

## Detection and Cloning of a Gene Involved in Zwitermicin A Synthesis from Plant Growth Promoting Rhizobacteria of *Bacillus* sp CR64

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### Abstract

Utilization of soil bacteria as biocontrol agent is becoming popular due to its valuable and effective mechanisms to suppress plant pathogenic microbes. We have previously isolated *Bacillus* sp, designated as *Bacillus* sp CR64, which exhibited effective plant growth promoting and antifungal activities. In this study, CR64 was examined in inhibiting the growth of *Rhizoctonia solani*, the causing agent of root rot disease. Partial sequence analysis of 16S rRNA gene revealed that this isolate similar with *Bacillus cereus* (94%). Furthermore, a gene designated *zmaR* was detected by means of specific amplification of DNA fragment approximately 950 bp. This fragment was then cloned onto pCRII-TOPO (3.9 kb) and sequenced using DNA sequencer ABI PRISM 310. Sequence analysis revealed that it had highest homology with the *ZmaR* protein (89% identity; 90% similarity) of *B. thuringiensis* serovar *kurstaki* (AAF82729.2). Alignment analysis with other *ZmaR* sequences from other antibiotic-producing Bacilli exhibited an almost fully conserved region within *ZmaR* sequences.

**Key words :** PGPR, *Bacillus* sp CR64, Zwitermicin A, Cloning, Antifungal.

### Introduction

Antibiotic-producing bacteria are continuously becoming an interesting topics for many researchers across the globe due to its remarkable application mostly in pharmacy and agriculture. Many assays for detecting antibiotics have been developed to improve its accuracy and reproducibility. Nowadays, the development of molecular approaches is have becoming applicable for many research fields, including antibiotics detection. Hence, in this study, we applied the molecular assays in order to detect the capability of bacteria to produce zwitermicin A.

Previously, we have already isolated *Bacillus* sp strain Cr64 that potential for its application as inoculants in crops. This strain is able to produce IAA hormone, solubilize phosphate, and promote soybean seed germination *in vitro* (Wahyudi *et al.* 2007). Moreover, Cr64 has no hypersensitivity response to tobacco leaves, so that this strain readily applicable. In order to explore plant growth promoting activities of this isolate, it is necessary to investigate their potential capability as biocontrol. Many biocontrol mechanisms have been recorded, so far, including antibiotic, HCN, siderophore, and hydrolytic enzymes (Haas & Defago 2005).

Zwitermicin A is a novel antibiotic that does not belong to any previously described class of antibiotics (Silo-Suh *et al.* 1994). It is built by linear aminopolyol compound that has a broad target range. It is able to inhibit the growth of many eukaryotes and prokaryotes, mainly certain plant pathogenic fungi such

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as *Phytophthora* and *Pythium* (Silo-Suh *et al.* 1998). Moreover, Zwittermicin A had been reported for its synergist interaction with Bt toxin to increase insecticidal activity of *Bacillus thuringiensis*. The mode of action of zwittermicin remarked unusual from other antibiotics, which does not appear to inhibit RNA transcription *in vivo* (Stabb & Handelsman 1998).

In order to investigate bacteria that are able to produce zwittermicin A, we have carried out a PCR technique by using specific primer of *ZmaR* obtained from Milner *et al.* (1996). *ZmaR* gene represents sequence of DNA that expressed protein for defending the cell from Zwittermicin A activity, and thus becoming a self resistance cell. *ZmaR* gene is always present in bacteria that produce zwittermicin A for protecting the bacteria from zwittermicin compound. Therefore, *ZmaR* gene is often used as reference sequence to detect zwittermicin-producing bacteria (Milner *et al.* 1996). This study demonstrated molecular detection and cloning of zwittermicin A gene of *Bacillus* sp CR64.

