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Construction of a Recombinant *Bacillus velezensis* Strain as an Integrated Control Agent Against Plant Diseases and Insect Pests

Roh, Jong Yul[1], Qin Liu[1], Jae Young Choi[2], Yong Wang[1], Hee Jin Shim[1], Hong Guang Xu[1], Gyung Ja Choi[3], Jin-Cheol Kim[3], and Yeon Ho Je[1]*

[1]*Department of Agricultural Biotechnology, College of Agriculture and Life Sciences, Seoul National University, Seoul 151-742, Korea*
[2]*Research Institute for Agriculture and Life Sciences, College of Agriculture and Life Sciences, Seoul National University, Seoul 151-742, Korea*
[3]*Chemical Biotechnology Research Center, Korea Research Institute of Chemical Technology, Daejeon 305-343, Korea*

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To construct a new recombinant strain of *Bacillus velezensis* that has antifungal and insecticidal activity *via* the expression of the insecticidal *Bacillus thuringiensis* crystal protein, a *B. thuringiensis* expression vector (pHT1K-1Ac) was generated that contained the *B. thuringiensis cry1Ac* gene under the control of its endogenous promoter in a minimal *E. coli–B. thuringiensis* shuttle vector (pHT1K). This vector was introduced into a *B. velezensis* isolate that showed high antifungal activities against several plant diseases, including rice blast (*Magnaporthe grisea*), rice sheath blight (*Rhizotonia solani*), tomato gray mold (*Botrytis cinerea*), tomato late blight (*Phytophthora infestans*), and wheat leaf rust (*Puccinia recondita*), by electroporation. The recombinant *B. velezensis* strain was confirmed by PCR using *cry1Ac*-specific primers. Additionally, the recombinant strain produced a protein approximately 130 kDa in size and parasporal inclusion bodies similar to *B. thuringiensis*. The *in vivo* antifungal activity assay demonstrated that the activity of the recombinant *B. velezensis* strain was maintained at the same level as that of wild-type *B. velezensis*. Furthermore, it exhibited high insecticidal activity against a lepidopteran pest, *Plutella xylostella*, although its activity was lower than that of a recombinant *B. thuringiensis* strain, whereas wild-type *B. velezensis* strain did not show any insecticidal activity. These results suggest that this recombinant *B. velezensis* strain can be used to control harmful insect pests and fungal diseases simultaneously in one crop.

Keywords: *Bacillus velezensis*, antifungal activity, *Bacillus thuringiensis*, *cry1Ac*, insecticidal activity

**Corresponding author*
Phone: +82-2-880-4706; Fax: +82-2-873-2319;
E-mail: btrus@snu.ac.kr

Bacillus species have been considered for use in industrial biotechnology, such as the production of antibiotics, enzymes, and microbial insecticides [25]. Some *Bacillus* species, like *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens*, have been extensively studied for use as biological control agents against crop fungal diseases [10, 15, 21, 27, 28, 31, 33].

In 2005, Ruiz-García *et al.* [23] identified two novel surfactant-producing bacteria, *Bacillus* species CR-502^T and CR-14b, and designated them *Bacillus velezensis* sp. nov., since they were isolated from the Vélez River in southern Spain. Based on differences in their phenotypic and chemotaxonomic characteristics and phylogenic distinctiveness, the *B. velezensis* strains were classified as novel *Bacillus* species. However, *B. velezensis* species is still very closely related to *Bacillus subtilis*, *Bacillus mojavensis*, and *Bacillus amyloliquefaciens*. In fact, one recent report has suggested that *B. velezensis* is a heterotypic synonym of *Bacillus amyloliquefaciens*, based on the lack of phenotypic distinctive characteristics, 16S rDNA and *gyrB* gene sequence similarity, and DNA–DNA hybridization between *B. velezensis* and *B. amyloliquefaciens* strains [32]. Bafana *et al.* [2] recently reported a *B. velezensis* strain that produced an azoreductase that was capable of decolorizing the Direct Red 28 (DR28) azo dye, a known toxic and carcinogenic compound.

