ANTIFUNGAL AND BIOCONTROL ACTIVITIES OF BACTERIA ISOLATED FROM JAPANESE FROG SKIN AGAINST PLANT PATHOGENIC FUNGI

2021.3

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Acknowledgement

I wish to acknowledge my profound gratitude and great appreciation to my supervisor Prof. Tsutomu Arie, for his invaluable guidance during my study in Japan, his kindness, patience, encouragement and continual help and advice. I would also like to express my gratitude and appreciation to Prof. Ken Komatsu for all his valuable comprehensive advice and encouragement. I am also thankful to Prof. Hiromitsu Moriyama (TUAT), Prof. Masami Nakajima (Ibaraki University) and Prof. Hisashi Nishigawa (Utsunomiya University) for their timely suggestions and contribution to the quality of my dissertation.

Sincere appreciation is expressed to Project Management Unit (PMU) 5000 Doktor Program of Indonesian Ministry of Religious Affairs for the financial support of my study. Thousand thanks are expressed to my institution, UIN Sunan Kalijaga Yoyakarta Indonesia, and colleagues for their great helps and supports. My special appreciation to my lab-mates for their great friendship and support in providing their relevant assistance during my work in laboratory. I truly appreciate all the advice from all lab members, past and presents of Arie Laboratory.

Deepest gratitude is expressed to my dear late mother and father. They have always been an integral part of my life and always mean a lot to me and I always be grateful to them. I am also thankful to my brothers and sister for their constant love and support.

学 位 論 文 要 旨

ANTIFUNGAL AND BIOCONTROL ACTIVITIES OF BACTERIA ISOLATED FROM JAPANESE FROG SKIN AGAINST PLANT PATHOGENIC FUNGI

日本産カエル類から分離した細菌の植物病原菌に対する抗菌活性および生物防除活性に関わる研究

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The bacterial communities on amphibians such as frogs play a pivotal role in host health. These communities of microbiota occupy the mucous layer forming a microbiological barrier for skin protection from invasive organisms such as fungi, bacteria, protozoa, and viruses by several mechanisms involved including competition of place or resources, production of antimicrobial compound production like volatile organic compounds and antimicrobial peptides. Frog-skin bacteria such as *Jatinobacterium lividum* and *Lysobacter gummomus* have been reported to have strong antifungal activity against the amphibian fungal pathogens. These bacteria having antifungal activity have the potential to be applied for other purposes, such as biocontrol agents for plant disease control. Concerns about chemical fungicides for controlling plant diseases are a social issue due to the burden on the environment and the emergence of resistant plant pathogenic fungi, and biological control is attracting attention as an alternative. Thus, employing some beneficial bacteria as environment-friendly "microbial fungicides" in agricultural fields could be applied for tackling plant diseases.

This study aimed to collect culturable bacteria from the skin of wild frogs sampled in Japan, and evaluate the antagonistic activity of the bacteria toward plant pathogenic fungi.

A collection of 106 bacterial isolates was obtained from three species of frogs, namely *Hyla japonica* (Japanese tree frog), *Pelophylax porosus porosus* (Tokyo daruma pond frog), and *Buergeria burgeri* (Kajika frog). Two most abundant phyla were detected. The predominant phylum from all three species of frogs was Proteobacteria which represented 79.3% of the total bacteria, followed by Bacteroidetes (15.1%). Bacteria in phyla Firmicutes and Actinobacteria were detected in a low abundance. In *H. japonica*, *P. p. porosus* and *B. buergeri*, the proportion of bacteria in the class Gamma-proteobacteria in the phylum Proteobacteria was 80.0, 62.5, and 60.0%, respectively. *Erwinia*, a genus in the phylum Proteobacteria, was dominant in *H. japonica* and *P. p. porosus* obtained from rice paddy fields, while *Cyrseobacterium* in the phylum Bacteroidetes and Acinetobacter in the phylum Proteobacteria were dominant in *B. buergeri* from a stream. These results suggested that host frog species and environment of habitat are significant factors influencing bacterial structure.

Using a dual-culture method on plate media three frog-skin bacteria, HjD52 and HjD92 from *H. japonica* and B341 from *B. buergeri* were selected based on their ability to significantly inhibit the growth of *Colletotrichum orbiculare*, the causal fungus of cucumber anthracnose disease. Among the 13 plant pathogenic fungi evaluated, the growth of 12 fungal species was significantly inhibited by HjD92 and B341. HjD57 inhibited the growth of five fungi. All three frog-skin bacteria strongly inhibited the mycelial growth of *Botrytis cinerea*, the gray mold pathogen, by 59.4, 55.0, and 63.1%, respectively. In contrast, none of the frog-skin isolates inhibited the mycelial growth of *Penicillium digitatum*, the green mold pathogen.

Furthermore, spray treatment with the suspensions (10^9 cfu/ml) of the three frog-skin bacteria effectively reduced the number of anthracnose lesions in greenhouse-grown, potted cucumber plants. Among the three frog-skin bacteria, B341 seemed to work the best in reducing symptoms. Moreover, pretreatment of tomato seedlings with isolates HjD57, HjD92 and B341 (10^9 cfu/ml) by soil drenching 1-week prior to inoculation with *Fusarium oxysporum* f. sp. *lycopersici* had significantly reduced symptoms of wilt disease. The three frog-skin bacteria also significantly reduced the severity of rice 'bakanae' symptoms, caused by *F. fujikuroi*, by submerging the infested rice seeds in their suspensions (10^9 cfu/ml), respectively. The three frog-skin bacterial isolates did not present negative influence on seed germination and plant growth of cucumber.

Based on the 16S rDNA sequence analysis and similarity search, isolates HjD57, HjD92 and B341 were identified as *Paenibacillus* sp., *Raoultella* sp. and *Citrobacter* sp., respectively.

Cell-free filtrates of isolates HjD57, HjD92 and B341 had strong antifungal activity against *C. orbiculare* mycelial growth which suggested that the possible mechanism of HjD57, HjD92 and B341 involved in the reduction of plant diseases was production of antifungal substances, respectively. The antifungal activity in cell-free filtrate did not lost after heat treatment.

In conclusion, *Paenibacillus* sp. HjD57, *Raoultella* sp. HjD92 and *Citrobacter* sp. B341 isolated from skins of two frog species, *H. japonica* and *B. buergeri*, are candidates for biocontrol agents of plant diseases caused by *C. obiculare*, *F. oxysporum* f. sp. *lycopersici* and *F. fujikuroi*. This is the first report showing the potential of *Paenibacillus* sp., *Raoultella* sp. and *Citrobacter* sp. from frog skin to serve as potent biocontrol agents against plant diseases. The mode of action responsible for the control of plant diseases by these three frog-skin bacteria seemed to be antibiose against pathogens by antifungal compounds produced by the bacteria, although further analyses are necessary to identify the compounds.

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inoculated with *C. orbiculare* A-29 and treated with a bacterial suspension (10^9cfu/ml) of isolate HjD57, HjD92 or B341 at 24-h post-inoculation. Yellow to brown spotted lesions were observed in the inoculated control (SDW). Treatments with isolate HjD57, HjD92 or B341 reduced the number of lesions (A). The number of lesions that developed on whole leaves was counted 7 days post-inoculation and compared with the control (SDW) (B). Data represents the mean (± standard error, SE, n=3) of four independent experiments, each performed in triplicate, and presented relative to control. Asterisks indicate statistically significant differences (***P<0.001, *P<0.05) by Student's t-test compared to the inoculated control (SDW).

Figure 4.3. Biocontrol activity of frog-skin bacteria against tomato wilt in a soil drenching test. Three-week old tomato (cv. Momotaro) seedlings were treated with a bacterial suspension (10^9cfu/ml) of isolate HjD57, HjD92 or B341 by soil drenching and inoculated with *F. oxysporum* f. sp. *lycopersici* race 3 KoChi-1 by soil drenching with a spore suspension 6 days after treatment. Photographs were taken at 30 days post-inoculation. Symptoms of yellowing and wilt were observed in the inoculated control (SDW) and treatment with isolate HjD57, HjD92 or B341 reduced symptom development (A). Symptoms of each plant were graded from 0 to 4, and the disease severity index for each treatment was calculated and compared with the control (SDW) (B). Data represents the mean (± standard error, SE, n=3) of three independent experiments, each performed in triplicate, and presented relative to control. Asterisks indicate statistically significant differences (***P<0.001) by Student's t-test compared to the inoculated control (SDW).

Figure 4.4. Biocontrol activity of frog-skin bacteria against rice 'bakanae' by a seed treatment test. Rice (cv. Tanginbouzu) seeds artificially infested with *Fusarium fujikuroi* Miyagi 92-10 were submerged in a bacterial suspension (10^9 cfu/ml) of isolate HjD57, HjD92 or B341 for 15 min and sown in sterile soil in pots. Photographs were taken 14 days after sowing. Symptoms of stunted, narrow and yellow leaves and irregular elongation of the leaves were observed in the inoculated control (SDW). Treatment with isolate

CHAPTER 1.
GENERAL INTRODUCTION

1.1. Amphibian's Microbiota and Their Benefits

1.1.1 Diversity of Amphibian Skin Peptides

Amphibian skin is always exposed to the environment including physical factors, microorganisms and predators (Xu and Lai, 2015). The amphibian skin is relatively thin and permeable (Varga et al., 2019), hence, their skin is vulnerable to pollutants (McCoy and Peralta, 2018) and pathogens (Madison et al., 2017). Therefore, the essential physiological processes occurred in their skin play an important role for maintaining their health (Varga et al., 2019). Skin is mainly responsible for respiration, heat transfer, camouflage, osmoregulation (Voyles et al., 2009) and thermoregulation (Demori et al., 2019).

Amphibian skins have several mechanisms to maintain their skin from the aforementioned situations. Their skin can secrete some remarkable substances such as antioxidants (Liu et al., 2010), and antimicrobial peptides (Conlon, 2011a; Rollins-Smith et al., 2005). Those substances are secreted in mucous glands and poison glands which have essential roles for gas exchange and host defense against some invasive organisms (Jared et al., 2018). A plethora of these two glands is located in amphibian dermis. Dermis layer is divided into two different layers, the stratum spongiosum and stratum compactum. Both stratums are located beneath the epidermis layer. The granular glands are equipped with a layer of myoepithelial cells with surrounded adrenoreceptors. The contraction of myoepithelial cells is induced by any kind of stress and squeezing the granular glands eventually release the glands content on the surface of skin (Demori et al., 2019).

Mucous glands produce substances for instance glycosylated mucin and mucopolysaccharides that can maintain skin humidity and can behave as a physical barrier to pathogen (Demori et al., 2019; Smith et al., 2018). Other glands namely poison glands or also called granular glands can release vary defensive bioactive molecules that are used for pathogen deterrence and host immunity, for instance antimicrobial peptides, alkaloids, steroids, amines, antibodies, and lysozymes (Daly, 1999; Rollins-Smith, 2005; Smith et al., 2018). Demori et al. (2019) suggested that each amphibian species produces a specific set of peptides with well-defined sequences. Xu and Lai, (2015) reported that 2000 peptides from amphibian skin have been well-characterized. Furthermore, their bioactive peptides have not only antimicrobial or antifungal activities but also have multiple functions such as wound-healing, mating, analgesia, antioxidant, immunoregulation, neuroendocrine regulation, antipredation, antimicrobe, and antiparasitism (Demori et al., 2019; Xu and Lai, 2015).

Those metabolites are released into skin when the amphibian is injured (irritation, mechanical pressure), or stressed, or by adrenaline stimulation (Smith et al., 2018; Xu and Lai, 2015). The higher concentration of peptides will be released if the degree of exposed stress is stronger (Rollins-Smith, 2005). Recently, the majority of studies has been increasingly focused on isolation and characterization of antimicrobial peptides due to its potential as therapeutic agents and protect the amphibian skin against pathogen (Libério et al., 2014; Won et al., 2004). Total of 1900 antimicrobial peptides (AMPs) from 178 amphibian species, mostly from frog species belonging to 28 genera, previously have been characterized (Xu and Lai, 2015). These AMPs are important for preventing infection.

Numerous studies have been reported previously on recovering the remarkable peptides from frog-skin with antimicrobial and antifungal activities. For instance, amolopins from *Amolops loloensis* (the rufous-spotted torrent frog) (Wang et al., 2008), temporin from *Pelophylax*

saharica (the Sahara frog) (Abbassi et al., 2008), brevinine-1, brevinine-2, esculantin-2 and temporin from *Hylarana erythraea* (the green frog) (Al-Ghaferi et al., 2010), temporin and brevinine-2 isolated from the edible frog, *Rana esculenta* (Ali et al., 2003) and magainin from *Xenopus laevis* (African clawed frog) (Zasloff, 1987). All of those AMPs effectively inhibited Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus* ATCC 2592, *Bacillus pumilus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii,* and also fungal *Candida albicans* ATCC 90028. Those studies indicated that these peptides from frog-skin considerably have attracted to be developed as a promising therapeutic option in the future.

An AMP namely esculentin-1 secreted by *R. esculenta* is known to inhibit a wide variety of microorganisms including *Phytophthora nicotianae* as reported by Ponti et al. (2003). *Nicotiana tabacum* (tobacco) which integrated with DNA coding for esculentin-1 was inoculated with *P. nicotianae* and presented the enhanced resistance to the pathogen.

1.1.2 Diversity of Amphibian Skin-Associated Bacteria

It has been known that the skin of amphibia are rich source of antimicrobial peptides and therapeutic agents (Daly, 1999; Pasteris et al., 2009) as innate immune defenses against the invasion of pathogen (Rollins-Smith, 2005). These antimicrobial compounds are secreted by microbial symbiont on amphibian skin (Mangoni et al., 2001). These communities of microbiota occupy the mucous layer forming a microbiological barrier for skin protection as illustrated in Fig. 1.1 (Varga et al., 2019). High diversity of microbes can grow and reside on amphibian skin surface because the skin provides a suitable habitats for them by producing a glycoprotein-containing mucus (Austin, 2000). Smith et al. (2018) and Woodhams et al. (2014) described the term of mucosome as a together mixture mucin glycoprotein, granular glands secretions and the skin microbiota.

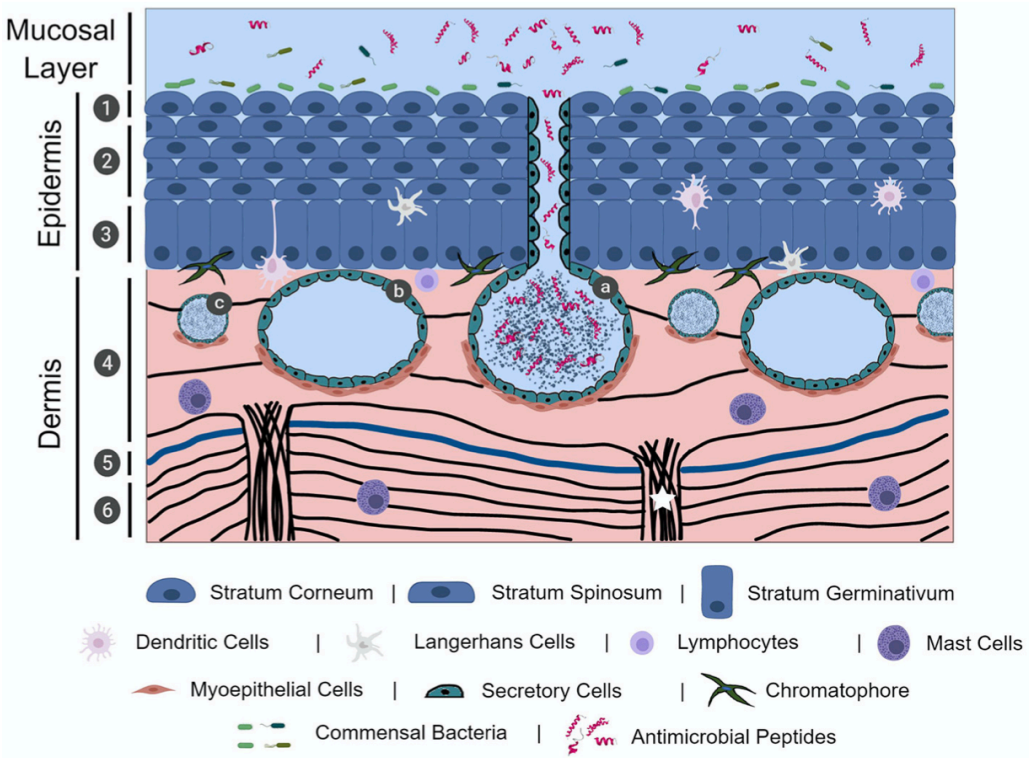

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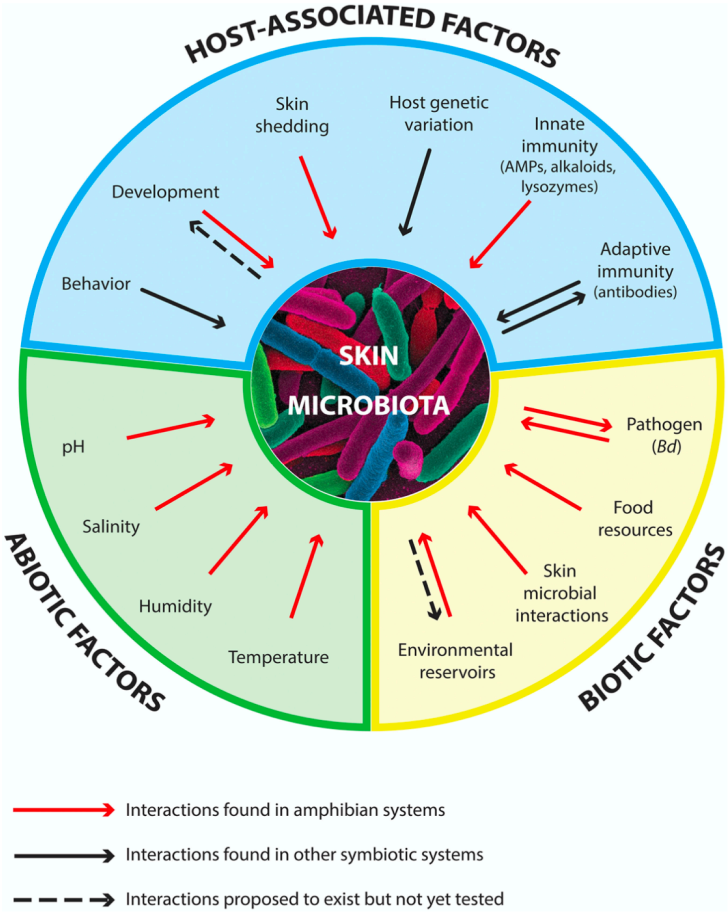
Figure 1.1. The physical, chemical, cellular and microbiological innate immune barriers of amphibian skin (e.g. frog). These four barriers that works altogether as host defense against pathogen. The communities of bacteria overlay the surface of skin forming the microbiological barrier. Frog-skin is composed of an epidermal and dermal layer. The epidermis is comprised of three different layers: (1) stratum corneum, (2) stratum spinosum, (3) stratum germinativum. The dermis is comprised of connective tissue formed by collagenous fibers (black lines) in two layers: (4) the spongious dermis, (6) the compact dermis connected with collagenous column (white star). In terrestrial frog, the Eberth-Kastschenko (EK, 5) layer separates the spongious and the compact dermal layer. (a) granular gland, (b) mucosal gland and (c) small mixed gland secretes metabolites compounds (antimicrobial peptides and mucus) (Varga et al., 2019; use permitted under the Creative Commons Attribution License (CC BY).

The bacterial communities on amphibian skin can greatly vary among the species, which mainly due to the result of complex processes including environmental factors and host-specific characteristics (Adair and Douglas, 2017) and moreover, Kueneman et al. (2014) and Varela et al. (2018) suggested that environment could be a significant factor in determining the bacterial community composition. These environmental factors are including biotic and abiotic factors, for abiotic factors such as temperature (Daskin et al., 2014), season (Longo et al., 2015; Woodhams et al., 2014), pH (Krynak et al., 2015), salinity and humidity (Antwis, et al., 2016). Pesticide (McCoy and Peralta, 2018) and captivity (Woodhams, et al., 2014) also can alter the composition of bacterial community on amphibian skin. But, one of the most important factors generating the composition of bacterial communities on amphibian skin is host species (McKenzie et al., 2012; Sabino-Pinto et al., 2016). Biotic factors include life stages (Rollins-Smith et al., 2011; Sanchez et al., 2017), diet (Antwis et al., 2014), pathogen (Jani and Briggs, 2014, 2018) and microbial interaction (Bates et al., 2018). All the main factors that influence the diversity of microbial community on amphibian skin are systematically illustrated by Rebollar et al. (2016a) (Fig. 1.2).

The variation of the skin microbial communities on amphibian skin can lead to higher mortality of amphibia (Harris et al., 2009). It suggested that the microbial structure relates to the host susceptibility and the presence of pathogens (Rebollar et al., 2016b). *Batrachochytridium dendrobatidis* and *B. salamandrivorans* are the most devastating fungal pathogens of amphibians, which cause chytridiomycosis, and possibly are responsible for the extinction of amphibian species worldwide (Becker et al., 2015; Bosch et al., 2001; Harris et al., 2009; Hyatt et al., 2010; Skerratt et al., 2007; Woodhams et al., 2016, 2018; Voyles et al., 2009; Zipkin et al., 2020). Campbell et al. (2019) reported that bacterial communities also protect amphibian skin (e.g. frog) from a viral pathogen, Ranavirus.



(Image source: Rebollar et al., 2016a, used under permission from Rebollar, E.A)

Figure 1.2. Three main factors that impact on the diversity and function of the amphibian skin microbes/microbiota includes biotic factors, abiotic factors and host-associated factors. Bidirectional interactions (indicated by arrow in both direction) might occur between skin microbiota and particular factor (Rebollar et al., 2016a)

1.1.3 Composition of bacteria on frog-skin

Some of bacteria which constitute the microbial communities on amphibian skin are culturable (Walke et al., 2015). Previous study has reported that frog-skin bacteria can be cultured by using the common techniques and media, and known as culture-dependent techniques (Medina et al., 2017; Walke et al., 2015), and by linking culture-independent and culture-dependent techniques (Rebollar et al., 2016a). Currently, molecular biology approaches have established by using for the culture-independent technique (Vartoukian et al., 2010). In amphibian skin system, the recent method used are by integrating "-omics" techniques with culture-independent such as metabolomics and the 16S amplicon sequencing. These approaches can identify the potential application of bacterial symbiont of amphibian skin as probiotics (beneficial skin-associated bacteria) (Rebollar et al., 2016a).

Numerous studies have been reported of the recovery of bacterial symbiont from frog-skin. For instance, Belden et al. (2015) successfully isolated diverse species of bacteria from the skin of frogs, *Craugastor fitzingeri* (Fitzinger's robber frog), *Dendropsophus ebraccatus* (pantless treefrog), and *Agalychnis callidryas* (red-eyed leaf frog) in Panama. Hillis et al. (2015) found the culturable bacteria from *Anaxyrus boreas* (boreal toad) and *Rana luteiventris* (Columbia spotted frog) from Wyoming, USA. Becker et al. (2015) found some culturable bacteria isolated from 11 Panamian frog species include *Allobates talamancae* (Striped rocket frog), *Atelonopus limosus* (Limosa harlequin frog), *Bufo typhonius* (Leaf letter toad), *Colostethus panamensis* (Panama rocket frog), *Craugastor crassidigitus* (Slim-fingered rain frog), *Dendrobates auratus* (Green and black dart-poison frog), *Hyalinobatrachium colymbiphyllum* (Plantation glass frog), *Silverstoneia flotator* (Rainforest rocket frog), *Smilisca sordida* (Drab treefrog), *Smilisca sila* (Panama cross-banded treefrog) and *Strabomantis bufoniformis* (Rusty robber frog), and

Woodhams et al. (2007) obtained some bacterial symbionts from skin of *Rana muscosa* (The mountain yellow-legged frog).

Most of the culturable bacteria from frog-skin were assigned to the class Gamma-proteobacteria in the phylum Proteobacteria (Sabino-Pinto et al., 2016; Hillis et al., 2015; Rebollar et al., 2016b). For instance, Belden et al. (2015) found that the predominant phylum of bacteria from species of frog in Panama were Proteobacteria (>60%), which did not contradict the report by Hillis et al. (2015) where they found that 68.3% of cultured bacteria from *Anaxyrus boreas* (boreal toad) and *Rana luteiventris* (Columbia spotted frog) from Wyoming, USA, were Proteobacteria. Longo et al. (2015) revealed phylum of Proteobacteria (65%) was dominated bacterial communities which isolated from species *Lithobates yavapaiensis* (The lowland leopard frog) and *Eleutherodactylus coqui* (Caribbean tree frog). Bie et al. (2019) detected the dominant phyla of the wild frog *Rana dybowskii* (Dybovsky's frog) in Northeast China, were Protobacteria (77.91%). Other predominant phyla were assigned as Bacteriodetes, Firmicutes, Actinobateria, Cyanobacteria, Verrucomicrobia and Acidobacteria (Belden et al., 2015; Bie et al., 2019; Walke et al., 2015). However, Proteobacteria were high proportion phyla discovered from several species of frog.

1.1.4 Current study on bacteria isolated from Japanese frog-skin

Bacterial communities on the several species of amphibian skin have been reported in several studies (Becker et al., 2009; Becker et al., 2015; Brucker et al., 2008; Harris et al., 2009). In those studies, frog species detected from different regions worldwide showed high diverse in species of bacteria. To date, only a few studies have empirically assessed on frog-skin-associated bacteria from Japanese species of frog and it has been rarely reported.

