Lateral-typed flagellin responsible for formation of a polar flagellum but not of lateral flagella in *Sphingomonas* sp. strain A1

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Alginate-assimilating *Sphingomonas* sp. strain A1 is the Gram-negative bacterium first identified to form a single polar flagellum containing lateral-typed flagellin (p6) in the filament. In addition to the p6 flagellin, two polar-typed flagellins (p5 and p5') are also included in the flagellum. Here we show the significant role of p6 as well as p5/p5' in flagellum formation and cell motility towards alginate. A p6 gene disruptant significantly reduced flagellum formation and it showed no cell motility, whereas each mutant with a disruption in the p5 or p5' gene exhibited cell motility through the formation of a polar flagellum containing p6. The ratio of p6 to p5 decreased in proportion to cell growth, suggesting that strain A1 changes flagellin ratios in the filament depending on the external environment. Each of purified recombinant p5 and p6 proteins formed the filament by *in vitro* self-assembly and an anti-p5 antibody reacted with the p5 filament but not with the p6 filament. Immunoelectron microscopy using an anti-p5 antibody indicated that strain A1 formed two types of the filament in a single polar flagellum: p6 alone in the entire filament and p5 elongation filament subsequent to the p6 proximal end. Immunoprecipitation with an anti-p5 antibody directly demonstrated that p5 and p6 coexist in a single filament. Strain A1 cells were also found to exhibit a chemotactic motility in response to alginate. This is the first report on function/location of the lateral-typed flagellin in a single polar flagellum and the bacterial chemotaxis towards alginate.

INTRODUCTION

Alginate, a heteropolysaccharide composed of α-L-guluronate and its C5 epimer β-D-mannuronate, is widely used as food additive, fibers and gel-forming polymers in various industries (Gacesa, 1988). Brown seaweeds and certain bacteria such as pseudomonads and *Azotobacter* are known to be potential producers of the polysaccharide. *Pseudomonas aeruginosa* produces extracellular biofilms containing alginate as virulence factors during lung infections in cystic fibrosis patients (May & Chakrabarty, 1994). Alginate is included in the outer layer of cysts transformed from vegetative cells of *Azotobacter vinelandii* (Campos et al., 1996). Due to the heterogeneity of alginate in composition and molecular mass, these bacteria are expected to produce tailor-made alginate with suitable homogeneity in molecular size or uronate composition by genetic engineering (Hay et al., 2013). In contrast, alginate produced by brown seaweeds is currently regarded as attractive marine biomass for microbial production of biofuels (Stockstad, 2012). Therefore, alginate-producing and/or -degrading microbes are of great interests from the viewpoint of energy, food, medical and polymer areas. However, to the best of our knowledge, no microbes showing a chemotaxis to alginate have been identified so far.

The Gram-negative and alginate-assimilating bacterium *Sphingomonas* sp. strain A1 (strain A1) directly incorporates the polysaccharide through the cell-surface pit and ATP-binding cassette (ABC) transporter (Hisano et al., 1995; Momma et al., 2000). The peculiar mechanism for import and degradation of alginate has been characterized in strain A1 cells (Hashimoto et al., 2000; Hayashi et al., 2014). The genome of strain A1 is approximately 4.6 Mb (GC content:...
et al. have been analysed in the strain A1 genome (Maruyama et al., 2015), although suitable and unsuitable substrates for strain A1 chemotaxis remain to be clarified. In the bacterial genome, there is a single cluster (flagellar gene set I) containing more than 30 genes required for flagellar formation, including those coding for basal body, hook, filament and other flagellum-related molecules. Distinct from this cluster, another several flagellar gene clusters are assembled at multiple sites in the genome (flagellar gene set II). Genes for chemotaxis are included in the set II but not in the set I.

Flagella are important organelles for cell locomotion and chemotaxis. Based on the flagellar location in bacterial cells, they are classified into four types: polar, subpolar, lateral and peritrichous (Haya et al., 2011). For example, P. aeruginosa has one or two polar flagella (Köhler et al., 2000), whereas Escherichia coli produces multiple peritrichous flagella (Van Houdt & Michiels, 2005). By contrast, Vibrio paraaerolyticus typically has a single polar flagellum. Importantly, the V. parahaemolyticus cell producing a polar flagellum synthesizes multiple lateral flagella to regain cell motility under various conditions such as a highly viscous milieu (McCarter et al., 1988). Lateral flagella are also involved in cell adhesion and motility (Belas & Colwell, 1982; Shinoda & Okamoto, 1977).

In spite of diversity in bacterial flagella, all bacterial flagellar filaments consist of certain proteins called flagellins. Flagellins in filaments, however, vary in composition and arrangement. For example, peritrichous flagella in Salmo-

nella enterica serovar Typhimurium are noncovalently formed by a single flagellin (FlIC) (Yonekura et al., 2003). In contrast, Bdellovibrio bacteriovorus has a single polar flagellum composed of six flagellins (FlC1–6) (Iida et al., 2009). Furthermore, V. parahaemolyticus produces a polar flagellum composed of six flagellins (FlA–F) and a multiple lateral flagella composed of a single lateral flagellin (LaFA) (Kim & McCarter, 2000; McCarter & Wright, 1993). Flagellar formation is strictly controlled through a gene expression cascade (Soutourina & Bertin, 2003). In E. coli, gene expression is altered depending on the growth phase (Dudin et al., 2013). Flagellar filaments of pathogenic Campylobacter jejuni are composed of two flagellins (FlA and FlB) and flagellin composition and filament length are modulated in a temperature-dependent manner (Wösten et al., 2010).

