Indonesian Journal of Biotechnology, June, 2010 Vol. 15, No. 1, pp. 9-14

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

Aris Tri Wahyudi[1]*, Rika Indri Astuti[1], Nisa Rachmania Mubarik[1], and Sarah Asih Faulina[1]

[1]Microbiology Division, Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University (IPB), Bogor, Indonesia

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 *zma*R 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 *Zma*R sequences from other antibiotic-producing Bacilli exhibited an almost fully conserved region within *Zma*R sequences.

Keyword : 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,

*)Corresponding Author : Aris Tri Wahyudi, Microbiology Division, Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University (IPB), Jl. Agatis - Kampus IPB Darmaga, Bogor 16680, Phone / Fax : (+62) 251 8622833; Email: aristri2003@yahoo.com or ariswa@ipb.ac.id

mainly certain plant pathogenic fungi such as *Phytophthora* and *Pythium* (Silo-Suh *et al.*, 1998). Moreover, Zwittermicin A had been reported for its synergistist 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 produced zwittermicin A, we have carried out a PCR technique by using specific primer of *Zma*R obtained from Milner *et al.* (1996). *Zma*R gene represents sequence of DNA that expressed protein for defending the cell from Zwitermicin A activity, and thus becoming a self resistance cell. *Zma*R gene is always present in bacteria that produce zwitermicin A for protecting the bacteria from zwitermicin compound. Therefore, *Zma*R gene is often used as reference sequence to detect zwitermicin-producing bacteria (Milner *et al.*, 1996). This study demonstrated molecular detection and cloning of zwitermicin 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, Indonsia. Bacillus species were isolated using dilution method with Nutrient Agar medium. First dilution of soil was boiled at 80°C for 10 minutes 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 Bergeys' 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) x 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 minutes at 94°C, denaturation for 30 seconds at 94°C, annealing for 30 seconds at 55°C, elongation for 1 minute at 75°C and post PCR

for 10 minutes at 75^{0}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 *Zma*R 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 minutes at 94^{0}C, denaturation for 1 minute at 94^{0}C, annealing for one minute at 50^{0}C or 60^{0}C, elongation for 1 minute at 72^{0}C and post PCR for 10 minutes at 72^{0}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_2O. The presence and yield of specific PCR product was visualized in 1% agarose (wt / vol) gel electrophoresis for 35 minutes 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 *Zma*R 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), ampicilin (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-*Zma*R, 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 *Zma*R gene was further aligned to the GenBank database using the BLASTX program (NCBI). Closest related sequences from the GenBank analysis to the *Zma*R gene were aligned to each other by using CLUSTALW program to reveal some conserved regions amongs *Zma*R 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). Amongs 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.

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

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

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 sphericus* (94%). Detection of antifungal gene using polymerase chain reaction (PCR) resulted in a specific band at approximately 950 bp, as shown in Figure 2.

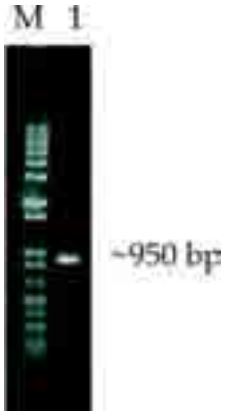

Figure. 2 . DNA fragment (~950 bp) yielded from PCR amplification of *Zma*R gene using Swt 677 and Swt 678 primer M: marker 1 kb DNA ladder, 1: *Zma*R gene from *Bacillus* sp. Cr-64

Previous findings by Milner *et al.*(1996) observed and sequenced a 1.2-kb fragment of DNA defined an open reading frame, designated as *Zma*R. In this study we only had partly amplified of the *Zma*R gene instead of the complete open reading frame.

Cloning and DNA Sequence Analysis

Partial *Zma*R 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 *Zma*R 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 *Zma*R gene was aligned to the other sequences of *Zma*R 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 *Zma*R gene could also be determined as positive zwitermicin producers. It is due to the fact that *Zma*R 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 *Zma*R 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 *Zma*R 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 thringiensis* 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 Zwitermicin A, and able to control the growth of *B. cereus* UW85. Zwitermicin A-producing strains can be isolated from soils that are geographically, physically and biologically diverse. Zwitermicin 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 the capability to produce zwitermicin A can be very useful as biocontrol agent.

