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Flagella and Motility in *Actinobacillus pleuropneumoniae*

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Actinobacillus pleuropneumoniae **has been considered nonmotile and nonflagellate. In this work, it is demonstrated that *A. pleuropneumoniae* produces flagella composed of a 65-kDa protein with an N-terminal amino acid sequence that shows 100% identity with those of *Escherichia coli*, *Salmonella*, and *Shigella* flagellins. The DNA sequence obtained through PCR of the *fliC* gene in *A. pleuropneumoniae* showed considerable identity (93%) in its 5′ and 3′ ends with the DNA sequences of corresponding genes in *E. coli*, *Salmonella enterica*, and *Shigella* spp. The motility of *A. pleuropneumoniae* was observed in tryptic soy or brain heart infusion soft agar media, and it is influenced by temperature. Flagella and motility may be involved in the survival and pathogenesis of *A. pleuropneumoniae* in pigs.**

Actinobacillus pleuropneumoniae is a gram-negative bacterium that is the etiologic agent of porcine pleuropneumonia, a disease associated with widespread morbidity and mortality among all ages of growing pigs. Animals become ill and often die due to the fibrino-hemorrhagic lesions produced. Survivors grow poorly and spread the infection to nonimmune herds (3, 16). *A. pleuropneumoniae* possesses both secreted and nonsecreted virulence factors (6, 14, 18).

A. pleuropneumoniae has been described as a nonmotile organism unable to produce flagellar locomotive appendages (10). A connection between virulence and flagellum-based motility has long been observed for many pathogens which require functional flagella for virulence (9, 12, 13). Nonmotile flagellated strains and mutants lacking flagellar filaments, as well as aflagellated strains of *Campylobacter jejuni*, were all rapidly cleared from the intestines following experimental infection of animals (17). However, in some pathogens motility must be suppressed to allow successful colonization (2). For example, *Bordetella* strains with a wild-type virulence control system, Bvg^+ strains, are motile under certain conditions, producing peritrichous flagella only in the presence of modulating signals, and they are able to colonize the trachea. However, Bvg^- (Δ*bvgAS* or Δ*bvgS*) strains are motile in the absence of modulators; thus, constitutive expression of this Bvg^- phenotype resulted in a defect affecting colonization (1).

This work aims to demonstrate the presence of flagella in *A. pleuropneumoniae* and their relevance for motility. The N-terminal amino acid sequence of the flagellin of this bacterium and both the 3′ and 5′ nucleotide sequences of the gene displayed strong homology with the flagellins and flagellin genes of other gram-negative bacteria, suggesting that flagellin is a conserved protein.

Methods. *A. pleuropneumoniae* serotype 1 (strain BC5235) was isolated from a lung abscess of a pig suffering acute porcine pleuropneumonia (13), and the bacterium was verified by biochemical characteristics and immunological reaction with specific antiserum. This strain and all *A. pleuropneumoniae* reference strains (15) were maintained on tryptic soy (TS) agar (Bioxón, Oaxaca, Mexico) or brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.), supplemented with 0.01% NAD plus 5% defibrinated sheep blood, at 37°C. To favor flagellum expression, bacteria were grown in the above-mentioned media but with 0.4% agar supplemented with sheep serum; bacteria from the colony edge were subcultured in the same media and under similar conditions in order to select for motile strains. To determine bacterial motility, a colony was inoculated by puncturing the center of a soft-agar plate or glass tube (TS or BHI plus NAD, with 0.3% agar). They were incubated at 22 or 37°C and checked every day. Radial growth or growth out of the line of puncture was considered a positive test (20). The enteropathogenic E2348/69 strain and a *fliC* mutant strain of *E. coli* were used as positive and negative controls, respectively.

To isolate *A. pleuropneumoniae* flagellin, selected motile bacteria were grown in BHI or TS broth plus NAD under stationary conditions for 48 to 72 h or on solid media supplemented with 5% defibrinated sheep blood. Cells were harvested without being washed and were suspended in 20 mM Tris-HCl (pH 8.0) containing 25 mM NaCl. The suspension was mechanically sheared for 5 min at 200 rpm and centrifuged (8,000 × *g* for 20 min) to remove flagella from cells. Flagella were obtained by differential centrifugation as described previously (8). Flagellum preparations were boiled for 5 min in buffer sample with 5% 2-mercaptoethanol before being separated by sodium dodecyl sulfate–14% polyacrylamide gel electrophoresis in a buffer system previously described by Laemmli (11).