B. thuringiensis is one of the *Bacillus* species frequently used industrially. *B. thuringiensis* strains are widely used for the control of agricultural and forest insect pests and their insecticidal protein genes (the *cry* family) have been used to generate insect-resistant transgenic crops (Bt crops) [22, 26]. In order to harness the insecticidal activity of *B. thuringiensis*, the *cry* genes have been transformed into other bacteria such as *Pseudomonas* [8], cyanobacteria

[19], *Rhizobium* [7], and *Bacillus* species that naturally colonize the plant phylloplane [29]. This approach may improve the persistence and maintenance of the insecticidal activity of the Cry proteins in crop fields. The native *B. subtilis* and *B. licheniformis* strains transformed with *cry1Ab* successfully expressed toxins and survived for at least 45 days on the surface of a tomato leaf [29]. Furthermore, in order to increase the efficacy and broaden the host spectrum of *B. thuringiensis*, other *cry* genes that have a greater toxicity against the same target, or are toxic to other insect orders, have been directly transformed into a *B. thuringiensis* strain [30, 35].

Here, we demonstrate that a new *Bacillus velezensis* isolate (Bv S3-5) has antifungal activities (more than 70% control efficacy) against several plant diseases, including rice blast (*Magnaporthe grisea*), rice sheath blight (*Rhizotonia solani*), tomato gray mold (*Botrytis cinerea*), tomato late blight (*Phytophthora infestans*), and wheat leaf rust (*Puccinia recondita*). In this study, we introduced the *B. thuringiensis cry1Ac* gene into a *B. velezensis* isolate in order to generate a recombinant *B. velezensis* strain capable of controlling plant fungal diseases and insect pests simultaneously.

Materials and Methods

Bacterial Strains and Culture

The *Bacillus velezensis* S3-5 isolate, which has high antifungal activities (more than 70% control efficacy) against several fungal phytopathogens, was kindly provided by Dr. Jin-Cheol Kim at the Korea Research Institute of Chemical Technology. *B. velezensis* S3-5 was cultured for 3 days at 37°C in tryptic soy broth (TSB) and washed with 0.025% Tween 20. The recombinant *B. velezensis* S3-5 was cultured in TSB medium supplemented with erythromycin (25 μg/ml). An acrystalliferous *B. thuringiensis* Cry$^-$B strain was used as a control strain for the expression of Cry1Ac protein following electroporation with pHT1K-1Ac. The *B. thuringiensis* Cry$^-$B transformant was cultured on nutrient agar plates supplemented with erythromycin for 5 days at 28°C and used as a positive control for the insect bioassay. According to our preliminary experiments, the *B. velezensis* S3-5 strain had no resistance to 25 μg/ml erythromycin and did not produce inclusion bodies in vegetative and sporulated cells.

Expression Vector and Electroporation

The expression vector (pHT1K-1Ac) used in this study was generated for the expression of the Cry1Ac protein in *Bacillus* spp. [12]. This vector was constructed with the insecticidal *cry1Ac* gene of *B. thuringiensis* under the control of its endogenous promoter in a minimal *E. coli–B. thuringiensis* shuttle vector. pHT1K-1Ac was introduced into the *B. velezensis* isolate by electroporation using a slightly modified protocol commonly used for the transformation of *B. subtilis* and *B. thuringiensis* [16, 20]. In order to generate electro-competent cells of the *B. velezensis* isolate, a 10-fold dilution of an overnight *B. velezensis* culture was incubated in 100 ml of PY salt medium (peptone 0.9%, yeast extract 0.5%, NaCl 0.5%, pH 7.0) for 4 h. Cells were harvested, washed with 30 ml of washing buffer (0.5 M sucrose, 0.01 M $MgCl_2 \cdot 6H_2O$, 0.01 M maleic acid, pH 6.5), recentrifuged, and resuspended in 1 ml of washing buffer. Competent cells (200 μl) were mixed with 1 μg of vector DNA and single-shocked in an 0.2-cm electroporation cuvette (Bio-Rad, U.S.A.) using a Bio-Rad Gene Pulser and the following conditions: volts, 2 kV; resistance, 100 Ω; capacitance, 25 μF. The electroporated cells were recovered in 1 ml of PY medium for 3 h at 37°C and plated on erythromycin-supplemented (25 μg/ml) PY agar plates. Transformed *Bacillus* colonies were examined 48 h post-transformation.

