Proteomics-Based Identification of Outer-Membrane Proteins Responsible for Import of Macromolecules in *Sphingomonas* sp. A1: Alginate-Binding Flagellin on the Cell Surface[†,‡]

Wataru Hashimoto,[§] Jinshan He,[§] Yushin Wada,[§] Hirokazu Nankai,[§] Bunzo Mikami,[||] and Kousaku Murata*[,§]

Laboratory of Basic and Applied Molecular Biotechnology, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan, and Laboratory of Food Quality Design and Development, Division of Agronomy and Horticultural Science, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan

Received May 11, 2005; Revised Manuscript Received August 25, 2005

ABSTRACT: A nonmotile Gram-negative bacterium, *Sphingomonas* sp. A1, directly incorporates macromolecules such as alginate through a "super-channel" consisting of a pit formed on the cell surface, alginate-binding proteins in the periplasm, and an ATP-binding cassette transporter in the inner membrane. Here, we demonstrate the proteomics-based identification of cell-surface proteins involved in the formation of the pit and/or import of alginate. Cell-surface proteins were prepared from the outer membrane released as vesicles during the conversion of intact cells to spheroplasts. Seven proteins (p1−p7) with acidic isoelectric points were inducibly expressed in the outer membrane of strain A1 cells grown on alginate and showed significant identity with bacterial cell-surface proteins (p1−p4, TonB-dependent outer-membrane transporter; p5 and p6, flagellin; and p7, lipoprotein). Each mutant with a disruption of the p1−p4 or p6 gene showed significant growth retardation in the alginate medium. Flagellin homologues (p5 and p6) were further analyzed because strain A1 forms no flagellum. p5 was found to be uniformly distributed on the cell surface by immunogold-labeling electron microscopy and to exhibit alginate binding with a nanomolar dissociation constant by a surface plasmon resonance sensor. The cell surface of the p6 gene disruptant differed from that of the wild-type strain A1 in that pit formation was incomplete and cell-surface structures shifted from pleats to networks. These results suggest that, distinct from bacterial flagellins constituting a helical filament of flagella, strain A1 cell-surface flagellin homologues function as receptors for alginate and/or regulators of cell-surface structures.

Alginate produced by brown seaweed and certain bacteria is a linear polysaccharide composed of α-L-guluronate (G)[1] and its C5 epimer β-D-mannuronate (M) and arranged in three ways: polyG, polyM, and heteropolymeric random sequences (*1*). Brown seaweed alginate is widely used in the food and pharmaceutical industries because the polymer chelates metal ions and forms a highly viscous solution (*2*). Extracellular biofilms including alginate are important virulence factors for pathogenic bacteria, such as *Pseudomonas aeruginosa*, during lung infections in cystic fibrosis patients (*3*). These biofilms often protect *P. aeruginosa* cells from phagocytic cells and/or antibiotics (*4*, *5*), making biofilm-dependent diseases difficult to treat. Alginate-depolymerizing enzymes are thus expected to become useful biochemicals in modifying or processing edible seaweed alginate and removing bacterial biofilm alginate (*6*).

Strain A1, isolated as a potent producer of alginate-depolymerizing enzymes (alginate lyases), is a nonmotile Gram-negative bacterium belonging to the genus *Sphingomonas* (*7*). Alginate lyase III (A1-III) of *Sphingomonas* sp. A1 (strain A1) is promising as a therapeutic agent for *P. aeruginosa* biofilm-dependent infectious diseases (*8*, *9*).

[†] This work was supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to K.M. (numbers 16013221, 15013228, and 14360052), and W.H. (number 15780053). This work was also supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) of Japan.

[‡] Nucleotide sequences of p1, p2, p3, p4, p5, p6, and p7 genes have been deposited in DDBJ/GenBank/EMBL databases under accessions AB211539, AB211540, AB211541, AB211542, AB183457, AB183458, and AB211543, respectively.

* To whom correspondence should be addressed. Telephone: +81-774-38-3766. Fax: +81-774-38-3767. E-mail: kmurata@kais.kyoto-u.ac.jp.

[§] Laboratory of Basic and Applied Molecular Biotechnology, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University.

[||] Laboratory of Food Quality Design and Development, Division of Agronomy and Horticultural Science, Graduate School of Agriculture, Kyoto University.

[1] Abbreviations: G, α-L-guluronate; M, β-D-mannuronate; strain A1, *Sphingomonas* sp. A1; A1-III, strain A1 alginate lyase III; ABC, ATP-binding cassette; A1-I, strain A1 alginate lyase I; A1-II, strain A1 alginate lyase II; A1-IV, strain A1 alginate lyase IV; AlgQ1, strain A1 periplasmic alginate-binding protein Q1; AlgQ2, strain A1 periplasmic alginate-binding protein Q2; AlgS, strain A1 ATP-binding protein S for alginate import; AlgM1, strain A1 transmembrane domain M1 for alginate import; AlgM2, strain A1 transmembrane domain M2 for alginate import; LB, Luria−Bertani; IPTG, isopropyl-β-D-thiogalactopyranoside; DTT, dithiothreitol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; 2D, two dimensional; IEF, isoelectric focusing; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; IPG, immobilized pH gradient; PCR, polymerase chain reaction; Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant; Cm^s, chloramphenicol sensitive; FliC, flagellin; SPR, surface plasmon resonance; RU, resonance units; pI, isoelectric point.

10.1021/bi050873b CCC: $30.25
Published on Web 09/28/2005

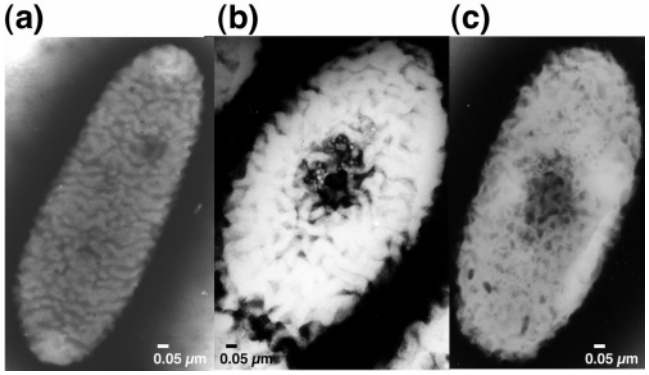

FIGURE 1: Cell-surface structure. Transmission electron microscope images after negative staining with phosphotungustic acid. (a) Strain A1 cell grown in the absence of alginate. (b) Strain A1 cell grown in the presence of alginate. (c) p6 gene disruptant cell grown on alginate. Cell-surface structures in three images (a−c) are typical (more than 80%) among about 20 cells in view fields.

Sphingomonades are distinct from other Gram-negative bacteria in that they contain glycosphingolipids instead of lipopolysaccharides in the outer membrane (*10*). This unusual characteristic provides their cell surface with pleat structures, hydrophobic circumstances responsible for degrading refractory environmental pollutants, and/or the ability to interact with the antibiotic polymyxin B (*11*−*13*). The cell surface of strain A1 also contains mono- and oligosaccharide glycosphingolipids instead of lipopolysaccharides (*7*) and is covered with many large pleats (*13*) (Figure 1a).

Strain A1 directly incorporates alginate into the cytoplasm simultaneously across the outer and inner membranes through what we term a "super-channel" (*14*), because alginate lyases are exclusively localized in the cytoplasm (*15*, *16*). The alginate incorporation pathway contains three components (Figure 2): a pit on the cell surface (*13*), alginate-binding proteins in the periplasm (*17*−*19*), and an ATP-binding cassette (ABC) transporter in the inner membrane (*20*). When strain A1 cells grow on alginate, a pit with a diameter of 0.02−0.1 μm forms on the cell surface, possibly through rearrangement of pleat structures, and concentrates the polymer (*13*) (Figure 1b). The pit disappears when cells are transferred to a medium in the absence of alginate (Figure 1a). The transport of alginate from the pit to the inner membrane is mediated by periplasmic alginate-binding proteins [AlgQ1 (59 kDa) and AlgQ2 (59 kDa)]. The ABC transporter localized in the inner membrane directly incorporates alginate into the cytoplasm. An ATP-binding protein [AlgS (40 kDa)] and transmembrane domains [AlgM1 (37 kDa) and AlgM2 (33 kDa)] constitute the ABC transporter. Incorporated alginate is then depolymerized to di-, tri-, and tetrasaccharides through the action of three cytoplasmic endotype alginate lyases, A1-I (65 kDa), A1-II (25 kDa), and A1-III (40 kDa). The alginate oligosaccharides thus formed are finally degraded by cytoplasmic exotype alginate lyase A1-IV (86 kDa) to monosaccharides, which are then nonenzymatically converted to α-keto acids. Genes for periplasmic alginate-binding proteins, the ABC transporter, and alginate lyases form a cluster in the genome of strain A1 and are inducibly expressed in the presence of alginate. This is, to our knowledge, the first known bacterial super-channel responsible for the direct import and depolymerization of macromolecules (*14*) (Figure 2).

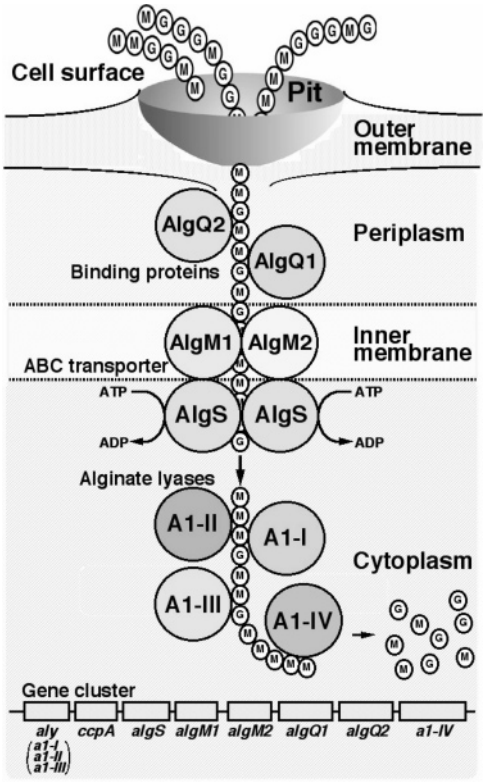

FIGURE 2: Alginate-import super-channel and alginate lyases in strain A1. Details are given in the text. G, α-L-guluronate; M, β-D-mannuronate; *aly*, gene for alginate lyases (A1-I, A1-II, and A1-III); *ccpA*, catabolite-control protein gene; *algS*, *algM1*, and *algM2*, ABC transporter genes for alginate import; *algQ1* and *algQ2*, genes for alginate-binding proteins; *a1-IV*, alginate lyase A1-IV gene.

