Hypothetical Protein Avin_16040 as the S-Layer Protein of Azotobacter vinelandii and Its Involvement in Plant Root Surface Attachment

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A proteomic analysis of a soil-dwelling, plant growth-promoting Azotobacter vinelandii strain showed the presence of a protein encoded by the hypothetical Avin_16040 gene when the bacterial cells were attached to the Oryza sativa root surface. An Avin_16040 deletion mutant demonstrated reduced cellular adherence to the root surface, surface hydrophobicity, and biofilm formation compared to those of the wild type. By atomic force microscopy (AFM) analysis of the cell surface topography, the deletion mutant displayed a cell surface architectural pattern that was different from that of the wild type. Escherichia coli transformed with the wild-type Avin_16040 gene displayed on its cell surface organized motifs which looked like the S-layer monomers of A. vinelandii. The recombinant E. coli also demonstrated enhanced adhesion to the root surface.

Azotobacter vinelandii is a Gram-negative free-living and obligate aerobic soil bacterium. It is well known to be a plant growth-promoting bacterium capable of fixing nitrogen and forming desiccation-resistant cysts under unfavorable growth condition (1, 2). The former activity requires it to house several oxygen-sensitive mechanisms while being an obligate aerobic bacterium (3). A. vinelandii also has characteristics such as production of plant growth hormones and antibiotics (4) as well as industrially important substances such as extracellular polysaccharide (EPS) alginate, poly-β-hydroxybutyrate (PHB), and siderophore compounds (5).

Many diverse genera of nitrogen-fixing bacteria are present in the plant rhizosphere. The effectiveness of their plant growth-promoting activity depends upon the establishment of their cells in the rhizosphere. This interaction depends upon many factors, one of them being plant exudates. As a diazotroph, A. vinelandii provides fixed nitrogen to the plant while acquiring sugars and other nutrients that leak from the roots (6).

The complete A. vinelandii genome (GenBank accession number NC_012560) has explained many biochemical pathways and structures of the bacterium (7). It has also revealed hypothetical genes with unannotated functions. The advances in proteomic technology have led to new understanding of and insights into many important proteins and their related mechanisms.

Studies have shown that plant-microbe communication is a two-way interaction involving various signal molecules that cause metabolic changes in both organisms (8–10). Nevertheless, there is limited information on the plant-bacterium interaction, especially with the roots and the rhizosphere. In this study, a proteomic approach was successfully used to study the interaction between a root-associated bacterium and rice plant in the rhizosphere (11–13).

In this study, a differential proteomic analysis of A. vinelandii ATCC 12837 in response to different conditions and at different locations within the Oryza sativa MR 219 rhizosphere was performed. By two-dimensional gel electrophoresis (2DE) followed by tandem mass spectrometry (MS/MS) analyses, several known and hypothetical proteins were found to be differentially expressed between A. vinelandii cells attached to rice root and the planktonic cells. Among these hypothetical proteins, a protein spot putatively identified as Avin_16040 was further studied. The expression of its gene during root surface colonization by A. vinelandii was analyzed by quantitative PCR (qPCR). An Avin_16040 deletion mutant was generated by homologous recombination, and the functional role of Avin_16040 was analyzed by conducting several phenotypic tests, such as a hydrophobicity test, root attachment assay, biofilm assay, and plant growth assay. Supported by bioinformatics information, we attempted to designate an identity for the hitherto-hypothetical protein Avin_16040. The ability to directly compare differential protein expression followed by comparative phenotypic analysis provides the means to identify potentially important proteins in the bacterial response plant-bacterium interaction.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. A. vinelandii Lipman ATCC 12837 was obtained from the American Type Culture Collection (ATCC), USA. Cultures of A. vinelandii was maintained in modified N-free Ashby medium (14). Sucrose 2% (wt/vol) was used as the sole carbon source, and the incubation was performed with continuous agitation at 200 rpm and 26 ± 2°C for up to 5 days. For the comparative proteomic analyses involving plant root-microbe interaction studies, a
modified formulation based on the Murashige-Skoog basal medium with vitamins was used (15). This modified medium (designated AMS+N+) consisted of NH₄NO₃ (1.65 g per liter) as the main nitrogen source, while K₂NO₃ was removed. To compensate for the removal of the latter, KH₂PO₄ was also removed, while K₂HPO₄ (522.51 mg per liter), KCl (223.65 mg per liter), and K₂SO₄ (435.67 mg per liter) were introduced. Other supplements in the original Murashige-Skoog basal medium remained the same. The medium was adjusted to pH 5.6. The N-free medium was devoid of NH₄NO₃ and was designated AMS–N. For studies not involving any plant root association, A. vinelandii was inoculated into AMS+N+ and AMS–N to a cell density of 10⁹ cells per milliliter and was incubated at 26 ± 2°C for 2 weeks.

