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RESEARCH PAPER

Screening, genetic diversity of *aii*A gene in AHL-lactonase producing bacteria and their potential to suppress the virulence factors of *Ralstonia syzygii* subsp. *indonesiensis*

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ABSTRACT

Ralstonia syzigii subsp. *indonesiensis* (Rsi) is a causal pathogen of Phylotype IV within the *Ralstonia solanacearum* species complex, commonly found in chili plants. This pathogenic bacterium uses a quorum sensing (QS) mechanism that relies on N-acyl homoserine lactone (AHL) signals to regulate the expression of virulence genes, such as those encoding exopolysaccharides (EPS). Biological control of Rsi can be achieved by disrupting its QS system. The aim of this study was to isolate AHL-lactonase-producing bacteria, analyze the genetic diversity of their *aiiA* gene, and evaluate their effectiveness in suppressing EPS production in Rsi. The research involved sampling, isolating, and screening bacterial candidates from chili plants as AHL-lactonase producers using bioassays and molecular techniques, followed by evaluation of their ability to inhibit EPS expression as a virulence factor of Rsi. Bacterial samples were isolated from Brebes, Bandung, and Garut Regencies. Molecular identification revealed that the twelve selected isolates belonged to the genus Bacillus. Sequencing results showed genetic diversity in the aiiA gene among isolates obtained from regions with different altitudes. All isolates demonstrated the ability to suppress Rsi virulence factors.

Key words: *Bacillus,* biocontrol agents, degrading enzymes, quorum quenching

INTRODUCTION

Chili peppers are crucial horticultural crops widely consumed by the Indonesian population. This commodity is extensively used as a spice in cooking, as well as in cosmetics and health products (Ashari, 2006). The productivity of chili pepper plants in Indonesia from 2021 to 2023 was 9.45, 10.16, and 10.71 tons/ha, respectively (Kementan, 2024). However, according to Rofidah et al. (2018), the productivity of red chili peppers in Indonesia remains low, despite its potential to reach up 22 tons/ha. One of the major challenges in chili pepper production in Indonesia is the attack of pests and plant pathogens that can cause significant yield losses, such as bacterial wilt disease.

Bacterial wilt is caused by the Gram-negative bacterium *Ralstonia syzigii* subsp. *indonesiensis* (formerly known as *R. solanacearum*) (Safni et al., 2014). *Ralstonia syzigii* subsp. *indonesiensis* (Rsi) is causal pathogen belonging to Phylotype IV within the *R. solanacearum* species complex, which the corresponds to isolates found in chili plants in Indonesia. This bacterium has a broad host range, affecting over 200 species from 20 different plant families across Asia and Australia (Hayward, 1986). Furthermore, Rsi has been reported to infect plants latently and can proliferate without causing visible wilting symptoms (Machmud & Hayward, 1992).

The virulence of Rsi is influenced by its ability to produce exopolysaccharides (EPS, which are regulated through a quorum sensing (QS) mechanism. Quorum sensing (QS) is a bacterial communication system that depends on cell population density (Rutherford & Bassler, 2012). It functions by transmitting chemical signals within bacterial communities (Zhao et al. 2020). These signaling molecules generally include: (1) acyl-homoserine lactones (AHLs), (2) auto-inducing peptides (AIPs), and (3) auto-inducer 2 (AI-2) (Huang et al., 2016). Gram-negative plant-pathogenic bacteria typically use AHLs as QS signals to regulate their pathogenicity and virulence (LaSarre & Federle, 2013).

Controlling Rsi can be achieved by preventing the accumulation of AHLs through their degradation. The anti-QS mechanism works by disrupting the communication pathways of bacteria, thus reducing the expression of virulence factors without killing the bacterial population within plant tissues. AHL-

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lactonase is an anti-QS enzyme that inhibits the quorum sensing process by enzymatically hydrolyzing the lactone ring in AHL molecules, thereby preventing the expression of pathogenicity genes (Dong et al., 2000). The AHL-lactonase-producing bacterium *Bacillus cereus* VT96 has been reported to control the production of the virulence factor pyocyanin in *Pseudomonas aeruginosa* PAO1 (Rajesh & Rai, 2016). Additionally, *B. cereus* DBK2 has been reported to suppress the production of the virulence factor EPS1 in *R. syzigii* subsp. *celebesensis* (Abidin, 2018).

The use of AHL-lactonase-producing bacteria is considered a promising and environmentally safe alternative for controlling Rsi without causing negative effects on host plants. However, the potential of AHL-lactonase-producing bacteria in chili pepper plants and their effectiveness as biological agents against Rsi have not been extensively studied or understood.

The aim of this study is to isolate AHL-lactonase-producing bacteria, analyze the genetic diversity of their *aiiA* gene, and assess their ability to suppress EPS production as virulence factor of Rsi.

