA, aerobically, 24h	16	256	64
J51TS3[g]	*Paenibacillus* sp.	LC588633	*Paenibacillus dokdonensis*	98.52	CSA, aerobically, 24h	2	64	2
J53TS1[g]	*Paenibacillus* sp.	LC588634	*Paenibacillus telluris*	98.21	CSA, aerobically, 24h	1	>256	128
J53TS2	*Paenibacillus rhizolycopersici*	LC588635	*Paenibacillus rhizolycopersici*	**99.72**	CSA, aerobically, 24h	8	32	0.5

[a] Underlined isolates were selected for genome sequencing.
[b] Bacterial species were identified by analyzing 16S rRNA gene sequences of the isolates using the EzBioCloud server (https://www.ezbiocloud.net).
[c] EzBioCloud analysis was performed in January 2024. The 16S rRNA gene sequence similarities higher than 98.65% (the threshold for differentiating two species [51]) are shown in bold.
[d] CSA, Columbia agar (Becton, Dickinson and Company, Franklin Lakes, NJ, United States) with 5% defibrinated sheep blood; GAMSA, modified GAM agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) with 5% defibrinated sheep blood.
[e] MIC, minimum inhibitory concentration; TS, tylosin; LCM, lincomycin; OTC, oxytetracycline. MICs for J33TS5 and the other isolates were assessed by agar dilution and broth microdilution methods, respectively, as described in Materials and Methods in Chapter I.
[f] The isolates were identified on a genus level because the taxonomic status of the closest type strain was an invalid name or preferred name (not correct name) in LPSN (https://lpsn.dsmz.de).
[g] The isolates were identified on a genus level because no type strains, which showed 98.65% or higher 16S rRNA gene sequence similarity, were found by EzBioCloud.
[h] The isolates were identified on a genus level because more than one closest type strains were detected by EzBioCloud.

Table 3. PCR primers used for sequencing of honey isolate-derived plasmids in the study in Chapter I

Primers	Sequence (5'-3')	Target	Description	Polymerase	PCR program
pJ45TS6_1	GTGTCTCTTAGACCGTGTCT	pJ45TS6 in isolate J45TS6	For amplification of partial regions of pJ45TS6 and sequencing of the amplified fragments.	ExTaq (Takara-Bio, Kusatsu, Japan)	95°C 2 min - 95°C 20 sec, 55°C 30 sec, 72°C 2-5 min (35 cycles) - 72°C 5 min
pJ45TS6_2	GGCTCACGATGTTATCGTGA				
pJ45TS6_3	CTTGAAACAGCAGGAAGCTG				
pJ45TS6_4	GGTATCGATTCAAAGTGGCG				
pJ45TS6_5	TGAGGGGTGTCTCTTAGACC				
pJ45TS6_6	TTCATTAAGCTAACGGGCAG				
pJ45TS6_7	ACAATTGGTGCGGAATGTCG				
pJ45TS6_8	TTATGACAGCTATGGCTGGG				
pJ45TS6_9	ATCCTGAACTAACACCGGAG				
pJ45TS6_10	TACCGGTGCTGGACATAATG				
pJ18TS1mac_1	CTCATTCCCTGATCTCGACT	pJ18TS1mac in isolate J18TS1	For amplification of partial regions of pJ18TS1mac and sequencing of the amplified fragments.		
pJ18TS1mac_2	CTCGCAGAGCACACACTTTA				
pJ18TS1mac_3	TCTCCTCCAGTTGCACATTG				
pJ18TS1mac_4	ATTTGTACTGCAATCGGGCG				
pJ18TS1tet_1	CCTGATCTCGACTTCGTTCT	pJ18TS1tet in isolate J18TS1	For amplification of partial regions of pJ18TS1tet and sequencing of the amplified fragments.		
pJ18TS1tet_2	CTCGCAGAGCACACACTTTA				
pJ18TS1tet_3	AAATGCATGTGAGGGGTTTG				
pJ18TS1tet_4	GGGTGAAAAGTCCATGTTTG				

Table 4. Macrolide resistance genes detected by Resistance Gene Identifier (https://card.mcmaster.ca/analyze/rgi) and Genome sequencing results of 50 representative honey-derived isolates [a]

Isolates[b]	Number of detected macrolide resistance gene			Macrolide resistance genes carried by MGEs[c]	Number of scaffolds	Depth	Total scaffold length (bp)	Number of CDS[d]	Accession number
	Total	Criteria							
		Perfect or strict	Loose						
J1TS1	2	2	0	–	99	1,387.2	4,302,142	4,265	BOQQ01000001-BOQQ01000042
J1TS3	4	1	3	–	98	1,013.6	4,682,804	4,518	BOQT01000001-BOQT01000086
J1TS5	6	0	6	–	190	752.3	7,335,197	6,440	BOSD01000001-BOSD01000138
J2TS4	4	1	3	–	88	884.5	6,674,740	5,881	BOSH01000001-BOSH01000073
J2TS5	2	1	1	–	99	1,246.9	4,389,838	4,521	BOQV01000001-BOQV01000093
J2TS6	2	0	2	–	87	724.9	6,757,555	6,138	BORQ01000001-BORQ01000065
J5TS1	2	1	1	–	30	1,209.3	4,372,756	4,526	BOQW01000001-BOQW01000026
J5TS2	2	0	2	–	97	984.7	5,090,001	4,547	BORK01000001-BORK01000082
J5TS4	2	2	0	–	24	1,305.8	4,162,733	4,166	BORG01000001-BORG01000019
J6TS1	5	1	4	–	102	1,068.9	5,316,142	4,944	BORJ01000001-BORJ01000073
J6TS2	3	1	2	–	140	1,176.1	5,310,951	5,266	BORH01000001-BORH01000112
J6TS7	1	0	1	–	740	890.8	6,831,239	6,628	BORY01000001-BORY01000617
J8TS2	2	0	2	–	207	1,179.5	4,567,145	4,288	BORB01000001-BORB01000188
J11TS1	5	0	5	–	101	1,267.7	4,018,736	3,908	BORO01000001-BORO01000094
J14TS2	5	0	5	–	115	103.2	5,863,450	5,383	BORE01000001-BORE01000104
J14TS5	4	2	2	–	89	1,663.5	7,329,731	6,602	BOSB01000001-BOSB01000064
J15TS10	2	0	2	–	94	967.4	5,819,404	5,196	BOSM01000001-BOSM01000070
J18TS1	4	1	3	*ermC* in plasmid pJ18TS1mac (accession number: LC586958)	106	1,088.9	4,691,447	4,455	BORN01000001-BORN01000099

Table 4. (Continued)

Isolates[b]	Number of detected macrolide resistance gene			Macrolide resistance genes carried by MGEs[c]	Number of scaffolds	Depth	Total scaffold length (bp)	Number of CDS[d]	Accession number
	Total	Criteria							
		Perfect or strict	Loose						
J19TS1	3	0	3	−	96	1,060.2	5,374,179	5,347	BOQX01000001-BOQX01000076
J19TS2	7	0	7	−	239	910.5	7,436,339	6,306	BORM01000001-BORM01000207
J21TS3	5	0	5	−	214	1,465.2	5,851,560	5,201	BORW01000001-BORW01000173
J21TS7	5	0	5	−	96	691.5	7,296,171	6,632	BORU01000001-BORU01000064
J22TS1	5	1	4	−	130	1,004.1	5,253,633	4,946	BORI01000001-BORI01000103
J22TS3	4	0	4	−	102	970.1	5,766,130	5,115	BOSF01000001-BOSF01000060
J23TS8	2	1	1	−	35	1,489.3	4,346,462	4,265	BOQY01000001-BOQY01000023
J23TS9	4	0	4	−	78	918.7	6,405,121	5,862	BOSG01000001-BOSG01000054
J25TS1	2	1	1	−	109	1,440.5	4,371,910	4,315	BOQZ01000001-BOQZ01000034
J25TS5	2	0	2	−	158	1,011.7	6,293,148	5,554	BORZ01000001-BORZ01000118
J26TS2	2	2	0	−	43	1,227.7	4,553,213	4,496	BOQR01000001-BOQR01000032
J27TS7	2	0	2	−	122	989.6	6,488,188	5,701	BORX01000001-BORX01000097
J27TS8	2	0	2	−	89	1,282.9	4,728,287	4,550	BORC01000001-BORC01000066
J28TS4	5	2	3	−	61	933.6	6,898,714	6,170	BOSC01000001-BOSC01000046
J31TS2	2	1	1	−	99	1,391.8	4,390,294	4,523	BOQU01000001-BOQU01000093

Table 4. (Continued)

Isolates[b]	Number of detected macrolide resistance gene			Macrolide resistance genes carried by MGEs[c]	Number of scaffolds	Depth	Total scaffold length (bp)	Number of CDS[d]	Accession number
	Total	Criteria							
		Perfect or strict	Loose						
J31TS3	5	1	4	−	147	857.3	6,795,756	6,065	BOSA01000001-BOSA01000098
J31TS4	2	0	2	−	72	2,138.3	5,090,261	4,669	BOSI01000001-BOSI01000060
J31TS6	2	1	1	−	106	985.2	6,564,183	6,449	BORL01000001-BORL01000071
J32TS2	2	2	0	−	98	1,287.6	4,238,113	4,201	BOQS01000001-BOQS01000080
J32TS6	3	0	3	−	46	1,017.7	4,698,878	4,274	BOSN01000001-BOSN01000039
J34TS1	2	0	2	−	186	898.2	7,242,150	6,453	BORT01000001-BORT01000146
J36TS2	2	1	1	−	71	1,127.0	4,579,420	4,534	BORA01000001-BORA01000057
J40TS1	3	0	3	−	104	1,314.9	6,058,380	5,352	BOSE01000001-BOSE01000074
J41TS2	3	0	3	−	48	958.4	4,797,974	4,895	BORD01000001-BORD01000042
J41TS4	3	0	3	−	138	1,128.0	5,473,147	4,963	BORS01000001-BORS01000097
J41TS8	2	1	1	−	93	1,184.4	4,464,729	4,441	BORF01000001-BORF01000071
J41TS12	3	0	3	−	160	888.6	5,577,454	5,089	BORR01000001-BORR01000121
J42TS3	2	0	2	−	129	804.4	5,640,808	5,167	BOSL01000001-BOSL01000099
J43TS3	3	0	3	−	40	1,488.9	3,476,337	3,463	BORP01000001-BORP01000031
J43TS9	4	0	4	*lsaB* and *oleC* in ICE	91	763.9	7,417,242	6,685	BORV01000001-BORV01000058

Table 4. (Continued)

Isolates[b]	Number of detected macrolide resistance gene			Macrolide resistance genes carried by MGEs[c]	Number of scaffolds	Depth	Total scaffold length (bp)	Number of CDS[d]	Accession number
	Total	Criteria							
		Perfect or strict	Loose						
J45TS6	2	1	1	*ermL* and *ermB* in plasmid pJ45TS6 (accession number: LC597664)	191	862.1	5,206,205	4,816	BOSJ01000001-BOSJ01000132
J53TS2	4	0	4	–	79	1,189.0	4,994,953	4,500	BOSK01000001-BOSK01000051

[a] The genome sequences were deposited in DDBJ under the DRA accession number DRA011480, BioProject accession number PRJDB11087 and BioSample accession numbers SAMD00277588-SAMD00277637.

[b] Genome sequence data of J14TS2 were obtained only by MiSeq (Illumina, Inc., San Diego, CA, USA), and those of J1TS1 and J25TS1 were obtained only by NovaSeq 6000 (Illumina). Genomes of the other isolates were sequenced by both MiSeq and NovaSeq 6000.

[c] MGEs, mobile genetic elements. ICE, integrative conjugative element. MGEs were identified by PlasmidFinder 2.1 (43), PHAST (42) and ICEberg 2.0 (41). Intact prophage was not detected.

[d] CDS, coding sequence.

Table 5. Macrolide, lincosamide, tetracycline resistance genes found in the genome of honey-derived isolates by Resistance Gene Identifier (RGI)

Isolates[a]	Species	RGI results[b]				
		Criteria	Antimicrobial resistance genes detected by RGI	Best identity	Drug class[c]	Resistance mechanism[c]
J1TS1	*Shouchella tritolerans*	Perfect	*clbC*	100	M, L, S, O, Ph, Pl	Antibiotic target alteration
		Strict	*erm*(34)	96.44	M, L, S	Antibiotic target alteration
		Loose	*ykkC*	58.72	Ag, T, Ph	Antibiotic efflux
		Loose	*tetA*(58)	57.28	T	Antibiotic efflux
		Loose	*ykkD*	56.86	Ag, T, Ph	Antibiotic efflux
J1TS3	*Siminovitchia fordii*	Strict	*emtA*	85.34	M, L	Antibiotic target alteration
		Strict	*tet*(45)	79.65	T	Antibiotic efflux
		Loose	*vgaC*	64.23	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*erm*(K)	60.21	M, L, S	Antibiotic target alteration
		Loose	*erm*(K)	60.21	M, L, S	Antibiotic target alteration
		Loose	*ykkC*	59.43	Ag, T, Ph	Antibiotic efflux
		Loose	*lmrB*	53.15	L	Antibiotic efflux
		Loose	*ykkD*	51.96	Ag, T, Ph	Antibiotic efflux
J1TS5	*Paenibacillus macerans*	Strict	*llmA*	85.66	L	Antibiotic target alteration
		Loose	*ermD*	69.04	M, L, S	Antibiotic target alteration
		Loose	*mel*	64.57	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*lmrB*	63.75	L	Antibiotic efflux
		Loose	*lsaB*	61.38	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*ykkC*	58.33	Ag, T, Ph	Antibiotic efflux
		Loose	*lmrB*	55.31	L	Antibiotic efflux
		Loose	*erm*(K)	54.83	M, L, S	Antibiotic target alteration
		Loose	*abeS*	53.92	M, Ac	Antibiotic efflux
		Loose	*oleC*	52.05	M	Antibiotic efflux
		Loose	*ykkD*	50	Ag, T, Ph	Antibiotic efflux
J2TS4	*Paenibacillus* sp.	Loose	*llmA*	75	L	Antibiotic target alteration
		Strict	*erm*(K)	68.09	M, L, S	Antibiotic target alteration
		Loose	*vgaC*	65.78	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*ykkC*	59.09	Ag, T, Ph	Antibiotic efflux
		Loose	*cfrA*	57.65	M, L, S, O, Ph, Pl	Antibiotic target alteration
		Loose	*lmrB*	53.88	L	Antibiotic efflux
		Loose	*tet*(L)	51.94	T	Antibiotic efflux
		Loose	*mphI*	51.63	M	Antibiotic inactivation (MPH)
		Loose	*tetA*(58)	50.79	T	Antibiotic efflux
J2TS5	*Bacillus licheniformis*	Strict	*ermD*	96.86	M, L, S	Antibiotic target alteration
		Loose	*ykkD*	72.12	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkC*	68.22	Ag, T, Ph	Antibiotic efflux
		Loose	*mphK*	61.51	M	Antibiotic inactivation (MPH)

Table 5. (Continued)

Isolates[a]	Species	RGI results[b]				
		Criteria	Antimicrobial resistance genes detected by RGI	Best identity	Drug class[c]	Resistance mechanism[c]
J2TS6	*Paenibacillus* sp.	Strict	*llmA*	84.32	L	Antibiotic target alteration
		Loose	*clbB*	73.82	M, L, S, O, Ph, Pl	Antibiotic target alteration
		Loose	*lmrB*	71.58	L	Antibiotic efflux
		Loose	*ykkC*	60	Ag, T, Ph	Antibiotic efflux
		Loose	*tet*(38)	55.61	T	Antibiotic efflux
		Loose	*oleC*	53.46	M	Antibiotic efflux
		Loose	*tetA*(58)	52.37	T	Antibiotic efflux
		Loose	*lmrB*	52.17	L	Antibiotic efflux
		Loose	*ykkD*	51.96	Ag, T, Ph	Antibiotic efflux
J5TS1	*Bacillus licheniformis*	Strict	*ermD*	93.71	M, L, S	Antibiotic target alteration
		Loose	*ykkD*	72.12	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkC*	68.22	Ag, T, Ph	Antibiotic efflux
		Loose	*mphK*	61.51	M	Antibiotic inactivation (MPH)
J5TS2	*Brevibacillus halotolerans*	Loose	*lsaB*	63.41	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*mphM*	57.49	M	Antibiotic inactivation (MPH)
		Loose	*ykkC*	54.37	Ag, T, Ph	Antibiotic efflux
J5TS4	*Bacillus tequilensis*	Perfect	*ykkC*	100	Ag, T, Ph	Antibiotic efflux
		Loose	*lmrB*	99.37	L	Antibiotic efflux
		Strict	*ykkD*	99.05	Ag, T, Ph	Antibiotic efflux
		Strict	*vmlR*	98.35	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Strict	*mphK*	97.39	M	Antibiotic inactivation (MPH)
J6TS1	*Siminovitchia terrae*	Strict	*clbA*	86.25	M, L, S, O, Ph, Pl	Antibiotic target alteration
		Loose	*lsaB*	83.54	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Strict	*tet*(45)	77.26	T	Antibiotic efflux
		Loose	*vgaC*	65.19	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*ykkC*	64.76	Ag, T, Ph	Antibiotic efflux
		Loose	*erm*(K)	60.85	M, L, S	Antibiotic target alteration
		Loose	*erm*(K)	60.14	M, L, S	Antibiotic target alteration
		Loose	*ykkD*	52.94	Ag, T, Ph	Antibiotic efflux
		Loose	*lmrB*	51.4	L	Antibiotic efflux
J6TS2	*Heyndrickxia sporothermodurans*	Strict	*clbA*	91.98	M, L, S, O, Ph, Pl	Antibiotic target alteration
		Loose	*tetB*(58)	85.61	T	Antibiotic efflux
		Loose	*lsaB*	83.74	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*tetA*(58)	81.76	T	Antibiotic efflux
		Loose	*ykkC*	63.72	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkD*	55.88	Ag, T, Ph	Antibiotic efflux
		Loose	*oleC*	52.97	M	Antibiotic efflux
		Loose	*Escherichia coli acrR* with mutation conferring multidrug antibiotic resistance	51.16	F, C, G, Pa, T, R, Ph, Tr	Antibiotic target alteration; antibiotic efflux

Table 5. (Continued)

Isolates[a]	Species	RGI results[b]				
		Criteria	Antimicrobial resistance genes detected by RGI	Best identity	Drug class[c]	Resistance mechanism[c]
J6TS7	*Paenibacillus dendritiformis*	Strict	*llmA*	81.53	L	Antibiotic target alteration
		Loose	*ykkC*	60.91	Ag, T, Ph	Antibiotic efflux
		Loose	*erm*(K)	58.57	M, L, S	Antibiotic target alteration
		Loose	*ykkD*	56.86	Ag, T, Ph	Antibiotic efflux
J8TS2	*Lederbergia ruris*	Loose	*ermG*	78.28	M, L, S	Antibiotic target alteration
		Loose	*ykkC*	66.67	Ag, T, Ph	Antibiotic efflux
		Loose	*vgaC*	65.83	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*ykkC*	55.24	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkD*	54.9	Ag, T, Ph	Antibiotic efflux
		Loose	*lmrB*	52.27	L	Antibiotic efflux
		Loose	*ykkD*	51.49	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkD*	51	Ag, T, Ph	Antibiotic efflux
J11TS1	*Oceanobacillus* sp.	Loose	*mphM*	70.34	M	Antibiotic inactivation (MPH)
		Loose	*mphM*	64.19	M	Antibiotic inactivation (MPH)
		Loose	*ermT*	60.08	M, L, S	Antibiotic target alteration
		Loose	*vgaC*	59.35	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*abeS*	56.25	M, Ac	Antibiotic efflux
		Loose	*ykkC*	50	Ag, T, Ph	Antibiotic efflux
J14TS2	*Lederbergia* sp.	Loose	*ermD*	67.97	M, L, S	Antibiotic target alteration
		Loose	*vmlR*	67.84	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*vgaC*	63.92	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*ykkC*	63.81	Ag, T, Ph	Antibiotic efflux
		Loose	*lmrB*	57.26	L	Antibiotic efflux
		Loose	*ermD*	54.65	M, L, S	Antibiotic target alteration
		Loose	*mphM*	52.53	M	Antibiotic inactivation (MPH)
		Loose	*ykkD*	51.96	Ag, T, Ph	Antibiotic efflux
		Loose	*lmrB*	51.62	L	Antibiotic efflux
		Loose	*ykkD*	51.49	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkC*	50	Ag, T, Ph	Antibiotic efflux
J14TS5	*Paenibacillus lautus*	Strict	*llmA*	99.65	L	Antibiotic target alteration
		Strict	*tetB*(58)	98.52	T	Antibiotic efflux
		Loose	*tetA*(58)	95.04	T	Antibiotic efflux
		Strict	*mphI*	94.81	M	Antibiotic inactivation (MPH)
		Strict	*cipA*	94.51	M, L, S, O, Ph, Pl	Antibiotic target alteration
		Loose	*lsaB*	61.79	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*ykkC*	60.58	Ag, T, Ph	Antibiotic efflux
		Loose	*lmrB*	58.86	L	Antibiotic efflux
		Loose	*ykkC*	58.42	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkD*	54.9	Ag, T, Ph	Antibiotic efflux
		Loose	*tet*(45)	53.15	T	Antibiotic efflux
		Loose	*oleC*	52.97	M	Antibiotic efflux
		Loose	*lmrB*	52.17	L	Antibiotic efflux

Table 5. (Continued)

Isolates[a]	Species	RGI results[b]				
		Criteria	Antimicrobial resistance genes detected by RGI	Best identity	Drug class[c]	Resistance mechanism[c]
J15TS10	*Paenibacillus woosongensis*	Loose	*tetB*(58)	85.61	T	Antibiotic efflux
		Strict	*llmA*	84.97	L	Antibiotic target alteration
		Loose	*tetA*(58)	75.38	T	Antibiotic efflux
		Loose	*ermD*	71.08	M, L, S	Antibiotic target alteration
		Loose	*ykkC*	67.65	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkD*	56.86	Ag, T, Ph	Antibiotic efflux
		Loose	*lnuG*	55.56	L	Antibiotic inactivation (MPH)
		Loose	*mphI*	53.33	M	Antibiotic inactivation (MPH)
		Loose	*lmrB*	52.93	L	Antibiotic efflux
J18TS1	*Oceanobacillus oncorhynchi* subsp. *incaldanensis*	Strict	*ermC*	95.08	M, L, S	Antibiotic target alteration
		Strict	*tet*(45)/*tetL*[d]	77.14	T	Antibiotic efflux
		Loose	*mphM*	69.26	M	Antibiotic inactivation (MPH)
		Strict	*tet*(W/N/W)	68.34	T	Antibiotic target protection
		Loose	ykkC	61.61	Ag, T, Ph	Antibiotic efflux
		Loose	ermA	58.46	M, L, S	Antibiotic target alteration
		Loose	*vgaC*	56.79	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*ykkD*	54.9	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkC*	54.55	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkD*	52.94	Ag, T, Ph	Antibiotic efflux
J19TS1	*Heyndrickxia oleronia*	Loose	*lsaB*	83.54	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*ykkC*	62.73	Ag, T, Ph	Antibiotic efflux
		Loose	*erm*(34)	57.35	M, L, S	Antibiotic target alteration
		Loose	*oleC*	52.51	M	Antibiotic efflux
		Loose	*ykkD*	51.96	Ag, T, Ph	Antibiotic efflux
J19TS2	*Cohnella xylanilytica*	Loose	*clbB*	80.76	M, L, S, O, Ph, Pl	Antibiotic target alteration
		Loose	*llmA*	77.35	L	Antibiotic target alteration
		Loose	*lsaB*	61.99	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*lmrB*	55.22	L	Antibiotic efflux
		Loose	*oleC*	54.38	M	Antibiotic efflux
		Loose	*adeN*	53.85	M, F, L, Ca, C, T, R, D, Ph, Pe	Antibiotic efflux
		Loose	*vmlR*	53.66	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*abeS*	53.47	M, Ac	Antibiotic efflux
		Loose	*oleC*	53.46	M	Antibiotic efflux
		Loose	*tetA*(58)	52.22	T	Antibiotic efflux
		Loose	*Escherichia coli acrR* with mutation conferring multidrug antibiotic resistance	51.52	F, C, G, Pa, T, R, Ph, Tr	Antibiotic target alteration; antibiotic efflux
		Loose	*ykkC*	51.38	Ag, T, Ph	Antibiotic efflux

Table 5. (Continued)

