Molecular cloning of two *Pseudomonas* flagellin genes and basal body structural genes

Craig Winstanley,¹† J. Alun W. Morgan,²‡ Roger W. Pickup³ and Jon R. Saunders¹

Author for correspondence: Craig Winstanley. Tel: +44 203 838408. Fax: +44 203 838282.

¹ Department of Genetics and Microbiology, University of Liverpool, Life Sciences Building, PO Box 147, Liverpool L69 3BX, UK

² Freshwater Biological Association, The Ferry House, Ambleside, Cumbria LA22 0LP, UK

³ Institute of Freshwater Ecology, The Ferry House, Ambleside, Cumbria LA22 0LP, UK

***Pseudomonas putida* strains PaW8 and PRS2000 produce flagellins with apparent molecular masses of 81 kDa and 50 kDa respectively. Two Tn5 insertion mutants of *P. putida* PaW8 lacking the ability to bind the flagellin-specific monoclonal antibody MLV1 were isolated. Mutant PaW8-*flg2* contained a Tn5 insertion within a 2.6 kb *EcoRI* fragment of the *P. putida* chromosome carrying putative basal body genes. DNA and deduced protein sequences suggested the presence on this fragment of two complete genes homologous to *flgH* and *flgI* from *Salmonella typhimurium*. The insertion of Tn5 occurred in the *flgI* locus and appeared severely to reduce expression of the *P. putida* flagellin gene. A Tn5-containing fragment of DNA from a second mutant, PaW8-*flg1*, was cloned and found to contain sequences that hybridized strongly with the *Pseudomonas aeruginosa* flagellin gene. A 2.3 kb *HindIII* fragment containing all but 62 bp of the *P. putida* PaW8 flagellin gene was cloned and used as a probe to identify clones carrying the equivalent gene from *P. putida* PRS2000. Flagellin genes from both *P. putida* strains were sequenced and their amino acid sequences deduced. Both flagellins were found to contain conserved amino- and carboxy-terminal regions when compared to other flagellins, with the central region being more variable. The epitope for MLV1 is likely to lie within this central region of *P. putida* PaW8 flagellin. The deduced molecular mass of *P. putida* PaW8 flagellin (68 kDa) differed significantly from its apparent molecular mass estimated by PAGE, possibly as a consequence of post-translational modification. This was not the case with flagellin from *P. putida* PRS2000, where the predicted and apparent molecular masses were similar.**

Keywords: *Pseudomonas putida*, flagellin, population analysis

INTRODUCTION

Flagella, consisting of a filament, hook and basal body, are organelles responsible for bacterial motility. The production and assembly of bacterial flagella are complex processes that can involve upwards of 40 genes (Macnab,

1992). The flagellum of *Pseudomonas putida* PaW8, a plasmid-free derivative of the TOL-plasmid-containing soil isolate PaW1, is polar, with the flagellar filament composed of a single flagellin.

Flagella are major antigenic determinants in many bacteria. Antigenic differences amongst bacterial flagellins have been widely reported and for example exploited in the serotyping of *Salmonella* spp. A number of flagellin genes from different bacterial species have been sequenced including *Salmonella* spp. (Joys, 1985; Wei & Joys, 1986), *Escherichia coli* (Kuwayama *et al.*, 1986), *Borrelia burgdorferi* (Gassmann *et al.*, 1991), *Caulobacter crescentus* (Gill & Agabian, 1983), *Roseburia cecicola* (Martin & Savage, 1988), *Rhizobium meliloti* (Bergman *et al.*, 1991), *Bacillus subtilis* (Albertini *et al.*, 1991), *Campylobacter jejuni* (Nuijten *et al.*,

† **Present address:** Division of Biological Sciences, Coventry University, Priory Street, Coventry CV1 5FB, UK.

‡ **Present address:** Horticulture Research International, Littlehampton, West Sussex BN17 6LP, UK.

The GenBank accession numbers for nucleotide sequence data reported in this paper are L15366 and L15367 for the *P. putida* PaW8 and *P. putida* PRS2000 flagellin genes, respectively, and L15385 for the putative basal body genes.

1990), *Serratia marcescens* (Harshey *et al.*, 1989) and *Pseudomonas aeruginosa* (Totten & Lory, 1990). Comparison of flagellin protein sequences from within and between different bacterial species indicates that these polypeptides are typically conserved at both ends, presumably because such regions are important for the correct assembly of filaments. Five phase-1 flagellin genes from *Salmonella* were shown to be 99% identical in nucleotide sequence for the first 300 bp at the 5' end and the first 200 bp at the 3' end, whereas the genes show decreasing homology towards the middle portion (Joys, 1985; Wei & Joys, 1986).

McDonough & Smith (1976) found variation of molecular mass amongst bacterial flagellins of between 28.6 and 63.2 kDa. We have previously reported that *P. putida* PaW8 possesses a flagellin of unusually large apparent molecular mass although the amino acid composition of the N-terminal sequence indicates a strong similarity to other flagellins (Morgan *et al.*, 1991). In this paper we describe the isolation following Tn5 mutagenesis of mutants of *P. putida* PaW8 no longer able to bind MLV1, a monoclonal antibody that reacts specifically with *P. putida* PaW8 flagellin (Morgan *et al.*, 1991). Sequences flanking the point of insertion of Tn5 on the bacterial chromosome of such mutants were cloned, enabling the sequencing of the flagellin gene and basal body genes of *P. putida* PaW8. The flagellin gene sequence of a second *P. putida* strain, PRS2000, was also obtained. The resulting information was used to evaluate the possibility of using flagellin sequence variation as a strain-specific marker in the analysis of bacterial populations.

METHODS

Bacterial strains and plasmids. These are described in Table 1.

Construction of Tn5 mutants. Plasmid pLG221, carrying Tn5, was introduced into *P. putida* PaW8 by conjugation followed by selection on minimal medium containing sodium succinate (5 mM) and kanamycin (100 µg ml⁻¹). Resulting colonies were patched on to nitrocellulose filters overlayed on nutrient agar plates containing kanamycin, and incubated at 30 °C overnight. Colonies were screened for the loss of response to MLV1 as described by Harlow & Lane (1988). Loss of motility was assessed microscopically and by using soft agar.

DNA manipulations. Whole-cell DNA was prepared as described previously (Winstanley *et al.*, 1993). Bulk plasmid DNA was prepared using Qiagen plasmid midi-kit solutions and columns (Hybaid). For small-scale plasmid isolations the method of Holmes & Quigley (1981) was used. Restriction endonucleases were obtained from Boehringer. Lambda DNA (Pharmacia) digested with *Hind*III or *Hind*III and *Eco*RI was used as a standard. Agarose gel electrophoresis was carried out by established procedures. Specific DNA fragments were eluted from agarose by the method of Girvitz *et al.* (1980).