## Materials and Methods

### *Bacterial strains, plasmids, and culture conditions*

Isolate of *Bacillus* sp. Cr64 was routinely grown in Nutrient Agar (NA). The fungal phytopathogen was maintained in Potato Dextrose Agar (PDA) and incubated for 3-5 days at room temperature. A plasmid pCRII-TOPO (Invitrogen, USA) was used for TA cloning, and *E. coli* TOP10 was used as a host cell for transformation. Molecular works such as plasmid isolation, transformation, and plasmid digestion were carried out according to the standard method (Sambrook & Russell 2001).

### *Isolation of Bacillus sp from the rhizosphere.*

Rhizosphere soil samples were collected from soybean growing fields in Plumbon, Cirebon, West Java, Indonesia. *Bacillus* species

were isolated using dilution method with Nutrient Agar medium. First dilution of soil was boiled at 80°C for 10 min in order to induce endospore formation of *Bacillus*. Each colony was assayed further for morphological and physiological characteristics including Gram reaction, endospore, and catalase enzyme activity. *Bacillus* species were estimated by morphologies and physiologies characteristics based on Bergey's Manual of Systematic Bacteriology.

### *Antagonism assay*

*Bacillus* sp. Cr64 isolate was assayed for antifungal activity against soil-borne fungal phytopathogens *Rhizoctonia solani* using Potato Dextrose Agar (PDA). *Bacillus* sp Cr64 was inoculated on PDA medium 3 cm in distance opposite to pathogenic fungi inoculated at the center of the medium. The barrier between Cr64 isolate and fungi indicated antagonist interaction between them. Antagonist activities were investigated for 4-7 days after inoculation. The value of inhibition was measured using the formula described by Kumar *et al.* (2002) which is  $1 - (a - b) \times 100\%$  (a : distance between fungi in the center of Petri dish to *Bacillus* isolate, b: distance between fungi in center of Petri dish and blank area without *Bacillus* isolate). *Bacillus* sp CR64 isolate that showed antagonist interaction toward *R. solani* was used for further assay.

### *Sequence analysis of 16S rRNA*

The isolation of genomic DNA was done with Cetyl Trimethyl Ammonium Bromide (CTAB) method (Wilson 1994). Amplification of 16S rRNA gene was carried out by PCR using specific primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387r (5'- GGG CGG WGT GTA CAA GGC-3') (Marchesi *et al.* 1998). The PCR condition was carried out for 30 cycles including pre-denaturation step for 2 min at 94°C, denaturation for 30 sec at 94°C, annealing for 30 sec at 55°C, elongation for 1 min at 75°C and post PCR for 10 min at

75°C. All of PCR products were purified and sequenced using ABI 310 (Perkin Elmer, USA). Similarity of the 16S rRNA gene sequence of the isolate was aligned against GenBank database by using the BLASTN program.

#### Detection of *ZmaR* gene by PCR

The isolation of genomic DNA from *Bacillus* isolate was done by using CTAB method. The amplification of *ZmaR* gene was conducted with pair of specific primers (Milner *et al.* 1996). Primer Swt 677 (5' TAA AGCTCGTCCCTCTTCAG-3') and primer Swt 678 (5'-ATGTGCACTTGTATGGGCAG-3') will amplify a specific band at approximately 951 base pairs. The PCR condition was carried out for 30 cycles including pre-denaturation step for 5 min at 94°C, denaturation for 1 min at 94°C, annealing for one min at 50°C or 60°C, elongation for 1 min at 72°C and post PCR for 10 min at 72°C. The PCR mixture total volume was 50 µl containing 1 µl of DNA template (1µg/µl), 1 µl each primer (10 pmol), 8 µl dNTPs, 25 µl Taq polymerase buffer, 0.5 µl LA Taq polymerase (Takara, Japan), and 4.5 µl ddH<sub>2</sub>O. The presence and yield of specific PCR product was visualized in 1% agarose (wt/vol) gel electrophoresis for 35 min at 100 V/cm in TAE 1X buffer. Amplicon was further purified by QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

#### Cloning of *ZmaR* gene in to pCRII-TOPO

Pure *ZmaR* DNA fragment was then ligated in to pCRII-TOPO (Invitrogen, USA) by using TA cloning procedure. The recombinant plasmid, designated as pCRII-TOPO-*zmaR*, was subsequently transformed into competent cells of *E. coli* TOP10. Transformant cells were plated on Luria Agar (LA) supplemented with kanamycin (50 mg/ml), ampicillin (50 mg/ml), and X-Gal (40 mg/ml). The recombinant plasmid from the several white colonies were subsequently isolated and verified for verification by using colony PCR method using *ZmaR* primers.

