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A new type of flagellin gene in *Pseudomonas putida*

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Previously established PCR amplification and Southern hybridization procedures were developed for the isolation of the 0.8-kb flagellin gene in *Pseudomonas putida.* The deduced protein sequence has significant homology to the N- and C-terminal sequences of other bacterial flagellins. We propose that *P. putida* flagellin genes can be divided at least into three size groups: type I (2.0 kb), type II (1.4 kb), and type III (0.8 kb). Type I and type II flagellin genes have been reported. The new 0.8-kb type III gene was expressed in *E. coli*, and the resulting protein was purified and used to raise polyclonal antibody to study whether this small gene encodes flagellin. The antiserum reacted with purified flagellin monomers from representatives of each flagellin type, as well as proteins of the same sizes in lysates of these organisms, on Western immunoblots. This antiserum was determined to be functional in a motility inhibition assay. Similar results were obtained from antiserum directed against purified type III flagellin, indicating that a new type of flagellin gene in *P. putida* has been found. Preliminary electron microscopic study revealed that *P. putida* isolate with the smaller flagellin gene type appeared to have a thinner flagellar filament.

Key Words——electron microscopy; flagellin gene; motility inhibition assay; polymerase chain reaction; *Pseudomonas putida*

Flagella are organelles responsible for motility and chemotaxis in many species of bacteria. Moreover, the importance of the flagellum as a potential virulence factor has been demonstrated for many pathogenic bacteria (Drake and Montie, 1988; McSweegan and Walker, 1986; Milton et al., 1996). Bacterial flagella consist mainly of three parts: a basal body, hook, and filament. The flagellar filament is composed of a single protein subunit called flagellin. DNA sequence analysis of flagellin genes from different genera of bacteria such as *Borrelia*, *Salmonella*, *Pseudomonas*, and *Shigella* demonstrated that the termini of flagellin proteins are conserved, whereas the central regions of the flagellin molecules are more variable. The conserved terminal and central variable sequences correlated with the basic structure of flagellin have been studied (Fedorov and Kostyukova, 1984; Namba et al., 1989; Vonderviszt et al., 1990). Flagellin folds into a hairpin conformation in which the conserved N- and C-termini lying inside are responsible for filament assembly, and the central domain, being surface exposed, is responsible for antigenic properties. These features have brought about several applications. PCR primers specific for N- and C-terminal conserved regions designed from a particular bacterial flagellin have been used to amplify flagellin genes from several related strains and species (Hales et al., 1998; Spangenberg et al., 1996; Tungpradabkul et al., 1998; Winstanley et al., 1994, 1996). Flagellin sequence comparison can be applied to phylogenetic studies (DeShazer et al., 1997; Fukunaga and Koreki, 1996). Furthermore, PCR/RFLP analysis of amplified flagellin genes allowed the typing of *Burkholderia cepacia* (Hales et al., 1998) and *Pseudomonas aeruginosa* strains (Winstanley et al., 1996). Flagellin genes have also been used as the specific targets for PCR detection or identification of specific bacteria (Oyofo and Rollins, 1993; Way et al., 1993). Besides accounting for antigenic diversity, the length and amino acid sequences of the central variable regions determine the flagellin molecular weights. Characterized wild-type eubacterial flagellin molecules have been reported to have M_r ranging from 29 to 69 kDa (Gill and Agabian, 1983; Lawn, 1977). Flagellin molecular masses are conserved within certain groups but those

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of some strains, even in the same species, are variable. In *Pseudomonas putida*, two flagellin genes with different sizes have been cloned and sequenced (Tungpradabkul et al., 1998; Winstanley et al., 1994). In this study, the PCR-based method for isolation of flagellin genes as previously described (Tungpradabkul et al., 1998) was applied to amplify a new type of *P. putida* flagellin gene. The cloned gene was analyzed for the properties of the expressed protein, and the functional activity of antibody raised against this protein was examined. Electron microscopy was used to visualize the width of flagellar filaments.

Materials and Methods

Bacterial strains and plasmids. *P. putida* DMS 0638, DMS 2704, DMS 3052, and DMS 3056 were obtained from National Institutes of Health, Nonthaburi, Thailand. *E. coli* JM109 (New England Biolabs, MA, USA) was used as a transformation recipient for recombinant plasmids. pUC19 (New England Biolabs) was used for cloning and sequencing. Plasmid pMal-c2 (New England Biolabs) was used for the construction of the fusion protein in *E. coli.*

PCR and Southern hybridization. Chromosomal DNA was isolated from *P. putida* by QIAGEN Genomic tip-100 (Qiagen, Hilden, Germany). Oligonucleotide primers OFA1 (5′-ATGGCCTTGACCGTCAACACCAACAT-3′) and OFA2 (5′-CAGAACCGACTGCGGCAGCTGTT-3′), obtained from BioService unit (Bangkok, Thailand), were used to amplify flagellin genes from *P. putida* genomic DNA. Amplifications were carried out in a DNA thermal cycler 480 (Perkin Elmer-Cetus, CA, USA), following published data (Tungpradabkul et al., 1998). Briefly, reaction mixtures contained 200 μM dNTPs, 0.4 μM of each primer, 1 U of Vent DNA polymerase (New England Biolabs), 1× PCR buffer (supplied with the enzyme), and 200 ng of DNA template in a 50-μl reaction volume. PCR amplification was preceded by an initial denaturation step at 100°C for 10 min. Each cycle consisted of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C (the last cycle, 72°C 5 min). After performing a total of 30 cycles, amplified products were separated on a 1% agarose gel. Hybridization was performed nonradioactively with the DIG-DNA labeling and detection kit (Boehringer, Mannheim, Germany) under high-stringency conditions, as previously described (Tungpradabkul et al., 1998).

