APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1992, p. 2783–2791
0099-2240/92/092783-09$02.00/0
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Vol. 58, No. 9

# A Model Study of Factors Involved in Adhesion of *Pseudomonas fluorescens* to Meat

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Received 11 March 1992/Accepted 11 June 1992

**A study was undertaken to investigate the factors involved in the adhesion of *Pseudomonas fluorescens* to model meat surfaces (tendon slices). Adhesion was fast (<2.5 min) and was not suppressed by killing the cells with UV, γ rays, or heat, indicating that physiological activity was not required. In various salt solutions (NaCl, KCl, $CaCl_2$, $MgCl_2$), adhesion increased with increasing ionic strength up to 10 to 100 mM, suggesting that, at low ionic strengths, electrostatic interactions were involved in the adhesion process. At higher ionic strengths (>10 to 100 mM) or in the presence of $Al^{3+}$ ions, adhesion was sharply reduced. Selectively blocking of carboxyl or amino groups at the cell surface by chemical means did not affect adhesion. These groups are therefore not directly involved in an adhesive bond with tendon. Given a sufficient cell concentration ($10^{10}$ CFU · $ml^{-1}$) in the adhesion medium, the surface of tendon was almost entirely covered with adherent bacteria. This suggests that if the adhesion is specific, the attachment sites on the tendon surface must be located within collagen or proteoglycan molecules.**

In the first study of bacterial attachment to meat (32), broilers were immersed in bacterial suspensions and then drained. The bacteria transferred from the suspensions to the meat surface during immersion were considered attached. The same immersion method, with minor modifications, was used in most subsequent studies of bacterial attachment to meat (2–4, 8–10, 12, 13, 21–24, 27, 30, 31, 33, 45, 46). From the results of these and other studies employing electron microscopy (26, 28, 41–44, 49), many factors that affect bacterial attachment to meat (strain, culturing method, and concentration of the organism, nature of the meat surface, contact time, temperature, pH, presence and concentration of ionic species in the adhesion medium, etc.) were identified.

Little is known of the precise nature of the mechanisms involved. Attachment was rapid in the first few minutes of immersion (1, 2, 4, 21, 24). During that time, large numbers of bacteria adhered to meat or became entrapped in channels and crevices at the meat surface (11, 34) but the respective contributions of adhesion and entrapment to surface contamination were unknown. What role, if any, flagella played at this stage remained unclear (1, 2, 10, 21, 23, 27, 30, 31). There is evidence that electrostatic interactions are involved in adhesion (8, 9, 24, 45), but it is not known whether there is also a specific mechanism of adhesion involving cell adhesins and attachment sites on the meat surface. It is also not known whether a primary polymer is involved in bacterial adhesion to meat, as is the case in the adhesion of a marine bacterium to solid surfaces (16). With time, adherent bacteria produce a secondary extracellular polymer (3, 12, 31). The role of this polymer in attachment remains conjectural.

We previously developed a new method to enumerate bacteria adhering to model meat surfaces (slices of beef, fat, and tendon [37]). This method was later used to study the adhesion of seven meat spoilage organisms (39). It was concluded that only limited information on the mechanisms of adhesion could be inferred from comparing the adhesion of various organisms with different characteristics. We subsequently selected *Pseudomonas fluorescens* for further studies, because this organism had been found to adhere to meat in large numbers (2, 10, 31, 39) and because of its prominent role in meat spoilage (10). Initially, we clarified the role of flagella in adhesion (38), and we now investigate the effects of surface charge, cell hydrophobicity, ionic environment and strength, and viability of cells on the adhesion of *P. fluorescens* to tendon slices, used as model surfaces for the epimysium layer surrounding muscles.

## MATERIALS AND METHODS

**Organisms and cultures.** *Enterobacter agglomerans* and *P. fluorescens* were isolated from refrigerated beef (10). Stock cultures were prepared in reconstituted skim milk (skim milk powder in deionized water, 20% [wt/vol] final concentration) and kept frozen at −80°C. Standardized cultures were obtained by growing the organisms at 25°C in brain heart infusion broth (BHIB; Difco Laboratories, Detroit, Mich.) for three consecutive 24-h periods, with transfers in fresh medium each day. The standardized cultures were then diluted 1:50,000 in fresh BHIB and incubated at 25°C for 24 h (working cultures). The *Lactobacillus* sp. was isolated from refrigerated vacuum-packed ham (37) and cultured in a similar way with Difco lactobacillus MRS broth as the growth medium. All incubations were without agitation.

**Measurement of adhesion.** Measurement of bacterial adhesion to tendon slices in specially designed flow chambers was described previously (37). In summary, a tendon slice was introduced into a flow chamber. The chamber was positioned to place the slice at the bottom, face up, and was filled with a suspension of the bacterial cells (ca. $10^7$ CFU · $ml^{-1}$). The suspension was left in the chamber to initiate adhesion (usually for 20 min) and rinsed away with deionized water (5 min, 110 μl · $s^{-1}$) before the remaining (adherent) bacteria were stained and enumerated.

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In some cases, photomicrographs of the bottom coverslip (bearing the meat slice) were taken before rinsing to determine the number of cells that reached the surface during the experiment.

**Influence of the contact time on adhesion.** The 24-h working culture was diluted 1:50 in sterile BHIB and introduced in a series of identical flow chambers containing tendon slices. The first chamber was immediately rinsed (contact time 0 min), and the bacteria adhering to tendon were stained and enumerated. The other chambers were rinsed after increasing contact times (2.5, 5, 10, 20 min) before the adherent bacteria were enumerated.