#### DNA sequence analysis

The recombinant plasmid, pCRII-TOPO-*ZmaR*, produced in the previous step was sequenced using DNA Sequencer ABI PRISM 310 with general M13 primers used for cycle sequencing. The DNA sequence of *ZmaR* gene was further aligned to the GenBank database using the BLASTX program (NCBI). Closest related sequences from the GenBank analysis to the *ZmaR* gene were aligned to each other by using CLUSTALW program to reveal some conserved regions amongs *ZmaR* sequences.

#### Results

##### Isolation of *Bacillus* sp and antagonism assay

In this study, a total of 50 *Bacillus* sp. isolates producing indole acetic acid (IAA) were isolated from the rhizosphere of soybean plant. All those isolates showed *Bacillus* sp characteristics either physiological or morphological criteria including endospore appearance (Bergey's Manual of Determinative Bacteriology). Among 50 *Bacillus* sp. isolates, one isolate was able to inhibit the growth of *R. solani* *in vitro*. This isolate designed as *Bacillus* sp. Cr64 had strong inhibition, with specific inhibition percentage was over than 40%, as shown in Figure 1.

This *Bacillus* sp CR64 was selected and applied for further analysis for detection of genes involved in antifungal activity.



Figure 1. Antagonism assay of *Bacillus* sp. Cr-64 against *Rhizoctonia solani* on PDA medium after 5 days of incubation at room temperature.

### Analysis of 16S rRNA sequence and detection of antifungal gene.

The amplification of 16S rRNA gene of the *Bacillus* sp Cr64 isolate resulted in a specific DNA fragment at approximately 1300 bp. DNA sequence analysis using the BlastN revealed high similarity with *Bacillus sphaericus* (94%). Detection of antifungal gene using polymerase chain reaction (PCR) resulted in a specific band at approximately 950 bp, as shown in Figure 2.

Previous findings by Milner and colleagues observed and sequenced a 1.2-kb fragment of DNA defined an open reading frame, designated as *ZmaR*. In this study we only had partly amplified of the *ZmaR* gene instead of the complete open reading frame.



Figure 2 . DNA fragment (~950 bp) yielded from PCR amplification of *ZmaR* gene using Swt 677 and Swt 678 primer M: marker 1 kb DNA ladder, 1: *ZmaR* gene from *Bacillus* sp. Cr-64

### Cloning and DNA sequence analysis

Partial *ZmaR* DNA sequence was successfully ligated in to pCRII-TOPO (3.9 kb) thus creating a recombinant plasmid, denoted as pCRII-TOPO-*ZmaR* (~4850 pb). The partial sequence of *ZmaR* gene was analyzed for its similarity to the database available at the GenBank. The BLASTX program showed a high similarity of this sequence to the *ZmaR* protein from *Bacillus thuringiensis* serovar *kurstaki* (89 identity; 90% similarity), as shown in. Furthermore, the partial *ZmaR* gene was aligned to the other sequences of *ZmaR* gene possessed by other

bacteria. The alignment analysis shown that mostly 97% of the partial sequences are the same, only 10 amino acids over 375 amino acids were different within *ZmaR* sequences originated from different bacteria (Figure 3).

### Discussion

The capability of *Bacillus* sp. CR64 to inhibit the growth of *R. solani* may involve many biocontrol mechanisms, which is not directly known. Haas and Defago (2005) previously described that antibiotics, HCN, hydrolytic enzymes, and siderophores are comprehended for its capability in inhibiting the growth of fungal pathogens. Nonetheless, this antagonist ability as of Cr64 would be an useful information for its application in controlling fungal pathogens *in planta*.