In the strain A1 genome, flagellar gene set I is structurally similar to the lateral flagellar gene clusters in Aeromonas and Vibrio spp., which have two distinct flagellar systems of polar and lateral flagella. The strain A1 cells have three flagellin genes in the genome: a flagellin (p6) gene in set I and two (p5 and p5′) genes in set II. p5 (39.8 kDa) and p5′ (39.5 kDa) resemble each other with a sequence identity of 85 %. They are closely related to a putative flagellin of Dech-

loromonas agitata with a single polar flagellum (p5, 76 % identity; p5′, 75 % identity) (Achenbach et al., 2001).

However, the arrangement of the flagellar gene cluster and molecular phylogenetic tree of flagellins suggest that p6 (31.1 kDa) is a member of the family of lateral flagellins (Maruyama et al., 2015). In fact, p6 shows a significant identity with the lateral flagellin LaF (Hashimoto et al., 2005). p5 binds specifically and with high affinity to alginates (Ks, nM), suggesting that it functions as an alginate receptor-like protein (Hashimoto et al., 2005).

Recently, motile cells designated as strain A1-M5 have been isolated by subculturing the strain A1 wild-type (nonmotile) cells on soft agar plates (Maruyama et al., 2015). The strain A1-M5 cells produce a single polar flagellum composed of p5, p5′ and p6. Both flagellar gene sets I and II, probably regulated by different sigma (σ) factors, are highly transcribed in the strain A1-M5 cells compared with the nonflagellated strain A1 wild-type cells. The sequence recognized specifically by sigma factor σ28 was located in the region upstream of the p5 gene, while the sequence located upstream of the p5′ gene is probably recognized by σ54. No consensus sequence for σ28 recognition was situated in set I including the p6 gene. The goal of this study was to elucidate substrates for strain A1 chemotaxis as well as the function and arrangement of the strain A1 flagellins in the flagellum through gene disruption and immunoelectron microscopy. Here we report the striking finding that the filament in a polar flagellum of the strain A1-M5 cells with a chemotaxis towards alginate contains the lateral-type flagellin p6.

**METHODS**

**Materials.** Sodium alginate with a mean molecular weight of 300 000 from Esi-

nia cycloides and agar were purchased from Nacalai Tesque. Pect-

in from citrus fruits was from Sigma-Aldrich. Xanthan was from Koh-

jin. These acidic polysaccharides were used for bacterial growth or chemotaxis assay. Restriction endonucleases and DNA-modifying enzymes were from Toyobo. DNase I was from Wako. Other analytical grade chemicals were from commercial sources.

**Bacterial strains and culture conditions.** To investigate assimila-

tion of acidic polysaccharides, bacterial cells were aerobically cultured at 30 °C in alginate or pectin minimal medium composed of 0.1 % (NH4)2SO4, 0.1 % KH2PO4, 0.1 % Na2HPO4 and 0.01 % MgSO4·7H2O (pH 7.2) containing 0.5 % sodium alginate or pectin as a carbon source. Strain A1-M5 and each flagellin gene disruptant (Table 1) were aerobi-

cally cultured at 30 °C in AY medium composed of 0.5 % sodium alginate, 0.1 % (NH4)2SO4, 0.1 % KH2PO4, 0.5 % Na2HPO4, 0.5 % yeast extract and 0.01 % MgSO4·7H2O (pH 7.2). To investigate chemotactic response, bacterial cells were aerobically cultured at 30 °C in PY or AY medium composed of 0.5 % pectin, 0.1 % (NH4)2SO4, 0.1 % KH2PO4, 0.1 % Na2HPO4, 0.5 % yeast extract and 0.01 % MgSO4·7H2O (pH 7.2). The gene disruptants were subcultured on soft agar plates before use in order to stimulate flagellum formation. The soft agar plates were prepared by solidification of the medium with 0.5 % agar. Approp-

riate antibiotics for the selection of each disruptant were added at the following concentrations: sodium ampicillin, 50 µg ml−1; kanamycin sulfate, 100 µg ml−1; tetracycline hydrochloride, 10 µg ml−1. E. coli cells were aerobically grown at 15, 30 or 37 °C in LB medium consisting of 1 % tryptone, 0.5 % yeast extract and 1 % NaCl (pH 7.0).
**Motility assay.** The bacterial cells (approximately 1.6×10^6 cells) were inoculated on yeast extract- and antibiotics-free YT plates solidified with 0.25% agar (soft agar plates), cultured for 1 week and subjected to a cell motility assay by measuring the spread of colonies.