**Effect of various lethal treatments on adhesion.** The cells from the 24-h working culture were harvested by centrifugation (5,000 × *g*, 10 min, 5°C), washed twice in phosphate-buffered saline (PBS; 0.1 M potassium phosphate buffer [pH 7.2] containing 0.85% [wt/vol] NaCl), and suspended in 0.1 M potassium phosphate buffer (pH 7.2). The cells were left untreated (control) or exposed to UV irradiation (20 ml of suspension in a petri dish [100 by 15 mm] exposed for 3 min under a 4-W shortwave UV Lab-Lite bulb [Lab-Aids Inc., Bohemia, N.Y.]), to γ rays (10 ml of suspension exposed for 10 min to a total dose of 0.33 kGy in the AECL irradiator of the Food Research and Development Centre, St. Hyacinthe, Québec, Canada), or to heat (10 ml of suspension at 50°C for 10 min). The treated and untreated cells were then diluted 1:50 in 0.1 M potassium phosphate buffer (pH 7.2) and introduced into flow chambers, and the extent of adhesion to tendon was determined.

**Detachment experiments.** The 24-h working culture was diluted 1:50 in sterile BHIB and introduced into a flow chamber. After 20 min of contact, the chamber was rinsed with deionized water for 5 min to remove nonadherent bacteria. To determine whether the remaining bacteria could be detached, the meat slice with adhering bacteria was subjected to successive rinses (each of 5 min) with increasing concentrations of various eluents. The eluents used were cations (0 to 1 M NaCl, KCl, $CaCl_2$, and $MgCl_2$ in 0.1 M increments; 10 μM, 100 μM, and 1 mM $AlCl_3$), salts of organic acids (0 to 1 M sodium citrate, sodium acetate, and sodium lactate in 0.1 M increments), surfactants (0 to 2% Tween 20 in 0.2% increments; 0 to 1% Tween 80 in 0.1% increments; 0 to 8% *n*-butanol in 1% increments; 0 to 50% methanol in 5% increments; 0.1, 0.2, and 0.3% sodium dodecyl sulfate), and a chelator (20 mM EDTA). After the last rinse with the eluent, the chamber was rinsed once more with deionized water to remove the residual eluent and the remaining adherent bacteria were stained and enumerated.

**Influence of ionic strength and cations on adhesion.** To determine the influence of ionic strength on adhesion, the 24-h working culture was first centrifuged (5,000 × *g*, 10 min, 5°C) and the sedimented cells were washed twice in PBS. The washed cells were suspended to a concentration of about $10^7$ CFU · $ml^{-1}$ in each of a series of potassium phosphate buffers (pH 7.2) of various ionic strengths (5, 50, 100, 250, and 500 mM), and the adhesion of the cells to tendon was measured in the flow chambers. The influence of cations on adhesion was determined in a similar way with salt solutions at various concentrations (0.1, 1, 10, 100, and 500 mM NaCl, KCl, $CaCl_2$, and $MgCl_2$; 0.1 and 1 mM $AlCl_3$) as adhesion media. The $AlCl_3$ solution was adjusted to pH 6 to 7 with 0.1 N NaOH before use. The concentration of sodium ions introduced in the solution as a result of pH adjustment was lower than 10 mM.

**Chemical modification of the cell surface and its effect on adhesion.** A method adapted from that of Fraenkel-Conrat and Cooper (18) was used to increase the net negative charge of bacteria. The cells from the 24-h working culture were washed twice in PBS and suspended (about $10^{10}$ CFU) in a mixture of 1 ml of 0.1 M citrate phosphate buffer (pH 2.2) and 4 ml of 0.1% (wt/vol) orange-G (The Coleman and Bell Co., Norwood, Oreg.) in deionized water. The suspension was shaken for 24 h on a reciprocating shaker (100 strokes per min) at room temperature. The suspension was then centrifuged (5,000 × *g*, 10 min, room temperature), the sedimented cells were washed twice in deionized water, and then the surface characteristics and adhesion to tendon were measured.

Neutralization of bacterial surface charges was done with 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride–glycinamide hydrochloride (EDC-G) by the method of Neihof and Echols (29). The 24-h PBS-washed cells (about $10^{10}$ CFU) were suspended in 40 ml of a 3 M solution of glycinamide hydrochloride (Sigma Chemical Co., St. Louis, Mo.) containing 2% (wt/vol) 1(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (Aldrich Chemical Co., Milwaukee, Wis.). The suspension, adjusted to pH 5.0 with 0.1 N NaOH, was left at room temperature for 20 to 24 h, during which time the pH remained unchanged. The suspension was then homogenized at high speed in a Sorvall Omnimixer (Ivan Sorvall Inc., Norwalk, Conn.) to break the cell clumps formed during treatment; the EDC-G-treated cells were collected (5,000 × *g*, 10 min, 5°C) and washed twice in deionized water before further use.

Bacterial cell surfaces were made positively charged by treating the cells with *N*-(2-hydroxy-ethyl)ethyleneimine (HEEI; Aldrich) by a method adapted from that of Gittens and James (19). The PBS-washed cells ($10^{10}$ CFU) were washed twice in 0.05 N HCl and twice in dioxan at about 20°C (dioxan freezes at 12°C) and finally suspended in 10 ml of dioxan. A 0.2-ml volume of HEEI was added, and the suspension was mixed and incubated at 40°C for 7 days. The treated cells were collected (5,000 × *g*, 10 min, 5°C) and washed twice in dioxan, and the cell clumps were broken at high speed in the Sorvall Omnimixer. Finally, the cells were washed twice in deionized water before use.