For generation of a disrupted mutant using homologous recombination, the kanamycin gene from plasmid pJR215 was used (16). Plasmid pDM4 was used to assemble the DNA construct containing the disrupted allele (17). For comparative analyses of the A. vinelandii mutant and wild-type strains, cells were grown in a modified Burk N-free medium with 2% sucrose as the carbon source (18). Burk N+ medium is the modified Burk N-free medium supplemented with 1 g of ammonium acetate, 2 g of tryptone, and 1 g of yeast extract per liter (19). Competent cells of Escherichia coli S17-1pir and E. coli DH5α ECOS 101 were purchased from Yeastern Biotech (Taiwan) and were maintained in Luria-Bertani (LB) medium (Merck, Germany). After transformation with plasmid vector pDM4 (20) or its derivatives, E. coli S17-1pir was grown in Blomfield medium containing the appropriate antibiotics (21) (see Table S1 in the supplemental material). E. coli DH5α carrying pJET1.2blunt (Promega, Lithuania) derivatives was maintained in LB medium containing 50 mg liter⁻¹ of ampicillin (Sigma-Aldrich, USA). E. coli was cultivated overnight at 37°C and 200 rpm.

Azo bacter vinelandii-Orzya sativa root association system. Seeds of O. sativa L. cv. MR 219 were dehusked and surface sterilized as previously recommended (22). Sterilized seeds were plated on 0.3% agar Bac teriological no.1 (Oxoid, United Kingdom) and germinated at room temperature for 2 weeks. Sterile polypolyethylene containers (100 mm [height] by 55 mm [upper radius] by 40 mm [lower radius]) containing 100 ml of AMS+ or AMS–N were inoculated with fresh A. vinelandii ATCC 12837 culture to a final bacterial count of 10⁶ CFU ml⁻¹ of ampicillin (Sigma-Aldrich, USA). E. coli was cultivated overnight at 37°C and 200 rpm.

Crude protein extraction and 2DE-MS/MS analysis. The O. sativa MR 219 roots with attached A. vinelandii ATCC 12837 cells were cropped and shaken vigorously in a lysis solution containing 2% SDS, 0.2% NaOH, 0.05 M EDTA, 2% 2-mercaptoethanol, and Complete protease inhibitor cocktail tablet (Roche, USA) to lyse the bacterial cells. After centrifugation for 15 min at 8,000 × g and 4°C, the supernatants containing the bacterial cell lysates were collected. Suspensions of free-floating A. vinelandii ATCC 12837 cells unbound to the roots were centrifuged at 8,000 × g for 15 min at room temperature before being resuspended in bacterial lysis solution.

Crude proteins were extracted by treating the cell lysates using the standard protocol (23). The protein samples from the root-attached cells involved in plant-microbe interactions in the AMS+ and AMS–N media were designated PM-root(N) and PM-root(0N), respectively. The protein samples from the free-floating cells from the AMS+ and AMS–N media were designated PM-cell(N) and PM-cell(0N), respectively. The proteins from the A. vinelandii ATCC 12837 cells not exposed to the plant roots in the AMS+ and AMS–N media were extracted as described for the free-floating cells in the plant-microbe interaction setup, and they were designated CELL(N) and CELL(0N), respectively.

First-dimension processing of crude protein was carried out according to the recommended procedure (Immobiline DryStrip manual) of GE Healthcare (Sweden). Briefly, crude protein pellet (~250 µg) was redissolved up to a volume of 340 µl in rehydration buffer (consisting of 8 M urea, 2% 3-[3-cholamidopropyl]-dimethylammonio)-1-propanesul fonate (CHAPS), 39 mM dithiothreitol (DTT), 1% Ampholyte 3–10 soluition (40%; Sigma-Aldrich, USA), and a trace amount of bromophen blue in MilliQ processed (18.2 MΩ) water). Isoelectric focusing (IEF) was conducted with the TV4000Y-2D-IEF-SYS horizontal 2DE system (Sciec Plas, United Kingdom) according to the manufacturer’s instructions, utilizing a Consort EV232 Power Pac (SciPlas, United Kingdom) programmed at 300 V for 1 h, 600 V for 1 h, 1,500 V for 1 h, 3,000 V for 12.5 h, and 300 V for 1 h. The proteins on each gel strip were then reduced and alkylated as described previously (24). For second-dimension gel electrophoresis, the proteins were resolved in 12% SDS-polyacrylamide gels using the Protean II xi system (Bio-Rad, USA) and stained with Coomassie blue according to the manufacturer’s instructions. Gel images were captured using the MultiImager II light cabinet (Alpha Innotech, USA). Protein spots of interest were excised from the 2D gel and immersed in 0.1% glacial acetic acid. Sample spots were analyzed by matrix-assisted laser desorption ionization–tandem time of flight (MALDI-TOF/TOF) conducted using the services of a commercial provider (Protein and Proteom ics Centre, National University of Singapore, Singapore). The peptide masses (m/z) were annotated using Matrix Science’s Mascot fingerprint tools (Table 1) (25). All peptide mass fingerprints were con fined to A. vinelandii sequence similarities. Semi-quantitative evaluation of 2DE protein spot intensities was conducted by ImageMeter 1.1.1 (Flash script, biz: http://www.files Sharp script.biz/AlR/imag emeter/ImageMeter.html) (the numeric values in Table 1 show the gel spot intensities).