MATERIALS AND METHODS

Research Site. The research was conducted at the Plant Bacteriology Laboratory and the Cikabayan Experimental Garden Greenhouse, Department of Plant Protection, Faculty of Agriculture, Bogor Agricultural University.

Collection of Plant Samples. Samples of AHL-lactonase-producing bacteria from chili plants were collected from Brebes (2 m above sea level), Garut (717 m above sea level), and Bandung (1400–1600 m above sea level) Regencies. Samples were taken from healthy chili plants, including stems, roots, and leaves, located among diseased plants. The parts collected included healthy stems, roots, and leaves.

Isolation of *Ralstonia syzygii* subsp. *indonesiensis* from Chili Plants. Stems from diseased chili plants showing with wild symptoms were collected from Bogor. These stems were cut transversely and placed into test tubes, then left for 30 min to allow the bacterial ooze to be released. The bacterial suspension was streaked onto a selective medium for Rsi, namely 2,3,5-triphenyl tetrazolium chloride (TZC) agar (containing peptone, sucrose, cassamino acid, and TZC solution), and incubated at room temperature for 24 hours. Pure isolates were then transferred to Nutrient Agar (NA) from the Himedia brand for further cultivation and characterization.

Identification of *Ralstonia syzygii* subsp. *indonesiensis* in Chili Plants. The bacterial isolates obtained were tested for growth on TZC medium and incubated at room temperature (29 ºC) for 48–72 hours. Identification was based on the morphological characteristics of the colonies on TZC medium (after 3 days) and confirmed using molecular techniques. The Rsi colonies were verified molecularly using PCR, following the method of Opina et al. (1997), with specific primers *Ralstonia solanacearum* 759R (5'-GTC GCC GTC AAC TCA CTT TCC-3') and 760F (5'-GTC GCC GTC AGC AAT GCG GAA TCG-3'), with amplify a 280 bp fragment.

Preparation of *Chromobacterium violaceum.* The *C. violaceum* isolate used in this study was obtained from the Plant Bacteriology Laboratory, Department of Plant Protection, IPB Unversity. The isolate was rejuvenated on Luria Agar medium (composed of tryptone, sodium chloride, yeast extract, and agar).

Isolation of AHL-Lactonase-Producing Bacteria. Isolation began by washing healthy plant samples with sterile distilled water, followed by grinding 1 g of each sample. The homogenates were serially diluted up to 10^{-5}, and 0.1 mL of each dilution was spread onto NA using glass beads. Colonies that grew were selected based on their morphology, including color, shape, margin, and elevation.

Anti-QS Activity Test against *Chromobacterium violaceum*. The anti-QS or quorum quenching (QQ) activity test was conducted to assess inhibition of violacein expression in *C. violaceum,* which is regulated via the quorum sensing mechanism. The assay was performed using the disc diffusion method with a double-layer culture plate technique, as describe by Song et al. (2012), with modifications.

Detection of the aii*A* Gene Encoding AHL-Lactonase. Detection of the *aiiA* gene began with total DNA extraction from bacterial isolates, following the method of Atashpaz et al. (2010). Amplification of the gene was performed using the forward primer aiiA1 (5'-ATG ACA GTA AAR AAR CTT TAT TTC-3') and the reverse primer aiiA2 (5'-TCA CTA TAT ATA YTC MGG GAA CTC-3') (R= A/G, Y = C/T, M = A/C), which target a 753 bp fragment (Pan et al., 2008). PCR products were sent to a commercial sequencing service. The sequencing results were processed using

BioEdit software and analyzed by comparing DNA sequence similarity in the GenBank database using the BLAST tool at NCBI. Phylogenetic relationships among the isolates were constructed using MEGA 11 (Molecular Evolutionary Genetics Analysis) software (Tamura et al., 2021).

In Vitro EPS Suppression by AHL-producing bacteria. Rsi isolates were grown in 10 mL of liquid medium supplemented with 1 mL of supernatant from AHL-lactonase-producing bacteria and shaken for 24 hours. A 2 mL aliquot of the suspension was transferred to a microcentrifuge tube and centrifuged at 4 ºC for 20 min at 6000 rpm. The resulting supernatant was transferred to a new microcentrifuge tube, and cold ethanol (95%) was added in a 2:1 ratio relative to the supernatant volume, then left overnight. The mixture was centrifuged again at 6000 rpm at 4 ºC for 20 min. The resulting pellet was dried in an oven at 55 ºC for 24 hours and then weighed to determine the dry weight of EPS.

RESULTS AND DISCUSSION

Ralstonia syzygii subsp. *indonesiensis* (Rsi) bacteria were isolated from 12-week-old chili pepper plants in Cikarawang, Bogor Regency. The plant samples exhibited typical symptoms of bacterial wilt disease, including wilting, stunted growth, and yellowing leaves leading to leaf drop. Thsese symptoms are consistent with the findings of Palupi et al. (2015), which reported that bacterial wilt in chili plants begins with apical wilting that spreads throughout the plant, followed by leaf yellowing, desiccation, and defoliation.