Adhesion of the chemically treated and untreated cells, at concentrations of about $10^7$ CFU · $ml^{-1}$, was measured in deionized water with a contact time of 20 min.

**Contact angles and electrophoretic mobilities of chemically treated and untreated cells.** The contact angles of the chemically treated and untreated cells were measured as previously described (39). In short, the cells were suspended in PBS ($A_{550}$ = 0.5) and filtered through a cellulose triacetate filter to form a uniform bacterial layer on the filter surface; the contact angle of a 2-μl drop of a 0.1 M NaCl solution on the bacterial layer was measured with a goniometer.

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

**Adhesion strength of chemically treated and untreated cells.** A qualitative estimation of the strength of adhesion of chemically treated and untreated cells to glass was obtained as follows. The cells were first introduced into a flow chamber to initiate adhesion. After the usual 20-min contact time and the subsequent rinsing step, the chamber was fixed on a microscope stage. The microscope was focused on the bottom glass coverslip to observe adherent bacteria, and the chamber was then emptied of liquid. A residual film of water remained on the glass surface, and this continuous film soon

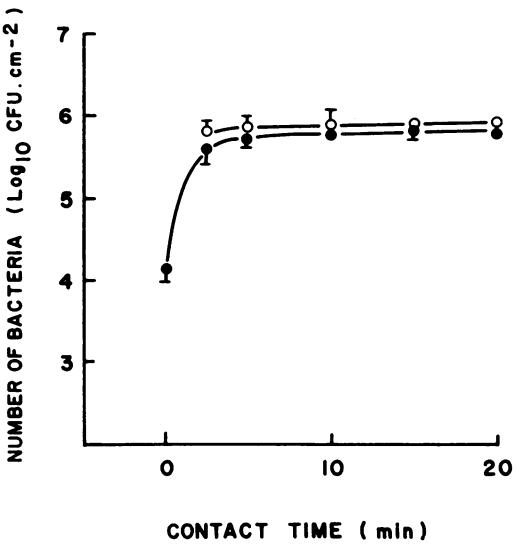


FIG. 1. Sedimentation (○) and adhesion to tendon slices (●) of *P. fluorescens* cells as functions of time. Points are the averages of two separate experiments with two replicates each.

coalesced into a multitude of small water droplets. Depending on the treatment applied to the cells, one of two events occurred during coalescence of the water film. Either the adherent cells were completely detached from the glass by the receding water front to be subsequently found in the water droplets formed, or the adherent bacteria remained in their original position after the droplet formation. The latter case indicated a stronger adhesion of the bacteria to glass.

**Influence of the cell concentration on adhesion.** The 24-h working culture was centrifuged (5,000 × *g*, 10 min, 5°C), and the cells were washed twice in PBS and suspended in 0.085 M NaCl (in deionized water, adjusted to pH 7.2) to a concentration of $10^{10}$ CFU · $ml^{-1}$. This suspension was serially diluted to obtain concentrations of $10^9$, $10^8$, $10^7$, and $10^6$ CFU · $ml^{-1}$. Each suspension was introduced into a separate flow chamber, and the adhesion to tendon was measured after 20 min of contact time.

## RESULTS

**Influence of contact time on adhesion.** With a population of $1.6 \times 10^7$ CFU · $ml^{-1}$ introduced into the flow chamber (corresponding to $1.3 \times 10^6$ CFU poised over 1 $cm^2$), a large number of cells ($6.3 \times 10^5$ CFU · $cm^{-2}$) reached the chamber bottom (and therefore the tendon slice) within 2.5 min of the introduction of the bacteria (Fig. 1). This number did not substantially increase with time. A slightly lower number of cells remained adherent to tendon after rinsing. Adhesion was fast and had nearly reached its maximum level after 2.5 min of contact time (Fig. 1).

**Effect of various lethal treatments on adhesion.** The three lethal treatments reduced the viable *P. fluorescens* populations in suspension by more than 3.6 $\log_{10}$ CFU · $ml^{-1}$ (Table 1). Regardless of the treatment, the number of cells adhering to tendon was at least 1.7 $\log_{10}$ units higher than the number of viable cells in the chamber, indicating that viability was not required for adhesion.

The effects of the three treatments on adhesion were not identical. Whereas the adhesion of UV-treated cells was similar to the adhesion of untreated cells, cells that had been killed by heat or by γ rays adhered in much lower numbers. This reflected a real decrease in adhesive properties, since the various treatments did not affect substantially the numbers of cells reaching the tendon surface during the experiment.

**Detachment experiments.** The number of cells adhering to tendon after the usual 5-min rinse in deionized water was 5.75 $\log_{10}$ CFU · $cm^{-2}$ (Table 2). This number was only marginally reduced when the rinsing time was increased to 55 min (total rinsing time in most elution trials), and no further release of cells occurred with the other rinsing solutions (Table 2).

