Interference of quorum sensing and virulence of the rice pathogen *Burkholderia glumae* by an engineered endophytic bacterium

Hyun-Soo Cho[1], Soo-Young Park[1,2], Choong-Min Ryu[1], Jihyun F. Kim[1], Jong-Guk Kim[2] & Seung-Hwan Park[1]

[1]Laboratory of Microbial Genomics, Systems Microbiology Research Center, KRIBB, Daejeon, Korea; and [2]Department of Microbiology, College of Nature Science, Kyungpook National University, Daegu, Korea

Correspondence: Seung-Hwan Park, Laboratory of Microbial Genomics, Systems Microbiology Research Center, KRIBB, Daejeon 305-806, Korea. Tel.: +82 42 860 4410; fax: +82 42 879 8595; e-mail: shpark@kribb.re.kr

Received 24 January 2006; revised 23 November 2006; accepted 23 November 2006. First published online 22 February 2007.

DOI:10.1111/j.1574-6941.2007.00280.x

Editor: Kornelia Smalla

Keywords
endophyte; *Burkholderia* sp.; rhizobacteria; biocontrol; *Burkholderia glumae*.

Abstract

Many bacterial species are known to thrive within plants. Among these bacteria, a group referred to as endophytes provide beneficial effects to the host plants by the promotion of plant growth and the suppression of plant pathogens. Among 44 putative endophytic isolates isolated from surface-sterilized rice roots, *Burkholderia* sp. KJ006 was selected for further study because of a lack of pathogenicity to rice, a broad spectrum of antifungal properties, and the presence of the *nifH* gene, which is an indicator for nitrogen fixation. In an attempt to control *Burkholderia glumae*, a casual pathogen of seedling rot and grain rot of rice, an *N*-acyl-homoserine lactonase (*aiiA*) gene from *Bacillus thuringiensis* was introduced into *Burkholderia* sp. KJ006 given that the major virulence factor of *Burkholderia glumae* is controlled in a population-dependent manner (quorum sensing). The engineered strain KJ006 (pKPE-*aiiA*) inhibited production of quorum-sensing signals by *Burkholderia glumae in vitro* and reduced the disease incidence of rice seedling rot caused by *Burkholderia glumae in situ*. Our results indicate the possibility that a bacterial endophyte transformed with the *aiiA* gene can be used as a novel biological control agent against pathogenic *Burkholderia glumae* that are known to occupy the same ecological niche.

Introduction

The interior of plant tissues provides an environment where other life forms can thrive as a result of the abundance of plant nutrients and the buffering against external environmental stresses. Many species of microorganisms are commonly found inside plant tissues and are referred to as endophytes (Hallmann *et al*., 1997; Hurek & Reinhold-Hurek, 2003). A number of studies of the endophytes have been conducted to understand better their possible role in the suppression of plant diseases and the promotion of plant growth (Hallmann *et al*., 1997; Hurek *et al*., 2002; Barac *et al*., 2004). Indeed, recent reports have indicated that some endophytic strains are capable of inducing a systemic resistance in plants that resulted in their protection against multiple plant pathogens (Ryu *et al*., 2003; Kloepper *et al*., 2004).

Burkholderia glumae is a Gram-negative and rod-shaped bacterium and an economically important pathogen that causes seedling rot and grain rot in rice nurseries and rice fields in eastern Asia, particularly in Korea, Japan and the Philippines (Uematsu *et al*., 1976; Cottyn *et al*., 1996; Jeong *et al*., 2003). The environmental conditions required for these diseases include a high temperature and high moisture typical of midsummer in these regions. Appropriate methods to manage these plant diseases have not been reported except for the example of transgenic plants (Iwai *et al*., 2002). *Burkholderia glumae* is often transmitted through seeds. The bacterium is then inherited within the plant tissue and infects rice flowers at the appropriate temperature (Uematsu *et al*., 1976; Cottyn *et al*., 1996). The yellow pigment toxin toxoflavin [1,6-dimethylpyrimidio (5,4-e)-1,2,4-triazine-5,7 (1H,6H)-dione] is a major virulence factor of *Burkholderia glumae* (Sato *et al*., 1989; Suzuki *et al*., 1998; Nagamatsu, 2001). Recent results have indicated that production of toxoflavin in *Burkholderia glumae* is regulated by a quorum-sensing mechanism that utilizes autoinducers such as *N*-acyl homoserine lactones (AHLs; Kim *et al*., 2004). AHLs in many Gram-negative bacteria harmonize target gene expression in a population density-dependent manner (Latifi *et al*., 1995; Kim *et al*., 2004). In Kim *et al*. (2004), a quorum-sensing signal-deficient mutant of *Burkholderia glumae* did not cause any symptoms when the bacterium was challenged on the rice grains, suggesting that

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

an autoinducer-mediated quorum-sensing mechanism plays an important role in the pathogenesis of *Burkholderia glumae*.

Inhibition of the quorum-sensing mechanism may be considered as a possible means to manage disease development caused by *Burkholderia glumae*. Recently, it was shown that *Bacillus* spp. and some Gram-negative bacteria have a counterpart mechanism to quorum sensing referred to as 'quorum quenching' that directly degrades quorum-sensing signal molecules (Dong *et al*., 2000; Lee *et al*., 2002). Many soil and root-associated bacteria have been reported to have the relevant AHL-degrading enzymes, including AHL lactonase or acylase (Dong *et al*., 2000; Lin *et al*., 2003; Xu *et al*., 2003; Park *et al*., 2005). Previous reports indicate that the introduction of an AHL lactonase (*aiiA*) into a root-associated bacterium, *Pseudomonas fluorescens* P3, successfully reduced symptom development of a soft-rot casual pathogen, *Erwinia carotovora*, which is dependent on the quorum-sensing-mediated production of cell-wall-degrading enzymes by the quorum-quenching mechanism (Molina *et al*., 2003). These results supported the notion that quorum quenching can be used as a reliable tool to control bacterial pathogens in agriculture (Zhang & Dong, 2004).

In this study, we have screened bacteria known to exist in the same environment as *Burkholderia glumae* and attempted to interrupt its quorum sensing for use as a biological control agent against rice seedling disease. Because there were no endophytes that demonstrated a quorum-quenching capacity in our study, we introduced a gene encoding the AHL-degrading enzyme (AHL lactonase, *aiiA*) from *Bacillus thuringiensis* into a selected endophyte, *Burkholderia* sp. strain KJ006. The new modified strain KJ006 (pKEA-*aiiA*) successfully attenuated the development of symptoms commonly caused by *Burkholderia glumae* in rice as well as *Erwinia carotovora* on potatoes. These results suggest that an endophyte expressing a useful gene such as *aiiA* can offer great potential benefits in controlling plant-pathogenic bacteria.