On TZC medium, the characteristics of the Rsi isolate were observed as irregular, mucoid colonies with a red center and white edges. Virulent Rsi isolates are typically white with a pale red center, broad and elevated, and exhibit a fluid consistency. Rsi has a rod-shaped, slightly curved morphology measuring 0.5–1.0 µm × 1.5–5.0 µm and moves using one or several polar flagella. It is classified as Gram-negative, aerobic bacterium (Safni et al., 2004).

Molecular confirmation was conducted using specific primers *Ralstonia solanacearum* 759R and 760F. PCR amplification was successful, indicated by the appearance of a 280 bp DNA band. This result aligns with Opina et al. (1997), who reported a similar fragment size in *R. solanacearum* from potato plants using the same primer pair (data not shown).

Bacterial isolates were also obtained from chili plant samples collected in Bandung, Brebes, and Garut Regencies. The goal was to obtain a diverse and abundant collection of AHL-lactonase-producing bacterial isolatesfor potential anti-quorum sensing (anti-QS) activity. A total of 382 isolates were successfully obtained from roots, stems, leaves, and soil. As shown in Table 1, Garut yielded the highest number of isolates (142: 28 from roots, 18 from leaves, 46 from stems, and 50 from soil), followed by Bandung (128: 28 from roots, 36 from leaves, 26 from stems, and 38 from soil), and Brebes (112: 34 from roots, 24 from leaves, 22 from stems, and 34 from soil).

Soil yielded the highest number of bacterial isolates (Table 1). According to Kafrawi et al. (2015), soil serves as a medium rich in microbial life, especially in the rhizosphere, which harbors beneficial microbes that interact directly or indirectly with plant roots. Niswati et al. (2008) also noted that microbial diversity

Table 1. Total number of bacterial isolates obtained from healthy chili plant samples collected from three different locations

Regency	Sub-regency	Number of isolates				Total isolate
		Root	Leaf	Stem	Soil	
Bandung	Marga Mekar	8	12	16	16	
	Marga Mulya	12	16	8	12	128
	Pangalengan	8	8	2	10	
Brebes	Jatibarang	16	14	8	8	
	Dukuhturi	6	2	8	16	112
	Brebes	12	8	6	8	
Garut	Margalaksana	16	8	16	16	
	Dawungsari	8	2	8	18	142
	Sukaratu	4	8	22	16	
Total		90	78	94	120	382

in the rhizosphere is generally higher than in bulk soil.

The subsequent screening of isolates was conducted using anti-QS activity tests against *C. violaceum*. Degradation activity was indicated by the absence of purple coloration around the filter paper. The non-purple inhibition zone observed in the anti-QS test during the screening of AHL-lactonase-producing bacteria signified the inhibition of violacein expression by the test bacteria (Figure 1). The production of violacein in *C. violaceum* is regulated by a QS mechanism (McClean et al., 1997).

From the 382 bacterial isolates screened, 78 showed degradation activity, as evidenced by the presence of an inhibition zone for violacein production around the filter paper treated with the bacterial supernatant. The degradation activity varied among the five selected AHL-lactonase-producing isolates, likely due to the differences in the ability of their compounds to degrade AHLs from *C. violaceum*. The largest average inhibition zone diameter, 16 mm, was observed in isolates 11 AP, 15 AP, and 80 BDS (Table 2).

Molecular confirmation for the presence of the *aiiA* gene, which encodes AHL-lactonase, was conducted using specific primers (Pan et al., 2008). Of the 78 isolates screened, 12 isolates successfully amplified the *aiiA* gene, producing a DNA band of 753 bp (Figure 2). Isolates 1–4 originated from Bandung,

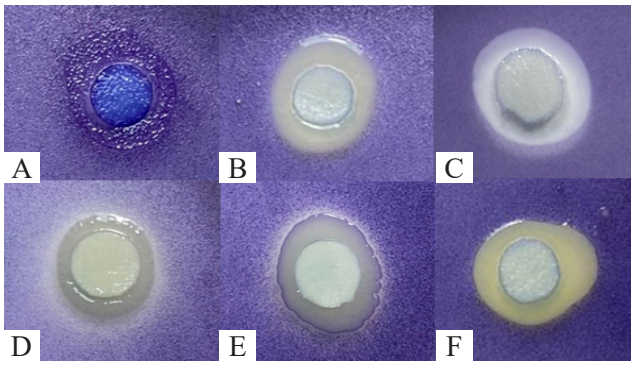

Figure 1. Bioassay of AHL degradation activity by AHL-lactonase-producing bacteria against *Chromobacterium violaceum*. Degradation activity is indicated by a clear (non-purple) zone, as marked by the arrow. A. Negative control with LB medium; B. Isolate 16AP; C. 17DB; D. 101DP; E. 27ASR; F 80BDS.

