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Biological control for rice blast disease by employing detachment action with gelatinolytic bacteria

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ABSTRACT

We screened collagenolytic/gelatinolytic bacteria from rice leaves and soil which degraded the fungal extracellular matrix to establish a novel biological control measure inhibiting germling adhesion on the host plant surface against airborne fungal diseases such as rice blast disease *Magnaporthe oryzae* B. Coch. Two different screening methods, i.e., screening from the leaf-associated bacterial library and direct screening from leaf and soil with or without collagen incubation, were conducted. Screening from the collagen treated material resulted in a higher number of gelatinolytic isolates than without collagen treatment. The selected bacteria were identified as *Acidovorax*, *Sphingomonas*, *Chryseobacterium*, and *Pseudomonas* genera by 16S rDNA sequence. Based on treatment with EDTA and addition of divalent cations, four of the five screened isolates tested produced a metalloproteinase. Furthermore, the enzymes produced by *Acidovorax* and *Sphingomonas* sp. were categorized as calcium-dependent metalloproteinases, and the enzymes produced by *Chryseobacterium* sp. were categorized as calcium/zinc-dependent metalloproteinases. The screened bacterial culture showed inhibitory effects on spore adhesion on the plastic cover glass, and disease protective effects on rice. This study suggests that bacteria inhibiting germling adhesion by phytopathogenic fungi may have promise as a biological agent.

1. Introduction

Biological control for plant disease has been investigated for more than 200 years (Le Berryais, 1785). Most approaches to biological control have been focused on primarily soil-borne diseases (Sanford and Broadfoot, 1931; Kloepper and Schroth, 1979). In contrast, little information has been reported about biological control for foliar airborne diseases because many effective chemical pesticides have been developed. Recently, plant pathologists and farmers have paid more attention to biological control as an alternative measure to using chemical pesticides, in response to consumer demand for safe farm products and concern about environmental pollution. Several successful reports about biological control for airborne diseases have been published, e.g., chitinolytic activity of *Serratia marcescens* for rice blast disease (Someya et al., 2002) and antimicrobial compounds produced by *Bacillus licheniformis* for tomato gray mold disease (Lee et al., 2006). Most biological control agents protect against disease utilizing antagonistic or killing effects against pathogens.

It is generally thought that biological control has some disadvantages, i.e., instability of the protection effect, difficulty in establishment of the biocontrol agents in the fields, and ambiguity of modes of protection (Enkerli et al., 2004; Barratt et al., 2010). This is why various kinds of biological control measures with diverse modes of protection are needed. We have been investigating a novel biological control measure, which has a different protective mechanism against airborne diseases. One of the promising targets for biological control is inhibition of the attachment of the pathogen to the host plant surface.

Adhesion of fungal spores to the host surface is considered as a critical first step to achieve infection (Deising et al., 1992; Braun and Howard, 1994). The spores of fungi secrete an extracellular matrix (ECM) as a factor in adhesion. Fungal ECM is involved not only in adhesion but also in signal exchange between plants and fungi (Nielsen et al., 2000; Vidhyasekaran, 2008). The attached spores develop a germination tube, an appressorium, and a penetration peg, and complete infection by signal exchange with the host plant via ECM. The ECM also offers a solid base for spores to penetrate the host cuticle. Consequently, inadequate adhesion of spores results in the failure of appressorium formation (Bae et al., 2007).

Many scientists have studied the composition of fungal ECM. The ECM of *Colletotrichum lindemuthianum* Briosi and Cavara is

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composed of a 110-kDa glycoprotein (Hughes et al., 1999). Fungal fimbriae of *Microbotryum violaceum* are composed of a type of collagen (Celerin et al., 1996).

The rice blast fungus *Magnaporthe oryzae* B. Couch (Couch and Kohn, 2002), used in this study, is one of the most devastating airborne diseases. The spores of this fungus attach to the host surface by spore tip mucilage secreted from the spore tip; the attached spores germinate producing ECM; and subsequently the germlings firmly attach to the plant surface (Hamer et al., 1988). The ECM of *M. oryzae* is composed of animal cell adhesion factor-like compounds such as collagen, laminin, and fibronectin (Inoue et al., 2007; Bae et al., 2007). Inoue et al. (2007) also demonstrated that collagenase and gelatinase, glycoprotein-degrading enzymes, had an inhibitory effect on spore adhesion of *M. oryzae* to the cellulose membrane, and suppressed disease occurrence. The inhibitory effect of collagenase on germling adhesion was also observed in *Alternaria alternata* Keissler Japanese pear pathotype (Hyon et al., 2009). These studies suggest that application of collagenase/gelatinase may have a wide spectrum of application in disease protection against spore-dispersing pathogenic fungi. Therefore, we attempted to screen collagenolytic/gelatinolytic bacteria from rice leaves and soil, and characterize their enzymatic activity to establish a new biological control measure using the detachment of pathogen from the host plant surface.