Positive detection of *ZmaR* gene could also be determined as positive zwittermicin producers. It is due to the fact that *ZmaR* is part of the zwittermicin A biosynthetic cluster, which involved in cell-resistance mechanism (Emmert *et al.*, 2004). In this study, Cr64 was positively having *ZmaR* gene as revealed by PCR amplification using *ZmaR* specific primer. The specific band resulted from the PCR was about 950 bp, while Milner *et al.* (1996) showed the whole ORF of *ZmaR* as 1.2 Kb sequence, so that we have only partially amplified this gene. According to the BLASTX analysis, the amplified sequence exhibited closest homology (89% identity; 90% similarity) to the *ZmaR* protein from *Bacillus thuringiensis* serovar *kurstaki* (AAF82729). Stabb *et al.* (1994) have previously reported diverse strains of *B. cereus* and *B. thuringiensis* isolated from variety of soils produced antibiotic Zwittermicin A, and able to control the growth of *B. cereus* UW85. Zwittermicin A-producing strains can be isolated from soils that are geographically, physically and biologically diverse. Zwittermicin A has diverse biological activities including suppression of oomycete diseases of plants and potentiation of the insecticidal activity of *B. thuringiensis* (Emmert *et al.* 2004). Therefore

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B. cereus AH1134      MIYELVKEKDEFKQISPLLVYKKNHPILNGIAHGYNKGHIYVDNPNNPRCALVWAEQEIF
B. thuringiensis GO3 MIYELVKEKDEFKQISPLLVYKKNHPILNGIAHGYNKGHIYVDNPNNPRCALVWAEQEIF
Bacillus Cr-64      MIYELVKEKDEFKQISPLLVYKKNHPILNGIAHGYNKGHIYVDNPNNPRCALVWAEQEIF
B. cereus UW85      MIYELVKEKDAFKQISPLLVYKKNHPILNGIAHGYNKGHIYVDNPNNPRCALVWAEQEIF
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B. cereus AH1134      YLLGDPTSNFVSSLPTFIKEVIAPEAQQIGDDFFQVELLPESKWKQVVEEQQLQMFIPKPY
B. thuringiensis GO3 YLLGDPTSNFVSSLPTFIKEVIAPEAQQIGDDFFQVELLPESKWKQVVEEQQLQMFIPKPY
Bacillus Cr-64      YLLGDPTSNFVSSLPTFIKEVIAPEAQQIGDDFFQVELLPESKWKQVVEEQQLQMFIPKPY
B. cereus UW85      YLLGDPTSNFVSSLPTFIKEVIAPEAEQIGDDFFQVELLPESKWKQVVEEQQLQMFIPKPY
*****

B. cereus AH1134      TRVTFTFDPEYNKLSKPNMPDEVTVERITIDMLSDDKFKAMVRDDIVDFWESTQDFIRNG
B. thuringiensis GO3 TRVTFTFDPEYNKLSKPNMPDEVTVERITIDMLSDDKFKAMVRDDIVDFWESTQDFIRNG
Bacillus Cr-64      TRVTFTFDPEYNKLSKPNMPDEVTVERITIDMLSDDKFKAMVRDDIVDFWESTQDFIRNG
B. cereus UW85      TRVTFTFDPEYNKLSKPNMPEEVTVERITIDMLSDDKFKAMVRDDIVDFWESTQDFIQNG
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B. cereus AH1134      FGYVVMVHEQVVTSCLSVFATDVEIGINTYDLFQRGKGYAWLAARAFDDCLKQGRTP
B. thuringiensis GO3 FGYVVMVHEQVVTSCLSVFATDVEIGINTYDLFQRGKGYAWLAARAFDDCLKQGRTP
Bacillus Cr-64      FGYVVMVHEQVVTSCLSVFATDVEIGINTYDLFQRGKGYAWLAARAFDDCLKQGRTP
B. cereus UW85      FGYVVMVHEQVVTSCLSVFATDVEIGINTYDLFQRGKGYAWLAARAFDDCLKQGRTP
*****

