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Cell aggregation is negatively regulated by *N*-acylhomoserine lactone-mediated quorum sensing in *Pantoea ananatis* SK-1

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***Pantoea ananatis* SK-1 produces *N*-acylhomoserine lactones (AHLs) and regulates expression of some virulence factors through AHL-mediated quorum sensing. In this study, we discovered that the strain SK-02I, which has a disrupted AHL-synthetic gene, showed constitutive cell aggregation. SK-1 has the ability to aggregate, and cell aggregation inhibitory factors are expressed under control of AHL-mediated quorum sensing. One of the transposon mutants, SK-33M, constitutively aggregates without defective AHL production. A homology search revealed that the transposon integration site was located in the adhesin-like *yeeJ* gene. Based on RT-PCR analysis, transcription of *yeeJ* is regulated by AHL-mediated quorum sensing. However, because both the wild-type and SK-33M strains induced necrotic symptoms in onion leaves, we conclude that the *yeeJ* gene is not involved in the pathogenicity of SK-1.**

[**Key words:** Quorum sensing; *N*-Acylhomoserine lactone; *Pantoea ananatis*; Cell aggregation; Adhesin]

Quorum sensing is a population density-dependent regulation mechanism used by bacteria to regulate gene expression. In many Gram-negative bacteria, several types of *N*-acyl-L-homoserine lactones (AHLs) (also called autoinducers) have been identified to be signal compounds involved in quorum sensing (1,2). When AHL concentration increases and reaches a threshold due to the accumulation of AHL derived from each bacterial cell, AHL receptor proteins belonging to the LuxR protein family bind AHL and regulate expression of many genes responsible for bioluminescence, the production of pigment or the production of antibiotics (1,2). AHL-mediated quorum sensing is highly conserved in plant pathogens (3). For instance, *Pectobacterium carotovorum* subsp. *carotovorum* (*Erwinia* subsp. *carotovora*) causes soft rot diseases in many plant species. *P. carotovorum* subsp. *carotovorum* regulates the production of various exoenzymes and plant tissue maceration by AHL-mediated quorum sensing (4). *Pantoea stewartii* also produces AHLs and regulates exopolysaccharide biosynthesis and infection of plants (5).

Pantoea ananatis causes disease symptoms in a wide range of economically important agricultural crops, including maize, rice and onion (6); however, the major virulence factors of *P. ananatis* are unknown. In a previous study, we determined that the *P. ananatis* strain SK-1 produced *N*-hexanoyl-L-homoserine lactone (C6-HSL) and *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL) (7). We cloned the LuxRI homolog EanRI from SK-1 and revealed that extracellular polysaccharide (EPS) biosynthesis, biofilm formation, and infection of onion leaves were regulated by AHL-mediated quorum sensing in SK-1 (7). In this study, we demonstrate the relationship between cell aggregation and AHL-mediated quorum sensing in SK-1. We also identify the genes related to cell aggregation and demonstrate transcriptional regulation of these genes by AHL-mediated quorum sensing.

MATERIALS AND METHODS

Bacterial strains, compounds, and culture conditions *Escherichia coli* DH5α were grown at 30°C in Luria–Bertani (LB) medium (8). *P. ananatis* strains were grown at 30°C in tryptic soy broth (TSB; Nippon Becton Dickinson, Tokyo, Japan). Solid bacterial media were made by the addition of agar to a final concentration of 1.5%. Antibiotics were added as required to final concentrations of 100 μg/ml ampicillin, 10 μg/ml chloramphenicol, 10 μg/ml tetracycline, or 10 μg/ml gentamicin. The AHL used in this study, *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL), was synthesized using a previously described method (9).

