

Title: Formation of a single polar flagellum by two distinct flagellar gene sets in *Sphingomonas* sp. strain A1

Authors: Yukie Maruyama^{1,2}, Masahiro Kobayashi¹, Kousaku Murata^{1,2}, Wataru Hashimoto^{1*}

Affiliations: ¹*Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan*

²*Present address: Department of Life Science, Faculty of Science and Engineering, Setsunan University, Neyagawa, Osaka 572-8508, Japan*

*Corresponding author: Tel: +81-774-38-3756

Fax: +81-774-38-3767

E-mail address: whasimot@kais.kyoto-u.ac.jp

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Abbreviations: Strain A1, *Sphingomonas* sp. strain A1; TEM, transmission electron microscopy; CBB, Coomassie Brilliant Blue R-250

Footnote: The nucleotide sequences of strain A1 flagellar and chemotaxis genes in this paper were deposited in the GenBank/EMBL/DDBJ databases under the following accession numbers: *sph1784-1824*, LC043068; *sph183-184*, LC043069; *sph223-224*, LC043070; *sph241-245*, LC043071; *sph539-544*, LC043072; *sph1045-1058*, LC043073; *sph3884-3889*, LC043074; *sph3931-3933*, LC043075, *sph3939-3951*, LC043076; *p5'*, AB968520; *sph183*, LC016694; *sph184*, LC016695; *sph1784*, LC016662; *sph1786*, LC016663; *sph1788*, LC016664; *sph1789*, LC016665; *sph1790*, LC016666; *sph1791*, LC016667; *sph1792*, LC016668; *sph1793*, LC016669; *sph1794*, LC016670; *sph1795*, LC016671; *sph1796*, LC016672; *sph1798*, LC017868; *sph1800*, LC016674; *sph1801*, LC016675; *sph1802*, LC016676; *sph1803*, LC016677; *sph1804*, LC016678; *sph1807*, LC016679; *sph1808*, LC016680; *sph1809*, LC016681; *sph1810*, LC016682; *sph1811*, LC016683; *sph1812*, LC016684; *sph1813*, LC016685; *sph1814*, LC016686; *sph1816*, LC016687; *sph1817*, LC016688; *sph1818*, LC016689; *sph1819*, LC016690; *sph1820*, LC016691; *sph1821*, LC016692; *sph1822*, LC016693; *sph241*, LC016780; *sph242*, LC016781; *sph243*, LC016782; *sph244*, LC016783; *sph245*, LC016784; *sph539*, LC016785; *sph540*, LC016786; *sph541*, LC016787; *sph542*, LC016788; *sph543*, LC016789; *sph544*, LC016790; *sph1046*, LC016791; *sph1047*, LC016792; *sph1048*, LC016793; *sph1049*, LC016794; *sph1050*, LC016795; *sph1051*, LC016796; *sph1052*, LC016797; *sph1053*, LC016798; *sph1054*, LC016799; *sph1057*, LC016800; *sph1058*, LC016801; *sph3884*, LC016802; *sph3885*, LC016803; *sph3886*, LC016804; *sph3887*, LC017869; *sph3888*, LC016805; *sph3889*, LC016806; *sph3931*, LC016807; *sph3932*, LC016808; *sph3933*, LC016809; *sph3939*, LC016810; *sph3940*, LC016811; *sph3941*, LC016812; *sph3942*, LC016813; *sph3944*, LC016814; *sph3945*,

47 LC016815; *sph3946*, LC016816; *sph3947*, LC016817; *sph3948*, LC016818; *sph3951*,
48 LC016819; *sph821*, LC016820; *sph1226*, LC016821; *sph1229*, LC016822; *sph1228*,
49 LC016823; *sph3661*, LC016824; *sph1225*, LC016825; *sph2312*, LC016826; *sph3660*,
50 LC016827; *sph3662*, LC016828; *sph3741*, LC016829; *sph3649*, LC016830; *sph2313*,
51 LC016831; *sph1230*, LC016832; *sph3659*, LC016833; *sph1231*, LC016834; *sph3658*,
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53 LC017709.

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A Gram-negative *Sphingomonas* sp. strain A1, originally identified as a non-motile and aflagellate bacterium, possesses two sets of genes required for flagellar formation. In this study, we characterised the flagellar genes and flagellum formation in strain A1. Flagellar gene cluster set I contained 35 flagellar genes, including one flagellin gene (*p6*), where the gene assembly structure resembled that required for the formation of lateral flagella in gamma-proteobacteria. The set II flagellar genes were arranged in eight shorter clusters with 46 flagellar genes, including two flagellin genes (*p5* and *p5'*) and *flhF*, which is required for polar flagella. Our molecular phylogenetic analysis of the bacterial flagellins also demonstrated that, in contrast to *p5* and *p5'*, *p6* was categorised as a lateral flagellin group. The motile phenotype appeared in strain A1 cells when they were sub-cultured on semisolid media. The motile strain A1 cells produced a single flagellum at the cell pole. DNA microarray analyses using non-motile and motile strain A1 cells indicated that flagellar formation was accompanied by increased transcription of both flagellar gene sets. The two flagellins *p5* and *p6* were major components of the flagellar filaments isolated from motile strain A1 cells, indicating that the polar flagellum is formed by lateral and non-lateral flagellins.

INTRODUCTION

Flagella are structures used for cell locomotion by various bacteria and there are several types, which depend on the number and position of the flagella. In general, flagella are classified into four types according to the flagellar position on the bacterial cell, i.e. peritrichous, polar, sub-polar and lateral flagella (Leifson, 1960). In general, one flagellar system is present but some bacteria, including *Aeromonas*, *Azospirillum*, *Rhodospirillum* and *Vibrio* species, possess two distinct flagellar systems (McCarter, 2006; Merino *et al.*, 2006). These bacteria constitutively express a polar flagellum via one flagellar system and environmentally-induced lateral flagella via the other system. Although the flagella produced by the one flagellar system often affect the transcription of genes required for the formation of the other flagella (Merino *et al.*, 2006), distinct flagellar systems are regulated primarily by different factors and they form flagella at different positions.

An individual flagellum comprises three major sub-structures: the filament, hook and basal body. The assembly of these sub-structures is controlled by a hierarchy that governs the transcription of flagellar genes. The regulatory hierarchies of flagellar gene expression have been analysed comprehensively in some bacterial flagella, such as the peritrichous flagella of *Salmonella enterica* serovar Typhimurium and *Escherichia coli* (Chilcott & Hughes, 2000), polar flagella in *Pseudomonas aeruginosa* (Dasgupta *et al.*, 2003) and the polar and lateral flagella in *Vibrio parahaemolyticus* (Stewart & McCarter, 2003; Kim & McCarter, 2000) and *Aeromonas hydrophila* (Wilhelms *et al.*, 2013; Canals *et al.*, 2006). The regulatory mechanism is different in each of these bacteria. For example, the flagellar genes are divided into three classes in *S. enterica*

(classes 1 to 3). The master gene operon *flhDC* in class 1 activates class 2 operons, which contain genes for the hook and basal body, with the help of a typical sigma factor σ^{70} . A flagellar-specific sigma factor (σ^{28}) called FliA is expressed from the class 2 operons, which then activates the class 3 operons to facilitate filament formation and chemotaxis. In *P. aeruginosa*, the flagellar genes are classified into four classes, which are governed by σ^{54} . The two distinct flagellar systems in *Aeromonas* and *Vibrio* species are regulated separately by different transcriptional hierarchies.