RT-PCR assay to study expression of the Avin_16040 gene. Reverse transcription (RT)-PCR was performed to quantify the transcription of the Avin_16040 coding gene. Total RNAs were extracted from the bacterial cells by using the acidic phenol approach (23). The bacterial cells were lysed in lysis solution as described earlier. RNAs were extracted by treating the cell lysates with saturated phenol (pH 4.3), precipitated with cold isopropanol, and dissolved in MilliQ processed (18.2 MΩ) water. The dissolved RNA was quantified and immediately processed for the reverse transcription reaction using the QuantiTect reverse transcription kit (Qiagen, Germany) according to the manufacturer’s instructions. The Avin_16040 coding gene was analyzed using custom-designed DNA primers Avin_16040 forward (5′-CTGGCCCTGAGCGGCTG-3′) and Avin_16040 reverse (5′-CCACCGCGGCTGTTCCG-3′). The hypervariable V3 region of the 16S rRNA gene was used as the internal control and amplified using DNA primers V3-forward (5′-CCTACGGAGGCGACGAGAGTA-3′) and V3-reverse (5′-ATATTACCGGGCTGCTG3′) (13). These primers were designed according to the full genome sequence of the reference strain A. vinelandii D1 (accession no. NC_012360). qPCRs were performed using the Quantifast SYBR green PCR kit (Qiagen, Germany) according to the manufacturer’s instructions, with 50 ng of cDNA as the starting template. The reaction was carried out with the CFX36 real-time PCR detection system (Bio-Rad Laboratories, USA). The relative gene expression data were analyzed by the comparative threshold cycle (Ct) method (26).

Construction and validation of the ΔAvin_16040 deletion mutant. The deletion mutant was constructed by homologous replacement. Three DNA fragments were generated by colony PCR (see Fig. S1 in the supplemental material). These were the DNA fragments located upstream and downstream from the Avin_16040 gene sequence and a kanamycin resistance (Km) gene which was PCR amplified from the pJR215 plasmid (see Table S1 in the supplemental material). All primers were designed with a restriction enzyme recognition sequence (underlined) at the 5′ end as follows: EcoRI-50F (5′-GGGGGGGAATCTACGGAGTGATA CTCGAGGTA-3′) and SalI-50R (5′-TCGAGGCTATCGTGCCAGCACACAGCCG-3′) amplified the upstream sequence (701 bp) and Avin_16040, XhoI-30F (5′-CGCGCGCTCTAGAAGTTGGAATCGTGAAGGATA-3′) and BamHI-30R (5′-TTTTTTGTTGAACTGCTAGGAG ATATCGAATCC-3′) amplified the downstream sequence (1,138 bp), and EcoRI-KanF (5′-GGGGGGGAATCTCAGGTAAGGTTGGGGAGGCGCGGTG-3′) and BamHI-KanR (5′-TTTTTTCGTTGATTCCGGAGGCCAGAATCCAAGC-3′) amplified the Km gene (928 bp). Each fragment was digested with its designated restriction enzyme (New England

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### TABLE 1 Intracellular peptide analysis results obtained with A. vinelandii Mascot peptide mass fingerprint search tool

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<th>Functional group</th>
<th>Protein spot</th>
<th>Mascot match affiliated with A. vinelandii DJ</th>
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TABLE 1 (Continued)

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<th>CELL (ON)</th>
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</table>

*a* The values presented are 2DE spot intensities according to ImageMeter 1.1.1 software. The protein spots were normalized based on spot 62.

BioLabs, United Kingdom) and fused (1:1:1 concentration ratio) to form a deletion construct of approximately 2.8 kb in size. The deletion construct was then ligated into pDM4 (doubly digested with XbaI and SalI) to form a replacement vector designated pDM4-1kanamycin. The deletion construct was then ligated into pDM4 (doubly digested with XbaI and SalI) to form a replacement vector designated pDM4-1kanamycin. The allelic exchange was confirmed by PCR using custom-designed primers UPSTREAM-50out (5'-GCGAATCCGGTTC-3') and DOWNSTREAM-50out (5'-TAGAGGTGTCGTTCGGCTAGATA-3'). The sequences of both primers were not involved in the assembly of the deletion construct and were located outside the recombinational homologous region. The mutant strain should produce a PCR fragment of 2.9 kb in length, shorter than the expected 3.3-kb fragment of the wild-type strain.