Materials and methods

Bacterial strains, plasmids and chemicals

All bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* was cultured in Luria–Bertani (LB) broth or on LB agar plates at 37 °C and *Burkholderia* spp. were cultured in LB medium at 30 °C. *Azoarcus indigens* (ATCC 51398; Reinhold-Hurek *et al*., 1993; Reinhold-Hurek & Hurek, 1998) was obtained from the American Type Culture Collection (www.atcc.org). For long-term storage, bacterial cultures were maintained at − 80 °C in nutrient broth (Difco, Sparks, MD) that contained 20% glycerol. *N*-hexanoyl-L-homoserine lactone (HHL) was purchased from Fluka Co. (Tokyo, Japan). Restriction endonucleases and chemicals used for DNA

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strain		
Azoarcus indigens ATCC 51398	Diazotrophic endophyte, isolated from surface-sterilized stem bases of Kallar grass	Reinhold-Hurek *et al*. (1993)
Burkholderia glumae	Rice grain rot, seedling rot and wilt pathogen, produces toxoflavin BGR1	Kim *et al*. (2004)
Burkholderia sp. KJ006	Endophytic bacteria isolated from surface-sterilized root of rice	This study
Erwinia carotovora	Soft-rot pathogen on several crops	National Institute of Agricultural Science and Technology, Korea
Plasmid		
pGEM-*egfp*	Ap^R; 0.73-kb promoterless *egfp* PCR product (designed for each end, 5′-*Eco*RI, *Bgl*II and 3′-*Bam*HI) inserted into pGEM-T vector	This study
pGEM-*aiiA*	Ap^R; 0.79-kb *aiiA* (synthetic *Bam*HI site of each end) PCR product inserted into pGEM-T vector	This study
pKT230	Km^R, Sm^R; broad-host-range vector in Gram-negative bacteria	Bagdasarian *et al*. (1981)
pKE	Km^R, Sm^R; 0.73-kb pGEM-*egfp* *Eco*RI–*Bam*HI fragment inserted into pKT230	This study
pKEA	Km^R, Sm^R; promoterless *bla* PCR gene containing *Bam*HI, each end inserted in pKE with *Bam*HI in the same orientation as *egfp*	This study
pKPEA	Km^R, Sm^R, Ap^R, GFP^+; 2-kb *Sau*3AI fragment containing TPR repeat into *Bgl*II site of pKEA	This study
pKPE-*aiiA*	Km^R, Sm^R, GFP^+, $AiiA^+$; *aii*A *Bam*HI fragment from pGEM-*aiiA* inserted pKPEA with *Bam*HI cleavage	This study

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

manipulations were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) and used as specified by the manufacturer.

Isolation and recovery of endophytes

After rice roots were washed thoroughly with tap water, the samples were sterilized using 1% NaOCl for 10 min. Surface-sterilized samples were washed three times with sterile deionized water. The third washing supernatant and washed roots were used as controls for surface sterilization. The samples were ground in a pestle and mortar thoroughly using a motorized homogenizer with 5 mL sterile water and plated on nutrient agar (NA). The plates were incubated for 2–5 days at 30 °C. Among colonies grown on the NA, the predominant colonies in number from each rice sample were chosen for further study.

To confirm an endophytic lifestyle, reisolation from rice seedlings inoculated with selected endophytes was carried out. Surface sterilization of rice seeds was performed as follows. The seeds were dehulled, treated with 5% NaClO for 20 min, and washed with sterile water five times. The treated seeds were dried on paper towels for 2 h. The sterilized seeds were laid on 0.6% water-agar plates and incubated at 30 °C in the dark. After seed germination, the shoots were cut and simultaneously inoculated with bacteria using scissors that were dipped in the bacterial suspension of the selected bacteria. Seven days after inoculation, reisolation was performed as described above. *Azoarcus indigens* and *Escherichia coli* DH5α were used as positive and negative controls, respectively. To validate that reisolated bacteria were identical with the introduced bacterial isolates, genomic fingerprinting of the strains was conducted using repetitive extragenic palindromic (REP) PCR (Alam *et al*., 1999).

Hypersensitive response and pathogenicity test

The tobacco plants were grown for up to 30 days in a growth chamber with 16 h of light at 27 °C and 8 h of darkness at 21 °C. The strains were cultured in 3 mL nutrient broth until a population density of 5×10^8 cells mL^{-1} was reached. The cells were harvested by centrifugation (1500 ***g***, 5 min, 4 °C), were washed in an equal volume three times and suspended with 0.5 mL phosphate-buffered saline. The cells were diluted to an $OD_{600\,nm}$ of 0.5 (about 10^8 cells mL^{-1}) prior to inoculation. The bacterial suspensions were infiltrated into abaxial surfaces of *Nicotiana benthamiana* leaves, and the appearance of a hypersensitive reaction was measured. Pathogenicity tests of strain KJ006 were performed using *A. indigens* as a negative control and *Burkholderia glumae* as a positive control pathogen. The rice flowers were dip-inoculated in each bacterial suspension (10^8 cells mL^{-1}). The treated plants were incubated for several days until disease symptoms were observed.

Suppression of fungal growth by strain KJ006

To select bacterial isolates that showed suppression of plant pathogenic fungi, we examined the antifungal capacity *in vitro* against eight soil-borne pathogenic fungi, *Rhizoctonia solani*, *Pythium aphanidermatum*, *Mucor ambiguus*, *Rhizopus oryzae*, *Alternaria* sp., *Fusarium oxysporum*, *Fusarium solani* and *Sclerotium rolfsii*. These fungi were obtained from the National Institute of Agricultural Science and Technology, Korea. Strain KJ006 was spot-inoculated on the plate edge at equal spacing around the perimeter of a PDA (potato dextrose agar) plate. After 1 day, a mycelial plug of each fungus grown on PDA was placed in the center of the plate. Suppression of fungal growth was measured 5 days after incubation as the diameter (mm) of clear zone between a bacterial colony and each fungus.

Analysis of the 16S rRNA gene and detection of the *nifH* gene

For identification of bacteria, 16S rRNA gene sequence analysis was performed as described previously (Wilmotte *et al*., 1993; Cheong *et al*., 2005). The 16S rRNA gene sequences of related taxa were obtained from GenBank (accession number): *Burkholderia cepacia* LMG 1222^T (AF097530), *Burkholderia gladioli* LMG 2216^T (X67038), *Burkholderia graminis* LMG 18924^T (U96939), *Burkholderia glumae* LMG 2196^T (U96931), *Burkholderia mallei* ATCC 23344^T (AF110188), *Burkholderia multivorans* LMG 13010^T (Y18703), *Burkholderia plantarii* LMG 9035^T (U96933), *Burkholderia pseudomallei* ATCC 23343^T (DQ108392), *Burkholderia stabilis* LMG 14294^T (AF148554) and *Burkholderia vietnamiensis* LMG 10929^T (AF097534). The *nifH* gene was detected using the methods described in Ueda *et al*. (1995) and Cheong *et al*. (2005).