Accumulated evidence indicates that the pit concentrates alginate and transports the polymer into the periplasm (*7*, *13*). Pit formation and the expression of periplasmic binding proteins, the ABC transporter, and alginate lyases are induced in the presence of alginate, suggesting the occurrence of a cell-surface alginate-sensing receptor involved in signal transduction. This, however, raises the following questions about the cell surface (outer membrane): What molecules recognize external alginate or constitute the pit? How does the pit concentrate alginate? What transporters are responsible for importing alginate across the outer membrane? No cell-surface molecules responsible for recognizing external alginate and transporting the polymer into the periplasm have been substantiated. Having recently determined the complete genome sequence of strain A1 [W. Hashimoto et al. (2003) Abstract for Annual Meeting of Japan Society for Bioscience, Biotechnology, and Agrochemistry, p 159], we are ready to conduct the proteome analysis. To clarify the overall macromolecule-import system (super-channel) in strain A1, this paper focuses on identifying cell-surface proteins involved in the formation of the pit and/or import of alginate by proteomics.

EXPERIMENTAL PROCEDURES

Materials. Alginate (sodium salt; average molecular mass, 26 kDa; M, 56.5%) from *Eisenia bicyclis* and hyaluronate (sodium salt) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). DEAE-Toyopearl 650M and SuperQ-Toyopearl 650C were purchased from Tosoh Corp. (Tokyo, Japan). Restriction endonucleases were obtained from Takara

Bio, Inc. (Otsu, Japan), and DNA-modifying enzymes were obtained from Toyobo Co., Ltd. (Tokyo, Japan). Gellan (average molecular mass, 500 kDa; deacetylated), pectin (average molecular mass, 350 kDa), and *Achromobacter* protease I (lysylendopeptidase) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Pyruvylated xanthan (average molecular mass, 2000 kDa) was a gift from Kohjin Co., Ltd. (Tokyo, Japan).

Microorganisms and Culture Conditions. To prepare the outer membrane, strain A1 cells were aerobically cultured at 30 °C in an alginate medium consisting of 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.1% Na_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.01% yeast extract, and 1.0% alginate (pH 7.2) or a yeast extract medium containing 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.1% Na_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, and 1.0% yeast extract (pH 7.2). To determine the growth of wild-type and gene-disruptant cells of strain A1, cells were aerobically cultured at 30 °C for 16 h in an alginate medium consisting of 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.1% Na_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.01% yeast extract, and 0.5% alginate (pH 7.2), and the turbidity of the culture was periodically measured at 600 nm. *Escherichia coli* strain BL21(DE3) (Novagen, Madison, WI) was used as the host for overexpressing the outer-membrane proteins of strain A1. To express proteins in *E. coli*, cells were aerobically precultured in Luria−Bertani (LB) medium (*21*) supplemented with ampicillin (0.1 mg/mL) at 37 °C. When turbidity reached 0.4 at 600 nm, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture (0.1 mM) and cells were further cultured at 16 °C for 42 h.

Isolation of the Outer Membrane. The outer membrane was prepared from strain A1 cells by two different procedures. (i) Preparation of the outer membrane from the total membrane fraction (*22*): briefly, strain A1 cells grown on alginate or yeast extract were converted to spheroplasts through treatment with lysozyme and EDTA, and resultant spheroplasts were ultrasonically disrupted (model 201M insonator, Kubota, Tokyo, Japan) at 0 °C and 9 kHz for 5 min. After intact cells and spheroplasts were removed by centrifugation at 20000*g* and 4 °C for 15 min, cell lysates were ultracentrifuged at 100000*g* and 4 °C for 2 h. Precipitants (total membranes, i.e., a mixture of outer and inner membranes) after washing were subjected to sucrose density gradient centrifugation. (ii) Isolation of the outer membrane during the conversion of intact cells to spheroplasts (*23*): unless otherwise specified, all operations were conducted at 0−4 °C. Strain A1 cells (wet weight of 8 g) were collected by centrifugation at 6000*g* for 5 min, washed with distilled water, and then resuspended in distilled water (29.7 mL). After 0.1 M Tris-HCl (pH 8.3) (17.9 mL), 2 M sucrose (15.1 mL), 1% EDTA (3.0 mL), and 0.5% lysozyme (3.0 mL) were added, the cell suspension was incubated at 30 °C for 1 h and then centrifuged at 20000*g* for 15 min to remove spheroplasts. The supernatant was ultracentrifuged at 100000*g* for 1 h. Resultant precipitants were washed with 0.1 M Tris-HCl (pH 7.5) containing 2% glycerol and 1 mM dithiothreitol (DTT), re-ultracentrifuged at 100000*g* for 1 h, and then resuspended in the same buffer (outer membrane).

Western Blotting. To detect the inner-membrane protein, membrane fractions were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (*24*), followed by Western blotting using anti-AlgS antibodies as described elsewhere (*25*). Anti-IgG conjugated with horseradish peroxidase (Pharmacia Biotech. Co., Uppsala, Sweden) and a POD immunostaining kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were used to visualize the protein band. The expression of an outer-membrane protein (p5) in strain A1 cells was also confirmed by Western blotting using anti-p5 antibodies.

Protein Assay. The protein concentration was determined by the methods of Bradford (*26*) for soluble proteins and Lowry et al. (*27*) for insoluble proteins, with bovine serum albumin as the standard.

Differential Display. (i) SDS−PAGE: outer-membrane proteins were separated on 12.5% SDS−PAGE gel. (ii) Two-dimensional (2D)-PAGE: the outer membrane was solubilized for isoelectric focusing (IEF) on ice for 10 min with a solution consisting of 7 M urea, 2 M thiourea, and 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). Insoluble materials were discarded by centrifugation at 10000*g* and 4 °C for 10 min. After 0.5% immobilized pH gradient (IPG) buffer, 20 mM DTT, and a trace of bromophenol blue were added, solubilized samples were loaded on immobiline dry strip gels (pH 3−10, 24 cm; pH 4−7, 24 cm) (Amersham Biosciences Corp., Piscataway, NJ) through rehydration and kept for more than 12 h under mineral oil. IEF was conducted at 20 °C for 96 000 Vh using IPGphor IEF (Amersham Biosciences Corp.). After equilibration with a buffer of 50 mM Tris-HCl (pH 6.8), 6 M urea, 30% glycerol, 2% SDS, and 0.25% DTT, strip gels were subjected to SDS−PAGE (12.5% gel) using Ettan *DALT II* (Amersham Biosciences Corp.). Proteins separated on 2D-PAGE gels were detected with Coomassie brilliant blue R-250 or silver.

Preparation of Internal Peptides of Outer-Membrane Proteins. Spots for outer-membrane proteins (p1−p7) inducibly expressed in the presence of alginate were cut out from 2D-PAGE gels and hydrolyzed at 35 °C for 20 h with lysylendopeptidase in 0.1 M Tris-HCl (pH 8.5). Resultant peptides were subjected to capillary HPLC using a 140B pump, a 785A UV monitor (Applied Biosystems Division, Perkin−Elmer, Foster City, CA), a microflow processor, a UZ flowcell, a microinjector (LC Packings, Amsterdam, Netherlands), and a Probot microfractionater (Bai, Lautern, Germany). Peptides were eluted for 100 min with a linear gradient of acetonitrile (2−90%) in 0.1% trifluoroacetic acid through a reversed-phase column (Symmetry C18 3.5 μm, 1.0 × 150 mm, Waters, Millipore, Billerica, MA) and detected by measuring absorbance at 210 and 280 nm.

N-Terminal Amino Acid Sequence. N-terminal amino acid sequences of internal peptides derived from p1−p7 were determined by Edman degradation with a Procise 494 HT protein sequencer (Applied Biosystems Division, Perkin−Elmer, Foster City, CA).

Sequencing and Manipulation of DNA. Nucleotide sequences of p1−p7 genes and their disrupted genes were determined by dideoxy-chain termination using automated DNA sequencer Model 377 (Applied Biosystems Division, Perkin−Elmer) (*28*). Subcloning, transformation, and gel electrophoresis were conducted as described elsewhere (*21*).