Bacteria have been isolated from wild and captive six Japanese frog species, including *Rana japonica* (Japanese brown frog), *Odorrana splendida* (Amami oshima frog), *Bufo japonicus* (Japanese common toad), *Bombina orientalis* (Oriental fire-bellied toad), *Cynops pyrrhogaster* (Japanese fire belly newt) and *Echinotriton andersoni* (Japanese warty newt) (Sabino-Pinto et al., 2016). Other studies showed that *Andrias japonicus*, the Japanese giant salamander, also harbor a number of skin bacteria (Bletz et al., 2017b). The predominant phyla found from *A. japonicus* skin were Proteobacteria (69%) followed by Bacteroidetes (14%), Actinobacteria (7%), Firmicutes (5%), and Verrucomicrobia (2%).

1.1.5 Contribution of frog-skin bacteria on antimicrobial peptides production

It was suggested that antimicrobial compound were produced by frog-skin bacteria (Mangoni et al., 2001; Rollins-Smith, 2009). The study on the beneficial use of these frog-skin bacteria on producing antimicrobial compound is still few. Moreover, several factors have been known to influence the AMPs gene expression. Secretion of AMPs onto the skin, and antimicrobial activity were reported (Varga et al., 2019). One of factors is the existence of frog-skin microbes. Thus, the dynamics of frog-skin communities may contribute to the AMPs production.

Mangoni et al. (2001) demonstrated that secretions of AMPs from *Rana esculenta* were activated by the presence of beneficial bacteria on frog-skin, while frogs that were kept on sterile environment did not produce AMPs. However, the interaction between microbe – frog-skin which influence AMPs production is still unclear. It suggested that likely via the initiation of signaling pathway known as NF- κB (nuclear factor kappa B), a transcription factor in the nucleus. As shown in *Bombina orienalis*, antimicrobial peptide Bombinin was secreted onto the surface of frog-skin (Miele et al., 1998).

Cell receptor on epithelial cell of frog-skin will recognize the microbes and eventually this signal leading to activate the granular glands for synthesis of antimicrobial peptides (Rollins-Smith, 2009). TLRs have pivotal roles in the innate immune system by pathogen-associated molecular pattern attained from various microbes include pathogenic microorganisms such as fungi, bacteria, virus, protozoa etc. It is hypothesized that these bacterial communities may provide a superior ability against pathogenic organism if host's AMPs showed low potency against pathogen (Conlon, 2011b). Thus, if the beneficial microbes are transferred to the susceptible species will enhance the host-defense from the invasion of pathogenic microorganisms.

Even though the findings of Matutte et al. (2000) studies showed that the changes of environment also could induce the production of antimicrobial peptides, this change sometimes promoted the growth of beneficial microbes and was consistent with their antimicrobial peptide production. Therefore, the microbial communities on frog-skin still have a pivotal role in the host defense by stimulating the antimicrobial peptide secretion.

1.1.6 Antifungal activity in frog-skin bacteria and their use

As I mentioned above, bacterial community on frog-skin can protect the host from some pathogen invasion. Most of these bacteria were focused on their fungicidal ability against amphibian pathogens namely *Batrachocytridium* spp., the most devastated pathogen which potentially implicated amphibians decline in worldwide (Voyles et al., 2009). The fungicidal activity of those bacteria can be ultimately applied in other fields for instance fungal disease control in wildlife, human and agriculture (Walke and Belden, 2016). For instance, those bacterial frog-skin can protect host from fungal invasion including *Nosema* sp. (bees), *Ophidiomyces*

ophiodiicola (snakes), *Pseudogymnoascus destructans* (bats), *Oryza sativa* (rice plant) and *Homo sapiens* (human) such as *Pycularia oryzae*, and *Trichophyton rubrum* (Walke and Belden, 2016).

The frog-skin-associated bacteria as the aforementioned, are highly suggested as probiotic to prevent the invasion of amphibian fungal pathogen, *B. dendrobatidis*, *B. salamandrivorans* (Becker et al., 2009; Becker et al., 2015; Brucker et al., 2008; Harris et al., 2009). The mechanisms involved in inhibition of pathogen invasion by bacteria are mainly antibiosis, including production of antimicrobial compounds like volatile organic compounds (VOCs) and bacteriocin, or competition (Brucker et al., 2008; Harris et al., 2009; Woodhams et al., 2018; Woodhams et al., 2016). Among the antimicrobial VOCs, violacein and indole-3-carboxaldehyde secreted by *Jatinobacterium lividum*, a bacterial symbiont in frog-skin, inhibited *B. dendrobatidis* (Woodhams et al., 2018). Also, 2-4-diacetylphloroglucinol (2,4-DAPG) produced by *Lysobacter gummomus* inhibited *B. dendrobatidis* (Brucker et al., 2008).

Myers et al. (2012) found that AMPs of *R. muscosa* and metabolite 2,4-DAPG from *P. fluorescens*, a bacterial species found on skin *R. muscosa* may work synergistically against the fungal pathogen *B. dendrobatidis*. Other notable AMPs include viscosin from bacterial frog-skin, *P. cichorii* may help against human fungal pathogen invasion *Aspergillus fumigatus* (Martin et al., 2019). Interestingly, the frog-skin bacteria themselves interacted each other synergistically to inhibit the fungal pathogen *B. dendrobatidis* (Loudon et al., 2014).

1.2. Crop production and its current constrains

1.2.1 Importance of crop production

It is inevitable that the demand for food globally predicted to increase as driven by the rising population growth. By 2050, the demand for food expected to double around 70%–100% (McKenzie and Williams, 2015; Ray et al., 2013). Several challenges, however, are faced for

meeting the food. According to FAO (2017), those are water scarcity, soil depletion, high level of greenhouse gas emission, resource-intensive farming system and high-input agriculture. To increase the agricultural productivity, some methods can be applied including the biological and agroecological aspects such as genetic plant improvement, sustainable land use, increasing irrigation, integrated nutrient managements, and controlling the pests, diseases and weeds (Spiertz, 2013).

Humans have cultivated the planet for decades to satisfy their needs for food, fiber, and energy. Nearly 40% of the global land surface is now being used for agriculture (Ramankutty et al., 2008). The high demand for food leads to the expansion of cropland and this is a major cause of biodiversity decline globally (Zabel et al., 2019). Thus, the balance of agricultural production and conservation goals are needed to be concerned.

1.2.2 Constrain of crop production

The yield loss in the agricultural sector due to plant diseases is a major problem worldwide. Several devastated diseases of important agricultural crops are caused by phytopathogenic fungi and bacteria (Suárez-Estrella et al., 2013; Oerke, 2006). It is estimated that fungi are the most major cause of plant disease (Chandrasekaran et al., 2016). Most common and economically important genera of phytopathogenic fungi are *Pyricularia oryzae*, *Botrytis cinerea*, *Puccinia* spp., *Fusarium graminearum*, *F. oxysporum*, *Blumeria graminis*, *Mycosphaerella graminicola*, *Colletotrichum* spp., *Ustilago maydis*, and *Melampsora lini* (Dean et al., 2012).

Colletotrichum is one of the most important fungal genera that causes economically important disease of cucurbitaceae production, especially *C. orbiculare* (Syn.: *C. lagenarium*) is a major cause of anthracnose disease on cucumber (*Cucumis sativus* L.) plant (Damm et al., 2013; Shimizu et al., 2009). The pathogen causes lesions on seedlings and all above ground tissue

including leaves, petioles, stems and fruits. Lesions are pale brown to reddish, crack in the center of leave and eventually fall out (Damm et al., 2013).

1.3. Importance of cucumber

Cucumber (*Cucumis sativus* L.) is a member of the family Cucurbitaceae which comprises of about 180 genera and 800 species (Ren et al., 2009) including economically important species like watermelon (*Citrullus lanatus* (Thunb.) Matsum. and Nakai), melon (*C. melo* L.), squash and pumpkins (*Cucurbita* spp). According to Tatlioglu, (1993) cucumber is the fourth most important vegetable worldwide crop after tomato (*Solanum lycopersicum*), cabbage (*Brassica* spp.) and onion (*Allium* spp.). In 2018, 75,219,440 tonnes of pickling and fresh cucumbers are produced worldwide (FAOSTAT, 2019). The top 20 countries for cucumber production (Table 1.1) have been listed as follows (FAOSTAT, 2018).

Likely that cucumber is one of the oldest cultivated crops, which originated from India and spread rapidly to Western Asia then to Southern Europe. Two botanical varieties were discovered including *C. sativus* var. *sativus* (domesticated cucumber) and *C. sativus* var. *hardwickii* (Royle) Alef (wild cucumber) (Lv et al., 2012). Cucumber plant is an annual herbaceous and have unisexual flowers and inferior ovaries (Ren et al., 2009). Since cucumber plant has a short life cycle, rich diversity, a small number of genes, suitability for vascular biology studies, accumulating resources in genetics and genomics, thereby this plant is being developed as a new model species in plant biology (Lv et al., 2012).

The fruits of Cucurbitaceae plants including cucumber provide vitamins and minerals, the source of phytochemicals, and dietary fiber that prevents some human health problems such as obesity and cardiovascular disease (Slavin and Lloyd, 2012). Cucumber fruits consist of more than 90% water, the taste is sweet, refrigerant, hemostatic, and tonic. Cucumber also contains

phytochemicals, that function as antioxidants, antiwrinkle, antimicrobial, antidiabetic, and hypolipidemic (Mariod *et al*., 2017). One cup of cucumber slices with the peel contains the total dietary fiber (TDF) nearly 0.60 g, insoluble dietary fiber (IDF) and soluble dietary fiber (SDF) are 0.50 g and 0.10 g, respectively, potassium (152.0 mg), energy (16.0 kcal) (Slavin and Lloyd, 2012), and vitamin C (Baudoin et al., 2017).

Table 1.2. Top 20 countries for cucumber production

Rank	**Country**	**Production (tonnes)**
1	China	56,240,428
2	Iran	2,283,750
3	Turkey	1,848,273
4	Russian Federation	1,604,346
5	Mexico	1,072,048
6	Ukraine	985,120
7	Uzbekistan	857,076
8	USA	700,819
9	Spain	643,661
10	Japan	550,000
11	Poland	538,676
12	Kazakhstan	460,110
13	Egypt	457,795
14	Indonesia	433,931
15	Netherlands	410,000
16	Republic of Korea	333,233
17	Germany	267,589
18	Cameroon	257,211
19	Sudan	240,405
20	Belarus	226,443

Cucumber can be planted all around year and will grow well under high temperature, high humidity, high intensity of light as well as the availability of nutrient (FAO, 2020; FAO, 2017). Cucumber is very sensitive to unfavorable environment such as temperature. Cucumber seedlings grow well under the temperature between 21°C and 29°C, while for best germination temperature is around 27°C – 28°C is required and these temperature should be maintained until germination stage (Badgery-Parker et al., 2010). This sensitivity of cucumber to the environmental factors may lead to multiple increases in disease if taking the inappropriate management.

The limitation of cucumber production occurs due to the presence of multiple pathogen attacks such as fungi, bacteria, viruses and nematodes. Numerous studies have reported the major diseases of cucumber plant (Chen et al., 2014; Damm et al., 2013; Harata et al., 2016; Ko et al., 2019; Kokalis-Burelle, 1980; Liu et al., 2008; Negishi et al., 2011; Palenchar et al., 2009; Shimizu et al., 2009; Sun et al., 2013; Zehnder et al., 2000).

1.4. Cucumber anthracnose disease and its control

1.4.1. Cucumber anthracnose disease

Colletotrichum is a large genera of phylum *Ascomycetes* which includes a number of important species causing devastating diseases of a wide variety of important agricultural commodities and ornamental plants (De Silva et al., 2017a; Hyde et al., 2009; Perfect et al., 1999) attacking up to approximately 3,200 species of monocot and dicot plants (O'Connell et al., 2012). This pathogen is not only known as a post-harvest pathogen of fruits and vegetables but also reported to cause diseases of tubers and all aboveground part of plant include leaves, stems and seedling in field (Cannon et al., 2012; Damm et al., 2013; De Silva et al., 2017). *Colletotrichum* has a major implication on plant biosecurity due to their difference in lifestyles during infection process. This life styles depend on host plant, species and physiology maturity of host plant and environment (De Silva et al., 2017b), Thus, *Colletotrichum* species have been widely studied as

a model organism for research into genetics (Cannon et al., 2012), and fungal-plant interactions (Perfect et al., 1999).

The most devastating species of *Colletotrichum* which attacks Cucurbitaceae is *C. orbiculare*. This pathogen causes anthracnose of *Cucurbitaceae* plants especially of cucumber (*C. sativus*), melon (*C. melo*), watermelon (*Citrullus lanatus*, pumpkins (*Cucurbita pepo*) and squash (*Cucurbita maxima*). More than 40 plant species are attacked by *C. orbiculare* (Damm et al., 2013). The pathogen can survive in infected seed, soil, crop debris and weeds and it can survive up to two years (Badgery-Parker et al., 2010; Palenchar et al., 2009).

All parts of aboveground host plant can be infected by the fungi (Agrios, 2005) and they present lesions on seedlings, leaves, petioles, stems and fruits of cucurbits (Damm et al., 2013; Qi et al., 2013). Initial symptoms on the leaves appears water-soaked pale brown to reddish lesions, the extend lesions then become rounded and sometimes the centers of lesions may crack and fall out (Damm et al., 2013; Palenchar et al., 2009), while on cotyledons the symptoms are begin with the small water-soaked lesions and turn to pale, chlorotic or necrotic, eventually the lesions become larger and the plants dry up then die (Palenchar et al., 2009). The pathogen can attack cucumber fruits when it begins to mature (Fig 1.3A). Fruit lesions appear circular sunken water-soaked and turn black in color in moist weather, eventually the fruits will be covered with pink spore masses (Damm et al., 2013; Palenchar et al., 2009). Infection with *C. orbiculare* on cucumber fruit resulting decrease in quality while in storage and /or transport, due to the development of infection which occur in field and continue latently after harvest (Palenchar et al., 2009). Initial symptoms on the leaves appears water-soaked pale brown to reddish lesions, the extend lesions then become rounded and sometimes the centers of lesions may crack and fall out as shown in Fig 1.3B (Damm et al., 2013; Palenchar et al., 2009), while on cotyledons the

symptoms begin to show the small water-soaked lesions and turn to pale, chlorotic or necrotic, eventually the lesions become larger and the plant dry up then die (Palenchar et al., 2009).

As a host invasion structure to host plants, *Colletotrichum* forms a dorm-shape and melanized appressoria (Kubo and Takano, 2013). They develop the melanized appressoria for penetration and performs a hemi biotrophic lifestyle as post invasive phases (Irieda et al., 2016) followed by symptomless biotrophic eventually a necrotrophic phase with severe symptoms (Kleemann et al., 2012). Appressorium is a structure that initially developed from germinated conidia on the plant surface, followed by turgor driven penetration of the cuticle (Deising et al., 2000) and could be sometimes of epidermal cells by infective hyphae (Cannon et al., 2012). This appressorium is pigmented with melanin. The melanization on appressoria is needed to activate its function for host penetration especially in *Pyricularia oryzae* and *Colletotrichum* species including *C. orbiculare*, thus the inhibition of melanization for instance due to the genetic mutation or chemicals causes a loss of pathogenicity (Asakura et al., 2012).

General Colletotrichum species have two major stages of infections, intracellular colonization and subcuticular intramural colonization (De Silva et al., 2017b; Perfect et al., 1999). The initial infection starts by melanized appressorium which is formed from a germinated conidium on plant surface and penetrate the cuticle directly. In the subcuticular intramural stage, the penetrated hyphae develop beneath the cuticle and spreads their infection by forming an intramural network of hyphae eventually infects within the epidermal cells. No detected biotrophic phase is shown by this strategy (Crouch et al., 2014; Perfect et al., 1999). Meanwhile, for intracellular colonization stage, following penetration, fungal hyphae grow within the cell lumen and produce specialized infection structure called primary hyphae for invading living host cells, with or without the initial formation of an infection vesicle (Kleemann et al., 2012; Perfect et al., 1999). This infection vesicle grows inside the epidermal and mesophyll cells. At this stage

the host cell and plant still alive and symptomless (De Silva et al., 2017a). The fungus produces bulbous primary hyphae enveloped by an intact host plasma membrane and develop inside living epidermal cells (biotrophic). Here the fungus switches to destructive phase (necrotropic) and differentiates thin, fast-growing hyphae that kill and destroy the host tissues (Crouch et al., 2014; De Silva et al., 2017b; O'Connell et al., 2012; Perfect et al., 1999).

A B

Figure 1.3. The part of cucumber plant infected with *Colletotrichum orbiculare*, a causal agent of cucumber anthracnose disease; A. symptoms of anthracnose lesions on cucumber leaf with rounded lesions and necrotic tissue eventually falling out; B. anthracnose symptom on fruit with water-soaked and sunken lesions (Photo of diseases fruit was taken by Charles Averre, North Carolina State University, Bugwood.org from URL http://www.pestnet.org/fact_sheets/cucurbit_anthracnose_200.htm accessed 11 February 2020).

1.4.2. Cucumber anthracnose disease control

In practice, application of the synthetic chemical fungicides is still a main strategy to control plant diseases including cucumber anthracnose, however, they sometimes promote rapid development of the fungal resistance to fungicides (Jamalizadeh et al., 2011) and possibly generate problems to the environment and human health (Heydari and Pessarakli, 2010).

Several alternative methods has been proposed for management of cucumber anthracnose disease, for instance as Palenchar et al. (2009) proposed of using a resistance cultivars, use healthy seeds and crop rotation, are important to avoid the disease (Zitter et al., 1998 *cit.* Palenchar et al., 2009). Those are some examples of effective technique in reducing the incidence of anthracnose.

Application of plant growth promoting rhizobacteria (PGPR) has been widely exploited and commercially used for plant protection especially to induce systematic resistance against various diseases such as anthracnose disease in cucumber (Ramamoorthy et al., 2001). Cucumber seed-treatment with PGPR *Pseudomonas fluorescens*, *P. aureofaciens*, *P. putida* and *Serratia plymuthica* resulted in a significant suppression of anthracnose disease caused by *C. orbiculare* (Wei et al., 1991). Furthermore, the application of PGPR showed highly consistent in reduction of anthracnose disease in the field by seed-treatment alone or by seed-treatment plus soil drenching (Wei et al., 1996). Mixture of strains PGPR activity, *Bacillus pumilus* INR7, *B. subtilis* GB03 and *Curtobacterium flaccumfaciens* ME1 effectively reduced the severity of anthracnose when applied to cucumber seeds before or at planting under field conditions (Raupach and Kloepper, 1998). The mechanisms involved was induction of systematic acquired resistance (SAR) (Raupach and Kloepper, 2000; Wei et al., 1991). Cell wall modification occurred by seed-treatment with PGPR resulted in the biochemical and physiological changes in cucumber, which

lead to synthesize of proteins and chemicals such as lipopolysaccharides, siderophores and salicylic acid (Ramamoorthy et al., 2001).

Several bacterial agents isolated from healthy plants or soil such as *Bacillus* spp., *Pseudomonas* spp., *Serratia* spp. and *Streptomyces* spp. have been reported to offer biocontrol efficacy against cucumber anthracnose diseases, caused by *C. orbiculare* (Jeun et al., 2004; Ji et al., 2013; Kim et al., 2016; Kim and Chung, 2004; Park et al., 2013; Shimizu et al., 2009), hypovirulent binucleate Rhizoctonia (HBNR) (Muslim et al., 2019), *Paenibacillus* spp. (Sang et al., 2014)

The application of biocontrol agents on cucumber anthracnose disease in field has been reported (Raupach and Kloepper, 1998; Raupach and Kloepper, 2000; Wei et al., 1996) and showed some efficacy and improvement of cucumber plant, however, more studies are required to achieve concrete effect of biocontrol. Novel agents, novel formulations, and understanding the environmental conditions where biocontrol agents work well may improve the efficacy of biocontrol (Heydari and Pessarakli, 2010).

Another strategies applied for controlling cucumber anthracnose disease have been reported by using several plant metabolites, such as the extract of cucumber leaves (*Cucumis sativus*) (Negishi et al., 2011). The combination of biocontrol agents and chemical compounds also have been applied to increase the efficacy of the control of anthracnose disease in cucumber plants, for instance, combined application of *B. subtilis* B4 and acibenzolar-S-methyl (benzo-(1,2,3)-thiadiazole-7-carbothioic acid S- methyl ester; ASM), a commercial systemic acquired resistance (SAR) inducer (Park et al., 2013), helps to reduce the progression of cucumber anthracnose disease (Raupach and Kloepper, 2000).

1.5. Purpose of this study

Various strategies for controlling plant pathogenic fungi especially cucumber anthracnose disease have been proposed, including the use of fungicide, resistance cultivars, physical stresses and extract from plants. Concerning the hazard effect of chemical fungicides, the application of biocontrol agents provides attractive alternatives to manage plant diseases because its environmentally safe and, in some cases could promote the growth of plant thus improve the high yield of crop production.

The origin source of candidate bioagent especially bacteria can be from various niche of environment such as soil, water etc. At current study, this is the first report of collecting bacteria as promising bioagent from wild Japanese species of from skin against plant pathogenic fungi *C. orbiculare*, a causal agent of cucumber anthracnose and other plant diseases, tomato wilt and rice "bakanae". This study was conducted with the following objectives: (1) to isolate bioagents candidates from wild species of Japanese frog-skin and to identify all collected isolate based on the 16S rDNA sequence analysis, (2) to primary screen their antifungal activities in vitro, (3) to verify their efficacy to suppress plant diseases, cucumber anthracnose, tomato wilt and rice "bakanae" under greenhouse experiment, (4) to analyze the putative mechanisms involved in antifungal activities.

1.6. Outline of this thesis

This thesis is composed of 5 chaptes. Chapter 1 presents a general introduction, chapter 2, presents isolation, morphological characterization and identification of all collected frog-skin bacteria, chapter 3, presents primary screening of antifungal activities of all isolated bacteria, chapter 4, presents the biological control test of three selected bacteria against some plant diseases under greenhouse experiment and analysis their putative mechanisms involved in biocontrol activity, chapter 5, presents conclusion. The outline of each chapter is as follows:

Chapter 1 is the general introduction. It deals with six sections; (1) Amphibian's microbiota and their benefits, (2) Crop production and its current constrains, (3) Importance of cucumber, (4) Cucumber anthracnose disease and its control, (5) Purpose of this study, (6) Outline of this thesis.

Chapter 2 has a title "Isolation and identification of culturable bacteria from skin of wild frog in Japan". It deals with isolation, purification, and molecular identification of culturable bacteria from frog-skin. It also presents the morphological colonies of bacteria.

Chapter 3 has a title "Screening of collected frog-skin bacteria for antifungal activities against *C. orbiculare*". It deals with the primary screening of antifungal activities of all collected bacteria from frog-skin in vitro.

Chapter 4 has a title "Antifungal activity of *Paenibacillus* sp. HjD57, *Raoultella* sp. HjD92 and *Citrobacter* sp. B341 against some important plant diseases and analysis of the putative mechanisms used in biocontrol". It deals with the efficacy of three selected bacteria for controlling cucumber anthracnose and two other plant diseases, tomato wilt and rice "bakanae" diseases under greenhouse experiment and analysis of its putative mechanisms involved in biocontrol test.

Chapter 5 is conclusion. It consists of two sections: (1) conclusion, and (2) prospect for future research.

CHAPTER 2.
ISOLATION AND IDENTIFICATION OF CULTURABLE BACTERIA FROM SKIN OF WILD FROG IN JAPAN

2.1. Abstract

Frogs carry bacterial communities on their skin (Harris et al., 2009) that benefit the host frogs by preventing harmful pathogen infections. This study aimed to collect culturable bacteria from the skin of wild frogs sampled in Japan, *Hyla japonica*, *Pelophylax porosus porosus,* and *Buergeria burgeri*, and identify their diversity by employing 16S rDNA amplicon sequencing of swab samples from three species of frogs that were wild-caught in the paddy fields and stream. A total of 106 bacterial isolates belonging to 4 different phyla were detected. The predominant phylum from all three species of frogs found was Proteobacteria, representing 79.3% of the total bacterial phyla, followed by Bacteroidetes (15.1%). Class Gamma-proteobacteria were highly represented among samples of frog, *H. japonica* (80.0%), *P. p. porosus* (62.5%), and *B. buergeri* (60.0%). Bacteria that belong Firmicutes and Actinobacteria were detected in a low abundance. Our result putatively suggests that host species and environment are significant variants in microbiota structure.

Key words: Japanese frogs, *Hyla japonica*, *Pelophylax porosus porosus, Buergeria burgeri*

2.2. Introduction

All living things host a diverse array of microorganisms, many of which are pivotally important for the host. Amphibians such as frogs are known to have distinct bacteria associated with their skin, and such bacterial communities have a different structure in comparison with other environmental sources including soil or pond water (Antwis et al., 2014; Belden et al., 2015; Bletz et al., 2017a; McKenzie et al., 2012). These bacterial floras are often protecting the hosts' skin from invasive organisms such as fungi, bacteria, and protozoa (Harris et al., 2009; Rollins-Smith, 2005). *Batrachochytridium dendrobatidis* and *B. salamandrivorans* are the most devastating fungal

pathogens of amphibians, which cause chytridiomycosis, and possibly are responsible for the extinction of amphibian species worldwide (Becker et al., 2015; Bosch et al., 2001; Harris et al., 2009; Hyatt et al., 2010; Skerratt et al., 2007; Woodhams et al., 2016, 2018; Zipkin et al., 2020). Several investigators have reported that some mutualistic bacteria protect amphibian skin from the invasion of *B. dendrobatidis* and *B. salamandrivorans* (Becker et al., 2009; Becker et al., 2015; Brucker et al., 2008; Harris et al., 2009). Campbell et al. (2019) suggested that bacterial symbionts also protect frog-skin from a Ranavirus, a viral pathogen.

