Revealing the proteomic profiles of antianthracnose-related proteins in *Serratia plymuthica* UBCR_12

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Abstract. Capacity of *Serratia plymuthica* UBCR_12 in suppressing *Colletotrichum gloeosporioides* is regulated molecularly through protein expression. Efficacy of this bacteria is greatly affected by various environmental factors. This study was aimed to investigate the expression profile of antianthracnose-related proteins in *S. plymuthica* UBCR_12 under different modified medium. Antagonistic activity against *C. gloeosporioides* was assessed using agar spot method in PDA medium supplemented with several concentrations of peptone or glucose. Co-culture was performed under the same condition as an antagonistic assay to induce the extracellular proteomes expression related to this antianthracnose trait. Proteome profiles were visualized using SDS-PAGE and 2-DE, then a highly differential protein spot was further analyzed using MALDI-TOF-MS. Prediction of protein classification and possible hypothetical pathway were performed in-silico. The highest suppression (42.3% at 9th-day post application) resulted in 2% glucose-supplemented medium, however 2% peptone-supplemented medium conferred a quicker suppression (at 5th-day post application) with quite lower inhibition efficacy (40%). Differential expression of 42 kDa protein band recorded during peptone addition was predicted to be flagellin protein, which might correlate with a rapid stimulus of suppression activity. This protein involved in the quorum-sensing mechanism by triggering the greater rate of cell division resulting in bacterial colonization and motility approaching the fungal pathogen.

1. Introduction

According to the central dogma of molecular biology, all phenotypes (traits) of an organism, including metabolites production, was initiated and regulated molecularly through the protein synthesis. This process plays a role as the first instruction determining the expression or production of certain metabolites by the cell. This process occurs dynamically and highly dependent on the interaction between genetic and environmental factor. Due to this characteristic, even a little change on the surrounding condition may easily change the achieved results of this protein synthesis based on the organism response. As a functional product of a gene and an active agent in the cell, protein is considered to be the most relevant approach [1] in the investigation on how a biological process is regulated at the molecular level. Since the last decades, studies on cellular mechanism related to interaction among species began to be investigated from the molecular point of view, particularly using the proteomic approach [2-6].
Regarding the development of a biocontrol agent, the use of proteomic approach would provide a relevant reference based on its naturally-occurred mechanism at the molecular level. It would result in a highly specific and persistent biocontrol effect, hence its resistance would be more unbreakable [4]. This present study investigated the profile of extracellular proteomes synthesized by biocontrol bacteria *Serratia plymuthica* UBCR_12 during its interaction with anthracnose causal agent *Colletotrichum gloeosporioides*. This rhizobacteria strain had revealed a promising antianthracnose activity in-vitro, both through its culture suspension and supernatant. The antianthracnose trait of this strain produced rapid growth inhibition effect in 1% peptone-supplemented medium, but resulted in higher suppression in 1% glucose-supplemented medium [7]. However, the molecular mechanism underlying these previous results had not been fully confirmed yet. Moreover, the interaction among *S. plymuthica* and *C. gloeosporioides* remained less studied using the proteomic approach. Therefore, the proteomic profile depicting the interaction among both organisms were further evaluated under various environmental conditions. This study was aimed to investigate the proteome profile of antianthracnose-related proteins during the interaction between *S. plymuthica* UBCR_12 and *C. gloeosporioides* under different modified medium.

2. Materials and Methods

2.1. Preparation of Pathogenic Fungus and Biocontrol Bacteria

Both *C. gloeosporioides* (isolated from anthracnose-infected chili pepper) and *S. plymuthica* UBCR_12 used in this study were collected from Laboratory of Biotechnology, Faculty of Agriculture, Andalas University. The pathogenic fungus was cultured onto PDA (potato dextrose agar) medium at RT for 7 days in darkness, while the biocontrol bacteria was grown using NA (nutrient agar) medium at 30°C for 18 hours.

2.2. Antagonistic Assay in Modified Medium containing Various Concentrations of Peptone or Glucose

Antianthracnose activity of *S. plymuthica* UBCR_12 against *C. gloeosporioides* was evaluated through antagonistic assay performed using agar spot method as described by Aisyah *et al.* [7]. This antagonistic assay used PDA medium (pH 7.0) supplemented with peptone or glucose as the sole nitrogen and carbon sources. The concentration of both peptone and glucose used in this assay ranging from 1 up to 4% (w/v).