Reaction conditions for ligation with T4 DNA ligase were as recommended by the supplier (Boehringer). Transformation into *Escherichia coli* ED8654 or *E. coli* JM103 was achieved either by the procedure of Cohen *et al.* (1972), or by electrotransformation using the Gene Pulser apparatus (Bio-Rad) and the method of Dower *et al.* (1988). For electrotransformation,

cuvettes with an electrode gap of 0.2 cm were used with a field strength of 12.5 kV cm⁻¹. Southern blot techniques and hybridizations were carried out as described previously (Winstanley *et al.*, 1993). DNA fragment probes were labelled with [³²P]dCTP (ICN Biomedicals) using a Random Primed DNA Labelling Kit (Boehringer). pUC19 gene banks were screened for flagellin clones by colony hybridization using standard procedures.

Flagellin protein purification and N-terminal sequencing.

Flagellin was purified from *P. putida* PaW8 and *P. putida* PRS2000 as described previously (Morgan *et al.*, 1991). N-terminal sequencing was carried out by Dr M. Wilkinson, Department of Biochemistry, University of Liverpool, UK.

SDS-PAGE, Western blotting and cyanogen bromide digestion.

SDS-PAGE was performed on 12% (w/v) slab gels as described by Laemmli (1970), using the molecular mass marker SDS-6H (Sigma). Western blotting (immunoblotting) was carried out by the method of Towbin *et al.* (1979). Following transfer, the nitrocellulose filter was submerged in 1% (w/v) bovine serum albumin (made in PBS: 0.1 M phosphate buffer containing 0.85% NaCl, pH 7.5) with gentle shaking for 1 h, washed in PBS and left overnight in tissue culture supernatant of cell line MLV1, which produces flagellin-specific monoclonal antibody (Morgan *et al.*, 1991). After three further washes in PBS the filter was placed in a 1:500 dilution (in PBS) of a peroxidase-complexed rabbit IgG to mouse IgG secondary antibody (Dako) for 1 h with gentle shaking. After final washes in PBS the filter was placed in a solution containing 20 ml of 3 mg ml⁻¹ 4-chloro-1-naphthol (dissolved in methanol), 80 ml PBS, and 3 µl hydrogen peroxide (50%, v/v, solution) until the reaction was completed.

Cyanogen bromide digestion was carried out by the method of Aitken *et al.* (1989). Digested samples were run on 10% SDS-PAGE gels and Western blotted on to Immobilon P Membrane (Dako) prior to N-terminal sequencing.

DNA sequencing. Double-stranded DNA sequencing of both strands of pUC18 and pUC19-based clones and sub-clones was carried out by the dideoxy-chain-termination method of Sanger *et al.* (1977) using United States Biochemicals Sequenase kits (Cambridge Bioscience) under the conditions recommended by the supplier, with either universal or synthetic primers. Single stranded DNA binding protein was used routinely to reduce secondary structure.

Computer sequence analysis. Identification of restriction sites, nucleotide sequence alignments, determination of amino acid composition, predicted protein molecular mass, alignments of predicted flagellin protein from *P. putida* PaW8 or PRS2000 with other flagellins (retrieved from EMBL or GenBank: Pearson & Lipman, 1988), and construction of sequence similarity dendrograms were carried out using the GCG sequence analysis software package (Genetics Computer Group, University of Wisconsin). Predictions of possible antigenic sites for MLV1 within the *P. putida* PaW8 protein sequence were obtained by using PEPTIDESTRUCTURE from the GCG sequence analysis software package to identify regions of high surface probability and antigenic index, and by using PREDICT (M. Partis, Horticultural Research International, Littlehampton, UK; personal communication), which identifies potential antibody-reactive peptides within a protein sequence by calculating hydropobic moment, flexibility, antigenicity and β -turn potential. Most likely candidate regions were taken as those scoring well on both tests.

Table 1. Bacterial strains and plasmids

Strain/plasmid	Description	Source/reference
<i>P. putida</i> PaW8	Plasmid-free derivative of TOL-containing soil isolate PaW1	Worsey & Williams (1975)
<i>P. putida</i> PaW8- <i>flg1</i>	MLV1 ⁻ mutant of PaW8	This study
<i>P. putida</i> PaW8- <i>flg2</i>	MLV1 ⁻ mutant of PaW8	This study
<i>P. putida</i> PRS2000 (ATCC 12633)	Widely-studied strain	
<i>E. coli</i> ED8654	Common transformation recipient	Winstanley <i>et al.</i> (1989)
<i>E. coli</i> JM103	Common transformation recipient for pUC vectors	Yanisch-Perron <i>et al.</i> (1985)
<i>E. coli</i> W3110(pLG221)	Tn5 donor	Boulnois <i>et al.</i> (1985)
pBR322	Cloning vector	Bolivar <i>et al.</i> (1977)
pBR325	Cloning vector	Bolivar (1978)
pUC18/19	Cloning vectors	Boehringer
pPT218	Cloned <i>P. aeruginosa</i> PAK type a flagellin gene	Totten & Lory (1990); gift of Prof. S. Lory
pLV1020	Contains Tn5	Winstanley <i>et al.</i> (1993)
pLV1026	Large Tn5-containing <i>EcoRI</i> fragment of PaW8- <i>flg1</i> DNA cloned into pBR322	This study
pLV1030/pLV1031	2.3 kb flagellin-related <i>HindIII</i> fragment of PaW8 DNA cloned into pUC19	This study
pLV1040/pLV1041	5 kb flagellin-related <i>KpnI</i> fragment of PRS2000 DNA cloned into pUC19	This study
pLV1042/pLV1043	1.85 kb <i>SacI</i> fragment sub-cloned from pLV1040 into pUC19	This study
pLV1044	Tn5-containing <i>EcoRI</i> fragment of PaW8- <i>flg2</i> DNA cloned into pBR322	This study
pLV1045	2.6 kb basal body-related <i>EcoRI</i> fragment of PaW8 DNA cloned into pUC19	This study

RESULTS

Tn5 mutagenesis of *P. putida* PaW8

Out of approximately 600 colonies screened, three MLV1⁻ mutants were identified. All three were found to be non-motile by microscopic analysis and the inability to

Fig. 1. Restriction enzyme maps of the flagellin gene regions of *P. putida* PaW8 (a) and *P. putida* PRS2000 (b). Maps were generated using the enzymes *EcoRI* (E), *HindIII* (H), *KpnI* (K), *PstI* (P), *SacI* (Sa), *SalI* (Sl), *SphI* (Sp), *StuI* (Su), *StyI* (St) and *XhoI* (X). The position of the flagellin gene is represented in both cases by the thick line. Arrows indicate the direction of transcription which, in pLV1030 and pLV1042, is in the same orientation as vector promoters.