Small portion (2.81–7.47%) of bacteria in microbial communities on amphibian skin are culturable (Walke et al., 2015). For instance, Belden et al. (2015) successfully isolated diverse species of bacteria from the skin of several frog species, including *Craugastor fitzingeri* (Fitzinger's robber frog), *Dendropsophus ebraccatus* (pantless treefrog), and *Agalychnis callidryas* (red-eyed leaf frog), in Panama. These investigators reported that bacteria in the phylum Proteobacteria were predominant (>60%) among the cultured isolates. This finding agreed with the report by Hillis et al. (2015) in which they found that 68.3% of the cultured bacteria from *Anaxyrus boreas* (boreal toad) and *Rana luteiventris* (Columbia spotted frog) from Wyoming, USA, were Proteobacteria. Moreover, most of the culturable bacteria from frog-skin were assigned to the class Gamma-proteobacteria in the phylum Proteobacteria (Sabino-Pinto et al., 2016; Hillis et al., 2015; Rebollar et al., 2016b). The major representative genera of the class Gamma-proteobacteria are *Citrobacter*, *Pseudomonas*, *Raoultella*, *Serratia*, and *Stenotrophomona* (Assis et al., 2017; Flechas et al., 2017; Hillis et al., 2015).

Bacteria from six Japanese frog species, including *Rana japonica* (Japanese brown frog), *Odorrana splendida* (Amami oshima frog), *Bufo japonicus* (Japanese common toad), *Bombina orientalis* (Oriental fire-bellied toad), *Cynops pyrrhogaster* (Japanese fire belly newt) and *Echinotriton andersoni* (Japanese warty newt), were reported previously by Sabino-Pinto et al. (2016). So far, however, no bacteria have been reported from *Hyla japonica* (Japanese tree frog;

Amphibia/Anura/Hylidae), *Pelophylax porosus porosus* (Tokyo daruma pond frog; Amphibia/Anura/Ranidae), and *Buergeria buergeri* (Kajika; Amphibia/Anura/Rhacophoridae), all of which are endemic in Japan and ubiquitously found primarily in agricultural paddy fields and streams (Osawa, 2008; Togane et al., 2018).

Similar to other vertebrates and human, the composition of bacterial communities on amphibian has distinct patterns (Sabino-Pinto et al., 2016). Multiples factors has been influenced of these patterns including host species and sampling sites (Belden et al., 2015; Kueneman et al., 2014); ontogenetic and seasonal changes (Longo et al., 2015). Additionally, the microhabitat associated with skin region also contribute to variation in frog-skin microbiota (Bataille et al., 2016).

It is essential to identify the composition of bacterial communities from frog-skin and specify of these communities in order to explore further as a promising candidate of bioagent against plant pathogenic fungi. In the present study, the variation of culturable bacteria from frog-skin of three native species in Japan were assessed. In addition, this study provides information on the putative composition of culturable bacteria isolated from the abovementioned frog-skin species which are still poorly studied.

2.3. Materials and Methods

2.3.1. Field Sampling

Three individuals of each Japanese frog species, *Hyla japonica*, *Pelophylax porosus porosus* and *Buergeria buergeri*, were used. All of them were identified based on their morphological features using identification book in Japan (Maeda and Matsui, 1999). Sampling was carried out in summer season 2016 and 2017 at different sites in the rice (*Oryza sativa*) paddy fields at the Field Museum Honmachi, TUAT, Fuchu, Tokyo for *H. japonica* and *P. p. porosus* (Fig. 2.1A), whereas *B. buergeri* was caught in the streams located at

Itsukaichi, Akiruno, Tokyo (Fig. 2.1B). All samples were captured and handled with gloved hands.

A **B**

Figure 2.1. Sampling site of three sampled species of frog. A) *Hyla japonica* and *Pelophylax porosus porosus* in the paddy field at the Field Museum Honmachi, TUAT, Fuchu, Tokyo. B) *Buergeria buergeri* in streams located at Itsukaichi, Akiruno, Tokyo.

2.3.2. Sampling procedure of isolation of frog-skin bacteria

In this study, the culture dependent characterization by plating onto R2A medium was performed. First, the sampled frogs were rinsed with sterile distilled water (SDW) three times to remove transient microbes on their skin surfaces (Woodhams et al., 2007), followed by wiping the skin with sterile tissue paper. Then the frogs were swabbed their dorsal and ventral regions twenty times each with sterilized cotton swabs (Medina et al., 2017). The dorsal swab was drawn from the posterior to the head and down to the pelvic region. The ventral swab

began from the forelimbs and extended posteriorly to the pelvic area. The swab buds were placed in 1.5-ml sterile plastic tubes and kept at 4ºC until use. Frogs were quickly released to the original site of collection. Each swab bud was dunked in 10 ml 0.85% (w/v) NaCl and vortexed. The suspension was then diluted (10^{-1} and 10^{-2}) with the same solution, and 100 µl of each dilution was spread onto an R2A medium plate (15 ml in a ø90-mm Petri dish; Antwis et al., 2014) and incubated at 28ºC for 2 days. The resulting bacterial colonies were sub-cultured on fresh nutrient agar (NA) medium plates and incubated at 28ºC for further study.

Bacterial morphotypes were defined according to the macroscopic characteristics of the obtained colonies (i.e. color, shape, elevation, margin and opacity). Single colonies of each bacterial morphotype were streaked on fresh NA plates until pure cultures were obtained. Each isolate was cryopreserved in 10% skim milk at −80°C.

2.3.3. DNA extraction

Genomic DNA of all recovered bacteria were extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Bacterial isolates were cultured in TSB at 28°C for 48 h. The bacterial cells were harvested by centrifugation (536 ×*g*) at 4°C for 15 min. DNA quantity and purity were spectrophotometrically evaluated using a NanoDrop 1000 UV-VIS microvolume spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the DNA concentration was adjusted to 50.0 ng/µl. An approximately 250-bp fragment of the bacterial V4 region of the 16S rRNA gene was amplified using the universal primer set 515F (5′-CACGGTCGKCGGCGCCATT-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Medina et al., 2017). The total reaction volume per vial was 10.0 µl and consisted of 0.05 µl Ex *Taq* (Takara Bio Inc., Shiga, Japan), 0.2 µl of each primer (20 µM), 0.2 µl of 2 mM (each) dNTPs, 1.0 µl of 10× buffer (Takara Bio) and 1.0 µl of the template bacterial DNA. Reactions

were held at 94°C for 3 min to denature the DNA, with amplification proceeding for 35 cycles at 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s, and a final extension at 72°C for 10 min. The amplicon was visualized in a 1% (w/v) agarose gel and subjected to DNA sequencing (ABI PRISM3130 Genetic Analyzer, Thermo Fisher Scientific). Primers used for sequencing were the same as those used for amplification of the 16S-V4 region (515F and 806R).

2.3.4. Sequence analysis

The sequence of each isolate was analyzed for its similarity with the sequences in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov) and analyzed using ATGC-MAC ver. 7 software and aligned by Clustal W (Thompson et al., 1994). A phylogenetic tree was constructed with Neighbor-Joining analysis (NJ) using MEGA Ver. X (Kumar et al., 2018; Stecher et al., 2020). Shared operational taxonomic units (OTUs) between groups of interest were visualized using Venn diagrams generated using the program Venny (Oliveros, 2007). The relative abundance of the top genera was generated using the function of heatmap.2 the gplots package in R (Warnes et al., 2009). Same package was used for displaying relative abundance in phylum and class level using the stacked bar chart.

2.4. Results

2.4.1. Isolation of bacteria from frog-skin and their macroscopic features

From the colonies appearing on the medium, those having a visually different phenotype (shape, surface, color, etc.) were picked. A total of 106 bacterial isolates were obtained, including 45 isolates from *H. japonica*, 16 isolates from *P. p. porosus* and 45 isolates from *B. buergeri* (Table. 2.1A, 2.1B and 2.1C). The feature of bacterial isolate from three sampled frog species shared in shape, margin and opacity characteristics, circular, entire and translucent. The color (Fig. 2.2.) and elevation of some colonies found differ among them.

Table 2.1A. Characteristic of the distinct colony morphologies of bacteria from frog skin (*H. japonica* = ニホンアマガエル) on TSB medium.

No	Isolate	Colour	Shape	Margin	Elevation	Opacity
1	HjV1	Cream	Circulair	Entire	Convex	Translucent
2	HjV8	Cream	Circulair	Entire	Convex	Translucent
3	HjV9	Yellow	Circulair	Entire	Flat	Translucent
4	HjV10	Yellow	Circulair	Entire	Flat	Translucent
5	HjV11	Cream	Circulair	Entire	Flat	Translucent
6	HjV12	Cream	Circulair	Entire	Flat	Translucent
7	HjV13	Cream	Circulair	Entire	Convex	Translucent
8	HjV14	Cream	Circulair	Entire	Flat	Translucent
9	HjV15	Cream	Circulair	Entire	Convex	Transparent
10	HjV16	Cream	Irregulair	Entire	Flat	Translucent
11	HjV20	Cream	Circulair	Entire	Flat	Translucent
12	HjV21	Cream	Circulair	Entire	Convex	Transparent
13	HjV22	Cream	Circulair	Entire	Flat	Translucent
14	HjV23	Cream	Circulair	Entire	Flat	Translucent
15	HjV25	Cream	Circulair	Entire	Flat	Translucent
16	HjV26	Cream	Circulair	Entire	Convex	Transparent
17	HjV29	Cream	Circulair	Entire	Flat	Translucent
18	HjV10b	Cream	Circulair	Entire	Flat	Translucent
19	HjV11a	Cream	Circulair	Entire	Convex	Translucent
20	HjV11b	Cream	Circulair	Entire	Convex	Translucent
21	HjV8a	Orange	Circulair	Entire	Rised	Translucent
22	HjV48b	Yellow	Irregulair	Entire	Flat	Translucent
23	HjV7a	Orange	Circulair	Entire	Convex	Transparent
24	HjV7b	Yellow	Circulair	Entire	Convex	Translucent
25	HjV23a	Cream	Circulair	Entire	Flat	Transparent
26	HjD53	Yellow	Circulair	Undulate	Flat	Translucent
27	HjD55	Cream	Circulair	Entire	Flat	Translucent
28	HjD56	Yellow	Circulair	Entire	Rised	Translucent
29	HjD57	Yellow	Circulair	Entire	Rised	Translucent
30	HjD58a	Cream	Circulair	Entire	Flat	Translucent
31	HjD59	Yellow	Circulair	Entire	Rised	Translucent
32	HjD61	Cream	Irregulair	Entire	Rised	Translucent
33	HjD64a	Cream	Circulair	Entire	Flat	Translucent
34	HjD90	Yellow	Circulair	Entire	Convex	Translucent
35	HjD91	Cream	Circulair	Entire	Flat	Translucent
36	HjD92	Cream	Circulair	Entire	Convex	Translucent
37	HjD93	Cream	Circulair	Entire	Flat	Transparent
38	HjD94a	Cream	Filamentous	Undulate	Flat	Translucent
39	HjV95	Cream	Circulair	Entire	Flat	Translucent
40	HjD97	Cream	Circulair	Entire	Flat	Translucent
41	HjD100	Cream	Circulair	Undulate	Flat	Opaque
42	HjD62a	Cream	Circulair	Entire	Flat	Translucent
43	HjD63a	Cream	Irregulair	Entire	Convex	Transparent
44	HjD65a	Cream	Circulair	Undulate	Rised	Translucent
45	HjV27	Cream	Irregulair	Entire	Flat	Translucent

Table 2.1B. Characteristic of the distinct colony morphologies of bacteria from frog skin (*P. p. porosus* = トウキョウダルマガエル) on TSB medium.

No	Isolates	Colour	Shape	Margin	Elevation	Opacity
1	PpV66	Cream	Circulair	Entire	Flat	Opaque
2	PpV67	Cream	Circulair	Entire	Flat	Opaque
3	PpV70	Cream	Irregulair	Undulate	Convex	Translucent
4	PpD78	Cream	Circulair	Entire	Convex	Translucent
5	PpD79	Cream	Circulair	Undulate	Rised	Opaque
6	PpV81	Cream	Circulair	Entire	Flat	Translucent
7	PpV83a	Cream	Circulair	Entire	Flat	Translucent
8	PpV84	Cream	Circulair	Entire	Rised	Translucent
9	PpV85	Cream	Circulair	Entire	Convex	Translucent
10	PpV82a	Cream	Circulair	Entire	Flat	Translucent
11	PpV82b	Yellow	Circulair	Entire	Convex	Opaque
12	PpV118	Cream	Circulair	Entire	Convex	Translucent
13	PpV119	Cream	Circulair	Undulate	Flat	Translucent
14	PpV120	Cream	Filamentous	Lobate	Flat	Translucent
15	PpV121	Cream	Filamentous	Lobate	Flat	Translucent
16	PpV122	Cream	Filamentous	Lobate	Flat	Translucent

Table 2.1C. Characteristic of the distinct colony morphologies of bacteria from frog skin (*B. buergeri* = カジカガエル) on TSB medium.

No	Isolates	Colour	Shape	Margin	Elevation	Opacity
1	MDB21	Cream	Irregulair	Entire	Convex	Translucent
2	MDB23	Yellow	Circulair	Entire	Convex	Translucent
3	MDB24	Yellow	Circulair	Entire	Flat	Translucent
4	MDB26	Cream	Circulair	Entire	Pulvinate	Translucent
5	MVB27	Yellow	Circulair	Entire	Convex	Translucent
6	MVB28	Yellow	Irregulair	Entire	Convex	Translucent
7	AFD1	Pink	Irregulair	Entire	Flat	Translucent
8	AFD2	Yellow	Circulair	Entire	Flat	Opaque
9	AFD3	Cream	Filiform	Entire	Flat	Translucent
10	AFD5	White	Circulair	Entire	Flat	Translucent
11	AFD6	Cream	Irregulair	Entire	Flat	Translucent
12	AFD7	Cream	Irregulair	Entire	Flat	Translucent
13	AFD8	Yellow	Irregulair	Entire	Flat	Translucent
14	AFD13	Yellow	Irregulair	Entire	Flat	Translucent
15	AFD14	Yellow	Irregulair	Entire	Flat	Translucent
16	FDB37	Cream	Circulair	Undulate	Flat	Translucent
17	FDB38	Yellow	Circulair	Entire	Convex	Translucent
18	FDB43	Yellow	Irregulair	Entire	Pulvinate	Opaque
19	FDB44	Cream	Circulair	Entire	Convex	Translucent
20	FDB39	Yellow	Circulair	Entire	Convex	Translucent
21	MD21	White	Irregulair	Undulate	Raised	Opaque
22	MD22	Cream	Irregulair	Entire	Convex	Translucent
23	MD23	Yellow	Circulair	Entire	Flat	Translucent
24	MD24	Yellow	Circulair	Entire	Convex	Translucent
25	MD25	Cream	Irregulair	Entire	Flat	Translucent
26	MD26	Cream	Circulair	Entire	Pulvinate	Translucent
27	MV27	Yellow	Circulair	Lobate	Flat	Translucent
28	MV28	Yellow	Circulair	Entire	Convex	Translucent
29	FD1B29	Cream	Circulair	Entire	Convex	Translucent
30	FD2B29	Cream	Circulair	Entire	Convex	Opaque
31	FDB40	Pink	Circulair	Entire	Flat	Translucent
32	FDB41	Cream	Irregulair	Undulate	Flat	Translucent
33	FDB42	Cream	Irregulair	Entire	Convex	Translucent
34	FV30	Pink	Circulair	Entire	Convex	Translucent
35	FV1B31	Yellow	Circulair	Entire	Convex	Translucent
36	FV2B31	Yellow	Circulair	Entire	Convex	Translucent
37	FV1B32	Yellow	Circulair	Entire	Convex	Translucent
38	FV2B32	Yellow	Circulair	Entire	Flat	Translucent
39	FV1B33	Cream	Circulair	Entire	Convex	Translucent
40	FV2B33	Yellow	Circulair	Entire	Flat	Translucent
41	B341	Cream	Circulair	Entire	Convex	Translucent
42	B342	Cream	Circulair	Entire	Convex	Translucent
43	FV35	Cream	Circulair	Entire	Flat	Translucent
44	FV2B36	Yellow	Circulair	Entire	Flat	Translucent
45	FV1B36	Yellow	Circulair	Entire	Convex	Opaque

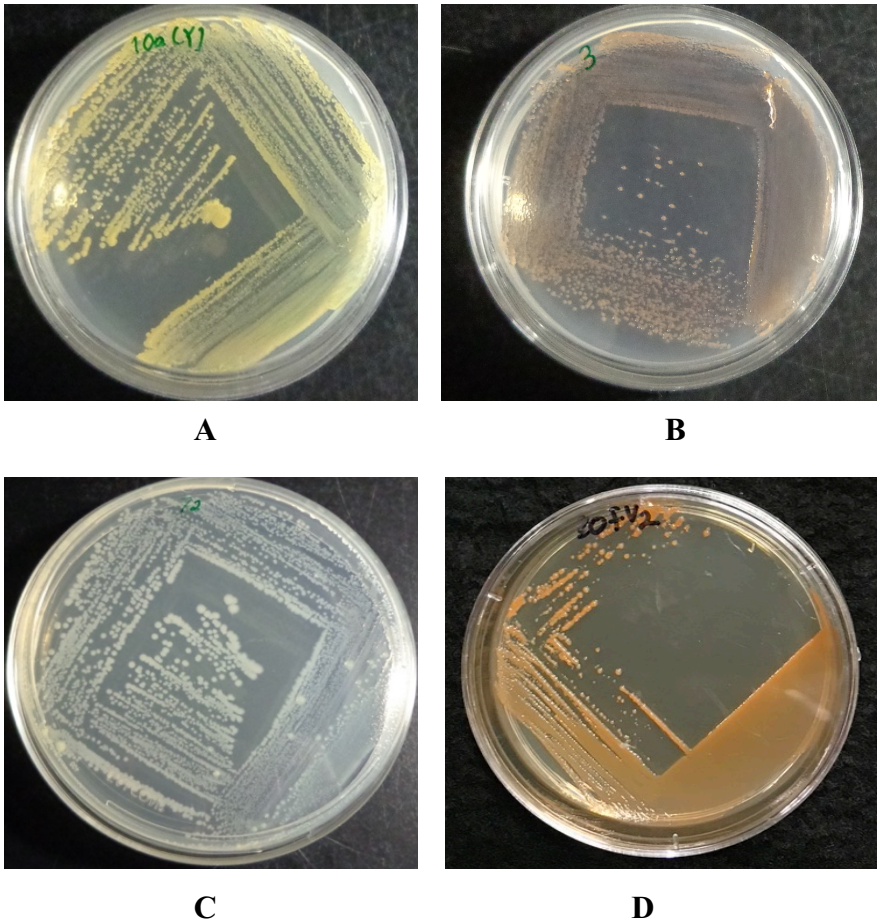


Figure 2.2. Colonies of some representative bacterial frog-skin with their morphological features on color isolated from *Hyla japonica* (A and B), *Pelophylax porosus porosus* (C) and *Buergeri buergeria* (D). All bacteria were grown on TSB medium plate 48 h 28°C.

2.4.2. Bacterial community composition

Many of the bacterial isolates from three species of frogs can be cultured on the media R2A. A total 106 isolates obtained were belonging to four phyla, Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria, and observed belongs to seven classes, Actinobacteria, Bacilli, Flavobacteriia, Sphingobacteriia, Alphaproteobacteria, Betaproteobacteria and Gamma-proteobacteria (Table. 2.2).

Based on 16S rDNA amplicon sequencing, the composition of frog-skin bacteria at the level of phylum is shown in Fig. 2.3. Prominent phyla included Proteobacteria Bacteroidetes, Firmicutes and Actinobacteria. The most dominant phyla presented by Proteobacteria and Bacteroidetes with relative abundance value 79.3% and 15.1%, respectively, followed by Firmicutes with 4.7% and the less abundant phyla was Actinobacteria (1%) from the total of bacterial phyla. Visual inspection of Fig. 2.4 reveals that Gamma-proteobacteria was the most abundant class found in three samples of frogs, *H. japonica*, *P. p. porosus* and *B. buergeri*. Class Flavobacteria presented more dominant in *H. japonica* and *B. buergeri* (8.9% and 22.2%, respectively), but few in *P. p. porosus* (6.2%). Interestingly, Beta-proteobacteria more abundant in *P. p. porosus* (18.8%) than *H. japonica* (2.2%) because they shared habitat in paddy field. The abundance of the most frequent bacterial genera in three samples frog species are displayed in heatmap as shown in Fig. 2.5. Bacteria belonging to the genus *Erwinia* was predominantly revealed in *H. japonica* and *P. p. porosus* (42.2% and 25%, respectively), and less abundant in *B. buergeri* (4.4%).

Venn diagrams revealed that there were more unique genera in the *H. japonica* than in two other frog species, *P. p. porosus* and *B. buergeri* (Fig. 2.6). There were five unique genera detected in *H japonica*, *Jantinobacteria, Paenibacillus, Pantoea, Raoultella* and *Staphylococcus.* Genus *Sphingobacterium* was a unique genus in *P. p. porosus*, whereas three

genera, *Comamonas, Delftia* and *Microbacterium* were revealed only in *B. buergeri.* The shared genera among three frog species were primarily *Erwinia* and *Chryseobacterium.*

Table 2.2. Frequency of occurrence, abundance relative (%) and taxonomic information for culturable of 106 isolates in skin bacterial community structure of three species of frog from Japan: *Hyla japonica*, *Pelophylax porosus porosus*, and *Burgeria buergeri*.

Phylum/Class/*Genus*	**Number (%) of each genus isolated from three frog species**		
	Hyla japonica	*Pelophylax porosus porosus*	*Burgeria buergeri*
Firmicutes			
Bacilli			
Bacillus	2 (4.4)	0	1 (2.2)
Paenibacillus	1 (2.2)	0	0
Staphylococcus	1 (2.2)	0	0
Actinobacteria			
Actinobacteria			
Microbacterium	0	0	1 (2.2)
Bacteroidetes			
Flavobacteriia			
Chryseobacterium	4 (8.9)	1 (6.2)	10 (22.2)
Sphingobacteriia			
Sphingobacterium	0	1 (6.2)	0
Proteobacteria			
Gamma-proteobacteria			
Acinetobacter	2 (4.4)	0	10 (22.2)
Aeromonas	3 (6.7)	3 (18.8)	0
Chimaeribacter	1 (2.2)	0	1 (2.2)
Citrobacter	3 (6.7)	0	7 (15.6)
Enterobacter	1 (2.2)	0	1 (2.2)
Erwinia	19 (42.2)	4 (25.0)	2 (4.4)
Pantoea	3 (6.7)	0	0
Pseudomonas	1 (2.2)	0	6 (13.3)
Raoultella	1 (2.2)	0	0
Stenotrophomonas	2 (4.4)	3 (18.8)	0
Beta-proteobacteria			
Alcaligenes	0	3 (18.8)	3 (6.7)
Comamonas	0	0	1 (2.2)
Delftia	0	0	1 (2.2)
Jantinobacterium	1 (2.2)	0	0
Alpha-proteobacteria			
Brevundimonas	0	1 (6.2)	1 (2.2)

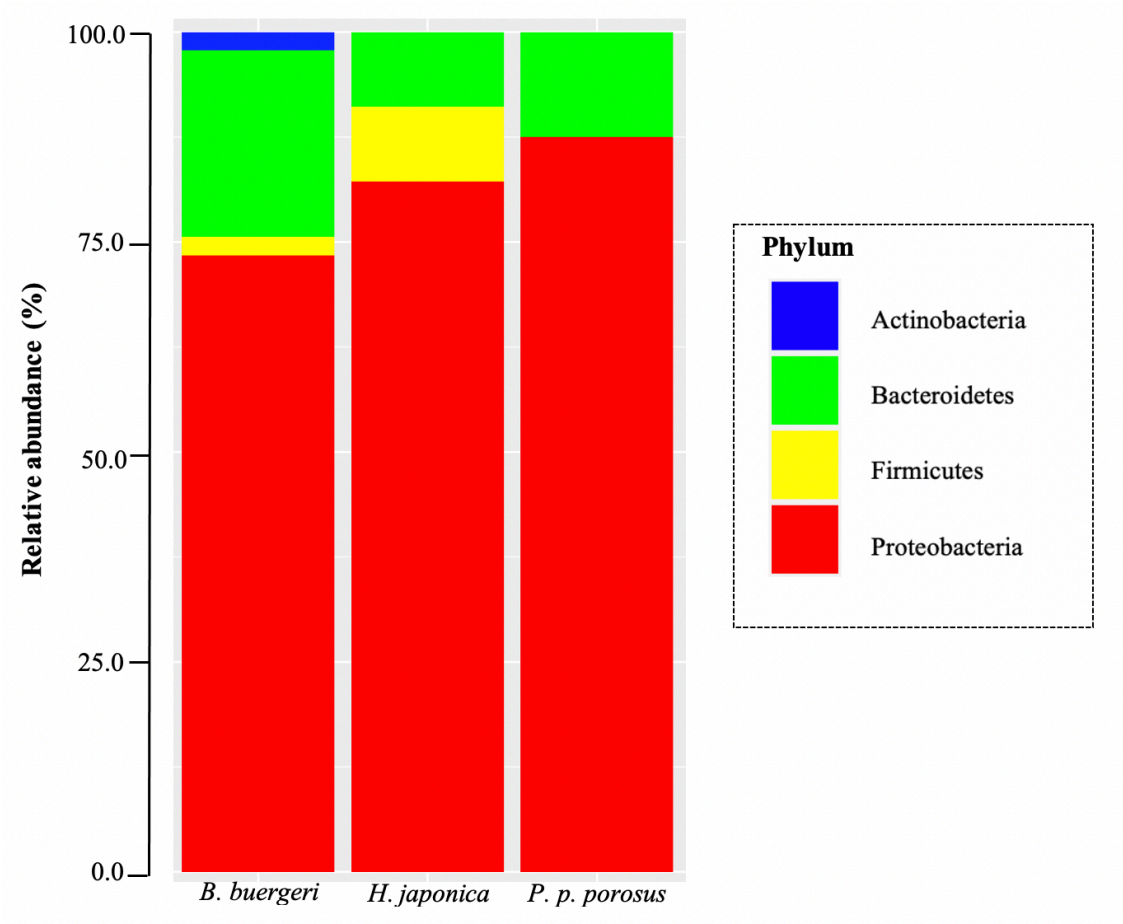


Figure 2.3. Stacked bar chart of the four most abundant phyla for *Hyla japonica*, *Pelophylax porosus porosus* and *Buergeria buergeri*. Sample sizes were n = 3 individuals/each species (adult frog).