2. Materials and methods

2.1. Screening from the library of rice leaf-associated bacteria

A library of rice leaf-associated bacteria, consisting of rice epiphytic and endophytic bacteria, is maintained at the National Institute for Agro-Environmental Sciences (Tsukuba, Japan). The bacteria were stored in skim milk medium at −80 °C. For this study, 2688 bacterial isolates were randomly selected, and each propagated in 100 μl NB liquid medium (Nutrient Broth, Becton Dickinson and Company, NJ, USA) on a 96-well microtiter plate for 3 days. Then, 30 μl of the culture was dropped on the gelatin medium (0.1% gelatin, 1% agar) and incubated overnight. The gelatin medium was stained with Coomassie Brilliant Blue (CBB) R-250 (Nacalai Tesque, Kyoto, Japan) for 30 min. After decolorization, the region where gelatin was degraded by bacterial colony showed as a clear plaque. The selected gelatin-degrading bacteria were further tested for gelatin-degrading ability on a 9-cm Petri dish containing the 0.1% gelatin medium.

2.2. Direct screening from leaves incubating with or without collagen

We examined the effect of pre-incubation with collagen on the microbial community. One gram of rice (*Oryza sativa* L.) leaves of each cultivar (cultivars Koshihikari and Nipponbare) was collected from the Food Resources Education and Research Center at Kobe University, and ground with 5 ml of 10 mM phosphate-buffered saline (PBS, pH 7.0) in a mortar. The leaf extracts were sequentially diluted with 10 mM PBS, spread onto NA medium (Nutrient Agar, Becton Dickinson), and incubated at 28 °C for 2 or 3 days. Ninety-six randomly selected bacterial isolates were stored in skim milk medium at −80 °C, as "pre-incubation genera." The leaf extracts were also incubated with 10 mM PBS with 0.4% collagen (sterilized at 70 °C for 3 days) (Wako, Osaka, Japan) for 24 h, and sequentially diluted with 10 mM PBS and spread onto NA medium. Ninety-six randomly selected bacterial isolates were stored at −80 °C as "collagen-incubation genera." The selected bacterial isolates from these two treatments were analyzed for the 16S rDNA sequence and preserved in −80 °C deep freezer in the laboratory of Kobe University.

2.3. Membrane-replica screening from leaves and soil

Microbial communities were also collected from 1 g of rice leaves at the Food Resources Education and Research Center and 1 g of soil in the field of Kobe University, and incubated with 10 mM PBS with 0.4% collagen for 48 h, and then sequentially diluted and spread onto NA medium. After colonizing, the colonies were transferred to sterilized nylon membranes (GE Health Care, Buckinghamshire, UK) as a replica. The replica membranes were put onto the gelatin plates and incubated for 2 or 3 days. The gelatin plates were stained with CBB. The bacterial isolates showing high gelatinolytic activity were analyzed for the 16S rDNA sequence and preserved in −80 °C deep freezer in the laboratory of Kobe University.

2.4. Measurement of gelatinolytic activity

Each bacterial culture was incubated for 3 days in NB medium and their OD 600 value adjusted to 0.8. Bacterial cultures (10 μl) of each isolate were dropped onto 9-cm Petri dishes containing gelatin medium and incubated for 3 days at 28 °C. After that, the Petri dishes were stained with CBB, and decolorized. The gelatinolytic activity was defined as the radius of the clear zone subtracted from the radius of the bacterial colony. These experiments were conducted in triplicate.

2.5. DNA extraction from the bacterial isolates and 16S rDNA sequence analysis

Bacterial genomic DNA was extracted by boiling with lysis buffer [200 mM Tris–HCl, 50 mM ethylenediaminetetraacetate (EDTA), 200 mM NaCl, 1% sodium lauroyl sarcosinate] and ethanol precipitation. Specific primers were used to amplify the 16S rDNA region, i.e., 63f (5′-CAGGCCTAACACATGCAAGTC-3′) and 1387r (5′-GGGCGGWGTGTACAAGGC-3′, W: A or T) (Marchesi et al., 1998). Polymerase chain reaction (PCR) was performed using a Mastercycler ep (Eppendorf Japan, Tokyo, Japan) as follows: 1 cycle at 95 °C for 5 min, 35 cycles at 95 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min), and 1 cycle at 72 °C for 5 min. The 1.3-kb PCR fragments were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Wisconsin, USA). The purified fragments were directly sequenced using Big Dye Terminator Sequencing Ver. 3.1 Kit (Applied Biosystems, California, USA) and analyzed using ABI-3100 Prism DNA Sequencer (Applied Biosystems). Sequences of each bacterial strain were identified using the BLAST program from GenBank (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov). The sequenced isolates showed 98–100% homology to the defined genus and/or species.

2.6. Effect of inhibitors on gelatinolytic activity

To characterize the gelatinolytic enzymes, bacterial cultures were co-incubated with protease inhibitors, i.e., EDTA (5 mM, Wako), antipain (10 μg/ml, Sigma, St. Louis, Missouri, USA), aprotinin (50 μg/ml, Sigma), and phenylmethylsulfonyl fluoride (PMSF; 1 mM, Wako), and gelatinolytic activity was measured as with in 2.4.

To examine the requirement of metal cations for gelatinolytic activity, bacterial cultures with or without EDTA (5 mM) were supplemented with metal cations, $CaCl_2$ (Nacalai Tesque), $ZnCl_2$ (Wako), and $MnCl_2$ (Wako) at concentrations ranging from 1 mM to 10 mM that did not affect fungal germination and attachment, and the recovery of gelatinolytic activity was evaluated. These experiments were conducted in triplicate.