PCR and SDS–PAGE

In order to confirm the introduction of the expression vector into *B. velezensis*, PCR was performed using a *cry1Ac*-specific primer set: cry1Ac forward primer (5'-TCACTTCCCATCGACATCTACG-3') and *cry1*-type common reverse primer, cry1-3 (5'-ATCACTGAGTC GCTTCGCATGTTGACTTTCTG-3'), with the following cycling parameters: 95°C for 5 min; 30 cycles of 95°C for 30 s; 50°C for 30 s; 72°C for 1 min; and 72°C for 5 min [11]. For the PCR template, 1 ml of *B. velezensis* culture was washed with ddH_2O two times, boiled at 100°C for 10 min, and the final supernatant was used. The amplified fragment was verified by DNA sequencing analysis.

The expression of the introduced protein in the transformants was confirmed by SDS–PAGE of 3-day cultures that washed three times with 0.025% Tween 20.

Electron Microscopy

Samples of 2 ~ 3-day cultured cells were harvested and washed with 0.025% Tween 20 three times. The harvested cells were initially fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) for 2 h at 4°C, followed by three washes with sodium cacodylate buffer. Post-fixation was carried out in 1% osmium tetroxide for 2 h at 4°C followed by two brief washes with ddH_2O. The samples were stained with 0.5% uranyl acetate overnight and dehydrated in increasing concentrations of ethanol. After dehydration, specimens for scanning electron microscopy were dried with 100% hexamethyldisilazane at room temperature. The samples were mounted on metal stubs and coated with gold. The infiltration of Spurr's resin was performed in propylene oxide for the transmission electron microscopy specimens and the specimens were embedded in Spurr's resin at 70°C for 24 h. Sections were cut with an ultramicrotome (MT-X, RMC, Tucson, AZ, U.S.A.) and were stained with 2% uranyl acetate and Reynolds' lead citrate. *B. velezensis* cells were observed with scanning (magnification ×20,000; JSM 5410LV, Jeol, Japan) and transmission (magnification ×60,000; Libra 120, Carl Zeiss, Germany) electron microscopes.

In Vivo Antifungal Activity

B. velezensis S3-5 and the derived recombinant strain were assayed against seven fungal phytopathogens: rice blast (RCB, *M. grisea*), rice sheath blight (RSB, *R. solani*), tomato gray mold (TGM, *B. cinerea*), tomato late blight (TLB, *P. infestans*), wheat leaf rust (WLR, *P. recondita*), barley powdery mildew (BPM, *Erysiphe graminis*), and red pepper anthracnose (PAN, *Collectotrichum gloeosporioides*). Tomato (*Lycopersicon seculentum* Mil., cv. Seokwang), barley (*Hordeum sativum* Jessen, cv. Dongburi), and wheat (*Triticum aestivum* L., cv. Chokwang) plants were grown in a greenhouse at 25±5°C for 1–3 weeks. The *B. velezensis* dilutions were sprayed on the plants, and fungal spores were inoculated after 24 h. Control plants were sprayed with 0.025% Tween 20. At 3–7 days post-inoculation, disease symptoms were rated. All treatments were performed in duplicate in a growth

chamber. The percentage of disease control was determined using the following equation: control activity (%)=100[(A-B)/A], where A and B indicate the area of infection (%) on leaves treated with Tween 20 alone (A) and the *B. velezensis* S3-5-treated leaves (B) [5].