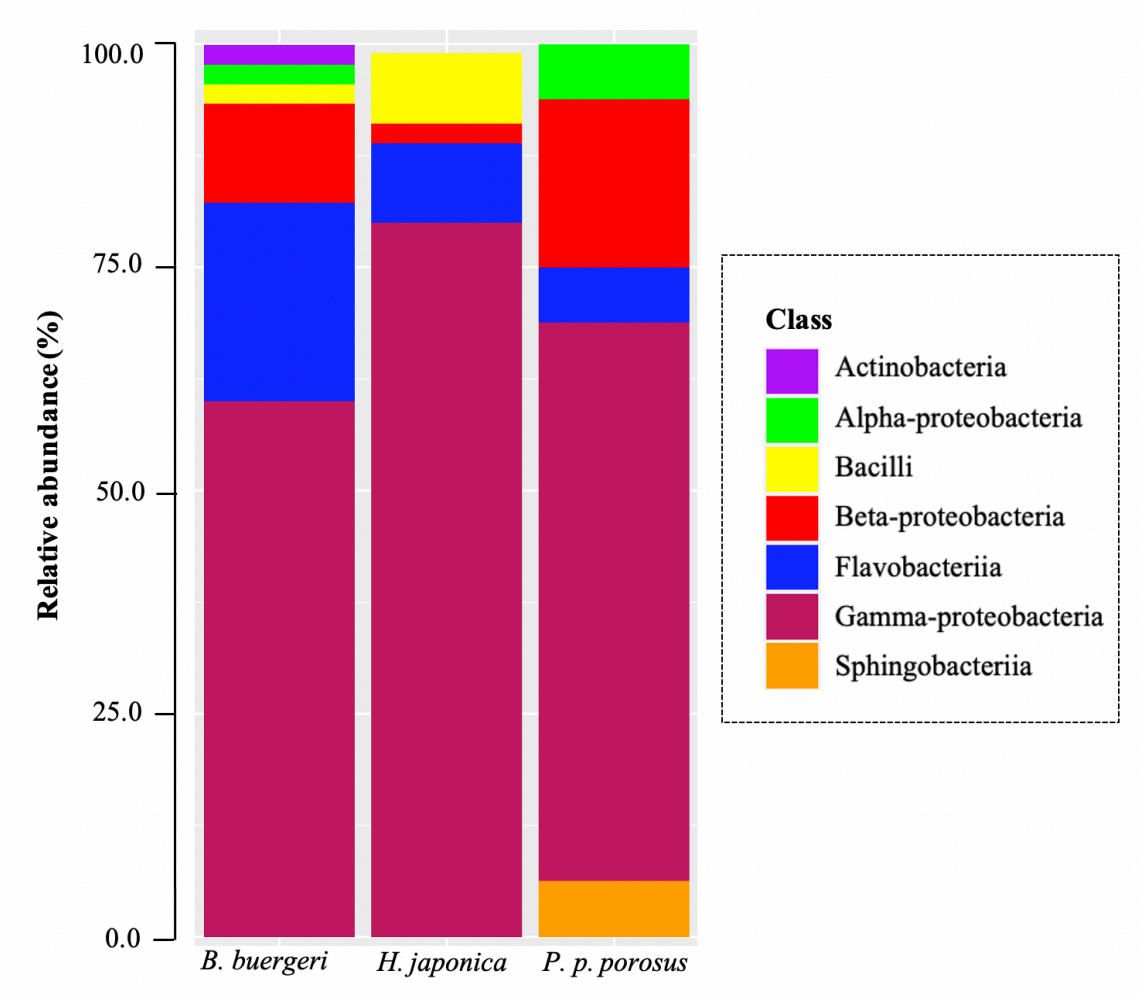


Figure 2.4. Stacked bar chart of the four most abundant class for *Hyla japonica*, *Pelophylax porosus porosus* and *Buergeria buergeri*. Sample sizes were n = 3 individuals/each species (adult frog).

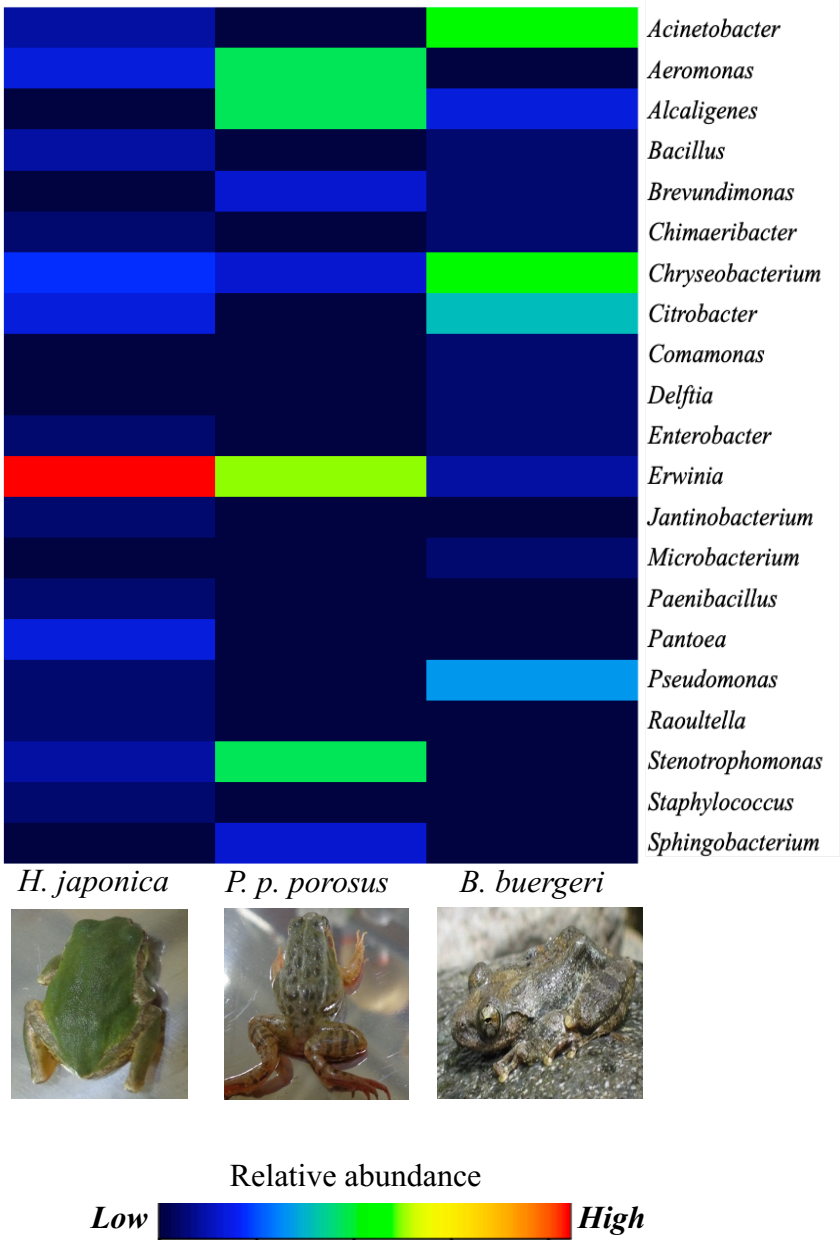


Figure 2.5. Heatmap displaying the relative abundance of the top 21 bacterial taxa for genus level from *Buergeria buergeri*, *Hyla japonica* and *Pelophylax porosus porosus*. Sample sizes were n = 3 individuals/each species (adult frog). (Photo of *B. buergeri* was taken from URL https://www.hitohaku.jp/material/l-material/frog/zukan/img/kajika2.jpg. accessed 13 March 2020).

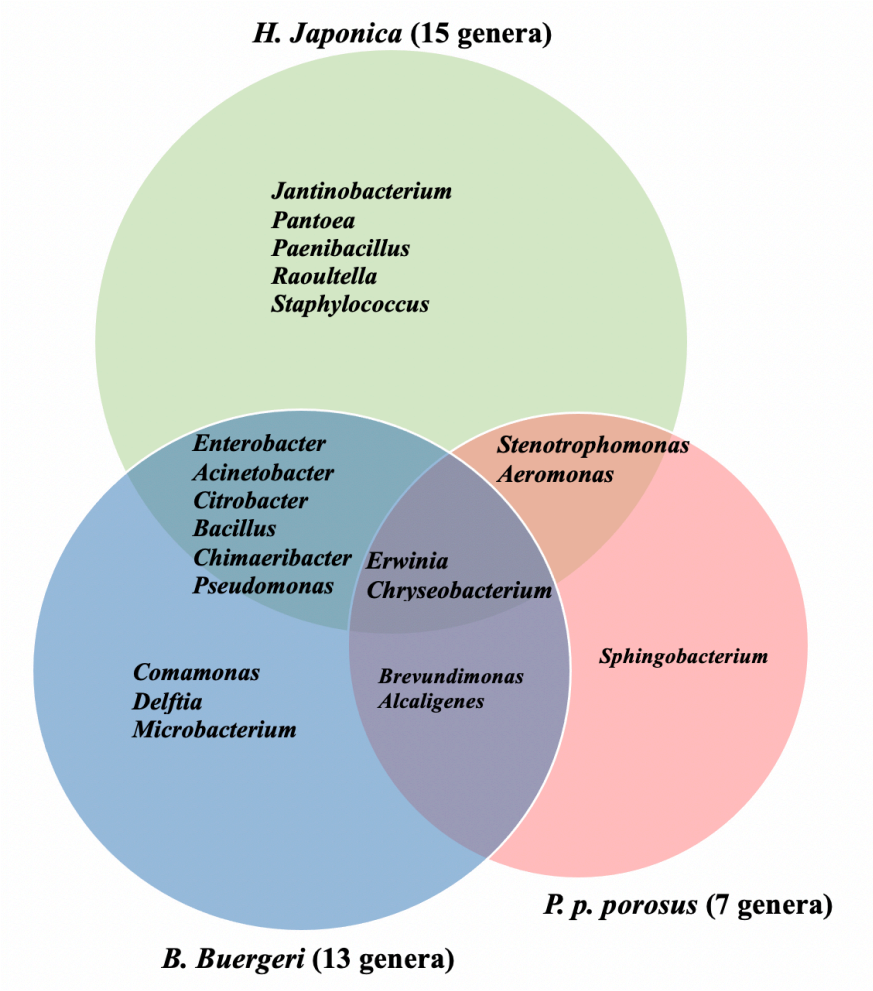


Figure 2.6. Venn diagram summarizing the number of shared and unique OTUs in each group of frog species, *Hyla japonica*, *Pelophylax porosus porosus* and *Buergeria buergeri*. Sample sizes were n = 3 individuals/each species (adult frog).

2.5. Discussion

Growing bacteria on low nutrient media such as R2A (Medina et al., 2017; Walke et al., 2015) results in a higher proportion and a more diverse population than could grow on high nutrient media like TSB and LB (Hugenholtz, 2002; Medina et al., 2017). Frog-skin bacteria vary significantly among host species (Belden et al., 2015; Kueneman et al., 2014; McKenzie et al., 2012; Sabino-Pinto et al., 2016), habitat (Belden et al., 2015; Kueneman et al., 2014; Varela et al., 2018) and seasonal changes (Longo et al., 2015). Adair and Douglas (2017) showed that the complex interaction between host species and environmental factors influences the bacterial community on amphibian. Environmental factors include not only biotic but also abiotic factors, such as humidity, pesticide, pH, season, salinity, and temperature (Daskin et al., 2014; Krynak et al., 2015; Longo et al., 2015; McCoy and Peralta, 2018; Rebolla, et al., 2016; Woodhams et al., 2014). Captivity also can alter the composition of bacterial community on amphibian skin (Loudon et al., 2014).

In this study, the culture-dependent techniques was used. This technique has a pivotal role in the study of bacteria associated with amphibian skin mainly in terms of conservation context to explore the possibility of probiotic-based conservation method (Medina et al., 2017). A total 106 bacterial isolates from all three analyzed species of frogs were obtained. Interestingly, only a smaller number of isolated bacteria from *P. p. porosus* (16) than *H. japonica* (45) and *B. buergeri* (45). The composition of bacterial genera from each frog species is shown in Table 2.2.

Bacterial communities of the skin three species of Japanese frogs were dominated by phylum Proteobacteria, Bacteroidetes and some genera in taxa Actinobacteria (Fig. 2.3), which is in agreement with findings from amphibians (frogs) in Panama (Belden et al., 2015), Wyoming, USA (Hillis et al., 2015), Arizona and Puerto Rico (Longo et al., 2015), and

Northeast China (Bie et al., 2019). All four most dominant phyla, Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes were present only in *B. buergeri*, which habitat is stream, whereas in *H. japonica* and *P. p. porosus* both were had different composition of phyla, three among four abundant phyla only Proteobacteria, Bacteroidetes and Firmicutes found in *H. japonica* but only two phyla detected in *P. p. porosus* (Proteobacteria and Bacteroidetes). Although *H. japonica* and *P. p. porosus* shared habitat in paddy field but their composition of bacteria was different. Herein, in this study, provides evidence that habitat and host species can be an important factor to influence the bacterial flora on frog-skin.

Seven bacterial classes (Actinobacteria, Alpha-proteobacteria, Bacilli, Beta-proteobacteria, Flavobacteriia, Gamma-proteobacteria, and Sphingobacteriia) were dominant across all three frog species, and all of the 106 isolates belonged to these class (Fig. 2.4). The phylum Proteobacteria was divided into classes Alpha-, Beta-, Delta- and Gamma proteobacteria and 73 isolates among 106 isolates belonged to the class Gamma-proteobacteria (Table 2.4), followed by class Flavobacteriia (15 isolates), and Beta-proteobacteria (9 isolates). The lowest number of OTUs belonged to the class Alpha-proteobacteria, Actinobacteria and Sphingobacteriia with only 2, 1 and 1 isolates, respectively. Similar to the findings of previous studies, the current study has shown that class Gamma-proteobacteria was the most abundant class detected in skin-associated bacteria on frog (Hillis et al., 2015; Rebollar et al., 2016b; Sabino-Pinto et al., 2016). The major representative genera of the class Gamma-proteobacteria are *Citrobacter*, *Pseudomonas*, *Raoultella*, *Serratia*, and *Stenotrophomonas* (Assis et al., 2017; Flechas et al., 2017; Hillis et al., 2015). In this study, mostly all of these representative genera except *Serratia* found in *H. japonica* only. Again, these findings provide evidence that host species could be one of the most important factors that influence the composition of bacterial flora.

All the bacterial genera determined from *P. p. porosus* were also present in the genera of *H. japonica* except *Alcaligenes*, *Sphingobacterium*, and *Brevundimonas*. *Aeromonas* and *Stenotrophomonas* (Fig. 2.5). *Erwinia* was found at the highest proportion in the genera from *H. japonica* with while most *Chryseobacterium, Acinetobacter, Citrobacter,* and *Pseudomonas* specifically seem to be dominant in the skin of *B. buergeri* which habitation, a stream, is different from the other species. These results suggested that habitation can be an important factor to influence the bacterial flora on frog-skin. In order to prove this, further studies using more individuals from more collection points are necessary.

From total 106 OTUs, 22 genera were identified. Few OTUs were shared among three analyzed frog species, only genus *Erwinia* and *Chryseobacterium* (Fig. 2.6) were share interestingly, 6 OTUs were shared among group species of *B. buergeri* and *H. japonica*, both are having different habitat, stream and terrestrial (paddy field), respectively. Meanwhile, bacterial composition in two frogs *H. japonica* and *P. p. porosus* found difference even though they shared habitat in paddy field sites. Apparently, *Sphingobacterium* was a unique genus found only in *P. p. porosus* and did not find in *H. japonica*. Our hypothesis was that since *H. japonica* and *P. p. porosus* shared habitat, the bacterial communities may present similar, but there are differences across paddy field, as well as for the host species, we observed that both species of frog had different bacterial communities. Here, again, we demonstrated strongly that species specific across different sites, even they co-exist in the same paddy field. These findings has been found in similar studies (McKenzie et al., 2012), they sampled two frog species, northern leopard frogs (*Lithobates pipiens*), western chorus frogs (*Pseudacris triseriata*) and salamander, tiger salamander (*Ambystoma tigrinum*) from multiple pond site in Colorado USA. Additionally, it may be indicative of selection the occurrence of specific bacteria in a specific environment (Bates et al., 2018).

2.6. Conclusion

A total of 106 isolates were recovered from three analyzed wild frog species in Japan. Most abundant 21 genera were identified. They were all belonged to the four abundant phyla and seven abundant class. Gamma-proteobacteria was the highest proportion class. In this study, the host species and habitat are important factors influencing frog-skin microbial communities.

CHAPTER 3.
SCREENING OF COLLECTED FROG-SKIN BACTERIA FOR ANTIFUNGAL ACTIVITIES AGAINST *C. orbiculare*

3.1. Abstract

A collection of 106 bacterial isolates was obtained from three species of frogs, namely *Hyla japonica*, *Pelophylax porosus porosus* and *Buergeria burgeri*. Using a dual-culture method, three isolates, *Paenibacillus* sp., HjD52, *Raoultella* sp. HjD92 and *Citrobacter* sp. B34, were selected based on their ability to significantly inhibit the growth of *Colletotrichum orbiculare*, the causal fungus of cucumber anthracnose disease. These three bacterial isolates also showed a broad-spectrum of antagonistic activity against 13 plant pathogenic fungi. Furthermore, no toxic effect of bacterial suspension treatment on cucumber seed germination.

Key words: Antifungal activity, *Colletotrichum orbiculare*, *Paenibacillus*, *Raoultella*, *Citrobacter*, Cucumber Anthracnose.

3.2. Introduction

The yield losses in the agricultural sector due to the crop disease is a major problem around the world. Several devastated diseases of important agricultural crops are caused by phytopathogenic fungi and bacteria (Suárez-Estrella et al., 2013), but the high losses are caused by fungi (Oerke, 2006). Approximately 10,000 plant species are affected by ~10% of known fungal species (Chandrasekaran et al., 2016). Most common and important genera of phytopathogens has been listed in the top 10 fungal pathogen that economically and scientifically important including *Colletotrichum* spp, *Fusarium graminearum, and Fusarium oxysporum* (Dean et al., 2012).

Colletotrichum is one of the most important fungal pathogens that economically impact on the limitation of *cucurbitacea* production, especially *Colltetotrichum orbiculare* (Syn.: *C. lagenarium*), is a major causes of anthracnose disease on cucumber plant (Damm et al., 2013;

Shimizu et al., 2009). The pathogen causes lesions on seedlings and all above ground tissue including leaves, petioles, stems and fruits. Lesions are pale brown to reddish, crack and eventually fall out (Damm et al., 2013).

In practice, application of the synthetic fungicides is still a main strategy for disease management, however, it promotes rapid development of the fungal resistance to fungicides (Jamalizadeh et al., 2011) and generates serious problems to environmental health (Heydari and Pessarakli, 2010). Furthermore, high demands for fungicide-free crop production in the market have led to explorations for alternatives to synthetic pesticides (Compant et al., 2005). Thus, employing some beneficial microorganisms, including bacteria or fungi, as environment friendly "microbial fungicides" in agricultural fields has been widely used for tackling plant diseases (Compant et al., 2005; Gotor-Vila et al., 2017).

Anthracnose caused by an ascomycete fungus, *Colletotrichum orbiculare*, is one of the most serious constraints in cucumber (*Cucumis sativus*) cultivation (Damm et al., 2013). Several bacterial agents isolated from healthy plants or soil such as *Streptomyces* spp. and *Bacillus amyloliquefaciens*, *B. pumilus*, *B. subtilis*, *Curtobacterium flaccumfaciens* have been reported to present the biocontrol efficacy to cucumber anthracnose disease (Ji et al., 2013; Kim and Chung, 2004; Raupach and Kloeeper, 1998; Shimizu et al., 2009). However, there has been no report on using of bacteria isolated from frog skin as biocontrol agent candidates.

Several bacterial agents isolated from healthy plants or soil such as *Bacillus* spp., *Pseudomonas* spp., *Serratia* spp. and *Streptomyces* spp. are reported to offer biocontrol efficacy against plant diseases (Jeun et al., 2004; Kim et al., 2016; Kim and Chung, 2004; Shimizu et al., 2009) and are used as biopesticides. Skin-frog bacteria sometimes produce secondary metabolites, including antifungal compounds (Harris et al., 2009). Therefore, the objective of this study was to evaluate antifungal activities of collected frog-skin bacteria from three species

of Japanese wild frogs against plant pathogen, *C. orbiculare*, causing agent of cucumber anthracnose disease.

3.3. Materials and Methods

3.3.1. Microorganisms and culture conditions

All collected bacterial isolates from three samples of Japanese wild frog were used in this study are listed in Table 2.1 (A, B, and C in Chapter 2). Pure bacterial cultures stored at –80ºC in 10% skim milk were revived on TSA (Trypticase Soy Agar) plates and incubated at 28ºC for 48 h in dark. The colonies appeared on media were collected using sterile disposal loops and used as liquid starter culture in 10ml of TSB (Trypticase Soy Broth) (Park et al., 2013) and incubated by shaking 120 rpm on rotary shaker. For further studies, the bacterial suspension was serially diluted using SDW.

Colletotrichum orbiculare A-29, the cucumber anthracnose fungus, and 13 other phytopathogenic fungi were used. Detailed information about those fungi are shown in Table 3.1. These fungi were cultured on PDA (potato dextrose agar) medium at 28°C in the dark (Kubota and Abiko, 2000). After 10 days, the plates were flooded with 10 ml SDW (sterile distilled water) containing 0.05% (v/v) Tween 20 (Kim et al., 2018; Zhang et al., 2016) and carefully scraped with a paint brush to harvest conidia. The conidial suspension was filtered through two layers of sterilized cheesecloth, and the density was adjusted to 10^6 conidia/ml using a hemocytometer under microscope (BX53, Olympus, Tokyo, Japan).

3.3.2. Primary screening of antagonistic bacteria by dual culture method

All bacterial isolates were initially screened for their antifungal activity against the cucumber anthracnose pathogen *C. orbiculare* A-29 by the dual-culture method (Damasceno et al., 2019) with a slight modification. Briefly, a 10-day old mycelial plug of *C. orbiculare*

(ø3 mm) obtained from the PDA culture was placed on the center of a fresh PDA plate in a ø90-mm Petri dish and incubated in the dark at 28ºC for 24 h. A 20 µl bacterial suspension (10^9 cfu/ml) was streaked 2.0 cm away from the fungal pathogen on the PDA plate, incubated in the dark at 28ºC for 7 days. Percent inhibition of mycelial growth (GI) was calculated as: GI (%) = $[(R-r)/R] \times 100$; where, R represents the colony radial size (mm) of the fungus distal to the bacteria (as a control) and r represents the colony radial size (mm) proximal to the bacteria (Lamsal et al., 2012). The tests were done in triplicates (n=3).

3.3.3. Antifungal activity assay for the selected bacteria

3.3.3.1. Mycelial growth inhibition

Mycelial plugs (ø3 mm) from a 10-day culture of *C. orbiculare* were prepared as mentioned in section *3.3.1.* and submerged in 5 ml of the bacterial culture diluted to three different densities (10^9, 10^8 and 10^7 cfu/ml) in a sterilized tube, and incubated at room temperature for 30 min. The plugs were recovered from the tube, air dried in a clean bench (laminar flow hood), placed on a fresh PDA plate, and incubated in the dark at 28°C. *C. orbiculare* submerged in SDW was used as control. Radial mycelial growth (mm) of *C. orbiculare* was observed after a 7-day incubation (Rahman et al., 2007). Each measurement was done in triplicate (n=3).

3.3.3.2. Conidial germination inhibition

A 100 µl conidial suspension (10^6 conidia/ml) of *C. orbiculare* was mixed with a 100 µl bacterial suspension (10^9, 10^8 or 10^7 cfu/ml) in a 1.5-ml sterile tube and incubated at 28°C for 24 h (Rahman et al., 2007). SDW was used instead of the bacterial suspension as control. The percentage of germinated conidia was calculated for each mixture by observing 100 conidia with a light microscope.