Pathogenic fungus was cultured onto peptone or glucose-supplemented PDA medium for 48 hours in darkness at RT. UBCR_12 cell suspension was collected from 18 hours of bacterial culture in LB medium when its density reached 1.00 at 600 nm wavelength. An aliquot (5 µl) of this bacterial culture was then spotted onto the antagonistic assay medium containing the previously cultured fungus. The spotting of UBCR_12 suspension was performed in 4 different positions located 3 cm from the center of fungal growth. Each treatment was carried out in five replicates. As a control, the antagonistic assay was performed in PDA medium without any supplemented peptone or glucose. Dynamics of the fungal suppression activity were observed routinely for 10 days by calculating the percentage of growth inhibition using the formula proposed by Islam *et al.* [8]. Concentrations of peptone and glucose resulting in the highest fungal suppression activity were subjected to co-culture.

2.3. Co-culture of Pathogenic Fungus and Biocontrol Bacteria

Co-culture was performed in PDB medium supplemented with 2% glucose or peptone using a protocol described by Zhang *et al.* [2] with slight modifications. Fungal mycelia (± 0.15 g) were co-cultured with 1 ml aliquot of UBCR_12 cell suspension (OD<sub>600nm</sub> = 1.0) for 24 hours at RT and 120 rpm in darkness. As a control, the bacteria were single-cultured using the same condition as the co-culture. After the 24 hours incubation, fungal mycelia were discarded thoroughly using Whatmann paper No. 1. Culture supernatant of UBCR_12 was then harvested and filtered using a 0.22 µm membrane filter. The collected filtrates were subsequently used for the analysis of proteome profiles.
2.4. Analysis of Bacterial Total Extracellular Proteome Profiles
Total extracellular proteomes of UBCR_12 (both single- and co-cultured) were extracted using a protocol adapted from Nouwens et al. [9] with the same execution as Aisyah et al. [7]. About 150 ng/µl UBCR_12 extracellular proteome was separated through SDS-PAGE using a 15% SDS-PAGE gel for 60 minutes at constant voltage (200 V). Two-dimensional electrophoresis (2-DE) was carried out for the treatment revealing the most differential proteome profile only. About 200 ng/µl bacterial extracellular proteome was firstly immobilized into 7 cm IPG strip (pI 3–10, Bio-Rad) using isoelectric focusing (IEF) under the setting of 7 cm broad and basic program (AA Hoefer). The immobilized strip was then subjected into a 15% SDS-PAGE for the second dimension separation with the same running condition as previously described. The gel visualization was performed using Coomassie staining before being documented using Canon CanoScan LiDE 110 scanner.

2.5. Protein Identification using MALDI-TOF-MS
Protein identification performed in this study was based on the peptide-mass fingerprinting method through MALDI-TOF-MS (Matrix Assisted Laser Desorption/Ionization – Time of Flight – Mass Spectrometer). Protein spot visualizing the most differential expression during 2-DE was cut and prepared for MALDI-TOF-MS. This identification was carried out by Proteomics Internasional (Australia) and the results were then analyzed using MASCOT software provided by http://www.matrixscience.com.

2.6. Prediction of Protein Classification and Possible Hypothetical Pathway
This predicted UBCR_12 antianthracnose-related protein was in-silico analyzed to identify its protein classification and involvement in the biological mechanisms. The analysis of protein classification was conducted in Gene Ontology website (www.geneontology.org), while the function of this predicted protein in the biological process was searched in KEGG (Kyoto Encyclopedia of Genes and Genomes) Pathway Database website (https://www.kegg.jp). These resulting information were then used to build the hypothetical pathway which was possibly associated with the mechanism used by UBCR_12 during its antianthracnose activity.

2.7. Statistical Analysis
Data of fungal growth inhibition was statistically analyzed using one-way ANOVA through SPSS version 23.0. Data were presented as the mean value of five replicates followed by the standard deviation (SD). The significance among treatments was analyzed using Duncan’s New Multiple Range Test (DNMRT) with a p<0.05.