**Influence of ionic strength and cations on adhesion.** Increasing the ionic strength of the adhesion medium from 5 to 100 mM caused a slight increase in the adhesion of *P. fluorescens* cells to tendon (Fig. 2). Further increases in ionic strength resulted in a sharp decrease in adhesion. A similar effect was observed when the concentration of monovalent cations in the adhesion medium was increased (Fig. 3). The effect of divalent cations on adhesion was also similar, except that maximum adhesion was reached at a lower concentration of the cations (1 to 10 mM). The sharp decrease in adhesion observed with high salt concentrations (>100 mM) was not due to a failure of the cells to reach the tendon surface. At 500 mM, the numbers of cells in contact with the chamber bottom before rinsing were 5.33 ± 0.10, 5.15 ± 0.17, and 5.27 ± 0.02 $\log_{10}$ CFU · $cm^{-2}$ in the KCl, $CaCl_2$, and $MgCl_2$ solutions, respectively.

Adhesion of *P. fluorescens* to tendon was markedly reduced in the presence of $AlCl_3$, even in low concentrations (0.1 and 1 mM).

**Chemical modification of the cell surface and its effect on adhesion.** Normal cells of *E. agglomerans*, *Lactobacillus* sp., and *P. fluorescens* are negatively charged (Table 3). Treatment with orange-G increased the net negative charge of the *P. fluorescens* cells but not that of the *E. agglomerans*

TABLE 1. Effects of various lethal treatments on the adhesion of *P. fluorescens* to tendon slices

| Treatment | No. of viable bacteria[a] | | Total no. of bacteria[a,b] ($\log_{10}$ CFU · $cm^{-2}$) | |
|---|---|---|---|---|
| | In suspension ($\log_{10}$ CFU · $ml^{-1}$) | Introduced in the chamber[c] ($\log_{10}$ CFU · $cm^{-2}$) | On the chamber bottom after 20 min[c] | Adherent to tendon |
| Heat | 3.51 ± 0.48 | 2.41 ± 0.33 | 5.53 ± 0.04 B | 4.14 ± 0.17 C |
| UV irradiation | 3.25 ± 0.56 | 2.15 ± 0.37 | 5.55 ± 0.05 B | 5.45 ± 0.02 B |
| γ irradiation | <2.07 | <0.97 | 5.48 ± 0.05 B | 4.30 ± 0.19 C |
| None (control) | 7.15 ± 0.12 | 6.05 ± 0.10 | 5.66 ± 0.06 A | 5.65 ± 0.05 A |

[a] Means ± standard deviations ($n \geq 3$).
[b] Values with different letters in the same column are significantly different ($P < 0.05$).
[c] Expressed as the number of viable bacteria poised over a unit area. The total number of bacteria introduced was the same in all chambers and was roughly equal to the number of viable bacteria in the control.

TABLE 2. Detachment of adherent *P. fluorescens* cells from tendon slices during rinsing with various eluents

| Eluent | Concns of eluent in the successive 5-min rinses | Total rinsing time (min) | Bacteria adherent to tendon after the last rinse ($log_{10}$ CFU · $cm^{-2}$)[a] |
|---|---|---|---|
| Controls | | | |
| Deionized water | | 5 | 5.75 ± 0.02 |
| Deionized water | | 55 | 5.60 ± 0.07 |
| Cations (chloride salts) | | | |
| NaCl | 0 to 1 M in 0.1 M increments | 55 | 5.63 ± 0.01 |
| KCl | 0 to 1 M in 0.1 M increments | 55 | 5.65 ± 0.01 |
| $CaCl_2$ | 0 to 1 M in 0.1 M increments | 55 | 5.59 ± 0.09 |
| $MgCl_2$ | 0 to 1 M in 0.1 M increments | 55 | 5.44 ± 0.10 |
| $AlCl_3$ | 10 μM, 100 μM, 1 mM | 20 | 5.57 ± 0.01 |
| Organic acids (sodium salts) | | | |
| Sodium citrate | 0 to 1 M in 0.1 M increments | 55 | 5.60 ± 0.03 |
| Sodium acetate | 0 to 1 M in 0.1 M increments | 55 | 5.61 ± 0.03 |
| Sodium lactate | 0 to 1 M in 0.1 M increments | 55 | 5.71 ± 0.03 |
| Surfactants | | | |
| Tween 20 | 0 to 2% in 0.2% increments | 55 | 5.69 ± 0.05 |
| Tween 80 | 0 to 1% in 0.1% increments | 55 | 5.76 ± 0.03 |
| *n*-Butanol | 0 to 8% in 1% increments | 45 | 5.67 ± 0.02 |
| Methanol | 0 to 50% in 5% increments | 55 | 5.75 ± 0.03 |
| Sodium dodecyl sulfate | 0.1, 0.2, 0.3% | 20 | 5.62 ± 0.07 |
| Chelator (EDTA) | 20 mM | 10 | 5.72 ± 0.04 |

[a] Means ± standard deviations ($n \geq 3$).

or *Lactobacillus* sp. cells. Treatments with EDC-G and HEEI caused the net surface charge of the three organisms to become nearly neutral and positive, respectively.

In general, changes in the surface charge resulted in an increase in contact angles, i.e., an increase in hydrophobicity; the positively charged HEEI-treated cells were the most hydrophobic (Table 3).

There was no substantial change in the adhesion of *E. agglomerans*, *Lactobacillus* sp., or *P. fluorescens* cells to tendon after treatment with EDC-G or HEEI (Table 4). In general, the small variation observed between treated and untreated cells merely reflected the changes in the numbers of cells that reached the chamber bottom during the experiment. There was also no change in the adhesion of *Lactobacillus* sp. and *P. fluorescens* cells to tendon after treatment with orange-G. However, orange-G-treated *E. agglomerans* cells adhered to tendon in much greater numbers than did untreated cells.