3.3.3.3. Analysis of fungal morphology

To examine the morphology of *C. orbiculare* after treatment with the frog-skin bacteria, a microslide culture technique (Shehata et al., 2016) was used with minor modification. A thin, 4 × 4 mm square section of the PDA layer was cut out, placed onto a sterilized glass slide. A small 10 day-old mycelial of *C. orbiculare* was placed in the center of one side of the PDA layer, and a 20 µl bacterial suspension (10^9 cfu/ml) was dropped at the center of the opposite side. A sterile cover glass was placed over the PDA layer, and the slide was maintained in a humidified Petri dish at 28ºC for 7 days. After incubation, the coverslip was removed, and the fungus was stained with 0.01% (w/v) trypan blue in lactophenol dye. Fungal morphology was observed using a light microscope.

3.3.4. Examination of the spectrum of antifungal activity

The spectrum of antifungal activity in each frog-skin bacterium, in other words the range of fungi affected by each bacterium, was examined using 13 phytopathogenic fungi (Table 3.1) by the dual-culture test as described in section *3.3.2*.

3.3.5. Effect of frog-skin bacteria on cucumber seed germination and growth

A blotter test evaluated the effect of selected bacteria on cucumber seed germination following the method of Mardanova et al. (2017). Cucumber (*Cucumis sativus* cv. Suyo; Kanda Seed, Nara, Japan) seeds were sterilized with 70% (v/v) ethanol for 30 sec and 2.5% (w/v) sodium hypochlorite for 5 min, rinsed several times with SDW, and dried. Sterilized seeds were submerged in a bacterial suspension (10^9 cfu/ml) at room temperature for 1 h, placed on moistened sterile filter paper in a Petri dish, and incubated in the light [intensity 361.2 lux (fluorescent lamps), 24 h a day] at room temperature. As a control, seeds were submerged in SDW only. Thirty seeds were used for each treatment with three replicates.

Table 3.1. Plant pathogenic fungi used in this study for antifungal spectrum in frog skin bacteria.

Fungal species	**Isolate number**	**Host plant**	**Disease**	**Origin**
Alternaria alternata				
tomato pathotype	As-27	Tomato (*Solanum lycopersicum*)	Alternaria stem cancer	M. Kodama[1)]
A. solani	Al-1	Tomato	Early blight	This laboratory
Botrytis cinerea	Str01	Strawberry (*Fragaria ananassa*)	Strawberry gray mold	This laboratory
Colletotrichum gloeosporoides	SP3	Sweet pepper (*Capsicum annuum*)	Anthracnose	Y. Morita[2)]
C. fructicola	SP2	Sweet pepper	Anthracnose	Y. Morita
C. orbiculare	A-29	Cucumber (*Cucumis sativus*)	Anthracnose	This laboratory
Fusarium fujikuroi	Miyagi 92-10	Rice (*Oryza sativa*)	'Bakanae'	H. Tateishi[3)]
Fusarium oxysporum				
f. sp. *lycopersici* race 3	KoChi-1	Tomato	Wilt	This laboratory
f. sp. *adzukicola*	96-3	Adzuki bean (*Vigna angularis*)	Wilt	N. Kondo[4)]
f. sp. *conglutinans*	Cong:1-1	Cabbage (*Brassica oleracea)*	Yellows	This laboratory
F. solani	KF1-2(5)	Taro (*Colocasia esculanta*)	Dry rot	This laboratory
Pyricularia oryzae	P2	Rice	Blast	This laboratory
Penicillium digitatum	Pd1	Citrus (*Citrus limon*)	Green mold decay	Y. Arimoto[5)]
Sclerotinia sclerotiorum	180508	*Aralia cordata* (Japanese spikenard)	White mold	This laboratory

[1)] Tottori Univ, [2)] Kochi Prefecture, [3)] Kureha Co., [4)] Hokkaido Univ, [5)]RIKEN

After a 7-day incubation, the number of germinated seeds were counted, and the lengths (mm) of shoots and roots of each plant were separately measured.

3.3.6. Statistical analysis

All the experiments were repeated three times for each treatment with three replications. Data were analyzed using Package R studio software version 3.5.1 (R Development CoreTeam). The significance of differences amongst all treatments was tested using Student's *t*-test (Rosner, 2016).

3.4. Results

3.4.1. Primary screening of bacterial frog-skin isolates for antagonistic activity against C. orbiculare

All isolates were screened for their antagonistic activity against *C. orbiculare* on PDA, and three isolates, HjD57 and HjD92 from *H. japonica* and B341 from *B. buergeri*, were selected based on their strong inhibitory activity toward the plant pathogen (Fig 3.1A). The percentage of mycelial growth inhibition achieved by HjD57 was 61.4% followed by B341 and HjD92 with 55.6% and 54.2% inhibition, respectively (Fig. 3.1B).

3.4.2. Antifungal activity against in culture suspension of frog-skin bacteria

Mycelial growth of *C. orbiculare* was inhibited by the frog-skin bacteria when mycelial plugs were submerged in their culture suspension (10^9, 10^8 and 10^7 cfu/ml) for 30 min (Fig. 3.2.). Isolate HjD92 and B341 significantly inhibited the mycelial growth of fungi. B341 exhibited the strongest inhibition at 10^9 cfu/ml, reducing mycelial growth by 87.7%. Moreover, *C. orbiculare* treated with B341 resulted in an irregular shape of the colony on PDA (Fig. 3.2A).

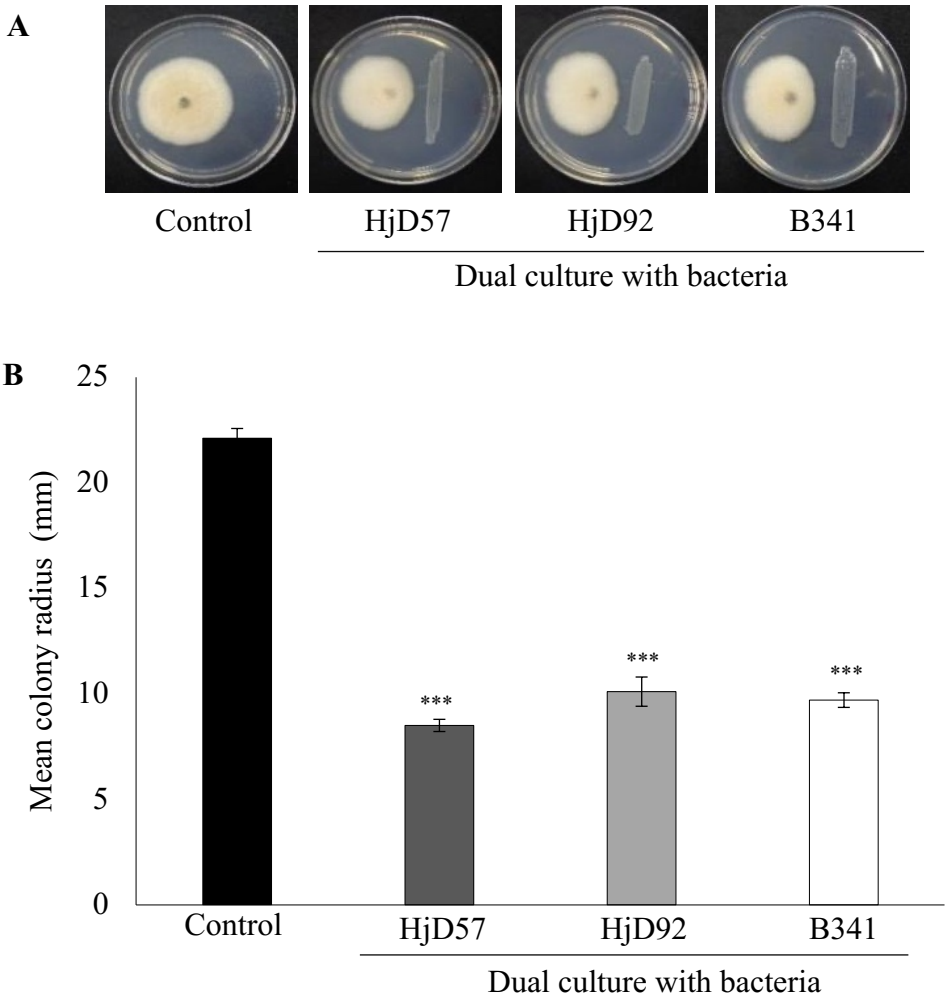


Figure 3.1. Frog-skin bacteria isolates, HjD57, HjD92 or B341, streaked on the right side of the PDA plates inhibited the mycelial growth of *Colletotrichum orbiculare* A-29 (left) in dual cultures incubated in the dark at 28°C. Colonies 7 days post-inoculation viewed from above the surface (A). The radius of the colony on the side nearest the bacteria was compared with the control (B). Data represents the mean (± standard error, SE, n=3) of three independent experiments, each performed in triplicate, and presented relative to control. Asterisks represent the level of significance (***P<0.001) by a two samples t-test compared to the control.

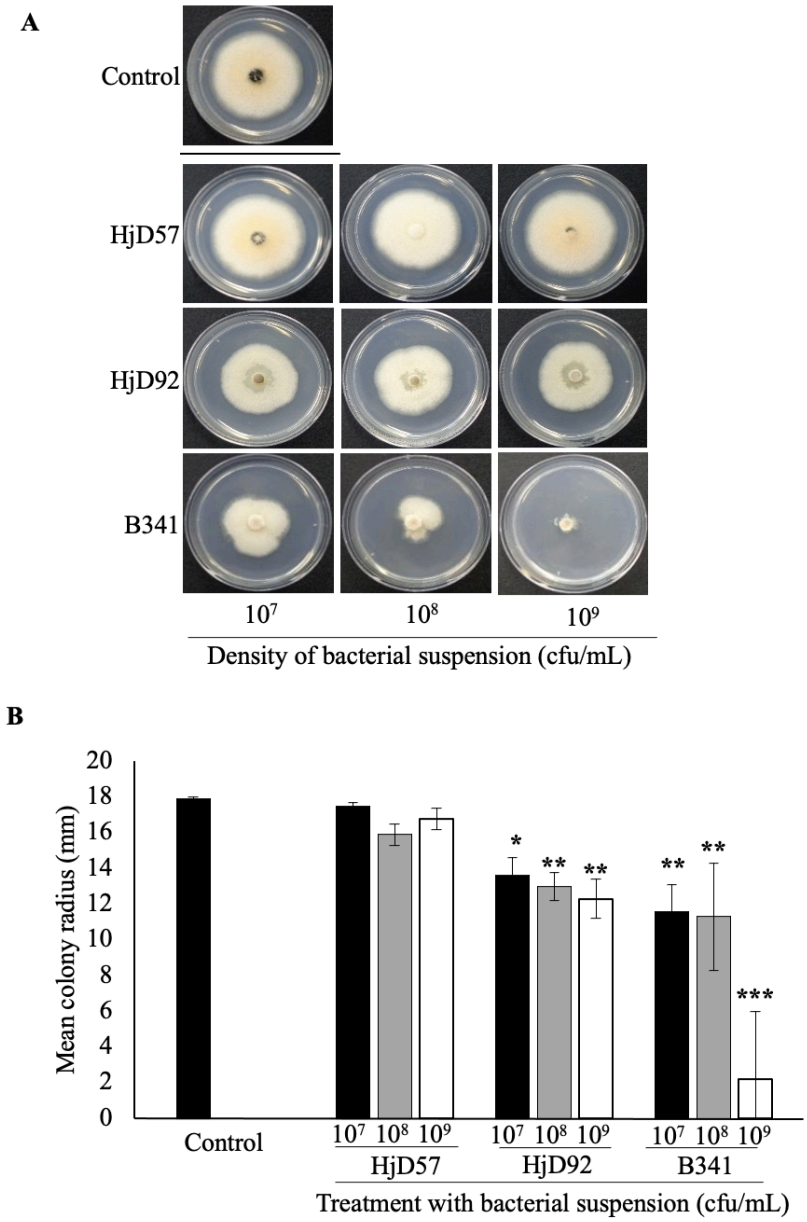


Figure 3.2. Mycelial plugs (3.0 mm) from a 10-day culture of *Colletotrichum orbiculare* A-29 were submerged in bacterial suspensions of isolate HjD57, HjD92 or B341 at three different densities (10^9, 10^8 and 10^7 cfu/ml) and incubated at room temperature for 30 min. The plugs were recovered, placed on fresh PDA plates, and incubated in the dark at 28°C for 7 days (A). The radius of each colony was measured at 3 points, the mean value was calculated and compared with the control (B) Data represents the mean (± standard error, SE, n=3) of three independent experiments, each performed in triplicate, and presented relative to control. Asterisks indicate the level of significance (***P<0.001, **P<0.01, *P<0.05) by a two samples t-test compared to the control.

In SDW, 84.7% of the *C. orbiculare* conidia germinated in 24 h at 28°C. In comparison, conidial germination was inhibited in suspensions of isolates HjD57, HjD92 and B341 at 10^9 and 10^8 cfu/ml (Fig. 3.3.). Most notably, isolate B341 had the strongest inhibition at 10^9 cfu/ml leading to a reduction in conidial germination of 97.6%, followed by a 91.7% reduction by 10^9 cfu/ml HjD92.

3.4.3. In vitro interactions between the frog-skin bacteria and C. orbiculare

After co-culture of the frog-skin bacteria and *C. orbiculare* on a microslide culture for 7 days, hyphal morphology was observed with a light microscope. *C. orbiculare* co-cultured with one of the isolates (HjD57, HjD92 or B341) resulted in curling (cr) , distortion (di), and swelling hyphae (sw in Fig. 3.4).

3.4.4. Spectrum of antifungal activity

Among the 13 plant pathogenic fungi evaluated, the growth of 12 fungal species was significantly inhibited by HjD92 and B341 (Fig. 3.5.). HjD57 inhibited the growth of five fungi. All three frog-skin isolates (HjD57, HjD92 and B341) strongly inhibited the mycelial growth of *Botrytis cinerea* by 59.4, 55.0, and 63.1%, respectively. In contrast, none of the frog-skin isolates inhibited the mycelial growth of *Penicillium digitatum*.

3.4.5. Effect of the frog-skin bacterial isolates on cucumber seed germination and growth

Submerging cucumber seeds in the bacterial suspensions (10^9 cfu/ml) for 1 h resulted in neither significant inhibition of germination (Fig. 3.6.A) nor obvious symptoms such as etiolation, stunting, or lesions on the seedlings (*in vitro* test). Shoot and root lengths of cucumber seedlings, however, exhibited differences compared with the control after being submerged in a suspension of either HjD92 or B341. HjD92 inhibited shoot and root growth in comparison to those of the control (Fig. 3.6.B). On the other hand, B341 stimulated root growth, shoot growth was not significantly enhanced (Fig. 3.6.B).

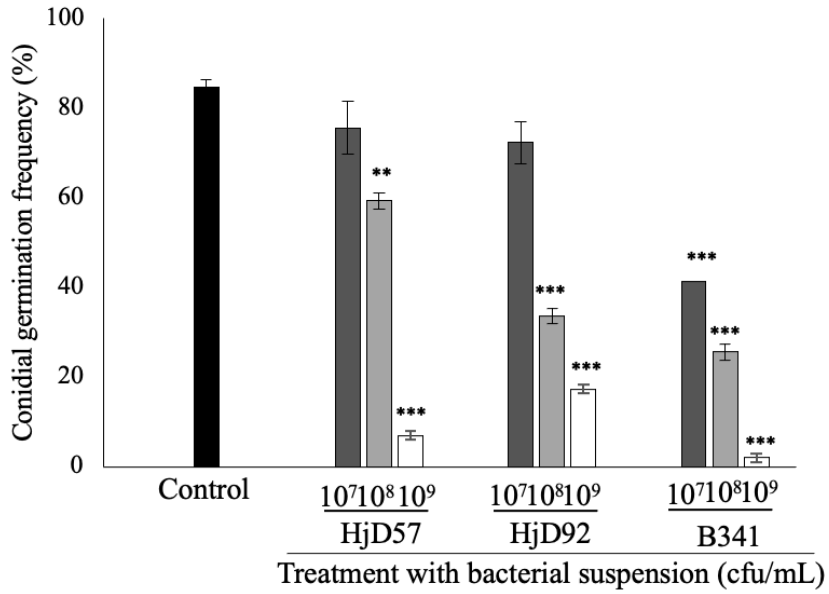


Figure 3.3. A conidial suspension (10^6 conidia/ml) of *Colletotrichum orbiculare* A-29 was mixed (v:v=1:1) with bacterial suspensions of isolate HjD57, HjD92 or B341 at three different densities (10^9, 10^8 and 10^7 cfu/ml) and incubated in the dark at 28°C for 24 h. The percentage of germinated conidia was calculated for each mixture by microscopic observation of 100 conidia. Vertical bars represent standard errors of the mean (n=3). Asterisks indicate the level of significance (***P<0.001, **P<0.01) by a two samples t-test compared to the control.

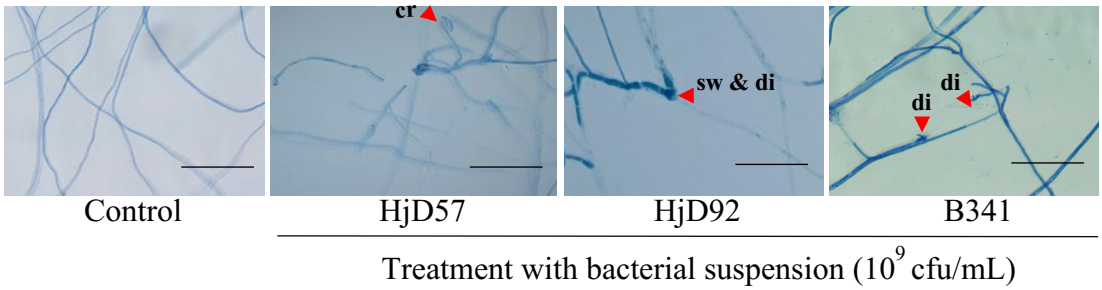


Figure 3.4. *Colletotrichum orbiculare* A-29 was co-cultured on a PDA microslide with a single, bacterial isolate (HjD57, HjD92 or B341) with density 10^9 cfu/ml in the dark at 28°C for 7 days. No abnormal hyphae were observed in the control without bacteria; whereas, in the presence of bacteria, some abnormal mycelia formed, including curled (cr), distortion (di), swollen hyphae (sw). Bars, 20 µm.

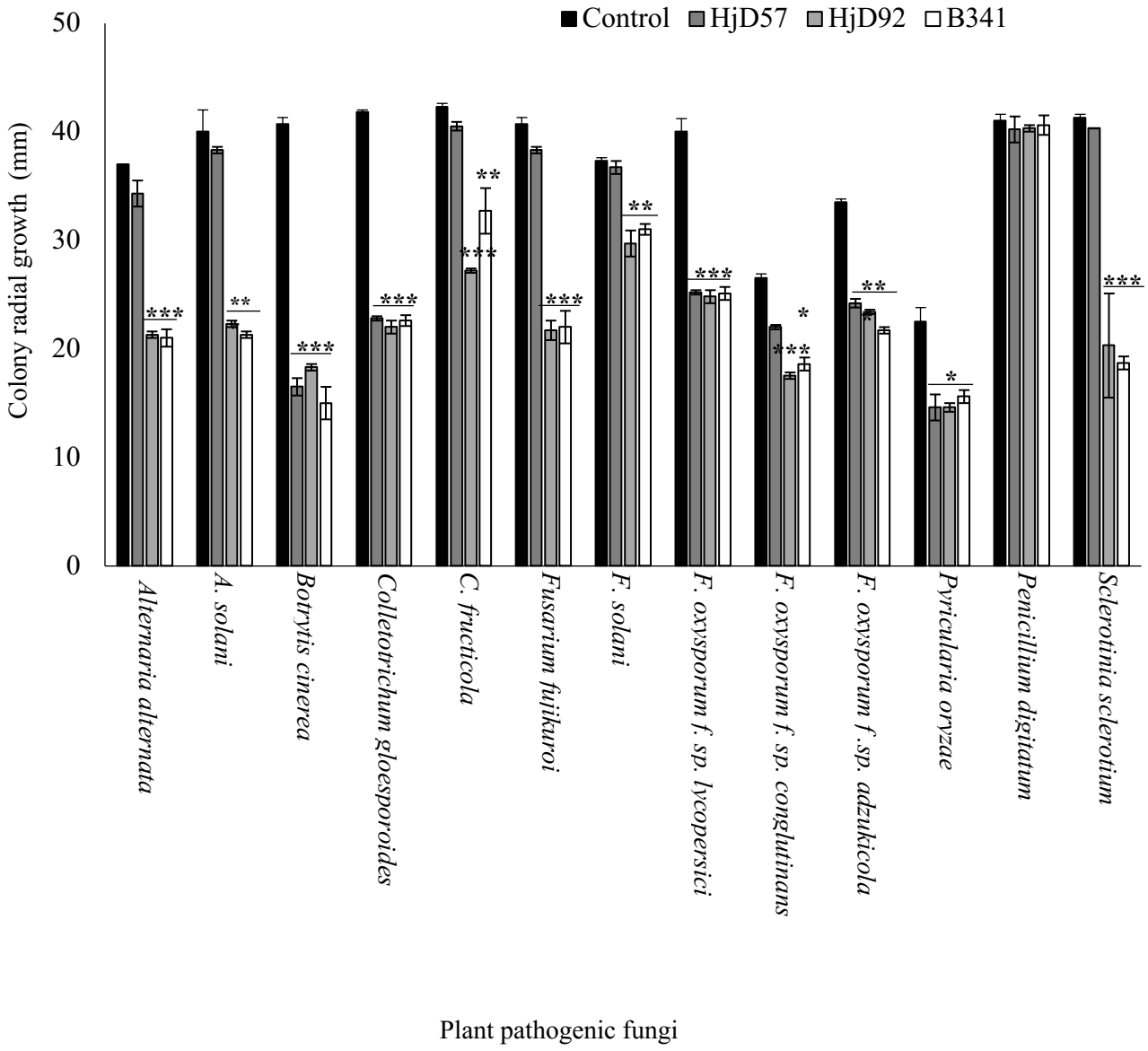


Figure 3.5. Spectrum of antifungal activity among frog-skin bacteria. The inhibition of mycelial growth of 13 plant pathogenic fungi was examined in dual cultures on PDA plates with isolate HjD57, HjD92 or B341 in the dark at 28ºC. The radius of the colony on the side nearest the bacteria was compared with the control. Vertical bars represent standard errors of the mean (n=3). Asterisks represent the level of significance (***P<0.001, **P<0.01, *P<0.05) by a two samples t-test compared to the control.

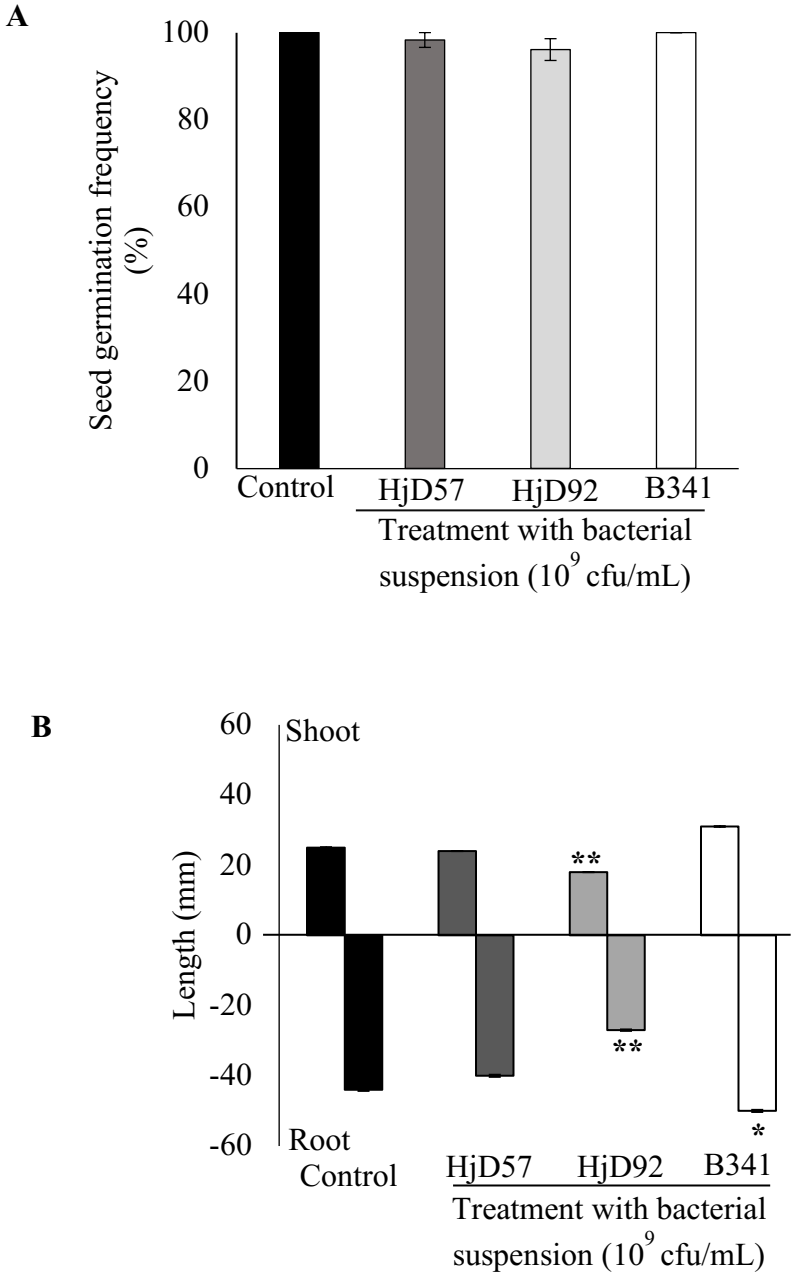


Figure 3.6. Germination frequencies of cucumber (cv. Suyo) seeds after submerging in a bacterial suspension (10^9 cfu/ml) of isolate HjD57, HjD92 or B341 for 1 h (A). Mean root and shoot lengths of the seedlings (B). Vertical bars represent standard errors of the mean (n=30). Asterisks indicate statistically significant differences (**P<0.01, *P<0.05) by Student's t-test compared to the control.