Gene Disruption. Plasmids for disrupting p1−p7 genes were constructed as follows: DNA fragments containing p1−p7 genes in the center were amplified by the polymerase chain reaction (PCR) using KOD polymerase (Toyobo Co., Ltd.), genomic DNA of strain A1 as a template, and two

synthetic oligonucleotides as primers. Oligonucleotides for cloning each gene were as follows: p1, forward 5′-GGCATATGAAATACCCGCACGAGGTAC-3′ and reverse 5′-GGCTCGAGCCACTTGTATTCGCCCGTCAGC-3′; p2, forward 5′-GGCATATGTCACGCCAGCATCGCTACC-3′ and reverse 5′-GGCTCGAGACGAGAAGCGTCGAAGCTGTAC-3′; p3, forward 5′-GGCATATGCAACAACGCTCGCTCAAGC-3′ and reverse 5′-GGCTCGAGGAAATCCACCGATGCCGAC-3′; p4, forward 5′-GGCATATGACCGGAGAGATTCACATGA-3′ and reverse 5′-GGCTCGAGGAACGTCCCCTGCATCCCCAGC-3′; p5, forward 5′-AAGGATCCCGAACGCTTGTTGATGGACGTG-3′ and reverse 5′-AAGGATCCGTCGTTGCTGATTTCGCCCAGT-3′; p6, forward 5′-AAGGATCCATGCTGAGTCTTCACACCAATGC-3′ and reverse 5′-AAGGATCCTTACTGCATCAGCGACAGCACGA-3′; and p7, forward 5′- AAGGATCCATGTTGTCCACCATGAAGCAATA-3′ and reverse 5′- AAGGATCCTCAGTCGAGCCCCAACAACCCCAC-3′. These oligonucleotides have a site (indicated by underlining) for the restriction enzyme (*Nde*I, *Xho*I, or *Bam*HI) added to 5′ regions. Fragments (p1−p7 genes) amplified by PCR were isolated and ligated with *Hin*cII-digested pUC118 (Takara Bio, Inc.). Resultant plasmids containing each of the p1−p7 genes were designated pUC118-pX (X, 1−7). Kanamycin-resistant gene cassettes for aminoglycoside 3′-phosphotranferase (Km^r) were amplified through PCR using pUC4K (Amersham Biosciences Corp.) as a template and two synthetic oligonucleotides as primers. Oligonucleotides for cloning the Km^r gene were as follows: for disruption of p1 and p3 genes, forward 5′-GGGATATCGGGGGCGCTGAGGTCTGCCTCGTG-3′ and reverse 5′-GGGATATCGGGGGGGAAAGCCACGTTGTGTCT-3′; for disruption of p2, p4, and p7 genes, forward 5′-GGAGCGCTGGGGGCGCTGAGGTCTGCCTCGTG-3′ and reverse 5′-GGAGCGCTGGGGGGGAAAGCCACGTTGTGTCT-3′; for disruption of the p5 gene, forward 5′-GGCCATGGGGGGGCGCTGAGGTCTGCCTCG-3′ and reverse 5′-GGCCATGGGGGGGAAAGCCACGTTGTGTCT-3′; and for disruption of the p6 gene, forward 5′-GGTGGCCAGGGGGCGCTGAGGTCTGCCTCGTG-3′ and reverse 5′-GGTGGCCAGGGGGGGGAAAGCCACGTTGTGTCT-3′. These oligonucleotides have a site (indicated by underlining) for the restriction enzyme (*Eco*RV, *Eco*47III, *Nco*I, or *Bal*I) added to 5′ regions. Km^r genes were introduced at the *Bal*I site of p1 and p6 genes in pUC118-p1 and pUC118-p6; at the *Eco*47III site of p2, p4, and p7 genes in pUC118-p2, pUC118-p4, and pUC118-p7; at the *Eco*RV site of the p3 gene in pUC118-p3; and at the *Hin*cII site of the p5 gene in pUC118-p5. Resultant plasmids with a disruption in each of the p1−p7 genes were designated pUC118-pX::Km^r (X, 1−7). pX::Km^r (X, 1−7) genes disrupted by insertion of the Km^r gene were amplified through PCR using pUC118-pX::Km^r (X, 1−7) as a template and two oligonucleotides (M13 forward and reverse primers) with a *Bam*HI site added to 5′ regions. Resultant PCR products were digested with *Bam*HI and ligated with *Bam*HI-digested pKTY320 with the gene for chloramphenicol acetyl transferase (Cm^r) (*29*). Resultant plasmids were designated pKTY320-pX::Km^r (X, 1−7) and introduced into *E. coli* strain DH5α (Toyobo Co., Ltd.). *E. coli* strain DH5α containing each pKTY320-pX::Km^r (X, 1−7) was used to transconjugate strain A1 cells through triparental mating in the presence of *E. coli* strain HB101 harboring pRK2013 (*30*). p1−p7 gene disruptants (Km^r and Cm^s) generated by homologous recombination were selected on a 0.5% alginate medium solidified with 1.5% agar containing 0.5% yeast extract, 5 μg/mL polymyxin B, and 25 μg/mL kanamycin.

Expression and Purification of Flagellin Homologues. Plasmids for overexpression of p5, p6, and *E. coli* flagellin (FliC) were constructed as follows: to introduce p5, p6, and FliC genes into an expression vector, pET21b or pET21d (Novagene), PCR was conducted using the genomic DNA of strain A1 or *E. coli* as a template and two synthetic oligonucleotides as primers. The following oligonucleotides were used for overexpression: p5, forward 5′-GGCATATGGCAATGACCATTAACACCAACG-3′ and reverse 5′-GGCTCGAGGCTCAGCAGCTTCAGCACTTGC-3′; p6, forward 5′-CCCATATGCTGAGTCTTCACACCAATGCCG-3′ and reverse 5′-CCCTCGAGCTGCATCAGCGACAGCACGAGC-3′; and FliC, forward 5′-GGCCATGGCACAAGTCATTAATACCAACAG-3′ and reverse 5′-GGCTCGAGACCCTGCAGCAGAGACAGAACC-3′. These oligonucleotides have an *Nde*I, *Nco*I, or *Xho*I site (indicated by underlining) in 5′ regions. p5 and p6 gene fragments amplified by PCR were digested with *Nde*I and *Xho*I and then ligated with *Nde*I- and *Xho*I-digested pET21b. The FliC gene fragment amplified by PCR was digested with *Nco*I and *Xho*I and then ligated with *Nco*I- and *Xho*I-digested pET21d. Resultant plasmids containing p5, p6, and FliC genes were designated pET21b-p5, pET21b-p6, and pET21d-FliC, respectively.

Unless otherwise specified, all purification was conducted at 0−4 °C. Cells of *E. coli* strain BL21(DE3) harboring pET21b-p5, pET21b-p6, or pET21d-FliC were grown in 1.5 L of LB medium (1.5 L/flask), collected by centrifugation at 6000*g* and 4 °C for 5 min, washed with 20 mM Tris-HCl (pH 7.5), and then resuspended in the same buffer. Cells were ultrasonically disrupted (model 201M insonator, Kubota) at 0 °C and 9 kHz for 20 min. Precipitants obtained on centrifugation at 15000*g* and 4 °C for 5 min were resuspended in 20 mM Tris-HCl (pH 7.5) containing 1 M arginine and solubilized by sonication. After supplementation with 1 mM phenylmethylsulfonyl furoride and 0.1 μM pepstatin A, solubilized materials were applied to a DEAE-Toyopearl 650M column (2.6 × 20 cm) previously equilibrated with 20 mM Tris-HCl (pH 7.5). Proteins were eluted with a linear gradient of 0−1.0 M NaCl (300 mL) in 20 mM Tris-HCl (pH 7.5), with a 3-mL fraction collected every 3 min. Fractions containing p5, p6, or FliC, which were eluted with 0.3−0.4 M NaCl, were combined and dialyzed against 20 mM Tris-HCl (pH 7.5). The dialysate was subjected to a SuperQ-Toyopearl 650C column (1.0 × 5.0 cm) previously equilibrated with 20 mM Tris-HCl (pH 7.5). Proteins were eluted with a linear gradient of 0−0.5 M NaCl (30 mL) in 20 mM Tris-HCl (pH 7.5), with a 1-mL fraction collected every 1 min. Fractions containing p5, p6, or FliC, which were eluted with 0.2 M NaCl, were combined and dialyzed against 20 mM Tris-HCl (pH 7.5). The dialyzate was used as purified p5, p6, or FliC.

Immunogold-Labeling Electron Microscopy. Antibodies to purified p5 were raised in a rabbit, and the serum was used as polyclonal antibodies against p5. Immunoelectron microscopy was conducted as described elsewhere (*31*). Briefly, strain A1 cells grown on alginate or yeast extract were fixed,

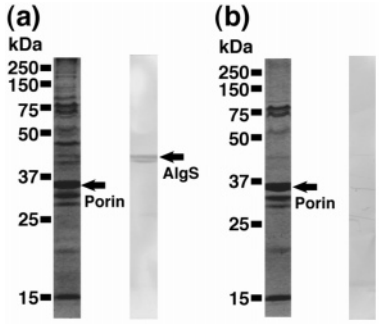

FIGURE 3: Isolation of the strain A1 outer membrane. Total membranes obtained from spheroplasts and vesicles released during the conversion of intact cells to spehroplasts were subjected to sucrose density gradient centrifugation. Each fraction with a high density from total membranes (a) and vesicles (b) is shown through SDS−PAGE analysis, followed by protein staining with Coomassie brilliant blue (left) and Western blotting using anti-AlgS antibodies (right).

dehydrated, and embedded in epoxy resin. Ultrathin sections were immunostained using anti-p5 antibodies and anti-rabbit IgG antibodies conjugated to 15 nm colloidal gold (Amersham Biosciences Corp.).

Surface Plasmon Resonance (SPR) Biosensor Analysis. To measure the interaction between flagellin homologues (p5, p6, and FliC) and polysaccharides, SPR biosensor analysis was conducted at 25 °C using CM5 sensor chips on a Biacore 3000 (Biacore International AB, Uppsala, Sweden), as described previously (*19*).

RESULTS

Outer Membrane of Strain A1. To isolate the outer membrane from Gram-negative bacteria such as *E. coli* and *Salmonella typhimurium*, we generally did as follows (*22*): bacterial cells are converted to spheroplasts through treatment with lysozyme−EDTA in a high osmotic solution, and resultant spheroplasts are ultrasonically disrupted. From a mixture of total membranes obtained after ultracentrifugation of speroplasts, outer and inner membranes are separated by sucrose density gradient centrifugation. Two membranes are divided on the basis of their density because the density of the outer membrane is well-known to be higher than that of the inner membrane. A mixture of total membranes obtained from strain A1 spheroplasts was subjected to sucrose density gradient centrifugation. The protein profile of membrane fractions with high to low densities (1.21−1.16 mg/mL) was analyzed by SDS−PAGE. Major protein bands with a molecular mass of about 35 kDa were observed in fractions with a high density (Figure 3a, left) and probably corresponded to outer-membrane porins based on the abundance of porins consisting of 300−420 amino acids in Gram-negative bacteria (*32*). Other than porins, a large number of faint bands with a high and low molecular mass were included in each fraction. Because ATP-binding protein AlgS of the ABC transporter for alginate is localized in the inner membrane of strain A1 cells (*20*), we examined the purity of the outer membrane by Western blotting through the absence of detection of the AlgS protein included in the fractions. We found the AlgS protein in almost all fractions, even in a fraction with a high density (Figure 3a, right), suggesting the difficulty in separating outer and inner membranes from total membranes.