3. Results and Discussion

3.1. Presence of Peptone and Glucose Directed the Mechanism of Fungal Growth Suppression in Different Way
Addition of various concentration of peptone or glucose showed that the resulting fungal growth inhibition appeared in a different pattern. The presence of peptone promoted a rapid stimulation leading to the achievement of the highest growth inhibition at the early days after bacterial application. Addition of 2 up to 4% peptone resulted in the highest inhibition at the 5th day after application (Table 1). Compared to the growth inhibition resulted from 1% peptone and non-modified PDA (Table 1), the increasing concentration of peptone had successfully doubled up the suppression efficacy of this strain. Of all concentrations tested, 2% peptone-supplemented medium revealed the highest suppression with 39.9% (Table 1). In contrast, the fungal suppression resulted from the glucose-supplemented medium at any concentration reached its optimum value at a slower rate. All glucose concentrations tested showed the highest inhibition at the 9th day after application (Table 1) and the best suppression was obtained from 2% glucose-supplemented medium with 42.3% (Table 1). Regarding the percentage of inhibition,
the presence of glucose triggered higher efficacy than the peptone-supplemented medium. However, peptone offered a quicker response in stimulating the antagonistic effect of UBCR_12.

**Table 1.** Effect of various concentrations of glucose or peptone on the fungal growth suppression at the 5th (peptone) and 9th day after *S. plymuthica* UBCR_12 application. Values are mean ± SD from five replicates.

| Peptone Conc. (%) | Growth Inhibition (%) | Glucose Conc. (%) | Growth Inhibition (%) |
|-------------------|-----------------------|-------------------|-----------------------|
| 1                 | 14.8 ± 3.0 c           | 1                 | 29.6 ± 4.0 b          |
| 2                 | 39.9 ± 2.4 a           | 2                 | 42.3 ± 3.4 a          |
| 3                 | 29.8 ± 10.1 b          | 3                 | 25.2 ± 4.5 c          |
| 4                 | 37.8 ± 7.5 ab          | 4                 | 14.0 ± 2.2 d          |
| Non-modified      | 5.2 ± 4.0 d            | Non-modified      | 7.1 ± 0.8 e           |

Numbers followed by the same lower case in the same column are insignificantly different based on DNMRT with a *p*<0.05.

Bacterial growth and metabolism required the presence of both nitrogen and carbon sources, however, these two elements played a different role in the bacterial metabolism. Regarding the biosynthesis of defense metabolites, such as antifungal compounds, both elements affected this activity in the opposite way. Ruiz *et al.* [10] stated that Gram-negative bacteria favored the absence of carbon source during the biosynthesis of its defense metabolites. The presence of an easily digested carbon source, such as a monosaccharide, was known to be highly supported for the bacterial growth, but potentially repressed the biosynthesis of most bacterial secondary metabolites [11]. However, the presence of exogenous carbon could trigger the faster accomplishment of bacterial stationary phase, thus leading to lower final culture density [12]. This condition was assumed to be strongly associated with the occurrence of metabolite biosynthesis repression at the early days after application which then resulted in slower suppression of UBCR_12. In line with this result, the biosynthesis of antibiotic in bacteria was mostly occurred under unfavorable growth condition due to carbon starvation and growth rate decrease, thus stimulating the activation of specific inducer [13].

Unlike carbon sources, nitrogen contributed specifically in the biosynthesis of bacterial metabolites where its kind depended on the type of biosynthesis pathway required by certain metabolites [14]. Certain nitrogen source, such N-acetyl-glucosamine (NAG), had been reported as one of the molecules regulating the bacterial host colonization, virulence and biofilm formation [15-18]. According to Table 1, the antianthracnose activity of UBCR_12 was rapidly triggered due to the addition of 2 up to 4% peptone. However, the previous study reported that the addition of 1% peptone stimulated the highest inhibition at the 2nd day after application [7]. Although the results of this study conferred longer period to achieve the highest suppression, this study had proven that the elevated concentration of peptone produced higher and more stable inhibition activity than at 1% addition one. Several *S. marcescens* strains also achieved its optimal production of antimicrobial metabolites in peptone-supplemented medium [19, 20].