DNA sequencing and computer analysis. Double-stranded DNA sequencing of both strands of pUC19-based clones and subclones was carried out by the dideoxy-chain-termination method of Sanger et al. (1977), using DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, CA, USA) on an automated DNA sequencer (Applied Biosystems, model 377). Identification of restriction sites, nucleotide sequence alignments, determination of amino acid composition, predicted protein molecular mass, and alignments of predicted flagellin protein from the experiment result with other flagellins (retrieved from GenBank) were carried out by using the PC/Gene Software Package (release 6.8, IntelliGenetics) or CLUSTAL W program (Thompson et al., 1994). For searches of related sequences, the BLAST program (Altschul et al., 1990) was used.

Construction of expression plasmid. A 831-bp blunt-ended fragment obtained by PCR amplification of the DNA of isolate DMS 3052 was inserted into the *Xmn*I site of vector pMal-c2 in *E. coli* to create pMPT3. Correct orientation was verified by restriction analysis. The insert DNA was located downstream of, and in frame with, the vector *malE* gene (*malE* encodes maltose-binding protein [MBP] and is under the control of an inducible *tac* promoter).

The expression of fusion protein in E. coli. An *E. coli* cell harboring the plasmid pMPT3 was used to generate the flagellin protein, EPT3. Overexpression and purification of the protein were performed according to the New England Biolabs protocol. Briefly, recombinant *E. coli* was grown and induced with isopropyl-β-D-thiogalactoside (IPTG), and the cells were harvested and frozen overnight at −20°C. After thawing, they were ruptured with a French pressure cell and the cell debris was removed by centrifugation. The supernatant solution was dialyzed against the column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4) and applied to an amylose resin column. The proteins were eluted with maltose-containing buffer, and the eluate was then concentrated. The expressed flagellin protein, EPT3, was then cleaved from the fusion protein by digestion with factor Xa protease overnight at 23°C. After separation by SDS-PAGE on a 10% polyacrylamide gel, all bands were visualized by soaking in cold 0.25 M KCl (Vaitukaitis, 1981). Blocks of the gel corresponding to the cleavage product was excised and homogenized to prepare it for injection.

Purification of flagellin. *P. putida* were grown in 1 L of Luria-Bertani (LB) broth overnight at 37°C. The cells were harvested by centrifugation at 7,000×*g* at 4°C for 20 min. Pellets were resuspended in 200 ml of 50 mM sodium phosphate buffer (pH 7.0) and blended in a Waring commercial blender for 1.5 min. Cells were removed by centrifugation at 12,000×*g* at 4°C for 20 min. The flagella were collected from the supernatant by centrifugation at 100,000×*g* for 1 h. Flagellin was purified by modification of the sequential acid pH disassociation, ultracentrifugation, and neutral pH reassociation procedure previously described (Brett et

al., 1994). The pellet containing flagella was resuspended in 3 ml of sodium phosphate buffer, adjusted to pH 3.0 with 2.0 M citric acid, and stirred at 0°C for 10 min. Insoluble material at pH 3 was removed by centrifugation at 100,000×*g* for 1 h, and the supernatant was brought to pH 7.0 by the addition of 5.0 M NaOH and dialyzed overnight against the 50 mM sodium phosphate buffer (pH 7.0).

Antibody production. A rabbit was immunized with 100 μg of *P. putida* DMS 3052 flagellin protein in complete Freund adjuvant (Sigma, St. Louis, MO, USA) by footpad injection. Antibody to expressed flagellin, EPT3, was prepared by an administration of gel homogenate, described above. The rabbit received a subcutaneous injection of 0.5 ml of the homogenate, without adjuvant, containing 100 μg of EPT3. The rabbits were boosted with the same preparation at 1, 2, 3, and 4 weeks, then bled at 5 weeks. These antisera were adsorbed with a lysate of *E. coli* containing pMal-c2 vector before use in the immunologic assays. Preimmune sera were taken and used as control serum.

SDS-PAGE and immunoblotting. SDS-PAGE was performed with a minigel apparatus (Bio-Rad, CA, USA) by the method of Laemmli (1970). Proteins were stacked in 5% acrylamide and separated in 10% acrylamide. Gels were run at a constant voltage of 100 V. Proteins were visualized by staining with Coomassie blue. Electrophoretic transfers were performed by the technique of Towbin et al. (1979). After blocking unbound sites for 2 h with PBS containing 5% skim milk, the membrane was incubated overnight with rabbit antiserum at a final dilution of 1 : 400. Bound antibodies were visualized by an alkaline phosphatase-conjugated goat antirabbit antibody (dilution 1 : 1,000; Sigma).