Sphingomonas sp. strain A1 (strain A1), one of the alpha-proteobacteria, is a Gram-negative bacterium, which was originally isolated from soil as an alginate-assimilating microbe (Yonemoto *et al.*, 1991). Strain A1 forms characteristic ‘pits’ on the cell surface to incorporate alginate, depending on the presence of extracellular alginate. Strain A1 has been identified as a non-motile and aflagellate bacterium, although flagellin homologous proteins (p5 and p6) are expressed on the bacterial cell surface, one of which possesses a potent alginate-binding ability (Hashimoto *et al.*, 2005). In the present study, we characterised flagellar formation in strain A1 through genome-based gene analysis, DNA microarrays and electron microscopy. The components of the strain A1 flagellar filaments were also analysed, which showed that the two distinct types of flagellins form a single polar flagellum.

METHODS

Bacterial strains and culture conditions. Non-motile wild-type and motile (A1-M5) strain A1 cells were used in this study. Bacterial cells were cultured aerobically at 30°C in ALY medium [0.1% (NH₄)₂SO₄, 0.1% KH₂PO₄, 0.1% Na₂HPO₄, 0.01%

MgSO₄·7H₂O, 0.01% yeast extract and 0.5% sodium alginate; pH 7.2] or AHY medium [0.1% (NH₄)₂SO₄, 0.1% KH₂PO₄, 0.1% Na₂HPO₄, 0.01% MgSO₄·7H₂O, 0.5% yeast extract and 0.5% sodium alginate; pH 7.2]. For plate culture experiments, agar was included in the AHY medium at a final concentration of 0.5 or 0.3%. For molecular identification of each flagellin (p5, p5', or p6) in the filament fraction, p5 and p6 gene-disruptants (Hashimoto *et al.*, 2005) were grown in the ALY medium. In the genome of these gene-disruptants, kanamycin resistant gene is inserted into the center region of the corresponding gene.

Isolation of motile strain. The motile cells were obtained using semisolid AHY plates with 0.5% agar as described previously (Negrete-Abascal *et al.*, 2003). A single colony of strain A1 (each non-motile strain of wild-type, p5 gene-disruptant and p6 gene-disruptant) grown on ALY plate solidified with 1.5% agar was sub-cultured on semisolid AHY plates and incubated at 30°C. The cells at the edge of a single colony formed were sub-cultured to a fresh semisolid AHY plate and were incubated at 30°C. In each experiment, cells of the motile strain were picked from the edge of colony formed on semisolid AHY plate which was sub-cultured periodically.

DNA microarray. Total RNA was extracted using the hot phenol method from wild-type strain A1 and A1-M5 cells grown in ALY medium to an OD₆₀₀ of 0.5 to 0.6 as described previously (Hayashi *et al.*, 2014). The resultant RNA was hybridized to strain A1 DNA microarray chip designed by Roche NimbleGen. The gene chip contains 3985 strain A1 genes fixed on a glass slide obtained by fixing two sets of 9 unique probes

composed of 60-mer synthetic oligonucleotides for each gene. Labeling with cyanine (Cy3), fragmentation, and hybridization were carried out by Roche NimbleGen in line with NimbleGen Array User's Guide: Gene Expression Arrays Version 5.1 (Roche NimbleGen, 2010). Arrays were scanned at 532 nm and a resolution of 2 μ m with a NimbleGen MS200 microarray scanner and analyzed by quantile normalization and robust multiarray averaging (Irizarry *et al.*, 2003a; Irizarry *et al.*, 2003b). The resultant about 18 raw expression data for each gene were subjected to statistical treatment. The normalized data were processed by a program of the NANDEMO Analysis 1.0.0 (Roche diagnostics). Student's t-test was adopted for analyzing the mean log ratios of two samples and subsequent Bonferroni adjustment for multiple testing (3985 genes on arrays) was used as a rigorous criterion for significant changes in signal intensity. Changes with $P < 0.05$ were considered statistically significant. The microarray data obtained in this study have been deposited in the Gene Expression Omnibus database at NCBI under accession number GSE62901.

Microscopy. Light microscopy was performed using an Olympus BX51 microscope. A1-M5 cells were grown overnight aerobically at 30°C in ALY medium. Transmission electron microscopy (TEM) (JEOL JEM-1200EX) was performed after negative staining with 2% phosphotungstic acid in a similar manner to that described previously (Hisano *et al.*, 1995).

Isolation of flagella. Flagellar filaments were isolated from the cells, as described previously (Iida *et al.*, 2009) but with some modifications. Briefly, A1-M5 cells were

cultured aerobically in AHY medium (10 mL) to an OD₆₀₀ of ca 1.0 and harvested by centrifugation at 4°C (6,000 × *g* for 10 min). The pellet was resuspended in 1 mL of 10 mM Tris-HCl (pH 7.5) and passed through a needle (22 gauge) 10 times to shear off the flagella from the cells. After removing the cells by centrifugation (10,000 × *g* for 10 min) at 4°C, the flagella were pelleted by ultracentrifugation (100,000 × *g* for 60 min) at 4°C and resuspended in 50 µL of 10 mM Tris-HCl (pH 7.5).

Western blotting. Bacterial cells grown in ALY or AHY medium at 30°C were harvested by centrifugation (6,000 × *g* for 10 min) at 4°C, resuspended in 20 mM Tris-HCl (pH 7.5) and lysed with SDS. The cell lysates or isolated flagellar filaments were subjected to SDS-PAGE and transferred to an Immobilon-P transfer membrane (Millipore) at 1.5 mA/cm² for 30 min. The immunoreactions were performed according to the rapid immunodetection method described in the manufacturer's instructions (Millipore) using rabbit anti-p5 antisera (Hashimoto *et al.*, 2005) or rat anti-p6 antisera (Kobayashi, Graduation Thesis of Kyoto University, pp9-10, 2013) as a primary antibody and donkey anti-rabbit or goat anti-rat immunoglobulin antibody (GE Healthcare) as a secondary antibody. Both antibodies to p5 and p6 purified from recombinant *E. coli* cells were raised in rabbit and rat, respectively. The resultant sera were used as polyclonal antibodies. Immunoreactive proteins were detected using a POD immunostain set (Wako) or EzWestLumiOne (ATTO).

Gene analyses. The MEME program (Bailey & Elkan, 1994) was used to extract the common motif from the upstream sequences of the flagellar genes. The primary

sequence identities of the flagellins were calculated using the BLAST program (Madden *et al.*, 1996). A molecular phylogenetic tree was constructed using CLUSTALW (Thompson *et al.*, 1994) and the TreeView program (Page, 1996).

RESULTS AND DISCUSSION

Two sets of flagellar genes in strain A1

The complete genome sequence of strain A1 was determined previously and the individual genes have been annotated, although the genome sequence data has not yet been published. The genome size of strain A1 is approximately 4.6 Mb with about 4000 genes. Strain A1 has been assigned as a non-motile and aflagellate bacterium (Yonemoto *et al.*, 1991), but we found that its genome contains at least 80 flagellar genes (Fig. 1). A large gene cluster from *sph1784* to *sph1824* (Fig. 1(a)) appeared to contain almost all of the genes required for flagellum formation. Other smaller clusters that contained 2–12 flagellar genes were also found in the genome (Fig. 1(b)). Most of the genes in the smaller clusters overlapped with the genes in the larger cluster and thus these genes alone appeared to be sufficient for flagellar formation. Therefore, we designated the larger cluster of flagellar genes as set I and the shorter clusters as set II.

The flagellar gene arrangement is known to differ among species, but the arrangement of lateral flagellar genes is somewhat conserved in gamma-proteobacteria (Merino *et al.*, 2006; Ren *et al.*, 2005). The arrangement of strain A1 gene cluster set I was similar to that of the lateral flagellar genes. *lafK* encodes a regulatory protein for lateral flagellar gene expression (Stewart & McCarter, 2003) and it is usually included in the *fliEFGHIJ* operon, but it was not present in the set I cluster (Fig. 1(b)).