Construction of a promoter-probing vector

To construct a promoter-probing vector, two reporter genes, an enhanced green fluorescent protein gene (*egfp*) and an ampicillin resistance gene (*bla*), were introduced sequentially into the broad-host-range vector pKT230 without their own promoters. For PCR amplification of the *egfp* gene we prepared two primers: EGFP-F (5′-*GAATTCAGATCTAGGAG*CCACCATGGTGAGCAAGG-3′) containing *Eco*RI and *Bgl*II restriction sites (underlined) and a Shine Dalgarno sequence (italicized) and a reverse primer EGFP-R (5′-*GGATCC*CTTTACTTGTACAGCTC-3′) containing the *Bam*HI restriction site (underlined). A pEGFP plasmid (Clontech, Palo Alto, CA,) was used as a template. The PCR-generated DNA fragment was first cloned into the pGEM®-T vector (Promega, Madison, WI) and then a

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

0.73-kb *Eco*RI–*Bam*HI fragment that contained the *egfp* gene was cut out and cloned into the relevant restriction sites of pKT230. The *bla* gene was amplified using two primers: forward primer amp-F (5′-*GGATCC*GAAAAAG-GAAGAG-3′) and reverse primer amp-R (5′-*GGATCC*T-TACCAATGCTTAATC-3′) with each *Bam*HI site (underlined) and pUC19 as a template. The PCR-generated DNA fragment containing the *bla* gene was cloned into the pGEM®-T vector and digested with *Bam*HI and inserted downstream of the *egfp* gene using the same restriction site, developing as a result the promoter-probing vector pKEA (Fig. 1).

Cloning of an indigenous promoter

Chromosomal DNA from *Burkholderia* sp. KJ006 was extracted and partially digested with *Sau*3AI, and 1–2-kb fragments were purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA). The *Sau*3AI fragments were ligated into the *Bgl*II site of pKEA. The ligation mixture was introduced into *Escherichia coli* DH10B by electroporation, and the *Escherichia coli* cells plated on LB agar that contained 20 μg mL^{-1} kanamycin and 100 μg mL^{-1} ampicillin. Plasmid DNA was extracted from the kanamycin- and ampicillin-resistant transformants using a QIAprep spin miniprep kit and then introduced into *Burkholderia* sp. KJ006 by electroporation. Transformants were selected on LB agar containing 40 μg mL^{-1} kanamycin. The DNA sequence of the *Sau*3AI fragment showing strong promoter activity was determined using the primer set PF (5′-TCAGCTTGCCGTAGGTGGC-3′) and PR (5′-ATGA AGCGCGAATATAGAG-3′), and the promoter structure was identified from the DNA sequence. The *aiiA* gene of *Bacillus thuringiensis* ssp. *kyushuensis*, kindly supplied by J.-K. Lee (Lee *et al*., 2002), was amplified with primer set *aiiA*-F (5′-*GGATCC*ATTCAAAAATGTAAAGGT-3′) and *aiiA*-R (5′-*GGATCC*TTATATATATTCAGGGAA-3′), which introduced the same *Bam*HI site (underlined), and cloned into pGEM-T vector. The 0.79-kb promoterless *aiiA* fragment was extracted with *Bam*HI from pGEM-*aiiA* and subcloned into pKPEA by substitution of the *bla* gene. The resulting plasmid was introduced into *Burkholderia* sp. KJ006 by electroporation.

Bioassay of the quorum-sensing signal degrading capacity

For the assay of HHL-degrading activity, *Burkholderia* sp. KJ006 and *Burkholderia* sp. KJ006 (pKPE-*aiiA*) were cultured overnight in LB, harvested and resuspended in 100 mM Tris-HCl (pH 7.0). Each suspension was disrupted by sonication and centrifuged (16 000 ***g*** for 10 min). Twenty microliters of each supernatant and 20 μL of 40 μM HHL were mixed and incubated at 30 °C with gentle agitation. After heat treatment at 90 °C for 5 min to stop the reaction, each reaction mixture was diluted to the appropriate concentration and loaded into the well of plates overlaid with *Chromobacterium violaceum* CV026 for HHL detection (McClean *et al*., 1997; Lee *et al*., 2002).

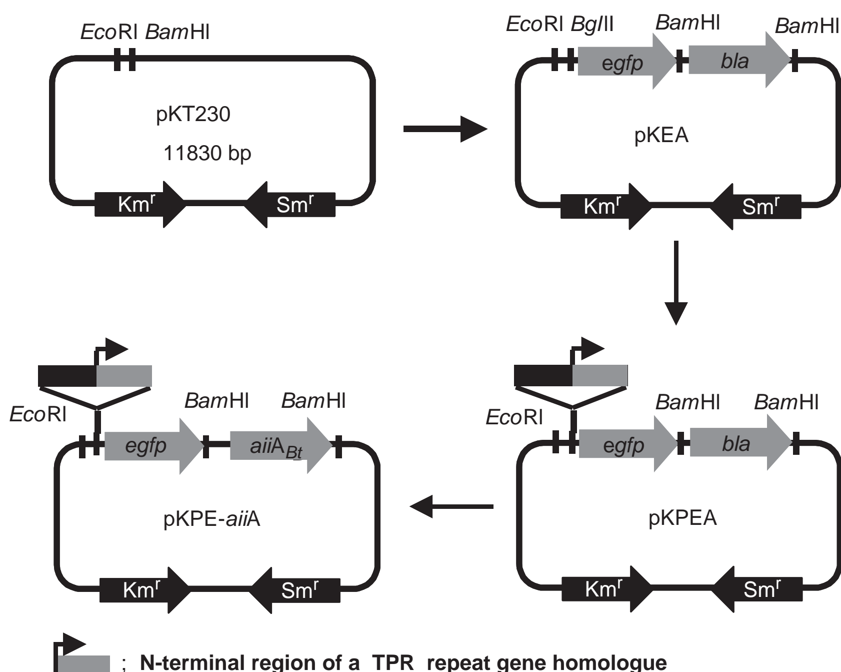

Fig. 1. Illustration of the plasmid construct. The pKT230 vector used for the construction of pKEA, pKPEA and pKPE-*aiiA*. The promoter for strain KJ006 was cloned into pKEA. In the pKPE-*aiiA*, *aiiA* was placed under the *TPR* (tetratrico peptide repeat) promoter and the *egfp* of strain KJ006 ($P_{TPR\text{-}egfp}$: *aiiA*) of vector pKPEA. Plasmid pKPE-*aiiA* carries a transcriptional *TPR-egfp-aiiA* fusion.