Motility inhibition assay. The motility inhibition assay was carried out according to a previous report (Brett et al., 1994). The wells in row A of a 24-well microtiter plate filled with agar were added with preimmune serum; row B contained anti-EPT3 antiserum from the same rabbit. Row C was added with preimmune serum, and the wells in row D contained anti-DMS 3052 flagellin antiserum. Rabbit antisera were diluted into 0, 1 : 25, 1 : 50, 1 : 100, 1 : 500, and 1 : 1,000. Each well was inoculated with *P. putida* DMS 3052, then incubated at 37°C for 24 h. Cell motility was examined by its ability to spread beyond the point of inoculation. *Burkholderia mallei*, nonmotile bacteria, was used to observe a growth phenomenon.

Electron microscopy. Bacteria were grown overnight at room temperature in LB broth. Cells were pelleted and resuspended in PBS buffer, and a drop of the suspension was directly applied to Formvar-coated carbon grids. The liquid was carefully removed with a filter paper. Subsequently, the samples were stained with 1% uranyl acetate for 10 min. The liquid was again carefully removed. The bacteria were observed under a Hitachi electron microscope (model H-7000, Japan).

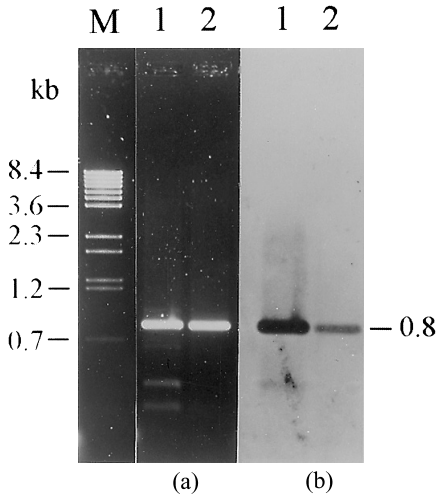

Fig. 1. Analysis by agarose gel electrophoresis (a) and by Southern hybridization of PCR-amplified products (b) from *P. putida* DMS 2704 (lane 1) and DMS 3052 (lane 2).
Lane M is *Hin*dIII-digested λ DNA.

Accession number. The nucleotide sequences reported in this paper have been submitted to GenBank and given the following accession numbers: AF077600 (DMS 2704) and AF077601 (DMS 3052).

Results

PCR and hybridization

Previous work from our laboratory demonstrated that oligonucleotide primers specific for N-terminal (OFA1) and C-terminal (OFA2) conserved regions can be used to amplify flagellin genes from different species of *Pseudomonas* (Tungpradabkul et al., 1998), including two *P. putida* isolates. With use of the same primers and the DIG-labeled *P. aeruginosa* ATCC 27853 flagellin gene probe (Tungpradabkul et al., 1998), a 0.8-kb fragment present in two other isolates of *P. putida* was thought to be the flagellin gene and was chosen for further analysis (Fig. 1). When equal volumes of PCR product were applied to an agarose gel, more intensity of hybridization signal was observed with the sample from DMS 2704 than that from DMS 3052 (Fig. 1b).

DNA sequence analysis of flagellin genes

The two 0.8-kb fragments were cloned and sequenced. Complete nucleotide sequences of 834 and 831 bp were obtained from DMS 2704 and DMS 3052, respectively. The DNA sequences were examined for

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DMS 0638    MALTVNTNITSLGVQKNLNRASDALGTSMSRLSSGLKINSAKDDAAGLQI    50
PaW8        MALTVNTNITSMSVQKNLNKSSDALGTTMGRLSSGLKINSAKDDAAGLQI    50
DMS 3056    MALTVNTNIASITTQGNLTKASNAQTTSMQRLSSGLRINSAKDDAAGLQI    50
PRS2000     MALTVNTNIASITTQGNLTKASNAQTTSMQRLSSGLRINSAKDDAAGLQI    50
DMS 2704    MALTVNTNITSLGVQKNLNRASEALSTSMTRLSSGLKINSAKDDAAGLQI    50
DMS 3052    MALTVNTNITSLGVQKNLNRASDALSTSMTRLSSGLKINSAKDDAAGLQI    50
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DMS 0638    SNRLTSQISGLNVAVKNANDGISIAQTAEGAMQASTNILQRMRELALQSA   100
PaW8        SNRLTTQIKGLSVAVKNANDGISIAQTAEGAMATSGNIMQRMRELALQSA   100
DMS 3056    ANRLTSQINGLGQAVKNANDGISIAQTAEGAMQASTDILQKMRTLALSSA   100
PRS2000     ANRLTSQINGLGQAVKNANDGISIAQTAEGAMQASTDILQKMRTLALCSA   100
DMS 2704    ATRMTSQIRGQTMAIKNANDGISIAQTAEGAMQEQTNILQRMRELAVQSR   100
DMS 3052    ATRMTSQIRGQTMAIKNANDGISIAQTAEGAMQEQTNILQRMRELAIQSR   100
            ..*.*.**.* . *.*****************.. ..*.*.**.**. *

DMS 0638    NGSNSDEDRTSLQQEFTALSGELTRISSTTTFGGRNLLDGTFTSTSFQVG   150
PaW8        NGSNSDDDRASMQQEFTALSGELTRIANTTTFGGRNLLDGTFSGSSFQVG   150
DMS 3056    TGSLSAEDRKSNNDEYQALTAELNRISDTTTFGGQKLLDGSYGTKAIQVG   150
PRS2000     TGSLSNADRKSNNDEYQALTAELNRICQTTTFGARSCWTVRTAPRPSRSA   150
DMS 2704    NDSNSTNDRVALDKEFQSMASELTRIANSTQLNGKNLLDGSASVMTFQVG   150
DMS 3052    NSSNSKEDRDALNTEFNAMSDELTRIAESTQLNGKNLLNASAT-MTFQVG   149
            ..* * .** . . *. ....**.**  .* .....  .   .  . . .