210 Most of the genes required for flagellar formation (Aizawa, 2014) were found
 211 in both the set I and II clusters. In addition to the essential genes required for flagellar
 212 production, the set II clusters contained *flhC* and *flhD*, which are present in the genomes
 213 of beta- and gamma-proteobacteria such as *Salmonella* and *Escherichia*, although strain
 214 A1 is a member of alpha-proteobacteria. Heterotetramer of FlhD2C2 is known to
 215 regulate the transcription of other flagellar gene operons, typically *fliAZ*, *flhBA*, *flgAMN*
 216 and *flgBCD* (Claret & Hughes, 2002). The sequence identities between strain A1 *flhC*
 217 and *flhD* genes and their corresponding genes of *E. coli* are 54 and 31%, respectively.
 218 We searched the common motives in the upstream sequences of the strain A1 *fliA*, *flhB*,
 219 *flgA* and *flgB* genes, but failed. Instead, the homologous sequence
 220 (5'-AAGCGGCCGAATAGCCggggcttttCCGCATTTATCCGGGC-3') to
 221 FlhD2C2-binding motif (5'-AATGGCAGAAATAGCG N₁₀₋₁₂
 222 CGCTATTTCTGCCATT-3') (Stafford *et al.*, 2005) was found only in the upstream
 223 sequence of the set II *flhB* (*sph3884*) gene. The *flhF* and *flhG* genes are involved in the
 224 orientation of the flagella at the cell pole and regulation of the number of flagella,
 225 respectively, and they are present in the genomes of bacteria with a single polar
 226 flagellum (Kazmierczak & Hendrixson, 2013, Kusumoto *et al.*, 2006). In strain A1, *flhF*
 227 and *flhG* were present in the set II genes, thereby suggesting that the set II flagellar
 228 genes are involved in the formation of a single polar flagellum.

229 Two flagellin homologues, p5 and p6, were identified previously in the outer
 230 membrane fraction of strain A1 (Hashimoto *et al.*, 2005). We found that another
 231 flagellin gene, *p5'*, was also encoded in the downstream region of *p5* (Fig. 1(b)). The
 232 shared amino acid sequence identities between *p5'* and *p5* and between *p5'* and *p6* were

85% and 40%, respectively. The amino acid length of *p5'* (384 residues) was also similar to that of *p5* (383 residues), but different from that of *p6* (297 residues). Although *p5* and *p5'* are neighbouring, a possible terminator sequence (5'-AACAAAAAACCCGCCTTGTGCGGGTTTTTTGTT-3') was inserted between them, thereby suggesting that there was no operon structure in *p5* and *p5'*. In fact, the sequence 5'-TAAGTTT X₁₁ GCCGATA-3' (Ide *et al.*, 1999), which is recognised specifically by sigma factor σ 28 during flagellar formation, was located in the region upstream of the start codon of *p5* (5'-TTCAAGTTTcaaagcgccggGCCGATG-3'), but not in that of *p5'*. Instead, the sequence 5'-CTGGCCcgccctTTGCA-3' located upstream of *p5'* is probably recognised by σ 54 (Barrios *et al.*, 1999). The consensus sequences for σ 28 recognition were situated in other sites in the strain A1 flagellar genes. All of the σ 28-related genes (*sph223*, *sph242*, *sph246*, *sph537* and *sph1057*) shown in Table 1 were included in set II, and none were found that belonged to set I.

Characterisation of strain A1 flagellin genes

A phylogenetic tree was constructed using flagellins from bacteria with two flagellar systems and from sphingomonads with analysed genomes (Fig. 2). Most of the bacteria with two flagellar systems constitutively produce a mono-polar flagellum and lateral flagella are induced by change in environmental conditions, such as viscosity, pressure and aeration (McCarter, 2004; Elo *et al.*, 2008; Shimada *et al.*, 1985; Moens *et al.*, 1995; Sachett *et al.*, 1997), except in the following three bacterial strains: *E. coli* strain 042 forms peritrichous flagella without expressing a second flagellar gene system (Ren *et al.*, 2005); *Rhodobacter sphaeroides* constitutively forms a single sub-polar flagellum,

but it produces a polar flagellum in aerobic growth conditions (Poggio *et al.*, 2007); *Bradyrhizobium japonicum* forms a single thick flagellum and multiple thin flagella in a sub-polar position, which are sometimes called lateral flagella (Kanbe *et al.*, 2007; Covelli *et al.*, 2013). Figure 2 shows that lateral and polar flagellins are classified as different branches, but some other flagellins belong to another branch. Interestingly, branches for the lateral and polar flagellins comprise flagellins from gamma-proteobacteria. The other bacteria, i.e. *Azospirillum brasilense*, *R. sphaeroides* and *B. japonicum*, are classified as alpha-proteobacteria.

Among the strain A1 flagellins, p5 and p5' were not categorised in the branch for lateral or polar flagellin. On the other hand, p6 was positioned at the lateral flagellin branch of gamma-proteobacteria (Fig. 2), although sphingomonads are classified as alpha-proteobacteria. These results are consistent with a previous analysis, which showed that p6 was most homologous to the lateral flagellin from *V. parahaemolyticus* (Hashimoto *et al.*, 2005). Data about flagellation and motility of other sphingomonads is limited. Both of motile and non-motile strains have been reported (Miake *et al.*, 1995; Yabuuchi *et al.*, 2001) and the motile strains express a single polar flagellum. Genome-sequenced sphingomonads, *Sphingomonas wittichii* and *Sphingomonas* sp. strain MM-1, have flagellar genes with two (*swit_1283* and *1284*) and five (*G432_0590*, *06930*, *06935*, *06940* and *06940*) flagellins, respectively. These flagellins were present at the branches of alpha-proteobacteria flagellins, but not at those between p6 and the lateral flagellins of gamma-proteobacteria. These observations suggest that the p6 gene was acquired via horizontal gene transfer after strain A1 diverged from other sphingomonads.

Similarities with the lateral flagellar genes were also detected for other genes in flagellar gene cluster set I. For example, BLAST searches against the UniProtKB and SwissProt databases using the nucleotide sequences of *sph1800* (*motB*), *sph1801* (*motA*) and *sph1802* (*fliA*) as query sequences showed that they shared high homology with *lafU*, *lafT* and *lafS* from *V. haemolyticus*, respectively.

The position in the flagellin phylogenetic tree, as well as the arrangement of the set I flagellar genes mentioned above, suggests that p6 is a lateral flagellin, which is typically designated as LafA, whereas p5 and p5' may be different from lateral flagellins based on their primary sequences.

Motility expression and flagellum formation by strain A1

The motility of cells was screened on semisolid AHY plates with 0.5% agar. First, the strain A1 cells streaked onto semisolid media exhibited no movement, but the cells began to spread. The cells at the edge of a single colony grown for a week were sub-cultured to a fresh semisolid AHY plate. In contrast to the wild-type strain A1 cells, sub-cultured strain A1 cells spread over the medium within a few days (Fig. 3(a)). We selected cells (designated as A1-M5) from the colony with the widest spread and used these cells in our further analyses. Western blotting analysis using anti-p5 antisera showed that A1-M5 cells produced a large amount of p5 compared with the wild-type strain A1 cells (Fig. 3(b)). The cross-reacted protein bands in A1-M5 cell extracts (Fig. 3(b) lanes 3 and 4) indicated the presence of other flagellins: p5' and p6. Both the wild-type and motile strain A1 cells grew well on alginate (Fig. 3(c)). A1-M5 cells were also confirmed to exhibit a swimming ability by light microscopy (Movie S1 and S2)

and this was supported by the cell movement on 0.3% agar plate (Fig. 3(d)). TEM analysis showed that about half of A1-M5 cells formed a single mono-polar flagellum (Fig. 4), which has not been observed previously. The length and thickness of the flagellar filament were determined as ca 8 μ m and 20 nm, respectively. Motility expression of strain A1 was confirmed to be reproducible. Furthermore, although initial experiment for the motility screening was undergone using semisolid AHY plates and cultivation in AHY liquid media increased the p5 expression in wild-type strain A1 (Fig. 3(b), lane 2), streaking of wild-type strain A1 onto semisolid ALY plates also induced cell motility, suggesting that high concentration of yeast extract is effective but not necessary on flagellation of strain A1.