Phenotypic evaluations of deletion and wild-type strains. (i) Colony morphology. The colony morphologies of the wild type and the ΔAvin_16040 mutant were evaluated using the bacterial adherence to hydrocarbons (BATH) assay as reported previously (29). Wild-type and mutant strains were independently cultured in Burk-sucrose and Burk-sucrose+N media at 30°C and shaken at 200 rpm until stationary phase (4 days). The relative cell surface hydrophobicity was determined for the wild type and the ΔAvin_16040 mutant in both Burk-sucrose and Burk-sucrose+N media. The test was performed in triplicates.

(ii) TEM. The bacterial colony was grown on 10 mM phosphate-buffered Burk medium containing 1% glucose (designated Burk-glucose medium) at 30°C with vigorous shaking (200 rpm) until an optical density at 600 nm (OD600) of 0.5 was achieved. Subsequently, 1 μg of purified pDM4-16040m was added to 50 μl of the bacterial culture. The mixture was incubated at 30°C under static conditions for 2 h, after which it was spread on Burk-glucose nitrogen-free buffer plus 1% sucrose at 30°C on a rotary shaker at 12837 rpm. The mixture was incubated at 30°C under static conditions for 2 h, after which it was spread on Burk-glucose nitrogen-free buffer plus 1% sucrose at 30°C on a rotary shaker at 12837 rpm. Before 2 days of incubation at 30°C, visible colonies on the agar surface were examined for chromosomal integration of the deletion construct. The allelic exchange was confirmed by PCR using custom-designed primers UPSTREAM-50out (5'-GCGAATCCGGTTC-3') and DOWNSTREAM-50out (5'-TAGAGGTGTCGTTCGGCTAGATA-3'). The sequences of both primers were not involved in the assembly of the deletion construct and were located outside the recombinational homologous region. The mutant strain should produce a PCR fragment of 2.9 kb in length, shorter than the expected 3.3-kb fragment of the wild-type strain.

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(iv) BATH assay. The relative cell surface hydrophobicities of the wild type and the ΔAvin_16040 mutant were evaluated using the bacterial adherence to hydrocarbons (BATH) assay as reported previously (29). Wild-type and mutant strains were independently cultured in Burk-sucrose and Burk-sucrose+N media at 30°C and shaken at 200 rpm until stationary phase (4 days). The relative cell surface hydrophobicity was determined for the wild type and the ΔAvin_16040 mutant in both Burk-sucrose and Burk-sucrose+N media. The test was performed in triplicates.

(v) Cell autoaggregation assay. The autoaggregation assay was performed as described previously (30). The wild-type and ΔAvin_16040 mutant cells were independently grown until stationary phase (4 days) in both Burk-sucrose and Burk-sucrose+N media. The assay was performed in triplicates.

Root attachment assay. The autoaggregation assay was performed as described previously (30). The wild-type and ΔAvin_16040 mutant cells were independently grown until stationary phase (4 days) in both Burk-sucrose and Burk-sucrose+N media. The assay was performed in triplicates.

Root attachment assay. The attachments of the A. vinelandii ATCC 12837 wild-type and ΔAvin_16040 mutant strains to the root surface were assayed as described previously (31). O. sativa MR 219 seeds were surface sterilized and germinated for 2 weeks with the bacterial cells. Individual roots were cropped and transferred to 1 ml of Burk-sucrose and Burk-sucrose+N media containing 106 CFU per ml of wild-type or mutant cells. After 5 days of incubation at room temperature, each root was immersed in 1 ml of sterile 1× phosphate-buffered saline (PBS) solution (Merck, Germany) and vortexed vigorously for 10 s to remove loosely bound bacterial cells. The root was immediately transferred to sterile Whatman paper and air dried for 1 min to eliminate excess water. Subsequently, the root was weighted before being immersed in 1 ml of sterile 1× PBS solution. Root-colonizing bacterial cells were dissociated at 25°C by two sonication pulses (40 kHz) of 1 min each with a pause period of 1 min between the pulses using a Bransonic 5510 ultrasonic bath (Branson, USA). Root colonization was quantified by counting the number of viable bacterial cells. The results were normalized to the weight of each root. Each test was carried out in triplicates.