**Gene disruption and DNA cloning.** The p5' single-gene (strain Δp5'), double-gene (strains Δp5/p5', Δp5/p6 and Δp5'/p6') and triple-gene (strain Δp5/p5'/p6') disruptants were constructed by insertion of an ampicillin resistance gene (Amp') into the p5' gene and a tetracycline resistance gene (Tet') into the p5' gene, as previously described (Hashimoto et al., 2005).

The Amp' gene cassette including the promoter was obtained from pACYC177 vector (Nippon Gene) by PCR. The p5' gene cloned into the plasmid pUC118 (Takara Bio) was digested by HinII and the Amp' gene cassette was ligated with the HinII-digested p5' gene in the plasmid. The resultant plasmid was designated pUC118-p5':Amp'. The p5' mutant gene was generated by insertion of the Amp' gene cassette in the middle of the p5' gene. The p5' gene was amplified from strain A1 cells by PCR using two primers (forward, 5'-GGGCGACAGCCTCTTTCGACGACGACAGCTTACAGG-3' and reverse, 5'-TTCGGTGCAGGACACGACTTACGAGGCGGCGG-3'). The amplified fragment was cloned into pUC18-2987s-ADH as a template and two primers (forward, 5'-GATGCAGGACAGCGTACGAGGCAGCC-3' and reverse, 5'-ATGCAGGACAGCGTACGAGGCAGCC-3'). To delete the centre region of the p5' gene, inverse PCR was carried out using pUC18-2987s-p5'-ADH as a template and two primers (forward, 5'-AAGCAGCGTACGAGGCAGCC-3' and reverse, 5'-ATGCAGGACAGCGTACGAGGCAGCC-3'). The resultant plasmid was designated pUC18-2987s-p5':Tet'-ADH. The p5' mutant gene was generated by insertion of the Tet' gene cassette in the middle of the p5' gene. The disrupted p5' or p5' gene fragment was ligated with the plasmid pKTY320 (Kimbara et al., 1989). The resultant plasmid was introduced into the E. coli strain DH5α. The transformant E. coli strain DH5α harbouring the plasmid was used to transconjugate strain A1 cells through triparental mating in the presence of the E. coli strain HB101 harbouring pRK2013 (Ruvkun & Ausubel, 1981) as a helper cell. The gene disruptant was screened on its resistance or sensitivity to antibiotics. Gene disruption was confirmed by the fragment size amplified by PCR and DNA sequencing. Each flagellin gene disruptant was found to produce no corresponding target flagellin by immunoblotting.

A plasmid for the overexpression of p6 with an addition of a histidine-tagged sequence at its C terminus was constructed by amplifying a linear fragment of the stop codon-truncated p6 gene by PCR using pET21b-p6 (Hashimoto et al., 2005) as a template. The fragment was digested with NdeI and XhoI and was ligated with NdeI- and XhoI-digested pColdI (Takara Bio). The resultant plasmid containing p6 was designated pColdI-p6 and was introduced into E. coli strain OrigamiB(DE3) (NovaGen). Standard DNA manipulations were conducted as described elsewhere (Sambrook et al., 1989).

**Protein expression and purification.** Unless otherwise specified, all purification procedures were conducted at 0–4°C. Recombinant p5 with a histidine-tagged sequence at its C terminus was expressed in the E. coli strain OrigamiB(DE3) cells and purified, as previously described (Maruyama et al., 2008). The E. coli cells harbouring pColdI-p6 were cultured at 30°C to an optical density at 600 nm (OD600) of approximately 0.4 in a total volume of 1.5 l of LB medium containing 34 μg ml⁻¹ sodium ampicillin. The culture broth was cooled to 15°C on ice. The cells, grown at 15°C for 12 h in the presence of 0.1 mM IPTG, were collected by centrifugation at 6700 g at 4°C for 5 min and were resuspended in 20 mM Tris/HCl (pH 7.5). To avoid protein aggregation, arginine/HCl (pH 7.2) was added to the cell suspension at a final concentration of 1 M. The E. coli cells were ultracentrifuged (Insonator Model 201 M, Kubota) at 0°C for 20 min and the clear solution obtained after centrifugation at 21 000 g at 4°C for 20 min was used as the cell extract. PMSF was added to the cell extract to give a final concentration of 0.1 mM. The cell extract was applied to 5 ml of TALON Metal Affinity Resin (Clontech) equilibrated with 20 mM Tris/HCl (pH 7.5) containing 500 mM NaCl. The TALON resin was washed with buffer A [20 mM Tris/HCl (pH 7.5), 500 mM NaCl and 10 mM imidazole] twice (first wash with 50 ml and second wash with 5 ml). The resin was packed into a column and washed again with 50 ml of buffer A. The protein was eluted with a linear gradient of imidazole (10–200 mM, 50 ml) in 20 mM Tris/HCl (pH 7.5) containing 500 mM NaCl and was fractionated each 2.1 ml every 3 min. The fractions containing p6 were identified by measurements of absorbance at 280 nm (A280). The resultant fractions were subjected to SDS-PAGE and p6 was confirmed purified to homogeneity. Antibodies against purified p6 were raised in rat (Kitayama Labes) and the serum was used as a polyclonal antibody against p6.

