Molecular Microbiology (2003) **50**(3), 809–824 doi:10.1046/j.1365-2958.2003.03740.x

A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in *Pseudomonas aeruginosa*

Nandini Dasgupta,[1†] Matthew C. Wolfgang,[2] Andrew L. Goodman,[2] Shiwani K. Arora,[1] Jeevan Jyot,[1] Stephen Lory[2] and Reuben Ramphal[1*]
[1]*Department of Medicine/Infectious Diseases, University of Florida,*Gainesville, Florida, 32610, USA.
[2]*Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts, 02115, USA.*

Summary

The single polar flagellum of *Pseudomonas aeruginosa* is an important virulence and colonization factor of this opportunistic pathogen. In this study, the annotation of the genes belonging to the *fla* regulon was updated and their organization was analysed in strains PAK and PAO1, representative type-a and type-b strains of *P. aeruginosa* respectively. The flagellar genes are clustered in three non-contiguous regions of the chromosome. A polymorphic locus flanked by *flgJ* and *fleQ* in Region I contains a glycosylation island in PAK. The expression and ordered assembly of the complex multicomponent flagellum is intricately regulated. Dedicated flagellar genes *fleQ*, *fleS*, *fleR*, *fliA*, *flgM and fleN* encode proteins that participate in the regulation of the flagellar transcriptional circuit. In addition, expression of the flagellum is coordinately regulated with other *P. aeruginosa* virulence factors by the alternative sigma factor σ^{54}, encoded by *rpoN*. In order to gain insight into the hierarchical regulation of flagellar genes, deletion mutations were constructed in *fleQ*, *fleR*, *fliA* and *rpoN*. The transcriptional impact of these mutations was examined by transcriptional profiling using a *P. aeruginosa* whole genome microarray. Analysis of the transcriptomes generated for each of these mutants indicates a four-tiered (Classes I-IV) hierarchy of transcriptional regulation. Class I genes are constitutively expressed and include the transcriptional regulator *fleQ* and the alternative sigma factor *fliA* (σ^{28}). Class II genes including *fleSR*, encoding a two-component regulatory system require FleQ and RpoN (σ^{54}) for their transcriptional activation. Class III genes are positively regulated by the activated response regulator FleR in concert with RpoN. The transcription of Class IV genes is dependent on the availability of free FliA following the export of the FliA specific antisigma factor FlgM through the basal body rod-hook structure (assembled from Class II and III gene products). Two previously uncharacterized genes, which are coordinately regulated with known flagellar genes have been identified by genome-wide analysis and their role in flagellar biogenesis was analysed.

Introduction

Flagella expressed on the surface of a number of bacterial species serve as a primary means of locomotion or motility. In many pathogenic bacterial species, flagellar motility is required in order to reach the site of infection as the first step in the establishment of a bacterial infection. A role for flagella in virulence has been demonstrated for numerous pathogenic bacteria including *Pseudomonas aeruginosa*, *Campylobacter jejuni*, *Proteus mirabilis*, *Vibrio cholerae* and *Helicobacter pylori* (Moens and Vanderleyden, 1996; Ottemann and Miller, 1997).

In *P. aeruginosa*, an opportunistic human pathogen that possesses a single polar flagellum, non-flagellated mutants have been shown to be defective in virulence in a burned mouse model of infection (Montie *et al.*, 1982) and demonstrate reduced invasion of cultured corneal epithelial cells (Fleiszig *et al.*, 2001). Moreover, disruption of *fliD*, the gene encoding the flagellar cap, reduces adhesion of *P. aeruginosa* to human respiratory mucin (Arora *et al.*, 1998). Despite the apparent importance of flagella in colonization and virulence, clinical isolates of *P. aeruginosa* from chronically colonized cystic fibrosis patients have a propensity to be non-motile (Mahenthiralingam *et al.*, 1994). Explanations for this phenomenon are still lacking but the absence of a flagellum during chronic colonization may be a means by which the organism evades a potent inflammatory response mediated by flagellin (Hayashi *et al.*, 2001) the most abundant flagellar protein. Thus, a complete understanding of the regulation of flagellum assembly in the organism may provide clues about the behaviour of this organism *in vivo* when more is known about genomic conservation in states of chronic colonization.

Accepted 25 July, 2003. *For correspondence. E-mail RAMPHR@medmac.ufl.edu; Tel. (+1) 352 392 2932; Fax (+1) 352 392 6481. †Present address: Department of Microbiology, University of Iowa, Iowa City, IA 52242, USA.

Expression of flagellar motility is an energetically expensive process, involving the expression of more than 40 different genes. The products of these genes include regulatory proteins, structural components of the flagellar secretion and assembly apparatus, proteins involved in generating the rotational motor force and the chemosensory machinery that controls flagellar-based chemotaxis. To ensure maximal efficiency and accuracy during flagellar biogenesis, bacteria utilize hierarchical regulatory networks involving transcriptional and post-translational mechanisms to control the ordered expression of the individual components of the flagellar organelle. Detailed studies of the regulation of flagellar assembly have been carried out in *Escherichia coli*, *Salmonella typhimurium* (Macnab, 1996), *Caulobacter crescentus* (Wu and Newton, 1997), *V. cholerae* (Prouty *et al*., 2001) and *V. parahaemolyticus* (Kim and McCarter, 2000; McCarter, 2001). A recent review has compared the different aspects of regulation in these organisms (Aldridge and Hughes, 2002). Whereas significant differences exist between the regulatory programs used by these organisms, a salient feature in all cases is that the flagellar genes can be classified based on their temporal expression and on their dependence on various nested transcriptional regulators.

In peritrichously flagellated *E. coli* and *S. typhimurium*, three classes of promoters have been described for the flagellar operons (Macnab, 1996). Class 1 consisting of the master regulator *flhCD*, is transcribed by σ^{70} (Kutsukake, 1997) and regulated by cAMP receptor protein CRP (Soutourina *et al*., 1999). The promoters directly activated by FlhCD comprise Class 2, which includes genes for various structural components and the alternative sigma factor FliA (σ^{28}). The Class 3 promoters require free FliA for their transcription and are dependent on the secretion of the FliA-specific antisigma factor FlgM through the assembled hook-basal body rod. The FliA–FlgM interaction thus regulates flagellar biogenesis via a post-translational mechanism.

In *C. crescentus*, a four-level gene hierarchy has been described in the biogenesis of its single polar flagellum. CtrA, the cell-cycle response regulator, occupies the first level and in turn activates Class II genes in response to cell cycle cues. The transcriptional factor FlbD and sigma factor RpoN, both Class II gene products, activate the transcription of the subsequent Class III and IV genes (Gober and England, 2000).

In the monoflagellate *V. cholerae*, a transcriptional hierarchy with four classes of genes has been described (Prouty *et al*., 2001). Class I consists of the transcriptional activator *flrA*. FlrA in association with σ^{54} (RpoN) transcribes the Class II genes, which code for structural and regulatory components including a two-component system (*flrBC*) and the alternative sigma factor σ^{28} encoded by *fliA*. Class III genes are dependent on FlrC and σ^{54} for their activation. They code for the structural components needed for the completion of the hook-basal body rod structure. Subsequent secretion of the anti-sigma factor FlgM through the assembled hook is postulated to allow transcription from the Class IV FliA-dependent genes.

In *P. aeruginosa*, transcription of flagellar genes has been shown to require a number of regulatory proteins including the transcription factor FleQ (Arora *et al*., 1997) and the two component system FleSR (Ritchings *et al*., 1995). Successful flagellar biosynthesis also requires three different sigma factors (σ^{70}, σ^{54} and σ^{28}) (Totten *et al*., 1990; Starnbach and Lory, 1992; Dasgupta *et al*., 2002). In addition, the FliA antisigma factor FlgM has been shown to modulate the activity of FliA and expression of the FliA-dependent flagellin gene fliC (Frisk *et al*., 2002). Although previous work has implicated these regulators in *P. aeruginosa* flagellar biosynthesis, interconnection of these regulatory components in a hierarchical expression circuit has not been established.

We took advantage of recently available resources and tools to gain an understanding of the interplay of the various flagellar regulators of *P. aeruginosa* and to construct a putative flagellar assembly pathway. These resources include the completed *P. aeruginosa* strain PAO1 genome sequence (Stover *et al*., 2000), the availability of an oligonucleotide microarray (GeneChip® *P. aeruginosa* Genome Array, Affymetrix) based on this sequence and a limited number of genes from other strains, and the increased collection of publicly available non-PAO1 sequence information. In this report, we present the organization of the various flagellar genes, their hierarchical regulation by RpoN, FleQ, FleR and FliA, and propose a model for the assembly of the flagellum in *P. aeruginosa*. In addition, we describe two novel *P. aeruginosa* genes involved in the biogenesis and function of the flagellum. Their role in flagellar biogenesis was hypothesized based on their coordinate regulation with known flagellar genes and confirmed by mutational analysis.

Results and discussion

Annotation and organization of the flagellar regulon in representative type-a and type-b P. aeruginosa *strains*

As a first step in studying the regulation of flagellar biogenesis, we carried out a thorough analysis to identify and annotate the genes present in the *P. aeruginosa* genome with a known or predicted role in flagellar biogenesis or function. This analysis was based on the complete genome sequence of *P. aeruginosa* strain PAO1 (Stover *et al*., 2000), information from the community-wide annotation project (available at http://www.pseudomonas.com), data deposited in GenBank by various investigators for other

P. aeruginosa strains and numerous studies carried out over the past decade. Previous studies have shown that *P. aeruginosa* strains can express one of two structurally distinct flagellin proteins encoded by the *fliC* gene, which are designated as type-a and type-b (Spangenberg *et al.*, 1996). The genome of the sequenced strain PAO1 encodes type-b flagellin. However strains expressing type-a flagellin represent the majority of clinical isolates surveyed (Arora *et al.*, 2001). As differences in flagellar components and genetic organization exist between type-b and type-a expressing strains, representative type-b strain PAO1 and type-a strain PAK were both annotated.

Based on the above resources, we were able to identify 41 genes encoding structural/assembly or regulatory components of the flagellar organelle in the sequenced PAO1 genome (Table 1). An additional nine

Table 1. Annotation of *P. aeruginosa* flagellar biogenesis genes.