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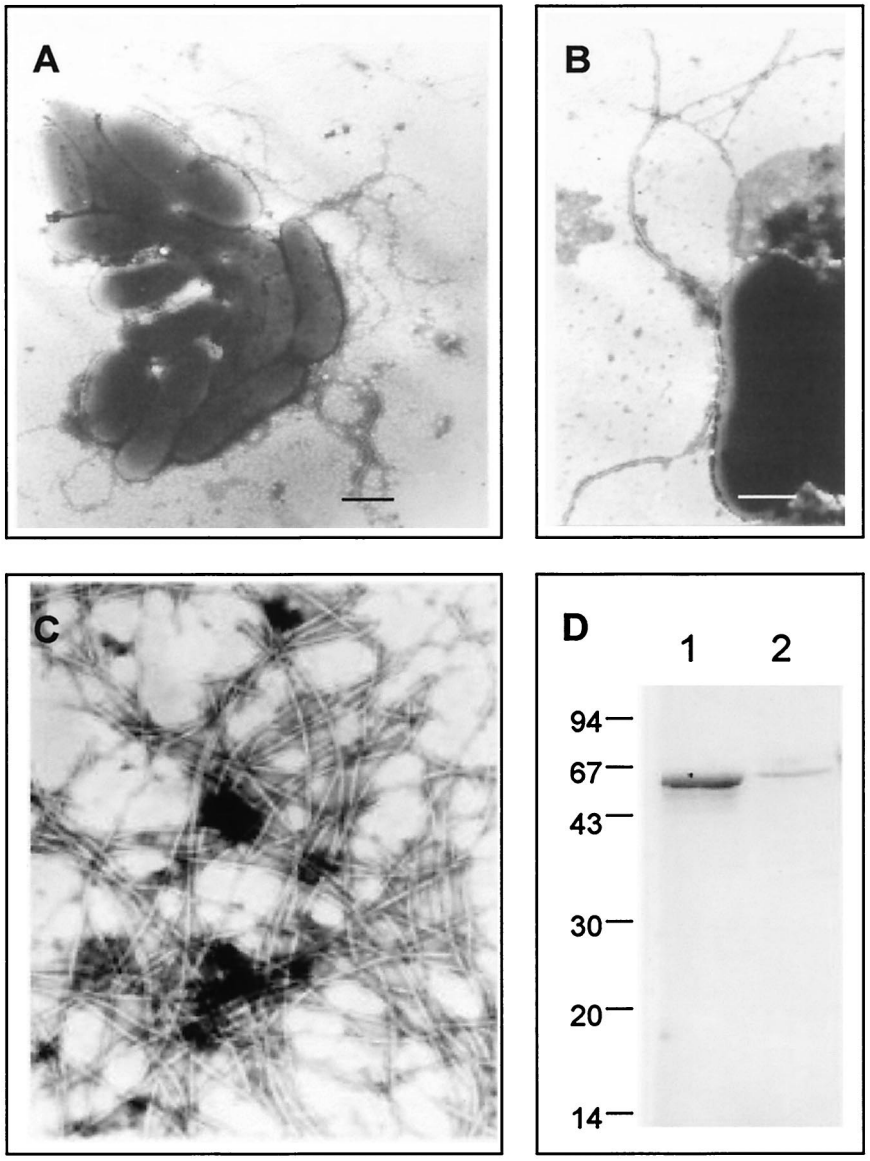

FIG. 1. (A and B) Electron micrographs showing flagella on the surfaces of *A. pleuropneumoniae* cells. Bars, 500 nm. (C) Purified *A. pleuropneumoniae* flagella. (D) Flagellar preparations electrophoresed in 14% acrylamide gels. Lane 1, flagellin from *E. coli*; lane 2, flagellin from *A. pleuropneumoniae.* Molecular mass standards (in kilodaltons) are indicated on the left.