3.4.6. Phylogeny tree of selected bacteria

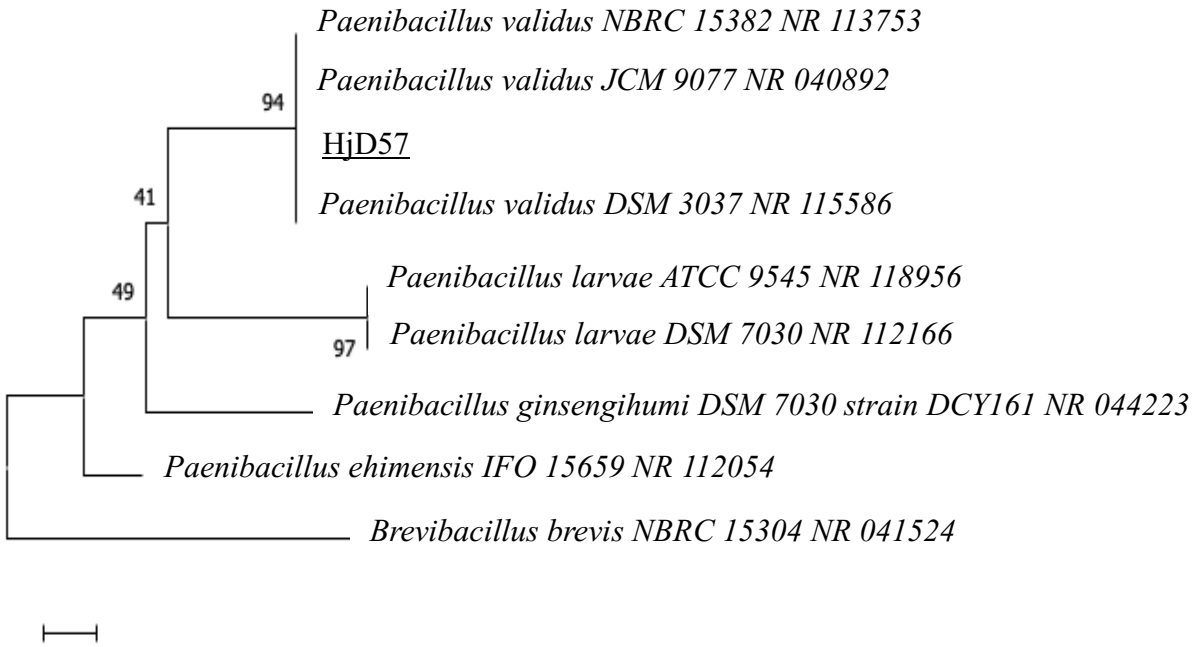
We sequenced the 16S rRNA-V4 region of HjD57 (237 bp), HjD92 (233 bp) and B341 (240 bp) and deposited the sequences in the NCBI database as accession numbers MT199188, MN258875 and MN258876, respectively. The sequences were 98.1% identical to those of *Paenibacillus validus* (for example, NR115586; Fig. 3.7.A), 100% identical to *Raoultella terrigena* (for example, LC060919; Fig.3.7.B), and 99.5% identical to *Citrobacter freundii* (for example MK847857; Fig. 12C) and *C. braakii* (NR117750; Fig. 3.7.C), respectively. We identified HjD57 as a *Paenibacillus* sp., HjD92 as a *Raoultella* sp. and B341 as a *Citrobacter* sp.

3.5. Discussion

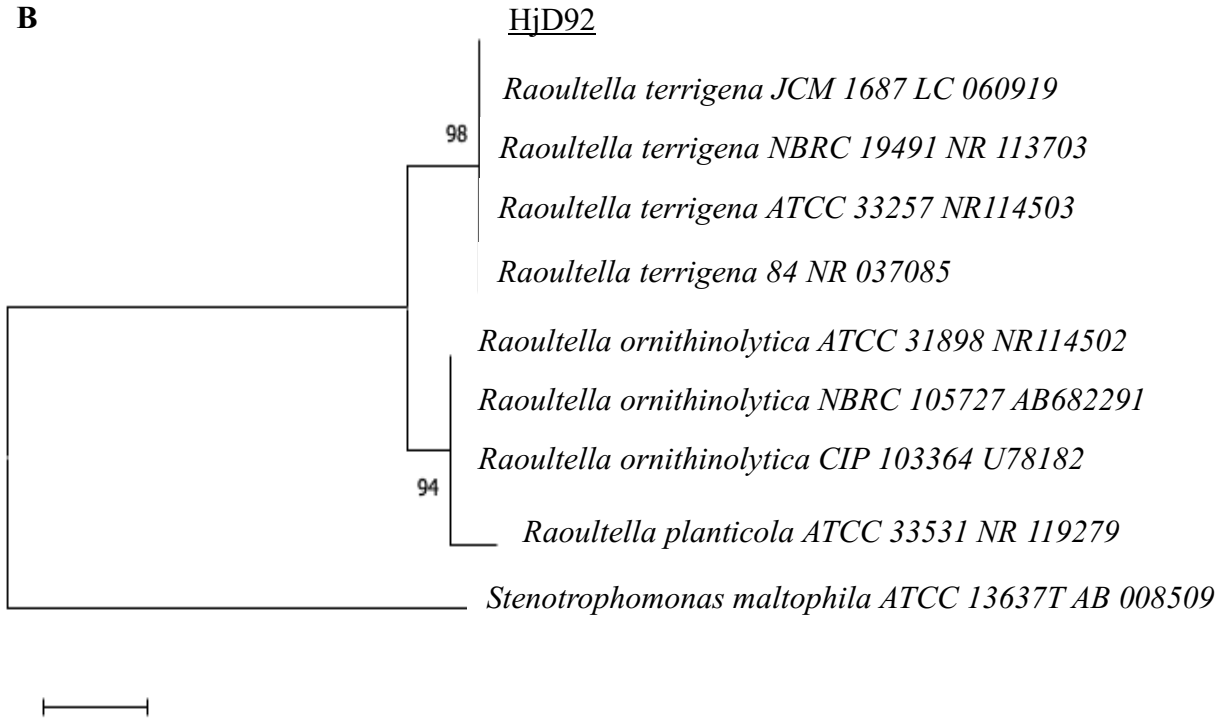
Bacteria on frog skin have been widely investigated and reported to protect the skin from fungal pathogens such as *Batrachochytridium dendrobatidis* and *B. salamandrivorans* by producing antifungal compounds (Woodhams et al., 2018). Among the 106 bacteria collected, three isolates were selected, HjD57, HjD92 and B341, presented antifungal activities against the cucumber anthracnose pathogen, *C. orbiculare* (Figs. 3.1–3.4). Those three isolates were identified as a member of Genus *Paenbacillus*, *Raoultella* and *Citrobacter*, respectively (Figs. 3.7.A–3.7.C).

The suppressive effect of the three bacterial isolates on mycelial growth and conidial germination of *C. orbiculare* was mostly dependent on the bacterial inoculum density (Fig 3.2), which suggested that some substance produced by HjD57, HjD92 and B341were involved in the suppression of conidial spore germination. It is therefore important to consider cell density when evaluating them as candidates for bioagent because some bacteria may use density-depenent mechanisms (eg quorum sensing) to regulate metabolite production (Yasumiba et al., 2016).

A


Paenibacillus validus NBRC 15382 NR 113753
Paenibacillus validus JCM 9077 NR 040892
94
HjD57
41
Paenibacillus validus DSM 3037 NR 115586
Paenibacillus larvae ATCC 9545 NR 118956
49
Paenibacillus larvae DSM 7030 NR 112166
97
Paenibacillus ginsengihumi DSM 7030 strain DCY161 NR 044223
Paenibacillus ehimensis IFO 15659 NR 112054
Brevibacillus brevis NBRC 15304 NR 041524

B


HjD92
Raoultella terrigena JCM 1687 LC 060919
98
Raoultella terrigena NBRC 19491 NR 113703
Raoultella terrigena ATCC 33257 NR114503
Raoultella terrigena 84 NR 037085
Raoultella ornithinolytica ATCC 31898 NR114502
Raoultella ornithinolytica NBRC 105727 AB682291
Raoultella ornithinolytica CIP 103364 U78182
94
Raoultella planticola ATCC 33531 NR 119279
Stenotrophomonas maltophila ATCC 13637T AB 008509

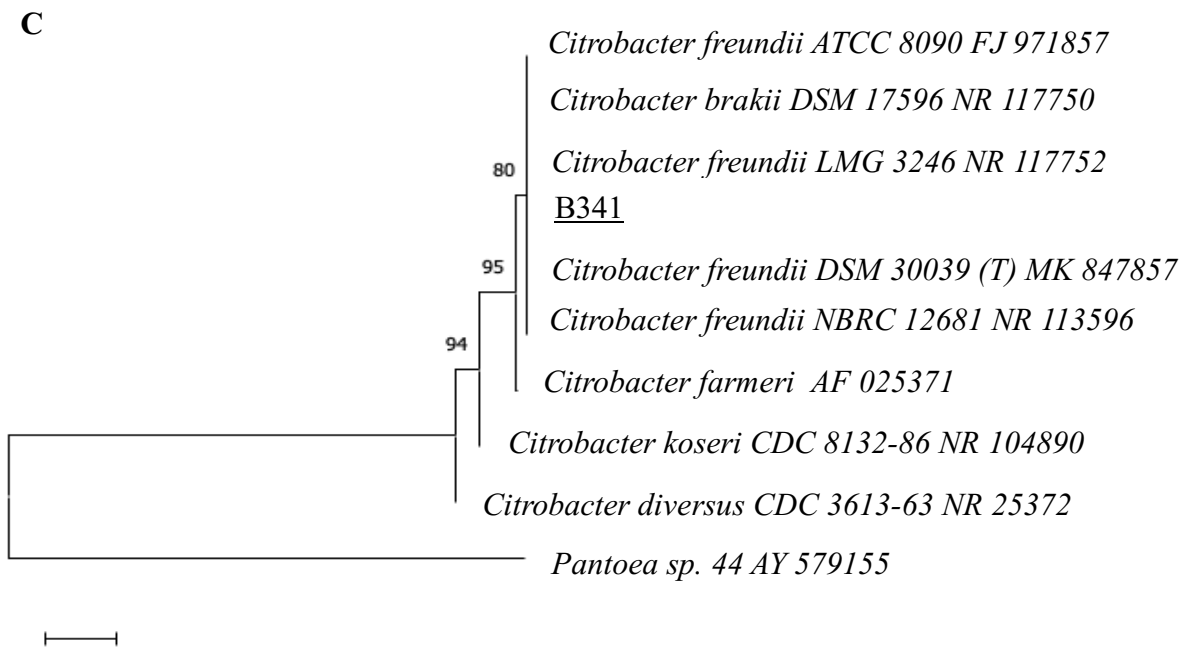


Figure 3.7. Neighbor joining (NJ) phylogenetic trees constructed based on bacterial V4 regions of the 16S rRNA gene sequence indicate relationship between HjD57 and *Paenibacillus* spp. (out group, *Brevibacillus brevis* NBRC 15304) (A), HjD92 and *Raoultella* spp. (out group, *Stenotrophomonas maltophilia* ATCC 13637) (B), and B34 and *Citrobacte*r spp. (out group, *Pantoea* sp. 44) (C), Values at the nodes represent the percentage NJ bootstrap values from 1000 replicates; values ≥ 50% are shown. Bars indicate phylogenetic distances of 0.5% (A), 2.0% (B) and 10% (C).

Paenbacillus sp. HjD57 and *Citrobacter* sp. B341 did not show any negative effects on host (cucumber seed) to germinate and grow, only *Raoultella* sp. HjD92 showed a slight effect on root and shoot length of cucumber seedlings (Fig. 3.6), conversely, isolate B341 promoted the growth of cucumber plant. Those isolates, HjD92 and B341 notably have been reported as opportunistic bacteria to mammals and humans, the further studies therefore are required to make sure the safety prior to using them as a potent biocontrol agent (Besset-Manzoni et al., 2019).

Paenibacillus is a gram-positive bacterium in the class Bacilli, *Paenibacillus* spp. can be isolated from widely divergent sources such as soil, the rhizosphere, plant tissues, insects, water, and mammals (Grady et al., 2016). *Paenibacillus* spp. have also been recovered from amphibian skin previously (Catenazzi et al., 2018). *Paenibacillus* spp. are known to promote crop growth and protect plants from insect herbivores and pathogens and, moreover, there are many reports of antifungal and biocontrol activities (Grady et al., 2016; Han et al., 2015).

Since *Paenibacillus* sp. form spores, the bacteria is highly resistant in the environment, and are, thus, predicted to be excellent bio-pesticide agents. In our study, for the first time, we demonstrated that *Paenibacillus* spp. from frog skin have shown potent antifungal activity against *C. orbiculare* and range of other phytopathogens. HjD57 had antifungal activity against not only *C. orbiculare* but also *C. gloesporioides*, *Botrytis cinerea*, *Fusarium oxysporum* and *Pyricularia oryzae* (Fig. 3.5) which also suggested that HjD57 could be used as a fungicide for a wider range of diseases.

The suppressive effect of *Paenibacillus* sp. HjD57 only presented strong on spore germination (Fig. 3.3) but did not show significant inhibition effect on mycelial growth on plate (Fig. 3.2B). However, the occurance of isolate caused the alteration of fungal hyphae (Fig. 3.4). Meanwhile, the inhibition of spore germination was dependent on the concentration of bacterial

suspension. The strongest suppressive effect presented with the consentration 10^9 cfu/ml. The minimal concentration (10^7 cfu/ml) did not inhibit the spore germination.

Raoultella and *Citrobacter* are gram-negative bacteria both of which belong to the class Gamma-proteobacteria. Previous studies demonstrated that several members of this class possessed biocontrol activity against plant diseases (Hillis et al., 2015). However, there are few examples of *Raoultella* sp. and *Citrobacter* sp. being reported as biocontrol agents. Therefore, this is the first report of using those two bacterial symbionts from frog skin as biocontrol agents.

Raoultella, previously known as *Klebsiella,* is often isolated from soil, the rhizosphere and water, but rarely from humans (Izard et al., 1981). *R. terrigena* was reported to be isolated from the skin of *Notophthalamus viridescens* (eastern newt, an amphibian endemic to eastern North America; Culp et al., 2007). Meanwhile, *Citrobacter* are ubiquitous bacteria and have also been isolated from some other species of frogs such as *Phyllomedusa distincta* (Monkey frog; Assis et al., 2017), *Agalychnis moreletii* (Morelet's tree frog; Antwis et al., 2014) and *Atelepus elegans* (elegant stubfoot toad; Flechas et al., 2017). To date, no previous studies reported on the presence of *Raoultella* spp. and *Citrobacter* spp. on Japanase wild frogs. Sabino-Pinto et al. (2016) and Bletz et al., (2017b) successfully collected bacteria from Japanese frogs and newt with different genera.

Similar to *Paenibacillus* sp. HjD57, *Raoultella* sp. HjD92 and *Citrobacter* sp. B341 also significantly inhibited the growth of *C. orbiculare* in vitro on plate (Fig. 3.1). Abnormalities on mycelial morphology and low frequency of conidial germination were also observed (Fig 3.3 and 3.4), indicating that the isolate HjD92 and B341 has a high potential as biocontrol agent. Moreover, HjD92 and B341 showed a broad spectrum of antifungal activity against not only *C. orbiculare* but also thirteen tested fungal pathogens except *Penicillium digitatum* (Fig. 3.5). Interestingly, mycelial growth of *C. orbiculare* showed the strongest inhibition effect after

treated with B341 suspension (10^9 cfu/ml) (Fig. 3.2.B), as well as in the frequency of conidial germination (Fig. 3.3).

Here, the primary screening of collected bacteria from three analyzed species frog has been done and provides evidence their high antifungal activities against *C. orbiculare*. However, their efficacy in plant disease reduction in pot and its mode of action are required to employ.

3.6. Conclusion

In summary, the results presented here suggest that three selected bacteria from frog skin, *Paenibacillus* spp. HjD57, *Raoultella* spp. HjD92 and *Citrobacter* spp. B341 has a high potent in reduction of growth of plant pathogenic fungi, *Colletotrichum orbiculare*, a causal agent of cucumber anthracnose disease.

CHAPTER 4.
ANTIFUNGAL ACTIVITY OF *Paenibacillus* sp. HjD57, *Raoultella* sp. HjD92 and *Citrobacter* sp. B341 AGAINST SOME IMPORTANT PLANT DISEASES AND ANALYSIS OF THE PUTATIVE MECHANISM USED IN BIOCONTROL

4.1. Abstract

This study evaluated the efficacy of three selected bacteria from Japanese frog skin, *Paenibacillus* sp. HjD57, *Raoultella* sp. HjD92, and *Citrobacter* sp. B341 for controlling cucumber anthracnose, tomato wilt, and rice 'bakanae' diseases caused by *Colletotrichum orbiculare*, *Fusarium oxysporum* f. sp. *lycopersici, and F. fujikuroi* respectively, under pot condition. In vitro detached-leaf assay has been carried out to only cucumber plants to rapidly screen the potential of tested bacteria in suppressing the disease. Application of bacterial suspension (10^9 cfu/mL) into three potted plants suppressed disease severity. Spray treatment onto the cucumber leaf with the bacterial suspensions effectively reduced the number of anthracnose lesions both in detached-leaf assays and pot-experiments. Likewise, the tomato wilt and rice 'bakanae' diseases were also significantly suppressed as compared to untreated pots. The putative mechanism of HjD57, HjD92, and B341 involved in the reduction of plant diseases was investigated. It revealed that undiluted cell-free filtrate of those bacteria considerably inhibited the conidial germination of fungi. Moreover, the antifungal activity in cell-free filtrate did not lost after heat treatment. Overall, the data indicated that bacterial frog skin could be a potential biocontrol agent.

Keywords: Biocontrol activity, detached-leaf, cucumber anthracnose, tomato wilt, rice "bakanae", Japanese frog.

4.2. Introduction

Host-associated symbiotic microbes have a pivotal role for host fitness and health (Adair and Douglas, 2017), also can protect host from pathogen invasions (Berg, 2009). For amphibian such as frog, the composition of bacterial symbiont has been reported from multiple species of frog and regions (Assis et al., 2017; Becker et al., 2014; Belden et al., 2015; Christian et al., 2018; Madison et al., 2017). Some bacterial symbiont demonstrated the incredibly antifungal activity against amphibian fungal pathogen, *Batrachochytridium dendrobatidis* such as *Jantinobacterium lividum* (Becker et al., 2009), *Serratia marcescens* (Madison et al., 2017), and *Stenotrophomonas maltophilia* (Robak and Richards-Zawacki, 2018). Yet, only a little is known about the application of bacterial symbiont from frog skin as a potent bioagent in agriculture field against fungal plant pathogen. However, most studies has focused on pathogen inhibition by symbionts on plant-associated microbes (Bakker et al., 2010; Berendsen et al., 2012).

Bacterial inhibition of pathogen invasion of frog skin is mainly by competition and antibiosis, including the production of antimicrobial compounds like volatile organic compounds (VOCs) and bacteriocin (Brucker et al., 2008; Harris et al., 2009; Woodhams et al., 2018; Woodhams et al., 2016). Among the antimicrobial VOCs, violacein and indole-3-carboxaldehyde are secreted by *Jatinobacterium lividum*, a bacterial symbiont on frog skin that inhibits *B. dendrobatidis* (Woodhams et al., 2018). Also, 2,4-diacetylphloroglucinol is produced by *Lysobacter gummomus* that inhibits *B. dendrobatidis* (Brucker et al., 2008).

Most of the culturable bacteria from frog skin were assigned to the class Gamma-proteobacteria in the phylum Proteobacteria (Sabino-Pinto et al., 2016; Hillis et al., 2015; Rebollar et al., 2016b). The major representative genera of the class Gamma-proteobacteria are *Pseudomonas*, *Serratia*, *Citrobacter*, and *Stenotrophomona* (Assis et al., 2017; Flechas et al., 2017; Hillis et al., 2015). *Raoultella* was also can be found as frog bacterial symbiont (Baron et al., 2018). *Citrobacter*

spp. found on *Phyllomedusa distincta* (Sao Paulo leaf frog), *Kalophyrnus sinensis* (Philippine sticky frog), *Limnonectes magnus* (Mindanao fanged frog), and *Megophrys stejnegeri* (Mindanao horned frog) had mutualistic relationships with the hosts. *Raoultella* spp. were predominantly found from *Dendropsophus labialis* (green dotted treefrog) and *Rheobates palmatus* (palm rocket frog) (Flechas et al., 2017). Bacteria belonging to the class *Bacilli* in the phylum *Firmicutes* are also reported among the bacterial flora found on frog skin. For example, isolation of *Paenibacillus* spp. from *Pristimantis diadematus* (Diadem robber frog) and *P. danae* (Cuzco robber frog) in the Peruvian Andes had been reported (Catenazzi et al., 2018). The investigation of genus *Citrobacter*, *Raoultella*, and *Paenibacillus* as a promising candidate bioagent against some plant pathogenic fungi is still poorly studied.

Several bacterial agents isolated from healthy plants or soil such as *Bacillus* spp., *Pseudomonas* spp., *Serratia* spp. and *Streptomyces* spp. are reported to offer biocontrol efficacy against plant diseases (Jeun et al., 2004; Kim et al., 2016; Kim and Chung, 2004; Shimizu et al., 2009) and are used as biopesticides. Frog bacteria sometimes produce secondary metabolites, including antifungal compounds (Harris et al., 2009). Using this knowledge, the use of bacterial symbiont from frog skin are proposed. In this study, three selected bacteria and have identified as *Paenibacillus* sp. HjD57, *Raoultella* sp. HjD92, and *Citrobacter* sp. B341 from Japanese frog skin *Hyla japonica*, *Pelophylax porosus porosus* and *Buergeria burgeri* were investigated for their possibility as biocontrol agents against cucumber anthracnose, tomato wilt and rice 'bakanae' disease caused by *Colletotrichum orbiculare*, *Fusarium oxysporum* f. sp. *lycopersici*, and *F. fujikuroi*, respectively. In addition, the putative mode-of-action underlying the suppressive activity is discussed.

4.3. Materials and Methods

4.3.1. Biocontrol activity in selected frog-skin bacteria

4.3.1.1. Cucumber anthracnose (detached leaf test)

Individual seeds of cucumber (cv. Suyo) were sown in 180 ml soil (Kumiai Nippi Engei Soil, Japan) in a 200-ml plastic pot and kept in a greenhouse at 25°C. Cucumber plants were used after reaching the 2–3-leaf stage (3–4-weeks old). The second-oldest leaves were detached, surface was washed with SDW, and dried in a clean bench (laminar flow hood). In this study we tested the curative activity of frog-skin bacteria. For that purpose, adaxial leaf surfaces were first sprayed evenly with the conidial suspension of *C. orbiculare* (10^6 conidia/ml), immediately placed in a transparent plastic box with moistened sterile filter paper to create high humidity, maintained in the environmental conditions mentioned in section *2.6*., and 24 h later, the adaxial leaf surface was again evenly sprayed with a bacterial suspension (10^9 cfu/ml) until run off and maintained in the same culture conditions. The leaves without any inoculation or treatment were used as healthy control. The number of lesions on each leaf was counted 7 days after treatment (Makovitzki et al., 2007). In this study three detached leaves were used for each treatment and three replications were performed.

4.3.1.2. Cucumber anthracnose (greenhouse test)

Potted plant tests were performed following the method of Negishi et al. (2011) and Shimizu et al. (2009). In this study we tested the curative activity of frog-skin bacteria. For that purpose, whole leaves of 4-week old cucumber seedlings (cv. Suyo) were evenly sprayed with a conidial suspension of *C. orbiculare* (10^6 conidia/ml) until runoff, and 24 hours later, whole leaves were sprayed with each frog-skin bacterial suspension (10^9 cfu/ml) until runoff. All treated plants were kept in a chamber with a photoperiod of 18 h light (500 lux)/6 h dark (light intensity 500 lux) and 100% relative humidity at 25°C for 48 h. After 48 h, all treated

plants were moved into a greenhouse maintained at approximately 25°C. The number of anthracnose lesions that developed on all of the leaves of a plant was counted at 7 days post-inoculation. Each treatment was done in triplicate (n=3) from four independent replications.

4.3.1.3. Tomato Fusarium wilt (greenhouse test)

In this study, we tested the preventive activity of frog-skin bacteria. The roots of a 3-week old seedling of tomato (*Solanum lycopersicum* cv. Momotaro; Takii & Co., Kyoto, Japan) potted in soil were injured by repeatedly (10 times) inserting a small stainless-steel spatula into the soil. The plants were treated with a soil drench of selected bacterial suspension at 25 ml/plant (10^9 cfu/ml). SDW was applied as the control.