Gene[a]	Flagellin Type[b]	Reference sequence[c] Type-b (PAO1)	Type-a (PAK)	Homology[d] (%)	Function[e]
flgB	a/b	PA1077	NA	NA	Basal body rod (H)
flgC	a/b	PA1078	NA	NA	Basal body rod (H)
flgD	a/b	PA1079	NA	NA	Hook cap scaffold (H)
flgE	a/b	PA1080	NA	NA	Hook (H)
flgF	a/b	PA1081	NA	NA	Basal body rod (H)
flgG	a/b	PA1082	NA	NA	Basal body rod (H)
flgH	a/b	PA1083	NA	NA	Basal body L ring (H)
flgI	a/b	PA1084	NA	NA	Basal body P ring (H)
flgJ	a/b	PA1085	unpublished	100	Flagellum specific muramidase (H)
flgK	a/b	PA1086	AF332547	87	Hook-filament junctional protein (H)
flgL	a/b	PA1087	AF332547	72	Hook-filament junctional protein (H)
vioA	a	NP	AF332547	NA	Flagellin glycosylation (E)
orfA	a	NP	AF332547	NA	Flagellin glycosylation (E)
orfB	a	NP	AF332547	NA	Flagellin glycosylation (E)
orfC	a	NP	AF332547	NA	Flagellin glycosylation (E)
orfD	a	NP	AF332547	NA	Flagellin glycosylation (E)
orfE	a	NP	AF332547	NA	Flagellin glycosylation (E)
orfF	a	NP	AF332547	NA	Flagellin glycosylation (E)
orfG	a	NP	AF332547	NA	Flagellin glycosylation (E)
orfH	a	NP	AF332547	NA	Flagellin glycosylation (E)
orfI	a	NP	AF332547	NA	Flagellin glycosylation (E)
orfJ	a	NP	AF332547	NA	Flagellin glycosylation (E)
orfK	a	NP	AF332547	NA	Flagellin glycosylation (E)
orfL	a	NP	AF332547	NA	Flagellin glycosylation (E)
orfM	a	NP	AF332547	NA	Flagellin glycosylation (E)
orfN	a	NP	AF332547	NA	Flagellin glycosylation (E)
fliC	a/b	PA1092	M57501 (flaA)	70	Flagellin (E)
fleL	a/b	PA1093	L81176 (flaG)	57	Filament length control (E)
fliD	a/b	PA1094	L81176	58	Filament cap, mucin adhesin (E)
fliS	a/b	PA1095	L81176	53	Filament elongation (H)
fliS′	a	NP	L81176 (orf126)	NA	Unknown
fleP[f]	a/b	PA1096	L81176 (orf96)	59	Type IV pili length control (E)
fleQ	a/b	PA1097	L81176	99	σ^{54} dependent transcriptional activator (E)
fleS	a/b	PA1098	L41213	99	Sensor kinase (H)
fleR	a/b	PA1099	L41213	99	σ^{54} dependent transcriptional activator
fliE	a/b	PA1100	L43507	99	Basal body component, MS ring/rod adapter (H)
fliF	a/b	PA1101	L43507	99	Basal body MS ring, mounting plate for motor/switch (E)
fliG	a/b	PA1102	L43507 (partial)	99	Motor/switch, mounted onto MS ring, rotor component (H)
fliH	a/b	PA1103	NA	NA	Negative regulator of FliI (H)
fliI	a/b	PA1104	NA	NA	ATPase (H)
fliJ	a/b	PA1105	NA	NA	Chaperone, export of hook proteins (H)
fliK	a/b	PA1441	NA	NA	Hook length control (H)
fliL	a/b	PA1442	NA	NA	Unknown
fliM	a/b	PA1443	L39832 (partial)	100	Motor/switch (H)
fliN	a/b	PA1444	L39832	99	Motor/switch (H)
fliO	a/b	PA1445	L39832	99	Flagellar export pathway (H)
fliP	a/b	PA1446	L39832 (partial)	100	Flagellar export pathway (H)
fliQ	a/b	PA1447	NA	NA	Flagellar export pathway (H)
fliR	a/b	PA1448	NA	NA	Flagellar export pathway (H)
flhB	a/b	PA1449	NA	NA	Flagellar export pathway (H)
flhA	a/b	PA1452	NA	NA	Flagellar export pathway (E)
flhF	a/b	PA1453	NA	NA	Polar flagellar site determinant (H)
fleN	a/b	PA1454	AF133657	100	Flagellar number regulator (E)

Table 1. *cont.*

Gene[a]	Flagellin Type[b]	Reference sequence[c] Type-b (PAO1)	Type-a (PAK)	Homology[d] (%)	Function[e]
fliA	a/b	PA1455	X61231	99	σ^{28}sigma factor (E)
cheY	a/b	PA1456	X61231	100	Switch regulator (H)
cheZ	a/b	PA1457	NA	NA	CheY phosphatase (H)
cheA	a/b	PA1458	NA	NA	CheY and CheB kinase (H)
cheB	a/b	PA1459	NA	NA	Chemoreceptor methylesterase (H)
motA	a/b	PA1460	NA	NA	Motor rotation (H)
motB	a/b	PA1461	NA	NA	Motor rotation (H)
cheW	a/b	PA1464	NA	NA	Positive regulator of CheA (H)
cheV	a/b	PA3349	NA	NA	Chemotaxis regulator (H)
cheR	a/b	PA3348	NA	NA	Chemoreceptor methylesterase (H)
flgA	a/b	PA3350	AY029221 (partial)	99	P ring assembly component (H)
flgM	a/b	PA3351	AY029221	99	σ^{28}-specific antisigma factor (E)
flgN	a/b	PA3352	AY029221	100	Initiation of filament assembly (H)

a. Unified nomenclature for genes encoding *P. aeruginosa* type-a and -b flagellar components and linked chemotaxis proteins. Genes are grouped according to their putative organization in operons.
b. Flagellin type specifies whether an allele of the gene is present in the type-a (a) and/or type-b (b) reference strain.
c. The source of the sequence for each gene is provided. Genes not present in a given reference strain are denoted NP. PA numbers refer to the unique identification number assigned to each gene as part of the PAO1 genome sequence annotation project (http://www.pseudomonas.com). GenBank accession numbers are given for strain specific sequences found in PAK. Parentheses indicate an alternative gene name was provided in the accession. NA indicates that the DNA sequence is not available for the PAK allele but that its existence is predicted based on the cross-hybridization of PAK genomic DNA to oligonucleotide probes derived from the corresponding PAO1 gene sequence.
d. Given is the per cent identity between type-a and -b strain alleles. NA signifies that only one sequence is available.
e. Function of the gene product was assigned based on studies conducted in *P. aeruginosa* (E) or predicted based on homology (H) to proteins of known function found in other flagellar systems.
f. The role of the *fleP* gene product in *P. aeruginosa* flagellar biogenesis is described in this work.

genes known or predicted to be involved in flagellar chemotaxis control were also included in the table of annotations based on their linkage to flagellar genes. Each of the 50 flagellar or linked chemotaxis genes identified in the PAO1 genome was assigned to one of 17 putative operons based on criteria described (Fig. 1; Table 1).

These operons are located in three regions of the chromosome (Fig. 1). Most of the structural genes coding for the basal body rod, rings, hook, filament, cap and basal body (*flgBCDE*, *flgFGHIJKL*, *fliCfleL*, *fliDS* and *fliEFGHIJ*) are clustered in Region I of the PAO1 genome. The transcriptional regulator *fleQ* and two-component system *fleSR* are also located within this region. Region II contains genes encoding the hook length regulator, switch, export apparatus, flagellar placement determinant, flagellar number regulator and alternative sigma factor FliA (*fliK*, *fliLMNOPQRflhB*, *flhA*, *flhFfleN*, *fliA*). This region also includes genes encoding motor and chemotaxis proteins (*cheYZ*, *cheAB*, *motAB*, *cheW*). Region III consists of genes coding for the flagellar export apparatus, antisigma factor (*flgA*, *flgMN*) and additional chemotaxis regulatory proteins (*cheVR*).

The complete genome sequence of a type-a strain is not currently available; however, from the numerous PAK-specific flagellar gene sequences that have been deposited in GenBank, sequence heterogeneity in the flagellar genes *flgK*, *flgL*, *fliC*, *fleL*, *fliD* and *fliS* found in Region I flanked by *flgJ* and *fleQ* have been reported between PAK and PAO1 (Table 1; Arora *et al*., 2001). This region also contains a polymorphic gene *fleP*, whose product plays a novel role in *P. aeruginosa* flagellar biogenesis (see below). Strain PAK as well as other type-a strains possess an additional gene in the *fliDS* operon, designated *fliS′* (because of its sequence similarity to *fliS*), which is located between *fliS* and *fleP* (Table 1; Fig. 1; Arora *et al*., 1998). The role of *fliS′* in flagellar biogenesis is unknown and it was included in this study because of its linkage and homology to *fliS*. Another defining difference between the studied type-a strain and the type-b strains is that all type-a strains carry a glycosylation island, a cluster of genes demonstrated to be involved in the glycosylation of the type-a flagellin (Arora *et al*., 2001). In strain PAK, the island is composed of 14 genes arranged in several putative operons and located between *flgL* and *fliC* (Table 1; Fig. 1). In the type-b strain PAO1, the island is replaced by three genes of unknown function and a putative glycosyltransferase (Arora *et al*., 2001).

Sequence comparison of 17 other PAK flagellar genes shows at least 99% identity to the equivalent PAO1 genes (Table 1). However, it remains to be determined whether additional evolutionary divergence has occurred between the type-a and -b flagellar systems as many PAK flagellar genes have yet to be sequenced. In order to examine potential sequence differences we have adopted a comparative hybridization approach utilizing GeneChip® *P. aeruginosa* Genome Arrays (Affymetrix). This commercial

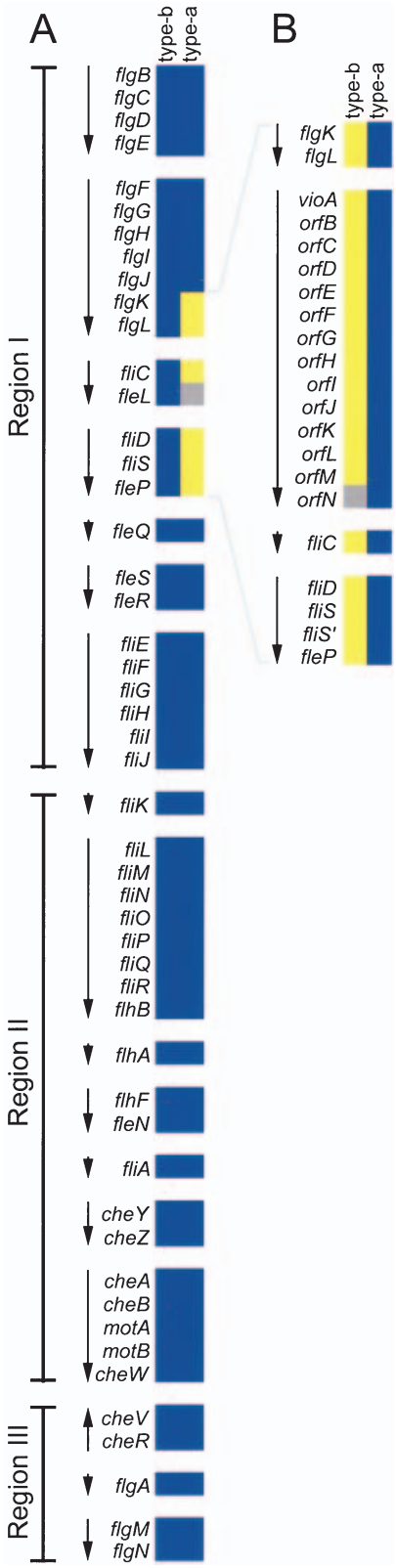

Fig. 1. Schematic representation of the organization of the flagellar and associated chemotaxis genes in three non-contiguous regions of the *P. aeruginosa* chromosome and their hybridization profiles (in colour) to type-b and type-a derived probes. Regions I, II and III span nucleotide position 1164275–1197833, 1570496–1593059 and 3760819–3763651, respectively, on the PAO1 chromosome. The rationale for assigning operon structures to groups of flagellar genes was based on the following criteria: (i) previous demonstration of their existence in a transcriptional unit; (ii) the lack of an appreciable intergenic region between genes; (iii) the presence of a rho-independent terminator sequence, and (iv) experimentally demonstrated activity of the putative promoter in the *P. aeruginosa* PAK background. A and B depict the organization and hybridization profile of the flagellar genes in PAO1 and a polymorphic locus in PAK respectively. Key to the colour coding for the hybridization profile: blue = hybridized, yellow = marginal hybridization and grey = hybridization not detectable. The arrows indicate the direction of transcription of the various operons.