Isolates[a]	Species	RGI results[b]				
		Criteria	Antimicrobial resistance genes detected by RGI	Best identity	Drug class[c]	Resistance mechanism[c]
J21TS3	*Paenibacillus cookii*	Strict	*llmA*	83.97	L	Antibiotic target alteration
		Loose	*clbB*	75	M, L, S, O, Ph, Pl	Antibiotic target alteration
		Loose	*lmrB*	71.85	L	Antibiotic efflux
		Loose	*erm*(K)	59.26	M, L, S	Antibiotic target alteration
		Loose	*ykkC*	59.09	Ag, T, Ph	Antibiotic efflux
		Loose	*oleC*	55.71	M	Antibiotic efflux
		Loose	*tet*(38)	55.33	T	Antibiotic efflux
		Loose	*oleC*	54.84	M	Antibiotic efflux
		Loose	*erm*(34)	54.84	M, L, S	Antibiotic target alteration
		Loose	*tetA*(58)	52.37	T	Antibiotic efflux
J21TS7	*Paenibacillus cineris*	Strict	*llmA*	87.11	L	Antibiotic target alteration
		Loose	*cipA*	83.04	M, L, S, O, Ph, Pl	Antibiotic target alteration
		Loose	*lmrB*	72.36	L	Antibiotic efflux
		Loose	*mel*	62.53	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*ykkC*	61.76	Ag, T, Ph	Antibiotic efflux
		Loose	*lsaB*	58.54	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*oleC*	55.76	M	Antibiotic efflux
		Loose	*lmrB*	55.72	L	Antibiotic efflux
		Loose	*oleC*	53.46	M	Antibiotic efflux
		Loose	*ykkD*	51.96	Ag, T, Ph	Antibiotic efflux
		Loose	*tetA*(58)	50.47	T	Antibiotic efflux
J22TS1	*Siminovitchia terrae*	Strict	*clbA*	86.25	M, L, S, O, Ph, Pl	Antibiotic target alteration
		Loose	*lsaB*	83.54	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Strict	*tet*(45)	77.04	T	Antibiotic efflux
		Loose	*vgaC*	65.38	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*ykkC*	64.76	Ag, T, Ph	Antibiotic efflux
		Loose	*erm*(K)	60.92	M, L, S	Antibiotic target alteration
		Loose	*erm*(K)	60.85	M, L, S	Antibiotic target alteration
		Loose	*ykkD*	52.94	Ag, T, Ph	Antibiotic efflux
		Loose	*lmrB*	51.62	L	Antibiotic efflux
		Loose	*ykkC*	50.67	Ag, T, Ph	Antibiotic efflux
J22TS3	*Paenibacillus* sp.	Strict	*llmA*	86.71	L	Antibiotic target alteration
		Loose	*clbB*	75	M, L, S, O, Ph, Pl	Antibiotic target alteration
		Loose	*lsaB*	63.41	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*ykkC*	57.14	Ag, T, Ph	Antibiotic efflux
		Loose	*lmrB*	56.9	L	Antibiotic efflux
		Loose	*tet*(38)	56.66	T	Antibiotic efflux
		Loose	*ykkD*	55.45	Ag, T, Ph	Antibiotic efflux
		Loose	*oleC*	54.38	M	Antibiotic efflux
		Loose	*lmrB*	52.61	L	Antibiotic efflux
		Loose	*tet*(L)	50	T	Antibiotic efflux
		Loose	*adeN*	50	M, F, L, Ca, C, T, R, D, Ph, Pe	Antibiotic efflux

Table 5. (Continued)

Isolates[a]	Species	RGI results[b]				
		Criteria	Antimicrobial resistance genes detected by RGI	Best identity	Drug class[c]	Resistance mechanism[c]
J23TS8	*Bacillus paralicheniformis*	Strict	*ermD*	99.65	M, L, S,	Antibiotic target alteration
		Loose	*ykkD*	72.12	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkC*	71.03	Ag, T, Ph	Antibiotic efflux
		Loose	*mphK*	60.73	M	Antibiotic inactivation (MPH)
J23TS9	*Paenibacillus dokdonensis*	Loose	*tetB*(58)	87.45	T	Antibiotic efflux
		Loose	*tetA*(58)	83.67	T	Antibiotic efflux
		Strict	*llmA*	83.62	L	Antibiotic target alteration
		Loose	*lsaB*	82.32	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*clbA*	77.84	M, L, S, O, Ph, Pl	Antibiotic target alteration
		Loose	*lmrB*	74.09	L	Antibiotic efflux
		Loose	*ykkC*	57.8	Ag, T, Ph	Antibiotic efflux
		Loose	*lmrB*	55.08	L	Antibiotic efflux
		Loose	*tet*(38)	54.98	T	Antibiotic efflux
		Loose	*oleC*	53.7	M	Antibiotic efflux
		Loose	*ykkD*	50.98	Ag, T, Ph	Antibiotic efflux
		Loose	*abeS*	50.5	M, Ac	Antibiotic efflux
J25TS1	*Bacillus paralicheniformis*	Strict	*ermD*	99.65	M, L, S	Antibiotic target alteration
		Loose	*ykkD*	71.15	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkC*	71.03	Ag, T, Ph	Antibiotic efflux
		Loose	*mphK*	60.73	M	Antibiotic inactivation (MPH)
J25TS5	*Paenibacillus faecis*	Strict	*llmA*	85.66	L	Antibiotic target alteration
		Loose	*lsaB*	61.79	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*ykkC*	58.1	Ag, T, Ph	Antibiotic efflux
		Loose	*lmrB*	56.3	L	Antibiotic efflux
		Loose	*ykkC*	55.45	Ag, T, Ph	Antibiotic efflux
		Loose	*mphI*	55.44	M	Antibiotic inactivation (MPH)
		Loose	*ykkD*	52.88	Ag, T, Ph	Antibiotic efflux
J26TS2	*Shouchella tritolerans*	Strict	*clbC*	98	M, L, S, O, Ph, Pl	Antibiotic target alteration
		Strict	*erm*(34)	96.8	M, L, S	Antibiotic target alteration
		Loose	*ykkC*	59.63	Ag, T, Ph	Antibiotic efflux
		Loose	*tetA*(58)	56.92	T	Antibiotic efflux
		Loose	*ykkD*	56.86	Ag, T, Ph	Antibiotic efflux
J27TS7	*Paenibacillus dendritiformis*	Loose	*clbB*	80.47	M, L, S, O, Ph, Pl	Antibiotic target alteration
		Strict	*llmA*	80.14	L	Antibiotic target alteration
		Loose	*ykkC*	60.91	Ag, T, Ph	Antibiotic efflux
		Loose	*lsaB*	60.8	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*ykkD*	55.88	Ag, T, Ph	Antibiotic efflux

Table 5. (Continued)

Isolates[a]	Species	RGI results[b]				
		Criteria	Antimicrobial resistance genes detected by RGI	Best identity	Drug class[c]	Resistance mechanism[c]
J27TS8	*Robertmurraya siralis*	Loose	*tetB*(58)	77.07	T	Antibiotic efflux
		Loose	*tetA*(58)	75.24	T	Antibiotic efflux
		Loose	*ermD*	73.5	M, L, S	Antibiotic target alteration
		Loose	*vgaALC*	62.6	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*ykkC*	58.72	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkD*	51.96	Ag, T, Ph	Antibiotic efflux
		Loose	*tet*(48)	51.22	T	Antibiotic target protection
J28TS4	*Paenibacillus lautus*	Strict	*llmA*	98.61	L	Antibiotic target alteration
		Strict	*cipA*	98.55	M, L, S, O, Ph, Pl	Antibiotic target alteration
		Loose	*tetB*(58)	92.99	T	Antibiotic efflux
		Strict	*mphI*	92.86	M	Antibiotic inactivation (MPH)
		Loose	*tetA*(58)	91.84	T	Antibiotic efflux
		Loose	*lsaB*	63.82	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*msrE*	60.54	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*ykkC*	59.62	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkD*	56.86	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkC*	53.64	Ag, T, Ph	Antibiotic efflux
		Loose	*oleC*	52.97	M	Antibiotic efflux
		Loose	*lmrB*	52.17	L	Antibiotic efflux
J31TS2	*Bacillus licheniformis*	Strict	*ermD*	96.86	M, L, S	Antibiotic target alteration
		Loose	*ykkD*	72.12	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkC*	68.22	Ag, T, Ph	Antibiotic efflux
		Loose	*mphK*	61.51	M	Antibiotic inactivation (MPH)
J31TS3	*Paenibacillus lactis*	Strict	*llmA*	94.08	L	Antibiotic target alteration
		Strict	*cipA*	93.93	M, L, S, O, Ph, Pl	Antibiotic target alteration
		Loose	*tetB*(58)	87.5	T	Antibiotic efflux
		Loose	*tetA*(58)	81.87	T	Antibiotic efflux
		Loose	*lsaB*	80.28	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*mphI*	74.75	M	Antibiotic inactivation (MPH)
		Loose	*ykkC*	59.81	Ag, T, Ph	Antibiotic efflux
		Loose	*msrE*	57.97	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*oleC*	54.84	M	Antibiotic efflux
		Loose	*ykkD*	53.92	Ag, T, Ph	Antibiotic efflux
		Loose	*lmrB*	52.61	L	Antibiotic efflux
		Loose	*ykkC*	52.29	Ag, T, Ph	Antibiotic efflux
J31TS4	*Paenibacillus* sp.	Loose	*llmA*	78.75	L	Antibiotic target alteration
		Loose	*ykkC*	59.63	Ag, T, Ph	Antibiotic efflux
		Loose	*mphI*	57.14	M	Antibiotic inactivation (MPH)
		Loose	*oleC*	53.92	M	Antibiotic efflux
		Loose	*Escherichia coli acrR* with mutation conferring multidrug antibiotic resistance	50	F, C, G, Pa, T, R, Ph, Tr	Antibiotic target alteration; antibiotic efflux

Table 5. (Continued)

Isolates[a]	Species	RGI results[b]				
		Criteria	Antimicrobial resistance genes detected by RGI	Best identity	Drug class[c]	Resistance mechanism[c]
J31TS6	*Brevibacillus reuszeri*	Strict	*clbB*	91.84	M, L, S, O, Ph, Pl	Antibiotic target alteration
		Loose	*tetB*(58)	81.18	T	Antibiotic efflux
		Loose	*tetA*(58)	80.73	T	Antibiotic efflux
		Loose	*lsaB*	64.02	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*ykkC*	61.47	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkD*	52.94	Ag, T, Ph	Antibiotic efflux
J32TS2	*Shouchella tritolerans*	Perfect	*clbC*	100	M, L, S, O, Ph, Pl	Antibiotic target alteration
		Strict	*erm*(34)	96.8	M, L, S	Antibiotic target alteration
		Loose	*ykkC*	58.72	Ag, T, Ph	Antibiotic efflux
		Loose	*tetA*(58)	56.79	T	Antibiotic efflux
		Loose	*ykkD*	55.88	Ag, T, Ph	Antibiotic efflux
J32TS6	*Virgibacillus pantothenticus*	Loose	*erm*(K)	64.16	M, L, S	Antibiotic target alteration
		Loose	*vgaA*	58.7	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*ykkC*	57.55	Ag, T, Ph	Antibiotic efflux
		Loose	*nalD*	52	M, F, Mo, Ca, C, Ce, Pa, T, Pt, Ac, D, Su, Ph, Pe	Antibiotic efflux
		Loose	*lmrB*	50.76	L	Antibiotic efflux
		Loose	*ykkC*	50	Ag, T, Ph	Antibiotic efflux
J34TS1	*Paenibacillus azoreducens*	Loose	*lsaB*	85.1	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Strict	*llmA*	84.67	L	Antibiotic target alteration
		Loose	*tetB*(58)	83.76	T	Antibiotic efflux
		Loose	*tetA*(58)	81.07	T	Antibiotic efflux
		Loose	*ykkC*	58.93	Ag, T, Ph	Antibiotic efflux
		Loose	*oleC*	53	M	Antibiotic efflux
		Loose	*tetA*(58)	52.29	T	Antibiotic efflux
		Loose	*lmrB*	52.17	L	Antibiotic efflux
J36TS2	*Bacillus paralicheniformis*	Strict	*ermD*	99.65	M, L, S	Antibiotic target alteration
		Loose	*ykkD*	72.12	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkC*	71.03	Ag, T, Ph	Antibiotic efflux
		Loose	*mphK*	60.4	M	Antibiotic inactivation (MPH)
J40TS1	*Paenibacillus montaniterrae*	Loose	*clbB*	84.5	M, L, S, O, Ph, Pl	Antibiotic target alteration
		Loose	*tetB*(58)	81.55	T	Antibiotic efflux
		Loose	*llmA*	77.08	L	Antibiotic target alteration
		Loose	*tetA*(58)	76.06	T	Antibiotic efflux
		Strict	*tet*(45)	73.64	T	Antibiotic efflux
		Loose	*mel*	67.51	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*ykkC*	65.71	Ag, T, Ph	Antibiotic efflux
		Loose	*lsaB*	59.84	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*ykkD*	53.85	Ag, T, Ph	Antibiotic efflux
		Loose	*tetA*(58)	52.05	T	Antibiotic efflux
		Loose	*tetA*(58)	51.8	T	Antibiotic efflux
		Loose	*lmrB*	51.4	L	Antibiotic efflux
		Loose	*lmrB*	50.65	L	Antibiotic efflux

Table 5. (Continued)

Isolates[a]	Species	RGI results[b]				
		Criteria	Antimicrobial resistance genes detected by RGI	Best identity	Drug class[c]	Resistance mechanism[c]
J41TS2	*Bacillus sonorensis*	Loose	*ermD*	85.05	M, L, S	Antibiotic target alteration
		Loose	*ykkC*	71.03	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkD*	70.19	Ag, T, Ph	Antibiotic efflux
		Loose	*mphK*	61.39	M	Antibiotic inactivation (MPH)
		Loose	*oleC*	54.34	M	Antibiotic efflux
J41TS4	*Paenibacillus apis*	Strict	*llmA*	87.06	L	Antibiotic target alteration
		Loose	*ykkC*	64.76	Ag, T, Ph	Antibiotic efflux
		Loose	*lsaB*	60.57	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*msrE*	58.56	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*oleC*	53.88	M	Antibiotic efflux
		Loose	*ykkD*	51.96	Ag, T, Ph	Antibiotic efflux
J41TS8	*Bacillus* sp.	Strict	*ermD*	99.65	M, L, S	Antibiotic target alteration
		Loose	*ykkD*	72.12	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkC*	71.03	Ag, T, Ph	Antibiotic efflux
		Loose	*mphK*	60.4	M	Antibiotic inactivation (MPH)
J41TS12	*Paenibacillus antibioticophila*	Strict	*llmA*	86.71	L	Antibiotic target alteration
		Loose	*lnuG*	81.89	L	Antibiotic inactivation (MPH)
		Loose	*ykkC*	64.76	Ag, T, Ph	Antibiotic efflux
		Loose	*lsaB*	60.37	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*msrE*	58.76	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*oleC*	52.97	M	Antibiotic efflux
		Loose	*ykkD*	52.94	Ag, T, Ph	Antibiotic efflux
J42TS3	*Paenibacillus vini*	Strict	llmA	85.66	L	Antibiotic target alteration
		Loose	*tetA*(58)	81.35	T	Antibiotic efflux
		Loose	*tetB*(58)	77.86	T	Antibiotic efflux
		Loose	*lmrB*	66.52	L	Antibiotic efflux
		Loose	*lsaB*	63.01	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*ykkC*	57.94	Ag, T, Ph	Antibiotic efflux
		Loose	*oleC*	52.97	M	Antibiotic efflux
		Loose	*ykkD*	50	Ag, T, Ph	Antibiotic efflux
J43TS3	*Ornithinibacillus bavariensis*	Loose	*erm*(K)	57.04	M, L, S	Antibiotic target alteration
		Loose	*ykkC*	55.96	Ag, T, Ph	Antibiotic efflux
		Loose	*vgaC*	54.98	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*oleC*	51.82	M	Antibiotic efflux

Table 5. (Continued)

Isolates[a]	Species	RGI results[b]				
		Criteria	Antimicrobial resistance genes detected by RGI	Best identity	Drug class[c]	Resistance mechanism[e]
J43TS9	*Paenibacillus cineris*	Strict	*llmA*	87.11	L	Antibiotic target alteration
		Loose	*cipA*	83.92	M, L, S, O, Ph, Pl	Antibiotic target alteration
		Loose	*lmrB*	72.36	L	Antibiotic efflux
		Loose	*ykkC*	61.76	Ag, T, Ph	Antibiotic efflux
		Loose	*lsaB*	58.54	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*oleC*	55.76	M	Antibiotic efflux
		Loose	*lmrB*	55.51	L	Antibiotic efflux
		Loose	*oleC*	54.84	M	Antibiotic efflux
		Loose	*ykkD*	51.96	Ag, T, Ph	Antibiotic efflux
		Loose	*tetA*(58)	50.47	T	Antibiotic efflux
J45TS6	*Paenibacillus* sp.	Strict	*ermB*	97.96	M, L, S	Antibiotic target alteration
		Strict	*lnuG*	95.13	L	Antibiotic inactivation (MPH)
		Strict	*llmA*	86.41	L	Antibiotic target alteration
		Strict	*tet*(45)/*tetL*[c]	76.71	T	Antibiotic efflux
		Loose	*mphM*	65.31	M	Antibiotic inactivation (MPH)
		Loose	*lmrB*	65.23	L	Antibiotic efflux
		Loose	*ykkC*	55.45	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkD*	53.85	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkD*	51.49	Ag, T, Ph	Antibiotic efflux
		Loose	*ykkC*	50	Ag, T, Ph	Antibiotic efflux
J53TS2	*Paenibacillus rhizolycopersici*	Strict	*llmA*	86.71	L	Antibiotic target alteration
		Loose	*tetB*(58)	82.29	T	Antibiotic efflux
		Loose	*tetA*(58)	80.51	T	Antibiotic efflux
		Loose	*clbB*	73.33	M, L, S, O, Ph, Pl	Antibiotic target alteration
		Loose	*ykkC*	61.68	Ag, T, Ph	Antibiotic efflux
		Loose	*lsaB*	61.18	M, L, S, T, O, Ph, Pl	Antibiotic target protection
		Loose	*ykkC*	57.94	Ag, T, Ph	Antibiotic efflux
		Loose	*lmrB*	56.86	L	Antibiotic efflux
		Loose	*oleC*	54.34	M	Antibiotic efflux
		Loose	*mphI*	51.31	M	Antibiotic inactivation (MPH)
		Loose	*lmrB*	50	L	Antibiotic efflux

[a] Genome sequence data of J14TS2 were obtained only by MiSeq (Illumina, Inc., San Diego, CA, USA) and those of J1TS1 and J25TS1 were obtained only by NovaSeq 6000 (Illumina). Genomes of the other isolates were sequenced by both MiSeq and NovaSeq 6000.
[b] Genes that showed >50% best identity and length of reference sequence of 60–140% were selected as antimicrobial resistance genes.
[c] M, macrolide antibiotic; L, lincosamide antibiotic; S, streptogramin antibiotic; O, oxazolidinone antibiotic; Ph, phenicol antibiotic; Pl, pleuromutilin antibiotic; T, tetracycline antibiotic; Ag, Aminoglycoside antibiotic; Ac, aminocoumarin antibiotic; F, fluoroquinolone antibiotic; C, cephalosporin; G, glycylcycline; Pe, penem; R, rifamycin antibiotic; Tr, triclosan; Ca, carbapenem; D, diaminopyrimidine antibiotic; Pa, penam; Mo, monobactam; Ce, cephamycin; Pt, peptide antibiotic; Su, sulfonamide antibiotic.
[d] Although these genes were detected as *tet*(45) by RGI, we used *tetL* for the genes in this study because most homologues of the products were annotated as TetL in the GenBank database.
[e] MPH, macrolide phosphotransferases.

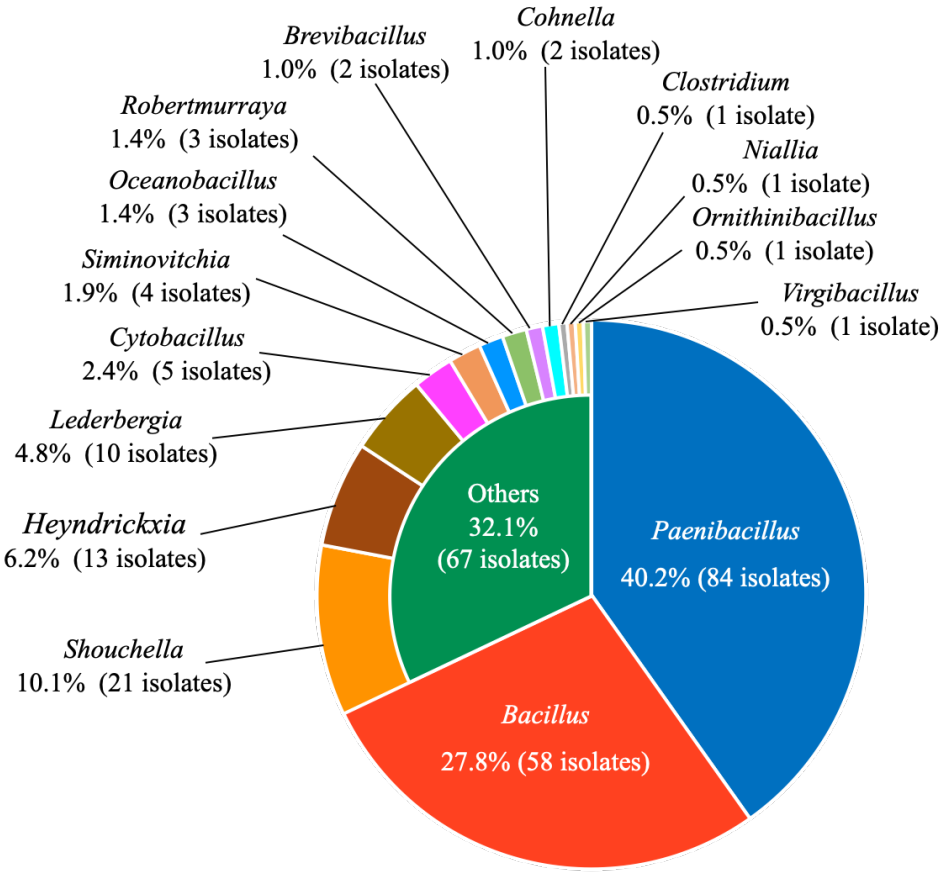

Figure 1. Proportion of genera found in the bacteria isolated from Japanese honey on 1 µg/ml-tylosin-containing agar media

Genera/species of the isolates was identified by 16S rRNA gene sequencing.

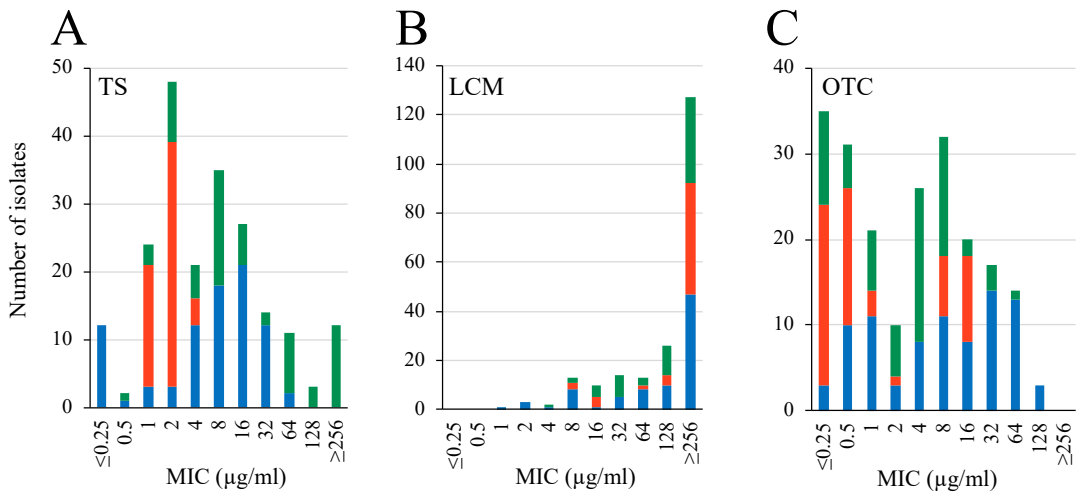

Figure 2. Susceptibility of the Japanese honey-derived isolates to tylosin (TS), lincomycin (LCM), and oxytetracycline (OTC), respectively (A, B, and C)

Minimum inhibitory concentrations (MICs) of TS, LCM, and OTC were measured by broth microdilution/agar dilution methods. The proportion of isolates with different genera in each MIC is indicated by different colors according to Figure 1.

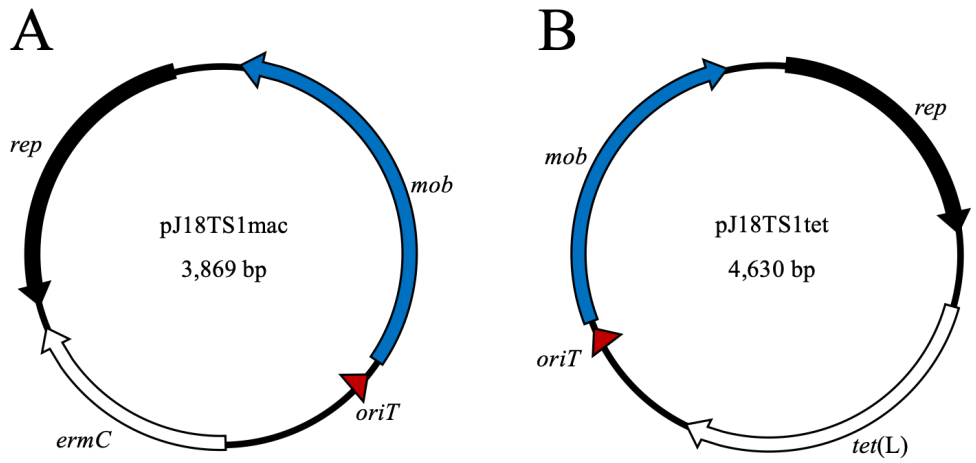

Figure 3. Map of (A) pJ18TS1mac, and (B) pJ18TS1tet

rep, replication protein gene; *mob*, mobilization protein gene; *ermC*, 23S rRNA methyltransferase gene (macrolide resistance gene); *tet*(L), efflux pump gene (tetracycline resistance gene); *oriT*, origin of transfer. The *oriT* sequences in the plasmids were identified using *oriT* finder (https://bioinfo-mml.sjtu.edu.cn/oriTfinder/).