DMS 0638    ANANETISFGMKSVSASELKGTYNEASVDAGTNKLSAVVTGKAGVITSNA   200
PaW8        ANSNESISFGMKDVSATSMKGNYNEASVAGGVATLQASVTGAAGKFGTNN   200
DMS 3056    ANANETINLTLDNVSAKSI----------------------------G   170
PRS2000     PTPTKPSTWRWTTFRPRA-------------------------------S   169
DMS 2704    SNT-----------------------------------------------   153
DMS 3052    SNS-----------------------------------------------   152
            ...

DMS 0638    NDVSSAKFVAQ------EKTLGAAGAGKITLG----TTDVDLAATDNLDS   240
PaW8        AGSTSASVVGTAGAGVFDKPTIGAAAGNLVLNVGTTTTTIAAAAGDTLQD   250
DMS 3056    SQQIKSLAVTASPTGL--------EANTITVTGNGQNTDVAIKAGDSAKA   212
PRS2000     ARSRSSRALSRLAQRV--------AAADLVVTGNGQSKTVSYDAGSSAKD   211
DMS 2704    ----------------------------------GSANQITIDLSAKFT-   168
DMS 3052    ----------------------------------GAENQIDIKLID-LK-   166
                                                .. ..    .

DMS 0638    IVTKIN-AAGVAGITASNQG--GVLRLNNTGA-DMALAGDAGTLTAIGLT   286
PaW8        VVDNINLETSNTGVTASIDSATGALKLDGTQAFTIDASTDDVLSTALGLA   300
DMS 3056    VAAS--LNGAIGGLTAT---ASTEVKF-----------------------   234
PRS2000     IAAK--LNGSIGGLTAS---ASTEVKL-----------------------   233
DMS 2704    -----------------------------------------------   168
DMS 3052    -----------------------------------------------   166

DMS 0638    AGATAIGGSANTDGTVQMGVQAIGAAGDLVIGSSAIRLAATDTLEDVVNK   336
PaW8        EAGGAQLSKTGT-ANLRDGVLGAGGAGNLTLGSTNIALVATDTLSSVVGK   349
DMS 3056    AVDTTKFTTTDPSANF-----------SMTVGGQTVEFVGVTDTASLADQ   273
PRS2000     AVASG---AAATPANF-----------DLTVGGTTVSFIGVTDTASLADQ   269
DMS 2704    -------------------------ASDLGVTS-SINIQGSDTTTA----   188
DMS 3052    -------------------------ADALSVDSDAIAISGADDAEN----   187
                                    .. ... .. . . .. ..

DMS 0638    VNQYSSSTGVTAKIADNGDGTGSLSLSSKTDFTIGQATSTAIATAFGYNT   386
PaW8        VNAQTGTTGVTASI---DSATGQLKLNSAAGFDVGGTAGTLTGLGL---T   393
DMS 3056    LKSNAAKLGISVNYDESKG--GSLSIKSDT---------------------   301
PRS2000     LKSNAAKLGISVNYDESTK---SLSVKSDT---------------------   296
DMS 2704    ----------------------------EANFS-----------------   193
DMS 3052    ----------------------------KTNFE-----------------   192

DMS 0638    AAADKAVTTTTGLTSDASIKVNGQSFSFKAGDTLSSIVASINDGAGAAGS   436
PaW8        AGSVAIAPQTTGLASAASIDINGTTFNFAQGDDLDAIVDNINNN-GAGAV   442
DMS 3056    ----------------------GENIEFSAAD-------------------   311
PRS2000     ----------------------GENLAFTS---------------------   304
DMS 2704    -----------------------------------------------   193
DMS 3052    -----------------------------------------------   192

DMS 0638    GAGTKATGVTASASADGRLILTSADGKDIKLENNSA----GALDTLGLTS   482
PaW8        GSGTALTGVTAK-NDNGRLVLTSANGQDIKLDNGSGVTGQGALAAVGLNS   491
DMS 3056    ------------------------------------KGSVDAL----   318
PRS2000     -------------------------------------KAGADAI----   311
DMS 2704    -----------------------------------------------   193
DMS 3052    -----------------------------------------------   192
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DMS 0638    GNTKAKLTEATSITVNNVEVKFKKDDDMAAIAAAINSSSSGVTASV---N   529
PaW8        GTTKAGLVADTSISLNGVEVKFKKGDDMDSIAASINAASTGVNASVVVNA   541
DMS 3056    -----------TMNVKGGDGKY--GTAAIKMQEADATATPPVTGKTVV--   353
PRS2000     -----------SVGARDGNGDF--PGTLTTL-------SATVNDKSTV--   339
DMS 2704    --------------------------------------------------   193
DMS 3052    --------------------------------------------------   192

DMS 0638    DDGTLGLFADQNIVIADGSNGTGLAALGLSAG---TTNAVTMNTSVNDLS   576
PaW8        GSSTLSLFADQDITVADGSNGTGLARRAGSDCCCRQTSALEMESTVSNLN   591
DMS 3056    -TGAISLDSAKGYSL----TGAGVTGLFDTG-----ASVTSQKTSISETN   393
PRS2000     -TGQISLDSAKGYSVANGATGTGATDLFGA------ASKSSAKTTIADTD   382
DMS 2704    --------------------------------------------------   193
DMS 3052    --------------------------------------------------   192

