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Role of Flagella in Adhesion of *Pseudomonas fluorescens* to Tendon Slices

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Tendon slices were used as model surfaces to investigate the role of flagella in the adhesion of *Pseudomonas fluorescens* to meat. The slices were introduced into a specially designed flow chamber, which was then filled with a suspension of the organism, and the tendon surface was observed at a ×640 magnification. The same events that occur during the colonization of glass surfaces (apical adhesion of cells with rotation around the contact point, longitudinal adhesion, detachment of apically and longitudinally adherent cells) were also observed on tendon. Mechanical removal of the flagella resulted in no change in the contact angles with 0.1 M saline or α-bromonaphthalene, in the electrophoretic mobility, or in the adhesion of the organism to hydrophobic and ion-exchange resins. In addition, cells from which flagella had been mechanically removed still adhered extensively to tendon. Nevertheless, under comparable conditions (bacterial concentration, contact time), flagellated cells adhered to tendon in larger numbers than did deflagellated cells. This was entirely due to the ability of the motile flagellated cells to reach tendon in greater numbers than deflagellated cells.

Conflicting results have been obtained regarding the involvement of flagella in the attachment of bacteria to meat surfaces. Motile gram-negative organisms were first reported to attach to meat more readily than nonmotile gram-positive organisms (1, 17), but this was challenged in later studies (5, 10, 13, 16). In fact, comparing the ability of various flagellated and nonflagellated bacteria to attach to meat surfaces is not appropriate to determine the role of flagella in the process of attachment, since many other species-dependent factors may also be involved (6).

Other approaches have also been tried. A nonflagellated mutant of *Escherichia coli* was found to attach to chicken skin (17) and to chicken breast meat (15) at a much lower rate than did the flagellated wild strain. Also, supplementation of the growth medium with 1% glucose resulted in both a decrease in flagellation and a decrease in attachment of *E. coli* to chicken skin (17). These results suggested a positive role of the flagellum in bacterial attachment to meat. In contrast, removal of the flagella by mechanical means did not alter the ability of *Salmonella typhimurium* to attach to broiler chicken skin (11).

The methods used in the above studies did not lend themselves to adequately resolve the controversy on the role of flagella in adhesion. Contamination of the meat surface was initiated by immersion of the meat in a bacterial suspension. During immersion, a fraction of the bacteria in suspension became associated with the meat surface; the bacteria that remained on the meat after rinsing were considered attached (1, 10, 16, 17). In reality, the so-called attached bacteria consisted of bacteria adhering to the meat surface and of bacteria physically entrapped in crevices and in networks of swollen collagen fibers formed at the meat surface during immersion (22–24). Under these experimental conditions, it is impossible to discriminate between the various possible effects of the flagella on attachment. By promoting motility, the presence of flagella first modifies the chances of the bacterium to encounter the surface and may provide the (kinetic) energy necessary to initiate adhesion. Flagella may also affect adhesion by virtue of their physicochemical characteristics (hydrophobicity, electrical charge) and may affect entrapment (increased bulk of the cell, ability to swim in or out of crevices).

We have previously developed a direct microscopic method for the study of bacterial adhesion to meat (18). This method overcomes some of the limitations inherent to the earlier immersion experiments. In particular, there is no entrapment and adherent bacteria are directly counted in situ. Also, the number of bacteria that actually get in contact with the surface during the experiment can be measured, which enables one to study separately the effect of cell mobility on adhesion. We now use the direct microscopic method to evaluate the role of flagella in the adhesion of *Pseudomonas fluorescens* to thin slices of beef tendon. Tendon was selected as the model surface because its structure and composition are similar to those of epimysium, one of the major surfaces exposed to bacterial contamination during dressing of a carcass.