**Preparation of flagellar filaments.** Flagellar filaments were prepared from bacterial cells as reported by Vonderviszt et al. (1990), with some modifications. The strain A1-M5 and the gene disruptant cells were aerobically cultured overnight at 30°C in 50 ml of YT medium. The cells were collected by centrifugation at 18 300 g at 4°C for 10 min and were resuspended in 10 mM Tris/HCl (pH 7.5). The supernatant was also collected and was used as the culture supernatant. The cell suspension was passed approximately 10 times through a 22G needle (outer diameter, 0.70 mm; inner diameter, 0.48 mm; length, 80 mm) with a syringe (Terumo) and the supernatant was collected. The precipitate was collected by ultracentrifugation at 100 000 g at 4°C for 1 h and was resuspended in a small volume of 10 mM Tris/HCl (pH 7.5). The resultant suspension was the flagellar fraction. The culture supernatant was also subjected to ultracentrifugation and the precipitate was collected and resuspended in a small volume of 10 mM Tris/HCl (pH 7.5). The resultant solution was used as the concentrate of the culture supernatant.

**Preparation of flagellar filament with hook basal body.** To examine the arrangement of flagellin in the strain A1-M5 flagellar filament, filaments with hook basal bodies (HBBs) were prepared as reported by Aizawa et al. (1985) with some modifications. The strain

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<tr>
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<tr>
<td>Δp5</td>
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r, Resistance; Amp, ampicillin; Km, kanamycin; Tet, tetracyclin.
A1-M5 cells were aerobically cultured at 30 °C to an OD$_{600}$ of 0.85 in 250 ml of AY medium. The cells were collected by centrifugation at 6700 g at 4 °C for 5 min and were resuspended on ice in 50 ml of 500 mM sucrose in 500 mM Tris (pH not adjusted). The solution was gently mixed on ice with a stirrer and 5 ml of 10 % Triton X-100, 6 ml of 100 mM MgCl$_2$ and 1 ml of 50 mg ml$^{-1}$ DNase I were sequentially and slowly added. After the addition of DNase I, the supernatant was collected by ultracentrifugation at 13 400 g and 4 °C for 15 min. The supernatant was adjusted to pH 12 with 5 M NaOH and the precipitate was collected by ultracentrifugation at 100 000 g and 4 °C for 1 h. After 8 ml of alkaline sucrose solution [500 mM sucrose, 200 mM Tris/HCl (pH 7.5) and 2 % Triton X-100 (adjusted to pH 12 with NaOH)] was added, the sample was left still. CaCl$_2$ powder was added to the solution at a final concentration of 33 % (w/v) and a target fraction was obtained by density gradient centrifugation at 46 000 g and room temperature for 10 h. TET buffer [10 mM Tris/HCl (pH 8.0), 5 mM EDTA and 0.1 % Triton X-100] was added and the precipitate was collected by ultracentrifugation at 100 000 g and 4 °C for 1 h. Finally, the precipitate was diluted with a small volume of TET buffer and was visualized using electron microscopy.

**SDS-PAGE and immunoblotting.** SDS-PAGE was performed using a 12.5 % polyacrylamide gel as described elsewhere (Laemmli, 1970), followed by protein staining with Coomassie brilliant blue R-250 (CBB). The intensity of protein band stained with CBB was measured with the ImageJ program (Schneider et al., 2012).

Immunoblotting was conducted by separating proteins by SDS-PAGE and transferring proteins to a PVDF membrane (Immobilon-P; Merck Millipore) at 1.5 mA cm$^{-2}$ for 25 min. The membrane was then immersed in methanol and air dried for 15 min. The membrane was incubated with rabbit anti-p5 antiserum (1 : 5000 dilution) or rat anti-p6 antiserum (1 : 1000 dilution) in TBS-T [10 mM Tris/HCl (pH 7.5), 150 mM NaCl and 0.1 % Tween 20] containing 1 % BSA for 1 h at room temperature. After washing twice with TBS-T, the membrane was incubated for 30 min at room temperature with donkey anti-rabbit IgG conjugated to horseradish peroxidase (HRP; 1 : 1000 dilution; GE Healthcare) or goat anti-rat IgG conjugated to HRP (1 : 1000 dilution; GE Healthcare). After washing twice more with TBS-T, the flagellins (p5, p5' and/or p6) were detected with POD Immunostain Set (Wako).

**In vitro assembly of recombinant flagellins to form filaments.** Filaments were constructed with recombinant flagellins according to the reference by Vonderviszt et al. (1989) with some modifications. Ammonium sulfate was added at a final concentration of 0.7 M to a solution containing each purified recombinant flagellin and this solution was incubated for 12 h at 25 °C.