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

```
B. cereus AH1134          MIYELVKEKDEFKQISPLLVDYKNHPILNGIAHGYNKGHIYVDNPNNPRCALVWAEQEIF
B. thuringiensis GO3      MIYELVKEKDEFKQISPLLVDYKNHPILNGIAHGYNKGHIYVDNPNNPRCALVWAEQEIF
Bacillus Cr-64            MIYELVKEKDEFKQISPLLVDYKNHPILNGIVHGYNKGHIYVDNPNNPRCALVWAEQEIF
B. cereus UW85            MIYELVKEKDAFKQISPLLVDYKNHPILNGIAHGYNKGHIYVDNPNNPRCALVWAEQEIF
                          ********** ********************.**************************

B. cereus AH1134          YLLGDPTSNFVSSLPTFIKEVIAPEAQQIGDDFFQVELLPESKWKQVVEEQLQMFIPKPY
B. thuringiensis GO3      YLLGDPTSNFVSSLPTFIKEVIAPEAQQIGDDFFQVELLPESKWKQVVEEQLQMFIPKPY
Bacillus Cr-64            YLLGDPTSNFVSSLPTFIKEVIAPEAQQIGDDFFQVELLPESKWKQVVEEQLQMFIPKPY
B. cereus UW85            YLLGDPTSNFVSSLPTFIKEVIAPEAEQIGDDFFQVELLPESKWKQVVEEQLQMFIPKPY
                          **************************:*********************************

B. cereus AH1134          TRVTFTFDPESYNKLSKPNMPDEVTVERITIDMLSDKKFAMVRDDIVDFWESTQDFIRDG
B. thuringiensis GO3      TRVTFTFDPESYNKLSKPNMPDEVTVERITIDMLSDKKFAMVRDDIVDFWESTQDFIRNG
Bacillus Cr-64            TRVTFTFDPESYNKLSKPNMPDEVTVERITIDMLSDKKFAMVRDDIVDFWESTQDFIRNG
B. cereus UW85            TRVTFTFDPESYNKLSKPNMPEEVTVERITIDMLSDKKFAMVRDDIVDFWESTQDFIQNG
                          **********************:***********************************::*

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



B. cereus AH1134          HWKTEDFRIPSIKLAGKVGFTNLQTYTAYVFPYNELDNFVFTAYHQLRYYSDFNKASEFV
B. thuringiensis GO3      HWKTEDFRIPSIKLAGKVGFTNLQTYTAYVFPYNELDNFVFTAYHQLRYYSDFNKASAFV
Bacillus Cr-64            HWKTEDFRIPSIKLAGKVGFTNLQTYTAYVFPYNELDNFVFTAYHQLRYYSDFNKASEFV
B. cereus UW85            HWKTEDFRIPSIKLAGKVGFTNLQTYTAYVFPYNELDNFIFTAYHQLRYYSNFNKASEFV
                          ****************************************:**********:***** **

B. cereus AH1134          QKARTIGDLNAWHHFLLSCGYSLIDRIDLSLKHMNLALDLGWNDVSDIRYVVDLVNLRKT
B. thuringiensis GO3      QKARTIGDLNAWHHFLLSCGYSLIDRIDLSLKHMNLALDLGWNDVSDIRYVVDLVNLRKT
Bacillus Cr-64            QKARTVGDLNAWHHFLLSCGYSLIDRIDLSLKHMNLALDLGWNDVSDIRYVVDLVNLRKT
B. cereus UW85            QKARTIGDLNAWHHFLLSCGYSLIDRIDLSLKHMNLALDLGWNDVSDIRYVVDLVNLRKT
                          *****:******************************************************

B. cereus AH1134          EEGRALLDRVEDSLR
B. thuringiensis GO3      EEGRALLDRVEDSLR
Bacillus Cr-64            EEGRALLDRVEDSLR
B. cereus UW85            EEGRALLDRVEDSLR
                          ***************
```

Figure 3. Alignment of several *Zma*R 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

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 zwitermicin 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 zwitermicin 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.

References

Emmert EAB, Klimowicz AK, Thomas MG, Handelsman J. 2004. Genetics of Zwittermicin A Production by *Bacillus cereus*. *Appl. Environ. Microbiol.* 70, 104–113.

Haas D, Defago G. 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nature Rev. Microbiol.* 1038,1-13.

Kumar NR, Arasu VT, Gunasekaran P. 2002. Genotyping of antifungal compounds producing plant promoting rhizobacteria, *Pseudomonas fluorescens*. *Curr. Sci.* 82, 1463-1466.

Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Dymok D, Wade WG. 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacteria 16S rRNA. *Appl. Environ. Microbiol.* 64, 795-799.

Milner JL, Stohl EA, Handelsman J. 1996. Zwitermicin A resistance gene from *Bacillus cereus*. *Jour. Bacteriol.* 178, 4266-4272.

Sambrook W, Russel DW. 2001. *Molecular Cloning: A Laboratory Manual*. Ed ke-3. New York: Gold Spring Harbor Laboratory.

Silo-Suh LA, Lethbridge BJ, Raffel SJ, He H, Clardy J, Handelsman J. 1994. Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85. *Appl. Environ. Microbiol.* 60, 2023-2030.

Silo-Suh LA, Stabb EV, Raffel SJ, Handelsman J. 1998. Target range of zwittermicin A, an aminopolyol antibiotic from *Bacillus cereus*. *Curr. Microbiol*.37,6-11.

Stabb EV, Jacobson LM, Handelsman J.1994. Zwittermicin A-Producing Strains of Bacillus cereus from Diverse Soils. *Appl. Environ. Microbiol.* 60, 4404-4412.

Stabb EV, Handelsman J. 1998. Genetic analysis of zwittermicin A resistance in *Escherichia coli*: effects on membrane potential and RNA polymerase. *Mol. Microbiol*. 27:311-322.

Wahyudi AT, Astuti RI, Rachmania N. 2007. Rizobakteria pemacu pertumbuhan tanaman kedelai (*Glycine max*): isolasi, karakterisasi, dan peningkatan pemacuan pertumbuhan. LPPM IPB: Laporan Akhir Hasil Penelitian Insentif Riset Dasar KNRT.

Wilson, HD, Doebly J, Duvall M. 1994. Chloroplast DNA diversity among wild and cultivated members of Cucurbita (Cucurbitaceae).
Jour.Am.Soc.Hor.Sci. 119, 110-115