Cloning of the Avin_16040 gene. The Avin_16040 gene was amplified by PCR using the primers Avin90 forward (5'-GACCAGCCCAGTAGCCCTTGCG-3') and Avin30 reverse (5'-GCTGGCCCTTTTCCGAGATCA-3'). Avin90 forward was located at nucleotides 51 to 32 upstream of the start codon, while Avin30 reverse was located at nucleotides 27 to 50
downstream of the stop codon. The amplified PCR product consisting of the open reading frame of \textit{Avin}_16040 was cloned into the pJET1.2blunt cloning vector (Fermentas, Lithuania) and transformed into \textit{Escherichia coli} DH5\textsubscript{x} (ECOS 101 competent cells; Yeastern Biotech, Taiwan). DNA sequencing analysis was performed to obtain the nucleotide sequence of \textit{Avin}_16040. DNA sequencing was carried out commercially by Medigene Sdn Bhd. with cloning vector primers.

The freshly cultivated recombinant clone was smeared on a glass slide and air dried. Gram staining was performed as described previously (23). The slide was viewed using a Primo Star upright microscope (Zeiss, USA) at a magnification of \times 1,000 with immersion oil. \textit{E. coli} DH5\textsubscript{x} without plasmid was also cultivated and used for comparison.

Bioinformatic analyses of the \textit{Avin}_16040 gene sequence. Gene sequences were analyzed with BLASTN, TBLASTX, and BLASTP (http://blast.ncbi.nlm.nih.gov/, last accessed 15 February 2013). Multiple-sequence alignment was performed with the ClustalW Multiple Alignment program (32). The presence of transmembrane helices was predicted with programs HMMTOP version 2.0 (33) and TMHMM server v. 2.0 (34). To predict the presence of protein translocation machinery, the translated amino acid sequence of \textit{Avin}_16040 was submitted to the SignalP 4.0 server of the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/, last accessed 15 February 2013). Subsequently, the amino acid sequence of the \textit{A. vinelandii} \textit{Avin}_16040 signal peptide was analyzed with the NCBI PSI-BLAST search tool (http://blast.ncbi.nlm.nih.gov/, last accessed 15 February 2013) against the nonredundant protein database to obtain its homology matches.

AFM. Atomic force microscopy (AFM) was conducted using a JPK-NanoWizard II system (JPK Instruments AG, Germany) to analyze the cell surface topography of wild-type \textit{A. vinelandii} ATCC 12837, the \textit{ΔAvin}_16040 deletion mutant, and the \textit{E. coli} DH5\textsubscript{x} transformant containing the \textit{Avin}_16040-coding gene. Samples were prepared by smearing the bacterial cells onto glass slides before subjecting them to a short fixation period of 45 s in glutaraldehyde (2% in PBS containing Ca\textsuperscript{2+} and Mg\textsuperscript{2+}) followed by an incubation of 20 min in paraformaldehyde (4% in.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Comparative analysis by 2DE of cell-bound proteomes of \textit{A. vinelandii} ATCC 12837 influenced by different growth conditions. (A) CELL(N); (B) CELL(0N); (C) PM-cell(N); (D) PM-cell(0N); (E) PM-root(N); (F) PM-root(0N). The figure shows the distinctive presence of hypothetical protein \textit{Avin}_16040 (spot 1, gel location highlighted with black circles) when \textit{A. vinelandii} adhered to the root surface. Arrows indicate 46 protein spots (including spot 1) which showed differential presence under the different growth conditions and spot 62, which was used to normalize the spots’ intensity. Detailed information for the protein spots is outlined in Table 1. The protein molecular mass ladder (at right side of each gel) indicates 225, 150, 100, 75, 50, 35, 25, 15, and 10 kDa.}
\end{figure}
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PBS containing Ca\(^{2+}\) and Mg\(^{2+}\) according to the manufacturer’s recommendation.

Nucleotide sequence accession number. The complete sequence of the Avin_16040 structural gene has been deposited in the NCBI GenBank database under accession number KF049202.

RESULTS
Proteomic analysis. The proteomes of the A. vinelandii planktonic and root-attached cells incubated under nitrogen-free and nitrogen-enriched conditions were compared. Overall, 46 cell-bound protein spots were excised from 2D gels (Fig. 1) and identified by MALDI-TOF/TOF peptide mass fingerprinting (Table 1). Among these proteins, 2DE spot 1 (identified as the hypothetical protein Avin_16040), which showed an intense response to root surface attachment (Fig. 1), was further analyzed.

Relative quantification of Avin_16040 mRNA using qPCR. The expression of the gene was quantified by qPCR (Fig. 2). Bacterial cells that were exposed to rice roots for 2 weeks were used. The Avin_16040 gene showed high levels of transcripts in the root-attached A. vinelandii ATCC 12837 cells relative to those in the free-floating cells. In the nitrogen-enriched medium, the expression was 37,122-fold higher, while in the nitrogen-free medium, it was 18,305-fold higher, compared to 1- and 3-fold, respectively, for the free-floating cells (Fig. 2).