Aggregation assay Cell aggregation was evaluated by a previously described method with slight modification (10). Briefly, bacterial strains were grown for 15 h and then inoculated into 4 ml of fresh TSB medium (1% inoculum) with C6-HSL or 3-oxo-C6-HSL at a concentration of 0, 0.1 or 1 μM. After incubation for 20 h, the OD_{600} of the culture was measured (OD_{total}). The culture was centrifuged at 700×*g* for 2 min and the OD_{600} of the supernatant was measured (OD_{sup}). The aggregation index (AI) was calculated as follows:

$$AI = 100 \times \{(OD_{total} - OD_{sup}) / OD_{total}\} \quad (1)$$

Transposon mutagenesis Transposon mutagenesis was performed using a previously described method with slight modification (11). Briefly, the transposon carrier plasmid pOT182 (12) was transformed into SK-1 by electroporation. Transposon mutants were selected by plating on LB agar plates containing tetracycline. In order to identify the insertion site of the transposon, chromosomal DNA from the mutant was extracted and digested with *Hin*dIII. The chromosomal fragments were self-ligated and transformed into *E. coli* DH5α. Transformants were selected by plating on LB agar plates containing ampicillin and tetracycline. Plasmids were extracted using the alkaline lysis method and were used for the sequencing reaction. DNA sequencing was performed by using BigDye Terminator ver. 3.1 and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan). The OT182-LT primer (5′-GAT CCT GGA AAA CGG

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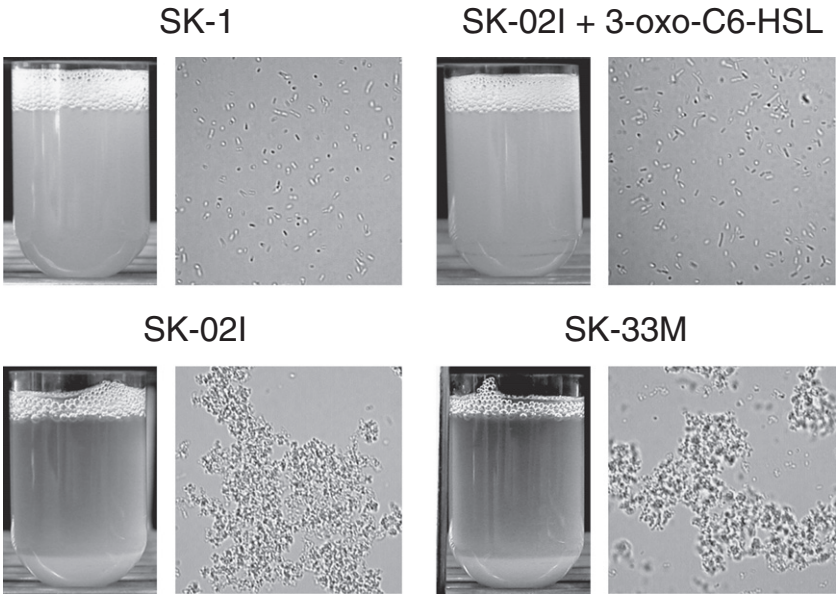

FIG. 1. Cell aggregation characteristics (right panels) and settling from static liquid suspensions (left panels) of SK-1, SK-02I, SK-02I with 1 μM of 3-oxo-C6-HSL, and SK-33M.

GAA AG-3′) was used to initiate the sequence reaction. DNA sequences were compared with the sequences in the DDBJ/EMBL/GenBank databases.

RT-PCR The full-grown cultures of bacterial strains were inoculated into 4 ml of fresh TSB medium (1% inoculum) with C6-HSL or 3-oxo-C6-HSL at a concentration of 0, 0.1 or 1 μM. After incubation for 10 h, 500 μl of each culture was mixed with 1 ml of RNAprotect Bacteria Reagent (Qiagen, Valencia, CA, USA). Total RNA was isolated from the bacterial pellet using ISOGEN (Nippon Gene, Tokyo, Japan). Residual DNA was removed by treatment with RQ1 RNase-free DNase (Promega, Madison, WI, USA). RT-PCR was performed using ReverTra Ace -α- (Toyobo, Osaka, Japan) in a 50 μl reaction mixture containing 80 ng total RNA. For amplification of the internal region of *yeeJ* we used the following primer set: (5′-CTG ACA TCC GGC AGG GTT TGT ATT GCT ACC-3′) and (5′-CGA CCA TAG CTG CCA CGT TGT TTG GCG ATA-3′). 16S rRNA was amplified using previously described primers (13). PCR cycling conditions were 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s for a total of 28 (for *yeeJ*) or 24 (for 16S rRNA) cycles.