Insecticidal Activity Against *Plutella xylostella*

The toxicity of the recombinant strain was determined against the diamondback moth (*Plutella xylostella*). *B. velezensis* was cultured for 3 days and serial dilutions from 1×10^7 CFU/cm^2 were used to treat a disc of Chinese cabbage leaf (2×2 cm^2). Ten third-instar larvae were introduced to each leaf surface and mortality was calculated by counting dead larvae at 24-h intervals for 2 days. The median lethal concentration (LC_{50}) was calculated by the Probit method using SoftTOX version 1.1 (Soft LabWare, U.S.A.). All assays were performed in triplicates at 25°C in 60–70% humidity with a 16 h/8 h light/dark cycle. As a negative control, 0.025% Tween 20 alone, which was used for dilution of *B. velezensis* cells, was used to treat the Chinese cabbage leaf.

RESULTS

Transformation of *cry1Ac* into *B. velezensis*

In a preliminary activity assay against crop fungal disease, the *B. velezensis* S3-5 strain was found to be an efficient agent. Before transformation with the *cry1Ac* expression vector, the susceptibility of the *B. velezensis* S3-5 strain to an antibiotic for Gram-positive bacterium (25 mg/ml erythromycin in TSB media) was examined. This strain did not show any resistance to erythromycin. Additionally, no inclusion or particle-like structure was detected in any cells of this strain under phase contrast microscopy (supplementary material).

The expression vector (pHT1K-1Ac) was transformed into competent *B. velezensis* S3-5 vegetative cells, and recombinant *B. velezensis* colonies were grown on PY agar medium supplemented with 25 μg/ml erythromycin. The *B. velezensis* transformant was confirmed by PCR and the PCR product was verified by DNA sequence analysis (Fig. 1A).

Stable Expression of Cry1Ac in *B. velezensis*

Expression of the introduced Cry1Ac protein in *B. velezensis* was detected by 12% SDS–PAGE and a protein of 130 kDa was detected (Fig. 1B). The Cry1Ac expression in *B. velezensis* appears to be stable, similar to the Cry protein expression in *B. thuringiensis*. The production of parasporal inclusion bodies in *B. velezensis*, and autolysed and sporulated cells were observed by electron microscopy. In the scanning electron micrographs, small and amorphous inclusion-like structures were observed (Fig. 2A). In order to further confirm this production, sections of sporulated cells were also examined by transmission electron microscopy (Fig. 2B). The presence of parasporal inclusion bodies was confirmed in the cells, although the inclusion bodies seem to be smaller than the typical *B. thuringiensis* Cry1Ac protein (~1 μm).

Antifungal Activity of the Recombinant *B. velezensis* Strain

The antifungal activities of the *B. velezensis* S3-5 transformant were assayed against crop pathogens in order to confirm the original activity of the *B. velezensis* S3-5 strain. Originally, the *B. velezensis* S3-5 strain exhibited high activity against *M. grisea* (RCB), *R. solani* (RSB), *B. cinerea*

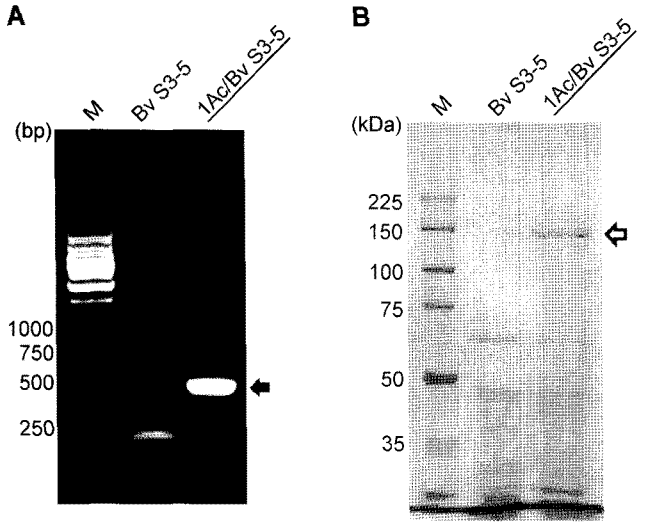

Fig. 1. PCR (**A**) and SDS–PAGE (**B**) analysis of *B. velezensis* S3-5 strain transformed with the expression vector for Cry1Ac, pHT1K-1Ac.