**Light and electron microscopy.** Cell motility was investigated by light microscopy (BX51; Olympus). Electron microscopy was performed by placing the samples on carbon-coated copper grids (Nisshin EM) followed by negative staining with two drops of 2 % phosphotungstic acid hydrate solution (pH 7.0). The samples were visualized by transmission electron microscopy (TEM) (H-7100; Hitachi High-Technologies). Immunogold electron microscopy analysis was conducted at room temperature by placing the samples on a grid. The grid was floated for 30 min on a drop of TBS [10 mM Tris/HCl (pH 7.5) and 150 mM NaCl] containing 2 % skimmed milk. The grid was incubated with rabbit anti-p5 antiserum (1 : 500 dilution) or rat anti-p6 antiserum (1 : 50 dilution) for 1 h. After incubation, the grid was floated on three drops of TBS. The grid was then floated on a drop of TBS containing 2 % skimmed milk and a 1 : 10 dilution of goat anti-rabbit or anti-rat IgG coated with colloidal gold particles. The particle size for anti-rabbit IgG was 5 and 10 nm (BB International) or 15 nm (GE Healthcare) and the particle size for anti-rat IgG was 10 nm (Ultra Biosols). After incubation for 1 h, the nonspecifically bound colloidal gold particles were removed by floating the grid on two drops of TBS and a drop of distilled water. The grid was then negatively stained and examined as described above.

**Immunoprecipitation.** Anti-p5 antiserum (1 µl) was added to 100 µl of the flagellar fraction of the strain A1-M5 or Δp6, and was mixed for 1 h at 4 °C by a rotator. After 50 µl of rmp Protein A Sepharose Fast Flow (50 % slurry in TBS; GE Healthcare) was added, the solution was mixed for 1 h at 4 °C. The precipitate was collected by centrifugation at 12 000 g and 4 °C for 20 s. After 1 ml of TBS was added, the precipitate was collected by centrifugation at 12 000 g and 4 °C for 20 s to wash the sample. This wash was repeated four times and trapped filaments were eluted twice with 40 µl of 150 mM glycine/HCl (pH 2.5) containing 1.0 mM EDTA and 40 µl of 1.5 M NaCl.

![Fig. 1. TEM of flagellar filaments. (a) Strain A1-M5 cell. (b) Strain Δp5 cell. (c) Strain Δp6 cell. (d) Strain Δp5’/p6 cell. (e) Strain A1-M5 flagellar filaments.](http://mic.microbiologyresearch.org)
500 mM NaCl. The resultant solution was neutralized with 10 µl of 1.7 M NaOH. Proteins were subjected to SDS-PAGE and were transferred to a PVDF membrane. After blocking with TBS-T containing 10% skimmed milk for 1 h at room temperature, the PVDF membrane was incubated with rabbit anti-p5 antisera (1:5000 dilution) in TBS-T for 12 h at 4 °C. After washing three times with TBS-T, the membrane was incubated for 40 min at room temperature with donkey anti-rabbit IgG conjugated to HRP (1:1000 dilution). After sequential washing with TBS-T, TBS and distilled water, the PVDF membrane was incubated with the Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) to visualize immunoreactive proteins. The PVDF membrane was visualized with ImageQuant LAS 4010 (GE Healthcare).

Chemotaxis assay. Chemotaxis assay was conducted as reported by Darias et al. (2014) with some modifications. The modified AY medium without yeast extract and sodium alginate (i.e. mineral medium) was solidified with 0.25 % agar. At the centre of the plates, 2 % chemical (chemoattractant or chemorepellent) solutions were spotted linearly at regular intervals. The plates were incubated at 4 °C for 16 h to create a concentration gradient. The bacterial cells (approximately $1.6 \times 10^6$ cells) were inoculated horizontally to each of the chemical spots and cultured at 30 °C for 5 days to investigate cell motility towards or from the chemicals. Concentration gradient of alginate was confirmed by staining of the plate with 5 % cetylpyridinium chloride solution for 15 min.