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Rice seedling rot and soft-rot disease assay

To evaluate the effects of quorum quenching, *Erwinia carotovora* and *Burkholderia glumae* were used as plant pathogens. In the potato test, *Erwinia carotovora*, *Burkholderia* sp. KJ006 and recombinant *Burkholderia* sp. KJ006 (pKPE-*aiiA*) were cultured until an $OD_{600\,nm}$ of 1.0 and then diluted with 0.9% NaCl. Equal volumes of the *Erwinia carotovora* ($OD_{600\,nm} = 0.01$), *Burkholderia* sp. KJ006 ($OD_{600\,nm} = 0.1$) and recombinant *Burkholderia* sp. KJ006 (pKPE-*aiiA*; $OD_{600\,nm} = 0.1$) were mixed. Mixtures of bacterial suspensions were inoculated to a wounded site in potato slices and incubated in petridishes at 30 °C for 48 h. The experiment to demonstrate that *Burkholderia* sp. KJ006 (pKPE-*aiiA*) could attenuate rice pathogenicity due to *Burkholderia glumae* was conducted with gnotobiotically growing rice seedlings. As described above, surface sterilized seeds were laid on 0.6% water-agar plates (square dish: 125 mm × 125 mm × 25 mm), sealed with laboratory sealing film and incubated vertically at 30 °C in the dark until seeds germinated. *Burkholderia* sp. KJ006, *Burkholderia* sp. KJ006 (pKPEA) and *Burkholderia* sp. KJ006 (pKPE-*aiiA*) were cultured in 3 mL nutrient broth ($OD_{600\,nm} = 1$), washed twice with equal volumes of phosphate buffer and diluted ($OD_{600\,nm} = 0.1$). The shoots of rice seedlings were cut and simultaneously infected using scissors soaked with each of *Burkholderia* sp. KJ006, *Burkholderia* sp. KJ006 (pKPEA) and *Burkholderia* sp. KJ006 (pKPE-*aiiA*). Seven days after inoculation, the shoots were cut and inoculated again using scissors soaked in a suspension of *Burkholderia glumae* (10^7 CFU mL^{-1}). After continuous incubation, the plates were placed under constitutive light conditions in the growth chamber at 27 °C. The rice seedlings were grown vertically. Twenty-five days after inoculation of *Burkholderia glumae*, disease severity was measured by scores of symptom development as follows: 0 = no symptoms, 1 = necrosis symptoms only at infected site, 2 = partial necrosis around the infected site, 3 = spread of mild symptoms over half the plant, 4 = spread of symptoms over entire plant, 5 = severe necrosis over entire plant. *In vivo* experiments were conducted with rice seedlings grown in a water-tray with standard paddy soil. Bacterial suspensions at 10^8 CFU mL^{-1} of *Burkholderia glumae* and *Burkholderia* sp. KJ006 or *Burkholderia* sp. KJ006 (pKPEA) or (pKPE-*aiiA*) were drenched on 2-week-old rice seedlings. Disease severity was measured 2 weeks after challenge: −, no symptoms; +, mild symptoms; ++, severe necrosis over entire plant.

Data analysis

Data were subjected to ANOVA using JMP software version 4.0.4 (SAS Institute Inc., Cary, NC). The effect of strain KJ006 and its engineered bacteria treatment on disease control was considered to be significant according to the magnitude of the *F* value ($P = 0.05$). When a significant *F* test was obtained for treatments, separation of means was accomplished using Fisher's protected least-significant difference (LSD).

Results

Isolation and nature of an endophyte, *Burkholderia* sp. KJ006

Burkholderia sp. KJ006 was selected from 44 preselected putative endophyte isolates following sterilization of the rice root surface. Cells of strain KJ006 were gram-negative rods and formed beige colonies on NA. The 16S rRNA gene sequence of strain KJ006 determined in this study was a continuous stretch of 1419 bp. (accession number EF128227). Strain KJ006 was determined as belonging to the genus *Burkholderia*, and highest levels of sequence similarity were with *Burkholderia vietnamiensis* LMG 10929^T (99.9%), *Burkholderia multivorans* LMG 13010^T (99.6%) and *Burkholderia cepacia* LMG 1222^T (99.5%). The *nifH* gene was amplified successfully from this strain. A BLAST search was performed at the NCBI site (http://www.ncbi.nlm.nih.gov/) against the partial reading *nifH* sequences (336 bp), indicating that the *nifH* (accession number EF128226) of strain KJ006 was closely related to that of the type strain of *Burkholderia vietnamiensis*, with 99% identity based on nucleotide sequence (accession number AJ512206) and 100% identity based on translated form (accession number CAD98763). Strain KJ006 suppressed the growth of all tested fungi except *S. rolfsii*, with a clear-zone diameter of from 11 to more than 16 mm (Table 2). Strain KJ006 was isolated with a frequency of up to $1 \pm 0.1 \times 10^5$ CFU g^{-1} fresh weight from the surface-sterilized roots of preinoculated rice seedlings (Table 2) whereas the population density of other *Burkholderia* spp. was 3×10^3–3×10^4 CFU g^{-1} fresh weight. Inoculation of *Burkholderia* sp. KJ006 and *A. indigens* did not cause any visual signs or symptoms on the rice seedlings. By contrast, severe grain rot symptoms were observed following challenge with *Burkholderia glumae* (Fig. 2). In addition, infiltration of bacterial suspensions of *Burkholderia* sp. KJ006 onto tobacco leaves did not result in any visible lesions, indicating that strain KJ006 may not be a pathogenic isolate (data not shown). To confirm that *Burkholderia* sp. KJ006 was a bacterial endophyte, we established a reisolation procedure based on Koch's postulates in a laboratory setting. *Burkholderia* sp. KJ006 and *A. indigens* as a positive control were reisolated at $10^{4\text{–}5}$ CFU g^{-1} fresh weight on NA without antibiotics. By contrast, treatments using buffer control and *Escherichia. coli* DH5α, as negative controls, did not detect any bacterial colonies. Using REP-PCR (Alam *et al*., 1999), the reisolated bacteria yielded the same genetic fingerprints as strain KJ006 (data not shown).

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Table 2. Effect of *Burkholderia* sp. KJ006 on suppression of plant pathogenic fungi *in vitro* and reisolation frequency

	Treatment	
Traits	KJ006	Water control
Suppression of fungal growth *in vitro**		
Rhizoctonia solani	++	–
Pythium aphanidermatum	+++	–
Mucor ambiguus	++	–
Rhizopus oryzae	++	–
Alternaria sp.	+++	–
Fusarium oxysporum	++	–
Fusarium solani	++	–
Sclerotium rolfsii	–	–
Reisolation frequency (CFU g^{-1} fresh tissue)†	$1 \pm 0.1 \times 10^{5a}$	0.0^{b}

*Fungal growth suppression by strain KJ006 was measured by the diameter of the clear zone caused by inhibition of fungal growth on PDA; the number indicates area of clear zone between bacterial inoculation and fungal growth: –, < 6 mm; +, 6–10 mm; ++, 11–15 mm; +++, > 16 mm.

†Numbers represent mean of four replications per treatment, four plants per replication. Different superscript letters indicate significant differences on Fisher's protected LSD test at $P = 0.05$.