Table 2. Diameter of the AHL degradation zone in by *Chromobacterium violaceum* caused by various AHL-lactonase-producing bacterial isolates

Isolates	Diameter of the AHL degradation zone (mm)	Isolat	Diameter of the AHL degradation zone (mm)	Isolates	Diameter of the AHL degradation zone (mm)
44TP	12	118DP	4	13TMGL	10
51AP	2	121BP	4	19AMGL	12
61BP	10	123BP	4	27ASR	12
62BP	14	124BP	2	41DMGL	8
63BP	2	125BP	2	43DMGL	4
72DP	4	2AJB	10	56TMGL	14
76DP	2	3AJB	12	65ADS	12
91BP	10	6AJB	10	66ADS	6
92BP	4	7AJB	12	67ADS	10
93BP	4	13TJB	8	68ADS	14
94BP	10	15TJB	10	69BMGL	10
95BP	10	16TJB	8	72BMGL	10
96BP	10	17DB	14	74BMGL	10
97BP	10	19BB	12	75BMGL	10
101DP	8	20BB	14	79BDS	12
102DP	10	21BB	10	80BDS	16

5–8 from Brebes, and 9–12 from Garut. The alignment of the aiiA gene sequences from the 12 isolates revealed several similarities in their nucleotide base sequences, indicating the presence of the aiiA gene in all twelve isolates (Figure 3)

Sequence alignment of the *aii*A gene from the 12 isolates revealed varying levels of homology. Using the ClustalW program, relatedness ranged from 40.2% to 99.8% data not shown. The highest similarity was observed between isolates 31DJB and 27ASR (99.8%), while the lowest was between isolate 11AP and others such as 27ASR, 68ADS, with 40.2% homology. This variation suggests high genetic diversity among isolates, likely influenced by environmental differences such as altitude, temperature, and sample origin. According to Nur & Syahruddin (2012), nucleotide differences can result from mutations influenced by environmental factors or mutagenic compounds.

The relationship among individuals and their evolutionary history can be inferred from genetic information of an organism (Young & Gillung, 2020). The genetic relationships among the 12 isolates were analyzed by constructing a phylogenetic tree using the Neighbor-Joining method with 1000 bootstrap replications in MEGA-X software. The cladogram grouped the isolates into two main clusters (Figure 4). Group 1 included 76BB, 13TMGL, 24AP, 65ADS, 75BB, 68ADS, 27ASR, 31DJB, and 11AP, while Group 2 comprised isolates 61BP, 112DP, and 17DB. According to Rahayu & Nugroho (2015), branches on a phylogenetic tree indicate degrees of genetic relatedness. Closely related species cluster on the same branch, and lower genetic distance suggests closer relationships (Rahayu & Handayani 2011; Campbell, 2008).

EPS production is a major virulence factor of Rsi. EPS is a high-molecular-weight compound produced both in vitro and within plant tissues, playing a key role in vascular obstruction that leads to wilting symptoms (Hayward, 1995). Bacterial

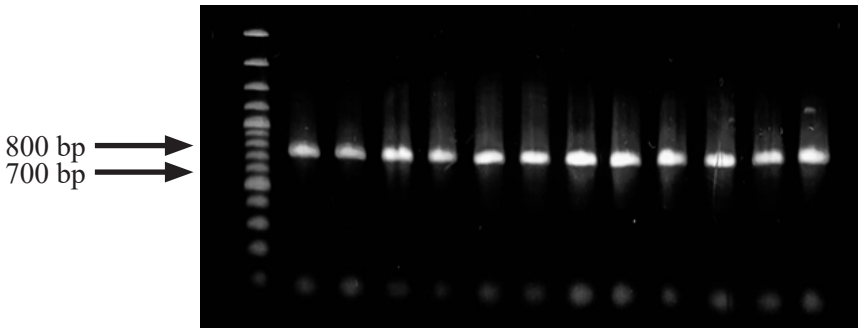

Figure 2. Electrophoresis of *aii*A gene amplicons produced by AHL-lactonase-producing bacteria. Lane 1: 100 bp DNA marker (Thermo Scientific, USA), Lanes 2–13: Bacterial isolates 11AP, 24AP, 61BP, 112DP, 31DJB, 75BB, 17DB, 76BB, 13TMGL, 27ASR, 65ADS, and 68ADS.