Mizushima and Yamada (*23*) reported that part of the outer membrane is released as vesicles from bacterial cells, while intact cells are converted to spheroplasts through treatment with lysozyme−EDTA in a high osmotic solution. We thus tried to isolate the outer membrane as vesicles. Strain A1 cells were converted to spheroplasts and then centrifuged to remove spheroplasts. The resultant supernatant was ultracentrifuged, and precipitants were recovered. Precipitants were also subjected to sucrose density gradient centrifugation. The protein profile of membrane fractions with high to low densities was analyzed by SDS−PAGE. Most fractions showed profiles similar to each other, that is, porins with a molecular mass of about 35 kDa were major proteins and some proteins with molecular masses of 75, 40, and 20 kDa were observed (Figure 3b, left). No AlgS protein was detected in any membrane fraction (Figure 3b, right), indicating that precipitants after ultracentrifugation contain the homogeneous outer membrane. The strain A1 outer membrane was determined to have a density of 1.20 mg/mL. Precipitants were used in further experiments as the isolated outer membrane.

Differential Display in Outer-Membrane Proteins. Outer membranes obtained from strain A1 cells grown in the presence and absence of alginate were first subjected to SDS−PAGE, and protein expression profiles in the outer membranes were compared (Figure 4a). Although profiles were basically similar to each other, some proteins with molecular masses of around 75 and 40 kDa were inducibly expressed in the outer membrane of alginate-grown cells, while a protein band (19 kDa) was detected only in yeast-extract-grown cells. We tried to identify these proteins through N-terminal amino acid sequence analysis but failed, possibly because of the low resolution of SDS−PAGE.

Two types of outer membranes were then subjected to 2D-PAGE. Because thiourea is reported to be an effective reagent for solubilizing outer membrane proteins from Gram-negative bacteria (*33*), strain A1 outer membrane proteins were solubilized with CHAPS in the presence of urea and thiourea. Solubilized outer membrane proteins were separated by 2D-PAGE consisting of IEF (pH 3−10) and SDS−PAGE (12.5% gel) and stained with silver (parts b and c of Figure 4). Interestingly, protein expression profiles in outer membranes on 2D-PAGE gels differed significantly between strain A1 cells grown in the presence and absence of alginate, although profiles on SDS−PAGE gels were similar to each other (Figure 4a). Comparing protein expression profiles in the outer membrane of alginate-grown cells to those of yeast-extract-grown cells, we found over 20 proteins to be expressed inducibly in outer membranes of alginate-grown cells (Figure 4b). Seven proteins (p1−p7) with acidic isoelectric points (pI values) were significantly abundant because they were readily detected even with Coomassie brilliant blue. Their pI and molecular mass were determined as follows: p1 (pI 5.7, 75 kDa), p2 (pI 5.2, 75 kDa), p3 (pI 5.2, 74 kDa), p4 (pI 4.9, 75 kDa), p5 (pI 4.8, 41 kDa), p6 (pI 5.7, 31 kDa), and p7 (pI 5.7, 27 kDa).

Identification of Outer-Membrane Proteins. To identify outer-membrane proteins (p1−p7) inducibly expressed in the outer membrane of strain A1 cells grown on alginate, we digested p1−p7 with lysylendopeptidase, isolated internal peptides, and then determined N-terminal amino acid sequences of peptides (Table 1). We analyzed internal peptides

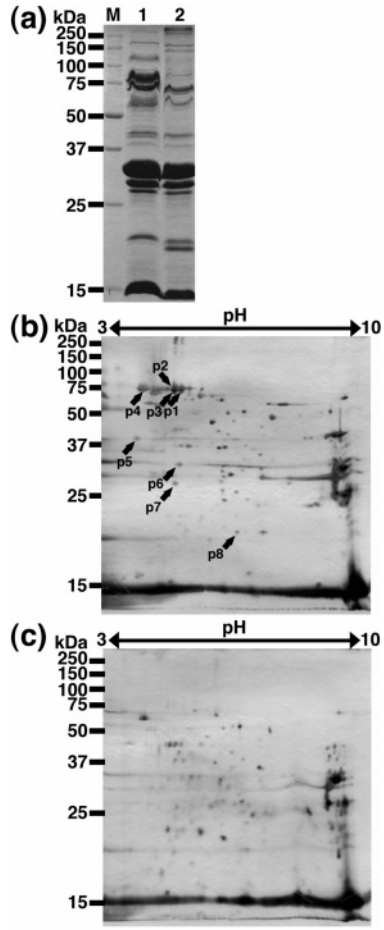

FIGURE 4: Protein expression profiles in outer membranes from strain A1 cells. (a) SDS−PAGE followed by protein staining with Coomassie brilliant blue. Lane M, molecular mass standards (from top): polypeptides with molecular masses of 250, 150, 100, 75, 50, 37, 25, and 15 kDa; lane 1, the outer membrane (40 μg of protein) from alginate-grown cells; and lane 2, the outer membrane (40 μg of protein) from yeast extract-grown cells. (b) Outer membrane proteins (0.7 mg) from alginate-grown cells were subjected to 2D-PAGE (IEF, pH 3−10; SDS−PAGE, 12.5%), followed by protein staining with silver. Eight proteins (p1−p8) indicated by arrows were inducibly expressed in the presence of alginate. (c) Outer membrane proteins (0.7 mg) from yeast extract-grown cells were subjected to 2D-PAGE (IEF, pH 3−10; SDS−PAGE, 12.5%), followed by protein staining with silver.

from p1−p7 after failing to determine the N-terminal amino acid sequences of p1−p4 possibly because of the N-terminal amino acid modification (e.g., pyroglutamylation) as is often seen in outer-membrane proteins in Gram-negative bacteria (*34*). In fact, all of p1−p4 after removal of signal peptides are predicted to have a glutamine residue as the N terminus through the primary structure analysis using a LipoP program (http://www.cbs.dtu.dk/services/LipoP/).

p1−p7 genes were identified on the basis of overall genomic information on strain A1 [W. Hashimoto et al. (2003) Abstract for Annual Meeting of Japan Society for Bioscience, Biotechnology, and Agrochemistry, p 159] and internal amino acid sequences of proteins (Table 1). The pI and molecular mass of p1−p7 were predicted from deduced amino acid sequences and confirmed to be comparable to those determined experimentally, indicating the correctness of p1−p7 identification. In homology analysis using primary structures of p1−p7, they show significant identity with bacterial cell-surface proteins as follows: p1−p4, TonB-dependent outer-membrane receptor/transporter; p5 and p6, flagellin; and p7, putative lipoprotein (Table 2).

Characterization of Gene Disruptants. To find clues to the involvement of p1−p7 in the formation of the pit and/or import of alginate, we constructed mutants with a disruption in each gene through an insertion of the Km^r gene. Gene disruption was confirmed through antibiotic selection and PCR amplification. Genomic PCR indicated that each gene disruptant had an enlarged gene of the outer membrane protein compared to wild-type strain A1 because of the insertion of the Km^r gene (Figure 5a). A mutant with a disruption of the p1, p2, p3, p4, or p6 gene shows significant growth retardation in the alginate medium (Figure 5b), while there was little difference in the growth on other carbon sources (e.g., pectin) between each mutant and wild-type strain A1 (data not shown). The growth of disruptants accelerated at the later stage, suggesting that disruptions might cause defects in adaptation to alginate.

Although little significant difference was observed in cell-surface structures between some disruptants and wild-type strain A1, the cell surface of the p6 gene disruptant differed from that of wild-type strain A1 in that formation of the pit was incomplete and cell-surface structures changed from pleats to networks (Figure 1c). In the case of the p5 gene disruptant, some cells show modulated surface structures observed in the p6 gene disruptant, but their frequency (50%) was lower than that of the p6 gene disruptant cells (over 80%). Among the proteins identified, i.e., p1−p7, the presence of flagellin homologues p5 and p6 was unexpected, because extensive examination by optical and electron microscopy failed to show the flagellation and motility of strain A1 cells grown under various conditions tested (*7*, *13*). We thus focused on functions of p5 and p6.

Genetic Features of Flagellin Homologues. The p5 gene of 1152 bp encodes a polypeptide composed of 383 amino acid residues with a molecular weight of 39 751 and a predicted pI of 4.79. The p6 gene of 894 bp encodes a polypeptide composed of 297 amino acid residues with a molecular weight of 31 066 and a predicted pI of 5.30. The homology of p5 and p6 against DNA/protein databases was analyzed using the BLAST program assisted by the GenomeNet server (http://www.genome.ad.jp/). p5 and p6 showed the most significant homology with a probable flagellin of *Xanthomonas axonopodis* (*35*) [*e* value, e-100; identity, 52%; accession number (acc. no.), AE011832] and a lateral flagellin of *Vibrio parahaemolyticus* (*36*) (*e* value, 4e-69; identity, 45%; acc. no., L06176), respectively. p5 and p6 are mutually similar (40% identity). Bacterial flagellins are generally known to constitute a helical flagellar filament through self-assembly and function as a propeller (*37*). The structure and function of *E. coli* and *S. typhimurium* flagellins have been well-characterized (*38*, *39*). N- and C-terminal domains highly conserved among bacterial flagellins are responsible for self-assembly (propeller shaft) in flagellar filaments, while the central domain forming a propeller is variable (*39*). Strain A1 p5 and p6 are highly similar to other bacterial flagellins in the N- and C-terminal domains, although little homology is observed in the central domain. Part (~100 amino acids) of the central domain of p5 and p6 and flagellins of *X. axonopodis* and *V. parahaemolyticus* is missing compared to *E. coli* (*38*) and *S. typhimurium* (*39*) flagellin structures.