B. cereus AH1134      HWKTEDFRIPSIKLAGVGFNTLQTYTAYVFPYNELDNFVFTAYHQLRYYSDFNKASEFV
B. thuringiensis GO3 HWKTEDFRIPSIKLAGVGFNTLQTYTAYVFPYNELDNFVFTAYHQLRYYSDFNKASEFV
Bacillus Cr-64      HWKTEDFRIPSIKLAGVGFNTLQTYTAYVFPYNELDNFVFTAYHQLRYYSDFNKASEFV
B. cereus UW85      HWKTEDFRIPSIKLAGVGFNTLQTYTAYVFPYNELDNFIFTAYHQLRYYSDFNKASEFV
*****

B. cereus AH1134      QKARTIGDLNAWHHFLSCGYSLIDRIDLSLKHMNALDLGWNDVSDIRYVVDLVNLRKT
B. thuringiensis GO3 QKARTIGDLNAWHHFLSCGYSLIDRIDLSLKHMNALDLGWNDVSDIRYVVDLVNLRKT
Bacillus Cr-64      QKARTVGDNLNAWHHFLSCGYSLIDRIDLSLKHMNALDLGWNDVSDIRYVVDLVNLRKT
B. cereus UW85      QKARTIGDLNAWHHFLSCGYSLIDRIDLSLKHMNALDLGWNDVSDIRYVVDLVNLRKT
*****

B. cereus AH1134      EEGRALLDRVEDSLR
B. thuringiensis GO3 EEGRALLDRVEDSLR
Bacillus Cr-64      EEGRALLDRVEDSLR
B. cereus UW85      EEGRALLDRVEDSLR
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Figure 3. Alignment of several *ZmaR* sequences (from GenBank) to the *ZmaR* sequence derived from *Bacillus* sp Cr64. \* similar amino acid between sequences (conserved region). Accession number of the selected *ZmaR* sequence : *B. cereus* AH1134= EDZ49432.1; *B. thuringiensis* = AAX84216.1; *B. cereus* UW85 = AAD40108.1

the capability to produce zwittermicin A can be very useful as biocontrol agent.

Alignment analysis of several *ZmaR* sequences showed that the 950 bp sequence was conserved within *ZmaR* sequence amongs different bacteria (Figure 3).

This result indicates a conserve sequence that relies in between Swt 677 and 678 primers are conserved amongs bacteria, mostly from the genera of *Bacillus*.

The detection of antibiotic that may be involved in biocontrol mechanism possessed by bacteria can be classified as preliminary

study, yet this step is still an essential. It is due to the fact that the successful use of existing microorganism or construction of improved variants for control of plant disease will be enhanced by understanding the mechanisms of biological control at the molecular and biochemical levels and by determining the basis for variability in biological control in the agroecosystem (Silo-Suh *et al.* 1994). The Cr-64 can be grouped as the potential inoculants according to its plant promoting characters. Instead of having the phytostimulator capabilities, Cr64 was

observed as the zwittermicin producers as the result from this study. Moreover, Cr64 has been also detected as the producer for another antibiotic compound which were iturin and bacilysocin (unpublished data). Thus, the application of Cr64 in the crop yield would be very promising in respect to its ability in producing antibiotic that may affect a broad range of pathogen targets. As previously revealed by Emmert *et al.* (2004), the antibiotic zwittermicin has a broad spectrum of activity, inhibiting certain gram-positive, gram-negative, and eukaryotic microorganisms. It also potentiates the insecticidal activity of the protein toxin produced by *Bacillus thuringiensis*, increasing mortality of insects that are typically recalcitrant to killing, such as gypsy moths reared on willow leaves.

#### Acknowledgement

This work was supported by Incentive Program for Basic Research from the Ministry of Research and Technology (KNRT-RISTEK) 2009 awarded to ATW. Part of this work was conducted at Tokyo University of Agriculture and Technology, Japan, especially at Department of Biotechnology and Life Sciences (Matsunaga Laboratory). Therefore, We are grateful to Prof. Tadashi Matsunaga for the laboratory facilities and valuable supports.

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