MATERIALS AND METHODS

Organism and growth conditions. *P. fluorescens* was isolated from beef stored at 4°C (5). A stock culture was prepared by suspending the cells, collected from an agar plate, in reconstituted skim milk (skim milk powder in deionized water at a final concentration of 20% [wt/vol]). The stock culture was stored at −80°C. Working cultures were prepared by inoculating stock cultures in brain heart infusion broth (BHIB; Difco Laboratories, Detroit, Mich.) and then incubating at 25°C without agitation for three consecutive 24-h periods with transfers into fresh medium each day. These cultures were subsequently diluted 1:5,000 in fresh BHIB and incubated at 25°C for 24 h (standardized cultures). The cells were harvested by centrifugation (5,000 × *g*, 10 min, 5°C) and washed twice in phosphate-buffered saline (PBS; 0.1 M potassium phosphate buffer [pH 7.2]

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containing 0.85% [wt/vol] NaCl) before measurement of contact angles, electrophoretic mobility, or adhesion to tendon or resins.

Removal of flagella. A 1.25-liter volume of the standardized culture was centrifuged (5,000 × *g*, 10 min, 5°C), and the cells were resuspended in 300 ml of PBS. The suspension was transferred to a 600-ml beaker and agitated for 30 min (initial temperature, 22°C; final temperature, 25°C) with a vibrating mixer (Vibro mixer E1; Chemapec Inc., Hoboken, N.J.) at the highest frequency to detach the flagella. The deflagellated cells were separated from the loose flagella by centrifugation (5,000 × *g*, 10 min, 5°C), and the cells were washed twice in PBS before use.

Transmission electron microscopy. Flagellated and deflagellated cells were harvested by centrifugation, washed twice with deionized water, and suspended in deionized water to a concentration of about 10^6 CFU ml^{-1}. One drop of the suspension was deposited onto a 400-mesh carbon-reinforced Formvar-coated grid and blotted with absorbent paper after 1 min of contact time. The bacteria adhering to the grid were stained for 1 min with one drop of a phosphotungstic acid solution (2% [wt/vol] in deionized water) containing bovine serum albumin (final concentration, 0.1% [wt/vol]). After removal of the stain, the grid was air dried and finally observed with a Philips EM 420 transmission electron microscope (Philips, Eindhoven, The Netherlands) operating at 80 kV.

Contact angles and electrophoretic mobility. Contact angles were measured as previously described (18). Briefly, washed flagellated or deflagellated cells were resuspended in PBS to an A_{550} of about 0.5. A 20-ml aliquot of the suspension was then filtered through a cellulose triacetate filter (20-mm diameter, 0.45-μm pore size) to form a uniform bacterial layer on the filter surface. The filter bearing the bacterial layer was mounted on a glass microscope slide with double-sided adhesive tape and stored in a desiccator for 30 min. The contact angles of 2-μl drops of saline (0.1 M NaCl in deionized water) or α-bromonaphthalene on the bacterial layer were measured with a goniometer within 2 s after the deposition of the drops.

The electrophoretic mobilities of washed flagellated and deflagellated cells, suspended in deionized water (pH 7.4) to a concentration of 10^7 to 10^8 CFU ml^{-1}, were measured at a potential of 100 V with a Laser Zee Meter 501 zetameter (Pen Kem Co., Bedford Hills, N.Y.).

Adhesion to hydrophobic and ion-exchange resins. Sepharose CL-4B resin and its hydrophobic phenyl and octyl variants were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) as suspensions of the swollen resins in 20% (vol/vol) ethanol. Ion-exchange resins (Dowex 1x8 and Dowex 50Wx8 from Serva, New York, N.Y.; Bio-Rex 70 from Bio-Rad Laboratories, Mississauga, Ontario) were obtained as dry powders (100-200 mesh). These resins were allowed to swell overnight in deionized water at 4°C and used in their chloride (Dowex 1x8), hydrogen (Dowex 50Wx8), and sodium (Bio-Rex 70) forms.

Aliquots (5 ml) of the various swollen resins (5 ml of PBS in controls) and 45 ml of the bacterial suspensions (washed flagellated or deflagellated cells, suspended in PBS to an A_{550} of about 1.0) were poured into 100-ml beakers. The suspensions and the resins were then mixed gently for 20 min on a rotary shaker (165 rpm, 2-cm amplitude) at room temperature. The resins were then allowed to sediment for 20 min, and the A_{550} of the supernatant bacterial suspensions were measured.