Table 3. Sequence homology of 12 AHL-lactonase-producing bacteria isolates

Isolates	Recovery (%)	Homology (%)	Species	Country	Accession code
11AP	92	97	*B. cereus* FORC087	Korea Selatan	CP029454.1
24 AP	99	97	*B. amyloliquefaciens* YB8 *AiiA*	China	OR423369.1
61 BP	97	94	*B. anthracis* Mn106-1	Afrika Selatan	CP126515.1
112 DP	100	96	*B. anthracis* MCC	China	CP031643.1
31 DJB	100	97	*B. subtilis strain 46 AHL-laktonase*	China	EF655619.1
75 BB	98	99	*B. thuringiensis* serovar *japonensis*	China	AY332612.1
17 DB	96	97	*B. thuringiensis* SCG04-02	China	CP017577.1
76 BB	99	100	*B. tropicus* EMB20	India	CP078081.1
13 TMGL	100	99	*B. subtilis* PEBS AHL-laktonase	China	FJ713590.1
27 ASR	99	99	*B. thuringiensis* SP-AB2	Africa Selatan	MW328525.1
65 ADS	96	99	*B. anthracis* HDZK-BYSB7	Amerika	CP026608.1
68 ADS	85	97	*B.cereus* A22	Vietnam	CP085498.1