Table 1: Genetic Characteristics of Outer-Membrane Proteins Inducibly Expressed in the Presence of Alginate

	internal amino acid sequence	ORF[a]	start[b]	stop[c]	bp[d]	chain[e]	amino acid[f]	MW[g]	pI[h]
p1	NH_2-VYWDALYRGHAV	SPH1326	1 547 388	1 545 160	2229	complement	742	81 267 (77 941)	5.76 (5.44)
p2	NH_2-GFSTDPDGWARQ	SPH2525	2 901 644	2 899 647	1998	complement	665	74 563 (71 073)	5.37 (5.11)
p3	NH_2-GDIVSASSGANN	SPH3146	3 630 592	3 628 436	2157	complement	718	76 341 (73 645)	5.36 (5.17)
p4	NH_2-LNNLFDAEVREH	SPH1886	2 161 953	2 164 103	2151	sense	716	76 113 (71 975)	5.14 (4.90)
p5	NH_2-VTDADFASETAN	SPH223	268 711	269 862	1152	sense	383	39 751	4.79
p6	NH_2-LSAAMTFQIGAA	SPH1824	2 091 496	2 090 603	894	complement	297	31 066	5.30
p7	NH_2-IEPIAELFSDLD	SPH726	842 948	842 124	825	complement	274	30 487 (27 971)	5.72 (5.31)

[a] ORF name in the strain A1 genome. [b] Location of the start codon in the strain A1 genome. [c] Location of the stop codon in the strain A1 genome. [d] Base pairs. [e] Indication of the encoded chain of the double-stranded strain A1 genome. [f] Amino acid residues from the deduced amino acid sequence. [g] Molecular weight predicted from the deduced amino acid sequence. The value in parentheses indicates the molecular weight calculated from the deduced amino acid sequence without a signal peptide predicted through a LipoP program (http://www.cbs.dtu.dk/services/LipoP/). [h] Isoelectric point predicted from the deduced amino acid sequence. The value in parentheses indicates the isoelectric point calculated from the deduced amino acid sequence without a signal peptide predicted through a LipoP program (http://www.cbs.dtu.dk/services/LipoP/).

Table 2: Homology Analysis of Outer Membrane Proteins Inducibly Expressed in the Presence of Alginate

	e value	identity (%)	homologous protein	MW[a]	producer	accession number
p1	e-118	35	putative TonB-dependent receptor protein	82 010	*Nitrosomonas europaea*	BX321863
p2	5e-52	26	putative TonB-dependent outer-membrane receptor protein	80 884	*Leptospira interrogans*	AE011607
p3	0.0	47	putative ferric siderophore receptor	78 708	*Bordetella pertussis*	BX640417
p4	e-170	47	putative TonB-dependent receptor	74 738	*Burkholderia mallei*	CP000011
p5	e-100	52	putative flagellin	40 880	*Xanthomonas axonopodis*	AE011832
p6	4e-69	45	lateral flagellin	29 746	*Vibrio parahaemolyticus*	L06176
p7	1e-95	66	putative lipoprotein	30 195	*Pseudomonas syringae*	AE016853

[a] Molecular weight.

Localization of Flagellin Homologues. Immunoelectron microscopy directly demonstrates detailed localization of antigenic proteins in cells. To examine the localization of flagellin homologues in strain A1 cells by immunoelectron microscopy, antibodies to flagellin homologues had to be prepared. An overexpression system for p5 and p6 was thus constructed in *E. coli* cells. After construction of a plasmid containing the p5 or p6 gene, the accuracy of their nucleotide sequences was confirmed by DNA sequencing (data not shown). A transformant of *E. coli* strain BL21(DE3) with plasmid pET21b-p5 or pET21b-p6 was grown at 16 °C in LB broth in the presence of IPTG at 0.1 mM to induce gene expression. Although both p5 and p6 were overexpressed as inclusion bodies in *E. coli* cells, flagellin homologues were solubilized with 1 M arginine and then purified through two anion-exchange column chromatographies. Purified p5 and p6 were confirmed to be homogeneous by SDS−PAGE (Figure 6a). Antibodies to p5 were obtained by immunization of the purified p5 in rabbit because we could purify much soluble p5. Western blotting analysis using anti-p5 antibodies showed a single protein band with a molecular mass of 40 kDa corresponding to p5 in strain A1 cells grown on alginate (lane 1 in Figure 6b) and also in the outer membrane of alginate-grown cells (lane 2 in Figure 6b). This evidenced a high specificity of anti-p5 antibodies for p5. Although p5 seemed not to be included in the outer membrane of yeast-extract-grown cells (lane 3 in Figure 6b), a faint band of p5 appeared after a prolonged immunostaining (lane 4 in Figure 6b), suggesting that p5 is constitutively expressed in strain A1 cells, although at a low level. Immunoelectron microscopy by immunogold labeling using anti-p5 antibodies showed gold particles on the surface of alginate-grown cells (Figure 6c, upper), but not in cells grown in the absence of alginate (Figure 6c, lower), indicating that p5 was inducibly expressed in alginate-grown cells and exclusively localized in the cell envelope.

Biosensor Analysis of the Interaction Between Flagellin Homologues and Polysaccharides. Because flagellin homologues were expressed on the cell surface of strain A1 grown in the presence of alginate, we studied the interaction between flagellin homologues and alginate using the SPR biosensor. p5 binding to alginate was detected at pH 4.0, 5.0, and 6.0, peaking at pH 4.0 (Figure 7a). In further interaction experiments at pH 4.0, the interaction between p5 and alginate increased with an increasing alginate concentration (Figure 7b). Because multiple binding data could not be fitted to 1:1 binding models provided with BIA evaluation software (Biacore International AB), we analyzed interaction kinetics using a hypothetical model with two alginate-binding sites, A and B, in p5. Dissociation constants (K_d) between p5 and alginate were estimated to be 1.3×10^{-7} M for site A and 2.6×10^{-9} M for site B. p5 bound alginate and xanthan but with a greater affinity to alginate (Figure 7c). Other polysaccharides such as pectin, gellan, and hyaluronate were inert as substrates. The alginate-binding ability of p6 was also confirmed to be comparable to that of p5.

To verify the universality of the alginate-binding ability in bacterial flagellins, we overexpressed, purified, and characterized *E. coli* FliC (Figure 6a), as is the case of p5 and p6. In addition to p5 and p6, FliC also bound alginate and is anticipated to have two alginate-binding sites with dissociation constants of 2.1×10^{-7} M and 1.5×10^{-9} M,

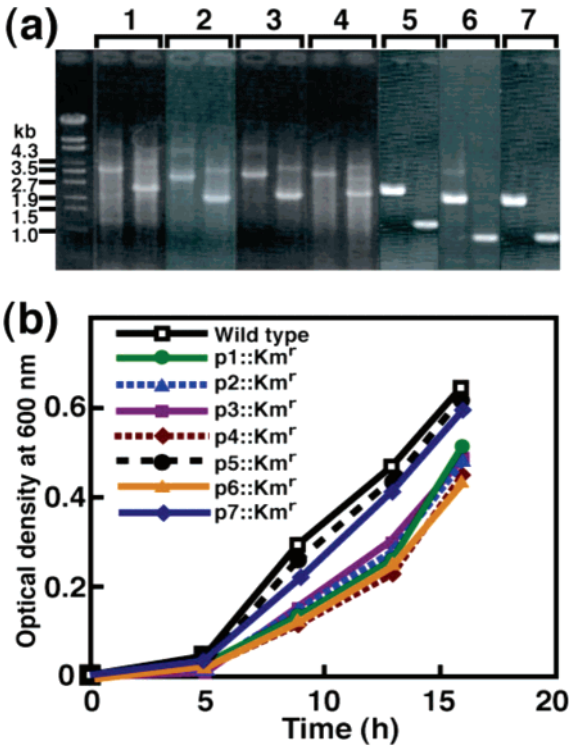

FIGURE 5: Characterization of outer membrane gene disruptants. (a) Confirmation of gene disruption. PCR products (outer membrane genes) from genomic DNAs of the gene disruptant (left) and wild-type strain A1 (right) were subjected to agarose gel electrophoresis. Block 1, p1 gene amplification; block 2, p2 gene amplification; block 3, p3 gene amplification; block 4, p4 gene amplification; block 5, p5 gene amplification; block 6, p6 gene amplification; and block 7, p7 gene amplification. (b) Growth of wild-type strain A1 and p1−p7 gene disruptants in the alginate medium.