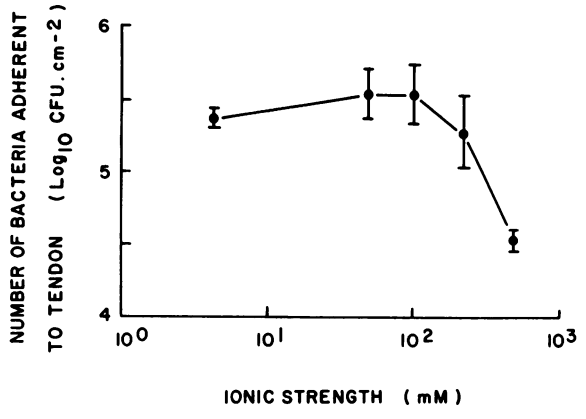


FIG. 2. Adhesion to tendon slices of *P. fluorescens* cells suspended in potassium phosphate buffers of various ionic strengths. Points are the averages of two separate experiments with two replicates each.

The effects of the various cell treatments on adhesion to glass were quite different. In particular, adhesion was largely increased when cells were treated with EDC-G (except for *P. fluorescens*) and even more so when cells were treated with HEEI (Table 4).

In only one case (i.e., with *P. fluorescens*) were there sufficient numbers of untreated and orange-G-treated cells adhering to glass for evaluation of the adhesion strength (Table 4). Here, the adhering bacteria were detached by the shear force of the receding water film. In contrast, EDC-G- and HEEI-treated cells were not displaced from the glass surface during receding of the water film into small droplets, indicating a stronger adhesion.

Attempts to evaluate the strength of adhesion to tendon by the same method failed because of difficulties in observing unstained bacteria on tendon. Observation of stained slices with adherent bacteria never showed cells packed in drop-like clumps. Whether this is due to strong adhesion of the bacterium to tendon or to the absence of droplet formation during the evaporation of the residual water film is unknown.

**Influence of the cell concentration on adhesion.** The number of *P. fluorescens* cells adhering to tendon increased almost linearly; the number of cells introduced in the chamber increased from $10^5$ to $10^8$ CFU · $cm^{-2}$ (Fig. 4). Above that level, the number of adherent cells gradually levelled off to reach a value of about $3 \times 10^7$ CFU · $cm^{-2}$ (Fig. 4). With this concentration, the tendon surface was entirely covered with bacteria (Fig. 5).

## DISCUSSION

One of the most striking results in the earlier studies of bacterial attachment to meat (1, 2, 4, 21, 24) was the rapidity with which attachment was initiated. Within 1 min of the

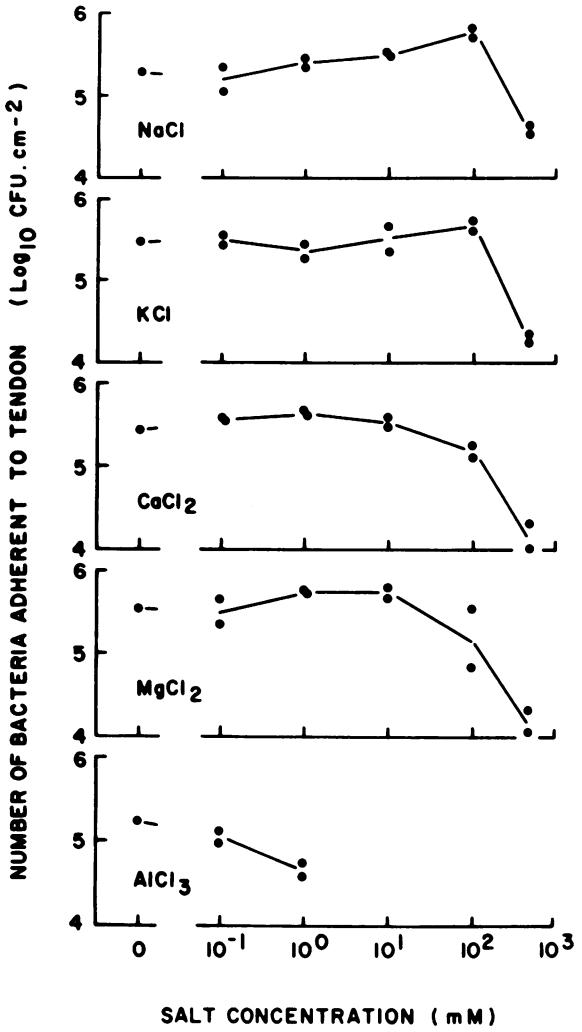


FIG. 3. Adhesion to tendon slices of *P. fluorescens* cells suspended in deionized water containing chloride salts of selected cations in various concentrations. Points represent the results of duplicate experiments with each salt concentration.

immersion of meat pieces in bacterial suspensions, large numbers of cells became associated with the meat surface. Whether these cells were adhering to the meat or merely entrapped in crevices or channels at the meat surface could not be resolved. In the present study, the use of a new method in which adherent bacteria can be precisely counted in situ (37) enabled us to show that the adhesion of *P. fluorescens* cells to model meat surfaces (tendon slices) is, indeed, very rapid. This implies that adhesion does not depend on physiological processes triggered by contact with the target surface. This is further demonstrated by the fact that cells which have been inactivated by UV, $\gamma$ rays, or heat adhere to tendon in large numbers. Therefore, if adhesion involves a specialized structure on the cell surface, this has to be constitutively expressed. A similar conclusion was reached by Stanley (42) in a study of colonization of stainless steel by *Pseudomonas aeruginosa*.