spread when patched on to soft agar. Two of the MLV1⁻ mutants proved to be identical. Chromosomal DNA from one of these mutants (PaW8-*flg1*) was prepared, digested with *EcoRI* (no cutting sites within Tn5) and hybridized against a Tn5-specific probe (a 9 kb *EcoRI* fragment of plasmid pLV1020: Winstanley *et al.*, 1993). *P. putida* PaW8-*flg1* was found to contain Tn5 within a large (approx. 15 kb) *EcoRI* fragment. This fragment was cloned from *P. putida* PaW8-*flg1* DNA into pBR322. The large insert carried by the resulting plasmid, pLV1026, hybridized strongly with a 1.7 kb *HindIII*-*EcoRI* fragment of pPT218 (Totten & Lory, 1990), carrying *P. aeruginosa* flagellin DNA. Labelled probes made from the cloned inserts of both pPT218 and pLV1026 were used to identify restriction fragments of *P. putida* PaW8 DNA that contained flagellin-related sequences. These included a *HindIII* fragment of approximately 2.3 kb.

Chromosomal DNA isolated from the third MLV1⁻ mutant (named PaW8-*flg2*) and digested with *EcoRI* was also hybridized against the Tn5 probe and found to contain Tn5 within an *EcoRI* fragment of approximately 7 kb.

Cloning of the *P. putida* PaW8 flagellin gene

P. putida PaW8 DNA digested with *HindIII* was cloned into pUC19, and the resulting recombinant plasmids were transformed into *E. coli* JM103 and screened by colony

Fig. 2. Western blot of *P. putida* PaW8 flagellin clone against MLV1. PAGE gel (a) and Western blot (b) of: (1) JM103(pUC19); (2) JM103(pLV1030); (3) JM103(pLV1031); (4) PaW8; (5) PaW8-*flg1*; (6) PaW8-*flg2*.

hybridization using the large *Eco*RI cloned fragment of pLV1026 as labelled probe. This resulted in the identification of pLV1030 and pLV1031, which contained a 2.3 kb *Hind*III cloned fragment in opposite orientations with respect to the pUC19 vector promoter (Fig. 1). Southern blots indicated that both the large *Eco*RI cloned fragment of pLV1026 and the 1.7 kb *Hind*III-*Eco*RI fragment of pPT218 (containing the *P. aeruginosa* flagellin gene) hybridized with the cloned *P. putida* PaW8 2.3 kb *Hind*III fragment of pLV1030 and pLV1031. Fragments of PaW8 DNA that hybridized with the pLV1030/pLV1031 cloned fragment (including a 2.3 kb *Hind*III fragment) were of similar size to those hybridizing with the large *Eco*RI cloned fragment of pLV1026 and the 1.7 kb *Hind*III-*Eco*RI cloned fragment of pPT218 (data not shown). A Western blot of *E. coli* JM103 containing pLV1030 and pLV1031, using MLV1 antibody, suggested that pLV1030 carries the *P. putida* PaW8 flagellin gene in the correct orientation for transcription from the pUC19 vector promoter (Fig. 2). No expression

was detectable when the *Hind*III fragment was in the opposite orientation with respect to the pUC19 *lac* promoter, suggesting that the flagellin gene promoter was recognized poorly or not at all by *E. coli* RNA polymerase.

Fig. 3. Restriction enzyme map of the 2.6 kb cloned *Eco*RI fragment of pLV1045. The map was generated using the enzymes *Eco*RI (E), *Kpn*I (K), *Pvu*II (Pv), *Pst*I (P) and *Sma*I (S). The arrow indicates the position of the Tn5 insertion in *P. putida* PaW8-*flg2*. The boundaries of the *flgG*, *flgH* and *flgI* genes are indicated by vertical broken lines.

(a)

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1  aagcttctgcctaocgagccattgtaggagcgggttcacccgcgaacacccggcaaagccggtgccacccatcgctct
   -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
80

81  ccttcttcgcagcctcaccgctccaccgacgggatgaggcattcagccagttcaaacagtttttgacgccaataaac
   -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
160

161  gcccttaacccttgatttctcagggctgccccatgccagtttttttttgaaaaaacctcaagcaaccgcgcac
   -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
240

241  ccgacgataaccattacgaaggttctctaggccacaccggctgtgccaggggccggaagccgcagtatccatacaacga
   -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
320

321  ggaattcgtcatggctttgacggttaacaccaacatcacctccatgagtggttcagaagaacctgaacaagtctcgacg
   -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
400
      M A L T V N T N I T S M S V Q K N L N K S S D A

401  ccttgggcaaccacatgggtcgtctgtcctctggcctgaaatcaacagtgccaaagacgacgccgaGGCCTGCAGATC
   -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
480
      L G T T M G R L S S G L K I N S A K D D A A G L Q I

481  TCCAAcgtctgaccactcaaatacaaggctctgagcgttgccgttcaagaacgccacgatggtatctctattgcgcaaac
   -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
560
      S N R L T T Q I K G L S V A V K N A N D G I S I A Q T

561  tgctgaaggcgccatggcgacttcaggcaacatcatgcagcgtatgcgtgaactggccctgcaatcgcggaacggctcga
   -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
640
      A E G A M A T S G N I M Q R M R E L A L Q S A N G S N

641  acagcgatgacgaccgcctccatgcagcaggagttaccgcgctgtcggcgagctcaccgctattgctaaccaccacc
   -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
720
      S D D D R A S M Q Q E F T A L S G E L T R I A N T T

721  actttcgggtggcgttaattctcgtgatggcacctctccggcagcagcttcagggtggcggaactccaacgaaagcat
   -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
800
      T F G G R N L L D G T F S G S S F Q V G A N S N E S I

801  ttogtttgcatgaaagatgtcagcgtcactccgatgaagggaactacaacgaagccagcgttgccggcggtgttgcta
   -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
880
      S F G M K D V S A T S M K G N Y N E A S V A G G V A T

881  ccctgcaagccagcgtcacccgtgcagccggttaagtccggcaccaacaatgctggcagcactctcgttcggttggtgg
   -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
960
      L Q A S V T G A A G K F G T N N A G S T S A S V V G

961  actgcaggcgccggcgctatttgataagccgaccatcggtgctgctgcggtaacttggtgctgaacgttggtaccaccac
   -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
1040
      T A G A G V F D K P T I G A A A G N L V L N V G T T T

1041  caccaccatcgctgctgcccagggcagccaccctgcaggatgtggttgacaatatcaacctggaaactagcaataccgggtg
   -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
1120
      T T I A A A A G D T L Q D V V D N I N L E T S N T G V