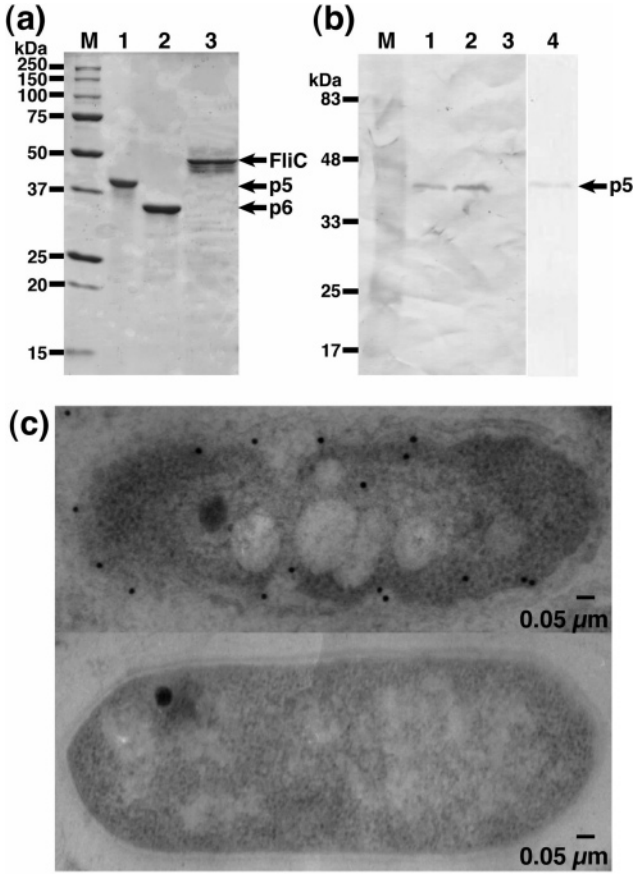

FIGURE 6: Expression of flagellin homologues. (a) SDS−PAGE followed by protein staining with Coomassie brilliant blue. Lane M, molecular mass standards (from top): polypeptides with molecular masses of 250, 150, 100, 75, 50, 37, 25, 20, and 15 kDa; lane 1, purified p5 (3 μg); lane 2, purified p6 (3 μg); and lane 3, purified *E. coli* flagellin (FliC) (3 μg). (b) SDS−PAGE followed by Western blotting using anti-p5 antibodies. Lane M, molecular mass standards (from top): 83, 48, 33, 25, and 17 kDa; lane 1, strain A1 cell lysates (2 μg of protein); lane 2, the outer membrane (2 μg of protein) from alginate-grown cells; lane 3, the outer membrane (2 μg of protein) from yeast extract-grown cells; and lane 4, the same sample as in lane 3, although prolonged immunostaining. (c) Localization of p5 in strain A1 cells. Immunogold electron microscopy image of strain A1 cells grown on alginate (upper) and yeast extract (lower).

comparable to those of strain A1 flagellin homologues (Figure 7d).

DISCUSSION

In this work, we have found strain A1 outer-membrane proteins inducibly expressed in the presence of alginate through proteome analysis and identified flagellin homologues as flagellum-independent cell-surface proteins responsible for alginate binding and/or modulation of cell-surface structures. This is, to the best of our knowledge, the first report on flagellum-independent cell-surface flagellins with alginate-binding ability.

Separation of outer and inner membranes from a mixture of total membranes by sucrose density gradient centrifugation was unsuccessful (Figure 3a), indicating that both membranes have similar densities within 1.16−1.21 mg/mL. Gram-negative bacteria generally differ greatly in outer- and inner-membrane density, e.g., 1.23 and 1.15 mg/mL for outer and inner membranes of *E. coli*, respectively (*40*). The close density in outer and inner membranes of strain A1 is probably due to the unusual cell-surface structures observed in sphingomonades. As described in the Introduction, sphingomonades have unusual outer membranes containing glycosphingolpids in place of lipopolysaccharides (*10*). The density of outer and inner membranes from *Sphingomonas puacimobilis* is determined to 1.19 and 1.14 mg/mL, respectively (*40*); therefore, the negligible difference in density between outer and inner membranes is thought to be common to sphingomonades.

Through a differential display between outer membranes from strain A1 cells grown in the presence and absence of alginate, seven proteins (p1−p7) with acidic pI values were found to be expressed in the outer membrane of alginate-grown cells (Table 1). Most homologous proteins with p1−p7 are bacterial cell-surface (outer membrane) proteins (Table 2), suggesting that the outer membrane was well-fractionated. p1−p4 and p7 are also predicted to have a signal sequence through the primary structure analysis using a LipoP program (http://www.cbs.dtu.dk/services/LipoP/). p1−p7 genes are scattered in the strain A1 genome (Table 1), while genes for the ABC transporter (AlgS, AlgM1, and AlgM2), periplasmic alginate-binding proteins (AlgQ1 and AlgQ2), and alginate lyases (A1-I, A1-II, A1-III, and A1-IV) thus far analyzed are assembled into a cluster in the strain A1 genome (location, 4 392 055−4 383 073) (Figure 2). Genes for p1−p7 are thus thought to be expressed independently of the genetic cluster, although all of p1−p7, the ABC transporter, periplasmic alginate-binding proteins, and alginate lyases are produced inducibly in the presence of alginate.

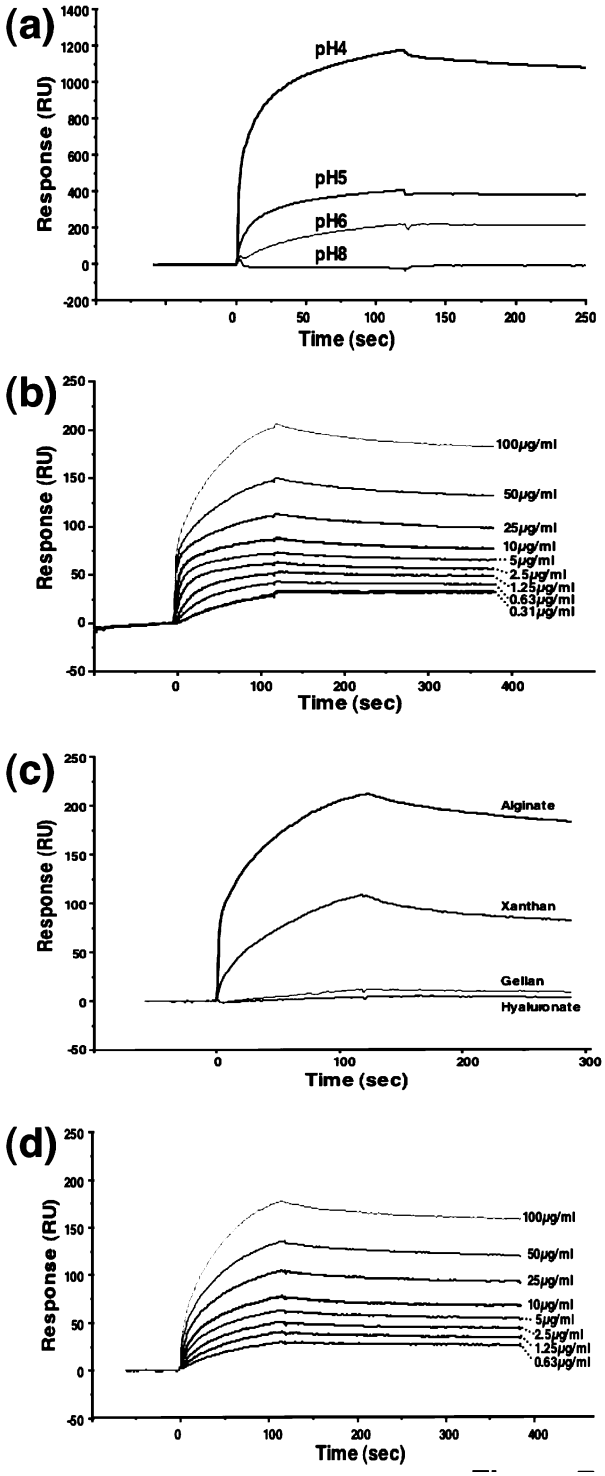

FIGURE 7: Interaction between flagellins and polysaccharides. The interaction was analyzed using an SPR biosensor. (a) Effect of pH values on the binding of p5 to alginate. Alginates (1 mg/mL) dissolved in different buffers were injected over p5-immobilized surfaces. pH 4, sodium acetate; pH 5, sodium acetate; pH 6, MES-NaOH; and pH 8, Tris-HCl. (b) Dependence of the alginate concentration (0.31−100 μg/mL) on p5 binding to alginate. (c) Specificity in p5 binding to 100 μg/mL polysaccharides (alginate, xanthan, gellan, and hyaluronate). (d) *E. coli* FliC binding to alginate (0.63−100 μg/mL) as in b.

p1, p2, p3, and p4 show significant identity with TonB-dependent outer-membrane transporters (Table 2). Transporters thus far analyzed incorporate siderophores (*41*−*44*) or vitamin B_{12} (*45*) into the periplasm with a proton-motive force generated from an inner-membrane complex (TonB−ExbB−ExbD) (*46*). *E. coli* FhuA, one of these transporters, functions as a receptor for siderophores and for antibiotics and phages (*41*). Siderophores such as ferrichrome, enterobactin, enterochelin, and citrate are well-known as TonB-dependent outer-membrane iron transporters. ExbB and ExbD are located in the inner membrane, while TonB anchoring the inner membrane extrudes into the periplasm to associate with TonB-dependent outer-membrane transporters (*47*). The TonB box highly conserved in the N-terminal region of outer-membrane transporters is responsible for the interaction of TonB and transporters (*48*). In fact, p1, p2, p3, and p4 show high homology with other TonB-dependent outer-membrane transporters in the N-terminal region (data not shown). We surmised that p1−p4 homologous with TonB-dependent outer-membrane transporters were overexpressed in the presence of alginate because (i) the import of alginate into the periplasm and/or (ii) iron limitation in the alginate medium as a result of the formation of an iron−alginate complex.

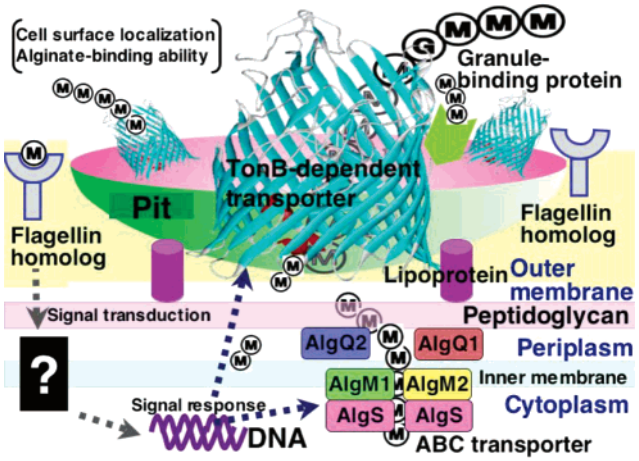

FIGURE 8: Cell-surface model of strain A1. Details are given in the text.