Adhesion to tendon slices. A detailed description of the preparation of the tendon slices and of the procedure used to measure adhesion in specially designed flow chambers has been published separately (18). Briefly, a tendon slice, mounted on a glass coverslip, was placed in a chamber and the chamber was positioned in such a way that the tendon slice was at the bottom, face up. Washed flagellated or deflagellated cells, suspended in fresh BHIB to a concentration of about 2×10^7 CFU ml^{-1}, were then introduced into the flow chamber. After 20 min of contact, the chamber was rinsed with deionized water for 5 min at a flow rate of 110 μl s^{-1} to remove nonadherent bacteria. The remaining (adherent) bacteria were then stained and enumerated. Results are expressed as the logarithm of the fraction of bacteria introduced that became adherent: $\log_{10}$ (adherent bacteria/bacteria introduced).

In a separate experiment, the chamber containing the tendon slice was placed on a microscope stage and the bacterial suspension (flagellated or deflagellated) was introduced. The deposition of bacteria on the coverslip bearing the meat slice was then followed over time by using a series of photomicrographs.

Statistical analysis. Analysis of variance and comparison of means were done by using the GLM procedure of the SAS statistical package (SAS Institute, Cary, N.C.).

RESULTS

The strain of *P. fluorescens* used in this study is characterized by a lophotrichous flagellation (Fig. 1A). Microscopic examination (hanging drop technique) of the culture before deflagellation indicated that most of the cells were motile at that time. All movement had ceased after about 15 min of vigourous agitation with the Vibro mixer. Transmission electron micrographs showed that, before deflagellation, about 80% of the cells were flagellated, with a range of 1 to 6 flagellar filaments per cell. Only short fragments of flagella remained on the cells after the first 15 min of agitation (Fig. 1B). Continued agitation for a further 15 min resulted in most of the cells (>95%) having no residual fragments of flagella (Fig. 1C).

The electrophoretic mobility of deflagellated cells was found to be similar ($P \geq 0.05$) to that of flagellated cells (both equal to $[-1.8 \pm 0.1] \times 10^{-8}$ m^2 V^{-1} s^{-1}). The contact angles of a layer of deflagellated cells with drops of saline (θ_s, 20 ± 2°) or α-bromonaphthalene (θ_b, 43 ± 3°) were also not different ($P \geq 0.05$) from those of flagellated cells (θ_s, 20 ± 1°; θ_b, 42 ± 3°).

Flagellated and deflagellated cells both adhered to anion-exchange resin (Dowex 1x8) in small and equal amounts (Table 1). In contrast, there was no significant adhesion to strongly acidic (Dowex 50Wx8) or weakly acidic (Bio-Rex 70) cation-exchange resin.

Flagellated and deflagellated cells adhered to both phenyl- and octyl-Sepharose (Table 1). Adhesion involved hydrophobic interaction of the organisms with the phenyl and octyl moieties, since there was no adhesion in the absence of these groups (Table 1). There was no substantial difference between the level of adhesion of flagellated and deflagellated cells to each resin.

Flagellated cells adhered to tendon slices more ($P < 0.05$) than did deflagellated cells after a 20-min contact time (Table 2). To determine whether the greater adhesion of flagellated cells was possibly related to motility, the rates of deposition of flagellated and deflagellated cells onto the glass coverslip bearing the tendon slice were measured at intervals over 30 to 40 min (Fig. 2). A large number of flagellated cells were