Filled and opened arrows indicate *cry1Ac* gene-specific PCR product and expressed Cry1Ac protein, respectively. The predicted size of the PCR product is 507 bp.

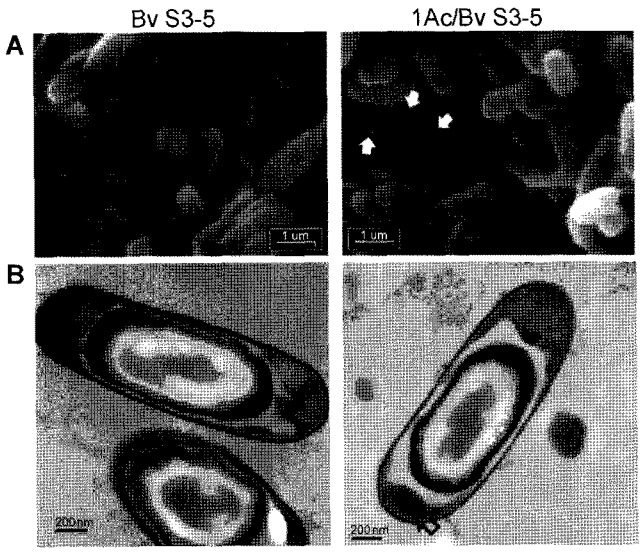

Fig. 2. Scanning (**A**) and transmission (**B**) electron microscopies of *B. velezensis* S3-5 transformant.

Left column represents *B. velezensis* S3-5 and right represents its transformant. Size bars indicate 1 mm (**A**) and 200 nm (**B**). White and opened arrows indicate Cry1Ac inclusions.

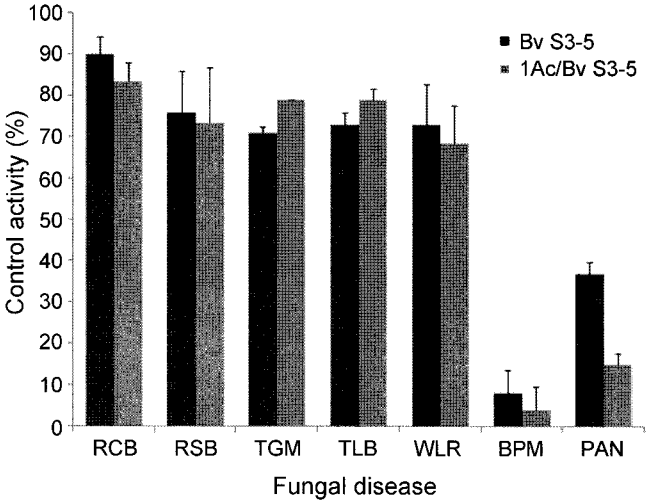

Fig. 3. *In vivo* antifungal activity of *B. velezensis* S3-5 transformant against seven fungal phytopathogens.

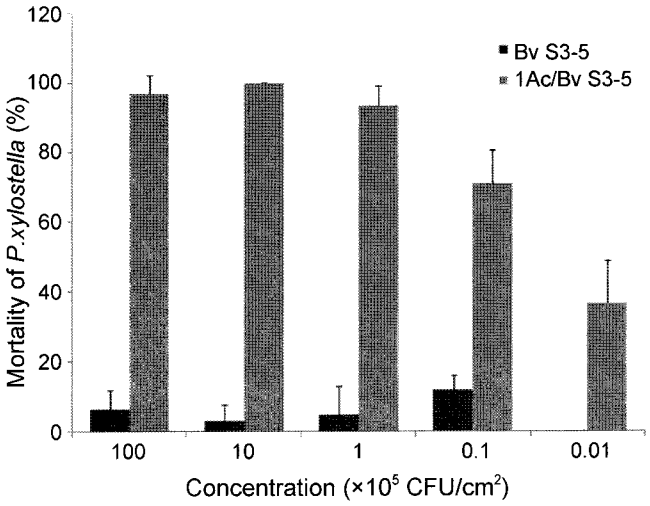

Fig. 4. Insecticidal activity of *B. velezensis* S3-5 transformant against third-instar larvae of *P. xylostella*. Mortalities were checked at 48 h after treatment.