oligonucleotide array carries probe sets for all annotated PAO1 genes as well as probe sets specific for the PAK alleles *flgK*, *flgL*, *fliC*, *fliD*, *fliS, fliS′, fleP* and the 14 glycosylation island genes *vioA-orfN.* Genomic DNA, prepared from both PAO1 and PAK, was fragmented, labelled and hybridized to separate GeneChips® as previously described (Wolfgang *et al*., 2003). Normalized hybridization intensities for each probe set have been included as supplemental data (Table S1). The hybridization profile is presented in Fig. 1. PAK genomic DNA showed levels of hybridization equivalent to that of PAO1 for all PAO1 flagellar gene probes with the exception of *flgK*, *flgL*, *fliC*, *fleL*, *fliD*, *fliS* and *fleP* mapping in Region I (Fig. 1A). The absence of hybridization to probes for these genes reflects the known sequence polymorphisms discussed above. The absence of, or marginal (*orfN*) hybridization of PAK DNA to the PAO1 sequence based probes indicates that significant sequence heterogeneity can be readily detected (Fig. 1B). Whereas minor sequence variation is likely to exist within the unsequenced PAK flagellar genes, relative to PAO1, the level of hybridization seen in Fig. 1A indicates that the set of genes sufficient for flagellar biogenesis in PAO1 are present in the PAK genome and they share a high degree of homology with PAO1 genes. However, this does not rule out the possibility that additional flagellar genes not identified in this study are present in type-a strains.

Transcriptional profiling in P. aeruginosa *and elucidation of the regulon controlling flagellar gene expression*

Previous reports have shown that the expression of some flagellar gene(s) necessary for the assembly of the flagellar organelle is under the control of several key regulatory factors including RpoN, FleQ, FleR and FliA (Totten and Lory, 1990; Starnbach and Lory, 1992; Ritchings *et al*., 1995; Arora *et al*., 1997; 1998; Jyot *et al*., 2002). The coordination of these regulators and their involvement in

the expression of other flagellar genes is unclear. In order to gain a more complete understanding of the regulatory pathways that control the expression of genes encoding flagellar components, we again utilized GeneChip® *P. aeruginosa* Genome Arrays (Affymetrix) to compare the expression of genes in wild type and isogenic *fleQ*, *fleR*, *fliA* and *rpoN* non-polar deletion mutants. Because most clinical isolates express type-a flagellin, analysis of the transcriptional regulatory network controlling flagellar expression was carried out in PAK, a model type-a *P. aeruginosa* strain. We have previously shown that of the 5900 probe sets included on the array 5678 hybridize to PAK genomic DNA (Wolfgang *et al*., 2003). For transcriptional profiling experiments only these 5678 probe sets were used. A complete list of the 5678 PAK specific probe sets used for transcriptional profiling is included as supplemental data (Table S2).

We first analysed the global effect of each of the deletion mutations on transcript levels (Table S2). Figure 2 summarizes the transcriptional profiling results, indicating the number of genes showing significantly altered transcript levels in each of the regulatory mutants relative to the wild-type parent. The identity of these genes and magnitude of transcriptional expression change in each mutant is provided as supplemental data (Tables S3-6). Although known flagellar genes exclusively showed reduced transcription in the mutants (consistent with previous studies), the genome-wide analysis showed additional genes significantly activated and repressed in the mutant backgrounds. Many of these genes showed altered expression in multiple mutants, indicating an overlapping regulatory network (Fig. 2).

Genes showing altered regulation in multiple mutants in the microarray studies were of particular interest. Forty-two genes showed significantly reduced expression in both *fleQ* and *rpoN* mutants (Fig. 2A). Of these, 26 showed reduced expression in the *fleR* mutant, indicating that their transcription was FleR dependent. The other 16 FleR-independent genes were not differentially regulated in the FliA mutant, therefore they could be categorized as genes directly regulated by FleQ. Because FleQ controls the expression of the *fleSR* operon, it is conceivable that FleQ indirectly controls the expression of the 26 FleQ/FleR/RpoN-dependent genes through the two-component system FleSR. 20 of these genes are known or predicted flagellar genes (Fig. 2A). They included 12 genes previously implicated in flagellar biogenesis, three genes that likely encoded motility/chemotaxis functions (PA1458, PA1459, PA2654), and PA1462 (which belongs to the ParA family of chromosome segregation proteins and may be part of the PA1458/59 operon) (Table 1). Of these *fliC*, *flgMN* and *fliS′* additionally displayed reduced transcription in the *fliA* mutant. Among the six genes not previously implicated in flagellar expression, five showed FliA dependence: PA1096 (*fleP*), PA3713, PA4310, PA4326, and PA4633 (Fig. 2B). Figure 3A shows the relative expression levels for 56 PAK genes that have been shown or implicated in flagellar biogenesis. Figure 3B indicates the location of previously mapped or deduced binding sites for the various transcription factors impli-

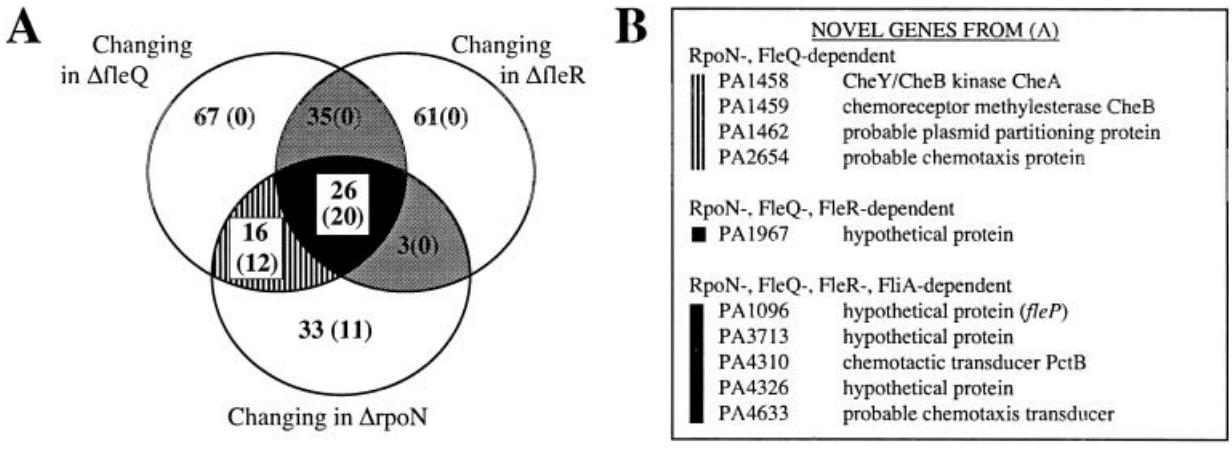

Fig. 2. Genomic analysis associates new genes with the flagellar biosynthesis regulatory hierarchy.
A. Venn diagram representation of genes showing altered expression between wild type and isogenic mutants. Numbers represent PAK-specific genes; numbers in parentheses represent the known flagellar biosynthesis genes annotated in Table 1. Standardized criteria were used to establish the subset of genes statistically changing between experiment and control; changing genes were filtered based on a minimum twofold change, a statistically significant difference from the control (*P*-value of 0.1), and a Present call in the upregulated condition as determined by Affymetrix Microarray Suite 5.0.
B. Genes coordinately regulated with known flagellar biosynthesis genes in two or more mutant backgrounds are listed. Area occupied by genes changing in (i) Δ*rpoN* and Δ*fleQ* mutants are hatched (ii) ΔrpoN, ΔfleQ and ΔfleR are darkened and (iii) either (ΔrpoN and ΔfleR) or (ΔfleQ and ΔfleR) are shaded. The third category of genes are not relevant in this study as there is a disparity in their pattern of expression and moreover none of the flagellar genes form a subset in it.

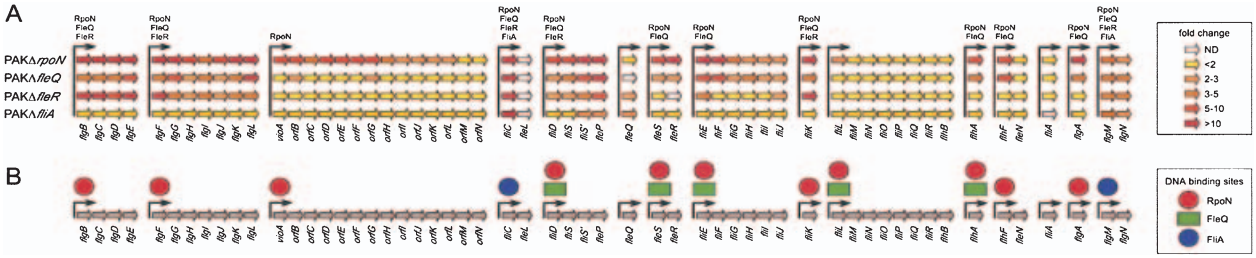

Fig. 3. Transcriptional profiling indicates hierarchical regulation of flagellar gene expression.
A. Presented is the average fold-change in expression of known flagellar biosynthesis genes in regulatory mutants relative to wild type. Values were derived from replicate transcriptional profiling experiments. Filled arrows represent Genes. Colour indicates the reduction in gene expression as fold-change relative to wild type. Genes shown in yellow are not transcriptionally altered whereas genes shown in shades of red are repressed in the indicated mutant strain. Values are defined in the key at the right. Data is not available for genes shown in white. Small black arrows represent the known or predicted promoter of the adjacent gene(s). Based on these results regulators predicted to contribute to the expression of each gene or operon are indicated above the corresponding promoter. Because of hierarchical regulation these predictions do not necessarily indicate direct effects. Numerical values and standard error of replicates can be found in supplemental data Table S1.
B. A summary of direct transcriptional control of flagellar gene promoters by known regulators is presented. Predicted or known DNA binding sites for each promoter are indicated for RpoN, FleQ and FliA. FleQ binding sites were determined based on gel shift analysis and DNase I footprinting experiments published elsewhere and discussed in the text. Binding sites for the sigma factors (RpoN and FliA) are predicted based on sequence analysis using experimentally derived consensus motifs.

cated in the regulation of expression of genes in the flagellar regulon. The chemotaxis genes mapping in Regions I, II and III were not included for further analyses.