Chapter II

Conferring tylosin resistance to *P. larvae* by macrolide resistance genes from honey-derived bacteria

Introduction

In the United States of America (USA), where *P. larvae* has already acquired resistance to all antimicrobials approved for use in the beekeeping industry, United States Department of Agriculture has approved the first-ever vaccine to prevent AFB in 2023 (52). The vaccine contains dead *P. larvae* and is administered by feeding it to the worker bees. Young worker bees that ingest the vaccine will secrete royal jelly containing fragments of the pathogen, and the queen bee ingests the vaccine component via the royal jerry. The pathogen information ingested by the queen bee is transferred to her eggs, and the larvae from those eggs become more resistant to AFB than larvae from eggs that have not received the pathogen information (53). It is expected to be a sustainable countermeasure as antibiotic treatment has limited effectiveness in the USA. However, it is not known whether this vaccine is truly effective in preventing AFB in the field. In addition, there are currently no plans to introduce it to Japan. Disinfection is also an important measure to prevent diseases; however, because *P. larvae* spores are highly resistant to physical and chemical stimulation, it is difficult to completely prevent AFB with disinfection alone. Therefore, maintaining the effectiveness of the prophylactic remains crucial for sustainable AFB control in Japan.

In Chapter I, four putative macrolide resistance genes (*lsaB*, *oleC*, *ermB*, and *ermC*) located on MGEs were found in Japanese honey-derived bacteria. The *ermB* and *ermC* genes were carried by plasmids. In particular, *ermC* was located on a putative mobilizable plasmid, pJ18TS1mac. Horizontal transfer of plasmids by conjugation requires multiple components; relaxase encoded by the *mob* gene, the type IV coupling protein (T4CP), and the components that assemble a type 4 secretion system (T4SS) (54). In the first step of the conjugation, the relaxase recognizes and cleaves a specific site within the origin of transfer (*oriT*) of the plasmid and bounds to the end of the cleaved DNA strand. The relaxase with the DNA then interacts with the T4CP and T4SS, a membrane-associated mating pair formation complex providing the mating channel to another bacterial cell, and the DNA is pumped into the recipient cell

through T4SS by the ATPase activity of the T4CP. Conjugative plasmids carry all the machinery needed for self-transfer. On the other hand, mobilizable plasmids carry only *oriT*, relaxase, and one or more auxiliary proteins. Therefore, the mobilizable plasmids cannot transfer to other bacterial cells on their own; however, if they coexist with conjugative plasmids in a bacterial cell, they can borrow part of the conjugative plasmids' machinery and transfer to other bacterial cells.

ICEs are also one of the main factors of gene transfer and have the same conjugation system as the conjugative plasmids (55); therefore, the mobilizable plasmid may also transfer to another host with the help of ICEs. ICEs are typically found integrated into the host chromosome and excise themselves from the genome when they transfer to recipient cells. In Chapter I, *lsaB* and *oleC* were found to be carried by a putative ICE, suggesting that these genes could also transfer to other hosts by conjugative transfer. Therefore, in Chapter II, to elucidate whether the four putative macrolide resistance genes on MGEs have the potential to confer macrolide resistance to *P. larvae*, these resistance genes were artificially introduced into a TS-susceptible *P. larvae* strain by using an expression vector. Moreover, for pJ18TS1mac, mating tests and stability tests of the MGE were carried out to investigate the transmissibility to and stability in *P. larvae*, respectively.

P. larvae strains are genetically diverse and have been classified into five enterobacterial repetitive intergenic consensus (ERIC) types (ERIC I–V) by repetitive-element PCR (56, 57) and 48 sequence types (STs) by multilocus sequence typing (MLST) (https://pubmlst.org/organisms/paenibacillus-larvae) (58). Strains with different genotypes differ in phenotypes, including virulence, at both the larval and colony levels (56, 57, 59, 60). Among the five ERIC genotypes, ERIC I and II are the major types among the strains isolated from AFB cases (7, 47, 56). In Japan, ERIC I and II are the only genotypes found in isolates from AFB cases, and isolation of ERIC II strains has recently increased (30). In addition, ERIC II strains are known to be more resistant to chlorine-based disinfectants (61); hence, this type may become more problematic in Japanese apiaries. Therefore, in the study of this chapter, an ERIC II

strain isolated in Japan was used as a representative strain of *P. larvae*.

Materials and Methods

P. larvae strain and honey-derived bacterial isolates used in this chapter

The *P. larvae* strain, DTK384 (originally called by strain N in Hirai et al. [62]), was used as the parent strain for introducing antimicrobial resistance genes. DTK384 was originally isolated from a diseased larva of an AFB case in Japan in 2012 (62). The strain is classified into ERIC II and ST10, one of the most frequently isolated genotypes in Japan in recent years (30). For cloning of putative antimicrobial resistance genes to be introduced into *P. larvae*, honey-derived isolates, J18TS1, J43TS9, and J45TS6 were used. The isolate J18TS1 possesses plasmid pJ18TS1mac carrying *ermC*, the isolate J45TS6 possesses plasmid pJ45TS6 carrying *ermB*, and the isolate J43TS9 possesses a putative ICE carrying the *lsaB* and *oleC* in the chromosome.

Construction of putative macrolide resistance gene expression vectors

Each putative macrolide resistance gene region (Figure 4) was amplified from the genomic DNA of the respective isolates by PCR using the primers and enzymes listed in Table 6. The primers were designed to produce amplicons that contained the antimicrobial resistance gene and its Shine-Dalgarno sequence and/or putative promoter region. pMX2-TA, which was originally developed as a stable gene expression vector for *M. plutonius* (63), was used. The PCR products were cloned into this plasmid vector via EcoRI and PstI or BamHI and PstI sites in *E. coli* MC1061 (64). *E. coli* was cultured in LB (Becton, Dickinson and Company) agar at 37℃ under aerobic conditions, and chloramphenicol (CP) was added to the media at 16 μg/ml for maintenance of the expression vectors. Accuracy of the cloned sequences was confirmed by PCR and sequencing analyses.

Introduction of the vectors into *P. larvae*

The expression vectors were then introduced into the *P. larvae* strain DTK384 by electroporation according to the procedures described by de Graaf et al. (47) except

that electrotransformed *P. larvae* cells were incubated in MYPGP broth (1.0% Mueller-Hinton broth [Oxoid, Hampshire, UK], 1.5% yeast extract, 0.3% K_2HPO_4, 0.1% sodium pyruvate and 0.2% glucose) (47) at 37℃ for 2 h 30 min–7 h 30 min without shaking, and transformants were selected on MYPGP agar (MYPGP broth supplemented with 2.0% agar) (47) supplemented with 16 μg/ml of CP. As a control, DTK384 possessing only pMX2-TA (DKT384control) was also constructed by introducing pMX2-TA into the strain. The resultant *P. larvae* strains are listed in Table 7.

Confirmation of introduced-gene expression by reverse transcription PCR (RT-PCR)

Total RNA of *P. larvae* strains was extracted from bacterial cells cultured on MYPGP agar (47) containing 16 μg/ml of CP at 35℃ for two days under air plus 5% CO_2 conditions using the RNeasy Mini kit supplemented with RNAprotect Bacteria Reagent and RNase-Free DNase Set, as recommended by the manufacturer (QIAGEN) with the following modifications: harvested and RNAprotect Bacteria Reagent-treated *P. larvae* cells were incubated in TE containing 10 mg/ml of lysozyme (Sigma-Aldrich) and 50 U/ml of mutanolysin (Sigma-Aldrich) at 37℃ for 20 min and then lysed by β-mercaptoethanol-supplemented Buffer RLT in the RNeasy Mini kit. Extracted RNA was transcribed to single-strand cDNA using the PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio). Expression of the introduced-antimicrobial resistance gene was confirmed by PCR using cDNA made from 10 ng of total RNA. To confirm the removal of DNA, the reverse transcriptase-untreated RNA was used as a template. All primers, polymerase and PCR conditions are listed in Table 6.

Filter Mating Test for transmission of macrolide resistance plasmids to *P. larvae*

Isolates J18TS1 and J45TS6, which possess macrolide resistance plasmids,

were used as donors, and MICs of mitomycin C (FUJIFILM Wako Pure Chemical Corp.) for the isolates were measured by broth microdilution methods using MYPGP broth as described in Chapter I. As the recipient, rifampicin (RIF)-resistant *P. larvae* was used. To prepare the recipient strain, *P. larvae* DTK384 was cultured on MYPGP agar containing 2 μg/ml of RIF (FUJIFILM Wako Pure Chemical Corp.), and a spontaneously generated RIF-resistant strain was isolated and named strain M20. The MIC of RIF for this strain measured by agar dilution methods was 128 μg/ml. For mating tests, J18TS1 and J45TS6 cultured on MYPGP agar with 1 μg/ml of TS at 35℃ for approximately 24 h under aerobic conditions and M20 cultured on MYPGP agar with 4 μg/ml RIF at 35℃ for 48 h under air plus 5% CO_2 conditions were separately inoculated into MYPGP broth and cultured at 35℃ with shaking (200 rpm). For donors, TS was added to the broth at a final concentration of 1 μg/ml. When the optical density of the cultures at 600 nm reached 0.4–0.6, donor cells from 5 ml of the culture were further incubated in fresh MYPGP broth (no mitomycin C) and that containing 1/2 and 1/4 MIC of mitomycin C. After incubation at 35℃ for 1 h, the donor cells were collected by centrifugation (11,000 rpm, 10 min), washed three times with sterile saline to remove mitomycin C, and suspended in 5 ml of fresh MYPGP broth. The donor and recipient cultures were then mixed at a ratio of 1:1, and the mixture was filtered through a 0.45-mm nitrocellulose filter (25 mm diameter). The filters were placed on MYPGP agar and incubated at 35℃ under air plus 5% CO_2 conditions. Two sets of filters were prepared under each condition, one was incubated for 24 h and the other for 48 h. To select recipient cells that acquired macrolide resistant plasmids, the donor and recipient cells on the cultured filters were suspended in 1 ml of sterile saline. One hundred microliters of the suspension was placed on MYPGP agar containing both RIF (4 μg/ml) and TS (1 μg/ml), and incubated at 35℃ for 48 h under air plus 5% CO_2 conditions.

Introduction of pJ18TS1mac into *P. larvae*

The plasmid pJ18TS1mac harbored by isolate J18TS1 was extracted using

the illustra plasmidPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions with the following modifications. Bacteria suspended in lysis buffer type 7 were treated with 0.2–1 mg/ml of lysozyme (Sigma-Aldrich) for 3–4 min at room temperature and then lysed by adding lysis buffer type 8. Extracted plasmids were introduced into *P. larvae* DTK384 by electroporation as described above. Transformants carrying pJ18TS1mac were selected on MYPGP agar containing 1 μg/ml of TS and a representative transformant (DTK384pJ18TS1mac) (Table 7) was used for further analyses.

Antimicrobial susceptibility tests for *P. larvae* strains

Putative macrolide resistance gene expression vector-introduced *P. larvae* DTK384 and pMX2-TA-introduced DTK384 (DTK384control) were cultured on MYPGP agar supplemented with 16 μg/ml of CP for two days under air plus 5% CO_2 conditions. The pJ18TS1mac-introduced DTK384 (DTK384pJ18TS1mac) was cultured on MYPGP agar supplemented with 2 μg/ml of TS under the same conditions. Antimicrobial susceptibility tests of the strains for TS, erythromycin (EM; FUJIFILM Wako Pure Chemical Corp.) and LCM were performed by agar dilution methods according to CLSI, M07-A10, except that MICs were measured on MYPGP agar plates after a 48-h incubation at 35℃ under air plus 5% CO_2 conditions. The antimicrobial concentration range tested was 0.0031–256 μg/ml. *S. aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 cultured on MH agar for 20 h at 37℃ under aerobic and air plus 5% CO_2 conditions, respectively, were employed as the quality control strains, and antimicrobials used were confirmed to have acceptable potency after a 20-h incubation of *E. faecalis* and *S. aureus*-inoculated MH agar plates at 35℃ under aerobic conditions.

pJ18TS1mac stability tests in *P. larvae*

To investigate pJ18TS1mac stability in *P. larvae* under nonselective (no

antimicrobial) conditions, DTK384pJ18TS1mac cultured for approximately two days on MYPGP agar containing 2 μg/ml of TS at 35℃ under air plus 5% CO_2 conditions was suspended in MYPGP broth to an optical density at 600 nm of approximately 0.2, and 10 μl of the suspension (inocula) was inoculated into 2 ml of non-selective MYPGP broth. After an approximately 24-h culture at 35℃ with shaking (200 rpm), 1–2 μl of the culture was inoculated into 2 ml of the fresh non-selective MYPGP broth and further cultured for 24 h under the same conditions. After 5, 10, 15 and 25 cycles of the non-selective broth culture steps, serial dilutions of the culture were inoculated onto selective (2 μg/ml of TS) or nonselective MYPGP agar plates. After 2–3-day culture of the plates at 35℃ under air plus 5% CO_2 conditions, plasmid retention rates were calculated by the following formula: the plasmid retention rate = the number of colonies grown on selective MYPGP agar plate/the number of colonies grown on nonselective MYPGP agar plate × 100%. Data were collected from five independent culture tubes. Differences in the average retention rate of pJ18TS1mac among inocula and bacteria at 5, 10, 15, and 25 passages were analyzed by Friedman's test. All tests were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan) (65), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). A value of P <0.05 was considered as the threshold for significance.

Results

Introduction of putative macrolide resistance genes into *P. larvae* DTK384

Among the five target resistance genes/region (blue lines in Figure 4), the *oleC* gene of isolate J43TS9, which encodes ATP-binding cassette (ABC) antibiotic efflux pump, was unable to be cloned into pMX2-TA in *E. coli*, possibly due to toxicity of the product to *E. coli*; therefore, this gene was not used for further analysis. As shown in Figure 5, the *ermC* from plasmid pJ18TS1mac of *O. oncorhynchi* subsp. *incaldanensis* J18TS1, *ermL-ermB* and *ermB* from plasmid pJ45TS6 of *Paenibacillus* sp. J46TS6, and *lsaB* from an ICE of *P. cineris* J43TS9 were successfully introduced into and expressed in *P. larvae* DTK384, and the resultant *P. larvae* strains were designated DTK384ermC, DTK384ermLB, DTK384ermB and DTK384lsaB, respectively (Table 7). As a control, DTK384 with the empty vector pMX2-TA (DTK384control) was also prepared.

Change in antimicrobial susceptibility in putative macrolide resistance gene-introduced *P. larvae* strains

In the antimicrobial susceptibility tests, in addition to the 16-membered ring macrolide TS, EM was used as a representative 14-membered ring macrolide. As macrolide resistance genes may confer cross-resistance to lincosamides, LCM was also used for the tests.

The parent strain DTK384 exhibited the expected antimicrobial susceptibility, with MICs of TS, EM, and LCM being 0.25, 0.125, and 0.25 μg/ml, respectively, which is consistent with the previous study (30). In addition, DTK384control (the empty vector-introduced DTK384) showed almost the same susceptibility (MICs of TS, EM, and LCM; 0.25, 0.0625, and 0.25 μg/ml, respectively) with DTK384 (Table 7). Among the putative macrolide resistance genes, *lsaB* conferred neither macrolide nor lincosamide resistance to *P. larvae* (MICs of TS, EM, and LCM for DTK384lsaB; 0.25, 0.125, and 0.25 μg/ml, respectively) (Table 7). In contrast, introduction of *ermB*

into DTK384 significantly reduced the susceptibility of the strain (DTK384ermB) to EM and LCM (MICs; 64 and >256 μg/ml, respectively). In addition, co-introduction of *ermL* and *ermB* into the strain made *P. larvae* (DTK384ermLB) more resistant to EM and TS (MICs; >256 and 1 μg/ml, respectively) than DTK384ermB (Table 7). Introduction of *ermC* into *P. larvae* DTK384 also significantly reduced the susceptibility of the strain (DTK384ermC) to EM and LCM (MICs; >256 and >256 μg/ml, respectively). Furthermore, DTK384ermC showed the lowest susceptibility to TS (MIC, 8 μg/ml) among the strains tested in this assay. Thus, among the genes tested, the *ermC* gene in pJ18TS1mac, and *ermL* and *ermB* genes in pJ45TS6 have the potential to confer macrolide and lincosamide resistance to *P. larvae*. Since pJ18TS1mac has the characteristics of mobilizable plasmids and *ermC* reduced the TS susceptibility of strain DTK384 the most, the *ermC* gene carried by pJ18TS1mac was considered the most important macrolide resistance gene in the emergence of prophylactic resistant strains in *P. larvae*.

Structure of plasmids carrying macrolide resistance genes found in honey-derived bacteria and their possible transmission to *P. larvae*

As introduced in the Introduction of this Chapter, horizontal transfer of plasmids by conjugation requires T4SS, T4CP, relaxase, and *oriT*. ICEs and conjugative plasmids have all the elements for self-transfer and therefore can transfer to another cell on their own. Even if the plasmid does not have T4SS and T4CP, it can transfer to another cell with the help of ICEs and conjugated plasmids if it has relaxase and *oriT*. As the pJ18TS1mac has *oriT* and the *mob* gene that encodes relaxase (Figure 3), it may transfer to other bacteria with the help of other MGEs. Because isolate J18TS1, the host of pJ18TS1mac, has putative ICEs containing T4SS and T4CP in the chromosome (Table 8), the isolate has the potential to horizontally transfer pJ18TS1mac to other bacterial strains/species via conjugation. Although isolate J45TS6 also possesses putative ICEs in the chromosome (Table 8), pJ45TS6 was not considered to be a transferable plasmid due to the absence of the *mob* gene.

In order to investigate whether J18TS1 and J45TS6 can directly transfer their macrolide-resistant plasmids to *P. larvae*, the author performed filter mating tests using the two honey-derived isolates as donors and *P. larvae* M20 as the recipient. In the filter mating tests, mitomycin C was used to induce ICEs on the donors' chromosomes and express their conjugation machineries. The MICs of mitomycin C in J18TS1 and J45TS6 were 0.03125 and 0.0625, respectively, and two conditions of mitomycin C (i.e., 1/2 and 1/4 MIC of mitomycin C) were tested for the induction. However, neither J18TS1 nor J45TS6 transferred the plasmids to *P. larvae* under the experimental conditions tested in this study.

Impact of pJ18TS1mac on antimicrobial susceptibility of *P. larvae* DTK384 and the plasmid stability in the *P. larvae* strain

Although the plasmids of J18TS1 and J45TS6 did not transfer to *P. larvae* in the filter mating tests, the possibility of horizontal transfer of the macrolide-resistant plasmids, particularly pJ18TS1mac, to *P. larvae* under natural environmental conditions cannot be completely ruled out. If transferred plasmids are stably maintained in *P. larvae*, TS-resistant *P. larvae* may spread in Japan like OTC-resistant strains in other countries. Therefore, in order to investigate whether pJ18TS1mac can replicate in and be stably inherited by *P. larvae*, the author introduced the plasmid into *P. larvae* DTK384 by electroporation and investigated plasmid retention rates in the strain under non-selective culture conditions.

The pJ18TS1mac-introduced *P. larvae* strain, DTK384pJ18TS1mac, became resistant to macrolide and lincosamide antibiotics as expected (MICs of TS, EM, and LCM for DTK384pJ18TS1mac: 16, >256 and >256 μg/ml, respectively) (Table 7). Even after 10 passages of the strain under nonselective (no antibiotic) culture conditions, almost all *P. larvae* cells stably retained the plasmid. After 15 passages, the average plasmid retention rate decreased to 61.11 ± 23.2%, but the rate remained approximately 70% in four of the five test culture tubes. Although the average retention rate decreased to 26.12 ± 27.80% after 25 passages, the rate varied depending

on the culture tubes (0.22–77.78%) and there were no significant differences in the average retention rate throughout the experimental period (Friedman's test, P = 0.0755) (Table 9 and Figure 6). This suggested that the macrolide resistance plasmid can be inherited in the *P. larvae* population for a long period even under nonselective conditions after acquiring the plasmid.

Discussion

Macrolides and lincosamides are antibiotics that inhibit protein synthesis, particularly the elongation step, by targeting the bacterial ribosome. In the ribosome, the peptidyl-transferase center (PTC) and adjacent nascent peptide exit tunnel (NPET) are the key players in protein elongation and function in catalyzing the peptide bond formation and the emergence of the nascent chain, respectively. Macrolides bind to a site within the NPET, whereas lincosamides are PTC-targeting antibiotics (66, 67, 68, 69). Among the four genes tested, *lsaB* located in an ICE of *P. cineris* J43TS9 is considered to encode an ATP binding cassette F (ABC-F) type ribosomal protection protein. ABC-F proteins are known to confer resistance to a number of clinically relevant antibiotics targeting the ribosome PTC/NPET region by interacting with the ribosome and displacing the bound drug (i.e., ribosomal protection mechanism) (70, 71, 72). However, expression of *lsaB* in *P. larvae* affected neither macrolide nor lincosamide susceptibility (Table 7). ABC-F proteins have been classified into three groups based on antibiotic resistance (Msr homologs [macrolides and streptogramin B], Vga/Lsa/Sal homologs [lincosamides, pleuromutilins and streptogramin A] and OptrA homologs [phenicols and oxazolidinones]) (72, 73). Although the gene of J43TS9 was detected as *lsaB* by RGI, its product was only loosely similar to the reference sequence of LsaB in CARD (Tables 4 and 5). This relatively low similarity to LsaB and our results suggest that neither macrolides nor lincosamides are the targets of the *lsaB* product of J43TS9.

In contrast, introduction of the *ermC* and *ermB* genes located in plasmids of J18TS1 and J45TS6, respectively, into *P. larvae* DTK384 markedly reduced the susceptibility of the strain to EM and LCM (Table 7). This was not surprising because the putative products of the two genes were similar to the methyltransferases that modify a specific adenine residue (A2058, *E. coli* numbering) of 23S rRNA and confer macrolide-lincosamide-streptogramin B (MLSB) resistance (74, 75). In the upstream region of *ermB*, an open reading frame (*ermL*) encoding the leader peptide was present, suggesting that expression of *ermB* is inducible. Leader peptide and inducible *erm*

genes are known to constitute an operon, and expression of the *erm* gene is normally repressed due to sequestration of the *erm* ribosome binding site in the mRNA secondary structure. In the presence of macrolides, ribosomes stall during the translation of the leader peptide. The stall triggers a conformational rearrangement in mRNA, resulting in the opening of the *erm* ribosome binding site and activation of *erm* translation (76). As expected from this, introduction of *ermL* further reduced the EM susceptibility of *P. larvae* (MIC of EM increased from 64 mg/ml to >256 mg/ml); however, the 16-membered macrolide TS was still relatively effective against *P. larvae* (MIC, 1 mg/ml) (Table 7). Most *erm* genes are known to be induced by the 14 or 15-membered macrolides and not by 16-membered macrolides (77); therefore, in *P. larvae*, expression of the *ermB* gene may not have been efficiently induced by TS. Of note, the hypothetical protein gene located in the *ermL* region was also introduced into *P. larvae* in this study (Figure 4); however, antimicrobial susceptibility of DTK384ermLB suggests that the gene does not have the ability to greatly reduce the TS susceptibility of *P. larvae*.

In contrast to *ermB* in pJ45TS6, no leader peptide gene was found in the upstream region of the *ermC* gene in pJ18TS1mac, suggesting constitutive *ermC* expression in bacteria. Indeed, the MIC of TS for *P. larvae* DTK384 increased 32-fold only by introducing the *ermC* gene (DTK384ermC in Table 7). However, TS was still more effective in DTK384ermC (MIC, 8 mg/ml) than EM (>256 mg/ml). Macrolides consist of a macrocyclic lactone ring carrying one or more sugar moieties (76). EM carries two monosaccharides, cladinose and desosamine, at the C3 and C5 positions of the ring, respectively. When it binds to the 50S subunit of ribosomes, the macrocyclic lactone ring associates the NPET wall formed by positions 2057–2059 of 23S rRNA, whereas the monosaccharide moiety at the C5 position points toward the PTC. However, it cannot span the distance to the catalytic center (74, 78). In contrast, TS has a disaccharide at the C5 position of the ring and reaches further toward to the PTC (74, 78). Such structural features of TS may have increased the affinity of the drug with ribosomes even in the presence of methylated A2058 in 23S rRNA and exhibited stronger antimicrobial effects against *P. larvae* than EM.

However, proliferation of *ermC*-positive *P. larvae* in larvae will hardly be arrested even by TS. In the previous study by Reynaldi et al. (79), beehives with 25,000 adult bees were treated with TS by dusting methods; i.e., the mixture consisting of 114 g of confectioner's sugar, 5 g of cherry jelly and 1 g of TS was divided into four, 250 mg of TS to 30-g portions, and each portion was sprinkled over the ends of the hives' top bars for four weeks at one-week intervals. According to the article, the mean concentration of TS in 2-day-old larvae measured by microbiological assay with *Geobacillus stearothermophilus* ATCC 12980 was always less than 4 μg/ml (79). In another previous study, honey bee colonies containing around 30,000 worker bees were fed 500 g of synthetic honey with 200 mg or 400 mg of TS for 48 h, and the maximum concentration of TS in 48-h-old larvae was less than 1 μg/g even when treated with 400 mg of TS (80). Similar to the study by Reynaldi et al. (79), TS is used by dusting methods in Japan, and only 150 mg of TS is used for 10,000 adult bees. The dose of TS in Japan is lower than and almost equivalent to those in the studies by Reynaldi et al. (79) and Bernal et al. (80), respectively; therefore, the TS concentration in young larvae is expected to be less than 1–4 μg/ml. In contrast, MICs of TS for DTK384ermC and DTK384pJ18TS1mac were 8–16 μg/ml, suggesting that *ermC*-positive *P. larvae* is clinically resistant to TS. Therefore, acquisition of *ermC* by *P. larvae* is one of the greatest threats to apiculture in Japan.