RESULTS

Role of each flagellin in flagellar formation and cell motility

The strain A1 flagellin gene disruptants were constructed by the insertion of an antibiotic resistance gene in the p5, p5’ and/or p6 genes. The strains were subjected to growth on soft agar plates, as previously mentioned to obtain strain A1-M5. The strain A1-M5 cells produced a single polar flagellum (Fig. 1a). Similarly, mutants with a disruption in the flagellin gene (strains $\Delta p5$, $\Delta p5'$, $\Delta p6$, $\Delta p5/p6$ or $\Delta p5'/p6$) produced a single polar flagellum (Fig. 1b–d), although the flagellation ratio differed (Table 2). There were no significant differences in morphology and motility speed between strain A1-M5 and each flagellin-deficient mutant with a cell motility. No lateral flagella were formed in any of the disruptants similar to strain A1-M5. To investigate flagellin components, flagellar filaments isolated from the strain A1-M5 and mutant cells were visualized by TEM (Fig. 1e). The components of flagellum in each strain were examined by SDS-PAGE followed by CBB staining (Fig. 2a) or immunoblotting with anti-p5 or -p6 antisera (Fig. 2b, c). The concentrates of the culture supernatant (fraction S) were also analysed by SDS-PAGE to examine the expression of filaments released from the cells to the culture supernatant. Fraction S from the strains A1-M5, $\Delta p5$ and $\Delta p5'$ all included p6, whereas no flagellins were detected in the fractions from the strains $\Delta p6$, $\Delta p5/p5'$, $\Delta p5/p6$, $\Delta p5'/p6$ and $\Delta p5/p5'/p6$. Since p5 and p5’ consist of 383 and 384 amino acid residues, respectively, it is difficult to identify each flagellin p5 or p5’ on the CBB-stained SDS-PAGE gel. However, p5 was much produced in strain $\Delta p5'$, while little expression of p5’ was observed in strain $\Delta p5$, suggesting that the expression level of p5’ was low even in strain A1-M5 and that the 40 kDa protein band in strain $\Delta p6$ mainly corresponded to p5. Since no flagement formation was observed in strain $\Delta p5/p5'$ (Table 2), the expression level of p6 was considered to be undetectable in both supernatant and flagellar fractions from this strain. Due to little expression of p5’ and low filament formation, no flagellins were observed in strain $\Delta p5/p6$. Following immunoblotting, anti-p5 antisera reacted with all flagellin types (p5, p5’ and p6), whereas anti-p6 antisera reacted with only p5 and p6. These cross-reactivities were considered to be due to primary structure similarities among flagellins (p5 and p5’ share 85 % sequence identity; p5 and p6 share 40 % identity). p5 and p5’ could barely be distinguished from each other based on their immunoreactivities and molecular weights. The flagellum of strain A1-M5 contained p5 and p6 as major components and p5’ as a minor component.

The flagellin components in the flagellum (Fig. 2a–c), cell motility on the soft agar plates (Fig. 2d) and flagellar formation observed by TEM are summarized in Table 2. Flagellar formation was measured by TEM of the cells cultured in test tubes (n is equal to approximately 300). In addition to the motility assay on soft agar plates, cell motility was also investigated by light microscopy. Microscopy showed that the strains A1-M5, $\Delta p5$ and $\Delta p5'$ cells are motile, whereas the strains $\Delta p6$, $\Delta p5/p5'$, $\Delta p5/p6$, $\Delta p5'/p6$ and $\Delta p5/p5'/p6$ cells are all non-motile (data not shown). This result is consistent with the results of the motility assay on soft agar plates. In the strain $\Delta p5$ mutant cells, flagellar formation slightly decreased while cell motility was still detected. By contrast, the strain $\Delta p5'$ mutant cells showed fewer changes in flagellar formation and cell motility compared with the strain A1-M5 cells. Thus, the effect of the p5’ gene disruption on flagellar production and movement in soft agar plates was reduced compared with that of the p5 gene disruption. Moreover, the strain A1-M5 filaments contained few p5’ proteins. In the case of the

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<th>Filament formation (%)</th>
<th>Motility</th>
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<tr>
<td>A1-M5</td>
<td>+</td>
<td>51</td>
<td>+</td>
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<tr>
<td>$\Delta p5$</td>
<td>–</td>
<td>35</td>
<td>+</td>
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<tr>
<td>$\Delta p5'$</td>
<td>+</td>
<td>58</td>
<td>+</td>
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<tr>
<td>$\Delta p6$</td>
<td>+</td>
<td>16</td>
<td>–</td>
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<tr>
<td>$\Delta p5/p5'$</td>
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<tr>
<td>$\Delta p5/p6$</td>
<td>+</td>
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<td>$\Delta p5'/p6$</td>
<td>–</td>
<td>13</td>
<td>–</td>
</tr>
<tr>
<td>$\Delta p5/p5'/p6$</td>
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+, Detected.

-, Not detected.
double-mutant strain Δp5/p5′ cells, both flagellar formation and cell motility were remarkably reduced, indicating that either p5 or p5′ is necessary to form the flagellum and contribute to cell motility. p5 may be a primary determinant in flagellar formation, whereas p5′ may have a secondary role in this process. In the strain A1 genome, the p5′ gene was located downstream of the p5 gene. The mechanism of regulation of gene expression remains to be clarified. Importantly, the inactivation of the p6 gene resulted in a significant decrease in flagellar formation and motility. Only 16% of the strain Δp6 mutant cells could form flagellum compared with 35% of the strain Δp5 mutant cells. Cell motility was undetectable in all the p6 gene disruptants (strains Δp6, Δp5/p6, Δp5′/p6 and Δp5/p5′/p6). These data indicated that the lateral-typed flagellin p6 as well as the polar-typed flagellins p5/p5′ are important for flagellum formation and cell motility in strain A1 cells.

