Production of Surfactin and Iturin by *Bacillus licheniformis* N1 Responsible for Plant Disease Control Activity

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*B. licheniformis* N1, previously developed as a biofungicide formulation N1E to control gray mold disease of plants, was investigated to study the bacterial traits that may be involved in its biological control activity. Two N1E based formulations, bacterial cell based formulation PN1E and culture supernatant based formulation SN1E, were evaluated for disease control activity against gray mold disease of tomato and strawberry plants. Neither PN1E nor SN1E was as effective as the original formulation N1E. Fractionation of antifungal compounds from the bacterial culture supernatant of *B. licheniformis* N1 indicated that two different cyclic lipopeptides were responsible for the antimicrobial activity of the N1 strain. These two purified compounds were identified as iturin A and surfactin by HPLC and LC-MS. The purified lipopeptides were evaluated for plant disease control activity against seven plant diseases. Neither PN1E nor SN1E was as effective as the original formulation N1E. The selection of effective biocontrol agents and development of formulations is one of the essential steps to develop effective biofungicides. Several mechanisms of biological control including antibiotics production, successful colonization and resistance induction on host plants have been reported (Emmert and Handelsman, 1999; Handelsman and Stabb, 1996). Especially, *Bacillus* species has been widely used to control multiple plant diseases with advantage of production of several antifungal compounds and long shelf-life as a result of endospore formation by the *Bacillus* species (Emmert and Handelsman, 1999).

Many strains of *Bacillus* species have been successfully developed as effective commercial biofungicides (Schisler et al., 2004). *Bacillus subtilis* GB03 has been used for seed treatment as a liquid formulation of endospores under the trade name of Kodiak® (Gustafson, USA) to control soil-borne disease (Brannen and Kenney, 1997; Mahaffee and Backman, 1993). A wettable powder type formulation, Serenade®, was produced using *B. subtilis* QST-713 to control foliar diseases of plants (AgraQuest, Davis, USA). Other strains of *Bacillus* species have been also used to control plant diseases and are in the process of commercial development (Fravel et al., 1988; Gueldner et al., 1988; Touré et al., 2004).

*Bacillus* strains often produce a vast array of biologically active compounds, including antibiotics which are inhibitory to plant pathogens (Emmert and Handelsman, 1999; Stein, 2005). Among the antimicrobial compounds, some cyclic lipopeptides have been described frequently from several biocontrol *Bacillus* species and have been shown to exhibit inhibitory activity against a number of plant pathogens. The three families of cyclic lipopeptides are iturin, surfactin, and fengycin (Ongena and Jacques, 2007), which are produced by *B. subtilis*, *B. amyloliquefaciens*, *B. coagulans*, *B. pumilus*, *B. licheniformis*, *B. cereus*, and *B. thuringiensis* (Bonnemain et al., 2003; Huszca and Burczyk, 2006; Jacques et al., 1999; Kim et al., 2004; Kounoutsi et al., 2004; Peypoux et al., 1999; Tsuge et al., 1999). These lipopeptides from *Bacillus* strains are structurally related compounds with different biological activities. The biocontrol strain *B.
subtilis QST-713 produces more than 30 different lipopeptide variants. Recent studies have revealed that the lipopeptides also play a role to enhance bacterial colonization on plant tissues and to induce plant resistance against pathogens (Ongena and Jacques, 2007).

Previously, we have shown that a specific formulation N1E developed by using bacterial fermentation culture of B. licheniformis N1 was effective to control gray mold diseases in tomato (Lee et al., 2006) and strawberry plants (Kim et al., 2007). Although a chitinase gene was found in the genome of B. licheniformis N1 strain, the gene was not functional due to the lack of expression (Lee et al., 2009). Our previous study using the cell-free formulation suggested that the production of antifungal compounds by the N1 strain may be involved in disease control activity on tomato plants (Lee et al., 2006). In this study, we have used biocontrol strain B. licheniformis N1 and its biofungicide formulation N1E to further investigate the biocontrol mechanism of N1 strain.