1121  ttacgcgtagcattgacagcgccactggtgcactgaagctcagcggcaccagggcattcaccatcgatgccagcactgat
   -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
1200
      T A S I D S A T G A L K L D G T Q A F T I D A S T D

1201  gacgtcttgagcactgccttgggtctgcccagggccgggtggtgcccagctgtcgaaaacccggtactgccaacctgcgtga
   -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
1280
      D V L S T A L G L A E A G G A Q L S K T G T A N L R D

1281  tgggtgattggggcgccggtggtgcccgttaacctgacgctcggctctaccaacattgcattggttgactgacaccctat
   -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
1360
      G V L G A G G A G N L T L G S T N I A L V A T D T L S

1361  cttcggtagtcggcaaggttaatgctcaaaccggcagcactggtgtaacggcttcattgacagtgccactgggcaactg
   -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
1440
      S V V G K V N A Q T G T T G V T A S I D S A T G Q L

1441  aaactgaactcggtgcgggcttcagctaggtggtactgcgggtacottgactggcctgggtctgactgcaggttctgt
   -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
1520
      K L N S A A G F D V G G T A G T L T G L G L T A G S V

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Fig. 4. For legend see p. 2025.

1521 tgccatcgcgctcagactaccggtctggcgctggctgcttcgatcgacatcaatggcactaccttcaacttcgctcaag 1600
 -----+-----+-----+-----+-----+-----+-----+-----+-----+
 A I A P Q T T G L A S A A S I D I N G T T F N F A Q G
 1601 gggatgacctggatgogatogttgacaacatcaacaacaacgggtgcaggtgctgtcggttcgggtactgcctgactggc 1680
 -----+-----+-----+-----+-----+-----+-----+-----+-----+
 D D L D A I V D N I N N N G A G A V G S G T A L T G
 1681 gtcactgcaaaaatgataacggtgcctgggtgctgacttcggccaatggccaggacatcaagttggacaatgggtctg 1760
 -----+-----+-----+-----+-----+-----+-----+-----+-----+
 V T A K N D N G R L V L T S A N G Q D I K L D N G S G
 1761 cgtgactgggcaaggggcactggctgacctgaggtctgaactcgggcaccaccaaggctgggttggtgctgatacctcca 1840
 -----+-----+-----+-----+-----+-----+-----+-----+-----+
 V T G Q G A L A A V G L N S G T T K A G L V A D T S I
 1841 tctctctgaacggcgtagaagtcaagttcaagaagggtgatgacatggatagcatcgagcctccatcaacgcgcgcagc 1920
 -----+-----+-----+-----+-----+-----+-----+-----+-----+
 S L N G V E V K F K K G D D M D S I A A S I N A A S
 1921 accggtgtcaacgccagcggttggttaatgccggcagcagcactctgtcgctggttcgctgaccaggacatcacogtgc 2000
 -----+-----+-----+-----+-----+-----+-----+-----+-----+
 T G V N A S V V V N A G S S T L S L F A D Q D I T V A
 2001 tgatggcagcaatggtacoggtctggcagcagagctgggtctgactgctgttcgaggcagacctctgcactcgagatgg 2080
 -----+-----+-----+-----+-----+-----+-----+-----+-----+
 D G S N G T G L A R R A G S D C C C R Q T S A L E M E
 2081 aatctaccgtttccaacctgaacatcacggacogcgaagtgctcagcaggctatccaggttctggatggtgcatgcag 2160
 -----+-----+-----+-----+-----+-----+-----+-----+-----+
 S T V S N L N I T D A Q S A Q Q A I Q V L D G A M Q
 2161 tcgctggactcccagcggtccagctgggtgcagtgagaaacogtcttcgatagcactgtcgctaacctgcagtcgatctc 2240
 -----+-----+-----+-----+-----+-----+-----+-----+-----+
 S L D S Q R S Q L G A V Q N R F D S T V A N L Q S I S
 2241 ggaaaactccacgcagcccgtagtcttatccaggtgcgcactttgctgcgaaactgcggagctgagcaagcagcaaa 2320
 -----+-----+-----+-----+-----+-----+-----+-----+-----+
 E N S T A A R S R I Q D A D F A A E T A E L S K Q Q T
 2321 ccctgcagcaagctccacogctatcctgtcgCAGGCGAACCGCTGCCatcttcggtactgaagctgctcggctaattc 2400
 -----+-----+-----+-----+-----+-----+-----+-----+-----+
 L Q Q A S T A I L S Q A N Q L P S S V L K L L G *
 2401 gattctgtgatgaatcggaagggtgcggcaacgtaccctttcgttttctgattctacgaggcaagcatcatggatatg 2480
 -----+-----+-----+-----+-----+-----+-----+-----+-----+
 2481 agtgtcaagctgaactctgttctctctgcgcccgcactcaagaagccagg 2531
 -----+-----+-----+-----+-----+-----+-----+-----+-----+
 (b) tttggagtgggtttttgacccaataaacogccttaacccttgatttatcgggctggcccatgcagcattttttttt 80
 1 -----+-----+-----+-----+-----+-----+-----+-----+-----+
 81 cgaaaaacccctcaagcatcccgccgacccgacgataactattacgaaggttctctaggccagtcggcggttgccaagg 160
 -----+-----+-----+-----+-----+-----+-----+-----+-----+
 161 ccggaagccgcagtaacctccaacgaggtatttcgtcatggctttaactgtaaacaccaacattgcgtcgatcactactc 240
 -----+-----+-----+-----+-----+-----+-----+-----+-----+
 M A L T V N T N I A S I T T Q
 241 agggcaacctgaccaaggctagcaacgctcagaccacttcgatgcagcgtctgtcctcgggtotgogtatcaacagcgt 320
 -----+-----+-----+-----+-----+-----+-----+-----+-----+
 G N L T K A S N A Q T T S M Q R L S S G L R I N S A
 321 aaagcagcagctgccGGCCTGCAGATCGCCAACcggtctgaccagccagatcaatggcctgggccaggtgtcaagaacgc 400
 -----+-----+-----+-----+-----+-----+-----+-----+-----+
 K D D A A G L Q I A N R L T S Q I N G L G Q A V K N A
 401 caacgacggtatctcgatcgctcagacogctgaaggtgcaatgcaggcttcgacogacatcctgcaaaaaatgcgtacgc 480
 -----+-----+-----+-----+-----+-----+-----+-----+-----+
 N D G I S I A Q T A E G A M Q A S T D I L Q K M R T L

Fig. 4. For legend see facing page.