In Gram-negative bacteria, external siderophores and vitamin B_{12} are transported into the cytoplasm by TonB-dependent outer-membrane transporters, periplasmic binding proteins, and inner-membrane ABC transporters (*49*, *50*). Because, in alginate import in strain A1, periplasmic binding proteins and the inner-membrane ABC transporter have already been identified (*19*, *20*), p1, p2, p3, and/or p4 may function as TonB-dependent outer-membrane transporters for the import of alginate across the outer membrane. In homology modeling (*51*), the transporter (p3) appeared to constitute a tunnel-like β-barrel structure spanning the outer membrane (Figure 8).

Alginate potentially chelates iron (ferric ions) (*52*), suggesting that iron available for growth is limited in the alginate medium. Under iron-deficient conditions, a large number of bacteria produce siderophores and outer-membrane iron transporters to import iron effectively; therefore, strain A1 cells may overproduce p1, p2, p3, and/or p4 in the outer membrane to incorporate limited iron in the alginate medium. The possibility that p1, p2, p3, and/or p4 transport iron into the periplasm using alginate as a siderophore cannot be ignored. In any case, a mutant with a disruption of the p1, p2, p3, or p4 gene shows significant growth retardation in the alginate medium, indicating that p1−p4 cooperatively

incorporate substrates into the periplasm. We are now constructing the overexpression system of p1−p4 in *E. coli* to clarify their intrinsic function.

One unexpected finding in this work concerns the localization and function of flagellin homologues. Bacterial flagellin is a helical filament, a component of the flagellum, and functions as a propeller (*37*). In spirochetes, flagellar filaments are present in the periplasm and filament rotation gives cells specific motility (*53*). In regard to the function of flagellin beyond that of a propeller, flagellum-dependent flagellins of a plant-pathogenic bacterium, *Pseudomonas syringae*, are reported to play an important role in inducing a hypersensitive reaction in plants (*54*), and some pathogenic bacterial flagellins cause a host innate immune response through the interaction with Toll-like receptors of hosts (*55*). These functions of flagellum-dependent flagellins, however, are not intrinsic but result from the interaction with bacteria and hosts. Strain A1 flagellin homologues are independent of flagellum formation and are exclusively localized on the cell surface. Although the p5 or p6 gene disruptant could grow on alginate, the growth of a mutant with a disruption of the p6 gene was retarded in the alginate medium. Distinct from wild-type strain A1, the p6 gene disruptant had unusual cell-surface and pit structures, which were also observed in some cells of the p5 gene disruptant. Our many attempts to construct the mutant with double disruptions of p5 and p6 genes from either p5 or p6 gene disruptant failed, suggesting that double disruptions of p5 and p6 genes cause strain A1 cells to be lethal and that p5 and p6 with high mutual similarity function complementarily as regulators for pleat structures and pit formation on the cell surface. The reason the growth of the p5 gene disruptant was comparable to that of wild-type strain A1 is possibly due to the fact that p6 effectively complements the function of p5 in the p5 gene disruptant.

Flagellin homologues further show high affinity (K_d in nanomolars) with alginate, suggesting that flagellin homologues are involved in alginate signaling as CD44-like receptors rather than in the harvesting of alginate by a cell-surface alginate-binding protein (p8) recently identified (*56*) and in the transport of alginate from the pit to the ABC transporter by periplasmic alginate-binding proteins (AlgQ1 and AlgQ2) (*19*). p8, AlgQ1, and AlgQ2 show lower affinity with alginate than flagellin homologues. CD44 is a mammalian transmembrane receptor (K_d in nanomolars) for hyaluronan, an acidic polysaccharide-like alginate (*57*). To clarify the universality of flagellin function (alginate binding), *E. coli* flagellin (FliC) was also subjected to SPR biosensor analysis. *E. coli* flagellin also binds alginate at a K_d in nanomolars, suggesting that bacterial flagellins possibly have a common characteristics to bind polysaccharides and that N- and C-terminal domains of flagellins are responsible for binding polysaccharides. This result implies two possibilities. One is that flagellin may first be created as a cell-surface receptor and then undergo change to a flagellar protein or vice versa. The other is that the binding ability of bacterial flagellins to polysaccharides might be related to glycosylation found in flagellins because several flagellins from Gram-negative bacteria and archaea have recently been shown to be glycosylated (*58*).

On the basis of results of proteome analysis of the strain A1 cell surface, a possible cell-surface model in strain A1 is shown in Figure 8. Although data are insufficient, cell-surface proteins cooperate to incorporate alginate as follows: alginate in the milieu is recognized by flagellin homologue receptors, and the resultant signals are transmitted to DNA through an unknown mechanism. The lack of a derivation of a mutant with double disruptions of p5 and p6 genes suggests that flagellin homologues play an important role in the construction of cell-surface structures and/or that the trigger in alginate import and depolymerization is the recognition of alginate by flagellin homologues. On the basis of signal transduction, genes for importing and depolymerizing alginate are expressed and then the super-channel, including the pit, binding proteins, and ABC transporter, is constructed. Cell-surface alginate-binding protein (p8) concentrates alginate and its granules in the pit (*56*), and TonB-dependent outer membrane transporters (p1−p4) with tunnel-like β structures incorporate alginate into the periplasm. p7 may function as a lipoprotein responsible for pit maintenance and stabilization through linkage between the pit and peptidoglycan, although supporting data for this assumption has yet to be obtained.

REFERENCES

1. Gacesa, P. (1988) Alginates, *Carbohydr. Polym. 8*, 161−182.
2. Onsøyen, E. (1996) Commercial applications of alginates, *Carbohydr. Eur. 14*, 26−31.
3. May, T. B., and Chakrabarty, A. M. (1994) *Pseudomonas aeruginosa*: Genes and enzymes of alginate synthesis, *Trends Microbiol. 2*, 151−157.
4. Schweizer, H. P., and Boring, J. R., III (1973) Antiphagocytic effect of slime from a mucoid strain of *Pseudomonas aeruginosa*, *Infect. Immun. 3*, 762−767.
5. Govan, J. R. W., and Deretic, V. (1996) Microbial pathogenesis in cystic fibrosis: Mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*, *Microbiol. Rev. 60*, 539−574.
6. Wong, T. Y., Preston, L. A., and Schiller, N. L. (2000) Alginate lyase: Review of major sources and enzyme characteristics, structure−function analysis, biological roles, and applications, *Annu. Rev. Microbiol. 54*, 289−340.
7. Hisano, T., Yonemoto, Y., Yamashita, T., Fukuda, Y., Kimura, A., and Murata, K. (1995) Direct uptake of alginate molecules through a pit on the bacterial cell surface: A novel mechanism for the uptake of macromolecules, *J. Ferment. Bioeng. 79*, 538−544.
8. Yoon, H.-J., Mikami, B., Hashimoto, W., and Murata, K. (1999) Crystal structure of alginate lyase A1-III from *Sphingomonas* species A1 at 1.78 Å resolution, *J. Mol. Biol. 290*, 505−514.
9. Sakakibara, H., Tamura, T., Suzuki, T., Hisano, T., Abe, S., and Murata, K. (2002) Preparation and properties of alginate lyase modified with poly(ethylene glycol), *J. Pharm. Sci. 91*, 1191−1199.
10. Kawahara, K., Kuraishi, H., and Zähringer, U. (1999) Chemical structure and function of glycosphingolipids of *Sphingomonas* spp. and their distribution among members of the α-4 subclass of Proteobacteria, *J. Ind. Microbiol. Biotechnol. 23*, 408−413.
11. White, D. C., Sutton, S. D., and Ringelberg, D. B. (1996) The genus *Sphingomonas*: Physiology and ecology, *Curr. Opin. Biotechnol. 7*, 301−306.
12. Wiese, A., and Seydel, U. (1999) Interaction of peptides and proteins with bacterial surface glycolipids: A comparison of glycosphingolipids and lipopolysaccharides, *J. Ind. Microbiol. Biotechnol. 23*, 414−424.
13. Hisano, T., Kimura, N., Hashimoto, W., and Murata, K. (1996) Pit structure on bacterial cell surface, *Biochem. Biophys. Res. Commun. 220*, 979−982.
14. Mishima, Y., Momma, K., Hashimoto, W., Mikami, B., and Murata K. (2001) Super-channel in bacteria: Function and structure of the macromolecule import system mediated by a pit-dependent ABC transporter, *FEMS Microbiol. Lett. 204*, 215−221.