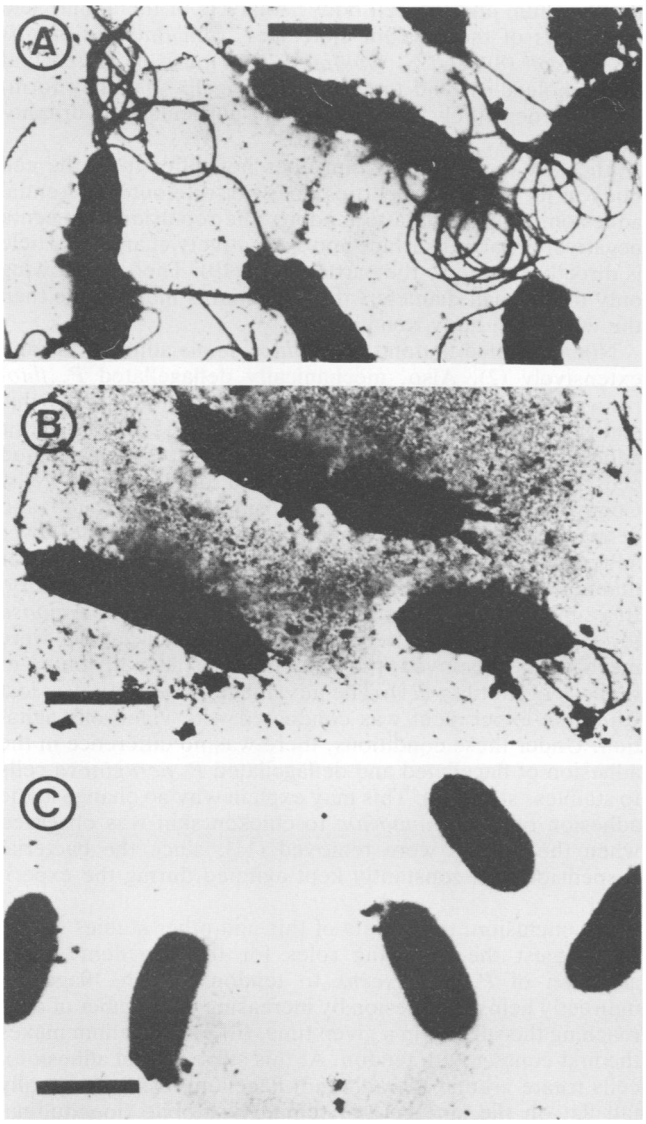

FIG. 1. Transmission electron micrographs of *P. fluorescens* cells before deflagellation (A), after 15 min of deflagellation (B), and after 30 min of deflagellation (C). The cells were negatively stained to demonstrate the presence of flagella. Bars, 1 μm.

seen to be deposited onto the coverslip immediately after introduction of the bacterial suspension into the chamber. The number of deposited cells increased for about 20 min and then reached a plateau. The deposition of deflagellated cells, which occurred only through sedimentation, was much slower. With the same number of flagellated and deflagellated cells introduced in the chamber (1.4×10^7 CFU ml^{-1}), the number of deflagellated cells in contact with the coverslip after 20 min was about 0.5 $\log_{10}$ units lower than the number of flagellated cells deposited during the same time.

The flow chamber containing the tendon slice and a bacterial suspension of flagellated cells was subsequently placed on a microscope stage, and the tendon surface was observed at a ×640 magnification. About 10% of the cells present on the tendon surface appeared to adhere by one pole only and to rotate around it, whereas the other 90% adhered longitudinally and remained immobile. The rotating cells were later seen to either swim away from the surface or fall flat on it and remain immobile in the longitudinal position. A fraction of the longitudinally adherent cells eventually reverted back to their rotating stage and swam away, or they swam away directly with no rotating stage.

TABLE 1. Effect of deflagellation on the adsorption of *P. fluorescens* to ion-exchange resins or hydrophobic resins

Resin and treatment	A_{550}[a]	
	Flagellated cells	Deflagellated cells
Ion-exchange resins		
Control (no resin)	0.86 ± 0.03 A	0.84 ± 0.06 A
Dowex 1x8 resin (Cl form)	0.75 ± 0.02 B	0.73 ± 0.04 B
Dowex 50Wx8 resin (H form)	0.85 ± 0.04 A	0.83 ± 0.05 A
Bio-Rex 70 resin (Na form)	0.84 ± 0.03 A	0.86 ± 0.06 A
Hydrophobic resins		
Control 1 (no resin)	0.84 ± 0.06 A	0.83 ± 0.07 A
Control 2 (Sepharose CL-4B resin)	0.85 ± 0.01 A	0.90 ± 0.03 A
Phenyl Sepharose CL-4B resin[b]	0.40 ± 0.06 C	0.48 ± 0.04 B
Octyl Sepharose CL-4B resin	0.39 ± 0.03 C	0.43 ± 0.07 BC

[a] Shown are the A_{550} values for the bacterial suspensions in the absence of resins (controls) or after 20 min of contact with resins. Within the same resin group (i.e., ion exchange or hydrophobic resins), means followed by the same letters are not significantly different (Duncan's multiple range test; $P > 0.05$).