Several attempts have been made to elucidate the mechanisms of adhesion from the results of experiments in which adherent cells were detached with various eluents. For

TABLE 3. Effects of various chemical treatments of the cell surface on the hydrophobicity (contact angle) and electric charge (electrophoretic mobility)[a]

| Organism and treatment | Electrophoretic mobility[b] ($10^8$ m$^2$ · V$^{-1}$ · s$^{-1}$) | Contact angle[c] (°) |
|---|---|---|
| *E. agglomerans* | | |
| None | −3.2 ± 0.1 C | 12 ± 1 C |
| Orange-G | −3.3 ± 0.1 C | 14 ± 1 C |
| EDC-G | −0.3 ± 0.1 B | 19 ± 2 B |
| HEEI | 1.5 ± 0.1 A | 70 ± 6 A |
| *Lactobacillus* sp. | | |
| None | −1.8 ± 0.1 C | 13 ± 1 B |
| Orange-G | −1.8 ± 0.1 C | 12 ± 1 B |
| EDC-G | 0.0 ± 0.2 B | 12 ± 1 B |
| HEEI | 0.6 ± 0.2 A | 37 ± 13 A |
| *P. fluorescens* | | |
| None | −1.9 ± 0.2 C | 20 ± 1 D |
| Orange-G | −3.9 ± 0.1 D | 37 ± 6 B |
| EDC-G | −0.2 ± 0.2 B | 26 ± 2 C |
| HEEI | 1.2 ± 0.1 A | 73 ± 3 A |

[a] For each organism, values with different letters in the same column are significantly different ($P < 0.05$).
[b] Means ± standard deviations ($n \geq 3$).
[c] Means ± standard deviation ($n \geq 12$).

example, Thomas and McMeekin (45) reported that rinses with physiological saline (but not rinses with deionized water) effectively detached large numbers of *Salmonella typhimurium* and *S. singapore* cells from collagen fibers. The authors suggested that sodium chloride interfered with a physiological interaction between the organism and collagen or the mucopolysaccharide matrix around it. Also, the fact that *Streptococcus faecium* could be desorbed from glass by a 2% Tween 80 solution but not by a 2 M NaCl solution (35) was interpreted as an indication that hydrophobic rather than ionic interactions were involved in the adhesion process. However, the present study indicates that the usefulness of detachment experiments in explaining adhesion mechanisms should be questioned. For example, the fact that no substantial removal of *P. fluorescens* cells adhering to tendon was obtained by rinsing with solutions containing various cations (0 to 1 M range) does not mean, in our view, that ionic interactions do not participate in the adhesion process. Indeed, adhesion was markedly affected by changes in the concentrations (0 to 500 mM range) of the same cations in the adhesion medium. Similarly, no relation was found between the conditions that control adhesion of bacteria to ion-exchange resins and the conditions that control detachment (48). Also, Jones et al. (20) clearly demonstrated that *S. typhimurium* cells could not be washed off of HeLa cells by low-ionic-strength solutions, although decreasing the ionic strength in the suspension medium sharply reduced adhesion.

Even though the results of the detachment experiments did not clarify the mechanism of adhesion (this study), they have an important practical implication: removal of bacteria from meat by rinsing with solutions compatible with the meat integrity (dilute salt and/or organic acid solutions) is ineffective. This adds to an already large amount of evidence (1, 5, 6, 33) pointing to the futility of adding more washing steps in processing lines to improve carcass hygiene. At best, the reduction in bacterial population achieved with rinsing procedures is expected to be around 1 $\log_{10}$ cycle,

TABLE 4. Effect of various chemical treatments of the cell surface on adhesion to tendon slices

| Organism and treatment | No. of bacteria ($\log_{10}$ CFU · $cm^{-2}$)[a] | | | | Adhesion strength[b] |
|---|---|---|---|---|---|
| | Introduced in the chamber[c] | On the chamber bottom after 20 min | Adherent to tendon | Adherent to glass | |
| *E. agglomerans* | | | | | |
| None | 5.98 ± 0.14 | 5.25 ± 0.04 | 4.55 ± 0.27 | 2.78 ± 0.50 | NA[d] |
| Orange-G | 5.94 ± 0.12 | 5.11 ± 0.46 | 5.21 ± 0.21 | <1.94 | NA |
| EDC-G | 5.90 ± 0.06 | 5.16 ± 0.07 | 4.32 ± 0.31 | 4.02 ± 0.39 | + |
| HEEI | 6.16 ± 0.12 | 4.94 ± 0.05 | 4.02 ± 0.21 | 4.87 ± 0.07 | + |
| *Lactobacillus* sp. | | | | | |
| None | 5.65 ± 0.38 | 4.67 ± 0.04 | 4.67 ± 0.15 | 2.18 ± 0.63 | NA |
| Orange-G | 5.77 ± 0.15 | 4.84 ± 0.02 | 5.01 ± 0.12 | 2.81 ± 0.36 | NA |
| EDC-G | 5.75 ± 0.06 | 4.90 ± 0.05 | 4.88 ± 0.03 | 4.93 ± 0.11 | + |
| HEEI | NT[e] | NT | NT | NT | NT |
| *P. fluorescens* | | | | | |
| None | 6.40 ± 0.15 | ND[f] | 5.31 ± 0.17 | 4.36 ± 0.21 | − |
| Orange-G | 6.23 ± 0.20 | ND | 5.16 ± 0.22 | 3.92 ± 0.47 | − |
| EDC-G | 6.26 ± 0.07 | ND | 5.35 ± 0.01 | 4.16 ± 0.24 | + |
| HEEI | 6.57 ± 0.09 | ND | 5.07 ± 0.05 | 5.20 ± 0.06 | + |

[a] Means ± standard deviations ($n \geq 3$).
[b] Bacteria were (−) or were not (+) displaced by the receding water film.
[c] Expressed as the number of bacteria poised over one unit area.
[d] NA, not applicable (not enough cells to measure adhesion strength).
[e] NT, not tested (clumps of chemically treated cells could not be broken apart).
[f] ND, not determined (cells were not sufficiently visible to permit enumeration).

which is clearly insufficient to bring about a meaningful improvement in the bacterial quality of meat (24).