Bioinformatics analyses show that Avin_16040 is similar to a surface layer protein. The DNA sequence of the structural gene of Avin_16040 revealed a complete open reading frame (ORF) of 1,368 bp in length (see Fig. S2 in the supplemental material) that matched the Avin_16040 gene of A. vinelandii DJ (GenBank accession no. NC_012560) with 97% identity (1,327/1,368 nucleotide bases). The predicted ORF of Avin_16040 for A. vinelandii ATCC 12837 was larger than that for A. vinelandii DJ by three nucleotides (one codon) (see Fig. S3 in the supplemental material). Its deduced amino acid sequence matched the Avin_16040 protein of A. vinelandii DJ with a 97% identity (see Fig. S4 in the supplemental material). A BLASTP analysis against the nonredundant protein database showed that the deduced amino acid sequence of Avin_16040 shared 39% identity with the surface layer protein of Aeromonas hydrophila (GenBank accession no. ACV89427), 38% identity with the paracrystalline surface layer protein of A. hydrophila (GenBank accession no. AAA67043), and 32% identity with the paracrystalline surface layer protein of Pseudomonas stutzeri DSM 4166 (accession no. YP_005940513) (see Fig. S4 in the supplemental material). Multiple-sequence alignment of these amino acid sequences showed highly conserved amino acid sequences at both the N and C termini (see Fig. S5 in the supplemental material). Further, predictions for surface protein transmembrane helices using two independent programs, HMMTOP version 2.0 and TMHMM v. 2.0, predicted a transmembrane helix of 18 amino acids at the N terminus (see Fig. S6 in the supplemental material). The hypothetical protein also contains a putative signal peptide of 20 amino acids (MKKSSLALLA VAALSANFA) which overlapped with the sequence of the predicted transmembrane helix structure. A signal peptidase cleavage site was detected directly after the amino acid alanine (A) (see Fig. S7 in the supplemental material). Using NCBI PSI-BLAST analysis, this predicted signal peptide showed the highest similarity (84%) to the signal peptide of the P. stutzeri DSM 4166 paracrystalline surface layer protein (see Table S2 in the supplemental material).

Allelic exchange mutagenesis to generate an Avin_16040 deletion mutant. PCR fragments of the Avin_16040 upstream and downstream regions and the interrupting kanamycin gene were successfully amplified and assembled into plasmid pDM4 to produce pDM4-16040m. Restriction digestion with XbaI and SalI produced a plasmid size of approximately 10 kb (see Fig. S8 in the supplemental material), while double digestion with XbaI and SalI produced two DNA fragments of 2.8 kb and 7 kb, representing the deletion construct and the pDM4 backbone, respectively. An Avin_16040 deletion mutant was then generated by transforming the replacement vector into A. vinelandii ATCC 12837, selecting for kanamycin resistance. Counterselection of sucB was achieved on Burk agar containing 10% sucrose to isolate colonies containing a successful double-crossover recombination event. PCR analysis amplified a single DNA band of 2.9 kb in size using the primers UPSTREAM-30out and DOWNSTREAM-30out, in contrast to a PCR band of 3.3 kb in size generated from the wild-type strain. The result verified the allelic replacement of the wild-type Avin_16040 gene with the deletion allele in the A. vinelandii ATCC 12837 genome (see Fig. S9 in the supplemental material). A further validation by 2DE confirmed the deletion, based on the disappearance of the Avin_16040 protein spot from its location on a 2D gel (see Fig. S10 in the supplemental material). The deletion mutant was designated the A. vinelandii ΔAvin_16040 strain.

Phenotypic changes of the ΔAvin_16040 mutant. (i) Colonial morphology. The mutant colony was whitish opaque in color, compared to the creamy yellow of the wild-type strain (Fig. 3). In addition, the mutant colony showed a unique appearance by having clear zones within it, as if spots of lysis had locally taken place (Fig. 3B, arrows). Both the wild-type and mutant colonies were covered with mucus. However, the mucoidal substance of the mutant appeared more opaque, more watery, and with a softer texture than that of the wild type.

(ii) TEM. Figure 4 shows the thin-section analysis by TEM of the interior structure of the wild-type and ΔAvin_16040 mutant strains. Both the wild-type and mutant strains showed large granules within the cells, which are suspected to be polyhydroxybu-
tyrate granules. However, the wild-type cell was completely bordered by a well-defined layer of cell membrane (Fig. 4A, arrow), while the mutant cells were only partially bordered (Fig. 4B). At higher magnification, this thick layer surrounding the wild-type bacterial cell resembled the S-layer matrix. This structure was noticeably absent in the mutant.