481	tgccctgtgctoggtactgctgctgagcaacgcagacgtaagtogaacaacgacgaataaccaggctctgacccgt	560
	A L C S A T G S L S N A D R K S N N D E Y Q A L T A	
561	gagctgaacggtatctgccaaccaccaccttcggtgccagaagctgctggacggttcgtacggcaccgaagccatccag	640
	E L N R I C Q T T T F G A R S C W T V R T A P R P S R	
641	gtcggcgccaacgccaacgaaccatcaacctggcgctggacaacggttcggccaagagcatcggtctgcagcagatcaa	720
	S A P T P T K P S T W R W T T F R P R A S A R S R S S	
721	gtcgggcactatcgcgcttagccagcgtgtggcgcgagctgatctggttgcactggcaacggtcagagcaaacccgtc	800
	R A L S R L A Q R V A A A D L V V T G N G Q S K T V	
801	agctatgatggggctcctcgggccaagacatcgctgccaactcaatggctccatcggtggcctgactgcttctgocag	880
	S Y D A G S S A K D I A A K L N G S I G G L T A S A S	
881	caccgaagtcaaacgtgttgcagtgccgctgcagctactccagccaactttgacctgactgtaggcggtacgaccg	960
	T E V K L A V A S G A A A T P A N F D L T V G G T T V	
961	tcagcttcatcggtgttactgacaccgcaagcctggctgatcagctgaagtccaacgcgcgaagctgggtatcagcgtt	1040
	S F I G V T D T A S L A D Q L K S N A A K L G I S V	
1041	aactacgacgaatcgaccaagagcctgtcggtgaagtcggacactggcgagaacctggccttcacctccaaggccggcgc	1120
	N Y D E S T K S L S V K S D T G E N L A F T S K A G A	
1121	agacgccatctcggtgggtgcacgcgacggaacggtgatttccaggctactctgaccacgcttccgctaccgttaacg	1200
	D A I S V G A R D G N G D F P G T L T T L S A T V N D	
1201	acaaatcgaccgttactggccagatttctctggactctgcgaaggttactcggtagctaacggcgccacgggtactggc	1280
	K S T V T G Q I S L D S A K G Y S V A N G A T G T G	
1281	gctaccgacgtgttcgggtgcagcttccaagtcctcgcccaagaccaccatcgccgataccgatgttacggaggccgtcaa	1360
	A T D L F G A A S K S S A K T T I A D T D V T E A V N	
1361	cgccagaacgcccctggcgttatcgacaaggccatcggttcgatcgacagcgtccggttcgggcctgggtgctaccaga	1440
	A Q N A L A V I D K A I G S I D S V R S G L G A T Q N	
1441	accgtctgcaaacaccgctcgacaacctgcagaacatccagaagaactccaccgctgcccgctccaccgtgcaggacgtg	1520
	R L Q T T V D N L Q N I Q K N S T A A R S T V Q D V	
1521	gacttcgcttcggaaactgcgagctgaccaagcagcagactctgcagcaggcttcgactgcgatcctgttgCAGGCTAA	1600
	D F A S E T A E L T K Q Q T L Q Q A S T A I L L Q A N	
1601	CCAGCTGCCatcttcgggtcctgaaactgtccagtaattcaggcgcctgagtaagggtgaaagg	1666
	Q L P S S V L K L L Q *	

Fig. 4. Nucleotide sequences of the flagellin genes of *P. putida* PaW8 (a) and *P. putida* PRS2000 (b). Nucleotide and deduced amino acid sequences for the flagellin genes and potential operator-promoter regions are shown. Known N-terminal sequences of both flagellins and peptide P1 are boxed. Possible RpoF-like recognition sequences are underlined. N-terminal sequences of P1 and PRS2000 flagellin (from purified flagellin) were obtained in this study. The N-terminal sequence of PaW8 flagellin has been reported previously (Morgan et al., 1991). Potential ribosome-binding sites are indicated by double underlining. Sequences corresponding to oligonucleotide primers that can be used for PCR of *Pseudomonas* flagellin gene sequences are capitalized.

Sequence analysis proved subsequently that the 2.3 kb *Hind*III fragment of pLV1030 does not contain 62 bp at the carboxy-terminal end of the flagellin coding sequence.

Thus *E. coli* JM103(pLV1030) produces a flagellin that is not quite complete. However, cloned flagellin, despite lacking some 20 amino acid residues at the carboxy-

	1				50					100
Paw8	MALTVNTNIT	SMsvQkNLNK	SSdALgTtMg	RLSSGLkINS	AKDDAAGLQI	sNRLTtQikG	LSvAVKNAND	GISIAQTAEG	AMatSgNIMQ	RMRELALQSA
PAK	.ALTVNTNIA	SLnTQrNLNn	SSasLnTSLQ	RLStGsrINS	AKDDAAGLQI	ANRLTSQVNG	LnVATKNAND	GISIAQTAEG	ALQqSTNIIQ	RMRLdLQSA
PRS2000	MALTVNTNIA	SitTQgNLtK	aSnAqtTSMQ	RLSSGLRINS	AKDDAAGLQI	ANRLTSQING	LgqAVKNAND	GISIAQTAEG	AMQaSTdILQ	hMRtLALCSA
	*****	* * *	* *	*** * *	*****	*** * *	* * *	*****	* *	*** * *
	101				150					200
Paw8	NGSNSDdDRa	SMqQEFtALs	gELtRIaNTT	TFGGRnLLDG	tFsgSSfQVG	ansNESISfG	mkDvSATSMk	GnyneAsvAG	gVatIqASvT	gaagkfgtnn
PAK	NGSNSDdERT	aLNgEakqLq	kELdRIaNTT	TFGGRkLLDG	sFgvasfQVG	sAaNEiISvG	idEmSAeSLn	GTyfkAdggG	avtaataSgT	vdia.....
PRS2000	tGSISnaDRk	SnNdEYqALT	aELnRIcQT	TFGaR.....	ScWtVr	tAprpSrSap	tpkpsTwrw	tTFrprasAr	srsrslSrI	aq.....
	** * *	* *	** * *	*** *						
	201				250					300
Paw8	aGsTsaSvVg	tagagvfdkp	tigaAAgnLV	lnVgttttTI	AaaAGdtlQD	VVdniNlets	ntgvtasids	atGALKldgT	qaFTidASTD	dvLstALGIA
PAK	iGiTggSaVn			VkVdmkgnet	AeqAaakiaa	aVndaNv...		giGAFsdGdT	isYvskAgkD	
PRS2000			..rvAAadLV	VtngngqskTV	sydAGssakD	IaakLn...		Gsi	ggLTasASTE	vkLavAsGaA
					*	*				
	301				350					400
Paw8	eaggaqlskt	gtanlrldgvl	gagGaGnLTl	GsTnIaLVat	dtlSSVvGkv	nAqTGtTGvt	asiDsaTgqL	klnSaaGfdv	gGTAgtlitgl	gltagSvAIA
PAK			..GsGaiT.		..SaVsGvV	iadtGstGV.		..GTaa.....		gVA
PRS2000			atpanfdLTv	GgTtVsFIgV	tdtsSladql	kснаaklGIs	vnyDesTksL	svkSdtGenl	aFT.....	SkAgA
			*			*			*	*
	401				450					500
Paw8	PqtTgLaSaa	siDinGTtfn	Faaggddldai	vdninnngag	avgsgtaltg	vtakndnrgl	vltsangqdi	kldngSgVTG	QgalaavglN	sgttkagIva
PAK	PsaTaFAk..									
PRS2000	daisvgArdg	ngDfpGTltt	Lsat.....					..vndkStVTG	Q.....	
	501				550					600
Paw8	dtisislngve	vkfkkgddmd	siaasinaas	tgvnasvvrn	agstlSLfa	dqdtItVADGS	nGTGLaRRAG	sdcccrqtSa	lemesTVsnl	nITDAqsAQq
PAK					t	ndtVakiDiS	TakaLsRRAG	dr.....		
PRS2000					iSLds	akgysVAnGa	TGTGatdlfG	a.....ask	ssaktTIadt	dvTEAvnAQn
							*			
	601				650					689
Paw8	AiqVldGAmq	SIDSqRSqLG	AVQNRFDtV	nLQksIsENS	TAARSRTQDa	DFAAETAELS	KQQTLLQAST	AILsQANQLP	SSVLKLLg	
PAK	ttaik	qIDasvptsv	AVQNRFDnTI	nNLKNIGENV	sAARGRIEdt	DFAAETAnLT	KnQvLQQAGT	AILsQANQLP	qSVLsLLr	
PRS2000	AlaViDkAig	SIDSvRSgLG	AtQNRLqtTV	dNLQNIqkNS	TAARSTvQDv	DFAaETAELT	KQQTLLQAST	AILLQANQLP	SSVLKLLq	
	**	*****	* *	* * *	***	* * *	* * *	* * *	***	**