The *A. pleuropneumoniae* flagellin N-terminal sequence was determined after the protein was electroblotted on a polyvinylidene difluoride membrane (Bio-Rad). The bands were excised from the membrane and sequenced on a model LF3000 protein sequencer (Beckman, Fullerton, Calif.) interfaced with an Applied Biosystems model 1120 online analyzer.

To determine the nucleotide sequence of the *fliC* gene, primers for PCR were synthesized on the basis of the N-terminal sequences of *A. pleuropneumoniae* flagellin and conserved motifs of C-terminal sequences of flagellins from other bacteria (*E. coli*, *Salmonella enterica*, *Serratia marcescens*, and *Shigella dysenteriae*). The upper primer sequence was 5′AGA GACAGAACCTGCTGC3′, and the lower primer sequence was 5′AATACCAACAGCCTCTCGCT3′. Genomic DNA was extracted as described previously (5). The reaction mixture contained 200 to 300 ng of DNA, 0.2 mM concentrations of each deoxynucleoside triphosphate, 25 pmol of each primer, 1.5 mM $MgCl_2$, 1.25 U of recombinant *Taq* polymerase (Promega), and 10× DNA polymerase buffer. Twenty-five amplification cycles were performed with a Perkin-Elmer model 480 thermocycler as follows: denaturalization at 94°C (1 min), annealing at 53°C (2 min), and extension at 72°C (3.5 min). The flagellin gene was subcloned into pCR2.1 vector, and the nucleotide sequence was obtained with a Perkin-Elmer genetic analyzer (ABI Prism 3100 sequencer) by using the T4 and M13 reverse universal primers with the *Taq* dye deoxy terminator and Dye primer sequencing protocols.

To obtain polyclonal antibodies against *A. pleuropneumoniae* flagellin, bands in polyacrylamide gels containing the flagellin were cut, dried, and suspended in 0.85% saline solution. Pro-