(TGM), *P. infestans* (TLB), and *P. recondite* (WLR). When compared with the wild-type *B. velezensis* S3-5 strain, the recombinant *B. velezensis* strain showed similar antifungal activity (Fig. 3). Although the recombinant strain showed decreased activity against some pathogens (*e.g.*, *M. grisea* and *C. gloeosporioides*), and increased activity against other pathogens (*e.g.*, *B. cinerea* and *P. infestans*), these results demonstrate that the original antifungal activities of the *B. velezensis* S3-5 strain were not affected by the introduction and expression of *B. thuringiensis* Cry1Ac.

Insecticidal Activity of the Recombinant *B. velezensis* Strain

In order to confirm the insecticidal activity of the recombinant strain, a bioassay against *P. xylostella* larvae was carried out (Fig. 4). Twenty-four hours after treatment, the recombinant strain showed insecticidal activity (58.8% mortality at the 1×10^7 CFU/cm^2 concentration), which increased 48 h after treatment (97.1% at 1×10^4 CFU/cm^2 concentration). As expected, the untransformed *B. velezensis* S3-5 strain did not show any activity against *P. xylostella* larvae. The median lethal concentration (LC_{50}) of the recombinant strain (7.86×10^3 CFU/cm^2) was approximately 7-fold greater than that of another recombinant *B. thuringiensis* Cry$^-$B strain that was transformed with the same expression vector (1.07×10^3 CFU/cm^2) (Table 1). These data demonstrate that the recombinant *B. velezensis* strain expressed the insecticidal Cry1Ac protein stably and functionally.

Discussion

Recently, many *Bacillus* species have been shown to have antifungal activity against phytopathogenic fungi. For example, a *B. amyloliquefaciens* strain was shown to produce seven antifungal compounds (iturins) that were active against *Colletotrichum dematium*, which causes mulberry anthracnose [9]. Additionally, a *B. licheniformis* strain was shown to produce an antagonistic lipopeptide (surfactin) that exhibited fungicidal activity against *M. grisea*, which causes rice blast [28]. A *B. subtilis* strain has also been shown to have inhibitory activity against the *Fusarium* head blight disease caused by *Fusarium graminearum* [31]. Moreover, an antifungal chitinase gene cloned from *B. subtilis* has been shown to exhibit inhibitory activity against *R. solani*, a phytopathogen that causes damping-off in radish seedlings [33]. However, there has been no report detailing the antifungal activity of a *B. velezensis* strain against phytopathogens, although at least one *B. velezensis* strain has been shown to produce surfactant molecules [23].

Table 1. Toxicity of *B. velezensis* S3-5 transformant against third-instar *P. xylostella* larvae.

Strain	LC_{50} ($\times10^3$ CFU/cm^2)	FL_{95}[a]
B. velezensis S3-5	-[b]	-
B. velezensis S3-5 transformant	7.86	(5.70–12.24)
B. thuringiensis Cry$^-$B transformant	1.07	(0.85–1.33)

[a]95% fiducial limits.
[b]6.1% mortality at 1×10^7 CFU/cm^2.

Interestingly, several reports describing the antifungal activities of *B. thuringiensis* strains against plant diseases have also been reported [4–6, 14, 34]. A *B. thuringiensis* strain (CMB26) has been shown to produce a fengycin-like lipopeptide that suppressed a phytopathogenic fungus, *Collectotrichum gloeosporioides* [14]. Choi *et al.* [5] have reported that four *B. thuringiensis* strains isolated from Japanese fecal samples exhibited strong activity against barley powdery mildew. Additionally, some reports have focused on antimicrobial compounds such as entomocin [4] and zwittermicin [34]. However, these reports only proposed the antifungal activity of *B. thuringiensis* and did not discuss the dual activities of the strains in detail. Therefore, as target phytopathogens and target insect pests can be co-harmful to one crop, we believe that one highly active *B. thuringiensis* strain may meet the requirements for use as an integrated control agent. Our rational transformation approach may be useful for real field applications because we can select a more applicable insecticidal protein based on the target of the antifungal activity.