DMS 0638    ILDAASAQQAILALDGAIQQVDSQRSQLGAVQNRFDSTVDNLQSIGENST   626
PaW8        ITDAQSAQQAIQVLDGAMQSLDSQRSQLGAVQNRFDSTVANLQSISENST   641
DMS 3056    VTDATTAQNALAVIDKAIGSIDSVRSGLGATQNRLQTTVDNLQNIQKNST   443
PRS2000     VTEAVNAQNALAVIDKAIGSIDSVRSGLGATQNRLQTTVDNLQNIQKNST   432
DMS 2704    --------AAVSAIDAALQTINSNRADLGAAQNRLTSTINNLQNINENAE   235
DMS 3052    --------KALSAIDDALQSINTTRADLGAAQNRLTSTINNLQNINENAE   234
                    *. ..* *.  ... *. ***.***. .*..***.* .*..

DMS 0638    AARSRVQDADFASETAELTKQQTLQQASTAILSQANQLPQSVL----   669
PaW8        AARSRIQDADFAAETAELSKQQTLQQASTAILSQANQLPSSVLKLLG   688
DMS 3056    AARSTVQDVDFASETAELTKQQTLQQASTAILSQANQLPQSVL----   486
PRS2000     AARSTVQDVDFASETAELTKQQTLQQASTAILLQANQLPSSVLKLLQ   479
DMS 2704    AARGRVQDTDFAAETAQLTKQQTLQQASTSVLAQANQLPQSVL----   278
DMS 3052    AARGRVQDTDFAAETAQLTKQQTLQQASTSVLAQANQLPQSVL----   277
            ***. .**.***.***.*.**********..* *******.***
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Fig. 2. Comparison of established *P. putida* flagellin protein sequences with the new flagellins DMS 2704 and DMS 3052. The identical amino acids are marked with an asterisk, and similar amino acids are indicated by dots. Underlines represent the primer sequences.

similarity to sequences reported previously in a nonredundant nucleotide sequences data bank by using the NCBI BLAST program (Altschul et al., 1990). The BLAST search results demonstrated that the two sequences showed similarities with those of hundreds of flagellin genes present in many bacteria. Because of the primer design, neither sequence contains 12 bp at the carboxy-terminal end of the flagellin-coding sequence.

A comparison of all published *P. putida* flagellins (Tungpradabkul et al., 1998; Winstanley et al., 1994) with the deduced protein product sequences of the two new flagellin genes was performed by using the CLUSTAL W program (Thompson et al., 1994). This alignment indicated that the N- and C-terminal regions of *P. putida* flagellins were highly similar. However, the amino acid sequences and the length of the middle portions were quite different (Fig. 2). The amino acid compositions of the deduced proteins were typical of other flagellin components, which lack cysteine and contain few or no histidine, tryptophan, or tyrosine (no residues in either flagellin). Taken together, these data confirmed that the 0.8-kb genes encoding the flagellins of *P. putida* were cloned. Two distinct flagellin gene sizes have been previously reported in *P. putida* (Tungpradabkul et al., 1998; Winstanley et al., 1994), which are 2.0 and 1.4 kb.

From these data, we propose that the flagellin genes from *P. putida* strains can be separated into three size groups. The first group has a gene size of 2.0 kb, and we are designating this group as type I flagellin gene. The flagellin gene size of the second group is 1.4 kb, and we are designating this group as type II flagellin gene. The third group is designated as type III flagellin gene with the newly found 0.8-kb gene. The predicted protein sequences from each type of *P. putida* flagellin genes have been compared (data not shown). DMS 0638 flagellin with accession number AF034767 (Tungpradabkul et al., 1998) shows 61% sequence identity with the PaW8 flagellin with accession number L15366 (Winstanley et al., 1994). DMS 3056 flagellin with accession number AF034766 (Tungpradabkul et al., 1998) shares 70% identity to the PRS2000 flagellin with accession number L15367 (Winstanley et al., 1994). A comparison of the deduced protein sequences from the smaller amplified products obtained from DMS 2704 DMS 3052 exhibits an identity of 83%. It is interesting that although DMS 2704 flagellin gene hybridized much stronger to the probe than DMS 3052 flagellin gene did, its nucleotide sequence homology with the probe (75%) was not much higher than that of the latter (73%) (data not shown).