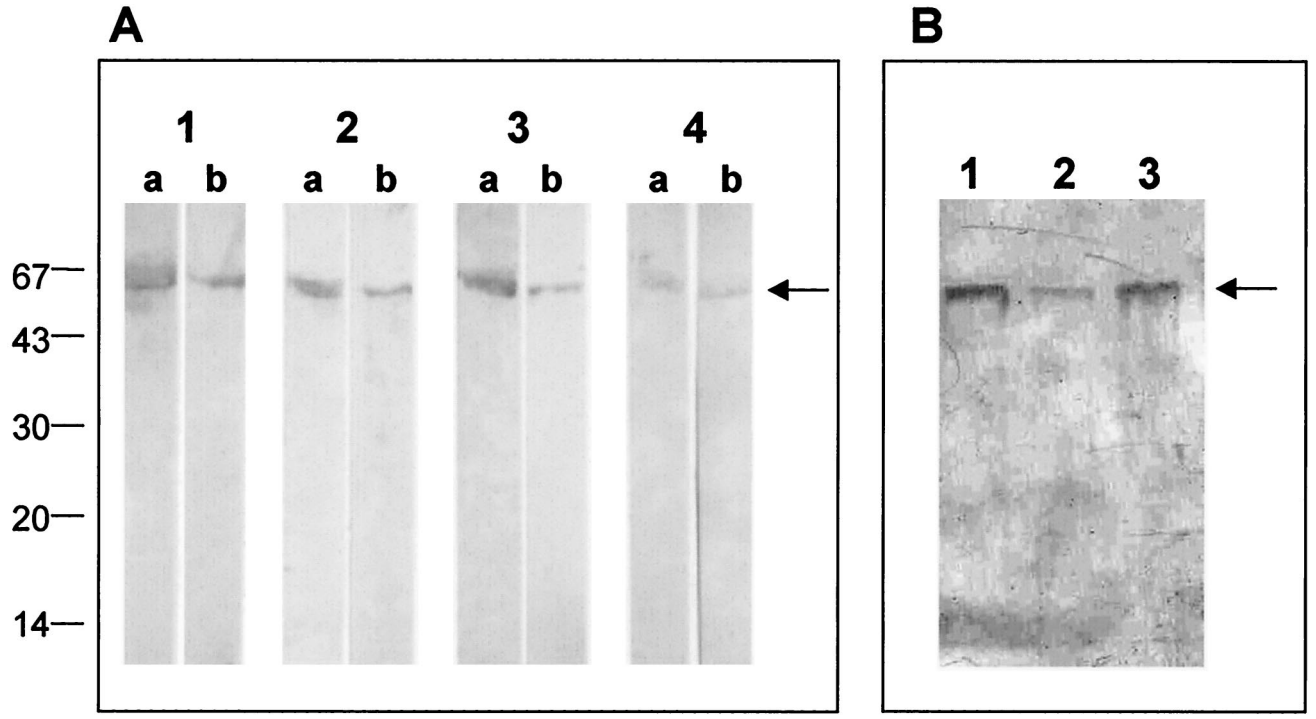

FIG. 2. (A) Reactivities of flagellar preparations with heterologous antisera as shown by immunoblotting. Flagellin samples were reacted with antiflagellum serum samples from *A. pleuropneumoniae* (lanes in panel 1), *E. coli* (lanes in panel 2), *Salmonella* (lanes in panel 3), and *Shigella* (lanes in panel 4). Lanes a, *E. coli* flagellin; lanes b, *A. pleuropneumoniae* flagellin. (B) *A. pleuropneumoniae* whole-cell extracts reacted with anti-*E. coli* flagellin serum. Lanes 1, 2, and 3, serotypes 3, 5b, and 7, respectively. Molecular mass standards (in kilodaltons) are indicated on the left

tein was mixed with Freund's complete adjuvant, and 75 μg of protein (4) was subcutaneously injected into two female New Zealand rabbits. Animals received three booster injections of Freund's incomplete adjuvant every 2 weeks. *A. pleuropneumoniae* flagellin was electrophoresed and transferred to nitrocellulose membranes (Bio-Rad), which were blocked with 5% skim milk and incubated with the anti-*A. pleuropneumoniae* flagellin serum. Control assays were performed with the antibodies against flagellins from *E. coli*, *Salmonella*, and *Shigella* (each in a 1:1,000 dilution) as previously described (8). Immune recognition was revealed with alkaline phosphatase-labeled goat immunoglobulin G anti-rabbit antibody. Purified *E. coli* flagellin was used as a positive control. Total cellular extracts from other serotypes were also tested for recognition by antiserum against either *A. pleuropneumoniae* serotype 1 or *E. coli* flagellin.

Negative staining and immune recognition were done with bacteria grown in TS broth or TS agar plus 5% sheep blood. The bacteria were suspended in sterile distilled water and placed on carbon- and Formvar-coated copper grids, stained with 1% (wt/vol) phosphotungstic acid, and observed with a JEM 2000 EX transmission electron microscope at 80 V. Purified flagella were also processed for negative staining. Cells and flagella were prepared for immunogold labeling as described previously (8) by using the aforementioned rabbit antiserum against *A. pleuropneumoniae* flagellin. Protein A-gold beads (5-nm diameter; Sigma) were used to detect the antibodies. A rabbit preimmune serum was used as a negative control.

***A. pleuropneumoniae* flagella.** *A. pleuropneumoniae* has been traditionally considered nonmotile and nonflagellate (10), and thus it would be interesting to know how it gains access to the lung. In some pathogenic bacteria, the expression of surface organelles for attachment may be induced only by factors related to a specific host microenvironment or under certain in vitro conditions. For example, enteropathogenic *E. coli* produces an inducible bundle-forming pilus under conditions that may simulate a small bowel habitat (7). Flagellum expression in *A. pleuropneumoniae* may be related to host conditions which are not commonly present in vitro. *A. pleuropneumoniae* produced these structures when it was grown at 37°C in TS broth or on agar medium plus sheep red blood cells or when it was grown in BHI under stationary conditions after 3 days of incubation (Fig. 1A and B). Similar results were observed when bacteria were grown in TS agar supplemented with blood and incubated in an atmosphere of 10% CO_2. This finding makes us think that there is an inducible agent that controls flagellum expression in *A. pleuropneumoniae* and may in part explain why these flagellum structures have eluded identification. *A. pleuropneumoniae* flagellin filaments have an average length of 5 μm and a width of 10 to 20 nm. The majority of the cells showed only polar flagella emerging from the cell surface. This polar distribution does not rule out the possibility of peritrichous flagellum expression under other in vitro conditions or in recently isolated bacteria.