As described above, strain A1 possesses three flagellin genes and two sets of flagellar genes. In order to determine the genes involved in the formation of the single polar flagellum in A1-M5, the gene expression levels were compared in wild-type and A1-M5 cells based on DNA microarray analyses. As shown in Table 2, all of the flagellar genes in the A1-M5 cells were expressed at much higher levels than those in the wild-type cells. The flagellar gene sets did not exhibit any obvious tendencies to be expressed preferentially. The expression levels of most of the chemotaxis-related genes also increased, but not all. The most dramatic increase (404-fold) was observed in *sph821*, which encodes a methyl-accepting chemotaxis protein that act as a receptor in chemotaxis. The expression levels of three flagellin genes, i.e. p5, p5' and p6, increased 67-, 57- and 93-fold, respectively, in A1-M5 cells, thereby indicating that all of them were transcribed inductively in A1-M5 cells. However, the transcription levels of these flagellins were different (p5, 39242; p5', 6054; p6, 44317). The transcriptions of *flhD*

and *flhC* genes whose products promote expression of the other flagellar genes in *Salmonella* and their related species were also highly upregulated in the A1-M5 cells. However, because the common motif for FlhD2C2 binding was not found in the strain A1 genome, the regulation of expression of flagellar genes remains to be clarified.

Because flagellar formation of the A1-M5 cells are stable, some kinds of genetic changes are supposed to occur. The similar phenomenon that flagellar formation in non-motile *Actionobacillus pleuropneumoniae* was facilitated in the soft agar media has previously been reported in the paper of Negrete-Abascal *et al.*, 2003. There is also a possibility that the strain A1 cell produces lateral flagella under the appropriate condition as reported in the case of second flagellar system of *R. sphaeroides* (Poggio *et al.*, 2007). However, we failed to find such condition to date and strain A1 cells producing lateral flagella

Flagellar filaments with two distinct types of flagellins

To investigate the flagellar constituents, the flagellar filaments were sheared off from A1-M5 cells and subjected to SDS-PAGE. Two major protein bands were observed with molecular sizes of 39 kDa and 30 kDa (Fig. 5). The N-terminal amino acid sequence of the 39-kDa protein was determined as NH₂-AMTINTN, which corresponded to 2–8 amino acid residues in p5 and p5'. However, we failed to determine the N-terminal sequence of the 30-kDa protein. The flagellar fraction was analysed further by western blotting using anti-p5 antisera. In addition to the major protein bands at 39 kDa and 30 kDa, a faint protein of 37 kDa was observed after staining with Coomassie Brilliant Blue R-250 (CBB) (Fig. 5(a)). The 37-kDa band was also detected by anti-p5 antisera

(Fig. 5(b)). Gene disruption analysis indicated that *p5* and *p6* gene-disruptants lacked the 39-kDa and 30-kDa proteins, respectively, in the flagellar fraction (Fig. 5(b) and (c)). These results demonstrate that the A1-M5 flagellar filaments mainly comprise *p5* (theoretical molecular mass, 39753 Da) and *p6* (31068 Da), while *p5'* (39542 Da) is a minor component. These findings are consistent with the results obtained in the DNA microarray analysis.

It is known that many bacteria possess multiple genes for flagellins in their genomes (Faulds-Pain *et al.*, 2011). These multiple flagellins with high shared sequence homology were probably derived via gene duplication and they often form a flagellum. For example, six flagellins are present in different positions in the flagellar filament of *Caulobacter crescentus* (Driks *et al.*, 1989). By contrast, the flagellins from different flagellar gene sets share lower sequence identities and they form different types of flagella, typically polar and lateral flagella (McCater, 2004). *V. parahaemolyticus* possesses seven flagellin genes, where six flagellins from two gene clusters in one chromosome form a single polar flagellum and one flagellin in the other chromosome forms lateral flagella. The six polar flagellins in *V. parahaemolyticus* are highly homologous in terms of their size (374–384 residues) and amino acid sequence (66–77%). By contrast, the lateral flagellin shares lower sequence identity with the polar flagellins and it contains fewer amino acids (284 residues).

The A1-M5 cells produced only a single polar flagellum, although they possessed two types (lateral and polar) of flagellar gene sets. In A1-M5 cells, the characteristic polar flagellar filaments comprised homologous flagellins (*p5* and *p5'*) as well as a lateral flagellin (*p6*). To the best of our knowledge, this is the first report of a

lateral flagellin forming a polar flagellum. At present, the relationships among flagella formation, the transcription of flagellin genes and the spatial arrangement of p5, p5' and p6 in the strain A1 flagellar filament are being analysed by characterising flagellin(s)-deficient cells.

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References

- Aizawa, S. (2014).** *The Flagellar World*. NY: Academic Press.
- Bailey, T. L. & Elkan, C. (1994).** Fitting a mixture model by expectation maximization to discover motifs in biopolymers. In *Proceedings of the Second International Conference on Intelligent Systems for Molecular Biology*, pp. 28-36. Menlo Park, CA: AAAI Press.
- Barrios, H., Valderrama, B. & Morett, E. (1999).** Compilation and analysis of σ^{54} -dependent promoter sequences. *Nucleic Acids Res* **27**, 4305-4313.
- Chilcott, G. S. & Hughes, K. T. (2000).** The coupling of flagellar gene expression to flagellar assembly in *Salmonella typhimurium* and *Escherichia coli*. *Microbiol Mol Biol Rev* **64**, 94-708.
- Canals, R., Ramirez, S., Vilches, S., Horsburgh, G., Shaw, J. G., Tomás, J. M. & Merino S. (2006).** Polar flagellum biogenesis in *Aeromonas hydrophila*. *J Bacteriol*

394 **188**, 542-555.

395 **Claret, L. & Hughes, C. (2002).** Interaction of the atypical prokaryotic transcription
396 activator FlhD2C2 with early promoters of the flagellar gene hierarchy. *J Mol Biol*
397 **321**, 185-199.

398 **Covelli, J. M., Althabegoiti, M. J., López, M. F. & Lodeiro, A. R. (2013).** Swarming
399 motility in *Bradyrhizobium japonicum*. *Res Microbiol* **164**, 136-144.

400 **Dasgupta, N., Wolfgang, M. C., Goodman, A. L., Arora, S. K., Jyot, J., Lory, S. &**
401 **Ramphal, R. (2003).** A four-tiered transcriptional regulatory circuit controls
402 flagellar biogenesis in *Pseudomonas aeruginosa*. *Mol Microbiol* **50**, 809-824.

403 **Driks, A., Bryan, R., Shapiro, L. & DeRosier, D. J. (1989).** The organization of the
404 *Caulobacter crescentus* flagellar filament. *J Mol Biol* **206**, 627-636.

405 **Eloe, E. A., Lauro, F. M., Vogel, R. F. & Bartlett, D. H. (2008).** The
406 deep-seabacterium *Photobacterium profundum* SS9 utilizes separate flagellar
407 ksystems for swimming and swarming under high-pressure conditions. *Appl*
408 *Environ Microbiol* **74**, 6298-6305.