Dynamic flagellin ratio in flagellar filaments

Flagellar filaments were collected from the strain A1-M5 cells at different growth phases (Fig. 3a) and these flagellar fractions were subjected to SDS-PAGE followed by CBB staining. Additionally, the band intensity of p5 and p6 was measured using electrophoretic profiles of the flagellar fractions and the
Filament formation by in vitro self-assembly of recombinant flagellins

Filaments were formed through in vitro self-assembly of recombinant p5 and p6 (Fig. 4a). These filaments were immunostained with anti-p5 or -p6 antisera and were subjected to TEM (Fig. 4b–e). The filaments were detected in both p5 and p6 samples, indicating that they both have an ability to form filaments individually. p5 filaments were immunostained with anti-p5 antiserum and gold colloids were observed on the filaments (Fig. 4b), whereas the particles were undetected on the filaments immunostained with anti-p6 antiserum (Fig. 4c). Additionally, the p6 filaments were not detected using either anti-p5 (Fig. 4d) or -p6 antisera (Fig. 4e). Cross-reaction of the anti-p5 antiserum against p6 by immunoblotting analysis was lost probably because of epitope coverage in the filamentous flagellins. The epitope of p6 was considered to be in parts of the flagellum not exposed to the anti-p6 antiserum since p6 was smaller than p5. As a result, the anti-p5 antiserum specifically recognized p5 in filaments. Hereafter, immunostaining with anti-p5 antiserum was used for the detection of p5 in filaments.

Flagellin arrangements in the flagellum

Flagellin arrangement is important for understanding flagellar formation. To further understand this, we performed immunoelectron microscopy of the strain A1-M5 filaments. However, the filaments collected by physical shearing of the cells were difficult to distinguish individually. Additionally, flagellar filaments near the cells were difficult to observe. To circumvent these difficulties, flagellar filaments with HBBs were obtained by the treatment of strain A1-M5 cells with lysozymes and detergents. The filament with HBB was immunostained with anti-p5 antiserum (Fig. 5a). Gold particles were observed on some filaments, excluding the regions near HBBs. These results indicated that the gold-labelled regions included p5. Because of high sequence identity between p5 and p5' (85%), the gold particles might also attach to p5'. Similarly, the gold particles were not entirely detected in the other filaments (Fig. 5b). The analysis of filament composition (Fig. 2) indicated that the strain A1-M5 filaments consisted of p5, p5' and p6. These results suggested the gold-unlabelled regions are composed of p6.

The filaments of flagellin gene disruptants (strains Δp5, Δp6 and Δp5'/p6) were also analysed by immunoelectron microscopy with anti-p5 antiserum (Fig. 5c–e). In strain Δp5, the gold particles were observed on a few filaments. The filament of strain Δp5 comprised p5' and p6 (Fig. 2).
Although we also examined the immunoelectron microscopy analyses of constructed filaments. Particle size of gold colloids was 5 nm. Filaments constructed by p5 were immunostained with anti-p5 (d) or anti-p6 (e) antisera. Filaments constructed by p6 were immunostained with anti-p5 (b) or anti-p6 (c) antisera. Filaments containing p5, the strain A1-M5 filaments containing p5 were collected by immunoprecipitation with anti-p5 antiserum and were subjected to immunoblotting (Fig. 5f, upper). In lane 1 (immunoprecipitate of the strain A1-M5 filament with anti-p5 antiserum), there were three protein bands (approximately 50, 40 and 30 kDa). In lane 2 (immunoprecipitate of the strain A1-M5 filament lacking anti-p5 antiserum), no protein bands were detected, indicating that the strain A1-M5 filament bound specifically to anti-p5 antiserum but not to Protein A Sepharose. In lane 3 (immunoprecipitate lacking the strain A1-M5 filament), there was a single protein band (approximately 50 kDa). It is possible that this band corresponded to anti-rabbit IgG. Consequently, the three protein bands observed in lane 1 were IgG, p5 and p6 (from top to bottom), indicating that the p6 flagellin, along with p5, existed within a single polar flagellum. Expectedly, no p6 was immunoprecipitated from the strain Δp6 filament with anti-p5 antiserum (Fig. 5f, lower).

Fig. 4. Filament composition in the recombinant flagellins. (a) SDS-PAGE of purified recombinant flagellins. M, Size markers; p5R, recombinant p5; p6R, recombinant p6. (b–e) Electron microscopy analyses of constructed filaments. Particle size of gold colloids was 5 nm. Filaments constructed by p5 were immunostained with anti-p5 (b) or anti-p6 (c) antisera. Filaments constructed by p6 were immunostained with anti-p5 (d) or anti-p6 (e) antisera.

and anti-p5 antiserum did not cross-react with p6 in filaments (Fig. 4). These results demonstrated that anti-p5 antiserum could recognize p5 and p5’ in filaments. These data also indicated that the gold-labelled regions include p5’, whereas the unlabelled region may contain only p6. On the other hand, there were particles on the filaments from the strain Δp6 cells, which primarily consisted of p5 (Fig. 2). However, the number of particles was less than that observed in the filaments of strain A1-M5, although the mechanism is unknown. The filament of strain Δp5'/p6 was also labelled by gold particles. This observation is consistent with the results of the in vitro self-assembled filament (Fig. 4b). One side of the strain A1-M5 filament was not recognized by anti-p5 antiserum, whereas the strain Δp5'/p6 filament was entirely recognized by anti-p5 antiserum. Although we also examined the immunoelectron microscopy of the strain Δp5/p6 filament, no filaments were observed, probably because of the low level of filament formation (Table 2).