15. Yoon, H.-J., Hashimoto, W., Miyake, O., Okamoto, M., Mikami, B., and Murata, K. (2000) Overexpression in *Escherichia coli*, purification, and characterization of *Sphingomonas* sp. A1 alginate lyases, *Protein Expression Purif. 19*, 84−90.
16. Hashimoto, W., Miyake, O., Momma, K., Kawai, S., and Murata, K. (2000) Molecular identification of oligoalginate lyase of *Sphingomonas* sp. strain A1 as one of the enzymes required for complete depolymerization of alginate, *J. Bacteriol. 182*, 4572−4577.
17. Momma, K., Mikami, B., Mishima, Y., Hashimoto, W., and Murata, K. (2002) Crystal structure of AlgQ2, a macromolecule (alginate)-binding protein of *Sphingomonas* sp. A1 at 2.0 Å resolution, *J. Mol. Biol. 316*, 1061−1069.
18. Mishima, Y., Momma, K., Hashimoto, W., Mikami, B., and Murata, K. (2003) Crystal structure of AlgQ2, a macromolecule (alginate)-binding protein of *Sphingomonas* sp. A1, complexed with an alginate tetrasaccharide at 1.6 Å resolution, *J. Biol. Chem. 278*, 6552−6559.
19. Momma, K., Mishima, Y., Hashimoto, W., Mikami, B., and Murata, K. (2005) Direct evidence for *Sphingomonas* sp. A1 periplasmic proteins as macromolecule-binding proteins associated with the ABC transporter: Molecular insights into alginate transport in the periplasm, *Biochemistry 44*, 5053−5064.
20. Momma, K., Okamoto, M., Mishima, Y., Mori, S., Hashimoto, W., and Murata, K. (2000) A novel bacterial ATP-binding cassette (ABC) transporter system that allows uptake of macromolecules, *J. Bacteriol. 182*, 3998−4004.
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
22. Osborn, M. J., Gander, J. E., Parisi, E., and Carson, J. (1972) Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of cytoplasmic and outer membrane, *J. Biol. Chem. 247*, 3962−3972.
23. Mizushima, S., and Yamada, H. (1975) Isolation and characterization of two outer membrane preparations from *Escherichia coli*, *Biochim. Biophys. Acta 375*, 44−53.
24. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature 227*, 680−685.
25. Hashimoto, W., Suzuki, H., Yamamoto, K., and Kumagai, H. (1995) Effect of site-directed mutations on processing and activity of γ-glutamyltranspeptidase of *Escherichia coli* K-12, *J. Biochem. 118*, 75−80.
26. Bradford, M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein−dye binding, *Anal. Biochem. 72*, 248−254.
27. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent, *J. Biol. Chem. 193*, 265−275.
28. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors, *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
29. Kimbara, K., Hashimoto, T., Fukuda, M., Koana, T., Takagi, M., Oishi, M., and Yano, K. (1989) Cloning and sequencing of two tandem genes involved in degradation of 2,3-dihydroxybiphenyl to benzoic acid in the polychlorinated biphenyl-degrading soil bacterium *Pseudomonas* sp. strain KKS102, *J. Bacteriol. 171*, 2740−2747.
30. Ruvkun, G. B., and Ausubel, F. M. (1981) A general method for site-directed mutagenesis in prokaryotes, *Nature 289*, 85−88.
31. Nishizawa, K., Maruyama, N., Satoh, R., Fuchikami, Y., Higasa, T., and Utsumi, S. (2003) A C-terminal sequence of soybean β-conglycinin α' subunit acts as a vacuolar sorting determinant in seed cells, *Plant J. 34*, 647−659.
32. Siritapetawee, J., Prinz, H., Krittanai, C., and Suginta, W. (2004) Expression and refolding of Omp38 from *Burkholderia pseudomallei* and *Burkholderia thailandensis*, and its function as a diffusion porin, *Biochem. J. 384*, 609−617.
33. Molloy, M. P., Herbert, B. R., Walsh, B. J., Tyler, M. I., Traini, M., Sanchez, J. C., Hochstrasser, D. F., Williams, K. L., and Gooley, A. A. (1998) Extraction of membrane proteins by differential solubilization for separation using two-dimensional gel electrophoresis, *Electrophoresis 19*, 837−844.
34. Veith, P. D., Talbo, G. H., Slakeski, N., Dashper, S. G., Moore, C., Paolini, R. A., and Reynolds, E. C. (2002) Major outer membrane proteins and proteolytic processing of RgpA and Kgp of *Porphyromonas gingivalis* W50, *Biochem. J. 363*, 105−115.
35. da Silva, A. C., Ferro, J. A., Reinach, F. C., Farah, C. S., Furlan, L. R., Quaggio, R. B., Monteiro-Vitorello, C. B., van Sluys, M. A., Almeida, N. F., Alves, L. M., do Amaral, A. M., Bertolini, M. C., Camargo, L. E., Camarotte, G., Cannavan, F., Cardozo, J., Chambergo, F., Ciapina, L. P., Cicarelli, R. M., Coutinho, L. L., Cursino-Santos, J. R., El-Dorry, H., Faria, J. B., Ferreira, A. J., Ferreira, R. C., Ferro, M. I., Formighieri, E. F., Franco, M. C., Greggio, C. C., Gruber, A., Katsuyama, A. M., Kishi, L. T., Leite, R. P., Lemos, E. G., Lemos, M. V., Locali, E. C., Machado, M. A., Madeira, A. M., Martinez-Rossi, N. M., Martins, E. C., Meidanis, J., Menck, C. F., Miyaki, C. Y., Moon, D. H., Moreira, L. M., Novo, M. T., Okura, V. K., Oliveira, M. C., Oliveira, V. R., Pereira, H. A., Rossi, A., Sena, J. A., Silva, C., de Souza, R. F., Spinola, L. A., Takita, M. A., Tamura, R. E., Teixeira, E. C., Tezza, R. I., Trindade, dos Santos, M., Truffi, D., Tsai, S. M., White, F. F., Setubal, J. C., and Kitajima, J. P. (2002) Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities, *Nature 417*, 459−463.
36. McCarter, L. L., and Wright, M. E. (1993) Identification of genes encoding components of the swarmer cell flagellar motor and propeller and a σ factor controlling differentiation of *Vibrio parahaemolyticus*, *J. Bacteriol. 175*, 3361−3371.
37. Aizawa, S.-I. (1996) Flageller assembly in *Salmonella typhimurium*, *Mol. Microbiol. 19*, 1−5.
38. Macnab, R. M. (1996) Flagella and Motility, in *Escherichia and Salmonella: Cellular and Molecular Biology* (Neidhardt, F. C., Curtiss, R., III, Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., and Umbargar, H. E., Eds.) Vol. 1, pp 123−145, American Society for Microbiology, Washington, DC.
39. Yonekura, K., Maki-Yonekura, S., and Namba, K. (2003) Complete atomic model of the bacterial flagellar filament by electron cryomicroscopy, *Nature 424*, 643−650.
40. Kawasaki, S., Moriguchi, R., Sekiya, K., Nakai, T., Ono, E., Kume, K., and Kawahara, K. (1994) The cell envelope structure of the lipopolysaccharide-lacking Gram-negative bacterium *Sphingomonas paucimobilis*, *J. Bacteriol. 176*, 284−290.
41. Coulton, J. W., Mason, P., Cameron, D. R., Carmel, G., Jean, R., and Rode, H. N. (1986) Protein fusions of β-galactosidase to the ferrichrome-iron receptor of *Escherichia coli* K-12, *J. Bacteriol. 165*, 181−192.
42. Pressler, U., Staudenmaier, H., Zimmermann, L., and Braun, V. (1988) Genetics of the iron dicitrate transport system of *Escherichia coli*, *J. Bacteriol. 170*, 2716−2724.
43. Lundrigan, M. D., and Kadner, R. J. (1986) Nucleotide sequence of the gene for the ferrienterochelin receptor FepA in *Escherichia coli*. Homology among outer membrane receptors that interact with TonB, *J. Biol. Chem. 261*, 10797−10801.
44. Koedding, J., Howard, P., Kaufmann, L., Polzer, P., Lustig, A., and Welte, W. (2004) Dimerization of TonB is not essential for its binding to the outer membrane siderophore receptor FhuA of *Escherichia coli*, *J. Biol. Chem. 279*, 9978−9986.
45. Heller, K., and Kadner, R. J. (1985) Nucleotide sequence of the gene for the vitamin B_{12} receptor protein in the outer membrane of *Escherichia coli*, *J. Bacteriol. 161*, 904−908.
46. Larsen, R. A., Thomas, M. G., and Postle, K. (1999) Proton motive force, ExbB and ligand-bound FepA drive conformational changes in TonB, *Mol. Microbiol. 31*, 1809−18024.
47. Postle, K., and Kadner, R. J. (2003) Touch and go: Tying TonB to transport, *Mol Microbiol. 49*, 869−882.
48. Larsen, R. A., Foster-Hartnett, D., McIntosh, M. A., and Postle, K. (1997) Regions of *Escherichia coli* TonB and FepA proteins essential for *in vivo* physical interactions, *J. Bacteriol. 179*, 3213−3221.
49. Raymond, K. N., Dertz, E. A., and Kim, S. S. (2003) Enterobactin: An archetype for microbial iron transport, *Proc. Natl. Acad. Sci. U.S.A. 100*, 3584−3588.
50. Locher, K. P., Lee, A. T., and Rees, D. C. (2002) The *E. coli* BtuCD structure: A framework for ABC transporter architecture and mechanism, *Science 296*, 1091−1098.
51. Peitsch, M. C. (1996) ProMod and Swiss-model: Internet-based tools for automated comparative protein modeling, *Biochem. Soc. Trans. 24*, 274−279.
52. Sreeram, K. J., Shrivastava, H. Y., and Nair, B. U. (2004) Studies on the nature of interaction of iron(III) with alginates, *Biochim. Biophys. Acta 1670*, 121−125.

53. Limberger, R. J. (2004) The periplasmic flagellum of spirochetes, *J. Mol. Microbiol. Biotechnol. 7*, 30−40.
54. Shimizu, R., Taguchi, F., Marutani, M., Mukaihara, T., Inagaki, Y., Toyoda, K., Shiraishi, T., and Ichinose, Y. (2003) The ΔfliD mutant of *Pseudomonas syringae* pv. *tabaci*, which secretes flagellin monomers, induces a strong hypersensitive reaction (HR) in non-host tomato cells, *Mol. Genet. Genomics 269*, 21−30.
55. Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., Eng, J. K., Akira, S., Underhill, D. M., and Aderem, A. (2001) The innate immune response to bacterial flagellin is mediated by toll-like receptor 5, *Nature 410*, 1099−1103.
56. He, J., Nankai, H., Hashimoto, W., and Murata, K. (2004) Molecular identification and characterization of an alginate-binding protein on the cell surface of *Sphingomonas* sp. A1, *Biochem. Biophys. Res. Commun. 322*, 712−717.
57. Turley, E. V., Noble, P. W., and Bourguignon, L. Y. W. (2002) Signaling properties of hyaluronan receptors, *J. Biol. Chem. 277*, 4589−4592.
58. Messner, P. (2004) Prokaryotic glycoproteins: Unexplored but important, *J. Bacteriol. 186*, 2517−2519.

BI050873B