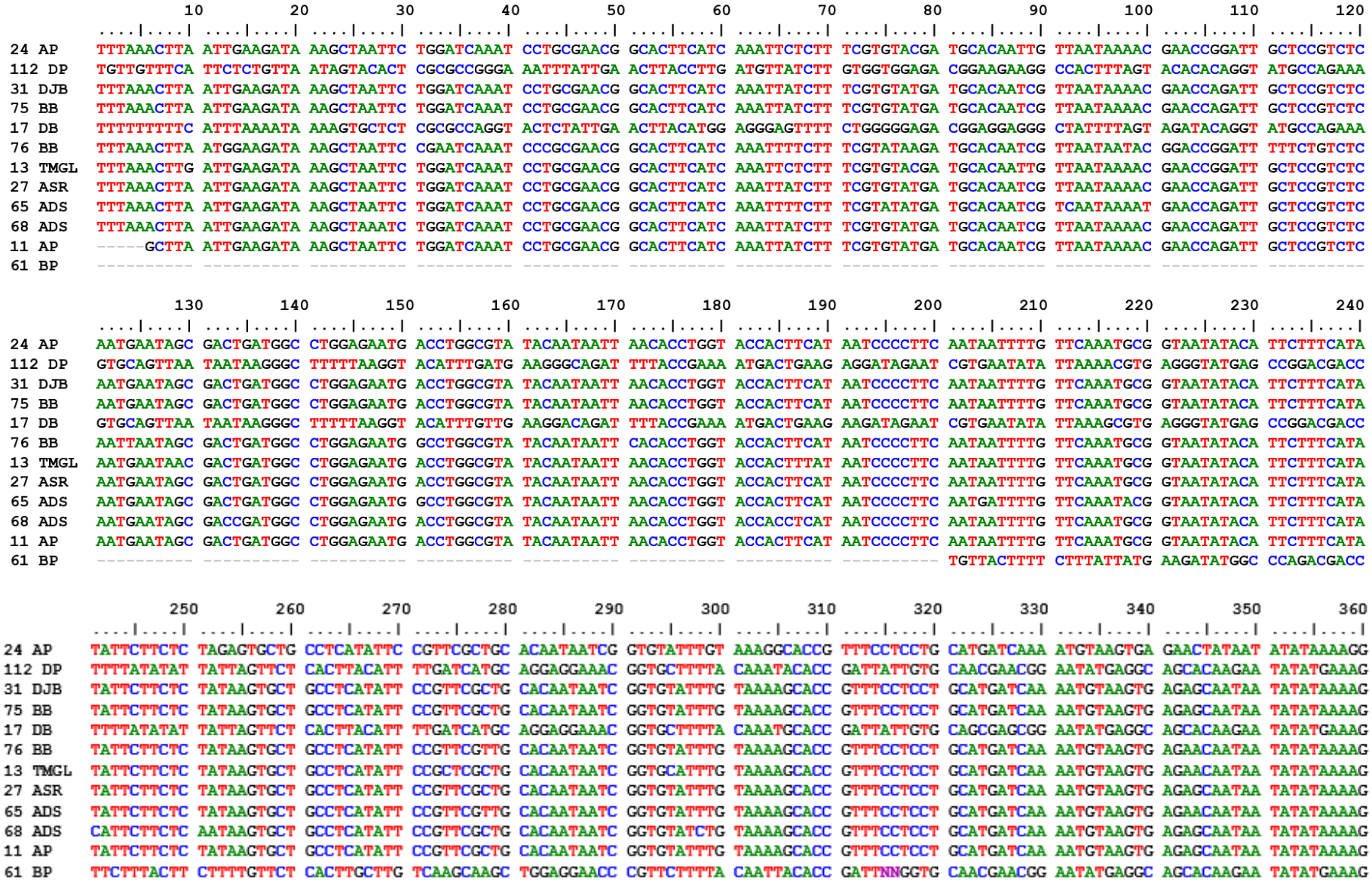

Figure 3. Multiple sequence alignment of *aii*A genes in 12 isolates bacteria

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                370        380        390        400        410        420        430        440        450        460        470        480
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
24 AP     TCGTCCGCTC ATACCCTACA CGCTTTAATA TATCACGATT CTATCTCAGT CATTTTCGTA AAATCTGTCT TCAACAAATG TACCGTTAGA AAGCCCTTCA TTATTAATGC ACTTTCTGGC
112 DP    AATGTATATT TGAACTACAA AAACATTGAA GGGATATGAA GGGGGAGCAG GTGTTCAATT ACTGTAAACG CCAGGCCATT CTCCAGGCCA TCAGTCGCTA TTCATTGCGG AGCAATCTGG
31 DJB    GTCGTCTGCT CATACCCTAC ACGCTTTAAT ATATCACGAT TCTATCTCAG TCATTTTGGT AAAATCTGTC TTCAACAAAT GTACCGTTAA AAAGCCCTTC ATTATTACTG CACTTTCTGG
75 BB     GTCGTCTGCT CATACCCTAC ACGCTTTAAT ATATCACGAT TCTATCTCAG TCATTTTGGT AAAATCTGTC TTCAACAAAT GTACCGTTAA AAAGCCCTTC ATTATTACTG CACTTTCTGG
17 DB     AATGTATATT TGAACTACAA AATTATTGAA GGGTTATAAA GTGGTACCAG GTGTTCAATT ATTGTATACG CCAGGTCATT CTCCAGGCCA TCAGTCGTTA TTCATTGCGG AGCAATCCGG
76 BB     GTCGTCCGCT CATACCCTAC ACGTTTTAAT ATATCACGAT TCTATCTCAG TCATTTTGGT AAAATCTGTC TTCAACAAAT GTACCGTTAA AAAGCCCTTC ATTATTACTG CACTTTCTGG
13 TMGL   GTCGTCCGCT CATACCCTAC ACGCTTTAAT ATATCACGAT TCTATCTCAG TCATTTTGGT AAAATCTGTC TTCAACAAAT GTACCGTTAA AAAGCCCTTC ATTATTACTG CACTTTCTGG
27 ASR    GTCGTCTGCT CATACCCTAC ACGCTTTAAT ATATCACGAT TCTATCTCAG TCATTTTGGT AAAATCTGTC TTCAACAAAT GTACCGTTAA AAAGCCCTTC ATTATTACTG CACTTTCTGG
65 ADS    GTCGTCCGCT CATACCCTAC ACGCTTTAAT ATATCACGAT TCTATCTCAG TCATTTTGGT AAAATCTGCC TTCAACAAAT GTACCGTTAA AAAGCCCTTC ATTATTACTG CACTTTCTGG
68 ADS    GTCGTCTGCT CATACCCTAC ACGCTTTAAT ATATCACGAT TCTATCTCAG TCATTTTGGT AAAATCTGTC TTCAACAAAT GTACCGTTAA AAAGCCCTTC ATTATTACTG CACTTTCTGG
11 AP     GACGTCTGCT CATACCCTAC ACGCTTTAAT ATATCACGAT TCTATCGCAG TCATTTTGGG AAAATCTGTC TTCAACAAAT GTACCGTTAA AAAGCCCTTC ATTATTACTG CGCTTTCTGG
61 BP     AATGTATATT TGAACTACAA AATCATTGAA GGGTTATGAA GTGGTACCAG GTGTTCAATT ATTGTATACG CCAGGCCATT CTCCAGGCCA TCAGTCGCTA TTCATTGCGG AGCAATCTGG