To our knowledge, this is the first report demonstrating that the Cry1Ac protein can be expressed in a *B. velezensis* strain while maintaining the intrinsic antifungal activity of the strain. There is no information about transformation to a *B. velezensis* strain. In our preliminary experiment, the transformation into *B. velezensis* S3-5 has not been accomplished with the method for *B. subtilis* [20] or *B. thuringiensis* [16]. Thus, the culture medium and electroporation condition based on a procedure for *B. subtilis* were modified and a recombinant *B. velezensis* strain harboring the expression vector (pHT1K-1Ac) was acquired. The stability of our expression vector (pHT1K-1Ac) in *B. velezensis* strain is an issue for practical use. The pHT1K is a minimal-sized vector derived from pHT3101 (based on *B. thuringiensis* resident plasmid pHT1030), and the stable expression of foreign gene (*cry1A*a) using pHT3101 was confirmed [16]. In addition, there is a previous report about the segregation stability of the plasmids derived from pHT1030. According to that report, after 30 generations without selective pressure, the plasmids from pHT1030 were stable (<10% segregation frequency) in *B. subtilis* strain at 30°C [17]. This reported result suggests that the expression vector (pHT1K-1Ac) will be stable in *B. velezensis* strain; however, the stable range of generation and temperature for pHT1K-1K without erythromycin will be need to be verified for further use.

It should be noted that the expression level of Cry1Ac in *B. velezensis* strain was low compared with the expression in *B. thuringiensis*. There are several examples of the expression in *B. thuringiensis cry* gene in cotton or tomato leaf-colonizing *Bacillus* species, including *B. megaterium* [3], *B. cereus* [18], *B. subtilis*, and *B. licheniformis* [29]. Similar to the results seen in this study, the expression of Cry proteins in these *Bacillus* species was frequently less than the expression level in *B. thuringiensis*. Importantly, the recombinant *B. velezensis* strain retained its antifungal activity after electroporation. This result suggests that the gene(s) controlling the antifungal activity may be located on the *B. velezensis* chromosome. Of note, an antifungal factor, a *B. subtilis* chitinase, has been cloned from chromosomal DNA [33].

In this study, the *B. velezensis* strain also exhibited activity against rice blast, rice sheath blight, tomato gray mold, tomato late blight, and wheat leaf rust, suggesting that the target crops for this bacterium are rice, tomato, and wheat. The *B. thuringiensis* insecticidal protein Cry1Ac is a well-known Cry protein that exhibits high insecticidal activity against lepidopteran larvae [26]. This protein had only a 2 ng/ml range of LC_{50} values against diamondback moth (*Plutella xylostella*) [24], and the toxicity spectrum of Cry1Ac against lepidopteran pests is known to be very broad. According to the target crop spectrum of the *B. velezensis* strain, for example, the LC_{50} value was 19.4 ng/mg in a diet incorporation bioassay against one notorious rice pest, the striped stem borer (*Chilo suppressalis*) [1], and the LC_{50} against the rice leaf folder (*Cnaphalocrocis medinalis*) was 21 ng/cm^2 [13]. This specificity of the Cry1Ac protein suggests that it may be more applicable in rice fields.

In conclusion, these results demonstrate that a recombinant *B. velezensis* strain may be used to control harmful insect pests and plant fungal diseases simultaneously in one crop. Furthermore, both culture broth and harvested cells of this strain can be used as individual biological control agents separately for integrated crop protection. Field trials using this recombinant *B. velezensis* strain as a dual control agent are now being performed by this laboratory.

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