Fig. 5. Alignment of *Pseudomonas* flagellins. The best-computer-assisted alignment of *P. putida* PaW8, *P. aeruginosa* PAK and *P. putida* PRS2000 flagellins is shown. Capital letters indicate consensus amino acid residues. Asterisks indicate residues common to all three proteins.

terminus, migrated on SDS-PAGE gels with a similar molecular mass to flagellin isolated from *P. putida* PaW8 (81 kDa).

Cloning of the *P. putida* PRS2000 flagellin gene

Using the 2.3 kb *Hind*III fragment of pLV1030 as probe, restriction fragments of *P. putida* PRS2000 chromosomal DNA containing flagellin-related sequences were identified, including a *Sa*I fragment (1.85 kb) and a *Kpn*I fragment (approximately 5 kb). Using colony hybridization for screening, the *Kpn*I fragment was cloned onto pUC19 in both possible orientations to create pLV1040 and pLV1041. The 1.85 kb *Sa*I fragment was sub-cloned from pLV1040 into pUC19 in both possible orientations to create pLV1042 and pLV1043 (Fig. 1). Southern blots of pLV1040 and pLV1041 DNA hybridized strongly against labelled probes of the 2.3 kb *Hind*III fragment of pLV1030 (containing almost all of the *P. putida* PaW8 flagellin gene) and the 1.7 kb *Hind*III–*Eco*RI fragment of pPT218 (containing the *P. aeruginosa* flagellin gene).

Cloning of a 2.6 kb *Eco*RI fragment containing flagellar-related DNA

The 7 kb *Tn*5-containing *Eco*RI fragment of chromosomal DNA from *P. putida* PaW8-*flag*2 was cloned into the *Eco*RI site of pBR322. When labelled, the 7 kb insert present in the resulting recombinant pLV1044 hybridized

with a 2.6 kb *Eco*RI fragment of *P. putida* PaW8 DNA. *P. putida* PaW8 DNA was digested with *Eco*RI and subjected to agarose gel electrophoresis. Fragments within the relevant size range were eluted and cloned into *Eco*RI-digested pBR325. Potential clones were screened subsequently by colony hybridization using the 7 kb insert of pLV1044 as a probe to identify flagellar gene sequences. pLV1045, containing a 2.6 kb *Eco*RI insert in pBR325, was thus identified. Fig. 3 shows a restriction map of pLV1045. The origin of the 2.6 kb insert DNA was confirmed by Southern blot analysis of digests of *P. putida* PaW8 DNA using the 2.6 kb fragment as a radioactively labelled probe (data not shown).

Flagellin gene and deduced protein sequences

The entire 2.3 kb *Hind*III cloned fragment of pLV1030 and pLV1031 was sequenced using a number of internal *Pst*I sub-clones for confirmation. The remaining 62 bp of the flagellin gene coding sequence was obtained by extending downstream of the 2.3 kb *Hind*III fragment using appropriate synthetic oligonucleotide primers and pLV1026 DNA as template (Fig. 4a). The *P. putida* PRS2000 flagellin gene was also sequenced using a number of internal *Pst*I sub-clones for confirmation (Fig. 4b). When compared with previously reported flagellin gene sequences, the *P. putida* genes exhibited greatest homology with that from *P. aeruginosa* (Fig. 5). The only possible reading frames for flagellin genes are indicated

Cyanogen bromide treatment of *P. putida* PaW8 flagellin yielded a peptide (P1) of 45–50 kDa that reacted with the strain-specific monoclonal antibody MLV1. The N-terminal sequence of P1 was obtained (MKGNYNEAS-VAGGVA) and used to locate the peptide within the deduced sequence of PaW8 flagellin (residues 169–519) (Fig. 4). These data confirm the assignment of the flagellin open reading frame (ORF).

The 2·6 kb *Eco*RI fragment was sequenced (GenBank accession number L15385) and potential ribosome-binding sites (GAGG for *flgH* and GGA for *flgI*) were identified. Computer-assisted DNA alignments suggested that most of a gene homologous to *Salmonella typhimurium flgG* (encoding a rod protein, FlgG: Homma *et al.*, 1990) was present within the 2·6 kb cloned fragment (60·2% identity in 674 bp overlap of DNA upstream from the *flgH* transcriptional start site; sequence alignments suggest that approximately 120 bases at the N-terminal end of *flgG* are not present on the fragment). Alignments also identified two complete ORFs downstream of the putative *flgG* gene aligning well to the *S. typhimurium flgH* (encoding L-ring protein, FlgH) and *flgI* (encoding P-ring protein, FlgI) gene respectively (Jones *et al.*, 1989). Sequencing of pLV1044 DNA showed that Tn5 in the mutant *P. putida* PaW8-*flg2* was located within the putative *flgI* gene (Fig. 3).