***A. pleuropneumoniae* flagellin.** Flagellar protein was dissociated from the cell body by mechanical shearing and purified by differential centrifugation. The presence of flagella was de-

termined by electron microscopy (Fig. 1C). The constituent monomeric subunits were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under denaturing conditions. The molecular mass of the flagellin was 65 kDa (Fig. 1D), very similar to that of *E. coli* flagellin. Cross-reactions among *A. pleuropneumoniae* and other flagellins were detected by Western blotting by using sera raised against flagellins of *A. pleuropneumoniae*, *E. coli*, *Salmonella*, and *Shigella* (Fig. 2A, lanes 1, 2, 3, and 4, respectively). The reactions with anti-*Shigella* flagellin serum were weak. However, no cross-reactions were observed by immunogold labeling with the same antisera in cells or in purified flagella (data not shown). This result was probably because the anti-*Shigella* flagellin serum was generated with denatured proteins. Tominaga et al. (21) found that serum specifically directed against the flagellin of *Shigella flexneri* also recognized *E. coli*, *Salmonella*, and other *Shigella* flagellins and concluded that flagellin is a highly conserved protein. In summary, immunoblot data with heterologous antiflagellum sera confirm the shared epitopes between *E. coli* and *A. pleuropneumoniae* flagellins. A similar immune recognition was observed when total cell extracts from other *A. pleuropneumoniae* serotypes were used (Fig. 2B, lanes 1 to 3). In Fig. 2, only the results obtained with extracts from serotypes 3, 5b, and 7 are shown.

***A. pleuropneumoniae* motility.** After we saw flagella on the bacterial surface, the logical question to answer was can *A. pleuropneumoniae* move? Motility was observed after 48 h of incubation in soft BHI or TS broth agar (Fig. 3) and was more evident in tubes inoculated with bacteria from the edge of a previous radial growth than from the center. In addition, cells incubated at 37°C were more motile than those incubated at room temperature (approximately 22°C) (Fig. 3B and A, respectively). Also, it appears that motility in *A. pleuropneumoniae*, in the same manner as in other bacteria, is influenced by temperature, because a longer radial and homogeneous growth was observed at 37°C than at 22°C. If bacteria are motile and positively chemotactic to one or more nutrients in the medium, they should sense the concentration generated by metabolism at the site of inoculation and swim in response to an increased attracting concentration (20). Motile chemotactic strains produce large circular swarms; motile but nontactic strains produce only small areas of radial growth near the site of inoculation; nonmotile strains produce small tight colonies at the inoculation site. If the medium is complex, several swarm rings may grow on the plate as different populations of bacteria metabolize and tactically respond to different nutrient sources within the plate (20). *A. pleuropneumoniae* motility was observed as several small swarm rings, which may indicate a motile but nontactic behavior. More experiments are needed to confirm the process behind the formation of swarm rings by *A. pleuropneumoniae*.

Amino acid and DNA sequences. The N-terminal amino acid sequence of *A. pleuropneumoniae* flagellin is AQVINTNSL SLI. This sequence evidences 100% identity with sequences of flagellins from *E. coli*, *S. enterica* serovar Typhimurium, *S. dysenteriae*, *Shigella boydii*, and *Serratia marcescens* and a strong similarity with those from *Proteus mirabilis*, *Bordetella bronchiseptica*, and *Pseudomonas aeruginosa.* PCR amplification allowed us to obtain a product of approximately 1,600 bp. The 5′ and 3′ nucleotide sequences exhibited high identity (93%) to