409 **Faulds-Pain, A., Birchall, C., Aldridge, C., Smith, W. D., Grimaldi, G., Nakamura,**
410 **S., Miyata, T., Gray, J., Li, G., Tang, J. X., Namba, K., Minamino, T. &**
411 **Aldridge, P. D. (2011).** Flagellin redundancy in *Caulobacter crescentus* and its
412 implications for flagellar filament assembly. *J Bacteriol* **193**, 2695-2707.

413 **Hashimoto, W., He, J., Wada, Y., Nankai, H., Mikami, B. & Murata, K. (2005).**
414 Proteomics-based identification of outer-membrane proteins responsible for import
415 of macromolecules in *Sphingomonas* sp. A1: alginate-binding flagellin on the cell
416 surface. *Biochemistry* **44**, 13783-13794.

417 **Hayashi, C., Takase, R., Momma, K., Maruyama, Y., Murata, K. & Hashimoto, W.**
418 **(2014).** Alginate-dependent gene expression mechanism in *Sphingomonas* sp. strain
419 A1. *J Bacteriol* **196**, 2691-2700.

420 **Hisano, T., Yonemoto, Y., Yamashita, T., Fukuda, Y., Kimura, A. & Murata, K.**
421 **(1995).** Direct uptake of alginate molecules through a pit on the bacterial cell
422 surface: a novel mechanism for the uptake of macromolecules. *J Ferment Bioeng*
423 **79**, 538-544.

424 **Ide, N., Ikebe, T. & Kutsukake, K. (1999).** Reevaluation of the promoter structure of
425 the class 3 flagellar operons of *Escherichia coli* and *Salmonella*. *Genes Genet Syst*
426 **74**, 113-116.

427 **Iida, Y., Hobley, L., Lambert, C., Fenton, A. K., Sockett, R. E. & Aizawa, S. (2009).**
428 Roles of multiple flagellins in flagellar formation and flagellar growth post
429 bdelloplast lysis in *Bdellovibrio bacteriovorus*. *J Mol Biol* **394**, 1011-1021.

430 **Irizarry, R. A., Bolstad, B. M., Collin, F., Cope, L. M., Hobbs, B., & Speed, T. P.**
431 **(2003a).** Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res*
432 **31**, e15.

433 **Irizarry, R. A., Hobbs, B., Collin, F., Beazer-Barclay, Y. D., Antonellis, K. J., Scherf,**
434 **U., & Speed, T. P. (2003b).** Exploration, normalization, and summaries of high
435 density oligonucleotide array probe level data. *Biostatistics* **4**, 249–264.

436 **Kanbe, M., Yagasaki, J., Zehner, S., Göttfert, M. & Aizawa, S. (2007).**
437 Characterization of two sets of subpolar flagella in *Bradyrhizobium japonicum*. *J*
438 *Bacteriol* **189**, 1083-1089.

439 **Kazmierczak, B. I. & Hendrixson, D. R. (2013).** Spatial and numerical regulation of

flagellar biosynthesis in polarly-flagellated bacteria. *Mol Microbiol* **88**, 655-663.

Kim, Y. K. & McCarter, L. L. (2000). Analysis of the polar flagellar gene system of *Vibrio parahaemolyticus*. *J Bacteriol* **182**, 3693-3704.

Kusumoto, A., Kamisaka, K., Yakushi, T., Terashima, H., Shinohara, A. & Homma, M. (2006). Regulation of polar flagellar number by the flhF and flhG genes in *Vibrio alginolyticus*. *J Biochem* **139**, 113-121.

Leifson, E. (1960). *Atlas of bacterial flagellation*. NY: Academic Press.

Madden, T. L., Tatusov, R. L. & Zhang, J. (1996). Applications of network BLAST server. *Meth Enzymol* **266**, 131-141.

McCater, L. L. (2004). Dual flagellar systems enable motility under different circumstances. *J Mol Biol* **7**, 18-29.

Merino, S., Shaw, J. G. & Tomás, J. M. (2006). Bacterial lateral flagella: an inducible flagella system. *FEMS Microbiol Lett* **263**, 127-135.

Miake, F., Murata, K., Kuroiwa, A., Kumamoto, T., Kuroda, S., Terasawa, T., Tone, H. & Watanabe, K. (1995). Characterization of *Pseudomonas paucimobilis* FP2001 which forms flagella depending upon the presence of rhamnose in liquid medium. *Microbiol Immunol* **39**, 437-442.

Moens, S., Michiels, K., Keijers, V., Van Leuven, F. & Vanderleyden, J. (1995). Cloning, sequencing, and phenotypic analysis of *laf1*, encoding the flagellin of the lateral flagella of *Azospirillum brasilense* Sp7. *J Bacteriol* **177**, 5419-5426.

Negrete-Abascal, E., Reyes, M. E., García, R. M., Vaca, S., Girón, J. A., García, O., Zenteno, E. & de la Garza, M. (2003). Flagella and motility in *Actinobacillus pleuropneumoniae*. *J Bacteriol* **185**, 664-668.

- Page, R. D. M. (1996).** TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357-358.
- Poggio, S., Abreu-Goodger, C., Fabela, S., Osorio, A., Dreyfus, G., Vinuesa, P. & Camarena, L. (2007).** A complete set of flagellar genes acquired by horizontal transfer coexists with the endogenous flagellar system in *Rhodobacter sphaeroides*. *J Bacteriol* **189**, 3208-3216.
- Ren, C. P., Beatson, S. A., Parkhill, J. & Pallen, M. J. (2005).** The Flag-2 locus, an ancestral gene cluster, is potentially associated with a novel flagellar system from *Escherichia coli*. *J Bacteriol* **187**, 1430-1440.
- Roche NimbleGen. (2010).** NimbleGen Arrays User's Guide: Gene Expression Arrays Version 5.1. Roche NimbleGen, Madison, WI.
- Sackett, M. J., Armitage, J. P., Sherwood, E. E. & Pitta, T. P. (1997).** Photoresponses of the purple nonsulfur bacteria *Rhodospirillum centenum* and *Rhodobacter sphaeroides*. *J Bacteriol* **179**, 6764-6768.
- Shimada, T., Sakazaki, R. & Suzuki, K. (1985).** Peritrichous flagella in mesophilic strains of *Aeromonas*. *Jpn J Med Sci Biol* **38**, 141-145.
- Stafford, G. P., Ogi, T. & Hughes, C. (2005).** Binding and transcriptional activation of non-flagellar genes by the *Escherichia coli* flagellar master regulator FlhD2C2. *Microbiology* **151**, 1779-1788.
- Stewart, B. J. & McCarter, L. L. (2003).** Lateral flagellar gene system of *Vibrio parahaemolyticus*. *J Bacteriol* **185**, 4508-4518.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994).** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting,

position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**,
4673-4680.

Wilhelms, M., Gonzalez, V., Tomás, J. M. & Merino, S. (2013). *Aeromonas*
hydrophila lateral flagellar gene transcriptional hierarchy. *J Bacteriol* **195**,
1436-1445.

Yabuuchi, E., Yamamoto, H., Terakubo, S., Okamura, N., Naka, T., Fujiwara, N.,
Kobayashi, K., Kosako, Y. & Hiraishi, A. (2001). Proposal of *Sphingomonas*
wittichii sp. nov. for strain RW1^T, known as a dibenzo-*p*-dioxin metabolizer. *Int J*
Syst Evol Microbiol **51**, 281-292.

Yonemoto, Y., Murata, K., Kimura, A., Yamaguchi, H. & Okayama, K. (1991).
Bacterial alginate lyase: characterization of alginate lyase-producing bacteria and
purification of the enzyme. *J Ferment Bioeng* **72**, 152-157.

Figure legends

Fig. 1. Flagellar genes in strain A1. (a) Large cluster designated as set I. (b) Smaller clusters designated as set II. The gene annotations of the flagellar genes and the sequence ID in the genome (*sphXXXX*) are also indicated. The genes related to chemotaxis are also shown for those that clustered with flagellar genes. Grey arrows indicate genes that are not related to flagella.