[b] No significant difference ($P > 0.05$) was found between the adsorption of flagellated and deflagellated cells to phenyl Sepharose when the Gabriel, Scheffé, and Tukey tests were used to compare the means.

DISCUSSION

Valuable information regarding the possible role of flagella in adhesion has been obtained from microscopic observations of bacteria on glass (9, 12, 14). During the colonization of glass surfaces by *P. fluorescens*, the first contact between the organism and the surface was apical (9), with the cell rotating around this point of contact (9, 12, 14). The rotation was apparently linked to flagellar activity, but it was not clearly established whether the flagellated or the nonflagellated pole made contact with the surface (9, 12). However, since a similar rotation was observed when cells of *E. coli* were purposely attached to glass by their flagella by using flagellar antibody (20), the assumption that the rotating *P. fluorescens* cells are attached to glass by their polar flagella seems reasonable (9, 14). Apical contact of *P. fluorescens* is temporary and is followed by one of two events. Either the cell detaches and swims away from the surface, or it stops rotating to become attached longitudinally. In different stud-

TABLE 2. Effects of deflagellation on the adhesion of *P. fluorescens* to tendon

Cell prepn	Bacteria ($\log_{10}$ CFU cm^{-2})[a]		Extent of adhesion[b]
	Introduced in the chamber	Adhering to tendon	
Flagellated	6.06 ± 0.02	5.43 ± 0.04	−0.64 ± 0.03 A
Deflagellated	6.20 ± 0.37	5.27 ± 0.39	−0.93 ± 0.05 B

[a] Expressed as the number of bacteria poised over one unit area.

[b] $\log_{10}$ (bacteria adherent/bacteria introduced). Means followed different letters in the same column are significantly different ($P \leq 0.05$).

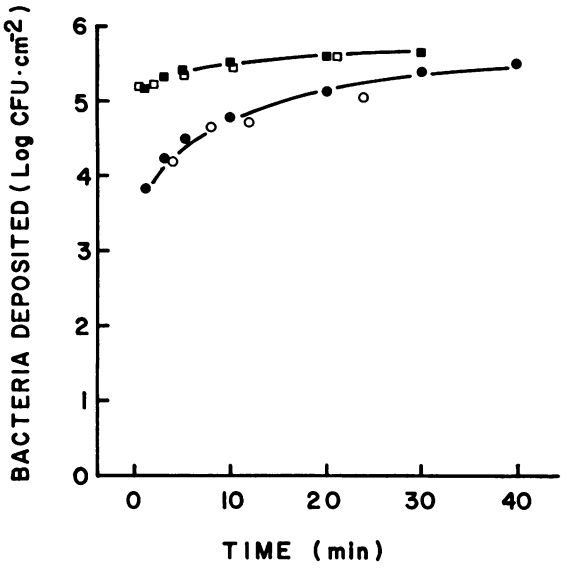

FIG. 2. Deposition rates of flagellated (□, ■) and deflagellated (○, ●) *P. fluorescens* cells on the bottom surface of the flow chamber. Populations introduced in the chamber (CFU poised over 1 cm^2 of surface): (■) 1.2×10^6, (□, ●) 1.1×10^6, (○) 5.9×10^5.

ies, longitudinal attachment was found to be irreversible (9) or reversible (14).

In the present study, the use of thin slices of tendon in a specially designed flow chamber (18) enabled us to make the first direct observations of live bacteria on meat. Results indicated that all the events previously observed on glass, i.e., apical adhesion with rotation around the contact point, longitudinal adhesion, and detachment of both apically and longitudinally adherent cells (9, 12, 14), also take place on tendon. This suggests that (i) as on glass, the *P. fluorescens* cells that rotated around one pole were possibly tethered to the tendon by their flagellum and that (ii) if there is, indeed, flagellar adhesion to tendon, it is a reversible process.