(iii) **Biofilm formation assay.** The ΔAvin_16040 mutant grown in the N-free Burk-sucrose medium demonstrated biofilm formation that was decreased by more than 50% compared to that of the wild type (Fig. 5). However, no significant difference was detected when grown in the N-rich Burk-sucrose +N medium.

(iv) **BATH assay.** The bacterial cell surface hydrophobicity was estimated based on the ability to bind with hydrocarbon hexane. When the ΔAvin_16040 mutant was grown in Burk-sucrose medium, the bacterial strain totally lost its cell surface hydrophobicity. In contrast, the wild-type strain demonstrated a hydrophobicity value of 21% (Fig. 6). Both the mutant and wild-type strains showed low levels of hydrophobicity (<5%) when grown in Burk-sucrose +N medium.

(v) **Autoaggregation assay.** Autoaggregation of the bacterial cells was estimated based on the sedimentation rate of the bacterial cells. Generally, the ΔAvin_16040 mutant demonstrated significantly reduced autoaggregation when grown in either the Burk-sucrose or Burk-sucrose +N medium. The effect was more evident for the cells grown in the Burk-sucrose +N medium, in which the ΔAvin_16040 mutant strain showed 18% lower autoaggregation ability than the wild-type strain. The results are shown in Fig. 7.

(vi) **Root attachment assay.** The root attachment ability of the Avin_16040 mutant was significantly reduced, by an order of magnitude, compared to that of the wild type when assays were performed in both Burk-sucrose and Burk-sucrose +N media (Fig. 8). Each strain showed an approximately 1-fold-higher root at-
attachment ability when grown in Burk-sucrose medium (N free) than when grown in Burk-sucrose + N medium (N rich). The mutant strain showed significantly reduced biofilm formation in Burk-sucrose medium (N free). No significant change in biofilm formation was observed in Burk-sucrose + N medium (N rich).

(vii) Optical microscopy analysis. Figure 10 shows optical microscopic views of bacterial clone E. coli DH5α containing recombinant plasmid pJET1.2blunt ligated with the full gene sequence of Avin_16040. When examined by Gram staining, the recombinant clones carrying both insert orientations displayed the same cell morphological change. In comparison to nontransformed E. coli DH5α cells, a recombinant clone with pPLN0009 showed elon-
gated (filamentous) cell morphology. In addition, transparent “tube-like” structures were also observed, and these were interspersed with single cells.

(viii) AFM analysis. Atomic force microscopy (AFM) analysis revealed different cell surface topographies for the A. vinelandii ATCC 12837 wild type and the ΔAvin_16040 deletion mutant (Fig. 11). The wild-type cell displayed a structure that resembled the S-layer matrix on the surface (Fig. 11A), while the surface of the mutant was smooth (Fig. 11B).

**DISCUSSION**

Plant-microbe interactions have drawn much attention because of their contribution to plant growth. Root attachment and colonization constitute an important stage, and little is known about their contribution to plant growth. Root attachment and colonization constitute an important stage, and little is known about their contribution to plant growth.

One of the proteins that was present exclusively in root-attached cells was annotated as hypothetical protein Avin_16040 in the A. vinelandii DJ genome database. Its occurrence suggested its possible involvement of this protein with root surface attachment. Subsequently, an Avin_16040 expression by root-attached cells indicated possible involvement of this protein with root surface attachment. According to Merrigan et al. (48), bacterial surface protein is one of the bacterial cell surface attachment elements. The role of bacterial surface protein as a surface adhesin matched the expression behavior of Avin_16040. Subsequently, an Avin_16040 deletion mutant, designated A. vinelandii ΔAvin_16040, was generated. The deletion mutant was subjected to a series of physiological tests related to bacterial cell surface properties.

Biofilms are microbial communities that adhere to biotic or abiotic surfaces coated with self-produced extracellular polysaccharide materials (49). The adherence of cells and biofilm formation by the mutant were not significantly different from those for the wild-type strain when they were grown in nitrogen-enriched medium. However, the A. vinelandii ΔAvin_16040 mutant strain demonstrated a significantly reduced biofilm formation on the...
polystyrene surface compared to the wild type when grown in nitrogen-deficient medium. The effects of nitrogen on the cell membrane composition and cell membrane structure of Azotobacter were previously reported (50, 51). In addition, increased EPS production by an N₂-fixing Rhizobium sp. in growth medium supplied with various N sources compared to an N-free control has been reported (52).