As the *ermC*-positive plasmid pJ18TS1mac possesses the *mob* gene (Figure 3), it is considered a mobilizable plasmid and may transfer to other bacterial cells using conjugation machineries encoded by other MGEs. Although pJ18TS1mac in *O. oncorhynchi* subsp. *incaldanensis* J18TS1 did not transfer to *P. larvae* DTK384 under the mating conditions tested in this study, we cannot exclude the possibility that the plasmid transfers to *P. larvae* under specific conditions in the natural environment. In Bergey's manual (81), *O. oncorhynchi* subsp. *incaldanensis* is described to be susceptible to EM and LCM; however, isolate J18TS1 was resistant to TS and LCM, and *ermC* in pJ18TS1mac was able to confer EM resistance. Therefore, J18TS1 is also considered to have acquired pJ18TS1mac from other bacteria horizontally, and thus, it is also possible that *P. larvae* acquires pJ18TS1mac or similar plasmids from other

bacteria in the natural environment. As demonstrated in this study, pJ18TS1mac can be maintained in the *P. larvae* population for a long period. Stable plasmid inheritance can be achieved by several means such as (i) integration into the host chromosome, (ii) incorporating genes that participate in plasmid stable inheritance, like the toxin-antitoxin systems, into the plasmid, (iii) incorporating partitioning systems such as the ParABS systems and (iv) reducing plasmid size and increasing plasmid copy number in bacterial cells (82). As pJ18TS1mac encodes a rolling circle replication initiator protein (Rep) (Figure 3; accession number LC586958), it is considered a rolling circle replicating-plasmid. Rolling circle replicating-plasmids usually do not carry specialized partitioning systems but ensure their stable inheritance because of their elevated plasmid copy numbers so that the probability of plasmid loss is reduced (82). Indeed, pJ18TS1mac possesses neither the toxin-antitoxin systems nor the ParABS systems and its size is small (3,869 bp) (Figure 3). Therefore, pJ18TS1mac may have been maintained stably in *P. larvae* because of its high copy number in the cells. Due to the stable inheritance, once *P. larvae* acquires this plasmid in the field, TS-resistant *P. larvae* may be selected by the use of the prophylactic for AFB and spread throughout Japan. A putative mobilizable plasmid carrying the *tet*(L) gene (pJ18TS1tet) was also found in J18TS1. Although the use of OTC in apiculture is not allowed in Japan, OTC-resistant *P. larvae* may also be selected and spread in Japan if the drug is used illegally. Furthermore, the use of these drugs as prophylactics may affect the antimicrobial susceptibility of another foulbrood pathogen, *M. plutonius*, as seen by the use of MRM in Japan (83). Although microorganisms in honey primarily originate from pollen, the digestive tracts of honey bees, dust, air, earth, and nectar, honey can also be contaminated with microorganisms during and after harvesting process by humans, equipment and buildings (26). Therefore, the isolates and their resistance genes found in commercially available honey in this study may not have necessarily existed in comb honey. However, our present study suggests the possibility of the future emergence of antimicrobial-resistant *P. larvae* by acquiring resistance genes from bacteria in honey. Therefore, it is necessary to use the prophylactic more carefully to prevent or delay the emergence. Development of novel molecular methods to detect

resistance genes on MGEs together with foulbrood pathogens in honey will be helpful to use the prophylactic more carefully. Elucidation of the TS and LCM resistance mechanisms of North American *P. larvae* (20) is also required to predict and prevent the further emergence of antimicrobial resistant *P. larvae*.

Summary

Genome analysis of TS-resistant Japanese honey-derived bacteria in Chapter I revealed the presence of many putative macrolide resistance genes in the genomes. Four of them (*ermC*, *ermB*, *oleC*, and *lsaB*) were located on MGEs, and the gene (*ermL*) that controls the expression of *ermB* was also found in the upstream region of *ermB*. To investigate whether these genes have the potential to confer TS resistance to *P. larvae*, they were artificially introduced to the TS-susceptible *P. larvae* strain DTK384 (MIC of TS, 0.25 μg/ml) by using the expression vector pMX2-TA. As a result, the *P. larvae* strain transformed with *ermB* and *ermC*, which encodes 23S rRNA methyltransferases, showed reduced susceptibility to macrolides. In particular, the introduction of *ermC* increased the MIC of TS for the *P. larvae* strain to 8 μg/ml. The *ermC* gene was on the small putative mobilizable plasmid, pJ18TS1mac, possessed by an *O. oncorhynchi* strain. Although pJ18TS1mac did not naturally transfer from *O. oncorhynchi* to *P. larvae* by *in vitro* mating tests, the *P. larvae* strain electrotransformed with pJ18TS1mac showed further reduced susceptibility to TS (MIC of TS, 16 μg/ml). Furthermore, *P. larvae* with pJ18TS1mac retained the plasmid stably even under non-selective conditions. These results suggest that, once *P. larvae* acquires pJ18TS1mac, TS-resistant *P. larvae* could persist for a long period in the field. Since the results of Chapters I and II also suggest that TS-resistant *P. larvae* may emerge in Japan, more careful use of TS is needed to prevent or delay the emergence.

Table 6. PCR primers used for cloning in Chapter II

Primers	Sequence (5'-3')	Target	Description	Polymerase	PCR program
For construction of the antimicrobial resistance gene expression vectors[a]					
ermC_F	ACCTCTGCAGCCTCAGCAAAGGGGGTTTTG	The *ermC* region in isolate J18TS1	For amplification and sequencing of the *ermC* region. PstI and EcoRI sites are underlined.	iProof (Bio-Rad Laboratories, Inc., Hercules, CA, USA)	98°C 1 min - 98°C 10 sec, 55°C 20 sec, 72°C 2 min 30 sec (35 cycles) - 72°C 5 min
ermC_R	TTTAGAATTCACAAGACAAGTTAAGGGGTG				
ermB_F	GCTGCTGCAGGCCTACGAGGAATTTGTATC	The *ermB*/*ermL-ermB* regions in isolate J45TS6	For amplification and sequencing of the *ermB*/*ermL-ermB* regions. PstI and EcoRI sites are underlined.		
ermB_F2	ATTTCTGCAGGTTAGATTAATTCCTACCAG				
ermB_R	AATGGAATTCATAGAATTATTTCCTCCCG				
lsaB_F2	CATGCTGCAGTTGAATGAATGTCGCTCGG	The *lsaB* region in isolate J43TS9	For amplification and sequencing of the *lsaB* region. PstI and BamHI sites are underlined.	KOD FX (TOYOBO Co., Ltd., Osaka, Japan)	94°C 2 min - 98°C 10 sec, 55°C 30 sec, 68°C 1 min 30 sec (30 cycles) - 68°C 2 min
lsaB_R	AACAGGATCCAAGGCGGATCGGTATAAATC				
oleC_F	CAAACTGCAGAGGCCATAAAAAAGGCAACG	The *oleC* region in isolate J43TS9	For amplification and sequencing of the *oleC* region. PstI and EcoRI sites are underlined.		
oleC_R	AAGCGAATTCCAAGCATAACGCCCATATCG				
For reverse transcription PCR[a]					
ermC_A_F2	AATTGGCTCAGGAAAAGGGC	*ermC*	For confirmation of introduced-*ermC* expression in *P. larvae*.	ExTaq (Takara-Bio, Kusatsu, Japan)	95°C 2 min - 95°C 20 sec, 55°C 30 sec, 72°C 30 sec (35 cycles) - 72°C 5 min
ermC_A_R2	TAGCAAACCCGTATTCCACG				
ermB_BCD_F	CATTTAACGACGAAACTGGC	*ermB*	For confirmation of introduced-*ermB* expression in *P. larvae*.	KOD FX (TOYOBO)	94°C 2 min - 98°C 10 sec, 55°C 30 sec, 68°C 30 sec (30 cycles) - 68°C 5 min
ermB_BCD_R	CTGGAACATCTGTGGTATGG				
lsaB_EF_F	ACCTGACGTTTGCCTATGAG	*lsaB*	For confirmation of introduced-*lsaB* expression in *P. larvae*.		
lsaB_EF_R	ACAACGTATTAAACGGGCGG				
ermL_F	GGTAACGGTTATTGCAGGTG	*ermL*	For confirmation of introduced-*ermL* expression in *P. larvae*.		
ermL_R	CTGGTAGGAATTAATCTAAC				

[a] Positions of the primers are shown in Figure 4.

Table 7. *P. larvae* strains used in Chapter II and their antimicrobial susceptibility

Strain	Description[a]	Introduced macrolide resistance genes[b]	Putative function of the introduced resistance genes[b]	MIC (μg/ml)[c]		
				TS	EM	LCM
DTK384[d]	The genotype ERIC II-ST10 strain isolated from a diseased *Apis mellifera* larva in Japan	NA	NA	0.25	0.125	0.25
DTK384ermC	The *ermC* expression vector-introduced DTK384	*ermC*	23S rRNA methyltransferase	8	>256	>256
DTK384ermLB	The *ermL-ermB* expression vector-introduced DTK384	*ermL* and *ermB*	23S rRNA methylase leader peptide (*ermL*) 23S rRNA methyltransferase (*ermB*)	1	>256	>256
DTK384ermB	The *ermB* expression vector-introduced DTK384	*ermB*	23S rRNA methyltransferase	0.25	64	>256
DTK384lsaB	The *lsaB* expression vector-introduced DTK384	*lsaB*	ATP-binding cassette F type ribosomal protection protein	0.25	0.125	0.25
DTK384pJ18TS1mac	pJ18TS1mac-introduced DTK384	pJ18TS1mac with *ermC*	23S rRNA methyltransferase	16	>256	>256
DTK384control	pMX2-TA-introduced DTK384	NA	NA	0.25	0.0625	0.25

[a] ERIC, enterobacterial repetitive intergenic consensus; ST, sequence type.
[b] NA, not applicable.
[c] MIC, minimum inhibitory concentration; TS, tylosin; EM, erythromycin; LCM, lincomycin.
[d] DTK384 was named strain N in the previous report (62).

Table 8. Integrative conjugative elements detected in isolates J18TS1 and J45TS6 by ICE berg 2.0

Isolate	Scaffold	Characteristics of detected ICE[a]									
		Location (nucleotide positions in the scaffold)	Length of ICE	GC content	Type	Major components					
						oriT	DRs (nucleotide positions in the scaffold)	T4SS components	T4CP	Relaxase	Integrase
J18TS1	NODE_2	166156..410059	243,904 bp	37.65%	Putative ICE with T4SS	−	+	attL (166156..166170 / tccaacttattttat) attR (410045..410059 / tccaacttattttat)	+	+	+	+
	NODE_10	88829..120210	31,382 bp	33.91%	Putative ICE without identified DR	−	−		+	+	+	+
J45TS6	NODE_2	108039..466549	358,511 bp	42.93%	Putative ICE with T4SS	−	+	attL (108039..108053 / cgttattcgcgatca) attR (466535..466549 / cgttattcgcgatca)	+	+	+	+
	NODE_13	78110..127552	49,443 bp	36.51%	Putative ICE without identified DR	−	−		+	+	+	+

[a] ICE, integrative conjugative element; DR, direct repeat; T4SS, Type IV secretion system; T4CP, Type IV coupling protein; +, detected; -, not detected.

Table 9. Stability of pJ18TS1mac in *P. larvae* under non-selective conditions[a]

Passage number under non-selective conditions	Average plasmid retention rate ± standard deviation (%)[b]
0 (inocula)	84.0 ± 22.25
5	84.15 ± 22.51
10	103.51 ± 21.87
15	61.11 ± 23.2
25	26.12 ± 27.8

[a] DTK384pJ18TS1mac was used for plasmid stability tests.

[b] Data were collected from five independent culture tubes. There were no significant differences in the average retention rate throughout the experimental period (Friedman's test, $P = 0.0755$).

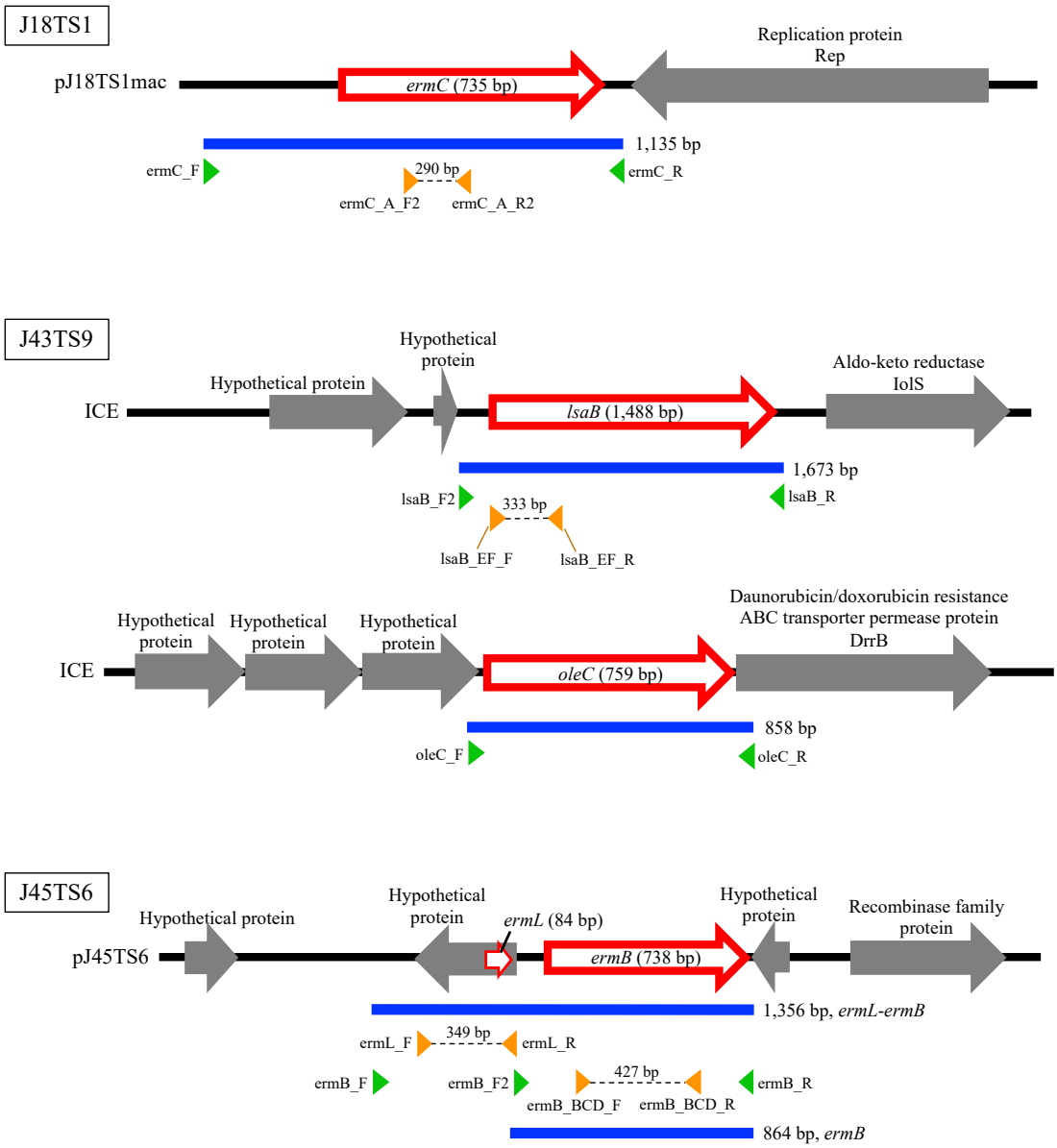

Figure 4. Schematic representation of macrolide resistance gene regions found in putative mobile genetic elements of honey-derived isolates

Blue lines indicate regions amplified by PCR for construction of antimicrobial resistance gene expression vectors, and green arrowheads represent primers used for PCR. Orange arrowheads represent primers used to confirm expression of the introduced macrolide resistance genes in *P. larvae*.

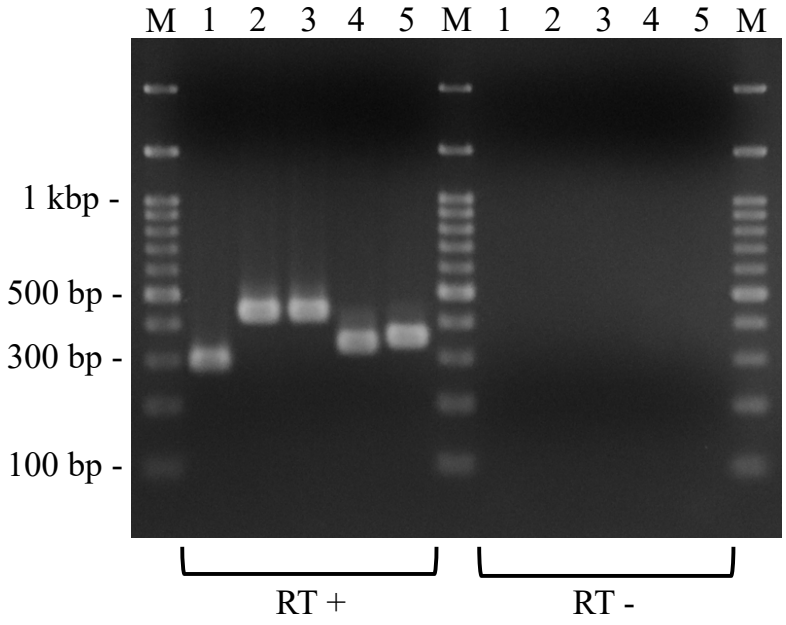

Figure 5. Expression of the introduced putative macrolide resistance genes in *P. larvae*

Total RNA was extracted from *P. larvae* strains cultured on MYPGP agar containing 16 μg/ml of chloramphenicol, and the expression of macrolide resistance genes was assessed by reverse transcription (RT)-PCR. To exclude DNA contamination in the RNA samples, total RNA, which was not treated with reverse transcriptase (RT -), was also used as the template for PCR. Lane 1: *ermC* expression in DTK384ermC; lane 2: *ermB* expression in DTL384ermLB; lane 3: *ermB* expression in DTK384ermB; lane 4: *lsaB* expression in DTK384lsaB; lane 5: *ermL* expression in DTL384ermLB. All introduced putative macrolide resistance genes were expressed in *P. larvae*.

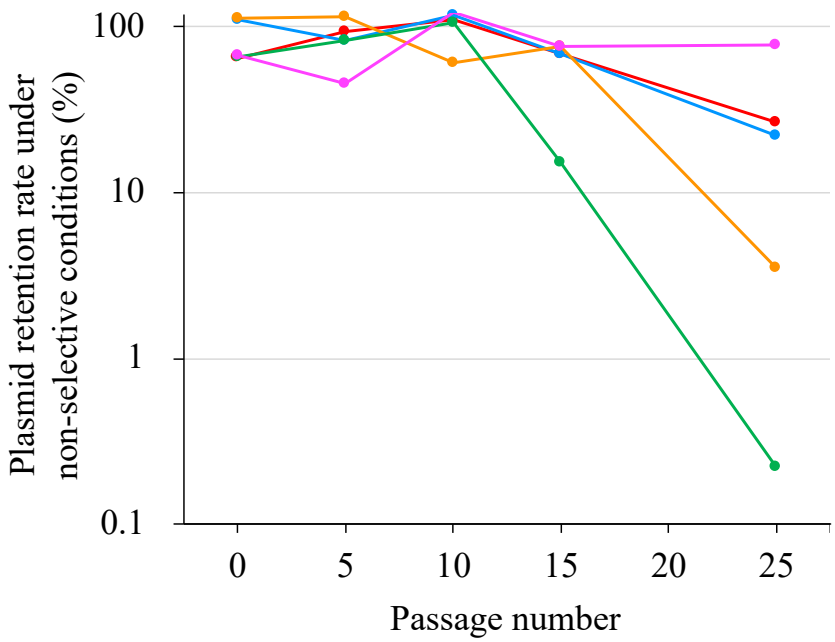

Figure 6. Stability of pJ18TS1mac in *P. larvae*

pJ18TS1mac-introduced DTK384 (DTK384 pJ18TS1mac) was cultured at 35℃ in MYPGP broth under non-selective conditions, and bacteria were subcultured every 24 h by inoculating 1–2 µl of the culture into a fresh non-selective MYPGP broth. After 5, 10, 15 and 25 cycles of the culturing steps, serial dilutions of the culture were inoculated onto selective (2 µg/ml of TS) and non-selective MYPGP agar plates, and plasmid retention rates were calculated by the following formula: the plasmid retention rate = the number of colonies grown on selective MYPGP plate/the number of colonies grown on non-selective MYPGP plate × 100%. Plasmid retention rates of inocula for the first culture step were also measured. Data was collected from five independent culture tubes and results of each culture tube are shown in different colors.

Chapter III

Development of PCR assays to detect different types of foulbrood pathogens and macrolide resistance genes in honey

Introduction

The results of Chapters I and II suggested that *P. larvae* may become resistant to TS by acquiring *ermC* from environmental bacteria in colonies where *P. larvae* and *ermC*-positive bacteria coexist. If the prophylactic were used under such circumstances, TS-resistant *P. larvae* could be selected in the larval midgut and spread throughout the apiary. Therefore, more careful use of the prophylactic is needed to maintain the effectiveness of the only approved prophylactic for AFB. However, because the prevalence of foulbrood pathogens and macrolide-resistant bacteria in Japanese apiaries has not been known, it has not been possible to properly determine which bee colonies should be treated with the prophylactic.

In *P. larvae*, levels of virulence and resistance to disinfectants vary widely among strains (56, 57, 59, 61). For example, *P. larvae* ERIC II strains are known to be more virulent for the individual larva (56, 57, 59) and more resistant to chlorine-based disinfectants (61) than ERIC I strains. Similarly, *M. plutonius* strains also exhibit varying levels of virulence and resistance to disinfectants depending on the strain type (84, 85, 86, 87). *M. plutonius* strains have been grouped into three clonal complexes (CC3, CC12, and CC13) by MLST, and CC3 and CC13 strains with fastidious cultural characteristics and CC12 strains with non-fastidious characteristics are also referred to as typical and atypical strains, respectively (12, 84, 88, 89, 90). In Japan, while typical (CC3 and CC13) and atypical (CC12) *M. plutonius* have both been detected in EFB cases, the isolation of the latter has been more frequent (12, 90). Atypical strains have higher toxicity against larvae (87) and higher resistance to chlorine-based disinfectants (61) than typical strains. Since *M. plutonius* is another important bacterial pathogen in the beekeeping industry, the spread of this pathogen in apiaries should also be monitored. Moreover, given the differences in characteristics between strains, it is important not only to confirm the presence or absence of foulbrood pathogens in apiaries, but also to identify the genotypes/phenotypes of the pathogens to accumulate epidemiological information, understand the ecology of pathogens, and plan comprehensive control measures based on characteristics of the pathogens present in

apiaries. For the prudent use of the prophylactic, it is also important to know the presence of macrolide resistance genes in beehives/apiaries before using the prophylactic. However, no rapid and easy method to detect and distinguish the major types of *P. larvae* and *M. plutonius* simultaneously has yet been reported. Moreover, no assay has been developed to detect macrolide resistance genes in honey that can potentially cause TS resistance in *P. larvae*.

P. larvae spores can remain for many years in honey bee materials (11) and exist not only in diseased colonies, but also in clinically healthy colonies. von der Ohe reported that honey in beehives may be contaminated by *P. larvae* spores 2−3 years before showing clinical signs (45). Although *M. plutonius* is not a spore-forming bacterium, it is also found in honey (14, 46). Therefore, honey is a useful material for surveying the contamination of beehives/apiaries by the pathogens. Honey can also be useful in surveying the contamination of apiaries by macrolide resistance genes, since honey may contain bacteria that harbor macrolide resistance genes as indicated in Chapters I and II.

Since the isolation and detection of foulbrood pathogens from honey using culture methods is time-consuming and not highly sensitive (14, 46, 47, 48, 49), the direct extraction of bacterial DNA from honey and the subsequent detection of specific genes using PCR techniques are useful as rapid and highly sensitive detection methods for foulbrood pathogens. Several DNA extraction methods for *P. larvae* spores in honey have been reported to date (27, 91, 92, 93, 94, 95), and honey has also been used as a material for the extraction of *M. plutonius* DNA (96). However, to the best of the author's knowledge, there are currently no optimized methods to simultaneously extract DNA from *P. larvae* spores and *M. plutonius* vegetative cells in one tube.

Therefore, in Chapter III, a multiplex PCR assay that enables the detection and differentiation of ERIC I and II *P. larvae* and typical and atypical *M. plutonius* in one reaction as well as the detection assays for *ermB* and *ermC* were constructed. Moreover, we selected commercially available kits that efficiently and simultaneously extract DNA from *P. larvae* spores and *M. plutonius* vegetative cells.

Materials and Methods

Bacterial strains

The bacterial strains used in this chapter are listed in Tables 10 and 11. As foulbrood pathogens, 15 *P. larvae* strains (9 ERIC I and 6 ERIC II strains) and 18 *M. plutonius* strains (9 typical and 9 atypical strains) isolated in Japan were selected in order to cover all STs found in AFB and EFB cases in Japan. ATCC 35311, the type strain of *M. plutonius* purchased from the American Type Culture Collection (Manassas, VA, USA) was also included. The representative strains of *P. larvae* and *M. plutonius* listed in Table 10 were used in sensitivity tests of the newly developed multiplex PCR assay. Sixty-five other bacterial strains, including those isolated from Japanese honey and honey bees (97), were used in addition to the foulbrood pathogens for specificity tests. Strains ATCC 6344 and ATCC 64, the type strains of *Paenibacillus alvei* and *Brevibacillus laterosporus*, respectively, were purchased from the American Type Culture Collection (Table 11). The four strains listed in Table 10 were also employed as positive controls for the tests.