Fig. 2. Molecular phylogenetic tree of flagellins in bacteria with dual flagellar systems and genome-sequenced sphingomonads. Abra, *A. brasilense*; Ahyd, *A. hydrophila*; Bjap, *B. japonicum*; Ecol, *E. coli*; Ppro, *P. profundum*; Rcen, *R. centenum*; Rsp, *R. sphaeroides*; laf, lateral flagellin; fla, flagellin-forming non-lateral flagella such as polar, sub-polar and peritrichous flagella. p5, p5', p6, Swit_#### and G432_#### are flagellins from sphingomonads. Grey-shaded branches and grey-dotted branches indicate the groups of lateral and polar flagellins from gamma-proteobacteria, respectively.

Fig. 3. Comparison of wild-type and motile strain A1 cells. (a) Swarming assay of wild-type strain A1 (left) and A1-M5 cells (right). Cells were inoculated onto semisolid AHY plates containing 0.5% agar and incubated at 30°C for 3 days. (b) Western blot analysis of bacterial cells (5×10^6 cells) using anti-p5 antisera. Lane 1, wild-type strain A1 cells grown in ALY medium; lane 2, wild-type strain A1 cells grown in AHY medium; lane 3, A1-M5 cells grown in ALY medium; lane 4, A1-M5 cells grown in AHY medium. (c) Growth curve of wild-type strain A1 cells (solid

line) and A1-M5 cells (dashed line) in AHY medium. The experiments were repeated three times. (d) Swimming assay of wild-type strain A1 (left) and A1-M5 cells (right). Cells were inoculated onto alginate plate (ALY medium without yeast extract) containing 0.3% agar and incubated at 30°C for 3 days.

Fig. 4. Transmission electron micrographs of A1-M5.

Fig. 5. Filament constitution of the A1-M5 flagella. (a) CBB-stained image after SDS-PAGE using the flagellar filament fraction from A1-M5 cells. (b) Western blot analysis of the flagellar filament fraction using anti-p5 antisera. Lane 1, wild-type strain A1; lane 2, A1-M5; lane 3, *p5* gene-disruptant; lane 4, *p6* gene-disruptant. (c) Western blot analysis of the flagellar filament fraction using anti-p6 antisera. Lane 1, wild-type strain A1; lane 2, A1-M5; lane 3, *p5* gene-disruptant; lane 4, *p6* gene-disruptant. In each lane of (b) and (c), the flagellar filament prepared from 200 μ L of bacterial cell culture (OD=1.0) was loaded.

Movie S1. Light microscopy of wild-type strain A1.

Movie S2. Light microscopy of A1-M5.

Table 1. σ^{28} recognition sequences at upstream of flagellar genes.

gene ID	Sequences
<i>sph223 (p5)</i>	TTCAAGTTT caaagcgccgg GCCGATG
<i>sph242 (motA)</i>	TTCAAGTTC gaacgaggtac GCCGTTA
<i>sph246 (cheY)</i>	CTAAAGGCC tacgccgtcgc GCCGATA
<i>sph539 (flgG)</i>	TTAAAGATT tgccctggccg GCCGTAA
<i>sph1057 (flgM)</i>	CTAAAGTTT tgtatcggtgg GCCGTTA

Table 2. Transcription of genes involved in flagellar formation and chemotaxis in wild-type (WT) and motile (M5) strain A1 cells.

gene ID	transcription level		change (fold)	p-value	annotation
	WT	M5			
<i>sph183</i>	76	581	7.7	5.0E-04	<i>flgL</i>
<i>sph184</i>	62	846	13.7	6.1E-06	<i>flgK</i>
<i>sph223</i>	581	39242	67.5	9.5E-08	<i>p5</i>
<i>sph224</i>	106	6054	57.3	2.2E-06	<i>p5'</i>
<i>sph241</i>	191	3387	17.8	1.4E-06	<i>motB</i>
<i>sph242</i>	240	6117	25.5	1.3E-05	<i>motA</i>
<i>sph243</i>	477	9410	19.7	5.6E-07	<i>flhC</i>
<i>sph244</i>	425	8446	19.9	1.1E-07	<i>flhD</i>
<i>sph245</i>	50	1921	38	2.8E-03	<i>cheY</i>
<i>sph539</i>	681	9367	13.8	1.3E-08	<i>flaG</i>
<i>sph540</i>	484	15956	32.9	3.1E-07	<i>fliD</i>
<i>sph541</i>	563	21240	37.7	1.5E-10	<i>fliS</i>
<i>sph542</i>	394	14235	36.2	1.1E-06	<i>fliT</i>
<i>sph543</i>	40	1197	29.9	1.3E-08	<i>fliK</i>
<i>sph544</i>	175	3504	20	7.8E-09	<i>fhlB</i>
<i>sph821</i>	38	15189	404.2	1.1E-06	<i>mcp</i>
<i>sph1045</i>	36	452	12.5	2.1E-03	<i>flgJ</i>
<i>sph1046</i>	72	1211	16.7	4.1E-04	<i>flgI</i>
<i>sph1047</i>	676	10850	16.1	1.2E-07	<i>flgH</i>
<i>sph1048</i>	389	7686	19.8	5.9E-07	<i>flgG</i>
<i>sph1049</i>	113	2899	25.6	2.6E-08	<i>flgF</i>
<i>sph1050</i>	351	7242	20.7	2.0E-07	<i>flgE</i>
<i>sph1051</i>	443	9126	20.6	2.9E-08	<i>flgD</i>
<i>sph1052</i>	1268	18799	14.8	1.1E-07	<i>flgC</i>
<i>sph1053</i>	354	2919	8.2	6.3E-08	<i>flgB</i>
<i>sph1054</i>	154	857	5.5	2.3E-04	<i>flgA</i>
<i>sph1057</i>	117	524	4.5	6.9E-07	<i>flgM</i>
<i>sph1058</i>	444	1977	4.5	4.1E-08	<i>flgN</i>
<i>sph1225</i>	74	8567	116.4	3.3E-05	<i>mcp</i>
<i>sph1226</i>	116	20446	176.5	6.3E-09	<i>cheY</i>
<i>sph1228</i>	53	7539	143.5	2.8E-06	<i>cheA</i>
<i>sph1229</i>	43	6462	151.1	2.9E-06	<i>mcp</i>
<i>sph1230</i>	36	2244	62.7	5.8E-05	<i>cheW</i>
<i>sph1231</i>	55	3297	60.5	4.8E-06	<i>cheR</i>
<i>sph1232</i>	50	1419	28.7	1.2E-07	<i>cheB</i>
<i>sph1784</i>	202	7494	37.1	1.7E-08	<i>flgL</i>
<i>sph1786</i>	141	5366	38.1	3.9E-09	<i>flgK</i>
<i>sph1787</i>	191	5083	26.6	3.2E-12	<i>flgJ</i>