F. oxysporum f. sp. *lycopersici*, the tomato wilt fungus, race 3 KoChi-1 (Table 3.1) was cultured in 100 ml PDB (potato dextrose broth) in a 300-ml flask at room temperature for 5 days by shaking at 120 rpm on a reciprocal shaker. The fungal culture was filtered with two layers of cheesecloth to remove mycelia, and the filtrate was centrifuged at 1631.5 ×*g* for 20 min to collect the bud-cell spores. The resulting pelleted spores were resuspended with SDW to a density of 10^6 spores/ml and used as a source of inoculum.

Six days after treatment with the bacteria, the tomato plants were challenged with 25 ml of *F. oxysporum* inoculum by soil drenching as described by Abdallah et al. (2016) and Inami et al. (2014). As a negative control, SDW was used instead of the spore suspension. Tomato plants were kept in a greenhouse at around 25°C. For each treatment, three replicates were used, and the entire experiment was repeated three times.

The external symptoms for each plant were evaluated 30 days post-inoculation by following the method of Inami et al. (2014): Grade 0, no wilt or yellowing; Grade 1, lower leaves yellowing; Grade 2, lower and upper leaves yellowing; Grade 3, lower leaves yellowing and wilting, and upper leaves yellowing; Grade 4, all leaves wilting and yellowing,

or dead (Inami et al., 2014). The disease severity index (DSI) was determined using the formula described by Jangir et al. (2018):

$$DSI = \Sigma \text{ (Grade} \times \text{Number of plants of that grade) / (4} \times \text{Total number of assessed plants)} \times 100$$

4.3.1.4. Rice 'bakanae' (greenhouse test)

A fungal spore suspension of *F. fujikuroi* Miyagi 92-10 (Table 3.1), the rice 'bakanae' pathogen, was prepared by cultured in 100 ml PDB (potato dextrose broth) in a 300-ml flask at room temperature for 5 days by shaking at 120 rpm on a reciprocal shaker. The fungal culture was filtered with two layers of cheesecloth to remove mycelia and the filtrate was centrifuged at 3000 rpm for 20 min to collect the bud cell spores. The resulting spore pellet was diluted with SDW to adjust to 10^6 spores/mL.

Rice (cv. Tanginbouzu) seeds were surface sterilized in 1% sodium hypochlorite for 5 min and followed by dipping in 70% ethanol for 5 min, washing several times with SDW, and air drying. Sterilized rice seeds were then artificially infested with a spore suspension of *F. fujikuroi* as described previously (Jeon et al., 2013; Matić et al., 2014) with slight modification. Briefly, seeds were submerged in the fungal spore suspension by shaking at 90 rpm at room temperature for 20 h. As a control, sterilized seeds were immersed in SDW instead of the spore suspension (10^6 conidia/ml). The infested seeds were then air dried for 2–3 h in a clean bench.

Infested rice seeds were submerged in the bacterial suspension (10^9 cfu/ml) by shaking at 90 rpm for 15 min at room temperature, and air dried for 2–3 h again in a clean bench. As a negative control, SDW was used instead of the bacterial suspension.

The seeds (50/pot) were sown in sterile soil. Seven pots were used with total 350 seeds/treatment. For each treatment, seven replicates and two repeats were performed. The

pots were placed in a greenhouse at 25–27°C. Disease symptoms were observed at 14 days after sowing. Symptoms of each plant were scored using a 0–5 scale following the procedure of Amatulli et al. (2010) and Saito et al. (2020): Grade 0, asymptomatic; Grade 1, stunted with narrow and yellow leaves; Grade 2, thin plants with narrow, pale yellow leaves; Grade 3, plants with narrow, pale yellow leaves, thin and long internodes and a short root system; Grade 4, severe 'bakanae' symptoms and some necrosis of the crown; Grade 5, dead plant. A disease severity index (DSI) was determined using the formula described by Jangir et al. (2018).

$$DSI = \Sigma\ (\text{Grade} \times \text{Number of plants of that grade}) / (5 \times \text{Total number of assessed plants}) \times 100$$

4.3.2. Evaluation of antifungal activity of frog-skin bacterial culture filtrate

4.3.2.1. Effect of frog-skin bacterial cell-free filtrates on C. orbiculare mycelial growth

Culture filtrate of each bacterium was prepared according to the method of Mardanova et al. (2017). Briefly, bacteria were individually cultured in TSB medium by shaking at 120 rpm at 28°C for 48 h. The bacterial culture was centrifuged at 536 ×*g* at 4°C for 20 min. The supernatant fraction was filtered through a 0.45 μm cellulose membrane (Merk Millipore, Darmstadt, Germany) to remove any remaining cells. Three 10-day-old PDA mycelial plugs of *C. orbiculare* (ø5.0 mm) were placed in a flask containing 20 ml cell-free supernatant without dilution and with dilution with PDB to 10 or 50-fold (10 or 2% v/v), and were cultured by shaking at 70 rpm at 28°C. As a control, PDB containing 10% (v/v) TSB instead of the cell-free filtrate was used. Flask contained three mycelial plugs treated with undiluted culture filtrate was performed. After a 7-day culture period, fungal mycelia were recovered by filtration using two layers of cheesecloth, oven-dried at 50°C for 72 h, and weighed (Singh et al., 2012). Each measurement was done in triplicate and two repeats were performed.

4.3.2.2. *Effect of frog-skin bacterial cell-free filtrate on C. orbiculare*, *F. oxysporum* f. sp. *lycopersici and F. fujikuroi conidial germination*

Undiluted, 10- or 50-fold diluted bacterial cell-free filtrate in PDB prepared as mentioned in section 4.3.2.1. was inoculated with 10^6 conidia of *C. orbiculare*, *F. oxysporum* f. sp. *lycopersici* and *F. fujikuroi*. After 24 h incubation in the dark at 28ºC, a portion of the culture was harvested, and at least 100 conidia were examined for evaluation of germination rate using a microscope (Rahman et al., 2007; Yoshida et al., 2001). As a control, PDB containing 10% (v/v) TSB without the cell-free filtrate fraction was used.

4.3.3. *Heat stability of antifungal activity of cell-free filtrate*

To determine the heat stability, the cell-free filtrate was heated at 50ºC, 100ºC for 1h (Kaur et al., 2016), and 121ºC for 20m by autoclaving. TSB (trypticase soy broth) medium without cell-free filtrate was prepared as a control. All treated samples were then placed of three 10-day-old PDA mycelial plugs of *C. orbiculare* (ø5.0 mm). The incubation condition and observation method were same as described in section 4.3.2.1.

4.3.4. *Statistical analysis*

All the experiments were repeated three times for each treatment with three replications (Seven replications with two repeats were performed for section 4.3.1.4, whereas for section 4.3.1.2 was done with four repeat experiments). Data were analyzed using Package R studio software version 3.5.1 (R Development CoreTeam). The significance of differences amongst all treatments was tested using Student's t-test (Rosner, 2016).

4.4. Results

4.4.1. Biocontrol activity of the frog-skin bacteria against C. orbiculare

4.4.1.1. Detached leaf test

Cucumber leaves inoculated with *C. orbiculare* showed necrotic lesions with a mean number of 32 lesions/leaf at 7 days after inoculation (SDW in Fig. 4.1). In contrast, spraying leaves with isolates HjD57, HjD92 and B341 (10^9 cfu/ml) 24-h post-inoculation resulted in a significant reduction in the number of lesions (Fig. 4.1.B). No lesions were observed on the detached leaves treated with SDW (negative control).

4.4.1.2. Greenhouse test

Cucumber seedlings inoculated with *C. orbiculare* had a mean number of 161 necrotic lesions at 7 days post-inoculation (SDW in Fig. 4.2). Cucumber seedlings treated with suspensions of isolates HjD57, HjD92 and B341 at 24-h post-inoculation had a reduced number of lesions (Fig. 4.2.B).

4.4.2. Biocontrol activity of frog-skin bacteria against other plant diseases

4.4.2.1. Tomato Fusarium wilt (greenhouse test)

Tomato plants inoculated with *F. oxysporum* f. sp. *lycopersici* presented yellowing and wilt symptoms and had a DSI of 67.5 at 30 days post-inoculation (SDW in Fig. 4.3). Pretreatment of tomato seedlings with isolates HjD57, HjD92 and B341 (10^9 cfu/ml) by soil drenching 1-week prior to inoculation with *F. oxysporum* f. sp. *lycopersici* had significantly reduced, and the DSIs were 12.5, 10.0 and 7.5, respectively (Fig. 4.3.B). Among the three frog-skin bacteria, B341 seemed to work the best in reducing symptoms.

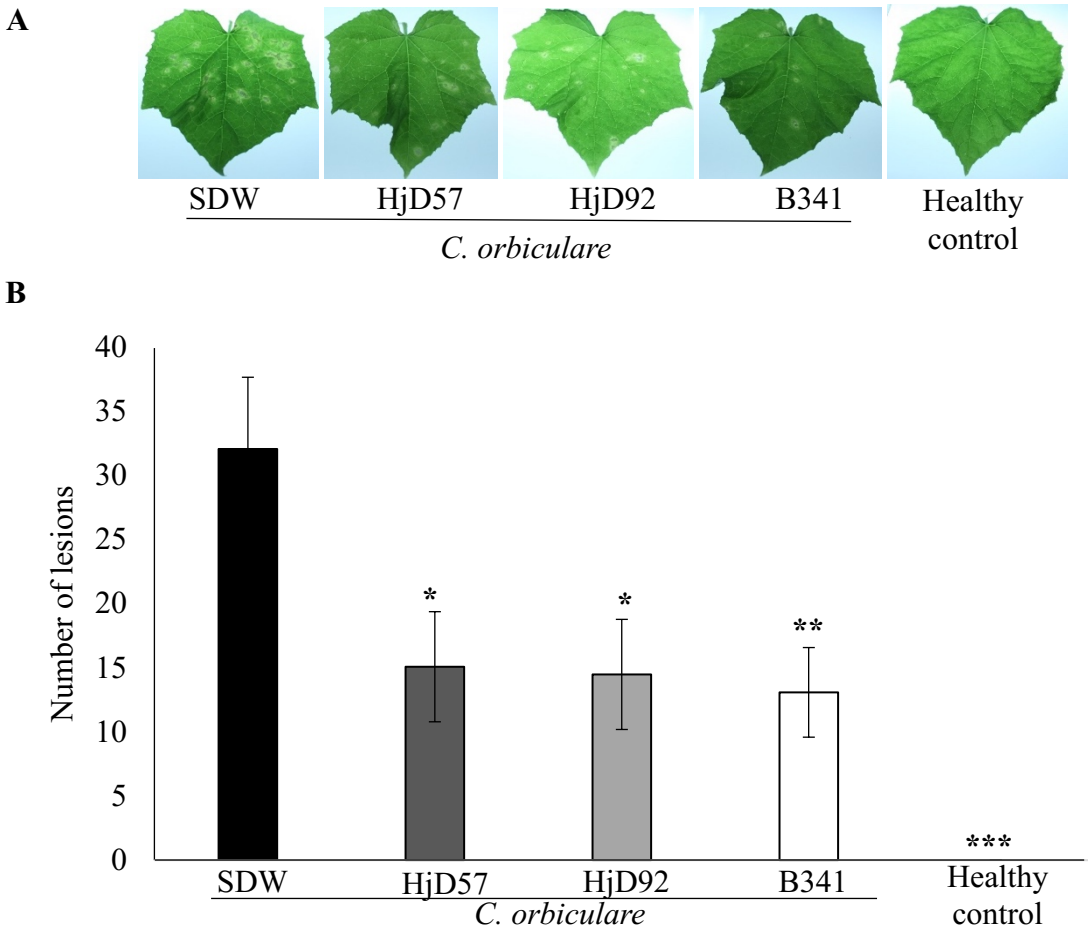


Figure 4.1. Biocontrol activity of frog-skin bacteria against cucumber anthracnose by the detached leaf test. Cucumber (cv. Suyo) leaves inoculated with *C. orbiculare* A-29 and treated with a bacterial suspension (10^9 cfu/ml) of isolate HjD57, HjD92 or B341 for 24-h post-inoculation. Yellow to brown spotted lesions were observed in the inoculated control (SDW). Treatment with isolate HjD57, HjD92 or B341 reduced the number of lesions (A). The number of lesions was counted 7 days post-inoculation and compared with the control (SDW) (B). Data represents the mean (± standard error, SE, n=3) of three independent experiments, each performed in triplicate, and presented relative to control. Asterisks indicate statistically significant differences (***P<0.001, **P<0.01, *P<0.05) by Student's t-test compared to the inoculated control (SDW).

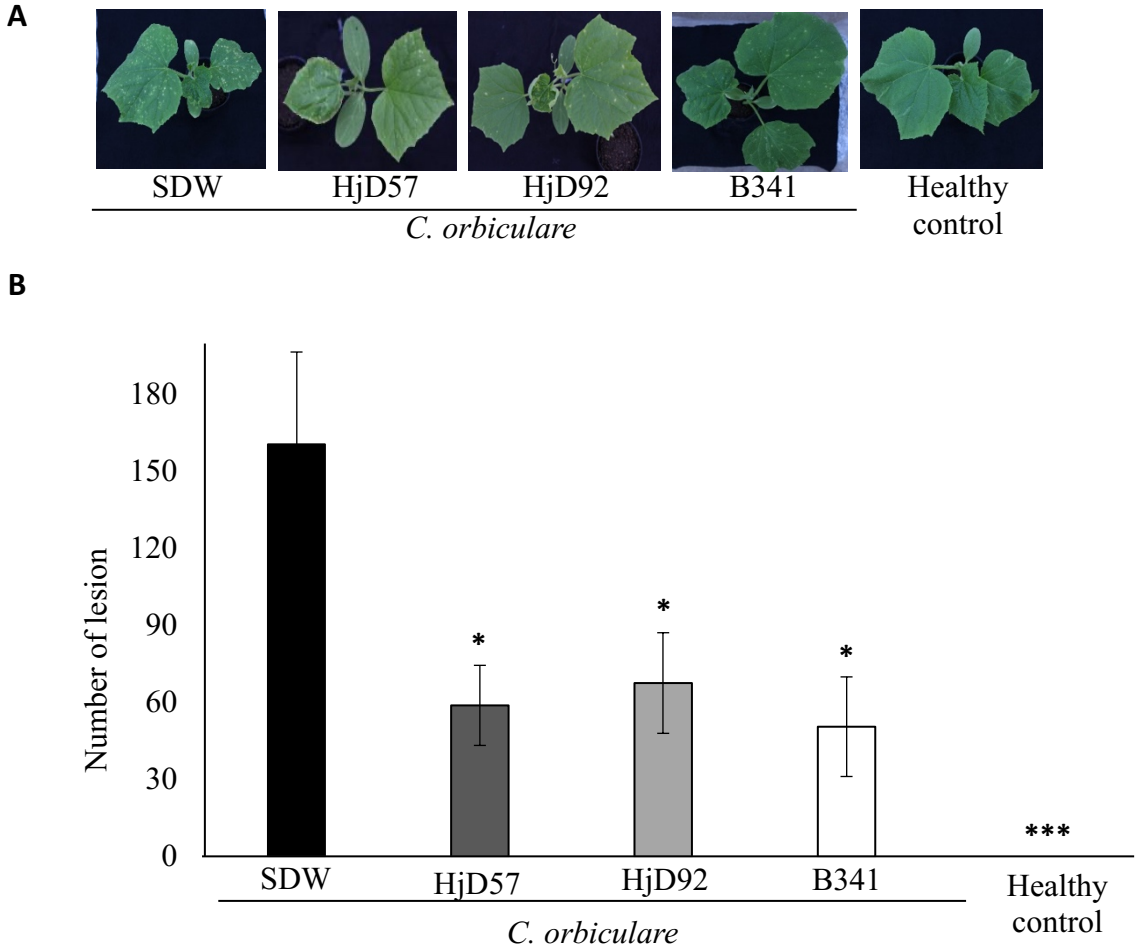


Figure 4.2. Biocontrol activity of frog-skin bacteria against cucumber anthracnose in a greenhouse test. Four-weeks old cucumber (cv. Suyo) seedlings were inoculated with *C. orbiculare* A-29 and treated with a bacterial suspension (10^9 cfu/ml) of isolate HjD57, HjD92 or B341 at 24-h post-inoculation. Yellow to brown spotted lesions were observed in the inoculated control (SDW). Treatments with isolate HjD57, HjD92 or B341 reduced the number of lesions (A). The number of lesions that developed on whole leaves was counted 7 days post-inoculation and compared with the control (SDW) (B). Data represents the mean (± standard error, SE, n=3) of four independent experiments, each performed in triplicate, and presented relative to control. Asterisks indicate statistically significant differences (***P<0.001, *P<0.05) by Student's t-test compared to the inoculated control (SDW).

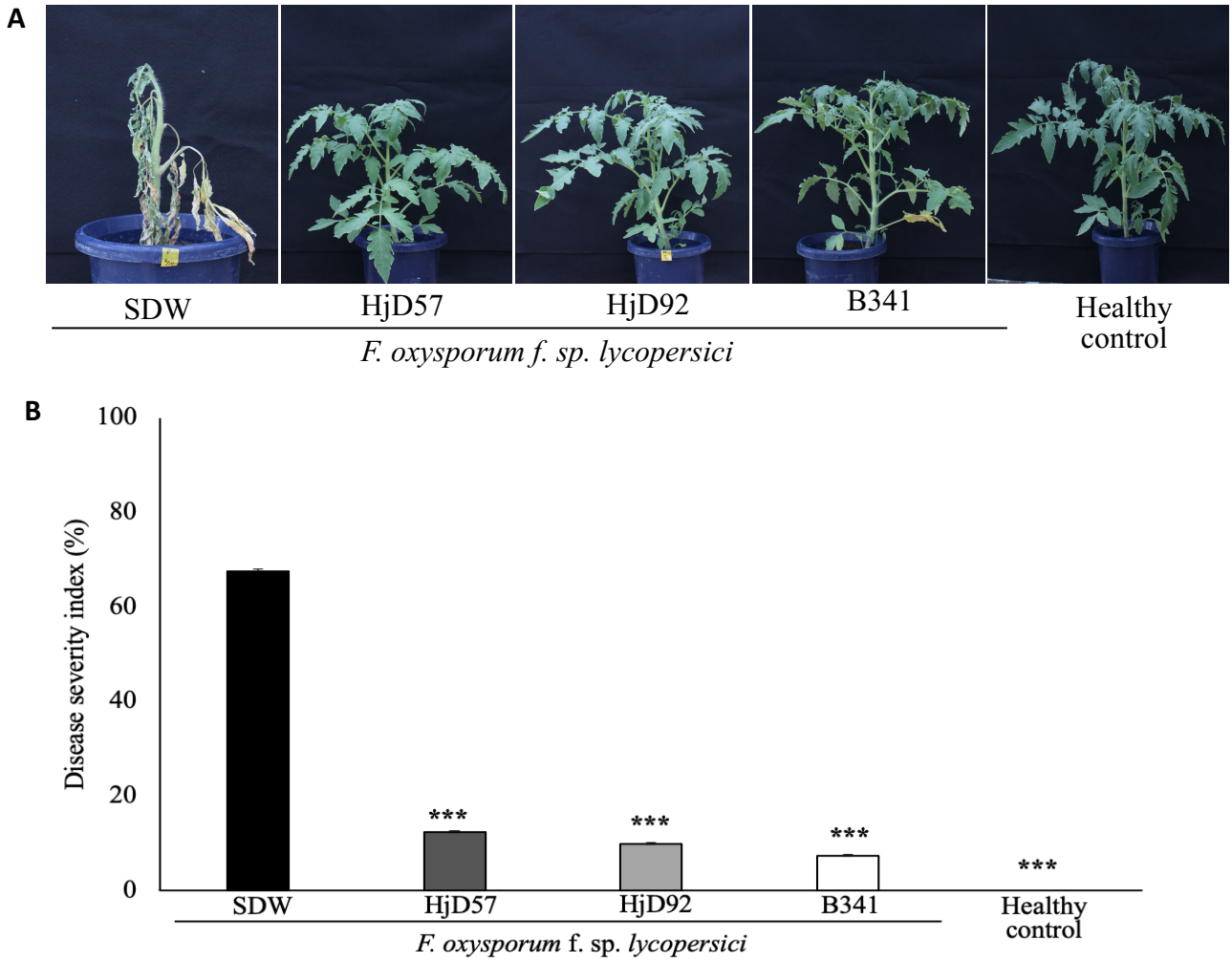


Figure 4.3. Biocontrol activity of frog-skin bacteria against tomato wilt in a soil drenching test. Three-week old tomato (cv. Momotaro) seedlings were treated with a bacterial suspension (10^9 cfu/ml) of isolate HjD57, HjD92 or B341 by soil drenching and inoculated with *F. oxysporum* f. sp. *lycopersici* race 3 KoChi-1 by soil drenching with a spore suspension 6 days after treatment. Photographs were taken at 30 days post-inoculation. Symptoms of yellowing and wilt were observed in the inoculated control (SDW) and treatment with isolate HjD57, HjD92 or B341 reduced symptom development (A). Symptoms of each plant were graded from 0 to 4, and the disease severity index for each treatment was calculated and compared with the control (SDW) (B). Data represents the mean (± standard error, SE, n=3) of three independent experiments, each performed in triplicate, and presented relative to control. Asterisks indicate statistically significant differences (***P<0.001) by Student's t-test compared to the inoculated control (SDW).

4.4.2.2. Rice 'bakanae' (greenhouse test)

The DSI of rice plants grown from *F. fujikuroi*-infested seeds was 44.7 (SDW in Fig. 4.4). Submerging the infested seeds in suspensions of either HjD57, HjD92 and B341 (10^9 cfu/ml) significantly reduced the severity of rice 'bakanae' symptoms with DSI values of 8.1, 13.1 and 7.8, respectively (Fig. 4.4.B).

4.4.3. Antagonistic activity in cell-free filtrates against C. orbiculare.

Cell-free filtrates of isolates HjD57, HjD92 and B341 had strong antifungal activity with dry (95.7% for all of them) against *C. orbiculare* mycelial growth in PDB (Fig. 4.5). In the presence of a 10-fold dilution of HjD57, HjD92 and B341 filtrates, mycelial growth (evaluated in dry weight) of *C. orbiculare* was inhibited by 44.8%, 12.0% and 19.0% in comparison to the control, respectively, and in a 50-fold dilution, *C. orbiculare* mycelial growth was inhibited by 7.4%, 11.7% and 11.7%, respectively.

Undiluted cell-free filtrates of isolates HjD57, HjD92 and B341 strongly inhibited conidial germination (100%, 99.0%, 93.4%, respectively) of *C. orbiculare* (Fig. 4.6.A). Similarly, conidial germination of *F. oxysporum* f. sp. *lycopersici* and *F. fujikuroi* were also significantly inhibited by undiluted cell-free filtrate of HjD57, HjD92 and B341 (97.2%, 67.8% and 84.3% for *F. oxysporum* f. sp. *lycopersici* and 92.5%, 79.5% and 89.0% for *F. fujikuroi*; Fig. 4.6.B and 4.6.C). However, 50-fold dilution of cell-free culture filtrate of B341 only suppressed significantly the conidial germination of *F. oxysporum* f. sp. *lycopersici* and *F. fujikuroi*. Meanwhile, 10-fold dilution of HjD92 cell-free filtrate showed the suppressive effect to all tested fungal conidial germination, *F. oxysporum* f. sp. *lycopersici, F. fujikuroi* and *C. orbiculare.* Cell-free filtrate of HjD57 and B341 showed similar inhibition effect of conidial germination of *F. oxysporum* f. sp. *lycopersici* and *F. fujikuroi.*

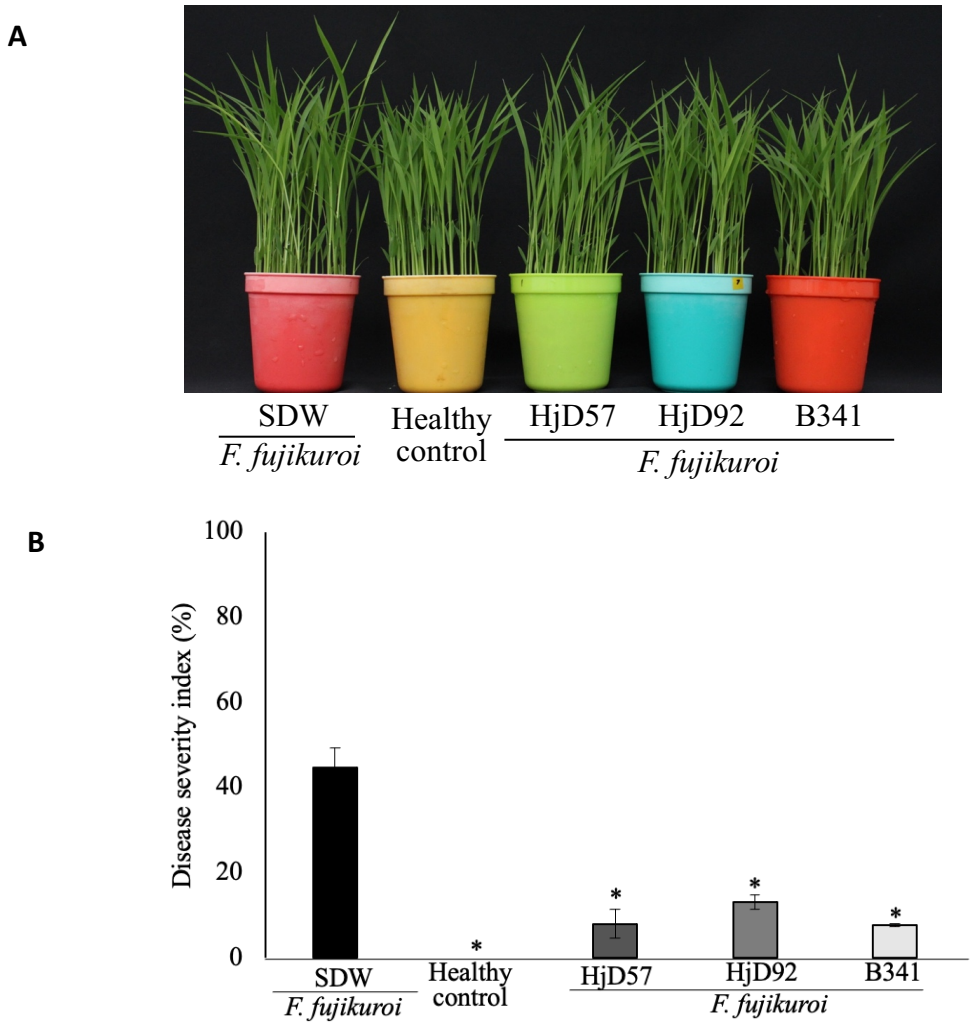


Figure 4.4. Biocontrol activity of frog-skin bacteria against rice 'bakanae' by a seed treatment test. Rice (cv. Tanginbouzu) seeds artificially infested with *Fusarium fujikuroi* Miyagi 92-10 were submerged in a bacterial suspension (10^9 cfu/ml) of isolate HjD57, HjD92 or B341 for 15 min and sown in sterile soil in pots. Photographs were taken 14 days after sowing. Symptoms of stunted, narrow and yellow leaves and irregular elongation of the leaves were observed in the inoculated control (SDW). Treatment with isolate HjD57, HjD92 or B341 reduced the symptom development (A). Symptoms of each plant were graded from 0 to 5 and the disease severity index of each treatment was calculated and compared with the control (SDW) (B). Data represents the mean (± standard error, SE, n=7) of two independent experiments, each performed in seven pots contained 50 seeds/pot and presented relative to control. Asterisks indicate statistically significant differences (*P<0.05) by Student's t-test compared to the control (SDW).