Plant infection assay Pathogenicity of the *P. ananatis* strain on onion leaves was determined as described previously (7). Briefly, a sterile needle was dipped into the bacterial colonies on TSB agar plates that were grown for 24 h. Then, the needle was inserted under the epidermis of an onion leaf. *P. ananatis* strains were inoculated at two sites per leaf. Inoculated leaves were incubated at room temperature and observed for the development of symptoms. All infection assays contained at minimum two leaves per treatment, and experiments were performed a minimum of two times.

RESULTS AND DISCUSSION

Cell aggregation is negatively regulated by quorum sensing To observe the ability of cells to aggregate, *P. ananatis* strains were grown at 30°C in TSB. Strain SK-02I, in which the AHL-synthetic gene *eanI* was disrupted, resulting in the strain being unable to produce any AHLs, was constructed in previous work (7). The full-grown culture was left standing for 30 min at room temperature. Wild-type SK-1 cells did not aggregate or settle from liquid suspensions (Fig. 1); however, the AHL-deficient mutant SK-02I was flocculated and settled, and aggregated cells were observed by microscopy (Fig. 1). To determine whether exogenous AHL represses SK-021 cell aggregation, SK-02I cells were cultured in TSB medium containing 1 μM of 3-oxo-C6-HSL. As a result of 3-oxo-C6-HSL addition, SK-02I did not aggregate or settle from liquid suspension (Fig. 1). SK-02I also showed cell aggregation in TSB medium containing 1 μM of C6-HSL (data not shown). These results demonstrated that SK-1 cell aggregation was negatively regulated by AHL-mediated quorum sensing. To investigate the effects of AHL on cell aggregation, the AI value of SK-1 and SK-02I was estimated. The AI value of SK-1 was approximately 45% (Fig. 2). By contrast, SK-02I had a much higher AI value (approximately 98%, Fig. 2). When 1 μM 3-oxo-C6-HSL was added to the TSB medium, the AI value of SK-02I decreased to the level of SK-1 (Fig. 2). The presence of 1 μM C6-HSL also decreased the AI value of SK-02I (data not shown).

The AI value of SK-1 and SK-02I was also determined throughout growth. Cultures of both strains showed the similar growth rates (Fig. 3A). The AI values increased until the late exponential phase in both strains. Although SK-02I had a high AI value (>80%) in stationary phase, the AI value of SK-1 was decreased compared to early stationary phase (Fig. 3B). These results suggested that SK-1 cells have the ability to aggregate and that putative aggregation inhibitory factors are expressed under control of AHL-mediated quorum sensing.

The adhesin-like *yeeJ* gene represses cell aggregation in SK-1 To identify the specific gene required for cell aggregation, we screened the constitutively aggregating SK-1 mutant. Transposon mutagenesis was performed by introducing the transposon carrier plasmid pOT182 into SK-1 by electroporation. Cell aggregation of approximately 3000 transposon mutants from the full-grown culture was checked after standing for 30 min. One of the transposon mutants, designated SK-33M, was flocculated and settled, as was SK-02I (Fig. 1). SK-33M also had a high AI value (approximately 96%) as did SK-02I (Fig. 2). To confirm that SK-33M is not an AHL-deficient mutant, AHL production by SK-33M was checked by an AHL-reporter strain, *Chromobacterium violaceum* CV026 (7). We found that SK-33M produced AHLs at the same level as the SK-1 wild type strain (data not shown).

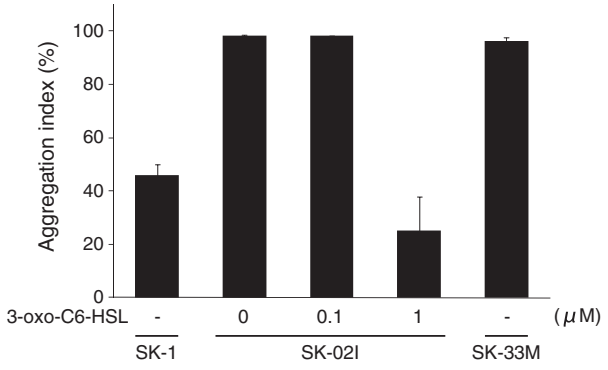

FIG. 2. Quantification of cell aggregation of SK-1, SK-02I, and SK-33M. The AI values were estimated after 20 h of cultivation. 3-oxo-C6-HSL was added to SK-02I cultures to a final concentration of 0, 0.1, or 1 μM. The results were reproduced in three experiments, and the error bars indicate standard deviations.