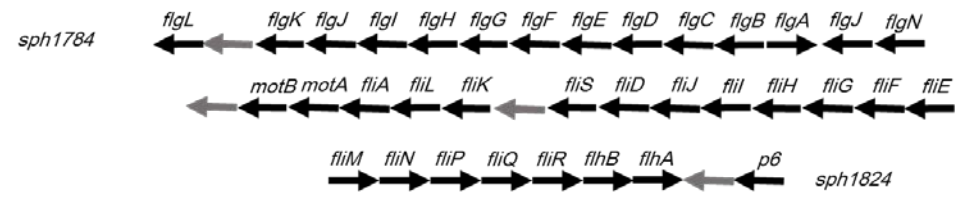
<i>sph1788</i>	68	1694	25	6.2E-06	<i>flgI</i>
<i>sph1789</i>	56	1565	28	2.6E-03	<i>flgH</i>
<i>sph1790</i>	239	6992	29.2	3.5E-06	<i>flgG</i>
<i>sph1791</i>	230	8279	36	1.5E-06	<i>flgF</i>
<i>sph1792</i>	178	8844	49.6	2.5E-06	<i>flgE</i>
<i>sph1793</i>	119	8486	71.5	2.4E-07	<i>flgD</i>
<i>sph1794</i>	203	10113	49.8	2.2E-08	<i>flgC</i>
<i>sph1795</i>	408	4009	9.8	9.1E-06	<i>flgB</i>
<i>sph1796</i>	447	3130	7	1.5E-07	<i>flgA</i>
<i>sph1797</i>	36	211	5.8	1.5E-06	<i>flgJ</i>
<i>sph1798</i>	25	156	6.2	2.8E-04	<i>flgN</i>
<i>sph1800</i>	38	987	25.8	4.6E-02	<i>lafU(motB)</i>
<i>sph1801</i>	29	1240	42.2	9.1E-07	<i>lafT(motA)</i>
<i>sph1802</i>	22	539	24.2	2.4E-05	<i>lafS(fliA)</i>
<i>sph1803</i>	75	4913	65.3	1.8E-06	<i>lafF(fliL)</i>
<i>sph1804</i>	32	1947	60.3	1.4E-05	<i>fliK</i>
<i>sph1807</i>	26	3723	144.6	7.0E-09	<i>fliS</i>
<i>sph1808</i>	52	10549	202.7	4.1E-06	<i>fliD</i>
<i>sph1809</i>	28	1119	39.6	5.1E-08	<i>fliJ</i>
<i>sph1810</i>	35	487	14.1	7.0E-01	<i>fliI</i>
<i>sph1811</i>	28	341	12.1	2.1E-05	<i>fliH</i>
<i>sph1812</i>	34	572	16.6	1.6E-04	<i>fliG</i>
<i>sph1813</i>	38	531	14	5.5E-04	<i>fliF</i>
<i>sph1814</i>	87	2563	29.4	3.4E-10	<i>fliE</i>
<i>sph1816</i>	27	408	15.2	3.0E-04	<i>fliM</i>
<i>sph1817</i>	37	1149	30.8	5.1E-07	<i>fliN</i>
<i>sph1818</i>	57	595	10.4	1.4E-08	<i>fliP</i>
<i>sph1819</i>	40	150	3.7	2.8E-04	<i>fliQ</i>
<i>sph1820</i>	31	172	5.5	1.1E-05	<i>fliR</i>
<i>sph1821</i>	39	255	6.6	7.5E-01	<i>flhB</i>
<i>sph1822</i>	28	301	10.8	7.9E-01	<i>flhA</i>
<i>sph1824</i>	475	44317	93.2	3.2E-08	<i>p6</i>
<i>sph2312</i>	31	3010	95.6	1.7E-07	<i>mcp</i>
<i>sph2313</i>	78	5868	75.4	3.5E-04	<i>mcp</i>
<i>sph2504</i>	255	2059	8.1	1.2E-06	<i>mcp</i>
<i>sph2859</i>	41	1557	37.6	7.3E-08	<i>mcp</i>
<i>sph2942</i>	168	5861	34.9	8.9E-06	<i>mcp</i>
<i>sph3649</i>	62	4762	77.1	5.1E-08	<i>cheR</i>
<i>sph3658</i>	179	9875	55.1	9.7E-05	<i>cheW</i>
<i>sph3659</i>	529	32640	61.7	1.2E-09	<i>cheW</i>
<i>sph3660</i>	366	32698	89.3	8.5E-12	<i>cheY</i>
<i>sph3661</i>	193	25684	133	1.1E-07	<i>cheZ</i>
<i>sph3662</i>	99	8030	81.3	1.1E-05	<i>cheA</i>
<i>sph3741</i>	101	8119	80.7	2.6E-06	<i>mcp</i>

<i>sph3884</i>	46	563	12.3	2.0E-04	<i>flhB</i>
<i>sph3885</i>	163	2155	13.2	1.0E-06	<i>flhA</i>
<i>sph3886</i>	58	841	14.6	8.5E-03	<i>flhF</i>
<i>sph3887</i>	128	1943	14.5	1.8E-06	<i>flhG</i>
<i>sph3888</i>	134	1943	14.5	2.0E-08	<i>fliA</i>
<i>sph3889</i>	137	1534	11.2	4.9E-10	<i>motA</i>
<i>sph3931</i>	178	303	1.7	2.3E-01	<i>fliR</i>
<i>sph3932</i>	46	199	4.4	8.4E-07	<i>fliQ</i>
<i>sph3933</i>	129	377	2.9	6.2E-05	<i>fliP</i>
<i>sph3939</i>	152	5072	33.3	2.4E-09	<i>fliN</i>
<i>sph3940</i>	161	4283	26.6	5.8E-04	<i>fliM</i>
<i>sph3941</i>	253	5033	19.9	4.9E-07	<i>fliL</i>
<i>sph3942</i>	419	1822	4.4	1.8E-04	<i>fliK</i>
<i>sph3944</i>	213	1347	6.3	3.0E-10	<i>fliJ</i>
<i>sph3945</i>	48	670	14	5.7E-05	<i>fliI</i>
<i>sph3946</i>	31	549	17.6	2.9E-07	<i>fliH</i>
<i>sph3947</i>	70	1062	15.3	3.4E-08	<i>fliG</i>
<i>sph3948</i>	56	710	12.6	1.1E-06	<i>fliF</i>
<i>sph3951</i>	165	2331	14.2	2.0E-08	<i>fliE</i>

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(a)



(b)