Fig. 2. Assessment of pathogenesis of *Burkholderia* sp. KJ006 on rice grains. Bacterial suspensions of *Burkholderia glumae*, *Azoarcus indigens* and strain KJ006 at 10^{8} CFU mL^{-1} were challenged on the rice grains. The image was taken 7 days after inoculation with pathogens.

Cloning of an indigenous promoter and expression of the *aiiA* gene in *Burkholderia* sp. KJ006

A promoter-probing vector was constructed using the broad-host-range vector pKT230 and two reporter genes, *egfp* and *bla*, and the plasmid was termed pKEA. To express a foreign AHL lactonase gene (*aiiA*) efficiently in *Burkholderia* sp. KJ006, we cloned an indigenous promoter using pKEA (Fig. 1). The plasmid isolated from the transformant was named pKPEA and the DNA sequence of the inserted *Sau*3AI fragment was determined. Analysis of the DNA sequence indicated that the insert contained a promoter and N-terminal region of a TPR (Tetratrico peptide repeat) gene homolog. To express the *aiiA* gene of *Bacillus thuringiensis* ssp. *kyushuensis*, a 0.79-kb *Bam*HI fragment containing *aiiA* was introduced into the region of *bla* in pKPEA using *Bam*HI sites, and the resultant plasmid, named pKPE-*aiiA*, was then used to transform *Burkholderia* sp. KJ006.

Characterization of the AiiA-producing derivative of *Burkholderia* sp. KJ006

Burkholderia sp. KJ006 (pKPE-*aiiA*) was compared with the parental strain (wild type) with regard to root colonization, antifungal activity and AiiA enzyme capacity. Using the reisolation method described previously, the population density of *Burkholderia* sp. KJ006 (pKPE-*aiiA*) inside rice tissues did not show any differences compared with that of the wild type (10^{4-5} CFU g^{-1} fresh root) at 17 days after inoculation. Moreover, the antifungal activity of the engineered strain against four fungi, *R. solani*, *F. oxysporum*, *Alternaria* sp. and *P. aphanidermatum*, was not different compared with its wild type strain (data not shown). Using epifluorescence microscopy, GFP-tagged bacterial cells were visualized in the rice root tissues. The bacterial cells were mainly localized outside the vascular bundles. The indicator strain *C. violaceum* CV026 was used to detect the AiiA-degrading capacity of the *Burkholderia* sp. KJ006 (pKPE-*aiiA*) to a C6 autoinducer, HHL. Incubation with both HHL and *Burkholderia* sp. KJ006 (pKPE-*aiiA*) for 2 h completely abolished any positive response of strain CV026 in the *in vitro* assay (Fig. 3a). We also conducted an *in vitro* test by coinoculating both *Burkholderia* sp. KJ006 (pKPE-*aiiA*) and *Erwinia carotovora*, a casual pathogen of soft-rot of potato and cabbage, and determined that the *Burkholderia* sp. KJ006 (pKPE-*aiiA*) treatment significantly reduced the development of soft-rot symptoms on potato slices (Fig. 3b).

Attenuation of seedling rot caused by *Burkholderia glumae* by AiiA-producing *Burkholderia* sp. KJ006

The direct application of *Burkholderia* sp. KJ006 (pKPE-*aiiA*) to the rice seedlings decreased the disease severity of rice seedling rot caused by *Burkholderia glumae* (Table 3,

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

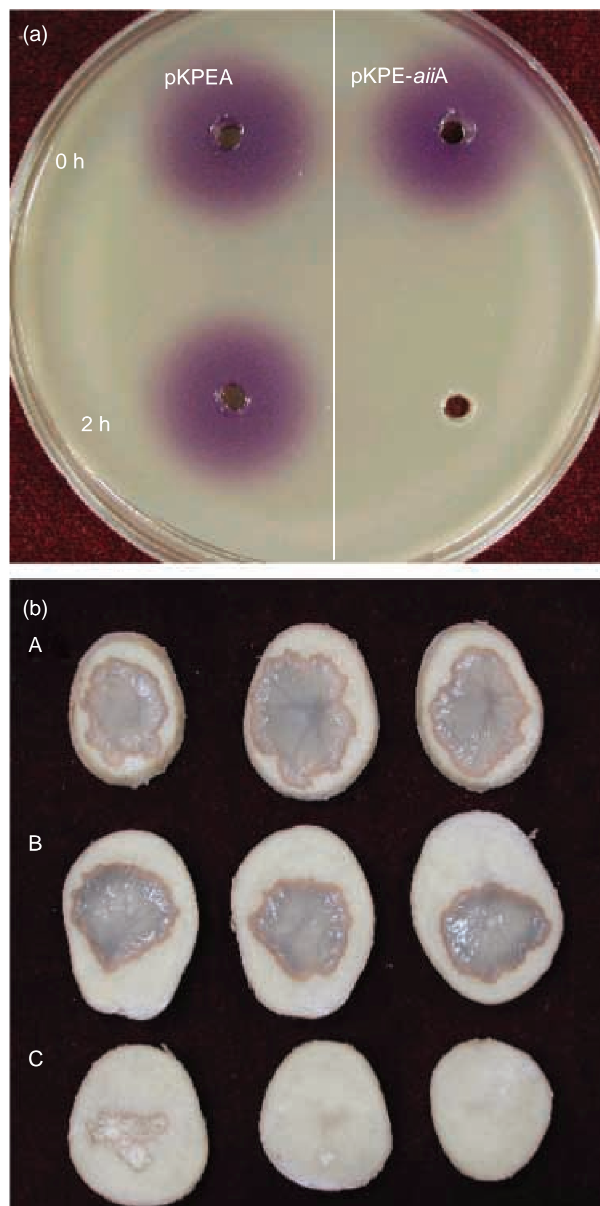

Fig. 3. Inhibition of quorum-sensing signals and suppression of symptom development caused by *Erwinia carotovora* by the engineered *Burkholderia* sp. KJ006 (pKPE-*aiiA*). (a) Degradation of acyl-homoserine lactone (AHL) 0 and 2 h after coinoculation of vector control (pKPEA) or acyl homoserine lactonase transformant (pKPE-*aiiA*) in *Burkholderia* sp. KJ006. The violet color indicates the presence of AHL detected by an indicator, *Chromobacterium violaceum* CV026. (b) Effect of *Burkholderia* sp. KJ006 (pKPE-*aiiA*) on soft-rot development on the potato slices by *Erwinia carotovora*. A, inoculation of *Erwinia carotovora* alone; B, coinoculation of *Erwinia carotovora* and vector control *Burkholderia* sp. KJ006 (pKPEA); C, coinoculation of *Erwinia carotovora* and engineered *Burkholderia* sp. KJ006 (pKPE-*aiiA*). The image was taken 2 days after pathogen inoculation.