The deduced protein product sequences of the putative *flgH* and *flgI* genes were obtained and analysed. Comparisons with previously reported protein sequences confirmed that the best alignments (Fig. 6) were against the flagellar L-ring protein precursor (FlgH) and flagellar P-ring precursor (FlgI) of *S. typhimurium*. Translational start sites were deduced by comparison with the corresponding *S. typhimurium* genes and by the location of possible ribosome-binding sites.

Flagellin genes and proteins

Analysis of the DNA sequence of pUC19, downstream of the *Hind*III cloning site, suggested that pLV1030 may produce a fusion protein consisting mostly of flagellin with 27 vector-sequence-derived amino acid residues at the C-terminus. The difference in size between this fusion protein and the native flagellin was not detectable by SDS-PAGE. The size of *P. putida* PaW8 flagellin (81 kDa) is larger than the predicted molecular mass (68.2 kDa) derived from the deduced protein sequence. Flagellins are known to migrate aberrantly in SDS-PAGE (Simon *et al.*, 1977). Post-translational modification may account for changes in molecular mass, as has been observed in flagellins from a number of bacterial species (Joys & Kim, 1978; Logan *et al.*, 1989; Wieland *et al.*, 1985). Kelly-Wintenberg *et al.* (1990) have reported the presence of phosphorylated tyrosines in the flagellin of *P. aeruginosa* PAO. The deduced molecular mass of the *P. putida* PRS2000 flagellin (49.2 kDa) was similar to the apparent molecular mass estimated by PAGE (50 kDa), suggesting that post-translational modification does not play a significant role in the flagellin of this strain. Computer-assisted analysis of deduced protein sequences confirmed that the *P. putida* flagellin genes showed strong homology

Fig. 7. A comparison of *P. putida* flagellins with other sequenced flagellin proteins. (a) Sequence similarity graph for all published flagellins including those of *P. putida* PaW8 and PRS2000. The length is derived from the longest flagellin, that of PaW8. (b) Dendrogram derived from the same flagellin protein sequences. Alignment of protein sequences was performed by the PILEUP Multiple Sequence Analysis program software package of the University of Wisconsin Genetics Computer Group. The clustering relationships created by PILEUP during multiple sequence alignment are presented.

with other flagellins at the amino- and carboxy-terminal ends while exhibiting more divergence in central regions (Fig. 7). Unlike previously reported flagellins, which have been found to lack cysteine residues, the *P. putida* flagellins both contained three cysteine residues, although in common with other flagellins both lack histidine residues

and have low numbers of tyrosine residues (one for PaW8; four for PRS2000).

Approximately 100 bp upstream of the translational start of both *P. putida* flagellin genes lies a 27 base sequence varying in two positions (TNAAGCANCCCGCGCA-CCCGACGATAA) which occupies an identical position and is very similar to the putative *P. aeruginosa* flagellin promoter sequence (TAAAGCTCCCCAGGGAA-GCGCCGATAA) (Totten & Lory, 1990). These sequences are similar to the promoter sequence for several *E. coli* flagellin genes (Bartlett *et al.*, 1988). The consensus sequence, TAAA-N₁₅-GCCGATAA, is recognized by a secondary sigma factor (σ^{28}) in *Bacillus subtilis* and RpoF in *E. coli* (Arnosti & Chamberlin, 1989). There are also sequences that conform to the GG-10-GC invariant consensus sequence for RpoN (σ^{54})-recognized promoters but none resemble the extended consensus sequence of CTGGYAYR-N₄-TTGCA (Totten *et al.*, 1990). As in *P. aeruginosa*, the flagellin gene initiating methionine codon in *P. putida* is preceded 8 bp by the sequence GAGG, a potential prokaryotic ribosome-binding site (Shine & Dalgarno, 1974; Fig. 4). It has been demonstrated that although *P. aeruginosa* requires the RpoN-encoded minor sigma factor (σ^{54}) for transcription of flagellin (Totten *et al.*, 1990), RpoF (encoding σ^{28}) is the likely functional consensus sequence for the promoter of the flagellin gene (Totten & Lory, 1990). Two possible explanations were proposed by Totten & Lory (1990): that RpoF is involved in the initiation of transcription of the flagellin and that RpoF exerts positive control of this transcription; or that RpoN may be required for transcription of the RpoF gene. In *P. putida* also the *rpoN* gene is required for production of flagella (Inouye *et al.*, 1990). In both of the *P. putida* strains used in this study, PaW8 and PRS2000, RpoF-like recognition sequences occur in an identical position with respect to the transcriptional start site to the RpoF recognition sequence reported for *P. aeruginosa* flagellin. The consensus promoter sequence recognized by RpoF (σ^{28})-containing RNA polymerase, TAAA-N₁₅-GCCGATAA, differs from the two *P. putida* RpoF-like recognition sequences by only two bases. This suggests that transcription of the flagellin gene may be regulated in *P. putida* in a similar manner to *P. aeruginosa*.

Our results from Southern hybridizations indicated that, as in *P. aeruginosa* (Totten & Lory, 1990), the *P. putida* flagellin gene is found in a single copy in the chromosome. This suggests that in *Pseudomonas*, antigenic variation between strains is due to inter-strain divergence rather than to alterations in the expression of multiple genes as occurs in *S. typhimurium* (Smith *et al.*, 1990), *Campylobacter jejuni* (Nuijten *et al.*, 1991) and *Rhizobium meliloti* (Bergman *et al.*, 1991). As anticipated, *P. putida* flagellin genes share considerable homology with other flagellins in their amino- and carboxy-terminal domains (Fig. 7a). In *S. typhimurium* such domains were found to be essential for export and polymerization of flagellin monomers (Homma *et al.*, 1987a; Kuwajima *et al.*, 1989). Central areas of the flagellins are less well conserved, containing hypervariable, non-essential regions (Fig. 7a). Indeed the toxin gene of *Vibrio cholerae* has been introduced into one

such region of the flagellin gene of *S. typhimurium*; the resultant modified flagellin was assembled into flagella and the cells remained motile (Newton *et al.*, 1989). *P. putida* PaW8 flagellin contains a substantial region (residues 425–545) of excess amino acid sequence not comparable to other sequenced flagellins [except for a 10 amino acid sequence (residues 472–481) matching with PRS2000 flagellin; Fig. 5]. Computer-assisted comparison of available flagellin protein sequences indicated that the two *P. putida* flagellins and the *P. aeruginosa* flagellin formed a closely-related distinct sub-grouping, although PaW8 flagellin appeared to be more closely related to *P. aeruginosa* flagellin than to PRS2000 flagellin (Fig. 7b). The presence of variable amounts of peptide sequence in the central areas of flagellins may make such studies difficult to interpret, with differences between strains swamping species differences in closely-related groups.