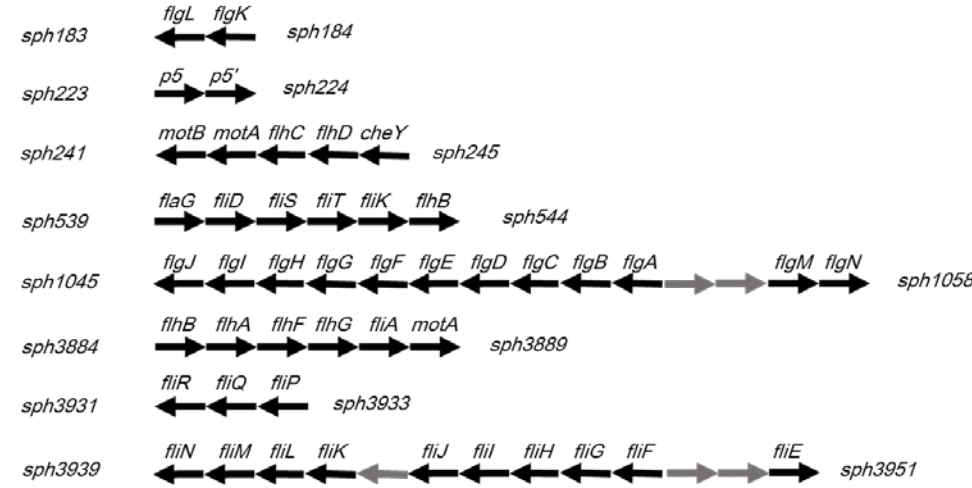


Fig. 1

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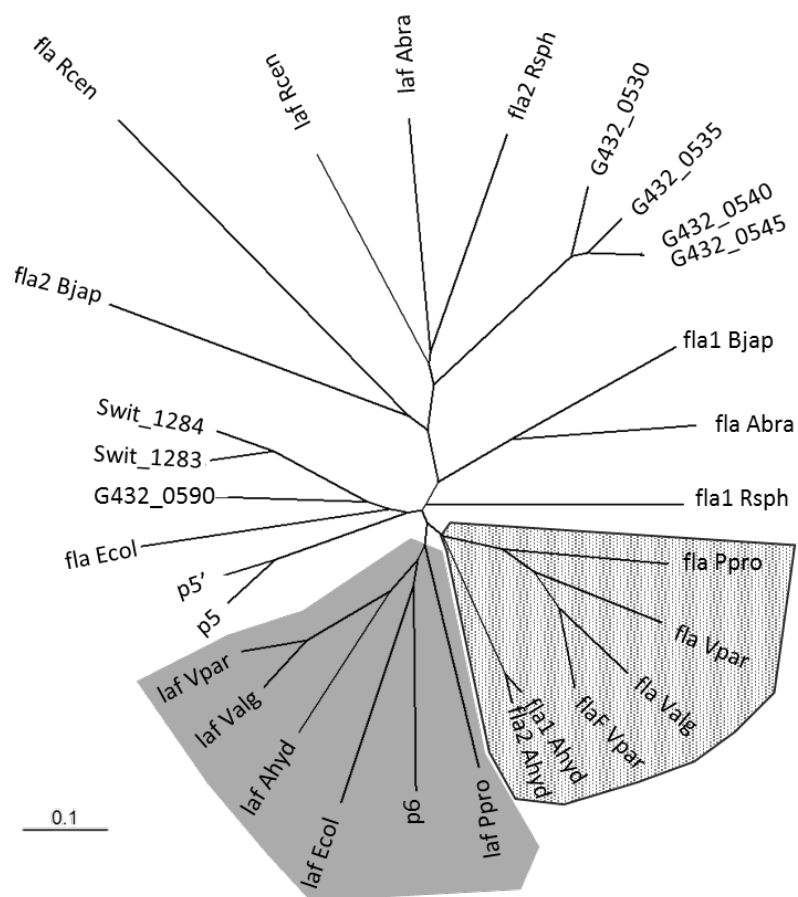


Fig. 2

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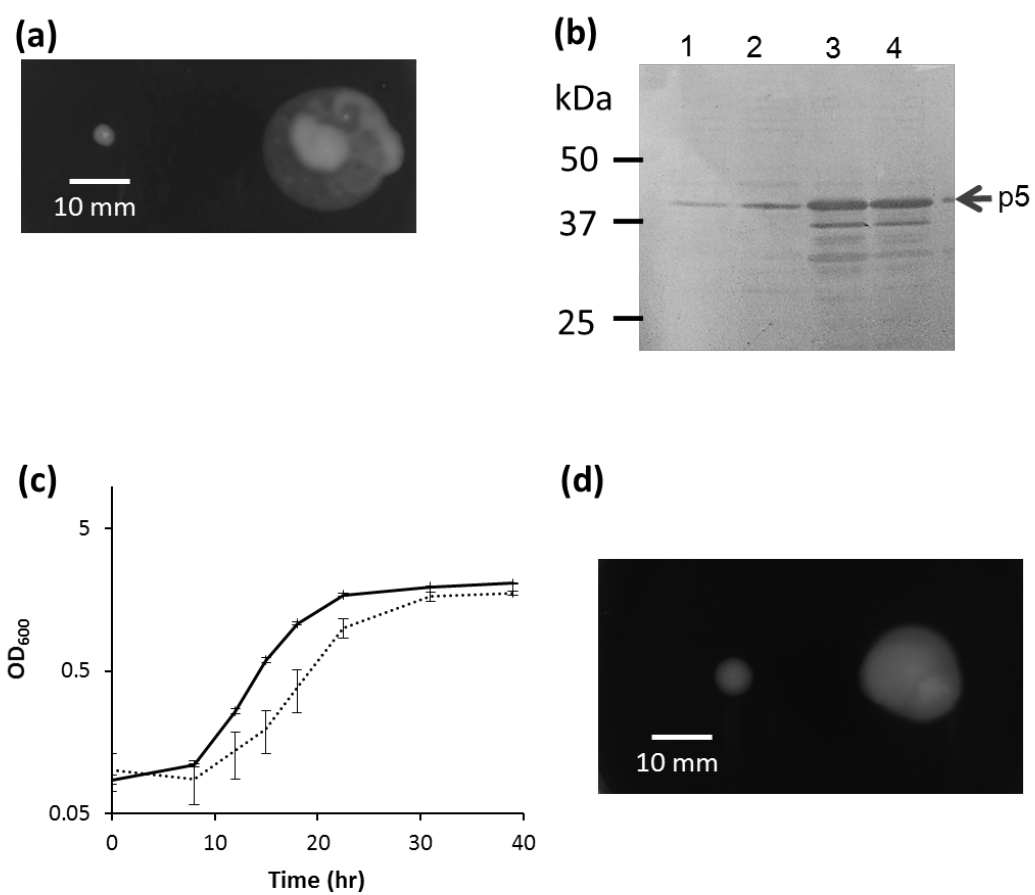


Fig. 3

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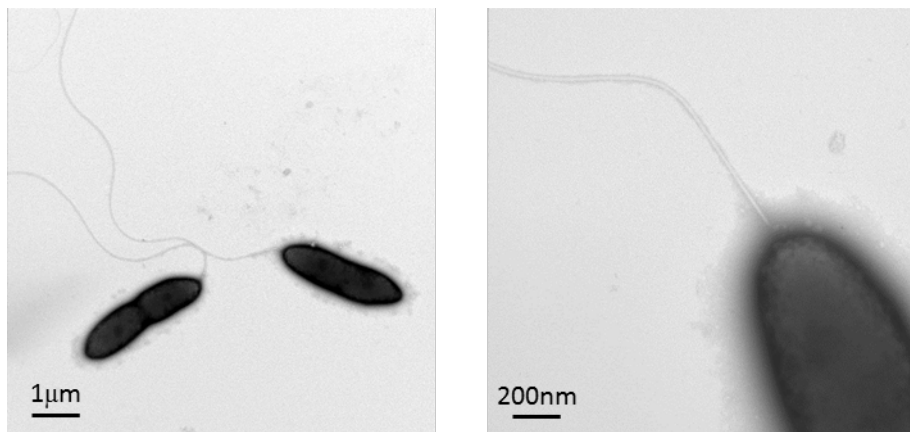


Fig. 4

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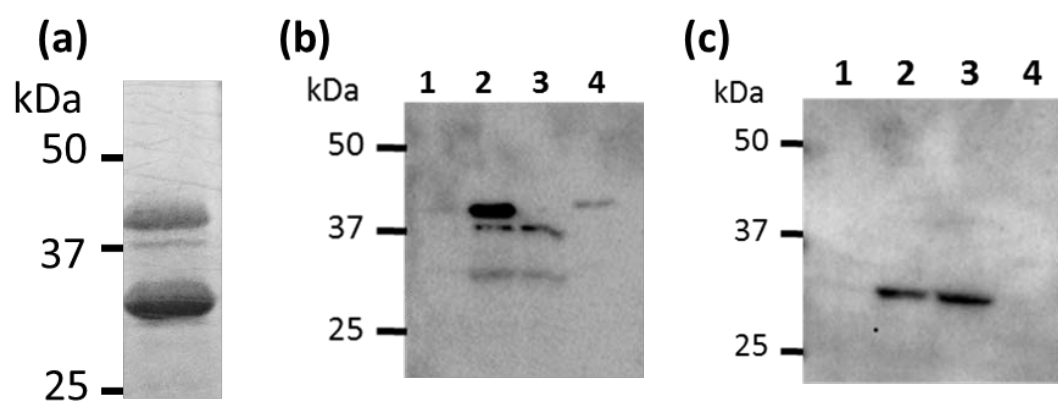


Fig. 5

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