Primers and conditions for the multiplex PCR assay for foulbrood pathogens

Primers for the multiplex PCR assay to detect and distinguish between different types of foulbrood pathogens are listed in Table 12. The multiplex PCR assay was performed using the QIAGEN Multiplex PCR Kit (QIAGEN) in a final reaction volume of 20 µl containing 10 µl of 2 × QIAGEN Multiplex PCR Master Mix, 2 µl of Q-solution, an appropriate concentration of each primer (Table 12), and 1 µl (for the sensitivity test) or 2 µl (for the other tests) of template DNA. Cycling conditions consisted of an initial denaturation step at 98℃ for 15 min, followed by 35 cycles of denaturation at 94℃ for 30 sec, annealing at 60℃ for 90 sec, and extension at 72℃ for 60 sec, and a final extension step at 72℃ for 10 min. All PCR amplifications were performed in T100^{TM} Thermal Cycler (Bio-Rad Laboratories). Six microliters of the amplification product were electrophoresed (100 V, 30 min) through a 2% agarose gel and visualized by staining the gel with ethidium bromide.

Bacterial DNA extraction

To assess the sensitivity of the multiplex PCR assay, genomic DNA was manually extracted from representative *P. larvae* strains cultured on MYPGP agar (47) at 35℃ for two days under air plus 5% CO_2 conditions and *M. plutonius* strains cultured on KSBHI agar (12) at 35℃ for three days under anaerobic conditions according to the methods as described in Chapter I and Arai et al. (12), respectively. In brief, bacterial cells cultured as described above were suspended in TE (10 mM Tris–HCl [pH 8.0] and 1 mM EDTA [pH 8.0]) or 5 × TE, treated with lysozyme and mutanolysin and lysed by 10% sodium dodecyl sulfate. The lysates were extracted with an equal volume of phenol, PCI, and chloroform at least once, three times, and once, respectively. Nucleic acids were then precipitated by ethanol and further treated with the following enzymes according to Chapter I and Arai et al. (12): RNase for *P. larvae*, and RNase and proteinase K for *M. plutonius*. After additional extraction with PCI and chloroform, DNA was precipitated and rinsed by ethanol and dissolved in Tris-based buffer. Genomic DNA was serially diluted to a final concentration of 10 fg/μl and used in sensitivity tests. Regarding specificity tests, genomic DNA was extracted from a broad range of bacterial species grown on culture media appropriate for each species (Table 11) using InstaGene Matrix (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions. Genomic DNA was diluted to a final concentration of 0.25 ng/μl and used in the multiplex PCR assay.

Preparation of honey spiked with *P. larvae* spores and *M. plutonius* cells

Spore suspensions of *P. larvae* DTK386 (ERIC I) and DTK384 (ERIC II) were prepared according to previously described procedures (61), and spores in sterile water were stored at 4℃ until used. The number of spores in a suspension was calculated using a hemocytometer under an inverted microscope (CKX41, OLYMPUS, Tokyo, Japan). To calculate the germination rates of spore suspensions, germinable spore concentrations were also measured by plating serial dilutions of spore

suspensions onto MYPGP agar after a heat shock treatment at 80, 85, 90, 95, and 100℃ for 10 min and counting colonies after the incubation of plates at 35℃ for six days under air plus 5% CO_2 conditions. *M. plutonius* typical strain DAT606 and atypical strain DAT561 cultured at 35℃ for three days on KSBHI agar under anaerobic conditions were collected using sterile cotton swabs, suspended in KSBHI broth containing 10% glycerol, and kept at –80℃ until used. Colony-forming units (CFU) per milliliter of *M. plutonius* cell suspensions were calculated by plating serial dilutions of suspensions onto Basal agar plates (14) and counting colonies after the incubation of plates at 35℃ for three days under anaerobic conditions. Since *M. plutonius* cells develop in chains (63), the author also calculated the average number of cells in a single chain in the suspensions after staining *M. plutonius* cells using Gram's method and counting cells in 100 chains under a microscope (CME, Leica, Wetzlar, Germany). A single colony on an agar plate originates from a single chain of cells; therefore, *M. plutonius* cell concentrations (cells/ml) in suspensions were measured using the following formula: cells/ml = CFU/ml × the average number of cells in a single chain. To prepare foulbrood pathogen-spiked honey, the author selected honey harvested in New Zealand where the occurrence of EFB has not been reported. The absence of *P. larvae* in honey was confirmed by plating sediments of honey onto MYPGP agar containing 20 μg/ml nalidixic acid and 10 μg/ml pipemidic acid (47) after a heat shock treatment at 80, 85, 90, 95, and 100℃ for 10 min and incubating the plates at 35℃ for six days under air plus 5% CO_2 conditions. The absence of foulbrood pathogens was also confirmed using the DNA extraction and multiplex PCR methods developed in this chapter. Foulbrood pathogen-free honey was then spiked with appropriate amounts of DAT606, DAT561, DTK386, and DTK384 cell/spore suspensions to yield final concentrations of 1,000, 100, 10, and 1 bacterial cell(s)/strain/ml of honey.

DNA Extraction from honey

Four commercially available DNA extraction kits were used for extraction of

bacterial DNA from honey: DNeasy Plant Mini Kit (QIAGEN), which is recommended for the extraction of *M. plutonius* DNA from honey in the "Standard methods for European foulbrood research (14)", DNeasy PowerSoil Kit (QIAGEN), which is designed to isolate DNA including that of bacterial spores from environmental samples, such as soil, and Johne-Spin ver. 2 and Johne Pure Spin (FASMAC Co., Ltd., Atsugi, Japan), which are the kits used to extract DNA from *Mycobacterium avium* subsp. *paratuberculosis* in bovine feces in Japan. Five milliliters of honey was diluted to 50% (v/v) with sterile water and centrifuged at approximately 12,000 × g for 15 min to obtain sediments. After sediments were suspended in 500 µl of sterile water, bacterial DNA was extracted from suspensions using the four kits according to each manufacturer's instructions. In extraction by DNeasy PowerSoil Kit, bacteria in sediments were mechanically lysed in PowerBead tubes by vortexing at the maximum speed for 10 min using the Vortex-Genie 2 mixer (Scientific Industries, Inc., Bohemia, NY, USA) and the vortex adapter 13000-V1-24 (QIAGEN). When DNA was extracted by Johne-Spin ver. 2 and Johne Pure Spin, bacteria in sediments were mechanically lysed in Beads tubes for the kits by vortexing at 4,000 rpm for 5 and 2 min, respectively, using the Multi-beads shocker MB3000 (Yasui Kikai Corp., Osaka, Japan).

Development of real-time PCR assays for the detection of *ermC* and *ermB*

To develop real-time PCR assays, multiple *ermC* and *ermB* gene sequences were retrieved from the GenBank database (Table 13), and specific primers were designed for the conserved regions of the retrieved genes (Table 14). Primer specificity was confirmed using a BLAST search (http://blast.ncbi.nlm.nih.gov). QuantiTect SYBR Green PCR kits (QIAGEN) were used to perform real-time PCR assays using a final reaction volume of 25 µL containing 12.5 µL of 2x QuantiTect SYBR Green PCR Master Mix, 0.3 µM of each primer, and 1 µL of template DNA. The PCR cycling conditions are listed in Table 14. All amplifications were performed using the QuantiStudioTM 3 Real-Time PCR System (Applied Biosystems). Each sample was

tested in duplicates. Genomic DNA of *ermC*-positive *O. oncorhynchi* subsp. *incaldanensis* strain J18TS1 and *ermB*-positive *Paenibacillus* sp. strain J45TS6 was extracted using InstaGene™ Matrix (Bio-Rad Laboratories) according to the manufacturer's instructions and used as positive controls. Positive controls and non-template negative controls were placed on each reaction plate.

Preparation of honey spiked with honey-derived macrolide-resistant bacteria

To confirm the detection of representative bacteria with *ermC*/*ermB* genes in honey samples, we prepared bacterial spore-spiked honey using *ermC*- and *ermB*-free honey harvested in Thailand. The absence of these genes in the honey sample was confirmed using the real-time assays described above. As representative bacteria with *ermC* and *ermB*, strains J18TS1 and J45TS6 were used, respectively (Table 15).

Spore suspensions of strains J18TS1 and J45TS6 were prepared according to previously described procedures (61), with certain modifications to the culture conditions (Table 15). Spores in sterile water were stored at 4℃ until used. The number of spores in each suspension was calculated using hemocytometers under an inverted microscope (CKX41, OLYMPUS). The spore suspensions were then added to honey samples to obtain final solutions of 1,000, 100, 10, and 1 spore(s)/mL honey. Genomic DNA was extracted from the spore-spiked honey samples using Johne Pure Spin as described above.

Results

Target genes and primers for the multiplex PCR assay for foulbrood pathogens

In this chapter, a multiplex PCR assay with five primer sets to detect and differentiate between the ERIC I, ERIC II, and the other ERIC-type strains of *P. larvae* and typical and atypical strains of *M. plutonius* in one reaction was designed. To detect typical *M. plutonius*, the multiplex PCR employed previously reported primers targeting the Na^+/H^+ antiporter gene (*napA*) (97). To detect ERIC I *P. larvae* and atypical *M. plutonius*, the author selected the toxin 1 gene (*plx1*) (DDBJ/EMBL/GenBank accession no. KC456421) and the Fur family transcriptional regulator gene (AP012282; locus tag, MPD5_0863), respectively, as targets based on previous studies (57, 97, 98) and designed new primer sets to give specific PCR products of easily distinguishable sizes. To select the target for ERIC II *P. larvae*, the author investigated nine sequences reported as ERIC II specific in the previous study (99) by the BLAST search (http:// blast.ncbi.nlm.nih.gov) and Sequencher ver. 5.4.6 (Gene Codes Corp., Ann Arbor, MI, USA) using *P. larvae* genome sequence data in the GenBank database (Table 16). As the sequence deposited in the database as neutral invertase-like protein gene (accession no. FI763267) was identified as the sole ERIC II-specific sequence among the nine, a newly designed specific primer set for the sequence was employed for the multiplex PCR assay. To detect *P. larvae* genotypes other than ERIC I and II, we additionally employed previously reported *P. larvae*-specific primers targeting the 16S rRNA gene (47, 100). The lengths of the primers ranged between 20 and 28 bp, and primer sequences and product sizes are listed in Table 12. The specificities of the primers against the DNA sequences of bacteria available in the GenBank database were confirmed by BLAST searches.

Specificity and sensitivity of the multiplex PCR assay

To evaluate the specificity of the PCR assay, we employed a wide range of bacterial strains, including those isolated from honey and healthy and diseased honey bee larvae (Table 11). Under the optimized conditions described in the Materials and

Methods section in this Chapter, multiplex PCR primers yielded specific PCR products of the expected sizes from all *P. larvae* ERIC I (973 bp and 554 bp) and ERIC II (973 bp and 333 bp) strains and all *M. plutonius* typical (187 bp) and atypical (257 bp) strains, while no products were generated from any other bacterial strains tested in the present study (Figure 7 and Table 11). Differently sized specific products were easily distinguishable from each other on agarose gels (Figure 7). The multiplex PCR assay detected the 16S rRNA gene of *P. larvae* from 100 fg of genomic DNA, and the other genes were detected from 1 pg of genomic DNA (Figure 8).

Comparison of DNA extraction efficiency from bacteria in honey by commercial DNA extraction kits

In this study, honey samples spiked with the representative strains of *P. larvae* and *M. plutonius* (Table 10) at the final concentration of 1,000 bacterial cells/strain/ml were prepared, and the efficiency of four commercial kits (DNeasy Plant Mini Kit, DNeasy PowerSoil Kit, Johne-Spin ver. 2, and Johne Pure Spin) to extract bacterial DNA in honey was compared using the multiplex PCR assay for foulbrood pathogens. As shown in Figure 9, *P. larvae* and *M. plutonius* DNA were not efficiently extracted from honey by DNeasy PowerSoil Kit or DNeasy Plant Mini Kit. Although the 16S rRNA gene of *P. larvae* and the typical *M. plutonius*-specific gene were barely detected in the DNA samples extracted by the two kits, the other products were invisible on agarose gels (Figure 9). In contrast, all target genes were detected from DNA extracted by Johne-Spin ver. 2 and Johne Pure Spin (Figure 9).

When Johne Pure Spin was used, all genes were detectable even in honey spiked at a concentration of 100 bacterial cells/strain/ml (Figure 10); therefore, we used Johne Pure Spin in subsequent experiments. Since most of the target genes were undetectable in honey spiked at concentrations of 10 and 1 bacterial cell(s)/strain/ml (Figure 10), the detection limit of this protocol (i.e., DNA extraction by Johne Pure Spin and the multiplex PCR assay) was 100 bacterial cells/strain/ml of honey. DNA extracted from 5 ml of spiked honey was eluted with 50 µl of elution buffer, and 2 µl

of the extract was used for one reaction of the multiplex PCR assay; therefore, any types of *P. larvae* and *M. plutonius* may be detected by the multiplex PCR assay when DNA from 20 cells is present in one PCR reaction. The germination rates of *P. larvae* DTK384 and DTK386 spore solutions used in the present study were 13.5% and 2.1% after heat shock treatment at 80℃ for 10 min and 90℃ for 10 min, respectively; therefore, their detection limits correspond to 13.5 and 2.1 CFU/ml of honey, respectively. In addition, the average numbers of cells in a single chain of strains DAT606 and DAT561 used for the preparation of *M. plutonius*-spiked honey were 5.26 and 3.43 cells, respectively, resulting in the detection limits of typical and atypical *M. plutonius* were equivalent to 19 and 29.2 CFU/ml of honey, respectively.

Detection of *ermC* and *ermB* in honey by the real-time PCR assays

Under the optimized conditions described in the Materials and Methods section in this Chapter and Table 14, the primers designed for the detection of *ermC* and *ermB* successfully detected the target genes from honey spiked with strains J18TS1 and J45TS6 spores, respectively, at concentrations of 10 spores/mL or higher. When *ermC* and *ermB* were detected from J18TS1- and J45TS6-spiked honey, the melting point temperatures were 75.0℃ and 75.6℃, respectively.

Discussion

The detection of foulbrood pathogens in clinically healthy colonies facilitates the control and prevention of diseases, and honey is one of the useful materials for assessing the contamination status of bee colonies. Although cultivation is a traditional and popular technique for detecting bacterial pathogens from materials, the detection of foulbrood pathogens from honey bee samples using this technique is time-consuming due to the relatively slow growth of pathogens. In addition, since *P. larvae* and *M. plutonius* require different fastidious culture conditions, they cannot be simultaneously isolated on the same culture medium. Furthermore, other bacteria contaminating honey overgrow and interfere with the isolation of foulbrood pathogens on agar media. To avoid the common limitations associated with culture methods, molecular diagnostic techniques, such as PCR, have been developed and are replacing culture methods. PCR techniques may be easily replicated in laboratories using commercially available reagents and detect more than one target in one reaction using multiple specific primer sets; therefore, they are useful for simultaneously detecting multiple bacterial species without needing to consider the culture characteristics of each bacterium.

Many PCR assays have been developed to detect foulbrood pathogens. Regarding *M. plutonius*, specific PCR assays that detect the 16S rRNA gene (96, 101, 102) and a real-time PCR assay for the manganese-dependent superoxide dismutase gene (*sodA*) (103) have been reported. In addition, Arai et al. (97) developed duplex PCR to easily detect and differentiate between typical and atypical *M. plutonius* strains. Many conventional and real-time PCR assays that target the 16S rRNA gene have been developed for *P. larvae* (91, 92, 93, 95, 100, 104, 105, 106). Moreover, Piccini et al. (94) and Alippi et al. (27) reported PCR assays for the specific detection of *P. larvae* subsp. *larvae* (i.e., ERIC I and II strains under the current taxonomic assignments of *P. larvae* [7, 56]), while Beims et al. (57) developed a triplex quantitative PCR assay that specifically detect ERIC I strains. However, PCR assays specific for ERIC II strains have not yet been developed. In the study of this chapter, the author identified

the sole ERIC II-specific sequence (accession no. FI763267) for the multiplex PCR assay; therefore, the PCR assay developed in this study is the first PCR that specifically detects *P. larvae* ERIC II strains. The multiplex PCR and triplex real-time PCR assays described by Garrido-Bailón et al. (107) and Dainat et al. (108), respectively, simultaneously detect *P. larvae* and *M. plutonius* in one reaction; however, these assays cannot distinguish between the genotypes of *P. larvae* and phenotypes of *M. plutonius*. Therefore, this newly developed multiplex PCR assay is also the first PCR assay that simultaneously detects and differentiates between the major ERIC types of *P. larvae* and typical and atypical strains of *M. plutonius*. However, the number of strains examined for the specificity test was still limited. Since *P. larvae* and *M. plutonius* are prohibited for import in Japan, strains isolated in Japan were mainly used for the specificity test, and, thus, further studies are needed to verify the specificity of our multiplex PCR assay using *P. larvae* and *M. plutonius* strains isolated in other countries.

Among the primers employed for the multiplex PCR assay, those for the 16S rRNA gene of *P. larvae* showed higher sensitivity (detection limit, 100 fg of genomic DNA) than the other primer sets (detection limit, 1 pg of genomic DNA) (Figure 8). This may be attributed to the copy numbers of the target genes because *P. larvae* strains have eight copies of the 16S rRNA gene in the chromosome (109). Therefore, if the number of *P. larvae* cells in a sample is low, even though the *P. larvae* 16S rRNA gene can be detected, its ERIC type may not be determined due to the lower sensitivity of PCR targeting ERIC I and II. Even in such cases, the ERIC type may be determined by performing an additional monoplex PCR using only ERIC I or II-specific primers (e.g., honey no. J113 in Table 17). However, even if the ERIC type cannot be determined, it is possible to determine whether the prophylactic should be used based on the presence or absence of *P. larvae* strains.

Johne-Spin ver. 2 and Johne Pure Spin were both developed for the efficient extraction of DNA from acid-fast bacteria, such as *M. avium* subsp. *paratuberculosis* in bovine feces. According to the manufacturer, Johne Pure Spin produces DNA with

higher quality than Johne-Spin by removing more PCR inhibitors. However, DNA extraction efficacy was almost comparable between Johne-Spin ver. 2 and Johne Pure Spin. Since both kits are widely used in local livestock hygiene service centers in Japan for the examination of Johne's disease, the inspection of foulbrood pathogens using the same kits will be easy to perform by these centers.

Regarding the extraction of DNA from honey using Johne Pure Spin, the detection limit of *P. larvae* DTK384 and DTK386 spores by the multiplex PCR assay was 100 spores/ml of honey. Considering the germination rates of the spore solutions used in the present study, the detection limits for DTK384 and DTK386 correspond to 13.5 and 2.1 CFU/ml of honey, respectively. The detection limits of *P. larvae* spores from honey were previously reported to be 283 spores of *P. larvae* subsp. *larvae*/g of honey (*P. larvae* subsp. *larvae* detection PCR developed by Alippi et al. [27]) and 9 CFU/ml of honey (nested PCR developed by Lauro et al. [92]). Although DNA extraction methods differed between previous studies and this study, the detection limit of *P. larvae* spores from honey by the methods developed in this study was superior or similar to those of previously reported methods. In contrast, the sensitivities of real-time PCR assays targeting the 16S rRNA gene of *P. larvae* reported by Martínez et al. (93) and Rossi et al. (95) were higher than that of the present PCR assay. When the UltraClean Soil DNA isolation kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA) with a bead beating procedure (93) and the NucleoSpin Tissue kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) (95) were used for DNA extraction from honey, these assays detected *P. larvae* from 2 spores/g of honey and 1 CFU/g of honey, respectively. However, since these methods cannot distinguish between the ERIC types of *P. larvae*, the developed multiplex PCR assay is useful for assessing the contamination status of beehives/apiaries by a pathogen.

Limited information is currently available on *M. plutonius* detection protocols from honey samples. McKee et al. (96) employed hemi-nested PCR described by Djordjevic et al. (101) and detected *M. plutonius* from honey. However, the detection limit was not investigated in that study. In this study, both typical (DAT606) and

atypical (DAT561) *M. plutonius* were successfully detected from honey samples with detection limits of 19 and 29.2 CFU/ml of honey, respectively. Although there are no reports referring to the detection limit of *M. plutonius* from honey by PCR assays, the present results imply the sufficiently high sensitivity of the methods developed in this study.

Various bacteria often have antimicrobial resistance genes as one of the survival strategies in nature. Bacteria contaminating honey also possess a wide variety of antimicrobial resistance genes including macrolide resistance genes as described in Chapter I. Among the macrolide resistance genes, the susceptibility of *P. larvae* strains to TS decreased by the acquisition of *ermB* and *ermC* as shown in Chapter II. In particular, *ermC* made *P. larvae* clinically TS-resistant. Therefore, in order to investigate a potential risk of the emergence of TS-resistant *P. larvae* in beehives and apiaries, the author designed real-time PCR primers for *ermB* and *ermC*. The real-time PCR primers were designed to be able to detect *erm* sequences from various Gram-positive and/or spore-forming bacteria (Table 13). However, because there is no information on the sequence diversity of the *erm* genes present in honey, it should be noted that the primers may not necessarily detect all *ermB* and *ermC* genes in honey. The melting point temperatures of the amplified *ermB* and *ermC* products by the real-time PCR assays, which were derived from *Paenibacillus* sp. J45TS6 and *O. oncorhynchi* subsp. *incaldanensis* J18TS1, respectively, were 75.6℃ and 75.0℃, respectively. However, since the melting point temperature is determined by the base composition of the amplified sequence, even if the target gene is accurately detected, the specificity cannot be distinguished by the melting point temperature alone if there are variations in the sequence. Therefore, when the melting point temperatures differ significantly among the amplified products, it may be necessary to sequence the amplified products to confirm that they are specific products. Despite these points that should be noted in its use, the real-time PCR assays developed in this study will be a useful tool to understand the contamination status of macrolide resistance genes in honey.

Summary

Honey has a potential as a reservoir of macrolide resistance genes which might confer TS resistance to *P. larvae*. As honey is also known to be contaminated with *P. larvae* and *M. plutonius*, honey is a useful material for assessing the risk of the emergence of macrolide-resistant foulbrood pathogens. In order to evaluate the presence of foulbrood pathogens and macrolide resistance genes in honey, a multiplex PCR assay and real-time PCR assays were developed, respectively. Furthermore, the optimal commercially available kit applicable to DNA extraction from *P. larvae* spores and *M. plutonius* vegetative cells in honey was selected. The newly developed multiplex PCR assay was able to detect important genotypes/phenotypes of foulbrood pathogens, i.e., *P. larvae* ERIC I and ERIC II strains and *M. plutonius* typical and atypical strains, in one reaction with high specificity and sensitivity. The real-time PCR assays for macrolide resistance genes were able to detect *ermB* and *ermC* in honey. Furthermore, among the commercially available DNA extraction kits tested, Johne Pure Spin kit, which was designed for DNA extraction of acid-fast bacteria in feces, was the most efficient in extracting DNA from both *P. larvae* spores and *M. plutonius* vegetative cells in honey. The combination of these methods will be new effective methods to assess the risk of the emergence of macrolide-resistance foulbrood pathogens in honey bee colonies and contribute to the prudent use of antibiotics in the apicultural industry.

Table 10. Strains used as positive controls for the multiplex PCR assay for foulbrood pathogens

Strain	Bacterial species	Genotype[a]	Source	References	Description
DTK386	*Paenibacillus larvae*	ST15, ERIC I	Diseased brood	(30, 62)	Strain I in reference (62).
DTK384		ST10, ERIC II	Diseased brood		Strain N in reference (62).
DAT606	*Melissococcus plutonius*	ST3, CC3	Diseased larvae	(12)	A typical strain.
DAT561		ST12, CC12	Diseased larvae		An atypical strain.

[a] ST, sequence type; ERIC, enterobacterial repetitive intergenic consensus; CC, clonal complex.