Flagellin protein variation in P. putida

Flagellin proteins from *P. putida* DMS 0638, DMS 3056, and DMS 3052 were isolated and analyzed by SDS-PAGE. Their molecular masses have been found

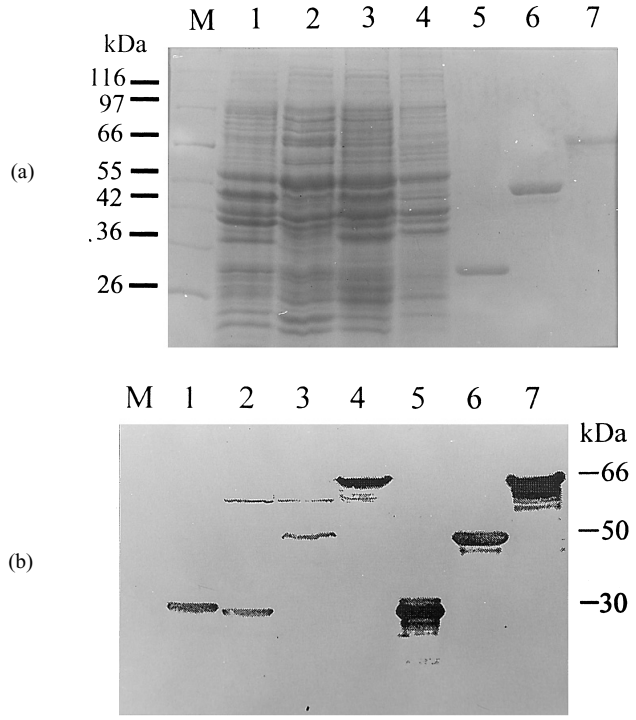

Fig. 3. SDS-PAGE (a) and Western blot analysis (b) of cell lysates and purified flagellin proteins from *P. putida* isolates.

The samples were electrophoresed on SDS-PAGE and immunoblotted with anti-EPT3 antiserum. Lanes: M, protein markers; 1, DMS 2704; 2, DMS 3052; 3, DMS 3056; 4, DMS 0638; 5, purified flagellin from DMS 3052; 6, purified flagellin from DMS 3056; 7, purified flagellin from DMS 0638.

to vary as follows: 66, 50, and 30 kDa, respectively (Fig. 3a). These sizes of flagellins are similar to the estimated molecular weight that correspond to each type of flagellin gene.

Detection of flagellins by Western immunoblot

The 831-bp flagellin gene from DMS 3052 was chosen for the synthesis of recombinant protein for antigenic analysis and functional analysis. Because of the lack of C-terminal sequence, *E. coli* JM109 harboring the plasmid pMPT3 produces a flagellin protein (EPT3) that is not quite complete. However, a protein band corresponding to the expected fusion protein size was synthesized (data not shown). After factor Xa cleavage, the flagellin band cut from a SDS-polyacrylamide gel was used to prepare anti-EPT3 anti-serum. Antibody to 30-kDa purified flagellin from DMS 3052 was also prepared in rabbit to use in control experiments.

To determine whether the 0.8-kb flagellin gene is expressed in *P. putida* DMS 3052, we tested these two antisera on Western immunoblots containing whole-cell lysates of DMS 3052. We also tested whether lysates of *P. putida* DMS 0638, DMS 2704, and DMS 3056 reacted with those antibodies. Anti-

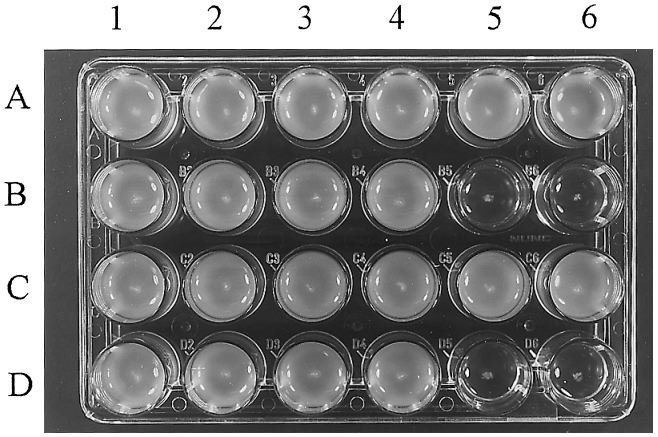

Fig. 4. Motility inhibition assay of *P. putida* DMS 3052 reacted with various dilutions of anti-EPT3 antiserum (row B) and anti-DMS 3052 flagellin antiserum (row D).

Respective preimmune antisera were added to rows A and C. Wells: 1, TE control; 2, 1 : 1,000 dilution; 3, 1 : 500 dilution; 4, 1 : 100 dilution; 5, 1 : 50 dilution; 6, 1 : 25 dilution.

serum raised against EPT3 reacted with a 30-kDa band of whole-cell lysates of DMS 3052 (Fig. 3b, lane 2). As expected, it also reacted with the purified flagellin from this isolate (Fig. 3b, lane 5). The 30-kDa band was also present in DMS 2704 (Fig. 3b, lane 1). The molecular mass of 30 kDa was similar to the mass predicted for the flagellin gene products of DMS 2704 and DMS 3052. Similar results were obtained with anti-DMS 3052 flagellin antiserum (data not shown). These data suggest that the 0.8-kb flagellin genes are expressed in *P. putida* DMS 2704 and DMS 3052.

The EPT3 antiserum also reacted with a 50- and 66-kDa protein from lysates of *P. putida* DMS 3056 (Fig. 3b, lane 3) and DMS 0638 (Fig. 3b, lane 4), respectively, and with their respective purified flagellin subunits (Fig. 3b, lanes 6 and 7). The antiserum against purified DMS 3052 flagellin also reacted with these bands of DMS 3056 and DMS 0638 (data not shown). These results demonstrate that two antisera are cross-reacting with other isolates of *P. putida* and are antigenically similar. Bands having M_r around 59 kDa were considered to be nonspecific because they were also present with each preimmune antiserum (data not shown).