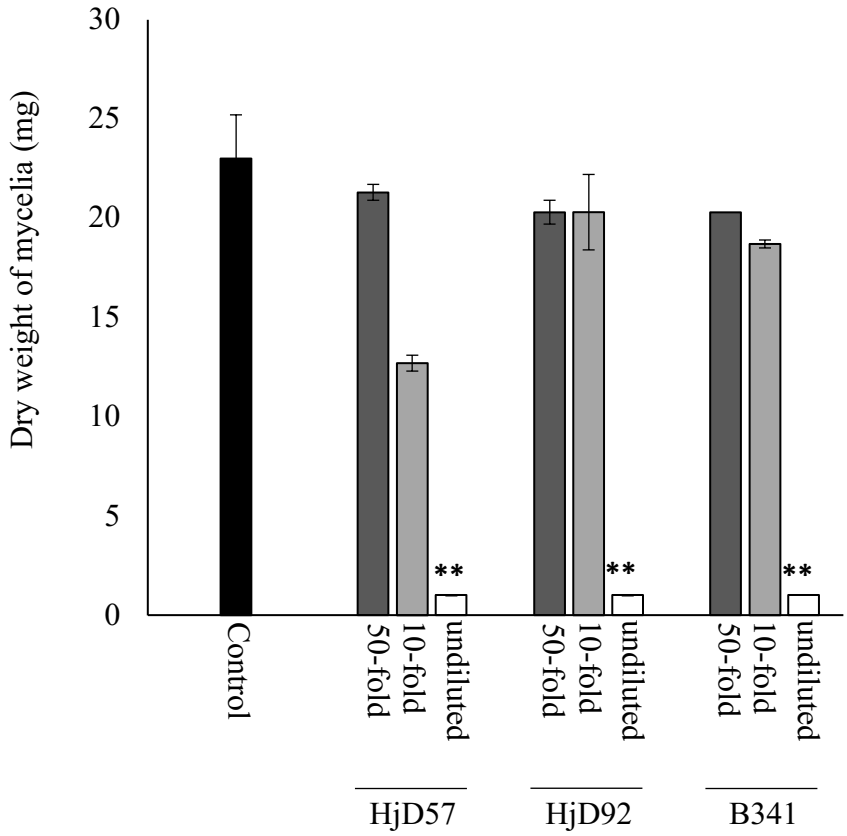


Figure 4.5. Mycelial growth of *Colletotrichum orbiculare* in PDB containing cell-free filtrates of isolate HjD57, HjD92 or B341. Treatment with the undiluted cell-free filtrate of isolate HjD57, HjD92 or B341 resulted in a reduction of mycelial growth in 7 days. Vertical bars represent standard errors of the mean (n=3). Asterisks indicate the level of significance (**P<0.01) by a two samples t-test compared to the control.

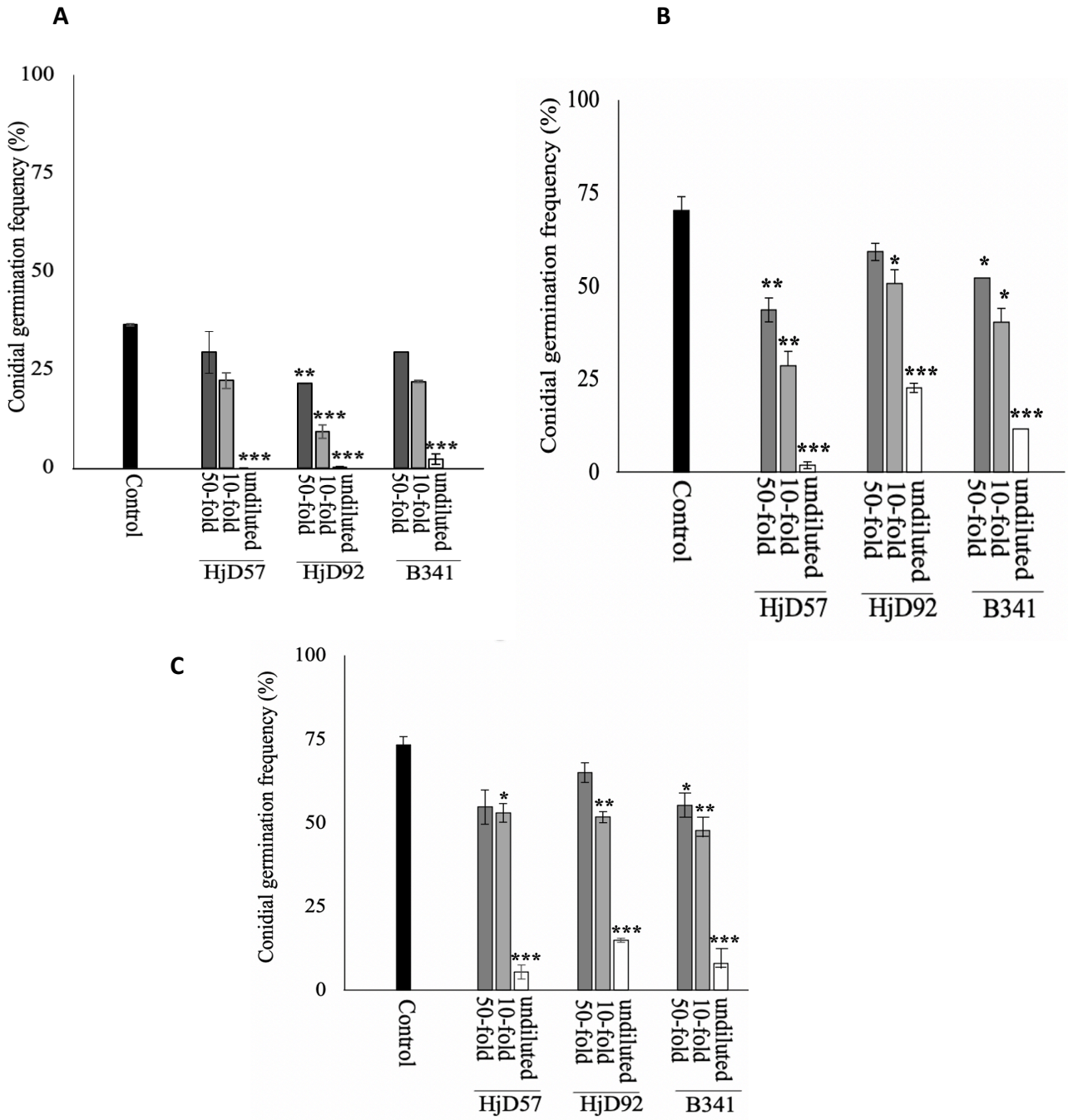


Figure 4.6. Evaluation of conidial germination of plant pathogenic fungi *Colletotrichum orbiculare* (A), *Fusarium oxysporum* f. sp. *lycopersici* (B), and *F. fujikuroi* (C) after treatment with different dilution fold of cell-free filtrate of bacterial isolate. The undiluted cell-free filtrate of isolate HjD57, HjD92 or B341 resulted in inhibition of conidial germination to all tested fungal pathogen in 24 h. Vertical bars represent standard errors of the mean (n=3). Asterisks indicate the level of significance (***P<0.001, **P<0.01) by a two samples t-test compared to the control.

4.4.4. Stability of antifungal activity

No loss in antifungal activity of cell-free filtrate was observed after its exposure to temperature up to 121°C (Fig. 4.7).

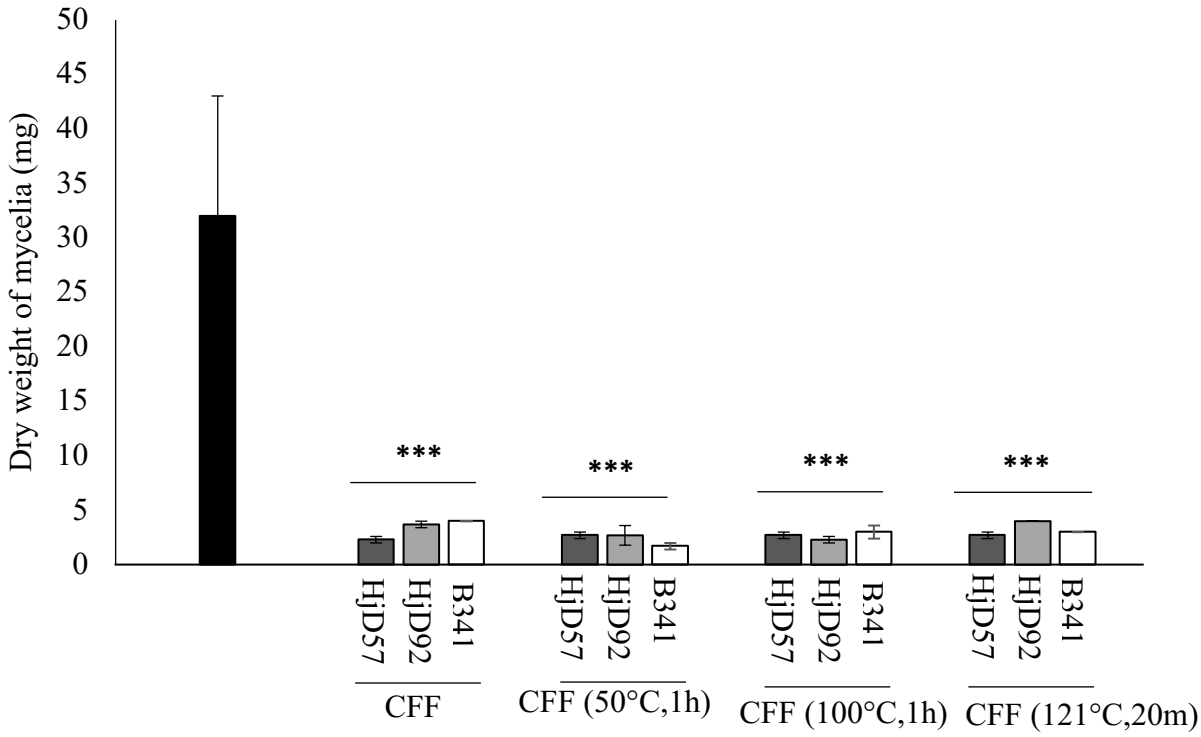


Figure 4.7. Effect of heat treatment on the antifungal activity in cell-free culture filtrate of isolate HjD57, HjD92 and B341. The cell-free culture filtrate was exposed to three different temperature by autoclaving it at 50° and 100°C for 1 h and 121°C for 20 m. Values represents the dry weight of *Colletotrichum orbiculare* A-29 after heat treatment. Vertical bars represent standard errors of the mean (n=3). Asterisks indicate the level of significance (***P<0.001, **P<0.01) by a two samples t-test compared to the control. CFF, Cell-free filtrate

4.5. Discussion

The resident beneficials of frog skin bacterial microbes form the first line of defense against the invasion of pathogen (Harris et al., 2009; Rollins-Smith, 2005). These bacterial frog skin symbionts are known to inhibit the important pathogenic fungi for amphibians, *Batrochocytridium dendrobatidis*, a causal agent of devastated disease called cytridiomycosis, which made an extinction of several species of frog around the world (Becker et al., 2015; Bosch et al., 2001; Harris et al., 2009; Hyatt et al., 2010; Skerratt et al., 2007; Woodhams et al., 2016, 2018; Zipkin et al., 2020). In this study, the exploration of other benefits of bacterial frog skin which may be control plant pathogenic fungi has been conducted. As Walke and Belden (2016) proposed that amphibian skin microbes could be used in suppressing fungal pathogens of humans, animals and plants. Thus, the finding in our study that *Paenibacillus* sp. HjD57, *Raoultella* sp. HjD92 and *Citrobacter* sp. B341, isolated from frog skin, are potential biocontrol agents towards plant pathogenic fungi is within our expectations.

Considering that anthracnose is the main disease in cucumber (Damm et al., 2013). Other phytopathogen, *Fusarium*, are also known causes a serious constraints to agricultural productivity globally (Khan et al., 2017), due to their high survival in the soil for extended period of time (Shlevin et al., 2004). *Fusarium* species can induce the necrosis, wilting, and producing mycotoxin, thus their infection on plant especially to many staple food crops causes massive economic losses worldwide. Mostly the available method to control disease is with fungicide excessively (Park et al., 2013), thus the use of alternative disease control methods can effectively replace the application of chemical fungicide. Here, the in vitro screening of collected bacteria from three species of Japanese frog skin has been done aiming the biological approach. These studies primarily were based on the antagonistic activities on plate and microscopic analysis of mycelial and conidial germination inhibition. It revealed that 3 isolates were selected among 106 isolates, which was collected from 3 species of frog

skin in Japan. Those bacteria were identified as *Paenibacillus* sp. HjD57, *Raoultella* sp. HjD92 and *Citrobacter* sp. B341.

This study has successfully demonstrated the potential of bacteria from frog skin as bioagents in controlling cucumber anthracnose, tomato wilt and rice "bakanae" diseases. The antifungal activity of the three bacteria obtained in this study is probably due to antifungal metabolites secreted by the bacteria because the cell-free culture filtrates of these bacteria reduced mycelial growth of *C. orbiculare* (Fig. 4.5) and conidial germination of *C. orbiculare*, *Fusarium oxysporum* f. sp. *lycopersici,* and *F. fujikuroi* when tested in vitro (Fig. 4.6). Moreover, no loss of antifungal activity in cell-free culture filtrate after heat treatment up to 121°C (Fig. 4.7).

Here, the detached leaf assay was adopted prior to performing the pot experiment under greenhouse condition. This method provides a useful and rapid indication of pathogenicity of a fungal causing foliar disease of plant (Pettitt et al., 2011). The treatment of foliar spray on cucumber detached leaf with *Paenibacillus* sp. HjD57, *Raoultella* sp. HjD92 and *Citrobacter* sp. B341 resulted in significant reducing the number of anthracnose lesion (Fig. 4.1), treated with B341 showed the highest ability to reduce the progression of anthracnose in cucumber leaves.

Genus of *Paenibacillus* has been studied as a promising biocontrol agent against some important plant diseases such as *P. ehimensis* KWN38 suppressed soilborne diseases caused by *Rhizoctonia solani*, *F. oxysporum* f. sp. *lycopersici* and *Phytophthora capsici* (Naing et al., 2014); *P. validus* inhibited the hyphal growth of the fungal pathogens *F. solani*, *Bipolaris oryzae*, *Aspergillus niger*, *A. fumigatus* and *A. oryzae* (Lorentz et al., 2006); *P. validus* ATY16 reduced symptoms of citrus greening disease caused by the bacterial pathogen, *Candidatus* Liberibacter asiaticus (Trivedi et al., 2011). In our study the number of lesions of cucumber anthracnose disease reduced by 63.4% (Fig. 4.2B) by treatment with 10^9 cfu/ml suspension of isolate *Paenibacillus* sp. HjD57, likewise, the tomato wilt and rice "bakanae" were also reduced by 81.5% and 81.9%, respectively (Fig. 4.3B and

4.4B), indicating that the isolate has a potential as a biocontrol agent against *C. orbiculare, Fusarium oxysporum* f. sp. *lycopersici,* and *F. fujikuroi*. *Paenibacillus* spp. often produce antifungal compounds, such as fusaricidin (Beatty and Jensen, 2002; Choi et al., 2008; Han et al., 2015; Li et al., 2013; Li and Chen, 2019; Yang et al., 2018), pelgipeptines (Wu et al., 2010) and VOCs for instance benzothiazole, benzaldehyde, undecanal, dodecanal, hexadecanal, 2-tridecanone and phenol (Raza et al., 2015). Further study is required to determine the antifungal substances produced by HjD57.

Raoultella spp. can be used as plant growth promoting bacteria (PGPB) (Sarron et al., 2018; Xu et al., 2015) and a highly potent as biofungicide against plant pathogenic fungi such as *Phytophthora fragariae* var. fragariae and *P. cactorum* (Anandhakumar and Zeller, 2008). Here, in our study, we used the bacteria for suppressing *C. orbiculare* and two other pathogens (*F. oxysporum* and *F. fujikuroi*) in a pot test. Similar to *Paenibacillus* sp. HjD57, *Raoultella* sp. HjD92 also significantly effective reduced the development of cucumber anthracnose disease by a pot test. HjD92 also presented biocontrol activities to tomato wilt and rice 'bakanae' diseases (Fig. 4.3 and 4.4). We presumed that HjD92 could also produce antifungal substances, yet at present, its mode of actions is unknown; however, it most likely is due to the production of an antifungal metabolite since mycelial growth and conidial germination of those three pathogenic fungi were inhibited after treatment with a cell-free culture filtrate (Fig. 4.6). Further studies, however, are required to determine its mode of action. Fiołka et al. (2013) reported a polysaccharide–protein (glycoprotein) complex from *R. ornithinolytica* exhibited antifungal activity against *Candida albicans*.

There are only a few reports mentioning that *Citrobacter* spp. have biocontrol activities against plant diseases. To our knowledge, this is the first report on *Citrobacter* sp. isolated from frog skin applied as a potent bioagent against *C. orbiculare*. *Citrobacter* spp. were previously reported as endophytes in the wild plant species *Zea* spp. (teosintes) and *Z. mays* (corn) (Johnston-Monje and

Raizada, 2011), *Saccharum officinarum* (sugarcane) (Magnani et al., 2013) and the root of *Solanum tuberosum* (potato) (Sturz et al., 2005). In our studies, here, we indicated that *Citrobacter* sp. B341 has also a high potent of antifungal activity against *C. orbiculare* similar to *Paenibacillus* sp. HjD57 and *Raoultella* sp. HjD92. Moreover, the lesion number of anthracnose disease on cucumber leaves were reduced. Likewise, fusarium wilt and rice 'bakanae' diseases were can be controlled effectively. The mode of action involved in this antifungal activity remained unknown, but it would have similar to other isolates (HjD57 and HjD92) since mycelial growth and conidial germination of *C. orbiculare* were inhibited after treatment with a cell-free supernatant. Again, further study is required to determine its mechanisms of activity. Several antifungal metabolites, such as iturin, surfactin and fengycin were produced by *Citrobacter* spp. (Mandal et al., 2013).

The antifungal metabolite that putatively contain in cell-free culture filtrate were further characterized and was shown to be relatively stable under high temperature (Fig. 4.7). This finding suggests that culture filtrate of HjD57, HjD92 and B341 probably contains heat stable antifungal compounds which are able to inhibit the fungal growth. This heat stability indicated that antifungal metabolite may not a protein (Darma and Purnamasari, 2016), but it may contain bioactive compound which has an antifungal activities known as lipopeptides with the major families includes iturin, surfactin, fengycin, and fusaricidin, almost all of those compound reported are stable to heat, pH and showed high capability of inhibiting various pathogens (Malviya et al., 2020). Further studies are needed to determine the chemical identity of the antifungal compound produced by HjD57, HjD92 and B341.

In this study we found that three bacteria obtained from frog skins are suitable candidates for biological control agents against foliar cucumber anthracnose, soilborne tomato fusarium wilt and seedborne rice 'bakanae' diseases (Figs. 4.2–4.4). Although *Citrobacter* sp. B341 promoted the growth of cucumber plants, *Raoultella* sp. HjD92 had a negative effect on plant growth (Fig. 3.6).

Moreover, some strains of these three genera have been reported as opportunistic, infectious bacteria to mammals and humans. As potential biological control agents (BCAs) are required to have not only a disease suppressive effect but also must not have any negative effect on host plant growth (Besset-Manzoni et al., 2019), it will also be necessary to monitor the potential pathogenicity of these bacteria to humans prior to their use as biopesticides.

To our knowledge, this is the first report on the beneficial use of *Paenibacillus* sp., *Raoultella* sp. and *Citrobacter* sp. isolated from frog skin as biocontrol agents that effectively control plant diseases.

4.6. Conclusion

Some important plant diseases such as cucumber anthracnose, tomato wilt and rice "bakanae" are the main diseases causing losses of yield. This is the first report of *Paenibacillus* sp. HjD57, *Raoultella* sp. HjD92, and *Citrobacter* sp. B341 as biocontrol agents collected from wild Japanese frog skin for controlling the cucumber anthracnose, tomato wilt and rice "bakanae", caused by *Colletotrichum orbiculare*, *Fusarium oxysporum* f. sp. *lycopersici*, and *F. fujikuroi*. Interestingly, the cell-free filtrates of those bacteria are thermostable and showed the strong inhibition on mycelial growth and conidial germination, it is possible that the bacteria may produce bioactive compound. Finally, *Paenibacillus* sp. HjD57, *Raoultella* sp. HjD92, and *Citrobacter* sp. B341 have significant potentials as the excellent candidate to replace the chemical fungicide for plant protection even though the safety is required to be evaluated prior to using them in the field.

CHAPTER 5.
CONCLUSION

5.1. Conclusion

1. A total 106 bacterial isolates were recovered from three species of frog skin, *Hyla japonica* (ニホンアマガエル), *Pelophylax porosus porosus* (トウキョウダルマガエル) and *Buergeria burgeri* (カジカガエル). Most abundant 21 genus have been identified, and they were all belonged to the four abundant phyla and seven abundant class. Gamma-proteobacteria found the highest proportion class.
2. Three bacterial isolates were selected, *Paenibacillus* sp. HjD57, *Raoultella* sp. HjD92 and *Citrobacter* sp. B341 had high potentials in reduction of growth of plant pathogenic fungus, *Colletotrichum orbiculare*, a causal agent of cucumber anthracnose disease in vitro.
3. *Paenibacillus* sp. HjD57, *Raoultella* sp. HjD92 and *Citrobacter* sp. B341 were potential candidates as biocontrol agents against *Colletotrichum orbiculare*, *Fusarium oxysporum* f. sp. *lycopersici*, and *F. fujikuroi*, causal agent of cucumber anthracnose, tomato wilt and rice "bakanae" respectively in pot experiment.
4. The putative reduction mechanisms the progress of plant diseases possibly due to the production of antifungal metabolite. To clarify the metabolite production, the further studies are needed.

5.2. Prospect for future research

Biological control method for controlling plant diseases become even more attractive because it provides a significant improvement for crop protection from pathogen, and it is environmentally safe. At current study, three isolates have been selected based on their high antifungal activities in vitro against anthracnose disease on cucumber plant. Moreover, those bacteria also showed the

efficacy in reduction the development progress of plant diseases include cucumber anthracnose, tomato wilt and rice “bakanae” diseases in pot experiment. The putative mechanisms of action associated with the biocontrol capacity was antibiosis by producing antifungal metabolite, and no loss of activity was shown after heat treatment. Further study is needed to determine the antifungal metabolite production.

In this study, the biocontrol strategy applied was a curative approach for controlling cucumber anthracnose and rice “bakanae”, whereas the preventive approach was applied only for tomato wilt. *Paenibacillus* sp. HjD57, *Raoultella* sp. HjD92 and *Citrobacter* sp. B341 showed the efficacy in the control of cucumber anthracnose, tomato wilt and rice “bakanae” diseases. Although curative activity was observed when using those three selected bacteria, studies about their preventive action are needed to establish the antagonistic potential of them.

The effectiveness of *Paenibacillus* sp. HjD57, *Raoultella* sp. HjD92 and *Citrobacter* sp. B341 in controlling plant diseases presented under controlled environment (greenhouse), however, it remains unknown whether their effectiveness still high in field area. Thus, field experiment is required for testing the consistency of antifungal activity.

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