Assuming that the first event leading to the adhesion of *P. fluorescens* to tendon is the tethering of the cells by their flagella (as on glass [9]), the flagellum must attach to tendon more readily than the rest of the bacterium. This might be due to different surface characteristics as is the case for type 1 fimbriae, which have strong adhesive properties related to a low net negative charge and a high degree of hydrophobicity (4). In the present study, no difference was found between flagellated and deflagellated cells in terms of hydrophobicity or surface charge, as determined by contact angles or electrophoretic mobility measurements, respectively. These measurements, however, give an average value for the whole cell surface (including the flagellum). Therefore differences in the surface characteristics of the flagellum and the cell may be undetected, because the contribution of the flagellum to the total bacterial surface is rather low (about 20%, assuming an average of two 0.02- by 5-μm flagellar filaments per 0.5- by 1.5-μm cell). For this reason, the relative surface charges and hydrophobicities of flagellated and deflagellated cells were also compared by using ion-exchange and hydrophobic resins, respectively. Since the interaction of bacteria with resins actually involves specific ligands, the use of resins is well suited to detect local differences in surface properties. The results pointed to no substantial differences between the surface characteristics of the flagellum and the cell body. Analysis of the amino acid sequences of the flagellin molecules from *Bacillus subtilis* (3), *E. coli* (8), and *S. typhimurium* (7) also indicated that these molecules (and therefore the flagella that they form) were not negatively charged and not particularly hydrophobic.

The fact that the flagellum does not differ from the cell body in its surface properties does not rule out preferential adhesion of the flagella to tendon. The repulsion between a negatively charged surface and a negatively charged particle is directly related to the particle size (19). Therefore, owing only to its small diameter, the flagellum is more prone than the whole cell body to adhesion.

Nonflagellated mutants of *P. fluorescens* adhered to sand extensively (2). Also, mechanically deflagellated *P. fluorescens* cells adhered to tendon in large numbers (this study). Therefore flagella do not appear to be necessary for adhesion. However, the extent of adhesion of deflagellated cells to tendon was lower than that of flagellated cells. Comparison of the rates of deposition of flagellated and deflagellated cells in the chamber indicated that the lower adhesion of deflagellated cells merely reflected the lower number of cells reaching the surface, due to loss of motility. In a similar study, the adhesion of *Pseudomonas aeruginosa* to stainless steel was reduced by 90% when flagella were mechanically removed; the reduction was also attributed to the loss of motility (21). The advantage of motility was lost when the experiment was conducted with vigourous agitation. Under these conditions, there was no difference in the adhesion of flagellated and deflagellated *P. aeruginosa* cells to stainless steel (21). This may explain why no change in the adhesion of *S. typhimurium* to chicken skin was observed when the flagella were removed (11), since the bacterial suspension was constantly kept agitated during the experiment.

In conclusion, the results of this and other studies (9, 11, 14) suggest the following roles for the flagellum in the adhesion of *P. fluorescens* to tendon. (i) The flagellum indirectly helps in adhesion by increasing the number of cells reaching the surface in a given time. (ii) The flagellum makes the first contact with tendon. At this stage (apical adhesion), cells rotate around the adherent flagellum. Cells eventually fall flat on the surface and remain immobile (longitudinal adhesion). (iii) Tethering of the cell by its flagellum does not involve special surface characteristics (high hydrophobicity, low surface charge), since the flagellum is no different from the cell surface in this respect. Rather, the small radius of the flagellum makes it more prone than the cell body to adhesion. (iv) Adhesion of the flagellum to tendon is reversible. Both apically and longitudinally adherent cells can swim away from the surface. (v) Even though the flagellum is implicated in adhesion (items i through iv), its presence is not essential for adhesion to occur. Deflagellated cells adhere to tendon in large numbers.

Since tendon slices are similar to epimysium in structure and composition, the above conclusions are expected to be valid on the thin epimysium layer surrounding muscles.

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