Motility inhibition assay

A motility inhibition assay was performed to determine the functional activity of both anti-EPT3 and anti-DMS 3052 flagellin antisera. *P. putida* DMS 3052 was inoculated with various dilutions of antisera. Incubation of the cell with the preimmune antisera did not inhibit motility. In contrast, both antisera at a dilution of 1 : 50 or less inhibited the cell motility of *P. putida* DMS

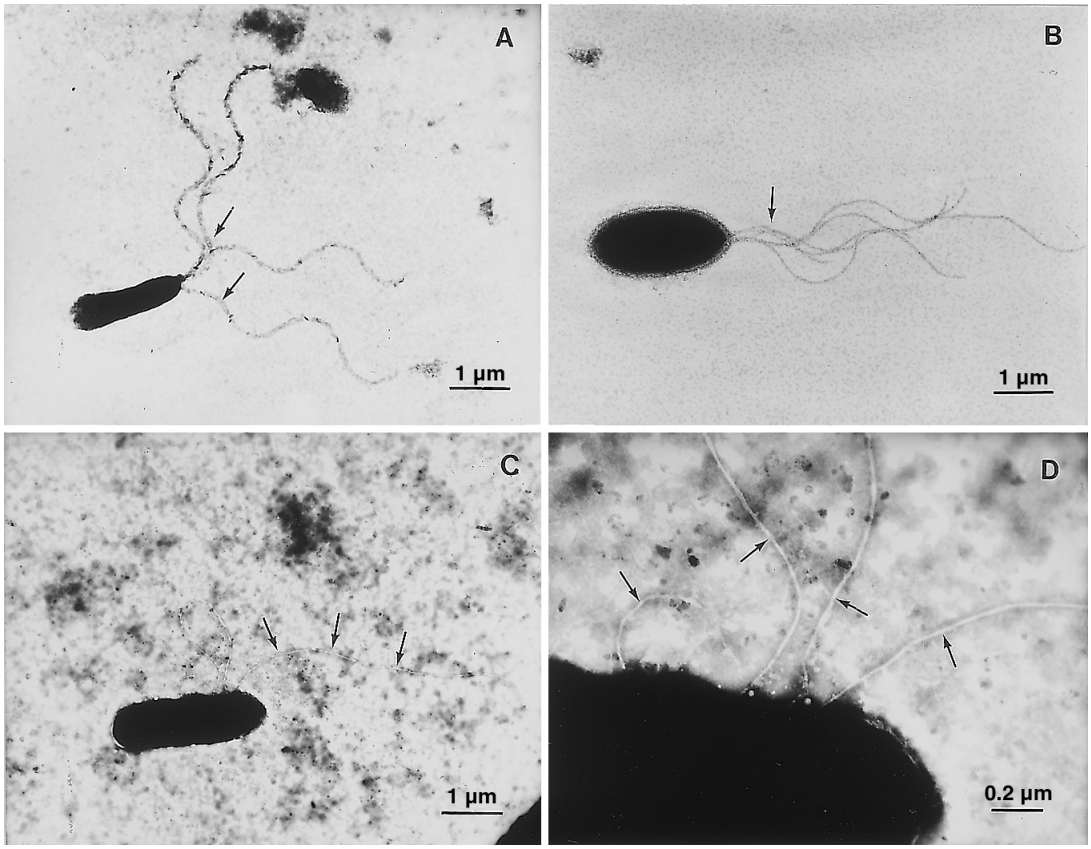

Fig. 5. Electron microscopic comparison of flagellar filament from three isolates of *P. putida.*
A, DMS 0638 (type I); B, DMS 3056 (type II); C and D, DMS 3052 (type III) and a micrometer bar (µm) are shown at a corner of each picture.

3052 (Fig. 4). Therefore these antisera were shown to be functional.

Electron microscopy

Structural analysis of *P. putida* flagellar filaments was done by electron microscopy. Although filament width measurement was not performed, variations in width of filaments among *P. putida* isolates were readily observed. Electron micrographs indicated that all filaments of the tufted polar flagella of isolate with the smaller flagellin gene type (type III, DMS 3052) appear to be thinner (Fig. 5C, D). The widest filaments were observed in DMS 0638 (Fig. 5A).

Discussion

Computer-assisted analysis revealed that deduced proteins of the 0.8-kb genes from *P. putida* DMS 2704 and DMS 3052 exhibit similarity to the sequences of other bacterial flagellins and have the common features of flagellin proteins. Consensus regions within the N- and C-terminal conserved domains were observed as previously described in *Pseudomonas* spp. flagellins (Tungpradabkul et al., 1998), which are RLSSGL-INSAKDDAAGLQI (N-terminal position 31 to 50), KNANDGISIAQTAEGAM (N-terminal position 66 to 82), KQQTLQQAST (C-terminal position −24 to −15), and QANQLP (C-terminal position −10 to −5). When flagellin protein sequences of different bacterial species are aligned (data not shown), these consensus sequences are apparently characteristic of *P. putida* flagellin gene.