Fig. 4). The protection rate afforded by the *Burkholderia* sp. KJ006 (pKPE-*aiiA*) treatment was estimated to be about twofold (1.86) greater than the vector control [*Burkholderia* sp. KJ006 (pKPEA); Table 3]. We also used *Burkholderia* sp. KJ006 (pKPE-*aiiA*) in an *in vivo* assay conducted in water-trays under greenhouse conditions. Inoculation of *Burkholderia glumae* alone and *Burkholderia glumae* plus *Burkholderia* sp. KJ006 (pKPEA) induced severe seedling rot symptoms whereas no symptoms were detected in the rice seedlings treated with *Burkholderia* sp. KJ006 (pKPE-*aiiA*) (Fig. 4, Table 3).

Table 3. Effect of AiiA-transformed *Burkholderia* sp. KJ006 (pKPE-*aiiA*) and vector control *Burkholderia* sp. KJ006 (pKPEA) on rice seedling-rot caused by *Burkholderia glumae*

	Disease severity	
Treatment	*In vitro* assay*	*In vivo* assay†
B. glumae	4.92[a]	++
B. glumae+KJ006 (pKPEA)	3.79[a]	++
B. glumae+KJ006 (pKPE-*aiiA*)	2.04[b]	–

*Numbers represent mean of 12 replications per treatment, one seedling per replication. Bacterial suspensions (10^8 CFU mL^{-1}) of *Burkholderia* sp. KJ006 (wild) or *Burkholderia* sp. KJ006 (pKPEA) or (pKPE-*aiiA*) were inoculated into 5-day-old seedlings on water-agar medium, and after 7 days suspensions (10^7 CFU mL^{-1}) of *B. glumae* were introduced. *Burkholderia glumae* was used as a control. Disease severity was measured by scoring symptom development on each plant at 25 days after challenge as follows: 0 = no symptoms, 1 = necrosis only at infected site, 2 = partial necrosis around infected site, 3 = spread of mild symptoms over half the plant, 4 = spread of symptoms over entire plant, 5 = severe necrosis over entire plant. Different superscript letters indicate significant differences among means using Fisher's protected LSD test at $P = 0.05$.

†*In vivo* experiment conducted with rice seedlings grown in water-trays with standard paddy soil. Bacterial suspensions (10^8 CFU mL^{-1}) of *B. glumae* and *Burkholderia* sp. KJ006 or *Burkholderia* sp. KJ006 (pKPEA) or (pKPE-*aiiA*) were drenched on 2-week-old rice seedlings. Disease severity was measured 2 weeks after challenge.

Discussion

In recent years, rice-growing fields of eastern Asia have reportedly suffered severe damage due to *Burkholderia glumae* infection, which causes rice grain rot or rice seedling rot (Cottyn *et al*., 1996). Appropriate means to control this disease have not been developed primarily due to the widespread compatible plant hosts and bacterial localization within seeds and plant tissues (Cottyn *et al*., 1996; Jeong *et al*., 2003). The results presented here provide a novel strategy to control this disease using an engineered strain of the endophyte *Burkholderia* sp. KJ006 (pKPE-*aiiA*) containing the *aiiA* gene from *Bacillus thuringiensis*. Our hypothesis was that the AiiA expression of *Burkholderia* sp. KJ006 would occupy a similar ecological niche as the target pathogen *Burkholderia glumae* and abolish the quorum-sensing mechanism which regulates toxoflavin, a major virulence factor involved in the damage and destruction of rice grain and rice seedlings. As predicted, seedling

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

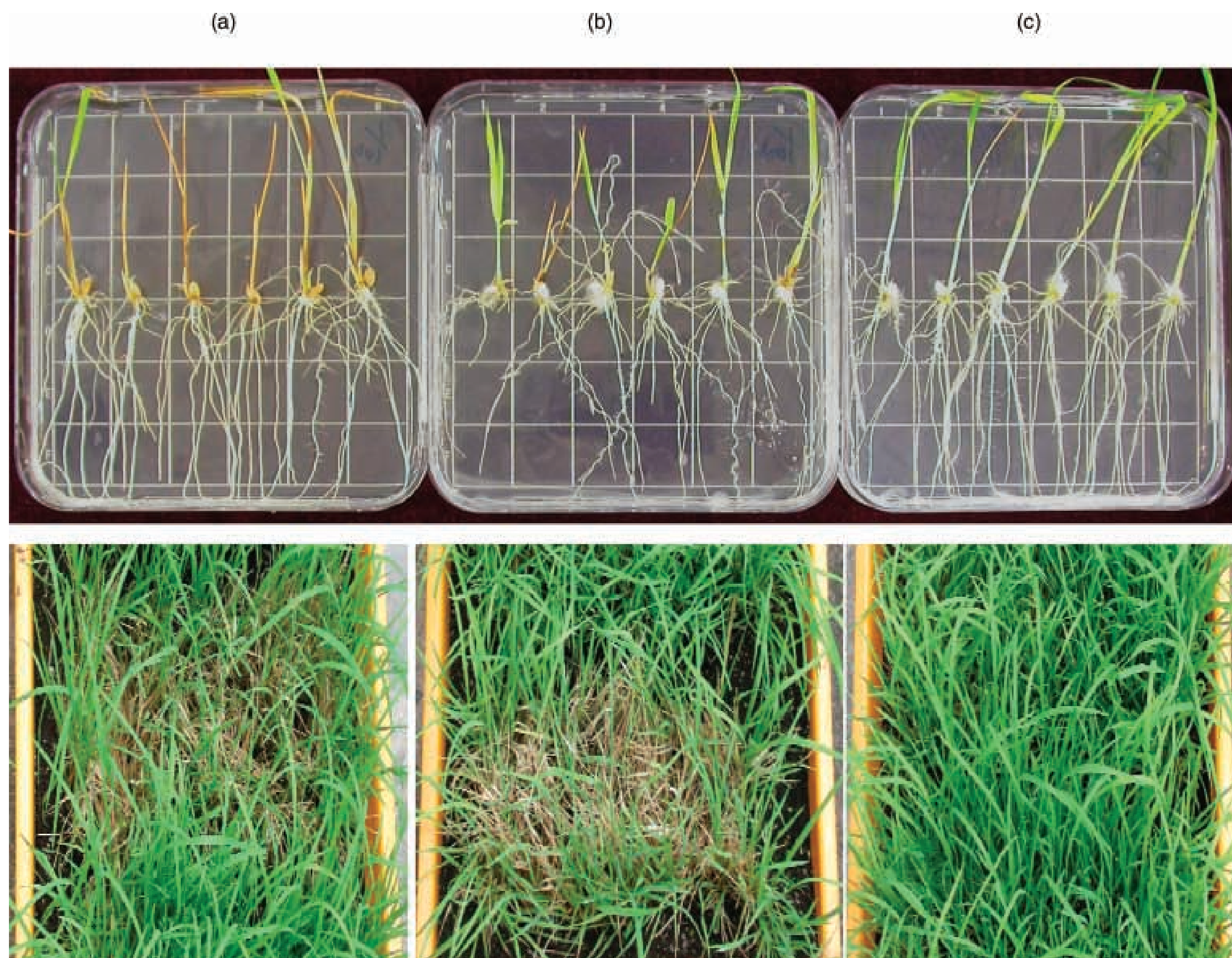

Fig. 4. Attenuation of rice seedling-rot caused by *Burkholderia glumae* by the engineered *Burkholderia* sp. KJ006 (pKPE-*aiiA*) and the vector control *Burkholderia* sp. KJ006 (pKPEA). The upper and lower panels represent *in vitro* and *in vivo* assays, respectively. (a) Inoculation of *Burkholderia glumae* alone; (b) Coinoculation of *Burkholderia glumae* and vector control *Burkholderia* sp. KJ006 (pKPEA); (c) Coinoculation of *Burkholderia glumae* and engineered *Burkholderia* sp. KJ006 (pKPE-*aiiA*). The images for the upper and lower panels were taken 25 and 14 days, respectively, after inoculation with pathogens.