Table 11. Strains used for the specificity test of multiplex PCR

Strains[a]	Genotype/ phenotype[b]	Bacterial species[c]	Culture conditions[d]	Results of the multiplex PCRassay[e]					Source[a]	References
				Paenibacillus larvae (target genotypes)			*Melissococcus plutonius* (target phenotypes)			
				ERIC I–V	ERIC I	ERIC II	Typical	Atypical		
J1TS1	n/a	*Shouchella tritolerans*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J1TS2	n/a	*Bacillus licheniformis*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J1TS3	n/a	*Siminovitchia fordii*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J1TS5	n/a	*Paenibacillus macerans*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J1TS6	n/a	*Paenibacillus dendritiformis*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J2TS1	n/a	*Heyndrickxia sporothermodurans*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J2TS2	n/a	*Bacillus* sp.	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J2TS4	n/a	*Paenibacillus* sp.	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J2TS6	n/a	*Paenibacillus* sp.	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J3TS1	n/a	*Cytobacillus kochii*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J3TS2	n/a	*Paenibacillus cineris*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J3TS4	n/a	*Cohnella* sp.	CSA, 5% CO_2, 24h	–	–	–	–	–	Japanese honey	This study
J4TS2	n/a	*Lederbergia ruris*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J5TS2	n/a	*Brevibacillus halotolerans*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J5TS4	n/a	*Bacillus tequilensis*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J6TS4	n/a	*Heyndrickxia oleronia*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J6TS6	n/a	*Paenibacillus* sp.	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J7TS1	n/a	*Lederbergia galactosidilytica*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J7TS3	n/a	*Bacillus paralicheniformis*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J7TS4	n/a	*Bacillus* sp.	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study

Table 11. (Continued)

Strains[a]	Genotype/ phenotype[b]	Bacterial species[c]	Culture conditions[d]	Results of the multiplex PCRassay[e]					Source[a]	References
				Paenibacillus larvae (target genotypes)			*Melissococcus plutonius* (target phenotypes)			
				ERIC I–V	ERIC I	ERIC II	Typical	Atypical		
J8TS7	n/a	*Paenibacillus timonensis*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J11TS1	n/a	*Oceanobacillus* sp.	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J12TS1	n/a	*Lederbergia* sp.	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J14TS5	n/a	*Paenibacillus lautus*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J15TS7	n/a	*Paenibacillus lactis*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J15TS10	n/a	*Paenibacillus woosongensis*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J18TS1	n/a	*Oceanobacillus oncorhynchi* subsp. *incaldanensis*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J19TS2	n/a	*Cohnella xylanilytica*	CSA, 5% CO_2, 24h	–	–	–	–	–	Japanese honey	This study
J21TS3	n/a	*Paenibacillus cookii*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J21TS8	n/a	*Paenibacillus vini*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J23TS3	n/a	*Oceanobacillus sojae*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J23TS9	n/a	*Paenibacillus dokdonensis*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J25TS5	n/a	*Paenibacillus faecis*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J27TS8	n/a	*Robertmurraya siralis*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J30TS3	n/a	*Niallia* sp.	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J31TS4	n/a	*Paenibacillus* sp.	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J31TS6	n/a	*Brevibacillus reuszeri*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J32TS6	n/a	*Virgibacillus pantothenticus*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J33TS5	n/a	*Clostridium beijerinckii*	GAMSA, anaerobically, 24h	–	–	–	–	–	Japanese honey	This study
J34TS1	n/a	*Paenibacillus azoreducens*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study

Table 11. (Continued)

Strains[a]	Genotype/ phenotype[b]	Bacterial species[c]	Culture conditions[d]	Results of the multiplex PCRassay[e]					Source[a]	References
				Paenibacillus larvae (target genotypes)			*Melissococcus plutonius* (target phenotypes)			
				ERIC I–V	ERIC I	ERIC II	Typical	Atypical		
J38TS3	n/a	*Bacillus glycinifermentans*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J39TS3	n/a	*Paenibacillus ginsengihumi*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J40TS1	n/a	*Paenibacillus montaniterrae*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J41TS2	n/a	*Bacillus sonorensis*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J41TS4	n/a	*Paenibacillus apis*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J41TS12	n/a	*Paenibacillus antibioticophila*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J43TS3	n/a	*Ornithinibacillus bavariensis*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J43TS5	n/a	*Paenibacillus yonginensis*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J45TS6	n/a	*Paenibacillus* sp.	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J46TS2	n/a	*Bacillus haynesii*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J47TS7	n/a	*Heyndrickxia* sp.	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
J48TS1	n/a	*Cytobacillus horneckiae*	CSA, aerobically, 24h	–	–	–	–	–	Japanese honey	This study
DAT702	n/a	*Bifidobacterium* sp.	MRS, anaerobically, 48h	–	–	–	–	–	A healthy Japanese honey bee larva	(97)
DAT703	n/a	*Lactobacillus melliventris*	MRS, anaerobically, 48h	–	–	–	–	–	A healthy Japanese honey bee larva	(97)
DAT706	n/a	*Staphylococcus hominis*	MRS, anaerobically, 48h	–	–	–	–	–	A healthy Japanese honey bee larva	(97)
DAT707	n/a	*Bifidobacterium asteroides*	MRS, anaerobically, 48h	–	–	–	–	–	A healthy Japanese honey bee larva	(97)
DAT709	n/a	*Streptomyces* sp.	BHI, aerobically, 24h	–	–	–	–	–	A healthy Japanese honey bee larva	(97)
ATCC 6344	n/a	*Paenibacillus alvei*	LB, aerobically, 24h	–	–	–	–	–	ATCC, Foulbrood of bees	
ATCC 64	n/a	*Brevibacillus laterosporus*	TSA, aerobically, 24h	–	–	–	–	–	ATCC	

Table 11. (Continued)

Strains[a]	Genotype/ phenotype[b]	Bacterial species[c]	Culture conditions[d]	Results of the multiplex PCRassay[e]					Source[a]	References
				Paenibacillus larvae (target genotypes)			*Melissococcus plutonius* (target phenotypes)			
				ERIC I–V	ERIC I	ERIC II	Typical	Atypical		
DAT820	n/a	*Dermacoccus* sp.	BHI, aerobically, 24h	–	–	–	–	–	A healthy European honey bee larva	(97)
DAT821	n/a	*Fructobacillus fructosus*	MRS, anaerobically, 24h	–	–	–	–	–	A healthy European honey bee larva	(97)
DAT822	n/a	*Apilactobacillus kunkeei*	BHI agar with 5% horse blood, 5% CO_2, 24h	–	–	–	–	–	A healthy European honey bee larva	(97)
DAT824	n/a	Gamma proteobacterium	GAM with 10% egg yolk saline solution, anaerobically, 24h	–	–	–	–	–	A healthy European honey bee larva	(97)
DAT868	n/a	*Enterococcus faecalis*	THA, 5% CO_2, 24h	–	–	–	–	–	Diseased European honey bee larvae	(97)
DAT817	n/a	*Enterococcus faecalis*	THA, 5% CO_2, 24h	–	–	–	–	–	A honey bee larva/larvae	(97)
DTK288	ST18, ERIC I	*Paenibacillus larvae*	MYPGP, 5% CO_2, 48h	+	+	–	–	–	Diseased European honey bee larvae	(30)
DTK400	ST2, ERIC I	*Paenibacillus larvae*	MYPGP, 5% CO_2, 48h	+	+	–	–	–	A diseased European honey bee larva	(30)
DAT989	ST15, ERIC I	*Paenibacillus larvae*	MYPGP, 5% CO_2, 48h	+	+	–	–	–	Diseased European honey bee larvae	(30)
DAT1051	ST15, ERIC I	*Paenibacillus larvae*	MYPGP, 5% CO_2, 48h	+	+	–	–	–	Diseased European honey bee larvae	(30)
DAT1052	ST10, ERIC II	*Paenibacillus larvae*	MYPGP, 5% CO_2, 48h	+	–	+	–	–	A diseased European honey bee larva	(30)
DAT1059	ST10, ERIC II	*Paenibacillus larvae*	MYPGP, 5% CO_2, 48h	+	–	+	–	–	Diseased European honey bee larvae	(30)
DAT1060	ST2, ERIC I	*Paenibacillus larvae*	MYPGP, 5% CO_2, 48h	+	+	–	–	–	Diseased European honey bee larvae	(30)
DAT1065	ST18, ERIC I	*Paenibacillus larvae*	MYPGP, 5% CO_2, 48h	+	+	–	–	–	A diseased European honey bee larva	(30)
DAT1125	ST18, ERIC I	*Paenibacillus larvae*	MYPGP, 5% CO_2, 48h	+	+	–	–	–	A diseased European honey bee larva	(30)
DAT1178	ST2, ERIC I	*Paenibacillus larvae*	MYPGP, 5% CO_2, 48h	+	+	–	–	–	A diseased European honey bee larva/larvae	(30)
DAT1224	ST10, ERIC II	*Paenibacillus larvae*	MYPGP, 5% CO_2, 48h	+	–	+	–	–	A diseased European honey bee larva	(30)

Table 11. (Continued)

Strains[a]	Genotype/ phenotype[b]	Bacterial species[c]	Culture conditions[d]	Results of the multiplex PCRassay[e]					Source[a]	References
				Paenibacillus larvae (target genotypes)			*Melissococcus plutonius* (target phenotypes)			
				ERIC I–V	ERIC I	ERIC II	Typical	Atypical		
DAT1259	ST24, ERIC II	*Paenibacillus larvae*	MYPGP, 5% CO_2, 48h	+	–	+	–	–	A diseased European honey bee larva	(30)
DAT1295	ST11, ERIC II	*Paenibacillus larvae*	MYPGP, 5% CO_2, 48h	+	–	+	–	–	A diseased European honey bee larva	(30)
ATCC 35311^T	ST1, CC13 Typical	*Melissococcus plutonius*	KSBHI, anaerobically, 72h	–	–	–	+	–	ATCC, honey bee larvae	
DAT351	ST12, CC12 Atypical	*Melissococcus plutonius*	KSBHI, anaerobically, 72h	–	–	–	–	+	A diseased European honey bee larva	(12, 83)
DAT567	ST25, CC12 Atypical	*Melissococcus plutonius*	KSBHI, anaerobically, 72h	–	–	–	–	+	A diseased European honey bee larva	(12, 83)
DAT571	ST12, CC12 Atypical	*Melissococcus plutonius*	KSBHI, anaerobically, 72h	–	–	–	–	+	A diseased European honey bee larva	(12, 83)
DAT580	ST26, CC13 Typical	*Melissococcus plutonius*	KSBHI, anaerobically, 72h	–	–	–	+	–	A diseased European honey bee larva	(12, 83)
DAT585	ST26, CC13 Typical	*Melissococcus plutonius*	KSBHI, anaerobically, 72h	–	–	–	+	–	Diseased European honey bee larvae	(12, 83)
DAT639	ST3, CC3 Typical	*Melissococcus plutonius*	KSBHI, anaerobically, 72h	–	–	–	+	–	A diseased European honey bee larva	(90)
DAT647	ST12, CC12/ Atypical	*Melissococcus plutonius*	KSBHI, anaerobically, 72h	–	–	–	–	+	A diseased European honey bee larva	(90)
DAT869	ST4, CC13 Typical	*Melissococcus plutonius*	KSBHI, anaerobically, 72h	–	–	–	+	–	A diseased Japanese honey bee larva	(90)
DAT885	ST27, CC12 Atypical	*Melissococcus plutonius*	KSBHI, anaerobically, 72h	–	–	–	–	+	A beehive of Japanese honey bees with clinical symptoms	(90)
DAT886	ST3, CC3 Typical	*Melissococcus plutonius*	KSBHI, anaerobically, 72h	–	–	–	+	–	A beehive of clinically healthy Japanese honey bees	(90)
DAT892	ST27, CC12 Atypical	*Melissococcus plutonius*	KSBHI, anaerobically, 72h	–	–	–	–	+	A diseased Japanese honey bee larva	(90)
DAT968	ST3, CC3 Typical	*Melissococcus plutonius*	KSBHI, anaerobically, 72h	–	–	–	+	–	A diseased European honey bee larva	(110)
DAT985	ST25, CC12 Atypical	*Melissococcus plutonius*	KSBHI, anaerobically, 72h	–	–	–	–	+	Diseased European honey bee larvae	(110)
DAT1148	ST29, CC3 Typical	*Melissococcus plutonius*	KSBHI, anaerobically, 72h	–	–	–	+	–	A diseased European honey bee larva	(83)
DAT1179	ST34, CC12 Atypical	*Melissococcus plutonius*	KSBHI, anaerobically, 72h	–	–	–	–	+	Diseased European honey bee larvae	(83)
DAT1263	ST29, CC3 Typical	*Melissococcus plutonius*	KSBHI, anaerobically, 72h	–	–	–	+	–	A clinically healthy European honey bee larva/larvae	This study

Table 11. (Continued)

[a] ATCC, American Type Culture Collection.
[b] n/a, not applicable; ST, sequence type; ERIC, enterobacterial repetitive intergenic consensus; CC, clonal complex.
[c] As of January 2024, the latest species names are shown.
[d] CSA, Columbia blood agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing 5% defibrinated sheep blood; GAMSA, modified GAM agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 5% defibrinated sheep blood; MRS, Lactobacilli MRS agar (Becton, Dickinson and Company); BHI, Brain Heart Infusion (Becton Dickinson and Company) agar; LB, LB (Becton Dickinson and Company) agar; TSA, Tryptic Soy agar (Becton Dickinson and Company); THA, Todd-Hewitt (Becton Dickinson and Company) agar; MYPGP, medium containing 1.0% Mueller-Hinton broth (Oxoid, Hampshire, UK), 1.5% yeast extract, 0.3% K_2HPO_4, 0.1% sodium pyruvate, 0.2% glucose, and 2% agar (47); KSBHI, BHI (Becton Dickinson and Company) agar with 0.15 M KH_2PO_4 and 1% soluble starch (12).
[e] +, PCR positive; −, PCR negative; Each target gene is listed in Table 12.

Table 12. PCR primers for the multiplex PCR assay to detect and distinguish between different types of foulbrood pathogens

Target			Primer				PCR product size (bp)
Bacterial species	Genotype /phenotype[a]	Gene/sequence (accession number)	Primer name	Sequence (5'-3')	Optimal final concentration for the multiplex PCR assay	Reference	
Paenibacillus larvae	ERIC I-V	16S rRNA gene (47, 100) (X60619)	Plarvae1	AAGTCGAGCGGACCTTGTGTTTC	0.1 µM	(47, 100)	973[b]
			Plarvae2	TCTATCTCAAAACCGGTCAGAGG	0.1 µM	(47, 100)	
	ERIC I	Toxin 1 gene, *plx1* (57, 98) (KC456421)	Pl-I-F1	GTAGCAGGGGTTTCTTCAGATGACAATG	0.2 µM	This study	554
			Pl-I-R1	AAAGCTACATGGGGATTACTCACACTCG	0.2 µM	This study	
	ERIC II	The sequence reported as neutral invertase-like protein gene (99) (FI763267)	Pl-II-F1	ACGGTCTCCCGTGTTACACTGACGTATG	0.2 µM	This study	333
			Pl-II-R1	GTTGCAAACTTCGCGATGATGATC	0.2 µM	This study	
Melissococcus plutonius	Atypical	Fur family transcriptional regulator gene (97) (AP012282; locus tag, MPD5_0863)	Mp-A-F1	TCCAACGGCAGATGAAATCTATCGAGCC	0.2 µM	This study	257
			Mp-A-R1	ACCCTGTTAACTTACTTACAACGCCTTC	0.2 µM	This study	
	Typical	Na^+/H^+ antiporter gene, *napA* (97) (AP012200; locus tag, MPTP_0420–0421)	Mp-T-F	TGGTAGCTTAGGCGGAAAAC	0.2 µM	(97)	187
			Mp-T-R	TGGAGCGATTAGAGTCGTTAGA	0.2 µM	(97)	

[a] ERIC, enterobacterial repetitive intergenic consensus.
[b] According to sequences in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/), a 972- or 974-bp product may be amplified from some *P. larvae* strains.

Table 13. Accession numbers of the reference sequences of *ermC* and *ermB* used for primer design

Gene	Accession number	Locus tag	Bacterial species
ermC	NC_001376	H4K10_RS00010	*Bacillus subtilis*
	M13761	–	*Bacillus subtilis*
	NG_047806	A7J11_00443	*Bacillus subtilis*
	MIOQ01000118	BHF98_11650	*Corynebacterium diphtheriae*
	NG_047817	A7J11_02291	*Lactobacillus reuteri*
	FJ489650	PA16_11	*Lactobacillus reuteri*
	NZ_MH423314	HTS84_RS00035	*Mammaliicoccus lentus*
	NZ_ATPV01000221	M703_RS06465	*Neisseria gonorrhoeae*
	LC586958	–	*Oceanobacillus oncorhynchi* subsp. *incaldanensis*
	NZ_AUPS01000034	M397_RS14135	*Staphylococcus aureus*
	NC_001395	HTN12_RS00010	*Staphylococcus aureus*
	NZ_LFXH01000034	AC235_RS12515	*Staphylococcus aureus*
	CP054554	FOB69_13025	*Staphylococcus hominis*
	NC_016139	HS754_RS00010	*Staphylococcus hyicus*
	LTIO01000074	HMPREF2695_03090	*Staphylococcus* sp.
ermB	AF480459	–	*Bacillus cereus*
	PNK22492	–	*Bacillus thuringiensis*
	CP052842	HIR78_19720	*Bacillus subtilis* subsp. *subtilis*
	CP041081	FJR70_32845	*Bacillus tropicus*
	AISD01000042	SC9_03208	*Enterococcus faecalis*
	NZ_LC597664	JNG29_RS00185	*Paenibacillus* sp.
	MH785229	BJL72_k00065	*Staphylococcus aureus*
	WXZD01000018	GT923_05810	*Streptococcus pyogenes*

Table 14. PCR primers and cycling conditions for the real-time PCR assays for macrolide resistance genes

Target	Primer name	Sequence (5'-3')	PCR conditions
ermC	ermC_qF	ATCGTGGAATACGGGTTTGC	95℃ 15 min-94℃ 15 sec, 55℃/56℃[a] 30 sec (45 cycles), 72℃ 30sec
	ermC_qR	CTGATAAGYGAGCTATTCAC	
ermB	ermB_qF	CCGCCATACCACAGATGTTC	95℃ 15 min-94℃ 15 sec, 55℃ 30 sec (45 cycles), 72℃ 30 sec
	ermB_qR	ACTTTGGCGTGTTTCATTGC	

[a] Presence of the *ermC* gene was assessed at two different annealing temperatures during the PCR cycle. All PCR results from the two conditions were used to calculate the *ermC* detection rates in Chapter IV.

Table 15. Bacterial strains used as positive controls for the real-time PCR assays and culture conditions for spore preparation

Strain name	Species	Macrolide resistance gene	Culture conditions for spore preparation			
			Medium[a]	Temperature	Atmosphere	Incubation time
J18TS1	*Oceanobacillus oncorhynchi* subsp. *incaldanensis*	*ermC*	BHI agar	20°C	Aerobic	30 days
J45TS6	*Paenibacillus* sp.	*ermB*	MYPGP agar	35°C	Aerobic	40 days

[a] BHI, Brain Heart Infusion (Becton Dickinson and Company); MYPGP, 1.0% Mueller-Hinton broth (Oxoid, Hampshire, UK), 1.5% yeast extract, 0.3% K_2HPO_4, 0.1% sodium pyruvate, and 0.2% glucose (47)

Table 16. The accession numbers of the complete genomes of *Paenibacillus larvae* strains used to select the PCR target gene for specific detection of ERIC II *P. larvae*

Strain[a]	Accession no.	ERIC type[b]
ATCC 9545	NZ_CP019687	I
DSM 7030	NZ_CP019651	I
DSM 25430	NC_023134	II
DSM 25430	NZ_CP019652	II
SAG 10367	NZ_CP020557	II
LMG 16252	NZ_CP019655	III
LMG 16247	NZ_CP019659	IV
ATCC 13537	NZ_CP019794	IV
CCM 38	NZ_CP020327	IV
DSM 106052	NZ_CP019717	V

[a] ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; SAG, Sammlung von Algenkulturen der Universität Göttingen; LMG, Laboratorium voor Microbiologie, Universiteit Gent; CCM, Czech Collection of Microorganisms.

[b] ERIC, enterobacterial repetitive intergenic consensus.

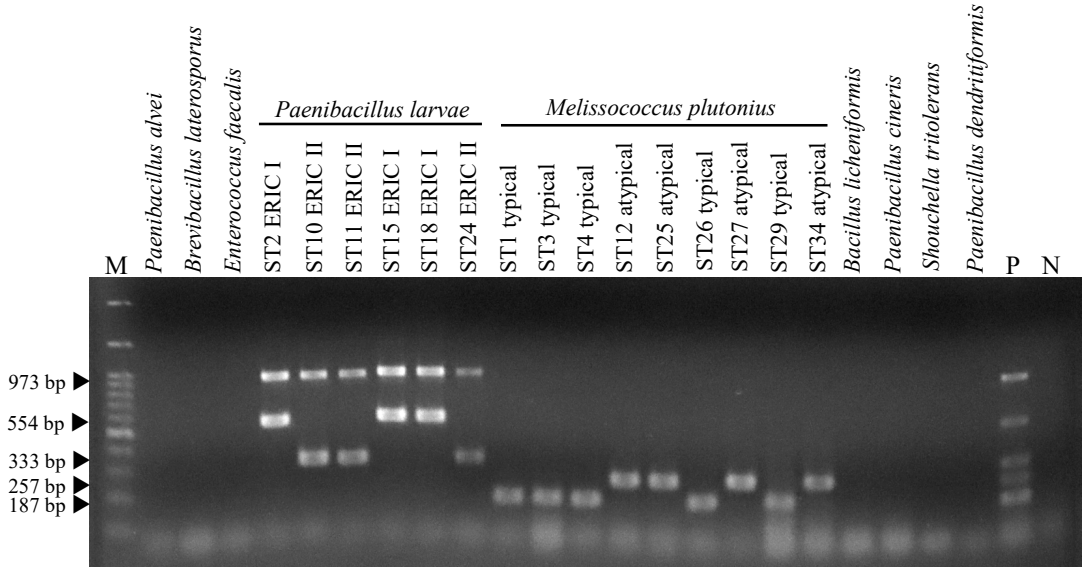

Figure 7. Representative results of the specificity test of the multiplex PCR assay for foulbrood pathogens developed in this study

DNA samples extracted from bacterial cultures were used for PCR. Bacterial species are indicated above each lane. ST, sequence type. ERIC, enterobacterial repetitive intergenic consensus. Six microliters of the PCR product was run on a 2% agarose gel and stained with ethidium bromide. M, molecular size marker (100-bp DNA ladder). P, positive control (a mixture of 0.5 ng of each DNA from *Paenibacillus larvae* DTK386 and DTK384 and *Melissococcus plutonius* DAT606 and DAT561). N, no template control.

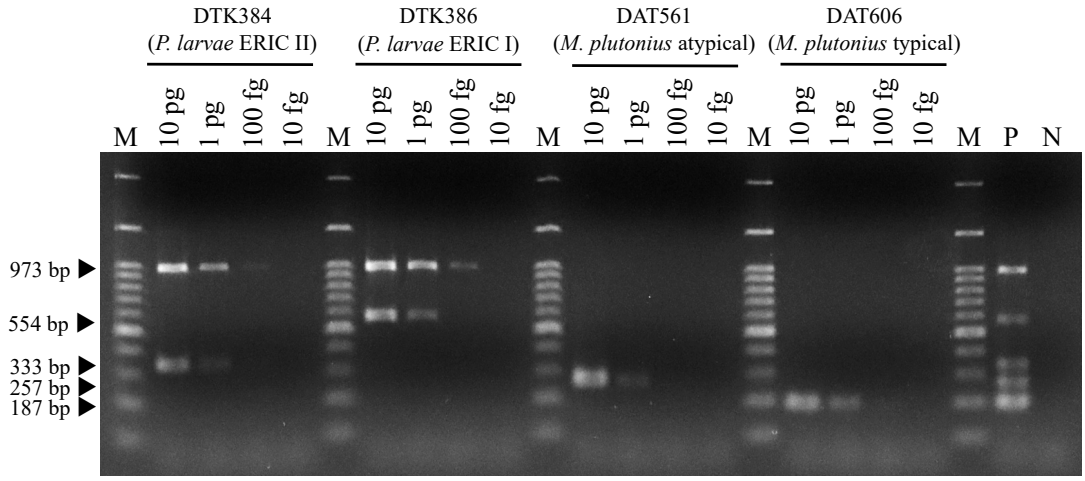

Figure 8. Sensitivity of the multiplex PCR assay for foulbrood pathogens developed in this study

Serial dilutions of DNA extracted from *Paenibacillus larvae* DTK384 (enterobacterial repetitive intergenic consensus [ERIC] II), *P. larvae* DTK386 (ERIC I), *Melissococcus plutonius* DAT561 (atypical), and *M. plutonius* DAT606 (typical) were used to investigate the sensitivity of PCR. The amount of DNA used as the template for each reaction is indicated above each lane. Six microliters of the PCR product was run on a 2% agarose gel and stained with ethidium bromide. M, molecular size marker (100-bp DNA ladder). P, positive control (a mixture of 0.5 ng of each DNA from *P. larvae* DTK386 and DTK384 and *M. plutonius* DAT606 and DAT561). N, no template control.

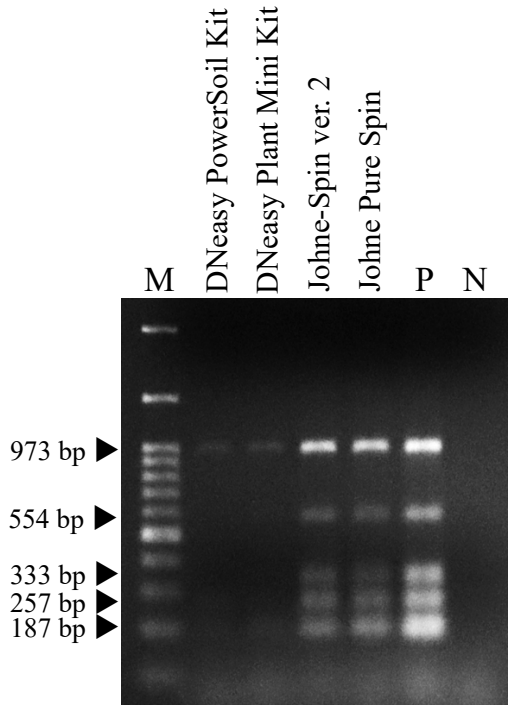

Figure 9. Comparison of DNA extraction kits

Foulbrood pathogen-free honey was spiked with the *Paenibacillus larvae* spores of enterobacterial repetitive intergenic consensus (ERIC) I and II types (DTK386 and DTK384, respectively) and typical and atypical *Melissococcus plutonius* strains (DAT606 and DAT561, respectively) at a final concentration of 1,000 cells/strain/ml of honey. DNA samples were extracted from sediments from 5 ml of the spiked honey using four different commercial kits (DNeasy PowerSoil Kit [QIAGEN, Hilden, Germany], DNeasy Plant Mini Kit [QIAGEN], Johne-Spin ver. 2 [FASMAC Co., Ltd., Atsugi, Japan], and Johne Pure Spin [FASMAC]) according to each of the manufacturer's instruction, and 2 µl of each DNA sample was used as a template for each reaction in the multiplex PCR assay. Six microliters of the PCR product was run on a 2% agarose gel and stained with ethidium bromide. M, molecular size marker (100-bp DNA ladder). P, positive control (a mixture of 0.5 ng of each DNA from *P. larvae* DTK386 and DTK384 and *M. plutonius* DAT606 and DAT561). N, no template control.