Almost the entire structural flagellin genes (lacking the 12 C-terminal bp) were obtained from two *P. putida* isolates. Since there is no significant variation in length and repeated sequences in the conserved C-terminal end of flagellin, a smaller *P. putida* flagellin gene is unlikely to be amplified from internal sequences of the gene. From data reported previously (Tungpradabkul et al., 1998; Winstanley et al., 1994), and observed in this study, we propose the presence of three flagellin gene types of *P. putida.* Distinct flagellin gene sizes in closely related *Pseudomonas aeruginosa* (Winstanley et al., 1996) and *Burkholderia cepacia* (formerly *Pseudomonas cepacia*) (Hales et al., 1998) have been reported. Despite having the same gene sizes, *P. aeruginosa* type a flagellin sizes are variable, which may be due to posttranslational

modification (Winstanley et al., 1996). Posttranslational modifications of flagellins, which have been observed in several bacterial species (Kelly-Wintenberg et al., 1990; Logan et al., 1989; Wieland et al., 1985), not only may generate antigenic variation, but also could result in changes in molecular mass. Therefore the similarity between predicted and observed molecular masses for *P. putida* DMS 0638, DMS 2704, DMS 3052, and DMS 3056 suggests that posttranslational modification does not play a significant role in the flagellins of these isolates. This was not so with flagellin from *P. putida* PaW8, in which posttranslational modification may be causing the size discrepancy between deduced (68 kDa) and apparent molecular mass (81 kDa), estimated by SDS-PAGE (Winstanley et al., 1994).

Besides the presence of the distinct types of flagellin genes among *P. putida* strains, comparison data revealed sequence heterogeneity in each type of flagellin gene. It is reasonable, therefore, that size variations of type I flagellins and different migration rates of type III flagellin molecules in SDS-PAGE gel were found. The 30-kDa flagellins of *P. putida* DMS 2704 and DMS 3052 are the smallest of all reported *Pseudomonas* spp. flagellins, previously shown to range from 42–53 kDa (Allison et al., 1985; Winstanley et al., 1994, 1996) and found with the unusual size of 81 kDa in *P. putida* PaW8 (Winstanley et al., 1994). However, small functional flagellins are found in other wild-type eubacteria e.g., 29 kDa for *Caulobacter crescentus* (Gill and Agabian, 1983) and 32 kDa for *Bacillus thuringiensis* (Lovgren et al., 1993).

In some groups of bacteria, central domain sequences and length are somewhat conserved (Wilson and Beveridge, 1993), indicating that some constraints exist and that these regions serve important functions. There have been reports that some bacteria inhabiting the mammalian gastrointestinal tract have large flagellins (Nuijten et al., 1990; Wei and Joys, 1985; Whitfield et al., 1988), which suggests that filaments with complex surfaces may enhance bacterial ability to move through mucus or bind to host tissues (Wilson and Beveridge, 1993). However, very little is known about any correlation between bacterial flagellin size and its source. Because of the use of a limited number of isolates, the distinct types of flagellin genes in *P. putida* from the data presented here cannot be used to determine a relationship to the bacterial source. Further study with a larger number of isolates is required to make any proposals.

Anti-EPT3 polyclonal antiserum cross-reacted with purified flagellins of DMS 0638 and DMS 3056 in Western immunoblot analysis and also reacted with the proteins of the same sizes in lysates of both isolates. This result suggests that these two isolates contain cross-reacting epitopes, which are from the flagellin subunits but not from other sources in the cells. The same assumption may be made for the *P. putida* DMS 2704 isolate. Because of the absence of a large central portion in the DMS 3052 flagellin gene, cross-reacting epitopes in other *P. putida* isolates are likely to be in the conserved N- and/or C-terminal parts of their flagellins. Moreover, the cross-reactivity of this antiserum to only one protein band in whole-cell lysates of each *P. putida* isolate on Western immunoblot suggests that each organism has a single type of flagellin.

A motility inhibition test demonstrated that anti-EPT3 polyclonal antiserum prevented the cell motility of *P. putida* DMS 3052 in vitro. The mechanism of antibody to inhibit motility is not well understood. However, it has been hypothesized that antibody aggregation at the surface of the flagella causes inefficient flagellar rotation (Montie et al., 1982). Control experiments using anti-DMS 3052 flagellin antiserum were used to confirm that the expressed protein from the 0.8-kb gene of *P. putida* DMS 3052 is flagellin protein. Applications of antiflagellum antibodies have been documented. For example, antibody to *P. putida* flagella was used in an immunocapture strategy to separate *P. putida* cells from other lake water microorganisms (Morgan et al., 1991), and antibody against *Burkholderia pseudomallei* flagellin protein can protect *B. pseudomallei* infection in a rat animal model (Brett et al., 1994). The variability in the flagellin sequences and length, however, may be crucial to the success of the immunoprotective study using antibody raised against flagellin.

A variation in flagellin gene size, which reflects a variation in filament width, was reported in *P. aeruginosa* (Winstanley et al., 1996) and *B. cepacia* (Hales et al., 1998). The preliminary electron microscopic analysis from our laboratory also indicated that *P. putida* isolates with the smaller flagellin gene type were found to have a thinner flagellar filament. Further work to determine filament widths is necessary to confirm this. It is proposed that flagellin monomers are transported through the filament's central channel during flagellum biosynthesis (Namba et al., 1989). Thus it is possible that the smaller flagellins, which constitute thinner filaments, are sufficient to construct a thinner channel to accommodate a smaller folded flagellin subunit as it passes through the channel. It is interesting that a *Vibrio anguillarum* mutant construct with a deleted flagellin gene produces a shortened flagellum (Milton et al., 1996). Additional study is needed to enlighten flagellar structural factors.

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