Determination of the precise location of the binding site for MLV1 was beyond the scope of this work. However, the most likely candidate areas for the location of the MLV1 epitope, indicated by computer analysis, are residues 273–280 (GALKLDGT), 289–300 (DDVLSTALGLA) and 513–517 (FKKG). Of the three most likely candidate sites for the MLV1 epitope, one lies within the area of excess amino acid sequence of PaW8 flagellin (residues 513–517) and the other two (residues 273–280 and 289–300) also lie within central areas of relatively poor sequence conservation. This is consistent with work on *S. typhimurium* (Smith & Selander, 1990) and *Borrelia burgdorferi* (Rasiah *et al.*, 1992) suggesting that the central variable areas of flagellins often account for antigenic diversity.

Preliminary experiments indicate that the sequence information obtained in this study can be used to design oligonucleotide primers for polymerase chain reaction (PCR) amplification of flagellin gene DNA from fluorescent pseudomonads. This has potential applications in the study of genetic diversity amongst mixed populations of pseudomonads or as a rapid assessment of strain type in cases, such as *P. aeruginosa*, where flagellin size can be used as an indicator (Allison *et al.*, 1985; Kelly-Wintenberg & Montie, 1989). Genes such as those encoding flagellin offer an alternative to the study of populations using PCR amplification of 16S rRNA genes because of their potential for higher resolution at the subspecies level.

Basal body genes and proteins

Basal body structures consist of a number of rings (four: L, P, S and M, in *S. typhimurium*; five: L, P, E, S and M, in *Caulobacter crescentus*) mounted on a central rod (Khambaty & Ely, 1992; Stallmeyer *et al.*, 1989a, b). The *S. typhimurium* genes encoding L-, P-, and M-ring proteins (*flgH*, *flgI*, and *fliF*) have been sequenced (Jones *et al.*, 1989), as has the *flgI* region of *C. crescentus* (Khambaty & Ely, 1992).

The order of the genes in *P. putida* (*flgG*–*flgH*–*flgI*) corresponds to the same order within the *flgB* operon of *S.*

typhimurium, although analysis of the sequence directly downstream of *flgI* in *P. putida* suggests that no equivalent gene to *flgJ* lies immediately downstream of *flgI*. The deduced molecular masses of the putative *P. putida* FlgH and FlgI proteins were 24.5 kDa and 38.2 kDa respectively, compared to 24.7 kDa and 38.1 kDa for *S. typhimurium* FlgH precursor and FlgI precursor respectively (Jones *et al.*, 1989). In *S. typhimurium*, the FlgH and FlgI proteins undergo cleavage during export to the outer membrane (Homma *et al.*, 1987b, c). The *S. typhimurium* FlgH and FlgI polypeptides contain a predicted trans-membrane α -helix in the region of their amino-terminal signal peptides (Jones *et al.*, 1989). N-terminal signal peptides have a number of possible features (Oliver, 1985). There is often a basic amino terminus containing one to three positively charged amino acid residues. *P. putida* FlgH (residue 3) and FlgI (residue 5) both feature an arginine residue at the N-terminus. This is generally followed by a hydrophobic core of 14–20 neutral, primarily hydrophobic amino acid residues. The protein sequences of FlgH and FlgI were analysed using the algorithms of Chou & Fasman (1978), Garnier *et al.* (1978) and Kyte & Doolittle (1982). The putative *P. putida* FlgH protein contained no predicted transmembrane α -helix in its N-terminal region although there is a possible hydrophobic core of approximately 20 amino acids. The putative *P. putida* FlgI protein contained a 22 amino acid N-terminal hydrophobic region within which an α -helix (in the area of residues 14–22) was predicted by both the Chou-Fasman (1978) and Garnier *et al.* (1978) analyses. The *P. putida* FlgH protein sequence, particularly at the N-terminus, differed far more from the equivalent *S. typhimurium* protein than did the *P. putida* FlgI protein sequence (48.8% similarity compared with 66.7%). Despite the differences observed, it is possible that *P. putida* FlgH and FlgI both contain N-terminal signal peptides. In FlgH there is a possible cleavage site adjacent to the sequence LAG (Cys-19). In FlgI there are three possible cleavage sites but alignment with the *S. typhimurium* FlgI sequence suggests a site adjacent to AQA (Glu-23) to be the most likely.

The overall amino acid similarity between the FlgI proteins of *P. putida*, *S. typhimurium* and *C. crescentus* (Khambaty & Ely, 1992) is high. Although the sequence is incomplete, it is clear that the putative *flgG* gene of *P. putida* PaW8 shares considerable homology with the *S. typhimurium* *flgG* gene. The FlgH protein appears to be less well conserved than FlgI or FlgG, suggesting that its structural role allows more scope for amino acid sequence variation. Flagellar genes are organized in transcriptional units located apart on the bacterial chromosome (Macnab, 1992). Such multicistronic operons often display complex patterns of gene regulation involving a number of transcription factors forming a regulatory hierarchy to control the relative stoichiometries of various gene products required for the production of macromolecular assemblies. It may not be surprising therefore that a mutation in a *P. putida* basal body structural gene (*flgI*, encoding P-ring protein) could severely reduce the production of the flagellar filament protein, flagellin,

which requires for its assembly an intact basal body structure. Alternatively, it could be that flagellin protein is synthesized, not assembled correctly and excreted from the cell. Temporal and spatial control of synthesis has been observed in the control of flagellar assembly in *C. crescentus* (Champer *et al.*, 1987), *E. coli* (Komeda, 1986) and *S. typhimurium* (Kutsukake *et al.*, 1990). Indeed two *flgI* mutants of *C. crescentus* have been shown to possess reduced intracellular levels of 25 and 27.5 kDa flagellins (Khambaty & Ely, 1992). In *S. typhimurium* the gap between the *flgG* and *flgH* genes is unusually large and contains an inverted repeat sequence thought to be involved in the formation of a stem-loop structure that may play a regulatory role (Jones *et al.*, 1989). No such structure was observed in the smaller gap (36 bp compared to 64 bp) between the putative *flgG* and *flgH* in *P. putida*. The absence of such similarities coupled with the apparent lack of a *flgJ* gene immediately downstream of *flgH* suggests that the organization and regulation of basal body structural genes in *P. putida* and *S. typhimurium* may differ.

ACKNOWLEDGEMENTS

We would like to thank Professor Stephen Lory for the provision of strains and M. Partis for assistance with computer analysis. This work was supported by Project Grant GR3/7466A from the Natural Environment Research Council.

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Received 7 October 1993; revised 22 December 1993; accepted 1 March 1994.