                490        500        510        520        530        540        550        560        570        580        590        600
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
24 AP     ATACCTGTAT TAAAATAGGC CCCTCTTTGT CTCCAAAAGA TAACACTACA GGTAAGTCAA TAAATTCTGT ACCATTAACA GAAGAATGAT CTAACATACA CCTGCTGGGA TGAAATAAAG
112 DP    TTCAATTTTA TGACGATTGA TGCATCTATA CGAAAGAAAA TTTTGAATGA AGTGCCTTCG CAGGATTTAG CTTTATCTTC AATTAAACGT TTAAAAGAAG TAGTGTTGAA AGAGAATCCA
31 DJB    CATACCTGTG CTAAAATAGG ACCTTCTCCG TCTCCAAAAG ATAACAACAC CGGCAATTTA ATAGTTTACG TACTGTTAAC AGACGAATGA TCCAACATGG ACCTGCTGGG ATGAAATAAA
75 BB     CATACCTGTG CTAAAATAGG ACCTTCTCCG TCTCCAAAAG ATAACAACAC CGGCAATTTA ATAGTTTACG TACTGTTAAC AGACGAATGA TCCAACATGG ACCTGCTGAG AAGAAATAAA
17 DB     TTCAGTTTTA TAACAATTGA TGCATCGACA CGAAAGAGAA TTTTGAATGA AGTGCCTTCG CAGGATTTAG CTTTATCTTC AATCAAACGT TTAAAAGGAG TTGTGGCGAA AGAGAAACCA
76 BB     CATACCTGTA CTAAAATAGG CCCCTCTCTG TCTCCAAAAG ATAACAATAC AGGTAATTCA ATAAATTGTG TACTATTAAC AGAAGAATGA TCTAACATAG ACCTGCTGGA ACGAAATAAA
13 TMGL   CATACCTGTA CTAAAATAGG CCCCTCTCTG TCTCCAAAAG ATAACAATAC AGGTAATTCA ATAAATTGTG TACTATTAAC AGAAGAATGA TCTAACATAG ACCTGCTGGG ATGAAATAAA
27 ASR    CATACCTGTG CTAAAATAGG ACCTTCTCCG TCTCCAAAAG ATAACAACAC CGGCAATTTA ATAGTTTACG TACTGTTAAC AGACGGATGA TCCAACATGG ACCTGCTGGG ATGAAATAAA
65 ADS    CATACCTGTG CTAAAATGGG ACCTTCTCCG TCTCCAAAAG ATAACAATAC AGGTAATTCA ATAAATTGTT TACTATTAAC AGAAGAATGA TCTAACATAG ACCTGCTGGA ATGAAATAAA
68 ADS    CATACCTGTG CTAAAATAGG ACCTTCTCCG TCTCCAAAAG ATAACAACAC CGGCAATTTA ATAGTTTACG TACTGTTAAC AGACGAATGA TCCAACATGG ACCTGCTGGG ATGAAATACA
11 AP     GATACATGTG CTAAAAAAGG ACCTTTTCCG TCCCCAGAAA GAAAAAGNGC CGGCACCCGG GCAGGTTATT TTTTCT---- ---------- ---------- ---------- ----------
61 BP     TTCAATTTTA TGACGATTGA TGCATCTATA CGAAAGAAAA TTTTGAATGA AGTGCCTTCG CAGGATTTAG CTTTATCTTC AATTAAATGT TTAAAAGAAG TAGTGTTGAA AGAGAATCCA

          ....|
24 AP     CTTCT
112 DP    ATTGT
31 DJB    GCTTC
75 BB     GTTTC
17 DB     ATTGT
76 BB     GCTTC
13 TMGL   GCTTC
27 ASR    GCTTC
65 ADS    GCTTC
68 ADS    GCTTC
11 AP     -----
61 BP     ATTGT
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Figure 3. (continued) Multiple sequence alignment of *aii*A genes in 12 isolates bacteria

production of EPS, along with enzymes like pectinase, cellulase, and protease, enhances pathogenicity. EPS consists of approximately 85% slime (without cells) and 15% capsular material surrounding bacterial cells (Arwiyanto et al., 2018). It not only blocks water and nutrient flow but also protects bacteria from host plant defense responses.

In this study, all 12 AHL-lactonase-producing isolates were able to suppress EPS production in Rsi to varying degrees. The most effective isolate was 65ADS, which inhibited EPS production by 74% (Figure 5). The degree of EPS suppression correlated with genetic relatedness among isolates. For instance, 61BP and 11AP showed different EPS suppression levels (71% and 19%, respectively), consistent with their low genetic similarity. Similarly, 61BP and 27ASR showed low homology and different EPS inhibition levels. These results suggest that genetic diversity among isolates influences their capacity to degrade AHL and suppress EPS.

All isolates significantly reduced EPS production compared to the control (Table 3, Figure 5), with isolates 65ADS and 61BP showing the highest inhibition and being closely related to *B. anthracis*.

BLAST-N analysis of the aiiA gene sequences against the GenBank database revealed that the 12 isolates showed high variability and matched with known AHL-lactonase-producing *Bacillus* species. These included *B. cereus* FORC087, *B. amyloliquefaciens* YB8, *B. anthracis* Mn106-1

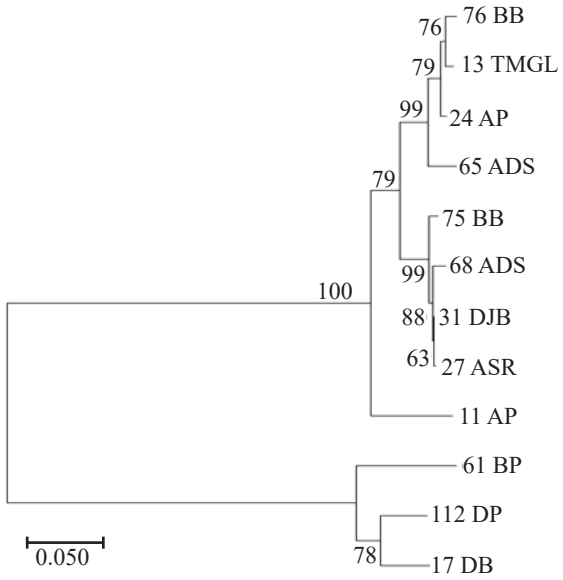

Figure 4. Phylogenetic tree of the *aiiA* gene sequences from 12 bacterial isolates, constructed using MEGA 11 software (Tamura et al., 2021). The tree was generated using the Neighbor-Joining method with Maximum Likelihood estimation and supported by bootstrap analysis with 1000 iterations.