We previously reported that the adhesion of *P. fluorescens* to tendon was higher in a 85 mM solution of NaCl than in deionized water (39). Now we show that it is ionic strength in general, rather than the concentration of sodium ions, that affects adhesion. Indeed, adhesion increased slightly with increasing ionic strengths (up to 10 to 100 mM), whether the adhesion occurred in potassium phosphate buffer or in various salt solutions containing monovalent (sodium, potassium) or divalent (calcium, magnesium) cations. Increases in the adhesion of organisms to various surfaces with increasing concentrations of monovalent or divalent cations were also reported by Stanley (42), Jones et al. (20), Ørstavik (35), and Marshall et al. (25). The increase in adhesion was attributed to the reduction in thickness of the electrical diffuse double layer resulting from the presence of ions in solution (20, 25). This, in turn, enables negatively charged bacteria to come closer to negatively charged surfaces before electrostatic repulsion comes into play (15), thereby increasing the number of chances to initiate adhesion through the action of attractive van der Waals forces or via cell surface appendages (40). Therefore, the variation of adhesion with ionic strength observed in this study suggests that, at low ionic strengths (<10 to 100 mM), electrostatic

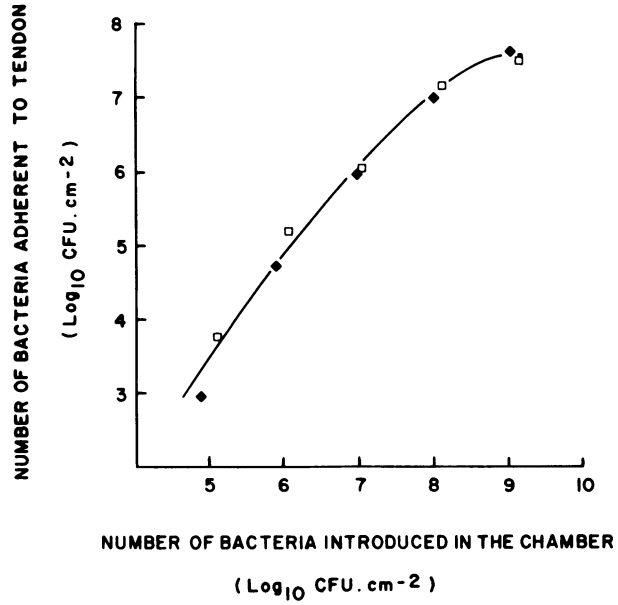


FIG. 4. Adhesion of *P. fluorescens* to tendon slices as a function of the number of bacteria introduced into the flow chamber. Open and closed symbols represent the results of duplicate experiments.

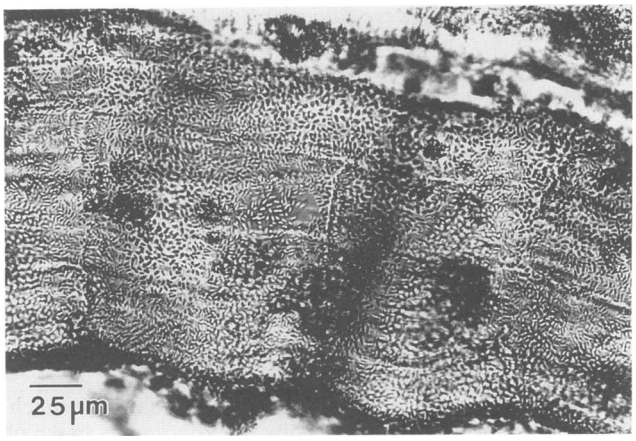


FIG. 5. Tendon slice covered with adherent *P. fluorescens* cells.

interactions are involved in the adhesion of *P. fluorescens* to tendon.

At higher ionic concentrations (>10 to 100 mM), a sharp decrease in the adhesion of *P. fluorescens* to tendon was observed. Stanley (42) also reported a decrease in the adhesion of *P. aeruginosa* to stainless steel when the electrolyte (NaCl, $CaCl_2$) concentration in the adhesion medium reached 10 to 100 mM. Also, a large decrease in the adhesion of *Salmonella* spp. to collagen fibers was observed when the experiment was performed in physiological saline (150 mM NaCl) instead of deionized water (24, 45). At these high salt concentrations, the outer wall layers of bacteria are altered (17) and structures possibly involved in adhesion may be denatured or released into the medium. The situation is even more complex when adhesion is studied on a biological surface, which can also be affected by the presence of ions in the adhesion medium. In particular, the mucopolysaccharide cementing matrix surrounding collagen fibers is partially solubilized in the presence of 50 mM concentrations of NaCl (45). Tendon, which is composed essentially of collagen, is also expected to be altered at high salt concentrations.