In order to directly demonstrate that p6 was included in the filaments containing p5, the strain A1-M5 filaments containing p5 were collected by immunoprecipitation with anti-p5 antiserum and were subjected to immunoblotting (Fig. 5f, upper). In lane 1 (immunoprecipitate of the strain A1-M5 filament with anti-p5 antiserum), there were three protein bands (approximately 50, 40 and 30 kDa). In lane 2 (immunoprecipitate of the strain A1-M5 filament lacking anti-p5 antiserum), no protein bands were detected, indicating that the strain A1-M5 filament bound specifically to anti-p5 antiserum but not to Protein A Sepharose. In lane 3 (immunoprecipitate lacking the strain A1-M5 filament), there was a single protein band (approximately 50 kDa). It is possible that this band corresponded to anti-rabbit IgG. Consequently, the three protein bands observed in lane 1 were IgG, p5 and p6 (from top to bottom), indicating that the p6 flagellin, along with p5, existed within a single polar flagellum. Expectedly, no p6 was immunoprecipitated from the strain Δp6 filament with anti-p5 antiserum (Fig. 5f, lower).

Chemotaxis towards alginate

To clarify physiological function of the strain A1 polar flagellum, the bacterial chemotaxis was investigated by using soft agar plates (Darias et al., 2014). The strain A1-M5 cells forming a single polar flagellum grew well on both alginate and pectin included in each minimal medium as carbon source (Fig. S1a, available in the online Supplementary Material). According to spot pattern shown in Fig. S1b, alginate was applied on the plate and after incubation at 4°C for 16 h and the following incubation at 30°C for 5 days, the plate was stained with cetylpyridinium chloride. After staining, alginate was clouded around the centreline (spotted site) of the plate (Fig. S1c). The stained plate indicated that the alginate concentration gradient was kept during the two steps of incubation. Thus, the strain A1 wild-type (non-motile) or A1-M5 cells precultured in AY or PY medium were subjected to chemotaxis assay. The strain A1 wild-type (non-motile) cells showed a growth at the cell-spotted site, especially at the site near the centreline of the plate (Fig. S1d), where the alginate concentration was considerably high (Fig. S1c). On the other hand, the strain A1-M5 cells moved towards the alginate-spotted site (Fig. S1e, g), while pectin was inert as a chemotacticant (Fig. S1f, h). The alginate concentration-dependent cell motility was observed in Fig. S2. Strain A1-M5 cells most close to alginate began to move towards the polysaccharide. These above-described results indicated that the strain A1-M5 cells exhibited a chemotactic motility towards alginate, but not towards pectin, although these cells were capable of assimilating alginate and pectin. Therefore, the strain A1-M5 cells are demonstrated to show flagellum-mediated chemotaxis specifically towards alginate. These results are consistent with the fact that the expression levels of most chemotaxis-related components are demonstrated to show flagellum-mediated chemotaxis specifically towards alginate. These results are consistent with the fact that the expression levels of most chemotaxis-related components.
genes increased in the strain A1-M5 cells compared with the strain A1 wild-type cells (Maruyama et al., 2015). The bacterial chemotaxis was further analysed using the plate simultaneously containing different acidic polysaccharides such as alginate, pectin and xanthan as shown in Fig. S1i. The strain A1-M5 cells showed a specific chemotaxis to alginate regardless of the effect of the preculture condition (AY or PY medium) (Fig. S1j, k).
**DISCUSSION**

In this report, we describe formation of a single polar flagellum including lateral-typed flagellin as well as polar-typed flagellins and the bacterial chemotaxis towards alginate. Immunoelectron microscopy and immunoprecipitation clearly indicated that strain A1 produces two types of flagellar filaments, one formed by three flagellins (p5, p5' and p6) and the other formed only by p6. p5 and p6 differ in molecular weight and type (polar or lateral). This composition is distinct because multiple flagellins within a single flagellum generally have similar molecular sizes (McCarter & Wright, 1993; Yonekura et al., 2003). Also, bacterial flagellar filaments extend from the originating cell to the extracellular space (Minamino et al., 2008). Therefore, flagellins located in the proximal end of filaments (p6 flagellin in strain A1) may be important to form functional flagella. To our knowledge, strain A1 is a peculiar bacterium forming a single polar flagellum containing the lateral-typed flagellin p6. The mechanisms of polar and lateral-typed flagellin regulation during the formation of a single flagellum filament are significantly more diverse and complex than previously thought.

A large number of alginate-degrading bacteria have been isolated from various sources such as soil, sea and waste water (Wong et al., 2000); to the best of our knowledge, there is no report on bacteria showing a chemotaxis towards alginate. Strain A1 is known to be capable of assimilating pectin as well as alginate and to incorporate alginate into the cytoplasm through KdgM porin and TogM-NAB ABC transporter (Blot et al., 2002; Hugouvieux-Cotte-Pattat et al., 2001). The strain A1-M5 cells are therefore first identified to exhibit a chemotactic motility towards alginate polysaccharide. The strain A1 chemotaxis-dependent alginate recognition system is now being analysed. This chemotaxis towards alginate is also expected to become a powerful machinery for microbes to readily approach alginate in order to convert marine biomass alginate into biofuels or to remove bacterial alginate-containing biofilms more efficiently.

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