Another property that has an effect on biofilm formation is the bacterial cell surface hydrophobicity. Cell surface hydrophobicity was determined by the ability of cells to adhere to hydrophobic hydrocarbon. In this study, a total loss of cell surface hydrophobicity was observed for the A. vinelandii ΔAvin_16040 mutant strain compared to the wild type when both strains were grown in the N-free medium. The lost cell surface hydrophobicity indicated either a loss or reduced adhesion of bacterial cells to external surfaces. In Staphylococcus aureus, the cell surface hydrophobicity was shown to be determined by the bacterial surface proteins (53). By disrupting its cell surface proteins with proteolytic enzymes, S. aureus showed reduced bacterial cell surface hydrophobicity. Another factor that was in direct correlation with the cell surface hydrophobicity was autoaggregation of bacterial cells (53). In this study, reduced autoaggregation of ΔAvin_16040 mutant cells compared to the wild type was observed.

The ability of the mutant to adhere to a biotic surface was also observed. The root attachment assay was performed to measure the ability to adhere to the plant root surface. Attachment of soil bacteria to plant cells is an important stage because it serves as the opening step required in plant-microbe interactions. This step is also necessary for the formation of microbial biofilms on plant roots (54, 55). The A. vinelandii ΔAvin_16040 mutant demonstrated a significantly reduced root surface attachment compared to the wild type. Just like adhesion to the polystyrene (abiotic) surface, the difference was more significant when the adherence assay was performed in the N-free medium. This finding suggests that the hypothetical protein Avin_16040 is involved in the adherence of the bacterial cells to the plant root surface. The capacity for microbial attachment to plant cells is very important to the competitiveness of microbes to colonize plant roots.

Introduction of the Avin_16040 gene into an E. coli DH5α host produced a morphological change in its customarily rod-shaped cells. Elongated filamentous cells and transparent “tube-like” morphologies were observed. The results were similar to a report

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FIG 11 AFM images of cell surface topography of wild-type A. vinelandii ATCC 12837 (A), the ΔAvin_16040 mutant (B), E. coli DH5α (C), colony E. coli DH5α(pET1.2blunt:Avin_16040) (D), and root-attached E. coli DH5α(pET1.2blunt:Avin_16040) (E). The images show increased S-protein density on the root-attached E. coli cell surface. Arrows indicate the S-protein monomer embedded on E. coli cell surface.
by Lederer et al. (56), who expressed the Lysinibacillus sphaericus JG-A12 S-layer-like protein SIIB gene in E. coli and observed filamentous cells with long, transparent, tube-like structures. The production of the heterologous S-layer protein possibly caused an alteration in cell morphology and a drastic change in the membrane property of the E. coli host.

Transmission electron micrographic (TEM) analysis of ΔAvin_16040 mutant cells revealed partial absence of their cell envelope as indicated by an incomplete border lining that enveloped the mutant cells. Enlarged views of the wild-type and mutant cells revealed a probable loss of the S-layer matrix in the mutant cells. The mutant strain appeared to develop a thick capsule-like layer of unknown identity. However, A. vinelandii is one of the bacterial species that produce both capsule and S-layer (57). Both components are important adhesion factors which contribute to the initial binding of bacterial cells to external surfaces for attachment (54). Structural characterizations of the tetragonal S-layer of A. vinelandii have been previously reported (35). The S-layer of A. vinelandii is an assembly of tetrameric structure located at the outermost surface of the bacterial cell (36). The organization consists of four identical subunits of a 60,000-molecular-weight protein (S protein), and the molecular weight of each tetrad unit was estimated to be 255,000. The assembly of the tetragonal surface array is induced by the divalent cations Ca\(^{2+}\) and Mg\(^{2+}\).

Despite S-layer proteins being one of the most abundant cellular proteins of archaea and bacteria, observations on mutants lacking the bacterial S-layer proteins are scarce. AFM observation revealed the absence of the S-layer matrix on the ΔAvin_16040 mutant cell surface. Surprisingly E. coli cells transformed with the full gene sequence of Avin_16040 showed a series of regularly arranged monomer motifs on their surface. Moreover, the acquired monomer size of approximately 13 nm was like that reported for A. vinelandii (36). This evidence substantiates the possibility that Luria et al. (56), who expressed the Lysinibacillus sphaericus JG-A12 S-layer-like protein SIIB gene in E. coli. The molecular weight of each tetrad unit was estimated to be 255,000. The assembly of the tetragonal surface array is induced by the divalent cations Ca\(^{2+}\) and Mg\(^{2+}\).

In conclusion, we have shown that an interesting protein was present only when A. vinelandii cells were attached to the O. sativa root surface and that it was encoded by the hypothetical gene Avin_16040. The proteins most similar to it are the bacterial S-layer proteins. The gene has been shown to be important for adherence, as shown by its induction when exposed to roots and the reduced adherence activity of its mutant. An E. coli host of the Avin_16040 gene showed enhanced adhesion to root surfaces, opening the prospect of enhancing root-adhesive properties of free-living plant-growth-promoting bacteria.

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