The microarray results were validated using *lacZ*-reporter constructs in the *fleQ*, *fleR* and *fliA* mutant backgrounds. Because the *rpoN* deletion has a pleiotropic effect it was not included in the *lacZ* reporter assays. Promoters of genes (*vioA*-*orfN*) predicted to be involved in the glycosylation of flagellin was not included in the reporter assay since they would be analysed in a separate study on regulation of glycosylation of flagellin. In general, most of the FleQ, FleR and FliA regulatory effects were verifiable by the reporter assay (Table 2). However, microarray analysis revealed two discrepancies with previously published reports and with the promoter-*lacZ* reporter analysis. The operon transcribed from the promoter preceding the *fliD* gene was FleR-dependent by microarray analysis (Fig. 3A), but not by reporter assay (Table 2). The pattern of RNA abundance corresponding to the individual genes in the operon (Table S2) suggests that the *fleR* mutation may have resulted in enhanced degradation of the entire RNA transcript from the 3′ end, which would not be apparent from the analysis of promoter activity in a fusion construct. Similarly, in the microarray studies *fliS′* and *fleP* belonging to the same

Table 2. Analysis of the fold upregulation or downregulation of various *P. aeruginosa* promoters in a β-galactosidase (LacZ)-reporter assay.

Operon	Promoter construct	Fold[a] down (–) or up (+) regulation of promoter activity[b] in: PAK-ΔFleQ	PAK-ΔFleR	PAK-ΔFliA	Promoter positively regulated by:
flgBCDE	placΩflgE	–17 ± 1.2	–28.0 ± 1.3	1.0 ± 0.2	FleQ and FleR
flgFGHIJKL	placΩflgF	–2.5 ± 0.3	–2.7 ± 0.2	+1.9 ± 0.2	FleQ and FleR
fliCfleL	pPT269	–14.8 ± 1.2	–8.4 ± 1.0	–21.5 ± 1.4	FleQ,FleR and FliA
fliDSS′fleP	placΩD	–3.1 ± 0.2	+1.2 ± 0.1	+1.1 ± 0.1	FleQ
fleQ	placΩQ	–1.2 ± 0.1	–1.7 ± 0.1	–1.4 ± 0.1	None
fleSR	placΩS	–6.8 ± 0.8	1.0 ± 0.1	1.0 ± 0.1	FleQ
fliEFGHIJ	placΩE	–3.0 ± 0.4	+1.4 ± 0.1	+1.1 ± 0.0	FleQ
fliK	pfliK-lacZ in attB site	–1.8 ± 0.2	–1.7 ± 0.2	1.0 ± 0.0	FleQ and FleR
fliLMNOPQRflhB	placΩL	–3.1 ± 0.6	–1.4 ± 0.1	+1.2 ± 0.1	FleQ
flhA	placΩA	–23.6 ± 1.0	1.0 ± 0.1	1.0 ± 0.0	FleQ
flhFfleN	placΩF	–9.1 ± 0.5	1.0 ± 0.1	1.0 ± 0.1	FleQ
fliA	pMS565	+1.3 ± 0.1	+2.6 ± 0.1	1.0 ± 0.0	None
flgA	placΩflgA	–8.2 ± 0.9	+1.6 ± 0.2	+1.6 ± 0.2	FleQ
flgMN	placΩM	–2.1 ± 0.2	–2.0 ± 0.2	–1.9 ± 0.2	FleQ,FleR and FliA

a. The promoter activities were determined in the wild-type PAK strain and various mutants and the fold change calculated with respect to the wild type.
b. The promoter activity was determined using the β-galactosidase assay. Each fold change depicted is an average of two assays along with the standard deviation.

operon as *fliD* show reduced transcription in the *fliA* mutant background probably because of the enhanced degradation of these gene transcripts at the 3′ end of the operon.

The other discrepancy pertained to the transcripts for the *fliLMNOPQRflhB* genes, which did not display significant changes by microarray in any of the mutants, suggesting that the transcription of these genes was independent of FleQ, FleR, RpoN and FliA. However, previous promoter activity studies conducted in our laboratory on the *fliLMNOPQRflhB* promoter indicated that it is dependent on FleQ (Dasgupta *et al*., 2000; Jyot *et al*., 2002). We verified the previous work by examining the activity of a *fliL-lacZ* promoter fusion in both wild-type PAK and a fleQ mutant where a threefold difference in β-galactosidase activity was detected (Table 2). The disparity in microarray and reporter results for this particular operon cannot be accounted for by the overall low levels of expression (Table S2) and thus represents an exception to the trend of agreement between these independent analysis methods.

Transcriptional profiling and reporter analysis in the various mutant backgrounds allowed us to identify and distinguish direct and indirect effects of the regulatory factors on the expression of the flagellar regulon. The *fliCfleL* operon and the *flgMN* operon show dependence on RpoN, FleQ, FleR and FliA. The regulatory regions of these operons contain a consensus FliA (σ^{28}) binding site and putative RpoN binding sites. The initiation of transcription at sites lying in close proximity to the predicted FliA binding sites in *fliCfleL* and *flgMN* has been demonstrated previously by primer-extension analysis (Starnbach and Lory, 1992; Frisk *et al*., 2002). We therefore conclude that their expression involves a regulatory cascade involving RpoN, FleQ, FleR and FliA. Very likely, FleQ controls the RpoN-dependent expression of *fleSR*, and FleR indirectly regulates the FliA dependent expression of the *fliCfleL* and *flgMN* promoters by a mechanism that we discuss below.

In *P. aeruginosa*, the activity of FliA is modulated by its antisigma factor FlgM (Frisk *et al*., 2002). In other flagellated bacterial species including *S. typhimurium*, FlgM directly binds to FliA and prevents it from activating transcription of FliA-dependent promoters (Aldridge and Hughes, 2002). The inhibition is postulated to be reversed following secretion of FlgM through the hook-basal body rod structure as demonstrated in *S. typhimurium* (Karlinsey *et al*., 2000). As the synthesis of components of the hook-basal body rod structure in *P. aeruginosa* is dependent on FleQ, FleR and RpoN (Fig. 3A; Table 2) and the secretion of FlgM is dependent on the completion of the hook basal body rod, we hypothesize that the secretion of FlgM would be compromised in a *fleQ* or *fleR* mutant background.

Completion of the hook substructure serves as a checkpoint for the transcription of FliA dependent genes such as fliC

In order to test the hypothesis that in *P. aeruginosa* FliA activity is dependent on completion of the basal body rod-hook structure and subsequent secretion of the FlgM anti-sigma factor, synthesis of flagellin from the FliA dependent *fliC* gene was assessed in various mutants by SDS-PAGE and immunoblotting with FliC-specific antisera (Fig. 4). Lysates from wild-type PAK displayed immunoreactivity to a cluster of protein bands suggestive of glycosylated flagellin (lane 1). These bands were not detected in the negative control lysates of the *fliC* mutant as expected (lane 8). Reduced amounts of flagellin were detected relative to wild type in equivalent lysates from the *fleQ* (lane 2), *fleR* (lane 3), and *fliA* (lane 4) regulatory mutants. This reduction in flagellin synthesis is consistent with the reduction in *fliC* expression seen in these mutants (Fig. 3A and Table 2). To further test the hypothesis that the flagellin synthesis defect results from reduced secretion of FlgM through the basal body rod-hook structure, insertion mutants were constructed in *fliF* and *flgE*. The structural proteins FliF (MS ring) and FlgE (flagellar hook) are implicated in the successful assembly of the basal body rod-hook structure (Chilcott and Hughes, 2000). We were unable to detect flagellin in lysates of the *fliF* mutant (Fig. 4, lane 5) and significantly reduced amounts were present in the *flgE* mutant (lane 7) compared to the wild type. As the expression of *fliF* and *flgE* are dependent on FleQ and FleR, respectively (Fig. 3A), we conclude that the reduction in *fliC* expression seen in *fleQ* and *fleR* mutants can be accounted for by the reduced expression of these and other basal body rod-hook structure components, resulting in the intracellular accumulation of FlgM and the inhibition of FliA activity. In the lysate of a PAO

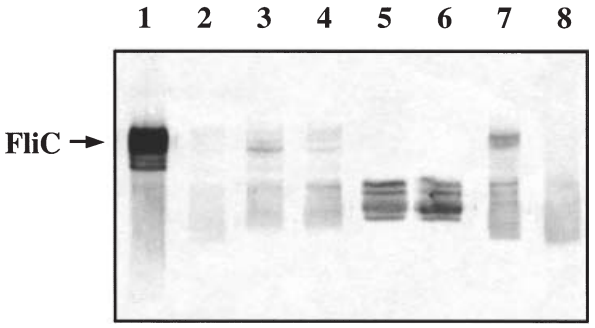

Fig. 4. Western blot analysis of various flagellar regulatory and structural mutants using anti-FliC antibodies. Whole cell lysates from wild-type PAK and isogenic mutants were separated by SDS-PAGE and subjected to Western blot analysis using FliC-specific antiserum. Lanes 1, wild type (PAK); 2 PAKΔFleQ; 3, PAK-ΔFleR; 4, PAK-ΔFliA; 5, PAK *fliF* mutant, 6, PAO *flhA* mutant; 7, PAK *flgE* mutant and 8, PAK-ΔFli*C*. The arrow shows the position of the immunoreactive glycosylated flagellin (FliC) in the PAK strain.

flhA mutant constructed for a previous study (Fleiszig and Arora, 2001) FliC was not detectable (Fig. 4, lane 6), thus indicating that in the absence of FlhA, predicted to be a structural component of the export apparatus, FliC expression was impaired. This can be best explained by the inability to export FlgM in the *flhA* mutant.