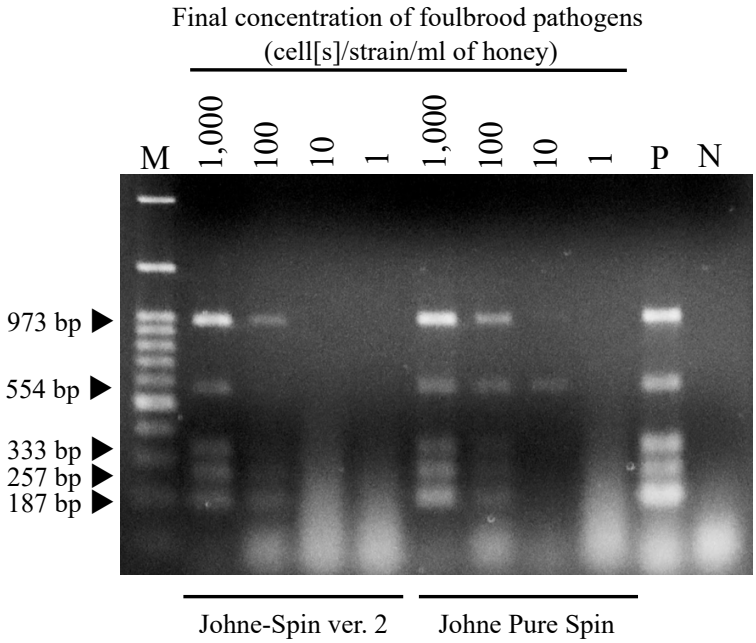

Figure 10. DNA extraction efficiency from foulbrood pathogens in honey by Johne-Spin ver. 2 (FASMAC Co., Ltd., Atsugi, Japan) and Johne Pure Spin (FASMAC)

Foulbrood pathogen-free honey was spiked with the *Paenibacillus larvae* spores of enterobacterial repetitive intergenic consensus (ERIC) I and II types (DTK386 and DTK384, respectively) and typical and atypical *Melissococcus plutonius* strains (DAT606 and DAT561, respectively). DNA samples were extracted from sediments from 5 ml of the spiked honey using the two kits, and 2 µl of each DNA sample was used as a template in each reaction of the multiplex PCR assay. Six microliters of the PCR product was run on a 2% agarose gel and stained with ethidium bromide. The final concentrations of bacterial cells in the spiked honey (cell[s]/strain/ml of honey) are indicated above each lane. M, molecular size marker (100-bp DNA ladder). P, positive control (a mixture of 0.5 ng of each DNA from *P. larvae* DTK386 and DTK384 and *M. plutonius* DAT606 and DAT561). N, no template control.

Chapter IV

A survey of foulbrood pathogens and macrolide resistance genes in Japanese honey using the developed PCR assays

Introduction

Foulbroods occur in most countries where beekeeping is practiced (5). Although foulbroods also occur in Japan every year, the distribution of *M. plutonius* and *P. larvae* in Japanese apiaries has not been investigated. In addition, the types of macrolide resistance genes, which may be possessed by bacteria present in the honey bee habitat in Japan, are also unknown. In the previous study conducted by Ueno et al. (30), TS-resistant *P. larvae* was not found among strains isolated between 1993 to 2017. Thus, Japan was probably free from TS-resistance *P. larvae* at least before the approval of TS as a prophylactic in 2017. Since TS was used in beekeeping in 2018, no isolations of TS-resistant *P. larvae* have been reported from AFB cases. Therefore, Japan is probably free from TS-resistant *P. larvae* even today. In order to maintain the effectiveness of the prophylactic and avoid the emergence of TS-resistant *P. larvae*, TS has to be used carefully on honey bee colonies.

In Chapter III, the author developed the multiplex PCR assay for foulbrood pathogens and the real-time PCR assays for *ermB* and *ermC*. In addition, the commercially available kits, Johne-Spin ver. 2 and Johne Pure Spin, which are easily accessible to local livestock hygiene centers, were found to be efficient in extracting bacterial DNA from honey. Using these assays and DNA extracted from honey, it is possible to determine the contamination status of foulbrood pathogens and macrolide resistance genes in beehives/apiaries. Based on the results, it will then be possible to determine the risk of TS-resistant *P. larvae* emergence and the need for prophylactic in the beehives/apiaries. For example, in the apiaries where both *ermC* and *P. larvae* are detected in honey, the use of prophylactics may select TS-resistant *P. larvae* because *P. larvae* may have acquired *ermC* in honey bee larvae that have eaten honey. In such cases, thorough disinfection and replacement of beekeeping materials is recommended rather than the use of prophylactics. In the apiaries where only *P. larvae* is detected, TS can be efficiently used in the control of AFB with a low risk of TS-resistant *P. larvae* selection. Moreover, in apiaries where *P. larvae* is not detected, beekeepers may be advised that it is not necessary to use antimicrobials to prevent

AFB, at least for that year.

TS is not used for the prevention of EFB. However, since TS is a prophylactic for AFB, it has been administered to honey bee colonies not showing symptoms of AFB. *M. plutonius* may exist in clinically healthy colonies (111, 112); therefore, although these macrolides are not approved for the control of EFB, *M. plutonius* may have been exposed to the drug in beehives, and this exposure may lead to the emergence of antibiotic-resistant *M. plutonius*. In fact, the analysis of antimicrobial susceptibility tests for *M. plutonius* strains isolated in Japan revealed that some *M. plutonius* strains showed resistance to MRM used as prophylaxis for AFB from 1999 to 2017 (83). Therefore, it is also important to understand the distribution of *M. plutonius* as this pathogen may also acquire TS resistance in the future.

Therefore, in Chapter IV, the distribution of foulbrood pathogens, ERIC I and ERIC II *P. larvae* and typical and atypical *M. plutonius*, in Japanese honey samples were investigated by using the multiplex PCR developed in Chapter III. Furthermore, the existence of *ermB* and *ermC* in the same honey samples was also investigated by using the real-time PCR assays developed in the previous Chapter.

Materials and Methods

Honey samples used in this study

To survey the contamination of Japanese honey by foulbrood pathogens and the distribution of macrolide resistance genes in honey, 116 Japanese honey samples were collected (Table 17). Except for honey no. J96, which was kindly provided by an apiary, samples were commercially available honey with different origins (i.e., different apiaries, producers, and/or floral origins) and purchased between 2017 and 2020. Three honey samples (J12, J24, and J42) were produced by Japanese honey bees, *A. cerana japonica*, and the others by European honey bees, *A. mellifera*. All samples were stored at room temperature until used. To extract DNA, sediments were obtained from 5 ml of each honey sample, and bacterial DNA was extracted from the sediments using Johne Pure Spin as described in Chapter III.

Detection of foulbrood pathogens and macrolide resistance genes

The multiplex PCR assay developed in Chapter III was performed against honey samples for the detection of *P. larvae* (ERIC I, ERIC II, and the other ERIC types) and *M. plutonius* (typical and atypical strains). In addition, the real-time PCR assays developed in Chapter III were also employed to detect *ermB* and *ermC* genes from the honey samples. The presence of the *ermC* gene was assessed at two different annealing temperatures during the PCR cycle, 55℃ and 56℃. All PCR results from the two conditions were used to calculate the *ermC* detection rates.

Confirmation of amplified PCR products

To confirm specific detection of the target genes of foulbrood pathogens, five honey samples (J67, J88, J97, J106 and J117), which showed positive results for all the targets, were randomly selected as representatives. Each target gene was reamplified individually from the selected samples under the same PCR conditions and directly sequenced as described previously (113). Briefly, the amplified products

were purified by the QIAquick PCR Purification Kit (QIAGEN), and their sequences were determined using the BigDye Terminator v3.1 cycle sequencing kit and 3130*xl* Genetic Analyzer (Applied Biosystems). Similarly, in order to confirm specific detection of *ermC*, amplified sequences of all *ermC* PCR products were determined as described above exceptionally using ExoSAP-IT™ Express PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA) for purification. Determined sequences were analyzed by the BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and CLUSTALW (https://www.genome.jp/tools-bin/clustalw). PCR primers were used for the sequencing.

Results

Foulbrood pathogen contamination rates in Japanese honey

P. larvae and/or *M. plutonius* were detected in 94.0% of honey samples (Figure 11A). Both pathogens were found together in 75.0% of samples, and 34.5% of samples were positive for all four targets (i.e., ERIC I and II *P. larvae* and typical and atypical *M. plutonius*) (Figure 11A and Table 17). ERIC I and II *P. larvae* were simultaneously detected in 46.6% of samples, while ERIC I and II were solely detected in 15.5% and 18.1% of samples, respectively (Figure 11B). Typical and atypical *M. plutonius* were simultaneously detected in 56.9% of the samples, and 31.9% of honey samples were positive for typical *M. plutonius* alone (Figure 11C). Of note, all the PCR products analyzed by sequencing were confirmed to be specific products, further supporting the specificity of the multiplex PCR and reliability of the survey results.

Distribution of *ermB* and *ermC* in Japanese honey

The *ermB* and *ermC* genes were detected in 30.2% and 86.2% of honey samples, respectively, and 25% of samples contained both genes (Figure 12 and Table 17). The *ermB* and *ermC* free honey was 8.6%. The *ermB* amplicons showed a single specific melting temperature at 75.5 ± 0.2℃ indicating negligible sequence variations. Since *ermC* showed an erratic melting temperature (71.7℃–75.2℃), the author determined the sequences of all *ermC* amplicons and found that *ermC* amplicons exhibited sequence variations with the homology of more than 94.4% (Figure 13).

Co-contamination of honey with *P. larvae* and *ermC*

P. larvae becomes TS-resistant if it acquires the *ermC* gene as shown in Chapter II; therefore, the co-contamination of honey with *P. larvae* and *ermC* suggests the potential risk of the emergence of macrolide-resistant *P. larvae* in beehives and apiaries. Surprisingly, both *P. larvae* and *ermC* were detected in 71.6% of honey samples (Figures 14 and 15 and Table 17). The *P. larvae* and *ermC* were detected alone

in 8.6% and 14.7% of the samples, respectively. The *P. larvae* and *ermC*-free honey was only 5.2%.

Discussion

The contamination of foulbrood pathogens in honey are reported in many studies. In previous studies using bacterial culture methods, the contamination rates of *P. larvae* spores in honey collected from Italy, Uruguay and Poland were 35.6–56% (50, 114, 115, 116). In a quantitative PCR assay, 59.2% of honey samples in Italy were revealed to be contaminated with *P. larvae* (117). Ribani et al. (118) also reported the detection of *P. larvae* in 49 and 79% of honey samples collected from Italy and other countries (North, Central, and South American, Asian, African, and Oceanian and other European countries), respectively, using a conventional PCR assay. They also demonstrated that *M. plutonius* was positive in 87% of all analyzed samples and that both pathogens were co-amplified from 50% of samples. According to these findings, both pathogens contaminate honey with high probability. In the present survey, the *M. plutonius*-positive rate (88.8%) in Japanese honey was similar to that reported by Ribani et al. (118), while the *P. larvae*-positive rate in the present study (80.2%) was higher than that in previous studies in other countries (50, 114, 115, 116, 117, 118). As all the honey-derived PCR products sequenced in the present study were specific products, these positive rates strongly suggest the wide distribution of foulbrood pathogens in Japan. Since commercial honey is generally dispensed from bulk honey collected from more than one honey bee colony and/or farm with a different infection status, the infectious status of each colony was unknown in most cases in the present study. However, J91 honey was a comb honey sample harvested from a single colony and showed positive for all of the target pathogens (Table 17), suggesting that a honey bee larva in a single colony may simultaneously encounter several pathogens.

In the survey of macrolide resistance genes, *ermB* and *ermC* were detected from 30.2% and 86.2% of the honey samples, respectively, and the results revealed the wide distribution of the macrolide resistance genes in Japanese apiaries. The *ermC* gene was detected from Japanese honey more frequency than the *ermB* gene and showed sequence variations in the amplicons (Figure 13). The higher detection rate and the sequence variations in *ermC* imply that *ermC* is carried by a wide variety of

bacteria in honey as compared to *ermB*. However, the possibility that different *ermC* genes are carried by a single or limited bacterial species cannot be excluded. As described in Chapter I, Japanese honey contains a wide variety of bacteria of different genus, such as *Bacillus*, *Paenibacillus*, and *Shouchella*. Among the bacterial isolates, the *ermC* and *ermB* genes have only been identified in *O. oncorhynchi* subsp. *incaldanensis* and *Paenibacillus* sp., respectively. However, information on other bacterial species in honey that carry *erm* genes remains unclear. Therefore, further investigation would be essential to elucidate the bacterial species that carry macrolide resistance genes and their distribution in Japanese honey.

In Japanese honey, both *ermC* and *P. larvae* were detected in 71.6% of honey samples (Figures 14 and 15 and Table 17). As described above, since most samples analyzed in this study were bulk honey, the detection rate did not represent the status of a single honey bee colony. In addition, as the present assays do not provide insight into the transferability of *ermC*, the detected gene may not have the potential to transfer to *P. larvae* and subsequently confer TS resistance to the bacterium. Moreover, as some of the *ermC* sequences detected in Japanese honey were expected to have mutations involving amino acid substitutions, some of the detected genes might not be functional. However, the high coexistence of *P. larvae* and *ermC* in Japanese honey suggests a high risk of future emergence of TS-resistant *P. larvae* in Japan, and once TS-resistant *P. larvae* emerges by acquiring functional *ermC* genes, the use of the prophylactic may result in the selection and spread of them within the apiary and the area. Therefore, in apiaries contaminated with both *ermC* and *P. larvae*, judicious use of TS is recommended to prevent the selection of macrolide-resistant *P. larvae* strains. The macrolide resistance determinant of TS-resistant *P. larvae* strains isolated in the USA (20) has not been revealed. Therefore, it is still unknown what kind of macrolide resistance genes *P. larvae* has acquired in the country. As various types of macrolide resistance gene groups, such as *msr*, *ole*, *vga*, *mph*, etc., were detected from honey-derived bacteria (Table 5), *P. larvae* may obtain TS resistance with genes other than *erm* genes. Nevertheless, in apiaries where only *P. larvae* but not *erm* genes are detected, TS can be efficiently used in the control of AFB with a low risk of TS-

resistant *P. larvae* selection. In order to be able to use prophylaxis more appropriately in the future, the overall mechanism of macrolide resistance in *P. larvae* needs to be understood. To this end, it will be necessary to identify macrolide resistance determinants when macrolide-resistant *P. larvae* strains are isolated.

In Japan, the number of apiaries including weekend beekeepers is approximately 11,000 houses. According to the annual statistics of foulbrood diseases conducted by the Ministry of Agriculture, Forestry and Fisheries in Japan, approximately 30–60 AFB/EFB cases were officially reported annually in recent years. Despite the high positive rate of foulbrood pathogens in Japanese honey, the number of reported foulbrood cases is relatively small, and this may be in part attributed to the disruption of diseased colonies before the diagnosis of foulbroods or the misdiagnosis of diseased colonies. Alternatively, the approved prophylactic, TS, may effectively control not only AFB, but also EFB. The growth of all Japanese *P. larvae* and *M. plutonius* isolates tested to date was inhibited by TS at low concentrations (30, 83). The hygiene behavior of honey bees, i.e., the removal of an infected brood before the infectious stage of *P. larvae*, is the main mechanism underlying resistance to AFB (119, 120, 121); therefore, the majority of honey bee colonies in Japan may be healthy and strong due to good beekeeping practices, which lead to efficient removal of infected brood by honey bees resulting in clinical healthy colonies without visible clinical signs regardless of the presence of foulbrood pathogens. Although further analyses are needed to identify the factors causing the relatively small number of reported foulbrood cases in Japan, countermeasures, such as the disinfection of beehives and the use of the prophylactic for AFB, will be required for prevention if pathogens are detected in honey. In contrast, if pathogens are not detected, the apiaries can be considered at a low risk of developing foulbroods in the year. In these apiaries, the amount of antibiotics can be reduced.

Long-term exposure of antimicrobials on honey bee colonies is known to affect the gut microbiota of the honey bees. According to Tian et al. (122), in the USA, where OTC has been used in beekeeping for many years, tetracycline resistance

determinants such as *tetH*, *tetL*, *tetM*, and *tetY* were detected often at high frequencies from the gut microbiota of adult worker bees. In contrast, only limited types of resistance genes were detected at low frequencies in bees from countries not using antibiotics in the industry. Besides, some *P. larvae* strains found in the USA become OTC-resistant through the acquisition of plasmids with *tet*(L) (21, 22). Therefore, the antimicrobial-resistant bacteria in honey bee gut could also be a reservoir of genes conferring antimicrobial resistance to *P. larvae*. It remains unclear how the gut microbiota of adult worker bees influences the acquisition of antimicrobial resistance in *P. larvae*. It is also unclear whether the continued use of antimicrobials in beekeeping also affects the prevalence of antimicrobial resistance genes in microbiota in the bee's habitat, such as those in honey. Nevertheless, it is important to consider that the use of antibiotics in honey bee colonies increases the level of antimicrobial resistance genes around and inside the honey bees.

Since MRM had been used for decades until TS was approved in Japan, macrolide resistance genes may have already accumulated in the honey and gut microbiota; therefore, *P. larvae* may have many opportunities to acquire macrolide resistance genes in Japan. Careful use of TS is much more important to avoid selecting macrolide-resistant bacteria in the adult and larval honey bee gut. Since macrolide resistance genes in the honey bee gut microbiota have not been analyzed in Japan, further investigation is needed to elucidate the mechanism by which foulbrood pathogens acquire antibiotic resistance.

Summary

In order to assess the risk of the emergence of macrolide-resistant foulbrood pathogens in honey bee colonies and contribute to the prudent use of antibiotics in the apicultural industry, it is important to know the contamination status of apiaries by foulbrood pathogens and antimicrobial resistance genes. Honey is a useful material to know the status; however, no survey using honey has ever been conducted in Japan. In Chapter IV, to reveal the contamination status of honey in Japan, the author investigated 116 Japanese honey samples by using detection methods developed in Chapter III, i.e., bacterial DNA extraction from honey by Johne Pure Spin kit, the multiplex PCR assay for foulbrood pathogens, and the real-time PCR assays for macrolide resistance genes, *ermB* and *ermC*. *P. larvae* and/or *M. plutonius* were detected in 94.0% of the Japanese honey samples tested, and both pathogens were found together in 75.0% of the samples. The *ermB* and *ermC* genes were detected in 30.2% and 86.2% of the samples, respectively. Furthermore, *ermC* and *P. larvae* co-existed in 71.6% of the samples. These detection rates, especially the high coexistence rate of *P. larvae* and *ermC*, suggest that there are many high-risk colonies/apiaries in Japan where TS-resistant *P. larvae* may emerge. Once TS-resistant *P. larvae* emerges, the use of the prophylactic may result in the selection and spread of them within the apiary and the area. To prevent such a situation from occurring, countermeasures against AFB other than prophylactics, such as disinfectants and replacement of beekeeping materials including brood combs, are needed for colonies at high risk of TS-resistant *P. larvae* emergence. Routine use of the honey-based testing methods developed in this study will not only reduce the risk of TS-resistant *P. larvae* emergence through appropriate use of the prophylactic in colonies, but will also help apiaries operate more efficiently by reducing the amount of TS used.

Table 17. Honey samples used in this study and PCR results

Lot no.	Species of honey bee	Floral origin	Results of the multiplex PCR assay[a]					Results of the real-time PCR assay[a]	
			Paenibacillus larva (target genotypes)			*Melissococcus plutonius* (target phenotypes)		Macrolide resistance genes	
			ERIC I–V	ERIC I	ERIC II	Typical	Atypical	*ermC*	*ermB*
J1	*Apis mellifera*	Multiflower	+	+	+	+	+	+	–
J2	*Apis mellifera*	Multiflower	+	+	–	+	–	+	–
J3	*Apis mellifera*	Multiflower	+	+	+	+	+	+	–
J4	*Apis mellifera*	Acacia	+	+	+	+	+	+	–
J5	*Apis mellifera*	Multiflower	+	+	+	+	+	+	–
J6	*Apis mellifera*	Japanese horse chestnut	+	+	+	+	+	+	–
J7	*Apis mellifera*	Acacia	+	+	+	+	+	+	+
J8	*Apis mellifera*	Apple	+	+	+	+	+	+	–
J9	*Apis mellifera*	Multiflower	–	–	–	–	–	+	–
J10	*Apis mellifera*	Japanese wax tree	–	–	–	+	+	+	–
J11	*Apis mellifera*	Mandarin orange	–	–	–	+	+	–	+
J12	*Apis cerana japonica*	Multiflower	+	–	+	–	–	+	–
J13	*Apis mellifera*	Multiflower	+	–	+	+	+	+	–
J14	*Apis mellifera*	Lotus	+	–	+	+	+	+	+
J16	*Apis mellifera*	Loquat	–	–	–	+	–	–	–
J17	*Apis mellifera*	Multiflower	–	–	–	+	–	+	–
J18	*Apis mellifera*	Japanese pagoda tree	+	–	+	+	+	+	–
J19	*Apis mellifera*	Acacia	+	+	+	+	–	+	–
J20	*Apis mellifera*	Japanese wax tree	+	–	+	+	–	+	–
J21	*Apis mellifera*	Cherry blossoms	+	+	+	+	+	+	+
J22	*Apis mellifera*	Japanese raisin tree	+	+	–	+	–	+	–
J23	*Apis mellifera*	Acacia	+	+	–	+	+	–	–
J24	*Apis cerana japonica*	Multiflower	–	–	–	–	–	+	–
J25	*Apis mellifera*	Multiflower	+	+	–	+	–	+	–
J26	*Apis mellifera*	Multiflower	+	+	–	+	+	+	–
J27	*Apis mellifera*	Japanese linden	+	+	–	+	+	–	–
J28	*Apis mellifera*	Lotus	–	–	–	–	–	+	–
J29	*Apis mellifera*	Lotus	+	+	+	–	–	+	–
J30	*Apis mellifera*	Multiflower	–	–	–	–	–	+	–
J31	*Apis mellifera*	Multiflower	–	–	–	–	–	–	–

Table 17. (Continued)

Lot no.	Species of honey bee	Floral origin	Results of the multiplex PCR assay[a]					Results of the real-time PCR assay[a]	
			Paenibacillus larva (target genotypes)			*Melissococcus plutonius* (target phenotypes)		Macrolide resistance genes	
			ERIC I–V	ERIC I	ERIC II	Typical	Atypical	*ermC*	*ermB*
J32	*Apis mellifera*	Acacia	+	–	+	+	–	+	–
J33	*Apis mellifera*	Acacia	+	+	–	+	–	+	–
J34	*Apis mellifera*	Multiflower	+	+	+	+	+	+	–
J35	*Apis mellifera*	Multiflower	+	+	+	+	+	–	–
J36	*Apis mellifera*	Multiflower	+	+	–	+	–	–	–
J37	*Apis mellifera*	Japanese horse chestnut	–	–	–	+	+	+	–
J38	*Apis mellifera*	Burr cucumber	+	+	–	+	–	+	+
J39	*Apis mellifera*	Sunflower	+	–	+	+	–	+	–
J40	*Apis mellifera*	Acacia	+	+	+	+	–	+	–
J41	*Apis mellifera*	Multiflower	+	+	+	+	–	+	–
J42	*Apis cerana japonica*	Multiflower	–	–	–	+	–	+	–
J43	*Apis mellifera*	Multiflower	+	+	+	+	–	–	–
J44	*Apis mellifera*	Acacia	+	+	+	+	–	+	+
J45	*Apis mellifera*	Multiflower	+	+	–	+	+	+	+
J46	*Apis mellifera*	Multiflower	+	–	+	+	+	+	+
J47	*Apis mellifera*	Apple	+	–	+	+	+	+	–
J48	*Apis mellifera*	Japanese horse chestnut	+	+	+	+	+	+	–
J49	*Apis mellifera*	Multiflower	+	+	+	+	+	+	–
J50	*Apis mellifera*	Lotus	+	+	+	–	–	+	–
J51	*Apis mellifera*	Horse chestnut	+	+	+	–	–	+	–
J52	*Apis mellifera*	Multiflower	+	+	+	+	–	+	–
J53	*Apis mellifera*	Multiflower	+	+	–	+	–	+	–
J54	*Apis mellifera*	Multiflower	–	–	–	+	–	+	–
J55	*Apis mellifera*	Acacia	+	+	+	+	–	+	–
J56	*Apis mellifera*	Lotus	+	+	–	+	–	+	–
J59	*Apis mellifera*	Acacia	+	–	+	+	+	–	–
J60	*Apis mellifera*	Cherry	+	–	+	+	–	+	–
J61	*Apis mellifera*	Multiflower	–	–	–	+	–	+	+
J62	*Apis mellifera*	Acacia	–	–	–	+	–	+	–
J63	*Apis mellifera*	Multiflower	+	+	+	+	+	+	+

Table 17. (Continued)