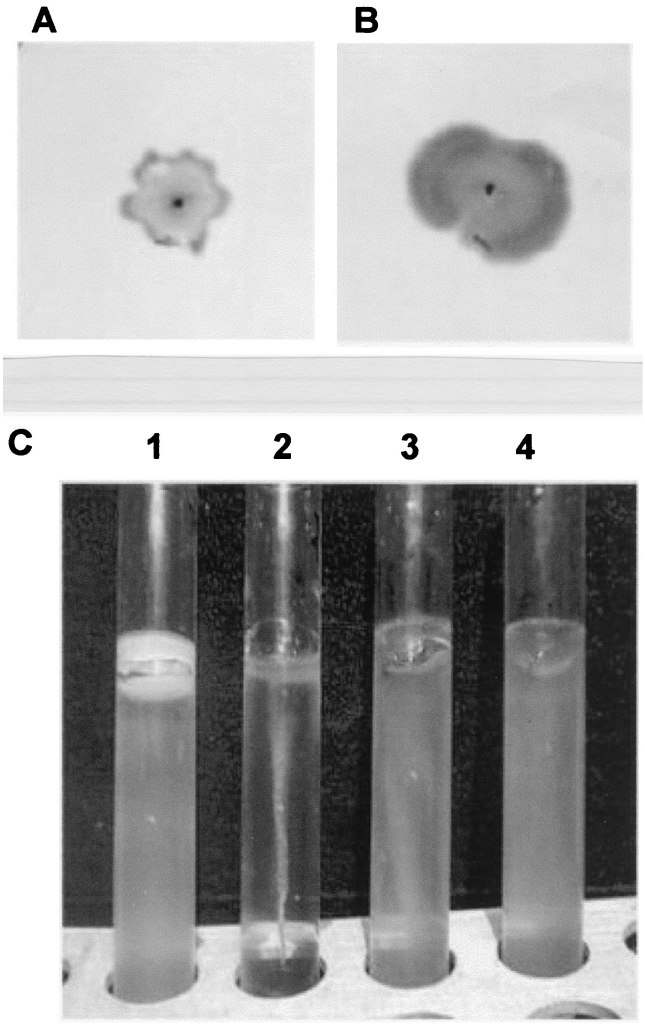

FIG. 3. *A. pleuropneumoniae* motility on soft agar in plates (A and B) and tubes (C). A comparison of cells incubated at 22°C (A) and those incubated at 37°C (B) shows the influence of temperature on motility. (C) Tube 1, *E. coli* E2348/69 positive control; tube 2, *E. coli fliC* mutant negative control; tubes 3 and 4, *A. pleuropneumoniae* serotypes 1 and 5, respectively. Motility in tubes was observed after 48 h of incubation at 37°C.

those of *fliC* genes in *E. coli* and different species of *Salmonella* and *Shigella* when the sequences were compared by using the BLAST program. This identity shows a genetic relationship between these genes. Akerley and Miller (1) showed that the product of *flaA*, a gene encoding flagellin in the swine respiratory pathogen *B. bronchiseptica*, is similar to the FliC flagellins of *S. enterica* serovar Typhimurium and *E. coli.* This similarity corroborates the similarity among flagellins from different gram-negative species.

The synthesis and function of the flagellar and chemotaxis systems require the expression of more than 50 genes (12, 19), which is metabolically costly; the payback comes in the ability of motile bacteria to seek out favorable niches by biasing their normally random movement towards a directed one in response to perceived chemical stimuli, a process called chemotaxis. Taxis allows motile bacteria to go toward and accumulate

in favorable environments, and it allows pathogenic bacteria to detect sites in hosts where an infection may be established (20). It is possible that flagellum expression and motility in *A. pleuropneumoniae*, in the same manner as in other bacteria, are driven by physiological and environmental factors. More studies have to be done in order to determine what role *A. pleuropneumoniae* flagella and motility play in this pathogenic scheme and what the heretofore nondescribed phenotypes have to do with the adaptation and survival of the organism in nature.

Nucleotide sequence accession numbers. The 5′ and 3′ nucleotide sequences of the *A. pleuropneumoniae fliC* gene have been deposited in the GenBank database under accession no. AF515472 and AF515473, respectively.

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