Coordinate regulation reveals new flagellar genes

Coordinate expression of genes can indicate functional relatedness between known and unknown genes. We used genome-wide transcriptional profiling to search for genes co-regulated with known flagellar biosynthesis operons. As shown in Fig. 2, we identified six genes not previously associated with flagellar expression or function whose expression was dependent on the flagellar transcriptional regulators FleQ, FleR, RpoN and in five cases FliA. Two of these genes (PA4310 and PA4633) have previously been annotated as chemotactic transducers. The remaining four genes (PA1967, PA1096, PA3713, PA4326) specify proteins of unknown function not previously implicated in flagellar biogenesis. In order to assess the potential role of the products of these genes, we have undertaken an analysis of strains carrying mutations in these hypothetical ORFs. We were able to successfully disrupt three out of the four genes; repeated attempts to engineer a mutation in PA4326 failed, suggesting that it may encode a protein whose basal level of expression is essential in *P. aeruginosa*. Among the engineered strains, mutations in PA1096 (PAK::*fleP*) and PA3713 (PAK::3713) resulted in reduced motility in the soft-agar motility assay (Fig. 5). Because of the effect of PA1096 on the *P. aeruginosa* pilus (below), we named this gene *fleP*. Motility of *P. aeruginosa* with a mutation in PA1967 was not reduced compared to the wild type.

To explore the basis for the observed motility phenotypes of these strains, we examined the *fleP* and PA3713 mutants by electron microscopy. Negative staining revealed that the average length of polar type IV pili in the *fleP* mutant (3.08 μm) were significantly longer than the pili in wildtype PAK (1.66 μm). The twitching motility phenotype of the *fleP* mutant was comparable to that of the wild-type PAK. The length of pili in a PA3713 mutant was comparable to the wild type. Complementing the *fleP* mutant with *fleP in trans* on a plasmid (pET-*fleP*) rescued the motility defect with an intermediate motility zone to that of the wild type and *fleP* mutant (Fig. 5). On electron microscopic examination in most of the *fleP* mutant bacteria, we observed that flagella had detached from the cells (data not shown). In the few cells that displayed an intact attached flagellum, the filament lacked the characteristic wavy contour. The polar type IV pili length and flagellar morphology in the pET-*fleP* complemented strain was comparable to the wild-type strain. We therefore infer that *fleP* is involved in a novel function responsible for maintaining the length of type IV pili and stable flagellar attachment to the bacterial pole. The flagellum of the PA3713 mutant appeared normal.

Flagellar structural genes are usually conserved across the genomes of flagellated bacteria, but differences in regulatory genes are not uncommon (Aldridge and Hughes, 2002). Peritrichous bacteria such as *E. coli* and *S. typhimurium* lack homologues of *fleQ*, *fleS*, *fleR*, *flhF*, *fleN*, and *fleL* found in the monoflagellates *V. cholerae*, *V. parahaemolyticus* and *P. aeruginosa*. More subtle differences in flagellar regulation may separate and distinguish the monoflagellate species from each other. *fleP* appears to represent a novel flagellar gene specific for genus *Pseudomonas*, although there appears to be sequence polymorphisms among the FleP homologues from different strains. The 96 amino acid FleP polypeptide from PAK is identical to a protein referred to as orf4 in strain DG1, a type-a strain (GenBank accession L43064) however, it

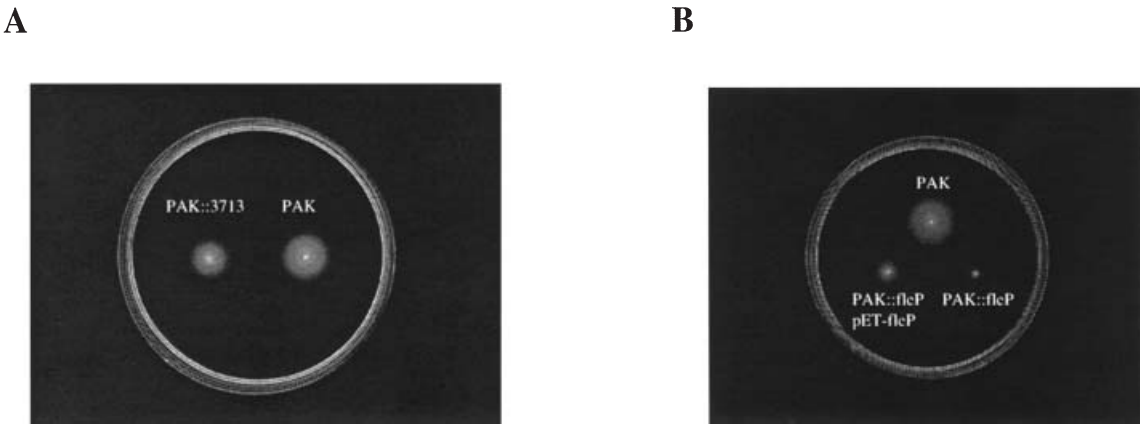

Fig. 5. Phenotypic characterization of the PAK::3713 and *fleP* mutants.
A. Motility phenotype of PAK wild type and PAK::3713.
B. Motility phenotype of PAK wild type, PAK *fleP* mutant and PAK *fleP* mutant containing pET-*fleP*.

shares only 43% identity with the corresponding protein encoded in the genome of PAO1 (Stover *et al*., 2000). Using the protein BLAST algorithm (Altschul *et al*., 1997), we identified two homologues of FleP in unfinished genome projects of *Pseudomonas putida* KT2440 and *Pseudomonas syringae* pv. syringae B728a, sharing 45% and 44% identity, respectively, with the *P. aeruginosa* PAK FleP. The genome of P. *fluorescens* Pf0–1 also contains a gene encoding a homologue of PAK FleP (36% sequence identity) (Casaz *et al*., 2001). In all of these *Pseudomonas* species, the *fleP* homologue is located in a large cluster of flagellar biogenesis genes, between homologues of *fliS* and *fleQ*.

The deduced sequence of PA3713 specifies a secreted protein of 620 amino acids. It showed significant level of relatedness (44% identity) to a hypothetical unknown protein identified as a translation product of a *Ralstonia metallidurans* gene (GenBank accession ZP00022954). Although its expression is dependent on RpoN, FleQ, FleR, and FliA, we were unable to identify any recognizable binding sites for RpoN and FliA upstream of this apparently monocistronic gene. The regulatory effects observed in the microarray analysis are therefore very likely indirect. Because no obvious morphological alterations were seen in the flagellar structure of the mutant lacking PA3713, we hypothesize that this gene product participates in flagellar function, perhaps in chemotaxis.

Transcriptional hierarchy of the P. aeruginosa *flagellar regulon consists of four classes of genes that are differentially regulated*

Peritrichously flagellated and monoflagellated bacteria show sequence similarities in flagellar proteins which suggest that their functions are conserved. The hierarchical organization of the regulatory networks that control flagellar biogenesis however, varies with flagellar number and placement. Bacteria with peritrichous flagella display a three-tiered hierarchy of transcriptional regulation of their *fla* regulon (Macnab, 1996), whereas the monoflagellates *V. cholerae* (Prouty *et al*., 2001), *V. parahaemolyticus* (Kim and McCarter, 2000) and *C. crescentus* (Gober and England, 2000) use a four-tiered hierarchy. The results presented here indicate that *P. aeruginosa,* a monoflagellate, utilizes a four-tier hierarchy (Fig. 6) involving the RpoN, FleQ, FleS, FleR, FliA and FlgM regulatory proteins to control and coordinate the transcription of the flagellar regulon.

Class I genes are externally regulated

Based on our present understanding of flagellar biogenesis, FleQ is the master regulator of the flagellar regulon in *P. aeruginosa*. It directly or indirectly regulates the expression of the majority of flagellar gene promoters (Fig. 3A;

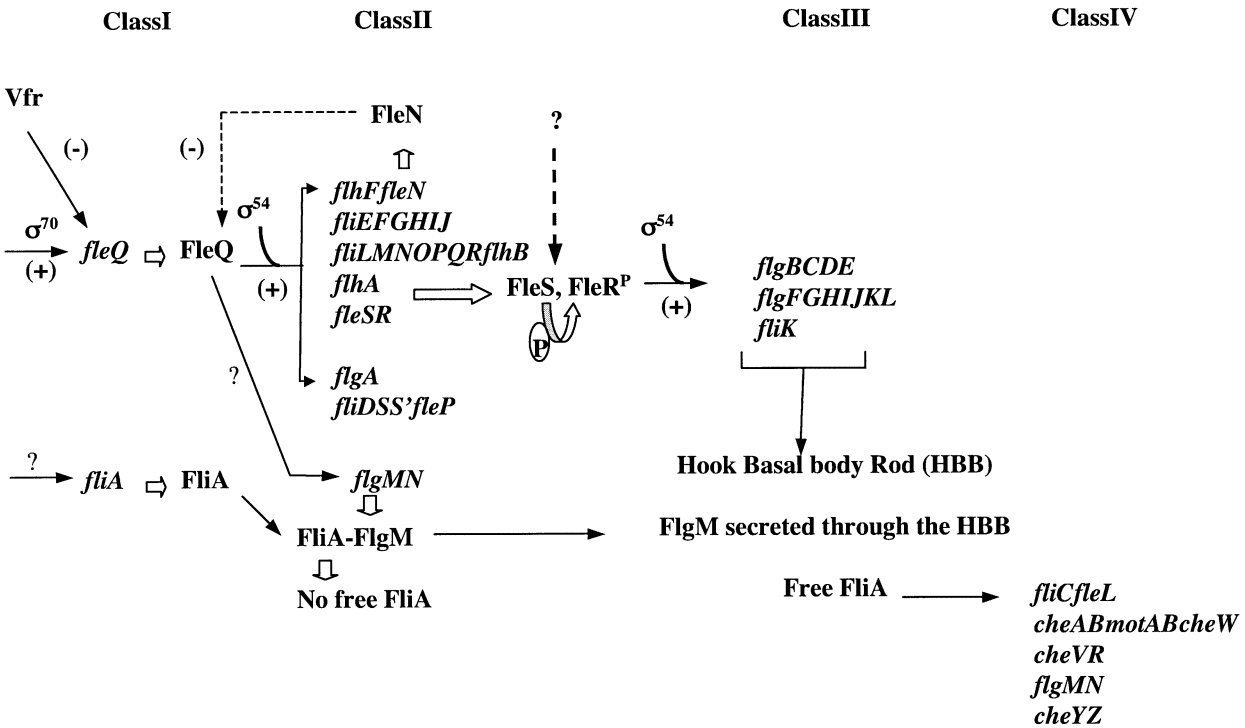

Fig. 6. Transcriptional hierarchy of the various flagellar genes (Class I–IV) in *P. aeruginosa*. (–) and (+) denote negative and positive regulation, respectively.? denotes an unknown factor(s). Phosphorylation of FleR by FleS is denoted by transfer of phosphate 'P'.

Table 2) with the exception of *fliA*. Consequently, we have grouped the promoters of *fleQ* and *fliA* as Class I. Although these promoters are primarily regulated by factors outside of the flagellar regulon (Fig. 6), they are independently controlled. In a recent report (Dasgupta *et al*., 2002) we provide evidence indicating the involvement of σ^{70} in the transcription of the *fleQ* gene and its repression by Vfr, the *P. aeruginosa* homologue of *E. coli* CRP. Additionally, *fleQ* expression appears to be modestly influenced by any disruption of the flagellar biogenesis pathway (Fig. 3A; Table 2).

The molecular mechanism that controls the expression of *fliA* remains unknown. The transcription of *fliA* appears to be constitutive and not dependent on σ^{54} or other flagellar regulators tested here (Fig. 3A; Table 2). The putative *fliA* promoter region was analysed for the presence of a σ^{70} consensus, although a complete consensus binding site was not identified, a putative –10 σ^{70} binding site with a four out of six match was found. The potential binding site was subjected to site directed mutagenesis and the activity of the altered promoter (placΩmutfliA) was compared to that of the wild type using a *lacZ* fusion. The mutation did not affect *fliA* promoter activity (data not shown). It therefore appears that *fliA* transcription is initiated by a novel mechanism, perhaps involving an as yet uncharacterized sigma factor.

Class II genes require FleQ and RpoN (σ^{54}) for transcriptional activation

Promoter fusions and microarray studies indicate that transcriptional activation of *fleSR, fliEFGHIJ*, *flhA, flhF-fleN, flgA* and *fliLMNOPQRflhB* genes are directly regulated by FleQ and σ^{54}, and consequently are classified as Class II (Fig. 3; Table 2; Fig. 6). The *fliDSS′fleP* operon shows dependence on FleQ in the reporter assays (Table 2, Arora *et al*., 1998) but the microarray data suggests that the operon is both FleR and FleQ dependent. However, Western blots show that the amount of the FliD protein synthesized in a *fleR* mutant is comparable to the amounts synthesized in the wild-type PAK strain, and it is drastically reduced in a *fleQ* mutant (unpubl. obs.). Therefore we have classified the *fliDSS′fleP* operon as FleQ dependent belonging to Class II (Fig. 6).

The Class II genes encode structural components of the basal body, MS ring, P ring, motor, switch, flagellar export apparatus and the filament cap. Regulatory proteins expressed from Class II genes include FlhF, FleN, FleS and FleR (Table 1). FlhF is the polar flagellar determinant in *P. putida* (Pandza *et al*., 2000) and its close homologues are present in the genomes of a variety of monoflagellated Gram-negative microorganisms. It is very likely that FlhF determines flagellar localization in other polar flagellates including *P. aeruginosa*. FleN, the antiactivator of FleQ, downregulates FleQ activity through direct interactions and thereby plays a crucial role in maintaining a single flagellum (Dasgupta *et al*., 2000; Dasgupta and Ramphal, 2001). FleS and FleR comprise a two-component system in which FleS is a sensor kinase for the response regulator FleR (Ritchings *et al*., 1995). In *V. cholerae*, the FleS homologue FlrB activates the FleR homologue FlrC by phosphorylation (Correa *et al*., 2000). We therefore hypothesize that in *P. aeruginosa*, the phosphorylation-dependent activation of FleR by its cognate sensor kinase FleS is necessary for the transcriptional progression from Class II to Class III promoters and serves as an additional check point in the flagellar biogenesis of this organism.

The majority of sensor kinases are integral membrane proteins, which activate their cognate response regulators in response to external stimuli. Analysis of the predicted secondary structure of FleS indicates that it is a cytoplasmic protein. The signal sensed by *P. aeruginosa* FleS is unknown; given its probable localization to the cytoplasm, the signal is unlikely to originate from the extracellular environment.

Class III genes require activated FleR and σ^{54}

The promoters of operons *flgBCDE*, *flgFGHIJKL* and *fliK* are grouped under Class III as their expression required RpoN, FleQ and FleR (Fig. 1A; Table 2; Fig. 6). The apparent involvement of FleQ is an indirect consequence of the *fleSR* promoter being FleQ dependent (Class II). The expression of the *fleSR* promoter also requires RpoN as do all other Class II promoters. In addition to its indirect effect RpoN is also required for the expression of Class III promoters as evidenced by the identification of σ^{54} binding sites in each promoter element (Fig. 3B). Class III promoters control the expression of genes coding for the basal body-rod, L ring, hook, hook-cap scaffold and hook-filament junctional proteins (Table 1).

Class IV genes require the gene products of the preceeding classes and FliA

Among the flagellar operons the transcription of two operons namely, *fliCfleL* and *flgMN* were significantly reduced in the *fliA* mutant (Fig. 3A; Table 2). This is consistent with earlier reports showing fliC and *flgMN* to be FliA (σ^{28}) dependent (Starnbach and Lory, 1992; Frisk *et al*., 2002). The *cheABmotABcheW*, *cheVR* operons showed a similar trend (Table S1, data not shown) and all genes showing a FliA dependence were grouped under Class IV.

Unlike the other Class IV gene, *fliC*, which lost most of its promoter activity in the *rpoN*, *fleQ*, *fleR* and *fliA* mutants, the *flgMN* promoter retained partial transcriptional activity in all the mutant backgrounds (data not

shown). A similar observation with the *flgMN* promoter was made in an earlier report (Frisk *et al*., 2002). This indicated that the observed basal level of its transcription was independent of RpoN, FleQ, FleR and FliA. It probably depends on another unknown transcriptional or sigma factor not examined in this study. The regulation of *flgM* is multifactorial in the enteric sysytem. In *S. typhimurium*, the *flgM* promoter is dually regulated by FlhCD and FliA and is classified under Class 2 and 3a respectively (Macnab, 1996). We hypothesize, that in *P. aeruginosa*, FlgM synthesized in a FliA independent manner binds to free FliA and inhibits its activity initially. Following the simultaneous expression of other Class II and III genes that contribute to the completion of the hook-basal body rod structure, FlgM is then secreted by a similar mechanism as described for *S. typhimurium* (Karlinsey *et al*., 2001) allowing for Class IV gene activation by free FliA (Fig. 6).

By adopting a detailed order of transcription of the flagellar genes as seen in other flagellated bacteria*, P. aeruginosa* functions to make flagellar synthesis more efficient by temporally and spatially regulating transcription to express proteins when needed in an ordered manner.

Model proposed for flagellar assembly in P. aeruginosa

In free-swimming *P. aeruginosa*, FleQ is the master regulator of flagellar biogenesis. However, there is no distinctive consensus sequence in the FleQ binding sites of the FleQ regulated Class II genes examined (Jyot *et al*., 2002). The lack of a FleQ binding consensus in these gene promoters suggests that FleQ probably has variable affinity for the different promoters it controls. This may represent a mechanism by which this single transcription factor could control the timing and level of expression of various components required at different stages in the flagellar assembly process. A similar mechanism controlling the expression of flagellar gene promoters has been described in *E. coli* (Kalir *et al*., 2001).

The proposed model for the assembly of the single polar flagellum *in P. aeruginosa* presented in Fig. 7 is based on the present understanding of transcriptional and post-translational regulation of the various flagellar genes. Initiation of the assemblage is most likely to begin with FlhF determining the polar placement site for the new flagellum. FlhF is thus an example of a Class II gene product that is required early on in the biogenesis of this organelle. The other Class II gene products comprising the MS ring, switch, basal-body, and export apparatus are subsequently assembled in the maturing flagellum. FliD, the filament cap protein, though expressed from a Class II gene is probably not assembled into the growing flagellar organelle until the hook and the hook-filament junctional proteins expressed from Class III genes are incorporated into the structure. Therefore though *fliD* is a

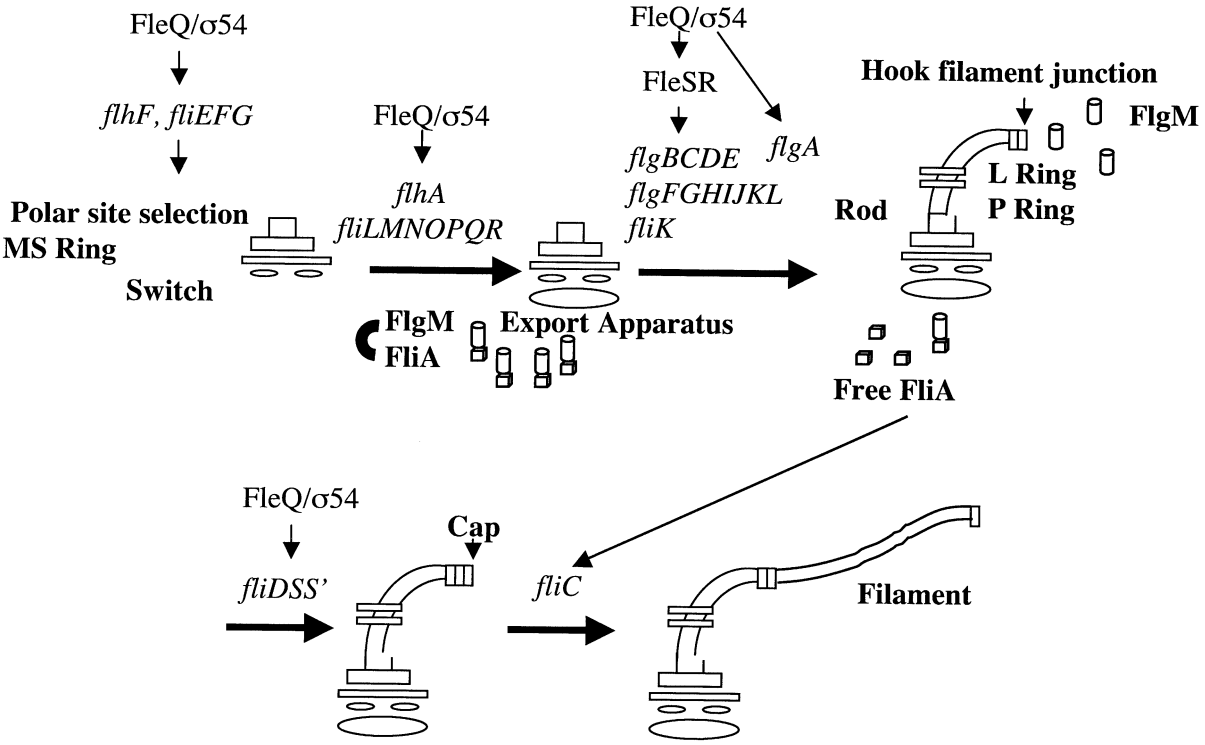

Fig. 7. Model proposed for flagellar assembly in *P. aeruginosa*.

class II gene, its product is required later in the assembly process. Meanwhile, simultaneous synthesis of FleN, another Class II gene product, helps maintain the monoflagellate status by inhibiting FleQ activity and down-regulating further synthesis of most of the structural components. The FleSR two-component system subsequently activates the Class III genes coding for proteins needed for the completion of the hook-basal body structure. This allows secretion of the antisigma factor FlgM and consequent transcription of FliA dependent genes coding for flagellin (FliC) and some chemotaxis proteins. *fleL,* a gene co-transcribed with *fliC*, implicated in the filament length control, maintains the flagellar filament length through a mechanism that yet remains to be understood. The *Pseudomonas* specific Class II gene product FleP influences stability of the mature flagellar filament by an unknown mechanism.

Experimental procedures

Promoter analysis and operon assignment

The TERMINATOR SEARCH program in the GCG Wisconsin package was used to search for factor independent transcriptional terminators. Intergenic regions greater than 100 base pairs were analysed for promoter activity, using the β-galactosidase reporter assay.

Genome content analysis

Genomic DNA hybridizations were preformed as previously described (Wolfgang *et al*., 2003). Genome arrays (Affymetrix) were washed and scanned according to the manufacturers cDNA protocol. Hybridization intensity data was extracted from the scanned array images and intrachip normalizations were performed using Affymetrix Microarray Suite 5.0 software. The mean average difference of the probe sets was scaled to 500 for interchip comparisons. A presence/absence determination was made by comparing hybridization intensity between strains. For PAO1 based probes values derived from the PAO1 genomic DNA hybridization were used as baseline. For the PAK based probes hybridization values for PAK DNA were used as baseline. Probe sets with a ratio of greater than 0.25 were considered present. Ratios between 0.1 and 0.25 were considered marginal and below 0.1 was designated absent.

Construction of P. aeruginosa *mutant strains*

The *fleQ* (PAKΔFleQ), *fleR* (PAKΔFleQ), *fliA* (PAKΔFliA) and *fliC* (PAKΔFliC) deletion mutants were derived from the wild-type strain PAK by introducing mutant alleles into the chromosome as described (Hoang *et al*., 1998). The mutant alleles for *fleQ* and *fleR* deleted for residues 2–475 for FleQ and 2–471 for FleR, respectively, were constructed in a plasmid by ligating two 1 kb fragments flanking the desired region to be deleted. The flanking regions were amplified by polymerase chain reaction (PCR) using the chromosomal DNA of PAK as template, specific primers enlisted in Table 3 and Taq DNA polymerase (Invitrogen). The fleQ flanking regions were amplified using primer pairs QKD1-QD2 and QD3-QD4. Primer pairs RD1-RD2 and RD3-RD4 were used to amplify the fleR flanking regions. The resulting PCR products were either directly cloned into the pCR2.1 TOPO vec-

Table 3. List of primers used in this study.

Primer	Sequence of the primer 5′→3′
For mutant construction	
QKD1	cccaaagaattccggtagccgaccgatatagagcg
QD2	ccggctcatgccgtacttgcgcggtaccattttgatcagctgccttgcatcg
QD3	cgatgcaaggcagctgatcaaaatggtaccgcgcaagtacggcatgagccgg
QD4	cccaaagaattcgtgtcgcgattgcacagcagctc
RD1	cccaaagaattcggagctggcggaaaaggagcgc
RD2	gaacatggcgagcactcagatggcgggatccatggggttactcctgaatcgcag
RD3	ctgcgattcaggagtaaccccatggatcccgccatctgagtgctcgccatgttc
RD4	cccaaagaattccagcgcctgttccttgtcgagg
Promoter cloning	
FlgA5′	cccaaagaattcgtcaggtaatgctcgcggccggcg
FlgA3′	cccaaaggatccggctgttgttagccgcgcacagg
FlgF5′	cccaaaggatccctggccaacttcgccaacatccag
FlgF3′	cccaaaggatccgatgttcgccaggttgttggcatg
CheW5′	cccaaagaattccttcgagtgcctgctgttcgacgtc
CheW3′	cccaaaggatccggaaggtgacccactgcaggatcgga
CheY5′	cccaaagaattctgcaccgcaacacgcgcat
CheY3′	cccaaaggatccccaagtcccgcaagaggttc
CheA5′	cccaaagaattcagcgcatcgaacagttcctc
CheA3′	cccaaaggatccattgctcgagaatctcgccg
CheV5′	cccaaaggatccactgccttcgcaactgatgcgc
CheV3′	gggtttggatccaagcagctccagacgggtctgtc
FlgL5′	cagctacgaattcgagctcagcgg
FlgL3′	cccaaaggatccggttcaggtccgcctagttgccg
MotA5′	cccaaaggaccaaagttgccgccgacgatggcgacga
MotA3′	cccaaagaattcaacttcccggcgccgctggtactgat
fliKbam25	cccaaaggatccgagcgctgggtaagggcgatg
fliKbam23	cccaaaggatccttcttcgcggtctccttggcatac

tor (Invitrogen) or digested with restriction enzymes to facilitate cloning into pUC19. These recombinants were further manipulated to ligate the respective fragments flanking either *fleQ* or *fleR*. The insert from the resulting plasmid was excised and cloned into pEXGwGm resulting in pEX-Q1234 and pEX-R1234 harbouring the *fleQ* and *fleR* mutant alleles respectively.

The mutant allele for *fliA* and *rpoN* was assembled by essentially deleting most of the coding region of the gene using a modified version of a PCR based technique termed splicing by overlap extension (SOE) (Warrens *et al*., 1997). Chromosomal DNA from strain PAK was used as template and the PCR carried out using the GC-Rich PCR system (Roche). Flanking primers were designed to anneal 800–1200 base pairs upstream and downstream of each deletion/splice junction respectively. These primers were tailed with *attB1* and *attB2* sequences, respectively, as described in the Gateway Cloning Technology Instruction Manual (Invitrogen). The resulting PCR products were cloned using gateway technology into pEXGmGW, which was adapted from pEX18Gm (Hoang *et al*., 1998) using the gateway Vector Conversion Kit (Invitrogen).

The *fliF* (Arora *et al*., 1996), mutant constructed by insertion of an antibiotic resistance marker into the coding region has been previously described. For the construction of the *flgE* and *fleP* mutants, a gentamicin cassette was inserted into a unique restriction site in the coding regions of the genes thereby disrupting the reading frame. This was achieved by allelic exchange of the wild-type chromosomal copy of the gene for a disrupted plasmid copy based on homologous recombination. pBS-*flgE*Gm and pKΔ4.5PGm containing the disrupted plasmid copy for *flgE* and *fleP* respectively were electroporated into the PAK strain and selected for double crossovers displaying gentamicin resistance, thus generating the *flgE* and *fleP* mutants respectively.

All the mutants were confirmed by PCR analysis for their respective deletion or insertion and tested in a motility assay for non-motility.

Rapid gene disruption

Candidate flagellar genes PA1967 and PA3713 were mutagenized by a previously described technique (Wolfgang *et al*., 2003). Briefly, an internal fragment of each target gene was amplified by PCR an tailed with *attB1* and *attB2* sequences by inclusion in the PCR primers. The products were cloned using Gateway Technology (Invitrogen) in pEXGmGW. Chromosomal integration was selected as described (Hoang *et al*., 1998) and maintained by the propagation in the presence of gentamicin (75 μg ml^{-1}).

*Construction of promoter-*lacZ *fusions in pDN19lacΩ*

Putative promoter containing regions (500–650 bp) located upstream of various genes including, *flgB*, *flgF*, *fleL*, *flgA*, *cheY*, *cheA* and *cheV* were amplified from the PAK genome by PCR using Pfu polymerase (Stratagene) and the products cloned in the appropriate orientation upstream of the promoterless *lacZ* in pDN19lacΩ yielding placΩflgB, placΩflgF, placΩfleL, placΩflgA, placΩcheY, placΩcheA, and placΩcheV, respectively. A 5′ and a 3′ primer designed to amplify the respective promoter regions of each gene is listed in Table 3. The results of the promoter studies with the chemotaxis genes have not been addressed in this study. The putative *fliK* promoter region was cloned into the mini-CTXlacZ vector upstream of the promoterless lacZ, resulting in pfliK-lacZ. This plasmid was integrated into the attB site of the various strains tested. The rest of the promoter constructs used in this study were available in the laboratory.

RNA extraction, labelling and hybridization

Overnight bacterial cultures were diluted to a starting OD_{600} of 0.01 in 100 ml of LB medium and grown with shaking (300 r.p.m.) at 37°C until an OD_{600} of 0.5. Cells harvested by centrifugation, RNA was isolated and cDNA was synthesized according to the Affymetrix Expression Analysis Protocol Guide. Briefly, 12 μg of RNA from each sample was converted to cDNA using Superscript II Reverse transcriptase (Invitrogen) and a semirandom decamer NS5. The reaction also included RNA spike controls provided by the Cystic Fibrosis Foundation. The resulting cDNA was purified, partially digested with DNase I and end-labelled with ddUTP-biotin according to the Expression Analysis Protocol (Affymetrix). The resulting targets were hybridized to GeneChip *P. aeruginosa* Genome Arrays. The arrays were washed, stained and scanned according to the manufacturer's recommendation.

Microarray analysis

Hybridization intensity data was extracted from the scanned array images and intrachip normalizations were performed using Affymetrix Microarray Suite 5.0 software.

Electron microscopy

Static cultures were grown overnight at 37°C with the respective antibiotics when needed. A drop of the culture was allowed to adhere to a carbon-coated grid for 10 s, drained off, the grid rinsed in a drop of saline and adherent cells negatively stained with a 2% aqueous solution of phosphotungistic acid for 10 s. Samples were examined with a Hitachi H-7000 transmission electron microscope. The average length of the type IV pilus was determined by measuring the lengths of 10–12 pili on an electron microscope.

Site directed mutagenesis

Primer pairs fliAmut1and fliAmutcomp2 were used in the Quick-change site directed mutagenesis kit (Stratagene, La Jolla, CA) to generate pBS-mutfliApr according to the protocol provided in the kit. Briefly, 20 ng of column purified (plasmid mini kit, Qiagen, Valencia, CA) plasmid template (pBS-fliApr) was used in a 50 μl amplification reaction containing 1 μl of dNTPs, 1 μl of Pfu polymerase, 1.25 μl of each primer and 5 μl of reaction buffer. It was subjected to a cycling profile of initial denaturation for 30 s at 95°C, followed by 13 cycles of denaturation (95°C for 30 s), annealing (60°C for 1 min) and extension (68°C for 8 min). The contents were then treated with *Dpn*I to digest the original plasmid template. One microlitre of the post-digestion amplification reaction

was used to transform *E. coli* XL1 blue cells and transformants were selected on LB ampicillin (100 μg ml^{-1}) plates. A clone with the desired site-specific mutagenesis confirmed by sequencing using the T7 primer was subsequently used for further characterization. The insert from pBS-mutfliApr was cloned upstream of the promoterless lacZ in pDN19lacΩ in the right orientation to yield placΩmutfliA.

β-Galactosidase assay

The promoter-*lacZ* fusions and the vector pDN19lacΩ were electroporated into wild-type PAK and mutants PAK-ΔFleQ, PAK-ΔFleR and PAK-ΔFliA. The strains were grown to late log phase (A_{600} of 0.7–1.0) in LB medium containing either streptomycin or tetracycline and their β-galactosidase activity assayed (Miller, 1972).

SDS-polyacrylamide gel electrophoresis (PAGE) and Western analysis

Three microlitres of the bacterial lysate (cell pellet from 1.0 ml of bacterial culture resuspended and boiled in 100 μl of SSB) was resolved on a 10% polyacrylamide gel in duplicate. Proteins in one set were stained with Coomassie brilliant blue and from the other transferred to a PVDF membrane (Sambrook *et al.*, 1989). The blot was developed using rabbit anti-FliC antibodies as the primary antibody and alkaline phosphatase conjugated anti-rabbit IgG as the secondary antibody. NBT and BCIP served as the substrate for the colour reaction.

Acknowledgements

This work was supported by NIH grant AI45014 to R.R. GeneChipâ *Pseudomonas aeruginosa* Genome Arrays were subsidised by Cystic Fibrosis Foundation Therapeutics. M.C.W. was supported by a Cystic Fibrosis Foundation Postdoctoral Research Fellowship. A.L.G. is a Howard Hughes Medical Institute Predoctoral Fellow.

Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi3740/mmi3740sm.htm

Table S1. Normalized hybridization intensities for each probe set.

Table S2. List of 5678 PAK specific probe sets used for transcriptional profiling.

Table S3. Identity of FleQ dependent PAK genes and magnitude of transcriptional expression change in each mutant.

Table S4. Identity of FleR dependent PAK genes and magnitude of transcriptional expression change in each mutant.

Table S5. Identity of FliA dependent PAK genes and magnitude of transcriptional expression change in each mutant.

Table S6. Identity of RpoN dependent PAK genes and magnitude of transcriptional expression change in each mutant.

References

Aldridge, P., and Hughes, K.T. (2002) Regulation of flagellar assembly. *Curr Opin Microbiol* **5:** 160–165.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25:** 3389–3402.

Arora, S.K., Ritchings, B.W., Almira, E.C., Lory, S., and Ramphal, R. (1996) Cloning and characterization of *Pseudomonas aeruginosa fliF* necessary for flagellar assembly and bacterial adherence to mucin. *Infect Immun* **64:** 2130–2136.

Arora, S.K., Ritchings, B.W., Almira, E.C., Lory, S., and Ramphal, R. (1997) A transcriptional activator, FleQ, regulates mucin adhesion and flagellar gene expression in *Pseudomonas aeruginosa* a cascade manner. *J Bacteriol* **179:** 5574–5581.

Arora, S.K., Ritchings, B.W., Almira, E.C., Lory, S., and Ramphal, R. (1998) The *Pseudomonas aeruginosa* flagellar cap protein, FliD, is responsible for mucin adhesion. *Infect Immun* **66:** 1000–1007.

Arora, S.K., Bangera, M., Lory, S., and Ramphal, R. (2001) A genomic island in *Pseudomonas aeruginosa* carries the determinants of flagellin glycosylation. *Proc Natl Acad Sci USA* **98:** 9342–9347.

Casaz, P., Happel, A., Keithan, J., Read, D.L., Strain, S.R., and Levy, S.B. (2001) The *Pseudomonas fluorescens* transcription activator AdnA is required for adhesion and motility. *Microbiology* **147:** 355–361.

Chilcott, G.S., and Hughes, K.T. (2000) Coupling of flagellar gene expression to flagellar assembly in *Salmonella enterica* serovar Typhimurium and *Escherichia coli*. *Microbiol Mol Biol Rev* **64:** 694–708.

Correa, N.E., Lauriano, C.M., McGee, R., and Klose, K.E. (2000) Phosphorylation of the flagellar regulatory protein FlrC is necessary for *Vibrio cholerae* motility and enhanced colonization. *Mol Microbiol* **3:** 5743–5755.

Dasgupta, N., and Ramphal, R. (2001) Interaction of the antiactivator FleN with the transcriptional activator FleQ regulates flagellar number in *Pseudomonas aeruginosa*. *J Bacteriol* **183:** 6636–6644.

Dasgupta, N., Arora, S.K., and Ramphal, R. (2000) *fleN*, a gene that regulates flagellar number in *Pseudomonas aeruginosa*. *J Bacteriol* **182:** 357–364.

Dasgupta, N., Ferrell, E.P., Kanack, K.J., West, S.E.H., and Ramphal, R. (2002) fleQ, the gene encoding the major flagellar regulator of *Pseudomonas aeruginosa* is σ^{70} dependent and is downregulated by Vfr, a homolog of *Escherichia coli* cyclic AMP receptor protein. *J Bacteriol* **184:** 5240–5250.

Fleiszig, S.M., Arora, S.K., Van, R., and Ramphal, R. (2001) FlhA, a component of the flagellum assembly apparatus of *Pseudomonas aeruginosa*, plays a role in internalization by corneal epithelial cells. *Infect Immun* **69:** 4931–4937.

Frisk, A., Jyot, J., Arora, S.K., and Ramphal, R. (2002) Identification and functional characterization of *flgM*, a gene encoding the anti-sigma 28 factor in Pseudomonas. *J Bacteriol* **184:** 1514–1521.

Gober, J.W., and England, J.C. (2000) Regulation of flagellum biogenesis and motility in *Caulobacter*. In *Prokaryotic Development.* Brun, Y.V., and Schimkets, L.J. (eds). Washington, D.C.: American Society of Microbiology Press, 319–340.

Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C.,

Goodlett, D.R., *et al.* (2001) The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* **410:** 099–103.

Hoang, T.T., Karkhoff-Schweizer, R.R., Kutchma, A.J., and Schweizer, H.P. (1998) A broad-host range Flp-FRT recombination system for site-specific excisiom of chromosomally located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* **212:** 77–86.

Jyot, J., Dasgupta, N., and Ramphal, R. (2002) FleQ, the major flagellar gene regulator in *Pseudomonas aeruginosa*, binds to enhancer sites located either upstream or atypically downstream of the RpoN binding site. *J Bacteriol* **184:** 5251–5260.

Kalir, S., McClure, J., Pabbaraju, K., Southward, C., Ronen, M., Leibler, S., *et al.* (2001) Ordering genes in a flagella pathway by analysis of expression kinetics from living bacteria. *Science* **292:** 2080–2083.

Karlinsey, J.E., Tanaka, S., Bettenworth, V., Yamaguchi, S., Boos, W., Aizawa, S.-I., and Hughes, K.T. (2000) Completion of the hook-basal body complex of the *Salmonella typimurium* flagellum is coupled to FlgM secretion and *fliC* transcription. *Moll Microbiol* **37:** 1220–1231.

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

Kutsukake, K. (1997) Autogenous and global control of the flagellar master operon, *flhDC. Salmonella typhimurium*. *Mol Gen Genet* **240:** 440–448.

Macnab, R.M. (1996) Flagella and Motility. In *Escherichia coli and Salmonella typhimurium Cellular and Molecular Biology.* Neidhardt, F.C. (ed.-in-chief). Washington, D.C: American Society for Microbiology Press, pp. 123–145.

Mahenthiralingam, E., Campbell, M.E., and Speert, D.P. (1994) Nonmotility and phagocytic resistance of *Pseudomonas aeruginosa* isolates from chronically colonized patients with cystic fibrosis. *Infect Immun* **62:** 596–605.

McCarter, L.L. (2001) Polar flagellar motility of the Vibrionaceae. *Mol Biol Rev* **65:** 445–462.

Miller, J.H. (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory. pp. 352–355.

Moens, S., and Vanderleyden, J. (1996) Functions of bacterial flagella. *Crit Rev Microbiol* **22:** 67–100.

Montie, T.C., Doyle-Huntzinger, D., Craven, R., and Holder, I.A. (1982) Loss of virulence associated with absence of flagellum in an isogenic mutant of *Pseudomonas aeruginosa* in the burned mouse model. *Infect Immun* **38:** 1296–1298.

Ottemann, K.M., and Miller, J.F. (1997) Roles for motility in bacterial–host interactions. *Mol Microbiol* **24:** 1109–1117.

Pandza, S., Baetens, M., Park, C.H., Au, T., Keyman, M., and Matin, A. (2000) The G-protein FlhF has a role in polar flagellar placement and general stress response induction in *Pseudomonas putida*. *Mol Microbiol* **36:** 414–423.

Prouty, M.G., Correa, N.E., and Klose, K.E. (2001) The novel sigma54- and sigma28-dependent flagellar gene transcription hierarchy of *Vibrio cholerae*. *Mol Microbiol* **39:** 1595–1609.

Ritchings, B.W., Almira, E.C., Lory, S., and Ramphal, R. (1995) Cloning and phenotypic characterization of *fleS* and *fleR*, new response regulators of *Pseudomonas aeruginosa* which regulate motility and adhesion to mucin. *Infect Immun* **63:** 4868–4876.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Springs Harbor, New York: Cold Spring. Harbor Laboratory Press.

Soutourina, O., Kolb, A., Krin, E., Laurent-Winter, C., Rimsky, S., Danchin, A., and Bertin, P. (1999) Multiple control of flagellum biosynthesis in *Escherichia coli*: role of H-NS protein and the cyclic AMP-catabolite activator protein complex in transcription of the *flhDC* master operon. *J Bacteriol* **181:** 7500–7508.

Spangenberg, C., Heuer, T., Burger, C., and Tummler, B. (1996) Genetic diversity of flagellins of *Pseudomonas aeruginosa*. *FEBS Lett* **396:** 213–217.

Starnbach, M.N., and Lory, S. (1992) The *fliA* (*rpoF*) gene of *Pseudomonas aeruginosa* encodes alternative sigma factor required for flagellin synthesis. *Mol Microbiol* **6:** 459–469.

Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J., *et al.* (2000) Complete genome sequence *of Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* **406:** 959–964.

Totten, P.A., and Lory, S. (1990) Characterization of the type a flagellin gene from *Pseudomonas aeruginosa* PAK. *J Bacteriol* **172:** 389–396.

Totten, P.A., Lara, J.C., and Lory, S. (1990) The *rpoN* gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. *J Bacteriol* **172:** 389–396.

Warrens, A.N., Jones, M.D., and Lechler, R.I. (1997) Splicing by overlap extension by PCR using asymmetric amplification: an improved technique for the generation of hybrid proteins of immunological interest. *Gene* **186:** 29–35.

Wolfgang, M.C., Lee, V.T., Gilmore, M.E., and Lory, S. (2003) Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway. *Dev Cell* **4:** 253–263.

Wu, J., and Newton, A. (1997) Regulation of the *Caulabacter* flagellar gene hierarchy; not just for motility. *Mol Microbiol* **24:** 233–239.