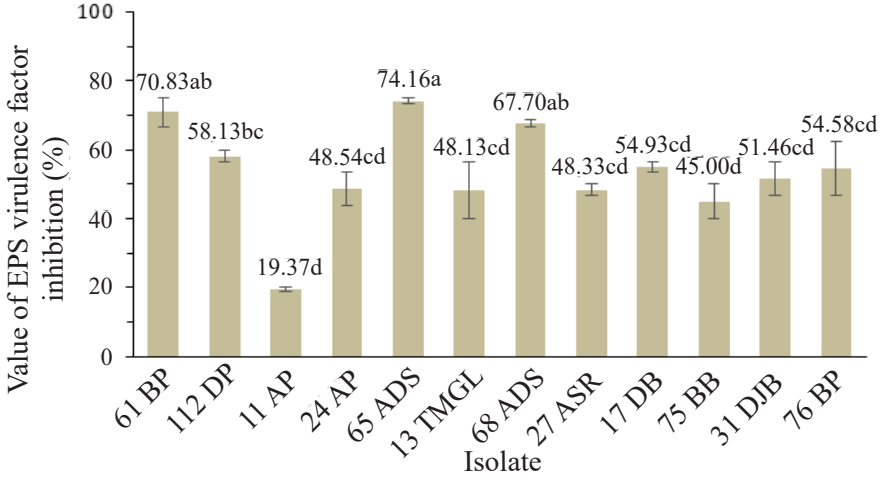

Figure 5. Percentage of EPS Production by Rsi Inhibited by AHL-Lactonase-Producing Bacteria

and MCC (from Bandung), *B. subtilis* strain 46, *B. thuringiensis* serovar *japonensis*, *B. thuringiensis* SCG04-02, *B. tropicus* EMB20 (from Brebes), *B. subtilis* PEBS, *B. thuringiensis* SP-AB2, *B. anthracis* HDZK-BYSB7, and *B. cereus* A22 (from Garut).

AHL-lactonase-producing bacteria are widely reported as effective biocontrol agents. Abidin (2018) reported that *Bacillus* isolate DBK2 suppressed blood disease in bananas with an inhibition rate of 92.97%. Khoiri et al. (2017) also reported that *Brevibacillus brevis* could suppress *Dickeya dadantii*, reducing soft rot in orchids and potatoes significantly (Khoiri et al., 2017).

CONCLUSION

The isolation of bacteria from Brebes, Bandung, and Garut Regencies yielded 12 isolates capable of producing AHL-lactonase, confirmed through bioassay using *C. violaceum* and further verified by *aiiA* gene detection. BLAST analysis from NCBI indicated that the isolates from Bandung (11AP, 24AP, 61BP, 112 DP) were homologous to *B. cereus* FORC087, *B. amyloliquefaciens* YB8 *AiiA*, *B. anthracis* Mn106-1, and *B. anthracis* MCC, respectively. Isolates from Brebes (31DJB, 75BB, 17DB, 76BB) were homologous to *B. subtilis* strain 46 AHL-lactonase, *B. thuringiensis* serovar *japonensis*, *B. thuringiensis* SCG04-02, and *B. tropicus* EMB20, respectively. Meanwhile, isolates from Garut (13TMGL, 27ASR, 65ADS, 68 ADS) were homologous to *B. subtilis* PEBS AHL-lactonase, *B. thuringiensis* SP-AB2, *B. anthracis* HDZK-BYSB7, and *B. cereus* A22, respectively. Sequence analysis of the *aiiA* gene revealed genetic diversity among the isolates, with homology levels ranging from 40.2% to 99.8%. These isolates also demonstrated varying abilities to suppress the expression of of the EPS virulence factor in Rsi, which is regulated through the quorum sensing (QS) mechanism. The highest suppression level was recorded at 74% by isolate 65ADS, while the lowest was 19.37%.

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AUTHORS' CONTRIBUTIONS

TE, GYT, and AAN jointly designed this research through discussion and idea exchange. GYT and AAN developed the research framework and methods and provided necessary materials for conducting the study. TE prepared the manuscript.

COMPETING INTEREST

The authors declare that there is no potential conflict of interest.

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