Lot no.	Species of honey bee	Floral origin	Results of the multiplex PCR assay[a]					Results of the real-time PCR assay[a]	
			Paenibacillus larva (target genotypes)			*Melissococcus plutonius* (target phenotypes)		Macrolide resistance genes	
			ERIC I–V	ERIC I	ERIC II	Typical	Atypical	*ermC*	*ermB*
J64	*Apis mellifera*	Multiflower	+	+	+	+	+	+	+
J65	*Apis mellifera*	Acacia	+	+	+	+	+	+	–
J66	*Apis mellifera*	Acacia	+	–	+	+	+	–	+
J67	*Apis mellifera*	Multiflower	+	+	+	+	+	+	+
J68	*Apis mellifera*	Acacia	+	+	–	+	+	+	–
J69	*Apis mellifera*	Japanese horse chestnut	+	+	+	+	+	+	–
J70	*Apis mellifera*	Multiflower	–	–	–	–	–	+	–
J71	*Apis mellifera*	Multiflower	+	–	+	+	+	+	–
J72	*Apis mellifera*	Multiflower	+	+	+	+	+	+	–
J73	*Apis mellifera*	Multiflower	+	+	+	+	+	+	–
J74	*Apis mellifera*	Citrus	+	+	+	+	+	+	–
J75	*Apis mellifera*	Chestnut	–	–	–	+	+	+	+
J76	*Apis mellifera*	Sumac	–	–	–	+	+	+	–
J77	*Apis mellifera*	Multiflower	+	+	–	+	+	+	+
J78	*Apis mellifera*	Acacia	+	+	+	+	+	+	+
J79	*Apis mellifera*	Multiflower	+	+	+	+	+	+	–
J80	*Apis mellifera*	Acacia	+	+	+	+	–	+	–
J81	*Apis mellifera*	Multiflower	+	+	+	+	+	+	+
J82	*Apis mellifera*	Multiflower	+	+	–	–	–	+	–
J83	*Apis mellifera*	Multiflower	+	+	+	+	+	+	+
J84	*Apis mellifera*	Acacia	+	+	+	+	–	+	–
J85	*Apis mellifera*	Multiflower	+	–	+	+	+	+	+
J86	*Apis mellifera*	Multiflower	+	+	+	+	+	+	+
J87	*Apis mellifera*	Acacia	+	+	+	+	+	+	–
J88	*Apis mellifera*	Acacia	+	+	+	+	+	–	+
J89	*Apis mellifera*	Multiflower	+	+	–	+	+	+	–
J90	*Apis mellifera*	Linden	+	+	+	+	+	+	+
J91	*Apis mellifera*	Multiflower	+	+	+	+	+	+	+
J92	*Apis mellifera*	Acacia	+	+	+	+	+	+	–
J93	*Apis mellifera*	Japanese horse chestnut	+	+	+	+	+	+	–

Table 17. (Continued)

Lot no.	Species of honey bee	Floral origin	Results of the multiplex PCR assay[a]					Results of the real-time PCR assay[a]	
			Paenibacillus larva (target genotypes)			*Melissococcus plutonius* (target phenotypes)		Macrolide resistance genes	
			ERIC I–V	ERIC I	ERIC II	Typical	Atypical	*ermC*	*ermB*
J94	*Apis mellifera*	Apple	+	+	+	+	+	+	–
J95	*Apis mellifera*	Multiflower	+	+	–	+	–	+	+
J96	*Apis mellifera*	Multiflower	–	–	–	+	+	+	+
J97	*Apis mellifera*	Clover	+	+	+	+	+	+	–
J98	*Apis mellifera*	Buckwheat	+	+	+	+	+	+	–
J99	*Apis mellifera*	Acacia	+	+	+	+	–	+	–
J100	*Apis mellifera*	Japanese linden	+	+	+	+	+	+	+
J101	*Apis mellifera*	Buckwheat	+	–	+	–	–	+	–
J102	*Apis mellifera*	Multiflower	+	–	+	+	–	+	–
J103	*Apis mellifera*	Multiflower	+	–	+	+	–	+	+
J104	*Apis mellifera*	Multiflower	+	+	–	+	+	+	–
J105	*Apis mellifera*	Acacia	+	+	+	+	–	–	–
J106	*Apis mellifera*	Clover	+	+	+	+	+	+	–
J107	*Apis mellifera*	Acacia	+	+	+	+	+	+	–
J108	*Apis mellifera*	Linden	+	+	+	+	+	+	+
J109	*Apis mellifera*	Acacia	–	–	–	+	+	–	–
J110	*Apis mellifera*	Multiflower	+	–	+	+	–	+	+
J111	*Apis mellifera*	Dandelion	+	–	+	+	–	+	+
J112	*Apis mellifera*	Acacia	+	–	+	+	+	+	+
J113	*Apis mellifera*	Multiflower	+[b]	–	+	+	–	+	+
J114	*Apis mellifera*	Multiflower	–	–	–	+	–	–	+
J115	*Apis mellifera*	Loquat	–	–	–	+	–	–	+
J116	*Apis mellifera*	Onion	–	–	–	–	–	+	–
J117	*Apis mellifera*	Multiflower	+	+	+	+	+	+	–
J118	*Apis mellifera*	Japanese linden	+	+	+	+	+	–	+
J119	*Apis mellifera*	Multiflower	–	–	–	+	+	+	–

[a] ERIC, enterobacterial repetitive intergenic consensus; +, PCR positive; −, PCR negative.

[b] Despite the positive result for ERIC II *P. larvae*, the 16S rRNA gene of *P. larvae* was not amplified from the DNA sample using the multiplex PCR assay. However, the 16S rRNA gene was amplified by a monoplex PCR assay using only the primers for the gene.

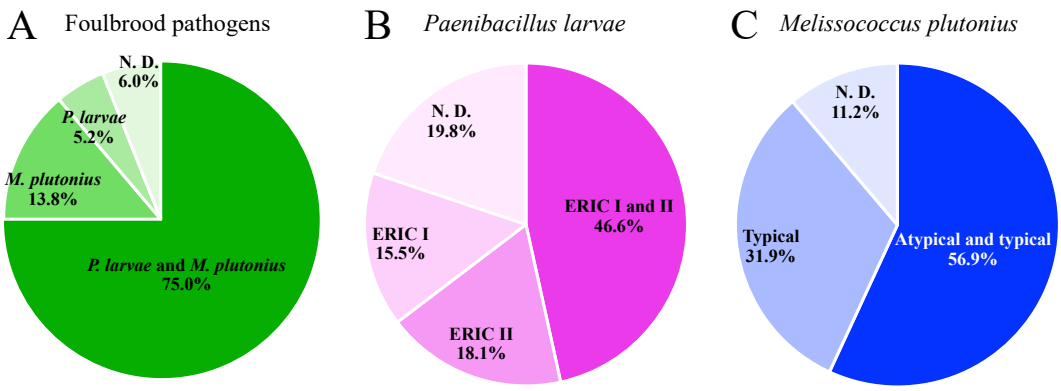

Figure 11. Detection rates of (A) *Paenibacillus larvae* and *Melissococcus plutonius*, (B) enterobacterial repetitive intergenic consensus (ERIC) I and II types of *P. larvae*, and (C) typical and atypical *M. plutonius* from Japanese honey

DNA samples were extracted from the sediments of 5 ml of honey by Johne Pure Spin (FASMAC Co., Ltd., Atsugi, Japan), and 2 µl of each DNA sample was used as a template in each reaction of the multiplex PCR assay. N. D., not detected.

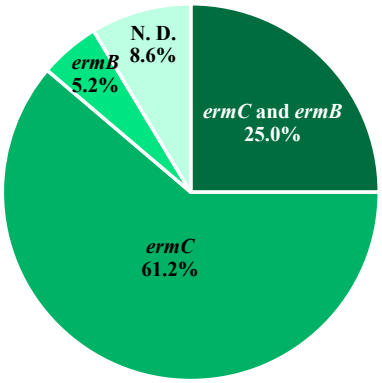

Figure 12. Detection rates of macrolide resistance genes, *ermC* and *ermB*

DNA was extracted from the sediments of 5 mL of honey using the Johne Pure Spin kit (FASMAC Co., Ltd., Atsugi, Japan), and 1 μL of the extracted DNA from each sample was used as a template in real-time PCR assay to detect *ermC*/*ermB*. N. D., not detected.

Figure 13. Sequence variations of the *ermC* gene fragments detected from Japanese honey samples

The *ermC* gene sequence of *Oceanobacillus oncorhynchi* subsp. *incaldanensis* (strain J18TS1) was retrieved from the GenBank database (accession no. LC586958) and compared with the amplified gene sequences from Japanese honey. The *ermC* gene sequence of J18TS1 is shown at the bottom. The numbers indicate the position of nucleotides in the *ermC* gene of strain J18TS1. Nucleotide differences are shown with colored backgrounds. R = A or G; Y = C or T.

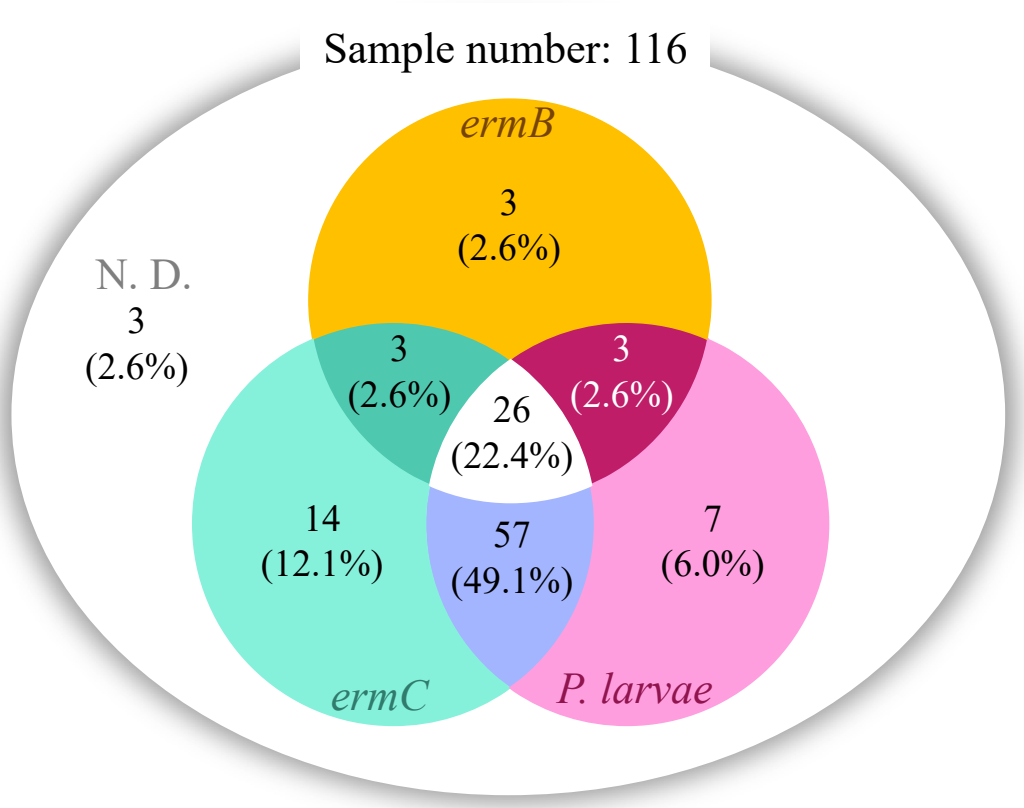

Figure 14. Venn diagram of detection numbers and rates of macrolide-resistance genes, *ermC* and *ermB*, and *Paenibacillus larvae* from Japanese honey samples

DNA samples were extracted from the sediments of 5 mL of honey by Johne Pure Spin (FASMAC Co., Ltd., Atsugi, Japan). Two microliters of each DNA sample was used as a template in each reaction of multiplex PCR for foulbrood pathogens. One microliter of each DNA sample was used as a template of real-time PCR for *ermC*/*ermB*. N. D., not detected.

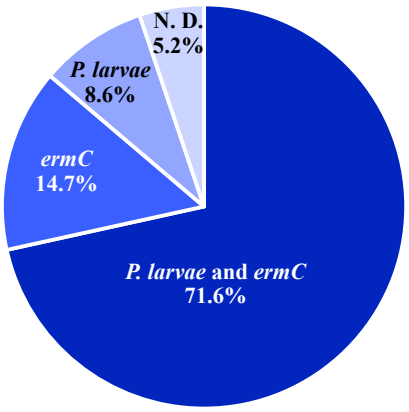

Figure 15. Detection rates of *Paenibacillus larvae* and *ermC* from Japanese honey

N. D., not detected.

Conclusion

TS is the only approved prophylactic for AFB in beekeeping in Japan. Although there are currently no reports of TS-resistant *P. larvae* being isolated in Japan, there is concern about the future emergence of TS-resistant *P. larvae*. Indeed, in North America, antimicrobial-resistant *P. larvae* has already been identified, and bacteria in honey are suspected as reservoirs that supply the antimicrobial resistance genes to *P. larvae*. However, the potential risk of *P. larvae* becoming TS-resistant via horizontal gene transfer from bacteria in honey in Japan had not been investigated. Therefore, in order to assess the risk of TS-resistant *P. larvae* in Japan, the author investigated macrolide-resistant bacteria latent in honey and developed detection methods that can evaluate the contamination status of foulbrood pathogens and macrolide-resistant genes in beehives/apiaries.

In Chapter I, 209 isolates belonging to at least 57 bacterial species including *Paenibacillus* and *Bacillus* spp. were isolated from Japanese honey samples by using TS-containing culture media, and most of them showed lower susceptibility to TS than *P. larvae* strains isolated in AFB cases in Japan. All 50 representative isolates whose genome sequences were analyzed possessed one or more macrolide resistance genes, and four of the resistance genes, *oleC*, *lsaB*, *ermB*, and *ermC*, were located on MGEs. These results indicate that Japanese honey has the potential to be a reservoir of macrolide resistance genes that confer TS resistance to *P. larvae* through horizontal transfer.

In Chapter II, in order to investigate whether the macrolide resistance genes found on MGEs can confer TS resistance to *P. larvae*, the resistance genes were artificially introduced into a *P. larvae* strain by using an expression vector. Antimicrobial susceptibility tests using the gene-introduced *P. larvae* revealed that *ermB* and *ermC* were capable of reducing TS susceptibility of *P. larvae*. In particular, the *ermC* gene cloned from the small mobilizable plasmid, pJ18TS1mac, reduced the TS susceptibility of *P. larvae* the most, and the direct introduction of pJ18TS1mac into *P. larvae* made the strain completely clinically TS-resistant. Moreover, pJ18TS1mac

was maintained in *P. larvae* stably even after 25 passages of the strain under nonselective culture conditions. Therefore, if *P. larvae* acquires *ermC* on MGEs such as pJ18TS1mac in the fields, the only prophylactic for AFB will lose its effectiveness.

There is a latent risk of TS-resistant *P. larvae* emerging in Japan, especially in colonies where *P. larvae* and *erm* genes coexist. However, the distribution of foulbrood pathogens and macrolide resistance genes in honey bee colonies was unknown in this country. Therefore, in Chapter III, in order to understand the contamination status in honey bee colonies, a multiplex PCR to detect and distinguish important types of foulbrood pathogens in Japan, including *M. plutonius*, and real-time PCR assays for macrolide resistance genes, *ermB* and *ermC*, were developed. In addition, the author found that Johne-Spin ver. 2 and Johne Pure Spin, which were originally designed for DNA extraction of acid-fast bacteria in feces, are appropriate for bacterial DNA extraction from honey.

In Chapter IV, with these assays and the Johne Pure Spin kit, 116 lots of Japanese honey samples were tested to estimate the distribution of foulbrood pathogens and macrolide resistance genes in Japanese apiaries. As a result, *P. larvae* and/or *M. plutonius* were detected in 94.0% of honey samples, and 34.5% of samples were positive for all types of targeted foulbrood pathogens, indicating the widespread distribution of the pathogens in honey bee colonies in Japan. Furthermore, both *ermC* and *P. larvae* were detected in 71.6% of samples, suggesting that Japanese apiaries are at high risk for the emergence of TS-resistant *P. larvae* strains with the use of the prophylactic.

Currently, there is no prophylactic available for the beekeeping industry in Japan other than TS. If TS-resistant *P. larvae* emerges and spreads, Japan would lose an important measure of AFB prevention. In this study, the author analyzed macrolide-resistant bacteria in honey for the first time in the world and strongly suggested that bacteria in honey are reservoirs of macrolide resistance genes that may be transmitted to *P. larvae.* These results indicate the need for more prudent use of the only prophylaxis in Japan. The multiplex PCR assay for foulbrood pathogens developed in

this study has been patented (Japanese patent No. 7390736) and has already been used to diagnose foulbroods in Japanese livestock hygiene service centers. The PCR is also beginning to be used for surveying the contamination status of foulbrood pathogens in honey at Japanese livestock hygiene service centers. Widespread use of the results of this research in the field will contribute to the further development of the beekeeping industry through appropriate hygiene management based on the presence of risk factors in honey bee colonies.

Acknowledgments

The author would like to acknowledge Prof. Y. Sakoda, Laboratory of Microbiology, Department of Disease Control, Faculty of Veterinary Medicine, Hokkaido University (Sapporo, Japan), for his guidance, encouragement, and detailed review of this manuscript.

Great appreciation is extended to Prof. C. Nakajima, Division of Bioresources, Hokkaido University International Institute for Zoonosis Control; Prof. T. Sato, Laboratory of Veterinary Hygiene, Faculty of Veterinary Medicine, Hokkaido University; Prof. N. Isoda, Laboratory of Microbiology, Department of Disease Control, Faculty of Veterinary Medicine, Hokkaido University for their critical review of the manuscript.

The author thanks to Hirotaka Fuyura and Ikuko Sugimoto for their technical assistance at the NIAH; Masahiko Kumagai and Hiroyuki Kanamori for their important support of the genome sequence analysis at the Science Research Supporting Programs of the Advanced Analysis Center and Institute of Crop Science, NARO; Ken Katsuda and Masahiro Kusumoto for their valuable support, advice, and discussion; and Daisuke Takamatsu for his kind and continuous guidance regarding the author's doctoral research and thesis writing.

The author owes her deepest gratitude to her family for their encouragement, love, and devoted support to her education.

This study was conducted by the regulatory research projects for food safety, animal health, and plant protection (JPJ008617.17935699) funded by the Ministry of Agriculture, Forestry and Fisheries of Japan. The user's manual, primers, and positive controls necessary for the multiplex PCR assay for foulbrood pathogens have been distributed to major livestock hygiene service centers in all prefectures and the Yokohama Head Office of the Animal Quarantine Service through the FY2022 Commissioned Project for Strategic Surveillance and Diagnosis System Development Promotion implemented by the Ministry of Agriculture, Forestry and Fisheries of Japan.

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Summary in Japanese (和文要旨）

ミツバチは多くの農作物の花粉交配を担う農業に欠かせない生物資源である。ミツバチは社会性昆虫であり、1 つの群は、女王蜂、働き蜂、雄蜂及びそれらに成長する蜂児で構成される。中でも働き蜂は、育児、巣作り、餌の採集など、繁殖以外の全ての仕事を担っており、蜂群の維持に欠かせない存在である。そのため、やがて働きバチへと成長する蜂児の健康に悪影響を与える要因は、蜂群崩壊への重大なリスクとなる。

蜂児に悪影響を及ぼす重大な要因として、*Paenibacillus larvae* によるアメリカ腐蛆病（American foulbrood: AFB）と *Melissococcus plutonius* によるヨーロッパ腐蛆病（European foulbrood: EFB）が知られている。どちらも家畜伝染病予防法で家畜伝染病に指定されており、発症した蜂群は焼却処分する必要がある。そのため、腐蛆病対策では発症予防が最も重要となるが、*P. larvae* は物理的・化学的に抵抗性の強い芽胞を形成することから、消毒による養蜂場からの排除が特に難しい病原体である。AFB の制御には抗生物質が使用される場合があるが、海外では薬剤耐性 *P. larvae* の出現が問題になっている。北米では AFB の治療にオキシテトラサイクリン（OTC）、タイロシン（TS）、リンコマイシンが使用されているが、全ての薬剤に対して耐性 *P. larvae* が確認されている。また、一部の OTC 耐性化は、テトラサイクリン耐性遺伝子を乗せたプラスミドの獲得によるものであることが明らかとなっている。ハチミツ中に混入する *Bacillus* 属菌からも同じテトラサイクリン耐性遺伝子が検出されていることから、*P. larvae* はハチミツ由来の薬剤耐性菌から薬剤耐性遺伝子を受け取り、OTC 耐性化した可能性が示唆されている。

日本では、マクロライド系抗生物質の TS が唯一の承認 AFB 予防薬として使用されている。現在のところ TS 耐性 *P. larvae* 株の分離は確認されていないが、今後、国内でも TS 耐性 *P. larvae* が出現する可能性は否定できない。北米で分離された TS 耐性 *P. larvae* の薬剤耐性化メカニズムについては解明されていないが、OTC 耐性化で示唆されたように、*P. larvae* がハチミツ中の細菌からマクロライド耐性遺伝子を受け取り、TS 耐性化する可能性が考えられる。しかし、国産ハチミツに混入する薬剤耐性菌の実態は明らかになってお

らず、日本における *P. larvae* の薬剤耐性化リスクは不明であった。そこで本研究では、国産ハチミツに潜むマクロライド耐性遺伝子の存在と、その遺伝子による *P. larvae* への影響について調査した。また、ハチミツを用いた腐蛆病菌の TS 耐性化リスクの評価系の開発と、その評価系を用いた国内における腐蛆病菌の TS 耐性化リスクの調査を行った。

第 I 章では、市販の国産ハチミツ 53 ロットから、TS 添加培地を用いて菌を分離したところ、57 菌種 209 株のハチミツ由来菌が分離された。TS に特に低感受性を示した株から代表株を 50 株選択してゲノム解析を行ったところ、全ての株でマクロライド耐性遺伝子を 1 つ以上保有することが明らかとなった。検出された遺伝子のうち、*ermC*、*ermL-ermB*、*lsaB* 及び *oleC* はプラスミドや integrative and conjugative elements 上に位置していたことから、国産ハチミツの中には可動遺伝因子を介して *P. larvae* に水平伝播する可能性のあるマクロライド耐性遺伝子が存在することが明らかとなった。

これらの遺伝子による *P. larvae* の TS 耐性化の可能性を調べるため、第 II 章では、クローニングベクターを用いて各遺伝子の *P. larvae* 株への導入を試みた。その結果、元の *P. larvae* 株の TS の MIC は 0.25 μg/ml であったのに対し、*ermC* および *ermL-ermB* を導入した株では、MIC がそれぞれ 8 μg/ml および 1 μg/ml まで上昇した。特に *P. larvae* の TS 感受性に大きな影響を与えた *ermC* は小型の可動性プラスミド pJ18TS1mac 上に位置していたことから、*P. larvae* に水平伝播する可能性が最も高い遺伝子であることが示唆された。pJ18TS1mac を *P. larvae* 株へ人工的に導入したところ、TS に対する感受性が大幅に低下し、MIC が 16 μg/ml まで上昇した。先行研究において、日本で承認されている用量と同程度以上の TS を蜂群に与えても、幼虫体内 TS 濃度は 4 μg/mL 程度までしか上昇しないことが報告されている。従って、pJ18TS1mac を獲得した *P. larvae* は臨床的にも耐性を示すことが示唆された。また、pJ18TS1mac は TS による選択圧がない条件下でも *P. larvae* の集団内で長期にわたって維持されたことから、本プラスミドによって *P. larvae* が TS 耐性化した場合、再び TS の有効性を取り戻すことが困難になることが示唆された。

これまで、国内の蜂群における腐蛆病菌やマクロライド耐性遺伝子の蔓延

状況について調査された報告は存在しない。また、調査に利用可能な検査法もなかったため、*P. larvae* の TS 耐性化リスクについて予測することは不可能であった。野外では、*P. larvae* だけでなく *M. plutonius* も予防薬の影響を受ける可能性があることから、ミツバチの生息環境における薬剤耐性菌のリスク評価には両腐蛆病菌について調査する必要がある。腐蛆病菌は健康な蜂群のハチミツにも混入することが知られており、マクロライド耐性菌もハチミツに混入することから、腐蛆病菌の薬剤耐性化リスクを評価する上でハチミツは最適な材料である。そこで第 III 章では、腐蛆病症例から分離される全ての型の腐蛆病菌を一度に検出し、識別するマルチプレックス PCR を世界で初めて開発した。また、マクロライド耐性遺伝子 *ermC*、*ermB* を検出するリアルタイム PCR を構築した。さらに、ハチミツに混入する細菌からの DNA の抽出には、糞便中の抗酸菌を標的とした DNA 抽出キットであるヨーネスピン ver.2 及びヨーネ・ピュアスピンが最適であることを明らかにした。

これらの技術を用いて、第 IV 章では市販の国産ハチミツ 116 ロットにおける腐蛆病菌とマクロライド耐性遺伝子の混入率を調査した。その結果、94.0%のハチミツからいずれかの腐蛆病菌が検出され、34.5%のハチミツでは国内で分離される全ての型の腐蛆病菌が同時に検出された。また、86.2%のハチミツから *ermC* が検出された。これらの高い検出率は、国内の蜂群には広く腐蛆病菌及びマクロライド耐性菌が蔓延していることを示唆している。さらに、71.6%のハチミツで *P. larvae* と *ermC* が同時に検出されたことから、国内の多くの蜂群では *P. larvae* と *ermC* を保有する菌が同時に存在し、予防薬の使用による TS 耐性 *P. larvae* が発生するリスクが高いことがわかった。

以上の結果から、国産ハチミツの中には *P. larvae* に予防薬耐性を与えるマクロライド耐性遺伝子を保有する菌が存在することが明らかとなった。また、国内の多くの蜂群において TS 耐性 *P. larvae* が発生する可能性が高い状況にあることが示唆されたため、唯一の AFB 予防薬である TS の有効性を維持するためには、より慎重な抗生物質の使用が求められる。本研究は、予防薬の有効性の維持による持続可能な AFB 対策の実践に資する知見と技術を提供するものであり、個々の蜂群や養蜂場に応じた予防薬の必要性と危険性を判

断する指標を提供することで、適切な飼養衛生管理とさらなる養蜂技術の向上に貢献する研究であると考えられる。