**3.2. Presence of Glucose or Peptone Affected The Expression of Possible Antianthracnose Inducer Proteins**

SDS-PAGE profile of UBCR_12 extracellular proteomes revealed the expression of an approximately 42 kDa protein band under both modified (2% glucose or peptone) and non-modified as well as single- or co-cultured conditions (Fig. 1). Interestingly, the expression level of this protein was nearly the same in all medium types and culture condition. It then indicated that this protein band might not be related directly to the antianthracnose activity of this strain. However, the difference of medium type and culture condition distinguished the expression of other protein bands, thus resulting in the differential pattern among treatments.
Figure 1. SDS-PAGE visualization of *S. plymuthica* UBCR_12 extracellular proteome profile after 24 hours under single- and co-cultured with *C. gloeosporioides* (Cg) conditions in both modified and non-modified mediums. **Upward arrow (↑)** indicated up-regulated expression; **Downward arrow (↓)** indicated down-regulated expression.

As the earliest achievement of optimal inhibition resulted from 2% peptone-supplemented medium, the proteome profile under this condition showed the up-regulated expression of 38, 50 and 60 kDa compared to the single-cultured UBCR_12 (Fig. 1). In contrast, both single- and co-cultured UBCR_12 in 2% glucose-supplemented medium did not reveal the expression of any other protein bands, except a 42 kDa band (Fig. 1). It implied that UBCR_12 was unable to express specific proteins related to its antianthracnose trait during 24 hours culture.

The expression profile of these protein bands was in line with the one resulted from Aisyah et al. [7]. However, the expression level of protein bands appeared in 1% peptone treatment [7] was still higher and clearer than in 2% peptone treatment resulted in this present study. It then indicated that the low amount of peptone could contribute faster stimulation on UBCR_12 antianthracnose activity, while higher concentration delayed the stimulation but produced higher suppression effect. It was also in accordance with Rokem et al. [21] mentioning that the production of microbial metabolites involved the role of certain kind of nitrogen sources at a certain amount. Several species of Gram-negative bacteria utilized this nitrogen acquisition to regulate cell motility. The process of nitrogen assimilation for this circumstance was regulated by the transcription factor σ^54. This transcription factor (TF) played an important role in stimulating cell response towards the occurring environmental changes [22]. It was also reported that this TF activity would trigger the production of N-acyl-homoserine lactones (AHL) as the signal molecule for the activation of quorum sensing mechanism [23]. At this phase, several important defense-related metabolites would be actively produced by the bacteria [24, 25].

Due to its stronger protein expression level, the 2-DE analysis was performed using 1% peptone-supplemented culture and non-modified culture was then used as control. As shown in Fig. 2, the 42 kDa protein band was found to be expressed in any kind of culture condition (both normal or peptone-supplemented). However, the expression level was varied where the presence of peptone stimulated a higher level of this protein expression. This 42 kDa protein band exhibited relatively weak expression in the non-modified medium, while the presence of fungal cells in this culture condition even weakened
the expression of this protein bands (Fig. 2a). In contrast, the presence of peptone also promoted other protein bands to be expressed. More numbers of protein bands seemed to be highly triggered to appear due to the presence of fungal cells in the culture (Fig. 2b). According to Fig. 2, it was also found that the pI of this protein band was predicted to be approximately 4.5 to 5. Based on the principle of molecular biology, all biological interactions among different organism were shaped by the synergistic association between the cellular and extracellular proteins [2]. However, several previous studies reported that extracellular proteins might play more critical role compared to the cellular one, such as in the regulation of bacterial virulence against other organisms [2, 26].

3.3. In-silico Prediction Analysis Revealed the Involvement of Flagellin Protein

Protein spot representing the expression of 42 kDa protein band (Fig. 2b) was identified to be similar to flagellin protein of S. plymuthica PRI-2C sized approximately 43.11 kDa and pI value of 4.87. The similarity coverage of this UBCR_12 putative flagellin was considered quite low (35%) indicating that the fragmented peptides resulted from this strain protein showed a hypervariable sequence with the previously known flagellin protein one. McDermott et al. [27] stated that the flagellin protein of Gram-negative bacteria composed of both highly conserved and hypervariable domains. The highly conserved domains were found at the end of its C and N-terminus containing approximately 235 residues. The center part of this protein was composed by hypervariable domain with different length, thus leading to the difference of its molecular weight among species.