application of *Burkholderia* sp. KJ006 (pKPE-*aiiA*) protected the rice plants under both *in vitro* and *in vivo* conditions, thereby suggesting that *Burkholderia* sp. KJ006 (pKPE-*aiiA*) is a promising biological control agent for the management of rice seedling rot disease.

The genus *Burkholderia* includes over 30 recognized species and occupies a wide range of ecological niches both on the surface of plants and within plant tissues (Coenye & Vandamme, 2003; Caballero-Mellado *et al*., 2004). *Burkholderia* species have also been reported to be plant pathogens in carnations (*Burkholderia carophylli*) and rice (*Burkholderia plantarii* and *Burkholderia glumae*) (Urakami *et al*., 1994; Coenye & Vandamme, 2003). By contrast, there are many strains of *Burkholderia* species that have been isolated from soil or rhizosphere that have been shown to control plant diseases (Parke & Gurian-Sherman, 2001). Previous reports have indicated that *Burkholderia* species, as well as *Pseudomonas* and *Bacillus* species, were also endophytes common to cotton, corn and cucumber plants (Hallmann *et al*., 1997). Recently, a genetically engineered endophyte, *Burkholderia cepacia*, was modified to improve its capacity to degrade toluene and hydrophobic volatile compounds (Barac *et al*., 2004). In a preliminary study, we determined that *Burkholderia* spp. were the most abundant species found within rice root tissues (data not shown). Among these putative endophytes, *Burkholderia* sp. KJ006 had a greater capacity to colonize inside rice tissues relative to other *Burkholderia* spp. and to a positive control, *A. indigens* (data not shown; Reinhold-Hurek *et al*., 1993; Reinhold-Hurek & Hurek, 1998). This colonizing capacity of *Burkholderia* sp. KJ006 in plant tissues may allow it to occupy similar ecological niches where *Burkholderia glumae* thrive.

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Burkholderia sp. KJ006 did not cause any lesioning on tobacco leaves within 24 h and any symptoms in rice grains, indicating that strain KJ006 was not a pathogenic bacterium on rice (Fig. 2). Our preliminary study proved that the capacity of strain KJ006 alone, however, could not adequately reduce the disease severity caused by *Burkholderia glumae* on the rice seedlings (data not shown).

To address this problem, we introduced a gene of the AHL-degrading enzyme (AiiA) into strain KJ006 using an *aiiA* expression vector following the indigenous promoter. Previously, researchers have investigated several methods in their attempts to protect plants against bacterial diseases. For example, transgenic tobacco and potato plants carrying the *aiiA* gene were protected against the wild-type *Erwinia carotovora* ssp. *carotovora* (Dong *et al.*, 2001). In addition, AiiA expression could attenuate the virulence of *Pseudomonas aeruginosa*, an opportunistic human pathogen that is known to cause cystic fibrosis in the *Caenorhabditis elegans* or *Drosophila* model systems (Waters & Bassler, 2005). Our engineered endophyte, *Burkholderia* sp. KJ006 (pKPE-*aiiA*), not only successfully degraded a reference quorum-sensing molecule *in vitro* but also decreased virulence of *Burkholderia glumae* on the rice seedlings *in vivo* and *in situ*. These results indicate that AiiA expression in an efficient endophyte is capable of suppressing symptom development caused by quorum sensing producing phytopathogenic bacteria such as *Burkholderia glumae*. However, large-scale application of strain KJ006 to control *Burkholderia glumae* will require detailed risk assessment towards animals and humans.

In conclusion, the introduction of a quorum-quenching gene, *aiiA*, into a rice endophyte, *Burkholderia* sp. KJ006, is a novel approach to control a pathogenic bacterium whose management can be very difficult. We have shown that to control bacterial diseases in plants effectively, there must be an understanding of the ecology of the target pathogens and the mechanisms of pathogenesis. However, the impact of *Burkholderia* sp. KJ006 harboring a quorum-quenching enzyme on soil microbial communities such as nontarget root-associated bacteria or fungi should be evaluated in the near future.

Acknowledgements

We thank Drs Sun-Yang Park and Jung-Kee Lee for critically reading our manuscript and providing the *aiiA* clone. We also thank Dr Ingyu Hwang for providing the *Burkholderia glumae* strain BGR1. This research was supported by grants from the Crop Functional Genomics Center of the 21st Century Frontier Research Program, funded by the Ministry of Science and Technology, and from the BioGreen 21 Program, funded by the Rural Development Administration, Republic of Korea.

Author contribution

S.-Y.P. and H.-S.C. contributed equally to this study.

References

Alam S, Brailsford SR, Whiley RA & Beighton D (1999) PCR-based methods for genotyping viridans group streptococci. *J Clin Microbiol* **37**: 2772–2776.

Bagdasarian M, Lurz R, Rückert B, Franckling FCH, Bagdasarian MM, Frey J & Timmis KN (1981) Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in Pseudomonas. *Gene* **16**: 237–247.

Barac T, Taghavi S, Borremans B, Provoost A, Oeyen L, Colpaert JV, Vangronsveld J & van der Lelie D (2004) Engineered endophytic bacteria improve phytoremediation of water-soluble, volatile, organic pollutants. *Nat Biotechnol* **22**: 583–588.

Caballero-Mellado J, Martinez-Aguilar L, Paredes-Valdez G & Santos PE (2004) *Burkholderia unamae* sp. nov., an N_2-fixing rhizospheric and endophytic species. *Int J Syst Evol Microbiol* **54**: 1165–1172.

Cheong H, Park SY, Ryu CM, Kim JH F, Park SH & Park CS (2005) Diversity of root-associated *Paenibacillus* spp. in winter crops from the southern part of Korea. *J Microbiol Biotechnol* **15**: 1286–1298.

Coenye T & Vandamme P (2003) Diversity and significance of *Burkholderia* species occupying diverse ecological niches. *Environ Microbiol* **5**: 719–729.

Cottyn B, Cerez MT, Van Outryve MF, Barroga J, Swings J & Mew TW (1996) Bacterial diseases of rice. I. Pathogenic bacteria associated with sheath rot complex and grain discoloration of rice in the Philippines. *Plant Dis* **80**: 429–437.