**Materials and Methods**

**Microorganisms and culture conditions.** B. licheniformis N1 was routinely grown in tryptic soy broth (TSB) or agar (TSA) medium at 30°C and cultured in the medium containing 5% dried soybean curd residue in distilled water (Biji meidum) for mass production. Soybean curd residue (‘Biji’ in Korean) was obtained from a Korean traditional tofu factory. Dried Biji was produced to make Biji medium as previously described (Lee et al., 2006). A fungal pathogen Botrytis cinerea LVF12, which causes gray mold on various plants, was routinely grown on potato dextrose agar (PDA) for 15 days at 25°C under a 12-hr light period condition to trigger conidia formation. The conidia were suspended in a 30% (v/v) tomato juice solution supplemented with 0.1 M KH₂PO₄ to a concentration of 1.6 × 10⁶ conidia/ml. Commercial tomato juice was purchased from the Gaya tomato juice company in Korea.

**Preparation of N1E formulations using B. licheniformis.** To generate formulations of B. licheniformis N1 using Biji broth culture (Lee et al., 2006), the 400 ml pre-cultured bacterial cells were added directly to 4 liters of Biji broth in a 7-liter jar fermenter with 10 ml of 10-fold diluted antibacterial cells were added directly to 4 liters of Biji broth in broth culture (Lee et al., 2006), the 400 ml pre-cultured bacterial culture. The other part was used to separate bacterial cells from the culture broth. Bacterial cells were harvested by centrifugation at 4°C for 10 min at 7,000 rpm. Bacterial pellet and culture supernatant were separated to produce different formulations. Before mixing with formulation materials, bacterial pellet was washed once with phosphate-buffered saline (PBS) and resuspended in PBS to the equivalent volume of the original culture. Both resuspended bacterial solutions and culture supernatant were separately used to generate different formulations by mixing with materials for N1E formulation. The mixtures were used to generate, PN1E and SN1E formulations, as previously described (Lee et al., 2006). Formulations, PN1E and SN1E, represent the formulations generated by using bacterial pellet suspension and culture supernatant, respectively. The formulations were stored at 4°C until use. A control formulation lacking bacterial cells was prepared with Biji broth mixed with corn starch for wettable powder (WP) formulation. Only fungal pathogen inoculation and the control formulation lacking bacterial cells were used as controls for pot experiments in a growth chamber.

**Plant growth and treatment.** The tomato plants and strawberry plants used in this study were the cultivar Kwang-Myeong (Dongbu HiTek Co., Korea) and the cultivar Reiko, respectively. The plants were grown in 15-cm-diameter pots containing the commercial horticulture nursery media soil (Punong Co., Ltd, Korea) in a plastic house until the four to six-leaf stages. The N1E-based formulations were diluted 100-fold with tap water before being sprayed on the plants. The fungicides used for standard chemicals treatment on tomato and strawberry plants were mixture of diethelorex (25%) and carbendazim (25%) (Dong-Bang Agro Co., Korea) and was iprodione (Rovral®) (Bayer CropScience Co., Korea), respectively. Both fungicides were used after 1,000-fold dilution with tap water.

**Pathogen inoculation and assessment of disease control.** Both tomato and strawberry plants were inoculated by spraying the conidial suspension of Botrytis cinerea LVF12 prepared in the tomato juice with 0.1 M of KH₂PO₄ onto tomato and strawberry leaves until runoff one day after chemical fungicide or microbial fungicide treatment. The plants were maintained in a controlled growth room for one day (20 ± 2°C, 90% RH) and then transferred into a plastic house (25 ± 5°C). Each treatment included 10 pots per treatment with five replications. The disease severity on plant leaves was rated on various days after pathogen inoculation. The disease severity index of gray mold on the tomato and strawberry plants was defined as the percentage of diseased leaf area, where 0 = no disease symptoms, 1 = 0.1-5%, 2 = 5.1-20%, 3 = 20.1-40%, and 4 = 40.1-100%.