![Figure 2](image.png)

**Figure 2.** 2-DE visualization of S. plymuthica UBCR_12 extracellular proteomes after 24 hours under single- and co-cultured with C. gloeosporioides (Cg) conditions in non-modified (a) and 1% peptone-supplemented (b) mediums. **Red arrow** indicated down-regulated expression; **Black arrow** indicated up-regulated expression.

Based on Gene Ontology, flagellin protein of S. plymuthica was localized in extracellular and its molecular function was related to the flagellar assembly which regulated the cell motility. During the antagonistic interaction between fungus and bacteria, cell motility was found to be the first response
expressed after successfully detected the volatile compounds released by the pathogenic fungus [6]. Signal receptor of bacteria was functioned in detecting this compound in order to determine the direction of its chemotaxis to a certain position considered as a favorable area for colonization [28].

Referred to its function in a more complex biological system, this cell motility was a part of the two-component system required for signal transduction processes. This system was a typical signaling mechanism mostly found in Gram-negative bacteria, such as Serratia. Through this system, bacteria enabled to stimulate a significant change in its cellular metabolism as the response toward the detected signal from its surrounding environment [29]. This flagellin protein played a specific role in the regulation of quorum sensing mechanism.

![Diagram](image)

**Figure 3.** Hypothetical pathway possibly related to the molecular mechanism triggering the expression of antianthracnose trait in *S. plymuthica* UBCR_12.

Quorum sensing (QS) was known to be a particular cell to cell communication mechanism used to initiate the cell response toward the increase of its own cell density [30, 31]. This mechanism was specifically induced by the presence of AHLs which were continuously accumulating along with the increase of bacterial population density. This increasing population growth would then stop when it reached a certain threshold value, thus leading to the change of cell physiology [29]. QS mechanism, particularly in *Serratia* species, also regulated the surface colonization process through its flagellar motility allowing bacteria to move closer to the area that might support its growth. This flagellar motility-mediated colonization would also trigger the biosurfactant production and several important antibiotics (e.g. prodigiosin) secretion. Related to the antagonistic interaction, flagellar movement also supported this bacterial virulence against pathogens through several ways, including adhesion, biofilm formation on the surface of host cells, biosurfactant production and antibiotic secretion [32-35]. In line with this statement, Williamson *et al.* [36] mentioned that the presence of biosurfactant along with the swarming motility contributed in the dispersal of prodigiosin, thus resulting in better capacity during the niche colonization against its competitors.

Referred to these explanations, the hypothetical pathway possibly related to the antianthracnose activity of *S. plymuthica* UBCR_12 (Fig. 3) was described as followed. Volatile compounds released by *C. gloeosporioides* was detected and captured by the receptor of extracellular signals. These signal
molecules would trigger the AHLs production and activate the bacterial QS mechanism through the expression of \( \text{QsecA} \) and \( \text{QsecB} \) proteins. It then stimulated the flagellar assembly involving the flagellin protein until the flagella were ready to move around. Along with the flagellar motility, the cell density would be continuously increased due to the nitrogen (case of this study: peptone) acquisition process. Flagella would direct the bacterial growth to colonize the area which was closer to the position of pathogenic fungus. This condition enabled the bacteria to dominate the space and niche utilization from the environment, thus affected the surrounding fungal growth and then considered as an antagonistic effect.

4. Conclusion

The antianthracnose trait of \( S. \text{plymuthica} \) \( \text{UBCR\textunderscore 12} \) was predicted to be regulated molecularly by the flagellar motility through the involvement of flagellin protein. Interaction between this bacterial strain and \( C. \text{gloeosporioides} \) enhanced the expression of this flagellin protein, thus triggering the expression of other antianthracnose-related proteins. The presence of peptone also contributed to rapidly induce the expression of flagellin protein of \( S. \text{plymuthica} \) \( \text{UBCR\textunderscore 12} \). Based on this present study, further investigation through in-vivo analysis was required to evaluate the validity of this in-silico prediction and provide empirically proven information regarding this molecular mechanism. Comprehensive information related to the molecular mechanism of this beneficial trait would be a powerful reference for the development of this strain as a biocontrol agent against anthracnose causal agent.

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