Dong YH, Xu JL, Li XZ & Zhang LH (2000) AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. *Proc Natl Acad Sci USA* **97**: 3526–3531.

Dong YH, Wang LH, Xu JL, Zhang HB, Zhang XF & Zhang LH (2001) Quenching quorum-sensing-dependent bacterial infection by an *N*-acyl homoserine lactonase. *Nature* **411**: 813–817.

Hallmann J, Quadt-Hallmann A, Mahaffee WF & Kloepper JW (1997) Bacterial endophytes in agricultural crops. *Can J Microbiol* **43**: 895–914.

Hurek T & Reinhold-Hurek B (2003) *Azoarcus* sp. strain BH72 as a model for nitrogen-fixing grass endophytes. *J Biotechnol* **106**: 169–178.

Hurek T, Handley LL, Reinhold-Hurek B & Piche Y (2002) *Azoarcus* grass endophytes contribute fixed nitrogen to the plant in an unculturable state. *Mol Plant Microbe Interact* **15**: 233–242.

Iwai T, Kaku H, Honkura R, Nakamura S, Ochiai H, Sasaki T & Ohashi Y (2002) Enhanced resistance to seed-transmitted bacterial diseases in transgenic rice plants overproducing an

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

oat cell-wall-bound thionin. *Mol Plant Microbe Interact* **15**: 515–521.

Jeong Y, Kim J, Kim S, Kang Y, Nagamatsu T & Hwang I (2003) Toxoflavin produced by *Burkholderia glumae* causing rice grain rot is responsible for inducing bacterial wilt in many field crops. *Plant Dis* **87**: 890–895.

Kim J, Kim JG, Kang Y, Jang JY, Jog GJ, Lim JY, Kim S, Suga H, Nagamatsu T & Hwang I (2004) Quorum sensing and the LysR-type transcriptional activator ToxR regulate toxoflavin biosynthesis and transport in *Burkholderia glumae*. *Mol Microbiol* **54**: 21–34.

Kloepper JW, Ryu C-M & Zhang S (2004) Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* **94**: 1259–1266.

Latifi A, Winson MK, Foglino M, Bycroft BW, Stewart GS, Lazdunski A & Williams P (1995) Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. *Mol Microbiol* **17**: 333–343.

Lee SJ, Park SY, Lee JJ, Yum DY, Koo BT & Lee JK (2002) Genes encoding the *N*-acyl homoserine lactone-degrading enzyme are widespread in many subspecies of *Bacillus thuringiensis*. *Appl Environ Microbiol* **68**: 3919–3924.

Lin YH, Xu JL, Hu J, Wang LH, Ong SL, Leadbetter JR & Zhang LH (2003) Acyl-homoserine lactone acylase from *Ralstonia* strain XJ12B represents a novel and potent class of quorum-quenching enzymes. *Mol Microbiol* **47**: 849–860.

McClean KH, Winson MK, Fish L *et al.* (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology* **143**: 3703–3711.

Molina L, Constantinescu F, Michel L, Reimmann C, Duffy B & Défago G (2003) Degradation of pathogen quorum-sensing molecules by soil bacteria: a preventive and curative biological control mechanism. *FEMS Microbiol Ecol* **45**: 71–81.

Nagamatsu T (2001) Syntheses, transformation, and biological activities of 7-azapteridine antibiotics: toxoflavin, fervenulin, reumycin and their analogs. *Recent Res Devel Org Bioorg Chem* **4**: 97–121.

Park SY, Kang HO, Jang HS, Lee JK, Koo BT & Yum DY (2005) Identification of extracellular *N*-acylhomoserine lactone acylase from a *Streptomyces* sp. and its application to quorum quenching. *Appl Environ Microbiol* **71**: 2632–2641.

Parke JL & Gurian-Sherman D (2001) Diversity of the *Burkholderia cepacia* complex and implications for risk assessment of biological control strains. *Annu Rev Phytopathol* **39**: 225–258.

Reinhold-Hurek B & Hurek T (1998) Interaction of gramineous plants with *Azoarcus* spp. and diazotrophs: identification, localization, and perspectives to study their function. *CRC Crit Rev Plant Sci* **17**: 29–54.

Reinhold-Hurek B, Hurek T, Gillis M, Hoste B, Vancanneyt M, Kersters K & De Ley J (1993) *Azoarcus* gen. nov., nitrogen-fixing proteobacteria associated with roots of Kallar grass (*Leptochloa fusca* (L.) Kunth), and description of two species, *Azoarcus indigens* sp. nov. and *Azoarcus communis* sp. nov. *Int J Syst Bacteriol* **43**: 574–584.

Ryu C-M, Hu C-H, Reddy MS & Kloepper JW (2003) Different signaling pathways of induced resistance by rhizobacteria in *Arabidopsis thaliana* against two pathovars of *Pseudomonas syringae*. *New Phytol* **160**: 413–420.

Sato Z, Koiso Y, Iwasaki S & Matsuda I (1989) Toxins produced by *Pseudomonas glumae*. *Ann Phytopathol Soc Jpn* **55**: 356–359.

Suzuki F, Zhu Y, Sawada H & Matsuda I (1998) Identification of proteins involved in toxin production by *Pseudomonas glumae*. *Ann Phytopathol Soc Jpn* **64**: 75–79.

Ueda T, Suga Y, Yahiro N & Matsuguchi T (1995) Remarkable N_2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of *nifH* gene sequences. *J Bacteriol* **177**: 1414–1417.

Uematsu T, Yoshimura D, Nishiyama K, Ibaragi T & Fujii H (1976) Pathogenic bacterium causing seedling rot of rice. *Ann Phytopathol Soc Jpn* **42**: 464–471.

Urakami T, Ito-Yoshida C, Araki H, Kijima T, Suzuki KI & Komagata K (1994) Transfer of *Pseudomonas planatarii* and *Pseudomonas glumae* to *Burkholderia* as *Burkholderia* spp. and description of *Burkholderia vandii* sp. nov. *Int J Syst Bacteriol* **44**: 235–245.

Waters CM & Bassler BL (2005) Quorum sensing: cell-to-cell communication in bacteria. *Annu Rev Cell Dev Biol* **21**: 319–346.

Wilmotte A, Van der Auwera G & De Wachter R (1993) Structure of the 16S ribosomal RNA of the thermophilic cynobacterium *Chlorogloeopsis* HTF (*Mastigocladus laminosus* HTF) strain PCC7518, and phylogenetic analysis. *FEBS Lett* **317**: 96–100.

Xu F, Byun T, Dussen HJ & Duke KR (2003) Degradation of *N*-acylhomoserine lactones, the bacterial quorum-sensing molecules, by acylase. *J Biotechnol* **101**: 89–96.

Zhang LH & Dong YH (2004) Quorum sensing and signal interference: diverse implications. *Mol Microbiol* **53**: 1563–1571.

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved