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Cloning and Expression of *Pseudomonas aeruginosa* Flagellin in *Escherichia coli*

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The flagellin gene was isolated from a *Pseudomonas aeruginosa* PAO1 genomic bank by conjugation into a PA103 Fla$^-$ strain. Flagellin DNA was transferred from motile recipient PA103 Fla$^+$ cells by transformation into *Escherichia coli*. We show that transformed *E. coli* expresses flagellin protein. Export of flagellin to the *E. coli* cell surface was suggested by positive colony blots of unlysed cells and by isolation of flagellin protein from *E. coli* supernatants.

Pseudomonas aeruginosa has been recognized for several years as a serious threat to certain compromised individuals (4). The virulence of *P. aeruginosa* is multifactorial in scope; factors include exotoxins, lipopolysaccharides, various mucoid polysaccharides, pili, and exoenzymes, especially proteases, to name the more prominent (37).

Genes encoding exotoxin A (13, 22), alginate (10, 12), pili (9, 33), and a porin protein of *P. aeruginosa* have been cloned using a triparental mating system and oligonucleotide probes (12, 30). Several investigators are presently examining the genes involved in regulation of the virulence factors of *P. aeruginosa* and the mechanism of regulation (10, 14, 15).

Our previous data indicated not only that flagella of *P. aeruginosa* contribute to virulence but also that flagellar protein serves as a protective antigen. Studies with nonmotile isogenic mutants showed severely reduced virulence (25, 27) and that the flagellar antigen was protective both as an actively administered immunogen (16) and in passive studies in compromised animals (6, 28). We have demonstrated by molecular weight determinations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and further confirmed by amino acid analysis the presence of two distinct protein antigenic types, a and b (1, 2, 26, 28). The a-type antigens have molecular weights (M_rs) of 45,000 to 52,000, while all of the b-type antigens characterized have an M_r of 53,000 (1).

Previous work performed by Tsuda and Iino (36) has shown by genetic mapping that the flagellar genes of this organism are clustered into two distinct regions. These regions are termed region I and region II and are located in the very late region of the linkage map of the chromosome (35). One motility (*mot*) and five flagellum (*fla*) cistrons were identified in region I, and ten flagellum (*fla*) and two chemotaxis (*che*) cistrons were identified in region II. By using multiflagellate (*mfl*) mutants and a mercury resistance sequence in Tn*501*, Tsuda and Iino determined the order of 1 *che* and 14 *fla* cistrons (35). The clustering effect of the flagellar genes of *P. aeruginosa* is also seen in *Escherichia coli* and salmonellae (18, 19, 31, 32). In *E. coli* and *Salmonella typhimurium*, more than 30 cistrons are clustered in three distinct regions of the chromosomal genome. In *P. aeruginosa*, genes coding for related functions usually do not show the close linkage characteristic of members of the family *Enterobacteriaceae*. This clustering of the *fla* cistrons suggests that expression of each *fla* cistron is coordinately regulated, constituting operons (35). As a first step in studying the composition and function of the Fla gene locus, we attempted to clone the structural flagellin gene into *E. coli*.

In the present study, we isolated DNA fragments containing the structural gene of the flagella from a library of *P. aeruginosa* PAO1 by utilizing triparental matings. Plasmids with DNA fragments containing the structural gene of the flagella were transformed into *E. coli* HB101 cells. Expression of the *Pseudomonas* flagellin protein in *E. coli* HB101 cells was examined by colony blotting and Western (immuno-) blotting of electrophoretically isolated protein.

P. aeruginosa PA103, originally described by Liu (21), was used as the recipient in triparental matings. This organism is deficient with respect to synthesis of a complete flagellar filament, although some flagellin is detectable. Well-characterized flagellin deletion mutants were not available and are not readily isolated (K. Kelly-Wintenberg and T. C. Montie, unpublished results). Since the flagellar operon is clustered in *P. aeruginosa*, we proposed to use PA103 as a vehicle to clone the cluster containing the structural gene by using a relatively large cosmid fragment (20 to 30 kilobases) (12). *E. coli* HB101, provided by Jeffrey Becker (University of Tennessee), was used in transformation experiments. The *P. aeruginosa* PAO1 genomic library pLAFR1-PAO1 harbored in *E. coli* HB101 cells was kindly provided by Dennis Ohman (12), as was the helper plasmid, pRK2013, used in the triparental matings.

Bacteria were grown in Luria broth (LB; 10% pancreatic digest of casein, 0.5% yeast extract, 0.5% NaCl per liter) for triparental matings, isolation of plasmids, and transformation experiments. Motility medium for assays and electron micrographs contained LB with 0.3% agar (GIBCO Laboratories, Grand Island, N.Y.). *P. aeruginosa* PA103 cells containing plasmids were selected in mineral salts medium [4 $\times 10^{-2}$ M K_2HPO_4, 2.2 $\times 10^{-2}$ M KH_2PO_4, 7.5 $\times 10^{-3}$ M $(NH_4)_2SO_4$, 2.0 $\times 10^{-4}$ M $MgSO_4$] with 0.4% sodium citrate and 300 μg of tetracycline (Sigma Chemical Co., St. Louis, Mo.) per ml. Luria agar was prepared with LB and 1.6% agar. For isolation of flagellin protein from supernatants of transformed *E. coli* cells, bacteria were grown in mineral salts medium with 0.4% sodium succinate and 0.3% Casamino Acids (Difco Laboratories, Detroit, Mich.), and 300 μg of tetracycline per ml was used.

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The pLAFR1-PAO1 genomic library (12) was constructed by an *Eco*RI partial digestion of PAO1 DNA followed by sucrose density centrifugation. DNA fragments of 15 to 30 kilobases were retrieved and ligated into a single *Eco*RI site of the broad-host-range vector pLAFR1 originally developed by Ditta et al. (5). The vector is 21.6 kilobases long and confers tetracycline resistance. The pLAFR1-PAO1 genomic library and pRK2013, both harbored in *E. coli* HB101 cells, were used in triparental matings to transfer conjugally the PAO1 library into the recipient strain, *P. aeruginosa* PA103 (12).

Triparental mating involved mobilizing the pLAFR1-PAO1 plasmids into *Pseudomonas* Fla$^-$ recipient cells. To transfer the pLAFR1-PAO1 plasmids, it was necessary to use a helper plasmid, i.e., pRK2013. The pRK2013 plasmid contains the conjugal transfer genes (*tra*) and determinants of kanamycin resistance (8).

Log-phase *E. coli* HB101 cells containing pRK2013 and the HB101(pLAFR1-PAO1) clone bank were grown in LB medium containing the appropriate antibiotics. The *E. coli* cells were mixed in a 1:1:1 ratio with 0.1 ml of log-phase *Pseudomonas* Fla$^-$ cells grown in LB. The suspensions were filtered on 0.45-μm-pore-size nitrocellulose membranes (Millipore Corp., Bedford, Mass.) and incubated on Luria agar plates (cell side up) at 37°C overnight. Following incubation, cells were removed from the filter by being vortexed in a 0.85% NaCl solution. The suspension was diluted on mineral salts medium containing 0.4% citrate and tetracycline (300 μg/ml) and incubated at room temperature for several generations. This method selected for *P. aeruginosa* PA103 cells containing pLAFR1-PAO1 molecules, not auxotrophic *E. coli* HB101 cells. Controls of *P. aeruginosa* PA103 and HB101(pLAFR1-PAO1) were processed separately and identically, and growth in the selection medium did not occur for either strain.

P. aeruginosa PA103 cells containing the recombinant plasmids complementing the Fla mutation were isolated by motility assays. The motility assay consisted of stab inoculation of cells which grew in the presence of tetracycline (300 μg/ml) on motility medium. For each assay, triplicate plates per strain were incubated at 37°C and examined periodically over 6 to 24 h for colony spreading and the distinctive rings characteristic of chemotactic bacteria. This assay detected any recombination event that involved the formation of a functioning flagellum. Upon screening the entire genomic library, we isolated eight clones which complement the Fla mutation of PA103. Only isolates that showed more than 80% of the motility of PAO1 were retained. The recipient strain, PA103 (Fla$^-$), routinely displayed 10% of the motility of PAO1.

Electron micrographs of complemented PA103 cells are shown in Fig. 1. On the basis of the electron micrographs and motility assays, it was evident that these cells produced a functional single-polar flagellum typical of wild-type *P. aeruginosa*. Amino acid analyses of purified flagella of PA103(pKW52) showed the content to be essentially identical to those of other *Pseudomonas* b-type flagella. Notably, there was no histidine low amounts of aromatic acids, and minute amounts of methionine.

Plasmid preparations from PA103 (designated pKW3, pKW8, pKW23, pKW52, pKW84, and pKW333) were examined by electrophoresis in 0.4% agarose gels and stained with ethidium bromide to visualize the plasmid clones. These plasmids were prepared by the method of Casse et al. (3) and were used in $CaCl_2$ transformation experiments with competent *E. coli* HB101 cells (24). Successfully transformed *E. coli* HB101 cells were isolated by their ability to grow in the presence of tetracycline (300 μg/ml) and anaerobically.

To determine whether *Pseudomonas* flagellin production occurred and was detectable in transformed *E. coli* cells, we utilized colony blots. This assay was modified from the method of Nicas et al. (29) and adapted from a published enzyme-linked immunosorbent assay for flagellar antigen (26). The method is specific for either a- or b-type flagellar antigen. Transformed HB101 cells were stab inoculated on Luria agar plates containing tetracycline (100 μg/ml) and incubated at 37°C for 24 h. After growth, the transformed colonies were blotted onto sterile nitrocellulose (Schleicher & and Schuell, Inc., Keene, N.H.) previously washed in a 0.2% sodium dodecyl sulfate solution. To eliminate nonspecific binding, a blocking step using a 3% gelatin–phosphate-buffered saline solution was included. Following washing, the absorbed antiserum was applied at a concentration of 1:1,000 to the nitrocellulose disks and incubated for 2 h. After washing, the secondary antibody, horseradish peroxidase conjugated to goat anti-rabbit immunoglobulin G (Hyclone Laboratories, Logan, Utah), was applied to the disk and incubated for 1 h. Following enzymatic development using H_2O_2 (0.3%) as the substrate, any colony of cells which possessed the flagellar antigen was considered positive by a yellow color on the nitrocellulose disk. The HB101 cells transformed with the positive clones appeared to produce the flagellar protein of *P. aeruginosa* (Table 1). It also appeared that the *E. coli* cells exported the flagellin protein at levels detectable by colony blotting, even when the colonies were not lysed with chloroform. As shown in Table 1, the *E. coli* cells containing pKW333 and pKW52 showed a reaction almost as intense as that obtained with *P. aeruginosa* PA103 cells which harbored the plasmid. However, reactions in HB101 cells containing pKW84 and pKW8, although readily detectable by blotting, were less intense than that detected in *P. aeruginosa* PA103 cells harboring these plasmids.

It is interesting that there are detectable differences in the intensities of the colony blots of the various transformed HB101 strains (Table 1). Since the assembly of a flagellum is complex and requires a large DNA-coding region (35), it is likely that the difference in intensities of the colony blots corresponded to different cloned regions of the flagellin DNA which affected expression.

To investigate the secretion of flagellin protein in *E. coli* HB101 cells, Western blots (34) of culture supernatant fractions were performed. Transformed HB101 cells were grown to late log phase in mineral salts medium-glucose with 0.3% Casamino Acids at 37°C in a gyratory shaker at 200 rpm. The supernatants were retained following centrifugation and dialyzed and concentrated by using lyophilization.

The prepared supernatant concentrates of the transformed *E. coli* cells were separated (20) in polyacrylamide gels by using the Phast System (Pharmacia Diagnostics, Piscataway, N.J.) (Fig. 2a) and stained with Coomassie brilliant blue. All of the HB101 supernatant samples containing positive clones displayed a protein band with approximately the same M_r (53,000) as the purified b-type PJ108 flagellin protein (Fig. 2a, lanes A, B, C, and E). In other gels, no stained bands were seen in the 53-kilodalton region when supernatant of non-transformed HB101 cells was used. Flagellin purified from PA103(pKW52) cells (Fig. 2a, lane D) appeared to migrate very closely to the standard b-type flagellin, and Western blotting analysis demonstrated that the flagellin of PA103(pKW52) cells was antigenic (Fig. 2b, lane D).

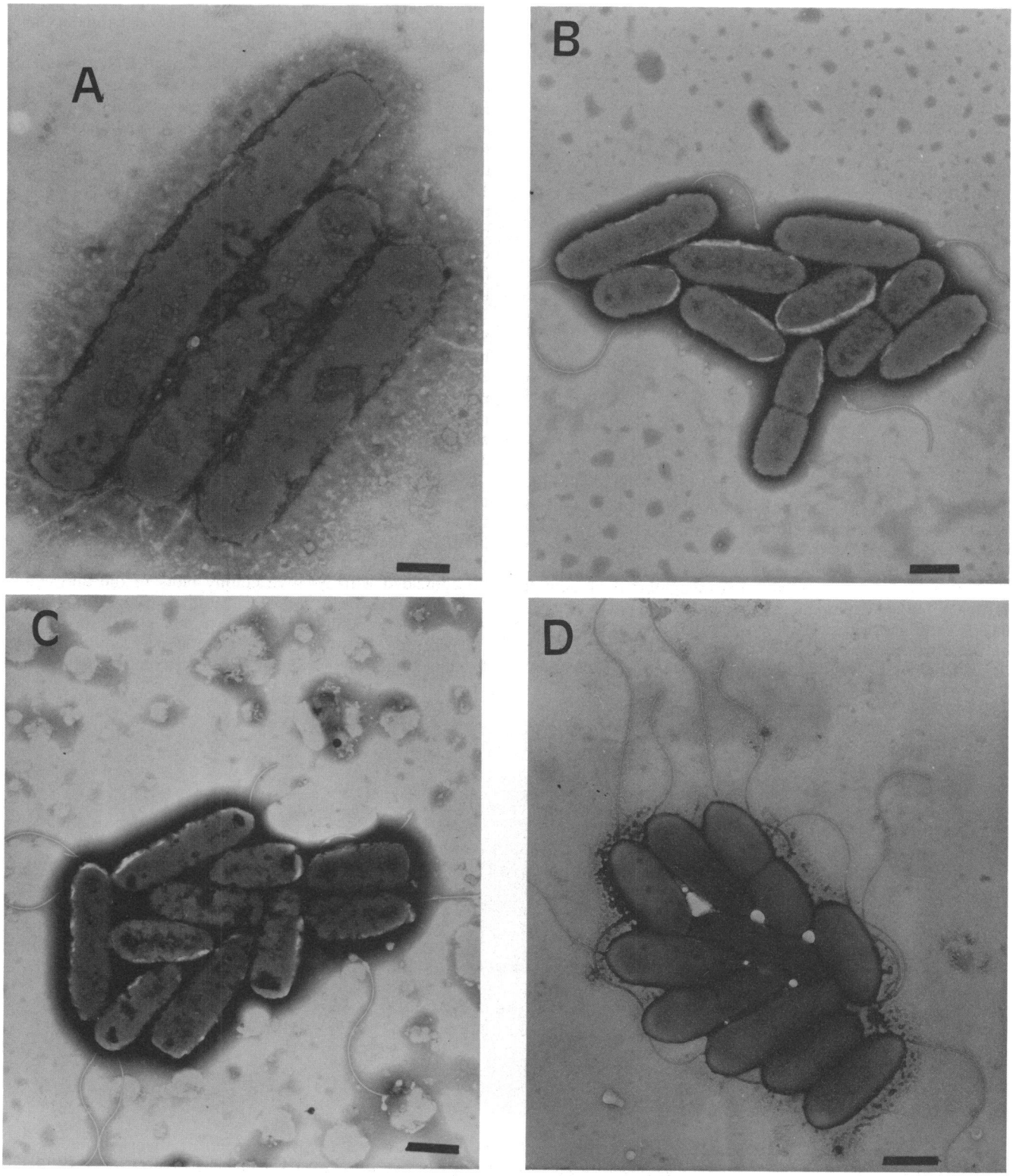

FIG. 1. Electron micrographs of PA103 Fla^+ cells. Cells were grown on motility medium for 18 to 20 h at 37°C. A loopful of cells was removed from motility plates and suspended in sterile phosphate-buffered saline. Cells were stained with 0.5% phosphotungstic acid, pH 7.0. The specimens were examined with an H-600 Hitachi transmission electron microscope. (A) PA103 Fla^-; magnification, ×20,000. (B) PA103(pKW333); magnification, ×10,000. (C) PA103(pKW52); magnification, ×10,000. (D) PA103(pKW84); magnification, ×10,000. The bar represents 1 μm except in panel A, where it represents 0.5 μm.

TABLE 1. Detection of *Pseudomonas* flagellar antigen in transformed *E. coli* HB101 cells by colony blotting[a]

Strain	Detection of flagellar filament protein[b] with the following antiserum:			
	Anti-b type		Anti-a type	
	Lysis	No lysis	Lysis	No lysis
E. coli				
HB101	−	−	−	−
HB101(pLAFR1)	−	−	−	−
HB101(pKW8)	++	++	−	−
HB101(pKW52)	+++	+++	−	−
HB101(pKW84)	++	++	−	−
HB101(pKW333)	+++	+++	−	−
P. aeruginosa PAO1	++++	++++	+	+
P. fluorescens	−	−	−	−

[a] The presence or absence of flagellar filament protein was determined by a reaction to either anti-M_2 (b-type) or anti-1210 (a-type) antiserum. Detection was verified by the color intensity of each blot compared with that of known positive and negative blots. Prior to application of the blocking solution the cells were lysed with chloroform (29), or after a blotting the blocking solution was added directly to the nitrocellulose disks (no lysis). Blots were done in triplicate.

[b] +++, Strong detection; ++, detection; −, no detection.

The proteins were transferred onto nitrocellulose by diffusion for 2 h at 37°C. The nitrocellulose blots were blocked with 3% bovine serum albumin in 10 mM Tris (pH 7.4)–0.9% NaCl. The blots were incubated with adsorbed antiserum (b type) diluted 1:1,000, followed by application of peroxidase conjugated to goat anti-rabbit immunoglobulin G diluted 1:10,000. Upon development, any band which possessed the flagellar antigen was considered positive by a yellow color on the nitrocellulose. In the Western blot shown in Fig. 2b (lanes B, C, and E), a single band of protein from each of the transformed *E. coli* supernatants and the control b-type flagellin reacted with the standard b-type polyclonal antiserum. In confirmation, the gel gave blotted bands with an approximate M_r of 53,000.

These experiments show that we cloned DNA fragments of *P. aeruginosa* containing the flagellin gene into *E. coli* HB101 cells. Upon analysis by colony blots, it appeared that most of the flagellin protein was expressed and transported to the cell surface, since the same results were seen in either the presence or the absence of cell lysis with chloroform. The expression and transport of flagellin out of the cell or to the surface was further indicated by results of Western blot reactions of *E. coli* extracellular supernatants. However, we cannot conclusively state that secretion into the extracellular fluid occurred. It is possible that a small number of cells lysed and released intracellular flagellin protein.

Preliminary experiments with transformed extracellular supernatants in nondetergent polyacrylamide gels followed by Western blots to detect antigen suggested that most of the exported flagellin is in a polymerized form. However, it is possible that self-aggregation of the secreted monomeric protein occurred since the phenomenon of self-aggregation at or near neutral pHs has been well documented with *Salmonella* flagellin (23).

Results with a number of *P. aeruginosa* mutant strains that synthesize flagellin but cannot assemble the intact filament showed that an apparent polymerized form of the flagellin is found in the extracelllular medium. In every strain tested, detection of the putative secreted polymerized flagellin coincided with positive colony blots of the unlysed *P. aeruginosa* (K. Kelly and T. C. Montie, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, P118, p. 90).

There appears to be no leader sequence requirement for flagellin protein export, and the C terminal of the flagellin

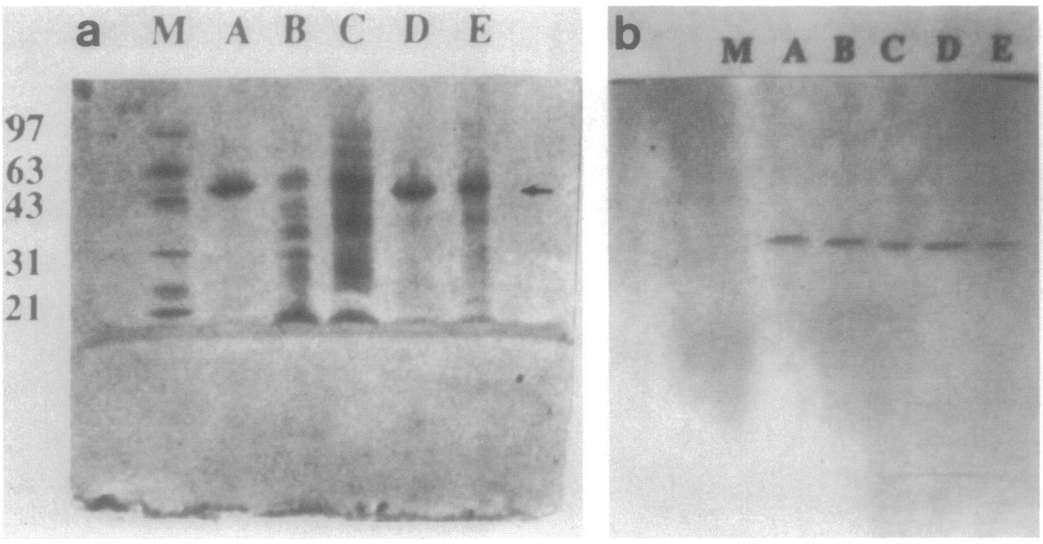

FIG. 2. (a) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of transformed *E. coli* HB101 supernatant preparations. Approximately 50 μg of the supernatant concentrates and 1.0 μg of pure flagellin protein were separated by electrophoresis in a 10 to 15% polyacrylamide gradient and stained with Coomassie brilliant blue. Lanes: M, molecular weight markers (10^3); A, PJ108 purified b-type flagellin; B, HB101(pKW333); C, HB101(pKW52); D, PA103(pKW52) purified flagellin; E, HB101(pKW84). The arrow indicates the *Pseudomonas* flagellin protein produced by transformed HB101 cells. (b) Western blot analysis of *Pseudomonas* flagellin protein detected in supernatant concentrates of HB101 cells. Following electrophoresis, the proteins were transferred by diffusion from a Phast gel to prepared nitrocellulose. The blots were developed as described in the text. Lanes: M, molecular weight markers; A, PJ108 purified b-type flagellin; B, HB101(pKW333); C, HB101(pKW52); D, PA103(pKW52) purified flagellin; E, HB101(pKW84). The immunoreactive band corresponded to the Coomassie blue-stained *Pseudomonas* flagellin.

portion is thought to be responsible for signaling (7, 17). The finding that the *P. aeruginosa* flagellin protein is expressed at the surface of the transformed *E. coli* cells in itself is not so surprising. Many flagellin proteins exhibit N- and C-terminal amino acid sequence homology (11, 17), so that recognition of export sequences could occur among genera. In this regard, the first nine amino acids of the N-terminal region are the same for both a- and b-type flagellins of *P. aeruginosa*. Also, homology within the region exists between *P. aeruginosa* and other gram-negative bacteria (T. C. Montie et al., unpublished data).

Similarly, unlike some of the secreted *P. aeruginosa* proteins, cloned pilin genes from *Bacteroides nodarus*, *Neisseria gonorrhoeae*, and *P. aeruginosa* show analogous sequences and are all expressed effectively in *E. coli* independent of a vector promoter (9). Although detected at the cell surface, *P. aeruginosa* pilin protein is not secreted extracellularly.

Using the clones in *E. coli*, we recently completed a limited endonuclease restriction map. The limited mapping has shown that the plasmids pKW333 and pKW52 probably contain the same *Pseudomonas fla*$^+$ DNA fragment, whereas plasmids pKW84 and pKW102 appear to contain overlapping but different *fla*$^+$ DNA. Experiments are in progress to subclone the DNA fragments containing the flagellin gene to reduce the size so that the flagellin gene can be sequenced and the inferred amino acid sequence can be determined.

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