The adhesion of *P. fluorescens* to tendon was reduced in the presence of $Al^{3+}$ cations. Marshall et al. (25) also reported a decrease in the adhesion of a marine pseudomonad to glass in the presence of trivalent cations ($Al^{3+}$, $La^{3+}$). This is surprising, since these cations, like the others, might be expected to promote adhesion by reducing the thickness of the electrical diffuse double layer. The mechanism by which trivalent cations interfere with the adhesion of pseudomonads is unknown. It has been suggested that these ions are involved in cross-linking polymeric substances the structural integrity of which is essential for adhesion (14).

Selectively blocking charged groups at the cell surface has been proposed as a means to evaluate the effect of surface charge on adhesion (48). In general, treatments that decreased the net negative charge (i.e., blocked the carboxyl and/or other anionic groups) promoted adhesion of *Staphylococcus aureus* to cation-exchange resins (48), whereas adhesion was reduced by treatments that blocked the cationic (essentially amino) groups. In the present study, the two treatments that were intended to esterify the carboxyl groups (EDC-G, HEEI) caused the organisms to become almost electrically neutral (EDC-G) or positively charged (HEEI), indicating a partial (EDC-G) or more complete (HEEI) neutralization of the anionic groups. Also, blocking of amino groups with orange-G was successful on one organism (*P. fluorescens*), increasing its net negative charge. Therefore, selective changes in surface charges were achieved with appropriate chemical treatments. However, since the changes resulted from the neutralization of charged groups, an overall increase in hydrophobicity (contact angles) was constantly obtained. As a result, any subsequent change in adhesion cannot be attributed specifically to electrostatic or hydrophobic interactions.

Even though the contribution of cell surface charge and hydrophobicity to adhesion could not be studied separately, the comparison of chemically altered and untreated cells provided useful information. First, the carboxyl and amino groups at the cell surface do not participate directly in the creation of an adhesive bond with the substrate. Otherwise, neutralization of these groups would have reduced adhesion of the organisms to tendon or glass, which was not observed. Second, electrostatic and/or hydrophobic interactions are involved in the adhesion of bacteria to glass, since cells that have become less negatively charged and more hydrophobic through chemical (EDC-G, HEEI) treatment adhered to glass in larger numbers than did untreated cells. The same effect was not observed on tendon. This is partially due to the fact that adhesion to tendon was much higher than adhesion to glass. In particular, all of the untreated *Lactobacillus* sp. cells that reached the tendon surface became adherent. Therefore, adhesion could not be increased further, even after improvement of the cell adhesive properties through chemical alteration. Similarly, untreated *P. fluorescens* cells adhered to tendon in large numbers and adhesion could not be substantially increased after chemical treatment. In contrast, only a fraction of the untreated *E. agglomerans* cells that reached the tendon became adherent. Yet, adhesion was not increased after EDC-G or HEEI treatment. This and the fact that orange-G-treated *E. agglomerans* cells adhered to tendon in larger numbers than did untreated cells, with a similar surface charge or hydrophobicity, confirmed that the knowledge of cell surface charge and hydrophobicity is not sufficient to accurately predict adhesion (39). Third, the strength of adhesion is influenced by the cell surface charge and hydrophobicity. In fact, it has been proposed that the cell surface characteristics have a greater influence on the adhesion strength than on the number of adherent bacteria (47). It is therefore possible that EDC-G- or HEEI-treated *E. agglomerans* cells adhere to tendon with a greater energy (and not in greater numbers) than do untreated cells. This could not be verified, however, since the qualitative method used to evaluate adhesion strength on glass was not applicable on tendon slices. Unfortunately, no simple and reliable method to measure quantitatively adhesion strength on biological tissues is yet available. Attempts made in our laboratory to measure the resistance to shear (by centrifugation) of bacteria adhering to tendon slices failed because the tendon disintegrated at high accelerations.

Earlier reports indicated that the number of bacteria adhering to meat increased with increasing cell concentration in the adhesion medium (4, 7, 11). Eventually, the meat surface was saturated with adhering bacteria, at surface concentrations of about $10^8$ CFU · $cm^{-2}$ (24, 28). Similarly, given a sufficient cell concentration in the adhesion medium, the surface of tendon was almost completely covered with adherent *P. fluorescens* cells ($3 \times 10^7$ CFU · $cm^{-2}$; this study). This suggests two possibilities for the mechanism of adhesion to tendon. (i) The adhesion is nonspecific and is entirely determined by general physicochemical properties of the cell and the tendon surfaces. Tendon is relatively homogeneous, so adhesion can occur anywhere on the tendon slice. (ii) Adhesion is specific and involves specialized structures at the cell surface that recognize and interact with attachment sites on the tendon slice. If so, the attachment sites must be very abundant and evenly distributed on the tendon slice. Accordingly, assumptions on the nature of the possible attachment site can be made from the known structure and composition of tendon (36). Tendon is essentially composed of parallel type I collagen fibrils (average diameter, 25 nm; maximum diameter, 300 nm; 90% of the tendon dry weight) closely packed in an hydrated proteoglycan matrix (1% of the dry weight). The main remaining component is elastin, which forms a network of fibers in the tendon mass. Only collagen and proteoglycans are ubiquitous and could therefore harbor the attachment sites.

## ACKNOWLEDGMENT

The technical assistance of Marie-Claude Collette, Food Research and Development Centre, Saint Hyacinthe, Québec, Canada, is greatly appreciated.

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